Opsonophagocytosis of Fluorescent Polystyrene Beads Coupled to *Neisseria meningitidis* Serogroup A, C, Y, or W135 Polysaccharide Correlates with Serum Bactericidal Activity

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We developed a polysaccharide-specific flow cytometric opsonophagocytic assay (OPA) for the simultaneous measurement of functional antibody to *Neisseria meningitidis* serogroups A, C, Y, and W135. OPA titers significantly correlated with serum bactericidal assay titers for all serogroups tested (mean r = 0.96; P < 0.001). OPA could be used in meningococcal vaccine evaluation.

Meningococcal disease continues to be a significant cause of morbidity and mortality in the United States and worldwide (3). The primary mechanism of protection from disease is through antibody- and complement-dependent bactericidal activity (9). Antibodies to capsular polysaccharides of groups A and C of *Neisseria meningitidis* have been shown to confer protection from meningococcal disease (1, 7).

*N. meningitidis* is efficiently phagocytosed by polymorphonuclear (PMN) cells (5, 8, 10), and antibodies specific for group A and C polysaccharides are bactericidal in the presence of peripheral blood polymorphonuclear leukocytes and complement (19). Monocytes can also phagocytose *Neisseria* species (12, 13, 16, 23). The relative importance of opsonophagocytic killing as a defense mechanism against N. meningitidis has been previously demonstrated (2, 4, 18, 20, 22). Opsonophagocytic assays (OPA) offer several advantages over the standard serum bactericidal assay (SBA). OPA for other pathogens such as *Streptococcus pneumoniae* have been developed which are semi-automated, use noninfectious targets, provide useful laboratory correlates of protection, and can be multiplexed (14).

This report describes a patent-pending multiplexed flow cytometric OPA for measurement of antibody- and complement-dependent cell-mediated antimeningococcal activity against serogroups A, Y, and W135. Postvaccination sera were collected 1 month after immunization with a quadrivalent meningococcal vaccine (Menomune; Connaught Laboratories, Philadelphia, Pa). Sera from nonimmunized adults (n = 15) and postvaccination adults (n = 12) were obtained through the Emory University Donor Services, Atlanta, Ga., and used as quality controls (15). Sera were stored at −70°C and heat inactivated at 56°C for 30 min just before being tested, to inactivate endogenous complement activity. We compare this flow OPA technique to a previously standardized SBA (15).

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Homologous capsular polysaccharide from meningococcal serogroups A, C, Y, and W135 (Connaught Laboratories) at a concentration of 1 mg/ml was oxidized by the addition of 218 µl of a 0.046 M sodium periodate solution for 30 min, protected from light, at room temperature. The reaction was stopped by the addition of 0.1 ml of glycerol per ml of reaction solution. The polysaccharide solutions were then dialyzed overnight against 0.1 M sodium bicarbonate buffer (pH 8.0).

Aliquots (100 µl) from the commercial preparation of 1-µm-diameter carboxylate-modified FluroSpheres (Molecular Probes, Eugene, Oreg.) were coupled with each oxidized meningococcal polysaccharide. Bead populations with different fluorescent spectra were used for each serogroup polysaccharide (serogroup A, 565 nm excitation/580 nm emission; serogroup C, 625 nm/645 nm; serogroup Y, 488 nm/525 nm; serogroup W135, 540 nm/560 nm). The beads were washed twice in 0.1 M sodium phosphate−0.15 M sodium chloride buffer for 15 min at 16,110 × g, after which the supernatant was removed. Then 1 ml of adipic acid buffer (containing 0.22 M adipic acid dihydrazide dissolved in 0.1 M sodium phosphate−0.15 M sodium chloride buffer) was added to the bead pellet. Water-soluble carboimide ECD (0.08 M) was added to the bead suspension, and the mixture was left at room temperature for 4 h. The activated beads were then washed three times for 5 min at 16,110 × g and resuspended in 0.1 M bicarbonate buffer (pH 8.0). The bead suspension was mixed with 1 ml of periodate-oxidized polysaccharide solution, and 10 µl of a 5 M sodium cyanoborohydride solution was added. This reaction mixture was then left in the dark for 2 h at room temperature to covalently bind the polysaccharide to the beads. The beads were washed four times in opsonophagocytosis buffer (Hanks balanced salt solution [Life Technologies, Rockville, Md.] with Ca2+ and Mg2+, 0.2% bovine serum albumin, 1× penicillin-streptomycin solution [Life Technologies]), resuspended in 1 ml of opsonophagocytosis buffer, and stored at 4°C, protected from light. All chemicals were of reagent grade and were purchased from Sigma Chemical Co., St. Louis, Mo.

