Development and Evaluation of a Tetraplex Flow Cytometric Assay for Quantitation of Serum Antibodies to Neisseria meningitidis Serogroups A, C, Y, and W-135

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A rapid and simple method for the simultaneous quantitation of serum immunoglobulin G (IgG) antibodies specific for Neisseria meningitidis serogroups A, C, Y, and W-135 was developed and evaluated. Four bead sets were generated, each conjugated with one of the meningococcal capsular polysaccharides (A, C, Y, or W-135) and serologically assessed by the use of antimeningococcal international reference sera. Cross-reactivity studies demonstrated no inhibition between monoplex and multiplex assays, and the assay was linear over a 24-fold serum dilution range. Inhibition studies demonstrated that the assay is specific, with <25% heterologous inhibition occurring. The assay was also found to have low intra- and interassay variations and limits of detection ≤650 pg/ml. A comparison of the meningococcal bead assay with the standardized meningococcal enzyme-linked immunosorbent assay showed a good correlation between the IgG concentrations obtained by each assay. The tetraplex assay has the potential to be an important addition to the serologic evaluation of meningococcal capsular polysaccharide conjugate vaccines.

Neisseria meningitidis is responsible for approximately 120,000 cases of meningococcal infections worldwide each year, with a fatality rate between 5 and 10% (5), caused mainly by five serogroups, A, B, C, Y, and W-135 (15). Meningococcal capsular polysaccharide vaccines are available as a bivalent (serogroups A and C) or a tetravalent (serogroups A, C, Y, and W-135) vaccine. However, these vaccines are of limited use due to the T-cell-independent nature of the serogroup C portion, which is poorly immunogenic in children aged less than 2 years (A. E. Taunay, P. A. Galvao, J. S. de Moraes, E. C. Gotschlich, and R. A. Feldman, Abstr. Pediatr. Res. 8:429, 1974) and which has been shown to induce hyporesponsiveness following multiple doses (20). Meningococcal serogroup C conjugate vaccines were introduced in the United Kingdom in 1999 (13), and these vaccines are immunogenic in all age groups studied and induce immunologic memory (1). The success of the conjugate vaccine technology is now being applied to the development of tetravalent conjugate vaccines specific for serogroups A, C, Y, and W-135; and trials are under way to determine the safety and immunogenicity of such vaccines (3).

Protection against meningococcal disease correlates with capsular polysaccharide-specific antibody for all serogroups (2) except serogroup B, for which the capsular polysaccharide is poorly immunogenic (21). Evaluation of the immune response to meningococcal vaccination therefore entails serogroup-specific serologic measurements. Assessment of the antibody response to tetravalent conjugate vaccines will involve measurement of an increased number of analytes. The standard methods used in meningococcal serology are the serum bactericidal assay, which measures functional antibody, and the enzyme-linked immunosorbent assay (ELISA), which quantitates meningococcal capsular polysaccharide-specific immunoglobulin G (IgG) (9). The ELISA is a specific, accurate, and reproducible assay that is well suited to the screening of many samples for a single analyte. However, a separate ELISA is required to determine each serogroup-specific antipolysaccharide antibody. Therefore, as the number of target organisms contained in vaccines increases, the ELISA will become increasingly time-consuming and laborious. Furthermore, due to the limited dynamic range of the assay, testing of multiple serum dilutions may be required. Flow cytometry-based multiplex assays incorporating fluorescent microspheres enable the simultaneous determination of multiple analytes in a single sample (23). The technology uses fluorescently distinct microspheres as a solid support for the antigen. This technique, which incorporates all the advantages of the ELISA, will also increase sample throughput and significantly reduce the sample volume required.

In this study, the development of a microsphere-based multiplex assay for the quantification of serum antibodies against meningococcal serogroups A, C, Y, and W-135, which has the potential to be a useful high-throughput assay and to aid in the evaluation of new multivalent polysaccharide-protein conjugate vaccines, is described. Assay characteristics such as accuracy, sensitivity, specificity, and robustness were determined; and the assay was evaluated by performing a comparison of the multiplex assay and the standardized ELISA.

