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Standardization of an Opsonophagocytic Assay for the Measurement of Functional Antibody Activity against *Streptococcus pneumoniae* Using Differentiated HL-60 Cells

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Host protection against pneumococcal disease is primarily mediated by phagocytosis. We developed and standardized an opsonophagocytic assay using HL-60 cells (human promyelocytic leukemia cells). Fifty-five serum samples were analyzed for the presence of functional antibody against seven pneumococcal serogroups or serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) by using differentiated HL-60 cells (granulocytes) and peripheral blood leukocytes (PBLs). Six of the 55 serum samples were from unvaccinated adult volunteers, 31 serum samples were from adults who received one dose of the 14-valent or the 23-valent polysaccharide vaccine, and 18 serum samples were from 16-month-old infants who received four doses of an investigational 7-valent polysaccharide-protein conjugate vaccine. The results of an opsonophagocytic assay with HL-60 cells correlated highly with those of an assay with PBLs as effector cells (median r for seven serotypes = 0.87; $P < 0.01$). Opsonophagocytic titers were compared with the immunoglobulin G antibody concentrations determined by enzyme-linked immunosorbent assay (ELISA). The r values for serogroups or serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F were 0.61, 0.60, 0.67, 0.90, 0.61, 0.39, and 0.57, respectively, when HL-60 cells were used as effector cells and 0.56, 0.47, 0.61, 0.90, 0.71, 0.31, and 0.62, respectively, when PBLs were used. The assay requires small amounts of serum (40 μl per serotype), making this test suitable for assaying infant sera. Culturable cells aid in assay standardization and likely reduce donor-to-donor variability. This standardized assay, in combination with the standardized ELISA, can be used to evaluate current and developing pneumococcal vaccines, in which functional opsonophagocytic antibody activity may correlate with protection against pneumococcal disease.

Streptococcus pneumoniae is an important bacterial pathogen of both children and adults worldwide. It is an important cause of death and illness in children and people with underlying medical conditions (7). Each year in the United States pneumococcal disease causes an estimated 3,000 cases of meningitis, 50,000 cases of bacteremia, 500,000 cases of pneumonia, and 7 million cases of otitis media (7). Fatality rates are approximately 20% for meningitis, 30% for bacteremia, and 5% for pneumonia (7, 34, 35, 42). In developing countries, pneumococcal pneumonia accounts for about 1.2 million deaths annually in children under 5 years of age; an additional 70,000 deaths are due to meningitis and bacteremia (35, 37). Vaccination has been encouraged as a method for protection against emerging multidrug-resistant pneumococci (5, 35, 41). The licensed 23-valent pneumococcal polysaccharide vaccine has been shown to be safe and, in the majority of studies, effective in reducing the incidence of invasive disease (7). To date, serologic correlates of protection for the evaluation of pneumococcal vaccines have not been established (12).

Immune responses to pneumococcal vaccines have been evaluated with assays that measure total binding antibody, such as radioantigen binding assays and enzyme-linked immunosorbent assays (ELISAs) (23, 29, 33). In addition, since host protection against pneumococcal disease is mainly mediated by phagocytosis (14, 16, 45), animal models and opsonophagocytic assays which measure functional antibodies are being developed (1, 30, 31, 40). Opsonophagocytic assays for *S. pneumoniae* are traditionally performed with peripheral blood leukocytes (PBLs) as effector cells and a variety of techniques (radioisotopic, flow cytometric, microscopic, and viability assays) to measure opsonophagocytic activity (1, 11, 14, 17, 21, 26, 38, 40, 44); however, a standard assay is not available.

Preliminary studies with culturable phagocytes (differentiated HL-60 cells) to measure complement-dependent opsonophagocytic activity in sera from individuals vaccinated with various pneumococcal vaccines have been described (18, 30, 43). Although in vitro complement-independent phagocytosis has been observed (17, 40), complement, especially the third component, has been demonstrated to be a necessary opsonin in the clearance of pneumococci in vivo (2, 6, 14, 44). Use of culturable phagocytes eliminates the need for human donors and decreases the interassay variability that occurs with random PBL donors. In this study, differentiated HL-60 cells were evaluated as an alternative to PBL effector cells for opsonophagocytosis of *S. pneumoniae* (serogroups or serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F). Although the measurement of functional antibody activity by opsonophagocytosis has not yet been definitively correlated with protection, comparison of results to those of future case-control studies may allow opsonophagocytosis to be used as a surrogate of protection for the evaluation of pneumococcal vaccines.