HL-60 human promyelocytic leukemia cells (CCL240; American Type Culture Collection, Rockville, Md.) were differentiated into monocytes by being cultured in the same medium supplemented with 1 mM n-butyric acid dissolved in

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Hanks buffered salt solution containing Ca$^{2+}$ and Mg$^{2+}$ (Life Technologies). The differentiation was allowed to continue for 4 days (21).

Eight twofold serum dilutions were made in opsonophagocytosis buffer from 10 μl of test serum. A 20-μl aliquot of bead suspension containing 10^5 beads (the bead suspension was counted using a hemacytometer) was added to each well, and the plate was incubated for 90 min at 37°C with horizontal shaking (200 rpm). Following this, 10 μl of test serum from 3- to 4-week old baby rabbits (Pel-Freez, Brown Deer, Wis.) was added to each well as a source of complement, with the exception of the HL-60 cell control wells, which received 10 μl of opsonophagocytosis buffer. After incubation of the mixture at 37°C in room air for 15 min with shaking, 40 μl of washed monocytes (10^5 cells) was added to each well, resulting in an effector-to-target ratio of 1:1, and the plates were incubated with shaking at 37°C in air for an additional 15 min. The final well volume was 80 μl. To provide sufficient volume for flow cytometry analysis, an additional 100 μl of opsonophagocytosis buffer was added to each well. The well contents were resuspended and transferred into titer tubes (Bio-Rad Laboratories, Richmond, Calif.). The titer tubes were placed inside 12- by 75-mm polystyrene disposable tubes (Fisher, Atlanta, Ga.) for flow cytometric analysis. The cytometer was optimized to collect data from four different fluorescence detectors (FL1, 525 nm; FL2, 560 nm; FL3, 580 nm; FL4, 645 nm) by adjusting instrument compensation to minimize spectral overlap.

Samples were assayed with a FACSCalibur immunocytometry system (Becton Dickinson, San Jose, Calif.). Data were collected and analyzed with CELLQuest software (Becton Dickinson, version 2.0 for Apple system 7.1). Samples with a maximum phagocytic uptake of <20% were considered negative and reported as a titer of 4 for analysis purposes.

The meningococcal SBA for all serogroups was carried out using a previously published method (15). Pearson’s product moment correlation coefficient, for normally distributed data, and the Mann-Whitney rank sum test, for non-normally distributed data, were used for statistical analysis of the data, with significance levels set at <0.05. Sigma Stat software program, version 2.0 (Jandel Scientific, San Rafael, Calif.) was used.

This standardized, multiplexed flow cytometric OPA can measure the opsonic activity of antibodies produced in response to the quadrivalent meningococcal polysaccharide vaccine. Homologous absorption studies using A, C, Y, or W135 polysaccharide (200 μg/ml) resulted in a mean decrease in titer of ≥98% when testing for specific antibodies against the absorbed serogroup. The geometric mean titers (GMTs) for post-vaccination sera using the SBA were as follows: A = 3,405, C = 2,409, Y = 3,405, and W135 = 2,864. The GMTs obtained on the postvaccination sera with the four-serogroup SBA were as follows: A = 3822, C = 3215, Y = 4288, and W135 = 2146. Although the SBA and OPA are different functional assays, no significant differences in GMTs were obtained by these two methods when tested by the Mann-Whitney rank sum method (all P > 0.64). Figure 1 shows the comparison between the SBA and the multicolor, multisero-group flow OPA. Correlations between the SBA and the multiplexed OPA on all sera (from nonimmunized and immunized persons) were very good (r > 0.94, P < 0.001). Our results clearly demonstrate that both the SBA and OPA may be used as indicators of functional antibody production in individuals vaccinated with meningococcal polysaccharide vaccines and that simultaneous measurement of OPA activity does not affect the observed OPA titers.

The current method of choice for measuring functional antibodies directed against N. meningitidis is the SBA. However, this assay is highly sensitive to complement sources, and marked differences in SBA titers have been observed depending on the complement used (15; G. Santos, B. Wacknov, R. Borrow, W. Leong, L. Danzig, A. Langenberg, D. Granoff, R. Deck, and J. Donnelly, Program Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 53, p. 238, 2000). A fluorescence-based SBA has also been developed and substantially reduces the assay time (17) compared with the standard SBA. Neither the standard SBA nor the newer fluorescence-based SBA is capable of simultaneously measuring functional antimeningococcal polysaccharide antibodies against different serogroups.