MATERIALS AND METHODS

Reagents. Methylated human serum albumin and the following N. meningitidis capsular polysaccharides were obtained from National Institute for Biological Standards and Control (NIBSC, Potters Bar, United Kingdom): serogroup A (NIBSC code 98/730), serogroup C (NIBSC code 98/730), serogroup Y (NIBSC code 01/426), and serogroup W-135 (NIBSC code 01/428). R-Phycocerythrin-
The samples and beads were incubated in a 96-well MV Multiscreen plate fitted with a fluid transfer unit (Biotek Instruments, Schwabach, United Kingdom) at 50 rpm for 20 min at room temperature in aluminum foil) was incubated on an orbital shaker (Polymax 1040; Heidolph Instruments, Schwabach, Germany) at 50 rpm for 20 min at room temperature in aluminum foil. Serum was then mixed with 25 μl of the microspheres (a cocktail of the four bead sets) and assayed as described above.

**Assay sensitivity.** The MFIIs generated from the blank wells were averaged (50 runs), and from this, 2 (positive) standard deviations were calculated for each serogroup. The limit of detection (LOD) was then determined for each serogroup by calculating the concentration (in picograms per milliliter) from the relevant standard curve (for serogroup A, C, Y or W-135). This concentration is equal to the assay LOD for that serogroup.

**Assay reproducibility.** Intra-assay variation was determined by assaying 15 samples, with each sample assayed on four wells within a plate; thus, four results were obtained for each sample. The coefficient of variation (CV; in percent) between each of these results for each serogroup was calculated and averaged. In order to determine the variation from assay to assay, the samples (n = 15) were run in three separate assays and the percent CV was calculated.

The reproducibilities of the results generated from separate batches of conjugated microspheres were also determined. Three sets of microspheres were conjugated (by using separate preparations of reagents) on the same day for each serogroup. Samples (n = 15) were assayed on the same day by using each preparation of the conjugated beads, and the mean value and the percent CV for each serogroup were calculated.

**ELISA for quantification of meningococcal antibodies.** Meningococcal serogroup C-specific ELISAs were performed as described previously for serogroup C (7). A standardized serogroup A-specific ELISA was performed as previously described by Carlone et al. (4), except that reference serum sample CDC 1992 was an individual conjugate, monoclonal-PAN-anti-human Fc purified antibody, were used.

Serogroup Y- and W-135-specific ELISAs were performed by a slight modification of the method described above for the serogroup A-specific ELISA. The serogroup Y and W-135 polysaccharides were coated onto microwell plates (Immulon 2; Thermolabsystems, South Trentham, United Kingdom) with final concentrations of 1 and 2 μg of polysaccharide/ml of methylated human serum albumin, respectively. Reference serum sample CDC 1992 was prediluted 1:75 and, in addition to the plates, was diluted twofold seven times. Following completion of the assay, the optical density was read with an Anthos 2001 plate reader (Labtech, Letchworth, United Kingdom) at 450 nM with a reference filter at 620 nm. Data acquisition was performed with SOFTmaxPRO (version 3.1.2) computer software (Molecular Devices, Sunnyvale, Calif.).

**RESULTS**

Development of a tetraplex assay for detection of meningococcal serogroup-specific IgG. The development of the meningococcal tetraplex assay required an initial assessment of the conjugation of the polysaccharides to the beads and the suitability of reference serum sample CDC 1992 as a standard. Each meningococcal polysaccharide was conjugated to an individual bead set, and seven fourfold dilutions of standard serum sample CDC 1992 were run in the meningococcal microsphere assay with each bead set (monoplex), two bead sets together (biplex), and all four bead sets (tetraplex) to assess the range of fluorescence generated by each individual bead set. Previously assigned anti-meningococcal IgG concentrations for reference serum sample CDC 1992 were used in this
Interference and cross-reactivity between the different bead sets was investigated by comparing the MFIs generated by the monoplex assay with those generated by the biplex assay. The plots in Fig. 1 demonstrate that no cross-reactivity was observed between the bead sets. The curves were similar for all four serogroups and demonstrated linearity over the seven fourfold standard dilution range. The MFIs generated from the tetraplex assay were virtually identical to those generated from the monoplex assays, and this was apparent for all serogroups (Fig. 2).