In vitro, an intact complement pathway is required for optimal serum bactericidal activity whereas only the early complement components (deposition of C3) are required for opsonophagocytic activity. Both mechanisms appear to operate in the host to clear meningococci (6, 7, 9, 23). To date, the opsonophagocytic mechanism of bacterial clearance has not been used as a measure of functional antibody production in response to meningococcal polysaccharide vaccines.

The flow cytometric OPA has several advantages over the standard SBA. This flow cytometric OPA requires less time, uses noninfectious targets, requires less test serum, uses fluorescent beads which are not sensitive to the presence of antibiotics or to the source of complement, and has been adapted to measure functional antibodies against multiple serogroups.
TABLE 1. Comparison between SBA and flow cytometric OPA for N. meningitidis serogroups A, C, Y, and W135 in postvaccination adult sera.

| Variable | Value for: |
|----------|------------|
| SBA vs sOPA | |
| GMT | 3,405/4,287 |
| r value | 0.73 |
| % ≥ 1 dilution | 58.3 |
| % ≥ 2 dilutions | 83.3 |
| SBA vs mOPA | |
| GMT | 3,405/3,822 |
| r value | 0.89 |
| % ≥ 1 dilution | 75 |
| % ≥ 2 dilutions | 83.3 |
| SOPA vs mOPA | |
| GMT | 4,287/3,822 |
| r value | 0.86 |
| % ≥ 1 dilution | 75 |
| % ≥ 2 dilutions | 75 |

a sOPA, single-serogroup OPA; mOPA, multiseroserogroup OPA.
b Linear-regression r value, all P ≤ 0.01.
c Percentage of titers within ±1 dilution of SBA mean titer.
d Percentage of titers within ±2 dilutions of SBA mean titer.
e Percentage of titers within ±1 dilution of sOPA mean titer.
f Percentage of titers within ±2 dilutions of sOPA mean titer.

simultaneously. These advantages make the flow cytometric OPA an attractive alternative to the SBA for assessing the protective potential of polysaccharide-based N. meningitidis A/C/Y/W135 vaccines. We developed but did not validate a serogroup-specific flow cytometric opsonophagocytic method to measure opsonizing antibodies produced in response to a quadravalent meningococcal polysaccharide vaccine. Our goal was to establish the concept of a multiseroserogroup N. meningitidis OPA capable of simultaneous measurement of opsonic antibodies to serogroups A, C, Y, and W135. Our method uses four different types of labeled fluorescent microspheres each covalently bound to one of either serogroup A, C, Y, or W135 meningococcal polysaccharide and HL-60 cell line-derived monoclonal antibodies to assess complement-dependent functional anti-meningococcal polysaccharide antibodies in healthy adults. This assay will eventually be used to evaluate opsonic activity in sera from individuals vaccinated with one or more doses of conjugate vaccine. While the use of fluorescent beads as targets in a flow cytometric assay is not unique (11, 24), to our knowledge this is the first assay to use multiple fluorescent bead populations to determine opsonophagocytic activity simultaneously against multiple specific bacterial antigens (patent pending #60/192,712). This type of methodology will require future validation and determination of assay sensitivity with low- and high-titer serum from vaccinated populations.

REFERENCES
1. Artenstein, M. S., R. Gold, J. G. Zimmerly, F. A. Wyle, H. Scheneider, and M. S. Artenstein. 1970. Prevention of meningococcal disease by group C polysaccharide vaccine. N. Engl. J. Med. 282:417–420.
2. Bredius, R. G. M., B. H. F. Derkx, A. P. Fijen, T. P. M. de Wit, M. de Haas, R. S. Weening, J. J. van de Winkel, and T. A. Out. 1994. Fc receptor Iia (CD32) polymorphism in fulminant meningococcal shock in children. J. Infect. Dis. 170:849–853.
3. Centers for Disease Control and Prevention. 2000. Recommendations of the Advisory Committee on Immunization Practices concerning the control and prevention of meningococcal disease. Morb. Mortal. Wkly. Rep. 49(RR07):1–10.
4. Estabrook, M. M., N. C. Christopher, J. M. Griffiss, C. J. Baker, and R. E. Mandrell. 1992. Sialylation and human neutrophil killing of group C Neisseria meningitidis. J. Infect. Dis. 166:1070–1088.
5. Fredlund, A., P. Olen, and D. Danielsson. 1988. A reference procedure to study chemoattractance in polymorphonuclear leukocytes by Neisseria meningitidis. Acta Pathol. Microbiol. Immunol. Scand. Sect. C 96:941–949.
6. Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus. II. Development of natural immunity. J. Exp. Med. 129:1237–1248.
7. Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus. I. The role of human antibodies. J. Exp. Med. 129:1307–1326.
8. Gomez-Lus, M. L., L. Aguilar, J. Vazquez, M. J. Gimenez, G. Ruiz, S. Berron, F. Fuentes, and J. Prieto. 2000. Ex vivo bacterial activity against group C Neisseria meningitidis in seronegative subjects. Infection 28:305–308.
9. Goldschneider, I. C., I. Goldschneider, and M. S. Artenstein. 1969. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. J. Exp. Med. 129:1367–1384.
10. Haltensper, A., H. Sjursen, S. E. Vollset, L. O. Froholm, A. Naess, R. Matre, and C. O. Solberg. 1989. Serum opsonins to serogroup B meningococci in meningococcal disease. J. Infect. Dis. 159:267–276.
11. Lehmann, A. K., A. Haltensper, and C. F. Basseo. 1998. Flow cytometric quantitation of human opsonin-dependent phagocytosis and oxidative burst responses to meningococcal antigens. Cytometry 36:406–413.
12. Lowell, G. H., L. F. Smith, M. S. Artenstein, G. S. Nash, and R. P. MacDermott, Jr. 1979. Antibody dependent cell-mediated antibacterial activity of human mononuclear cells. J. Exp. Med. 150:127–137.
13. Lowell, G. H., L. F. Smith, J. M. Griffis, B. L. Brandt, and R. P. MacDermott, Jr. 1980. Antibody dependent mononuclear cell-mediated antimeningococcal activity. J. Clin. Invest. 66:260–267.
14. Martinez, J. E., S. Romero-Stener, T. Pilisivili, S. Barnard, J. Schinsky, D. Goldblatt, and G. M. Carlone. 1997. Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. Clin. Diag. Lab. Immunol. 4:156–167.
15. Maslanka S. E., L. L. Gheesling, D. E. Libutti, K. B. Donaldson, H. S. Harakeh, J. K. Dykes, F. F. Arhin, S. J. Devi, C. E. Frasch, J. C. Huang, P. Kri-Kuzemenska, R. D. Lemmon, M. Lorange, C. C. Peeters, S. Quataert, J. Y. Tai, and G. M. Carlone. 2000. Ex vivo bactericidal activity against multiple serogroups to determine opsonophagocytic activity simultaneously against multiple specific bacterial antigens (patent pending #60/192,712). This type of methodology will require future validation and determination of assay sensitivity with low- and high-titer serum from vaccinated populations.
16. Mezzatesta, J. R., and R. F. Rest. 1983. Phagocytic killing of Neisseria gonorrhoeae by human monocytes. Infect. Immun. 42:99–103.
17. Mountzouros, K. T., and A. P. Howell. 2000. Detection of complement-mediated antibody-dependent bactericidal activity in a fluorescence-based serum bactericidal assay for group B Neisseria meningitidis. J. Clin. Microbiol. 38:2878–2884.
18. Raff, H. V., D. Devereux, W. Shuford, D. Abbot-Brown, and G. Maloney. 1988. Human monoclonal antibody with protective activity for Escherichia coli K1 and Neisseria meningitidis group B infections. J. Infect. Dis. 157:118–126.
19. Roberts, R. B. 1970. The relationship between group A and group C meningococcal polysaccharides and serum opsonins in man. J. Exp. Med. 131:499–513.
20. Ross, S. C., P. J. Rosenthal, H. M. Berberich, and P. Densen. 1987. Killing of Neisseria meningitidis by human neutrophils: implications for normal and complement-deficient individuals. J. Infect. Dis. 155:1266–1275.
21. Rovera, G. D. Santoli, and C. Damsky. 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc. Natl. Acad. Sci. USA 76:2779–2783.
22. Schlesinger, M., R. Greenberg, J. Levy, H. Kaythy, and R. Levy. 1994. Killing of meningococci by neutrophils: effect of vaccination on patients with complement deficiency. J. Infect. Dis. 170:449–453.
23. Smith, L. F., and G. H. Lowell. 1980. Antibody-dependent cell-mediated antibacterial activity of human mononuclear cells. II. Immune specificity of antimeningococcal activity. J. Infect. Dis. 141:748–751.
24. Uff, C. R., A. G. Pockley, and R. K. S. Phillips. 1993. A rapid microplate-based fluorometric assay for phagocytosis. Immunol. Invest. 22:407–413.