**Assay specificity.** The specificities of the assays were evaluated in order to determine whether the tetraplex assay was measuring serogroup-specific capsular polysaccharide antibodies only. Following addition of one of the serogroup polysaccharides to the reaction mixture, percent inhibition of the MFIs compared to that for the control was calculated. Dilutions of reference serum sample CDC 1992 (NIBSC code 99/706), the meningococcal control serum sample (NIBSC code 96/562), and control serum sample 89-S were preincubated with one of the four polysaccharides for 1 h prior to assaying the sera by the tetraplex microsphere assay. Serum containing no polysaccharide was used as a control. The MFIs generated following addition of the inhibitor are shown in Fig. 3 for all four serogroups and each reference serum sample. Good specificity was observed for serogroups A, C, Y, and W-135 for all serum samples. The percent reduction in the MFI on addition of the homologous serogroup was above 60% for all serogroups. Addition of heterologous polysaccharide resulted in inhibition of <25%.

**Assay sensitivity.** The sensitivity of the tetraplex assay was assessed for each serogroup by using the values obtained from the blank wells. The MFIs generated from the blank wells were averaged (50 runs), and 2 standard deviations were calculated. The LOD of the assay was then obtained from the standard curve. The calculated LODs of the bead assay suggest that the bead assay has a greater sensitivity than the ELISA and are as follows: 650 pg/ml for serogroup A, 370 pg/ml for serogroup C, 260 pg/ml for serogroup Y, and 590 pg/ml for serogroup W-135.
**Assay reproducibility.** The reproducibility of the microsphere assay was assessed by obtaining both intra- and inter-assay variability data and also by observing the variations in the results obtained with different preparations of conjugated microspheres. The mean percent CVs obtained from duplicates of 15 samples within a plate were 4.6% for serogroup A, 4.7% for serogroup C, 5.1% for serogroup Y, and 8.9% for serogroup W-135. Interassay variation was calculated by comparison of the antibody concentrations obtained for samples (n = 15) run in three separate assays. The percent CVs were as follows: 7.8% for serogroup A, 10.9% for serogroup C, 17.5% for serogroup Y, and 13.6% for serogroup W-135. The variation between titers for samples (n = 15) assayed with three different preparations of conjugated microspheres was also investigated (Table 1). For each sample, the mean IgG concentration from the three assays was calculated, and the percent CV between these three results was also determined. For all four serogroups, minimal batch-to-batch differences in the microspheres were observed and the mean percent CV was within the acceptable limit of ≤25% for all serogroups and for most samples. The exceptions were the percent CVs obtained for serum sample men 4 for serogroups A and W-135, men 5 for serogroup W-135, and men 10 for serogroup C, the CVs for all of which were >25% but <32%.

**Comparison of the microsphere assay with the ELISA.** To evaluate the performance of the tetraplex microsphere assay compared with that of the standardized meningococcal ELISA, a panel of adult postvaccination serum samples, pediatric pre- and postvaccination serum samples, and control serum samples (NIBSC codes 96/562 and 89-S) were run by both assays (serogroup A, n = 90; serogroup C, n = 54; serogroup Y, n = 96; serogroup W135, n = 80) and the IgG concentrations were compared. Those results were analyzed by linear regression, and correlation coefficients (R values) were determined (Fig. 4). A good correlation between the results of the ELISA and those of the bead assay was achieved for all serogroups. The individual correlation coefficients were as follows: serogroup A, R = 0.93; serogroup C, R = 0.96; serogroup Y, R = 0.87; and serogroup W-135, R = 0.94.

To compare the performances of both assays at the lower
end of the standard curve, a panel (n = 8) of pediatric pre-vaccination sera were assayed for serogroups C, Y, and W-135 by the tetraplex assay and the ELISA. Those data (not included in the correlation charts) demonstrated that the concentrations obtained by the tetraplex assay were lower than those obtained by the ELISA due to the increased range of the microsphere assay (Table 2). The IgG concentrations obtained by the ELISA, which were less than the lower limit of quantitation (LLQ), were recorded as half of the LLQ (or LOD). The LODs for the ELISA were as follows: serogroup A, 0.08 μg/ml; serogroup C, 0.06 μg/ml; serogroup Y, 0.075 μg/ml; and serogroup W-135, 0.075 μg/ml. Even though some of the values for the pediatric sera were assigned the LOD by the ELISA, a result was obtained by the tetraplex assay for all samples analyzed.

**DISCUSSION**

The meningococcal tetraplex assay developed in the study described herein uses the multiplexing capabilities of flow cytometric microsphere-based assays to simultaneously quantitate the levels of IgG antibodies to four meningococcal sero-

### Table 1. Mean IgG concentrations and CVs obtained by testing samples with three different preparations of conjugated microspheres

| Sample | Mean IgG concn (μg/ml) | % CV | Mean IgG concn (μg/ml) | % CV | Mean IgG concn (μg/ml) | % CV | Mean IgG concn (μg/ml) | % CV |
|--------|------------------------|------|------------------------|------|------------------------|------|------------------------|------|
| men 1  | 22.5                   | 11.0 | 60.8                   | 16.9 | 163.4                  | 3.1  | 31.3                   | 11.9 |
| men 2  | 13.9                   | 5.9  | 103.8                  | 12.4 | 34.1                   | 12.7 | 38.0                   | 12.4 |
| men 3  | 27.9                   | 15.8 | 18.5                   | 5.4  | 23.3                   | 24.3 | 13.1                   | 12.4 |
| men 4  | 80.6                   | 31.4 | 15.3                   | 6.5  | 9.0                    | 10.2 | 15.6                   | 26.9 |
| men 5  | 4.8                    | 13.4 | 60.8                   | 23.4 | 1.5                    | 10.1 | 1.0                    | 25.5 |
| men 6  | 3.9                    | 17.4 | 3.9                    | 17.0 | 1.4                    | 13.4 | 1.4                    | 13.4 |
| men 7  | 10.0                   | 24.0 | 7.8                    | 20.5 | 4.4                    | 15.2 | 1.8                    | 28.6 |
| men 8  | 12.9                   | 5.7  | 30.3                   | 11.8 | 3.1                    | 16.7 | 4.4                    | 15.0 |
| men 9  | 6.5                    | 15.4 | 5.7                    | 8.9  | 0.6                    | 13.2 | 0.8                    | 16.7 |
| men 10 | 31.3                   | 23.5 | 89.0                   | 28.4 | 8.6                    | 17.5 | 4.2                    | 11.9 |
| men 11 | 3.5                    | 20.2 | 2.4                    | 12.6 | 1.6                    | 18.5 | 5.6                    | 8.3  |
| men 12 | 7.6                    | 19.1 | 14.0                   | 6.6  | 1.5                    | 18.5 | 0.5                    | 7.9  |
| men 13 | 6.5                    | 18.4 | 3.1                    | 7.2  | 1.4                    | 6.3  | 1.3                    | 9.5  |
| men 14 | 2.5                    | 12.1 | 2.2                    | 2.4  | 0.5                    | 9.2  | 0.9                    | 1.9  |
| men 15 | 32.3                   | 8.5  | 13.8                   | 3.9  | 1.5                    | 10.6 | 6.3                    | 8.4  |

**Mean** | 16.1 | 11.9 | 11.9 | 13.5

FIG. 3. MFIs generated with NIBSC (black bar), CDC 1992 (striped bar), and 89-S (grey bar) sera following incubation with each of the four meningococcal serogroup capsular polysaccharides. (A) Serogroup A beads; (B) serogroup C beads; (C) serogroup Y beads; (D) serogroup W-135 beads.
groups in a single sample. Such simultaneous quantitation means that greatly reduced sample volumes are needed, along with greatly reduced amounts of time and labor to assay the sera.

The two-step carbodiimide method used for conjugation of polysaccharide to microspheres is both simple and reproducible. Optimization of the conjugation procedure included variation of the incubation times between the polysaccharide-PLL conjugate and the activated microspheres. It was discovered that less time was required to reach optimal conjugation for serogroup A (1.5 h) than for the other serogroups. For serogroups C, Y, and W-135, saturation was not achieved until at least 2.5 h. The meningococcal microsphere assay demonstrated linearity over a seven fourfold standard serum dilution range, which compares to a dynamic range of seven threefold dilutions (serogroup A) and seven twofold dilutions (serogroups C, Y, and W-135) in the ELISA. Such an increase in the dynamic range should reduce the number of sample dilutions required, increase the accuracy of the assay, and permit much lower levels of antibody to be detected.

The possibility of interference and cross-reactivity between bead sets is an important consideration for multiplex assays. Comparison of the MFIs generated by the meningococcal monoplex, biplex, and tetraplex assays demonstrated, however, that no interference between bead sets occurred and multiplexing did not appear to alter the qualities or the sensitivities of the assays.

Competitive inhibition-of-binding studies confirmed that the meningococcal tetraplex assay is specific, and this suggests that the conjugation technique does not have a significant detrimental effect on the important antigenic moieties on the cap-

**FIG. 4.** Comparison of IgG concentrations obtained by the meningococcal ELISA and the microsphere bead assay for serogroup A (A), serogroup C (B), serogroup Y (C), and serogroup W-135 (D).

| Sample | Serogroup C | Serogroup C | Serogroup W-135 |
|--------|-------------|-------------|-----------------|
|        | ELISA       | Tetraplex   | ELISA           | Tetraplex       | ELISA           | Tetraplex       |
| 585    | 0.1400      | 0.0007      | 0.212           | 0.0006          | 0.065           | 0.0009          |
| 589    | 0.0600      | 0.0010      | 0.075           | 0.0003          | 0.15            | 0.0027          |
| 597    | 0.0600      | 0.0009      | 0.38            | 0.0006          | 0.24            | 0.0040          |
| 599    | 0.0600      | 0.0013      | 0.47            | 0.0045          | 0.31            | 0.0063          |
| 601    | 0.1500      | 0.0040      | 0.075           | 0.0004          | 0.065           | 0.0035          |
| 602    | 0.0600      | 0.0120      | 0.075           | 0.0003          | 0.065           | 0.0025          |
| 604    | 0.1300      | 0.0008      | 0.075           | 0.0004          | 0.15            | 0.0030          |
| 616    | 0.0600      | 0.0007      | 0.075           | 0.0030          | 0.12            | 0.0030          |
sular polysaccharide. Addition of homologous polysaccharide (2.5 μg/ml) resulted in a signal reduction, with the levels of inhibition ranging from 60.6 to 95% (for all serogroups). The MFI s generated from serogroup Y- and W-135-specific beads were low for NIBSC serum sample pool 96/562 (Fig. 3) because only naturally acquired antibody is present in this serum pool. Nevertheless, a high level of homologous inhibition was observed for both serogroup Y (94.8%) and serogroup W-135 (75.3%). Addition of up to 80 μg of polysaccharide per ml (data not shown) did not significantly change the percent inhibition obtained. These data suggest that the bead assay is likely to have a higher specificity than the ELISA, in which a minimum of 200 μg of homologous polysaccharide per ml is required to inhibit specific antibody binding (data not shown).

The level of heterologous inhibition in the bead assay was low and varied between 0 and 20.6%. Due to similarities in the bacterial capsular polysaccharide structures, a low level of cross-reactivity between the four serogroups is not unexpected. The specificity arises from the differences in the monosaccharides: the serogroup A polysaccharide is composed of an α1-6-linked N-acetyl-d-mannosamine-1-phosphate repeating unit, whereas the serogroup C polysaccharide contains α2-6-linked N-acetyl neuraminic acid (sialic acid) (18). Serogroup W-135 and Y polysaccharides also contain sialic acid and differ from one another only by the presence of a galactose (serogroup W-135) or a glucose (serogroup Y) (11).

The LOD is the lowest level of antibody response that can be accurately distinguished from a true-negative result, and this can be determined statistically by using values obtained from the blanks. The LOD concentrations calculated for the tetraplex assay ranged from 370 to 650 pg/ml. This implies that the bead assay has a sensitivity much greater than that of the standardized ELISA, with the LODs of the bead assay being approximately 100-fold lower than those of the ELISA.

The variability of the assay on a run-to-run basis was minimal (CV < 18% for all serogroups). The results for replicates of standards from several separate runs also exhibited minimal variation (data not shown), suggesting that the tetraplex assay exhibits good precision. Intra-assay variation was also shown to be very low (CV < 9% for all serogroups). Furthermore, the variation in the data obtained by using separate preparations of conjugated beads was low, suggesting that the two-step carbodiimide methodology, which incorporates PLL, is highly reproducible. Although some of the CVs exceeded the cutoff limit of 25% (Table 1), these samples contained very high or very low levels of antibody; and the readings generated from these areas of the standard curve are likely to be somewhat more variable, especially between separate preparations of beads. The fluorescent readout from the Bio-plex reader was also shown to be consistent across the 96-well plate (data not shown) and is likely to be more stable than the colorimetric readout of the ELISA, which relies on enzyme amplification.

The IgG concentrations obtained with sera tested by the microsphere assay demonstrated a good correlation with those obtained by the standardized ELISA. The results of the microsphere assay for adult sera and also pediatric pre- and postvaccination sera correlated well with those of the ELISA for serogroups A, C, and W-135. For serogroup Y the overall correlation of the results obtained by the tetraplex assay and the ELISA was reasonable. However, a number of pediatric vaccination sera from a particular study gave a result of half the LLQ by the ELISA yet generated IgG levels in the range of 0.01 to 0.9 μg/ml in the tetraplex assay. This observation appears to be serum sample specific, because analysis of pediatric vaccination sera from a different study demonstrated that for serogroup Y, the tetraplex assay is more sensitive than the ELISA and gave acceptable results for these samples compared to the results obtained by ELISA, which were less than the LLQ. The reason for this discrepancy between the two assays is unclear; however, it is likely that the tetraplex assay detects serogroup Y polysaccharide-specific IgG at an extremely low level that is undetectable by the ELISA. It should be noted that these IgG levels are below the protective level of 2 μg/ml against serogroup A determined in Finnish efficacy trials (12, 14).

Use of the multiplexing technique in place of the ELISA for quantification of meningococcal antibodies in serum would significantly reduce the amounts of time and labor expended. For example, by using the present standardized ELISA, 20 samples can be tested for all four serogroups in 1.5 days, whereas the microsphere assay would require approximately 3 h. Minimal user training is required to use the Bio-plex instrument, as the software is simple and data acquisition is performed in real time; hence, the rapidity of the assay is enhanced even further.

An advantage of microsphere assays is their flexibility for expansion to include new targets of interest. As 100 bead sets are available, in theory, a single sample could be analyzed for 100 analytes. The assay could be expanded to include detection of antibodies to Streptococcus pneumoniae (16) and H. influenzae and could be fully validated in a manner to similar that for the ELISA. For example, a recent study developed a multiplex flow cytometric assay for quantitation of antibodies to the pathogens that cause tetanus and diphtheria and antibodies to H. influenzae (17).

In conclusion, the multiplex assay developed in the present study is sensitive and rapid and shows good specificity, and the results of the assay demonstrate a good correlation with those of the ELISA for quantitation of the serum IgG antibody response to meningococcal capsular polysaccharides. The microsphere assay has the potential, therefore, to become a viable alternative to the standard ELISA, especially since the number of analytes is set to increase with the development of tetravalent meningococcal conjugate vaccines.

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