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TABLE 1. Strains of *S. pneumoniae* used in this study

Serogroup or serotype	Strain	Source
4	DS2341-94	CSF[a]
	DS1690-95	Purpura
	DS2221-94	Blood
	DS2382-94	Blood
	0215-95[b]	Blood
6B	DS1756-94	B/A[c]
	DS2161-94	Nasal
	DS2230-94	Blood
	DS2215-94	Blood
	DS2631-94	Purpura
	DS2212-94[b]	Blood
9V	DS2187-94	CSF
	DS2250-94	Blood
	DS400-92[b]	Blood
14	DS2231-94	Blood
	DS2249-94	Blood
	DS2235-94	Blood
	DS2232-92	Blood
	DS2214-94[b]	Blood
18C	DS07-95	Blood
	DS54-95	Blood
	GP116[b]	Unknown
19F	DS2228-94	Ear
	DS2171-94	Blood
	DS2333-94	Ear
	DS1692-94	B/A
	DS2217-94[b]	Blood
23F	DS2167-94	Blood
	DS2186-94	Blood
	DS1689-94B	Blood
	DS1689-94A	B/A
	DS2238-94	Blood
	DS2216-94[b]	Blood

[a] CSF, cerebrospinal fluid.
[b] Reference strain used in the comparative study of PBLs and HL-60 cells as effector cells for opsonophagocytosis of *S. pneumoniae.*
[c] B/A, blood isolate that was passed through BALB/c mice.

MATERIALS AND METHODS

Bacteria. All pneumococcal isolates used in this study are listed in Table 1. The strains were recent clinical isolates sent to the Centers for Disease Control and Prevention for serotyping with type-specific antiserum by the Quellung reaction (24). Strains were sensitive to optochin (BBL, Becton Dickinson and Co., Cockeysville, Md.), alpha-hemolytic on 5% sheep blood agar, and soluble in 2% sodium desoxycholate. Highly encapsulated strains, as judged by the Quellung test with rabbit OMNI serum (Statens Seruminstitut, Copenhagen, Denmark), were used. For the opsonophagocytic assay with HL-60 cells, bacterial strains were grown in 50 ml of Todd-Hewitt broth supplemented with 0.5% yeast extract to an optical density at 420 nm of 0.5 to 0.6 (mid-logarithmic growth phase for the majority of strains tested by using a 13-mm [outer diameter] path length) and inoculated with a 500-μl aliquot from an overnight culture in the same growth medium. Fifteen percent glycerol was added to each culture before dispensing 1-ml aliquots in cryovials for flash freezing in a 95% ethanol–dry ice bath. Frozen cultures were kept at −70°C for further use. Viable counts of randomly chosen aliquots were performed to determine the concentration of pneumococci before and after the freezing protocol. Dilution schemes were determined for all strains to ensure the use of ~1,000 CFU/20 μl in the functional assay. The same frozen lot per strain was used throughout the entire investigation.

Serum samples. Thirty-seven serum samples (6 prevaccination and 31 postvaccination serum samples from healthy adult volunteers who were vaccinated with the 14-valent [$n = 10$] or the 23-valent [$n = 21$] polysaccharide vaccine) were used in this study. The adult volunteers varied in race, sex, and age (age range, 24 to 61 years). In addition, 18 serum samples (postvaccination) from 16-month-old infants who had received four doses of an investigational polysaccharide-protein conjugate vaccine (Merck & Co., Inc., West Point, Pa.) were used. Sandoglobulin, purified immunoglobulin G (IgG) from 16,000 donors at a concentration of 6% (Sandoz Pharmaceuticals Co., East Hanover, N.J.), was used as a reference preparation in each microtiter plate. Six of the 21 adult postvaccination (23-valent) serum samples were designated reference sera to be used in the standardization of opsonophagocytic assay variables. The opsonophagocytic activity of the reference serum sample 89-SF used in ELISA (29) was determined and compared with that of Sandoglobulin. Serum samples were aliquoted (200 μl) and stored at −70°C. Although serum samples may have been exposed to temperatures that inactivate complement, any remaining endogenous serum complement was inactivated by heating at 56°C for 30 min prior to testing.

IgG antibody level by ELISA. IgG antibody levels for seven serogroups or serotypes of *S. pneumoniae* (4, 6B, 9V, 14, 18C, 19F, and 23F) were measured by a modified ELISA protocol (29). Antibodies to the common cell-wall polysaccharide (CPS) were absorbed with purified CPS (Statens Seruminstitut) by incubation (30 min, room temperature) of the diluted serum (1:50) in a CPS solution (10 μg/ml). The IgG antibody concentration (in micrograms per milliliter) in serum was calculated by measuring the absorbance (optical density at 490 nm) against a standard curve. The standard reference serum used on each microtiter plate was serum sample 89-SF (29). Data were analyzed by using a four-parameter logistic log curve-fitting technique (28).

Growth and differentiation of HL-60 cells. Cells of the tissue culture cell line HL-60 (promyelocytic leukemia cells; CCL240; American Type Culture Collection, Rockville, Md.) which is of human origin, were used as the effector cells (8). A frozen stock (passage 20; 5.6×10^6 cells) was diluted 1:20 and expanded in tissue culture flasks (T-75; Corning, Corning, N.Y.) to a cell density of $\sim 6 \times 10^5$ cells/ml in 80% RPMI 1640 medium containing 1% L-glutamine (Life Technologies, Grand Island, N.Y.) supplemented with 20% fetal bovine serum (Hyclone, Logan, Utah) and antibiotics (1× penicillin-streptomycin solution; Life Technologies). Subsequent cultures were grown in 100-ml volumes of the same growth medium supplemented with 10% fetal bovine serum (FBS). Cells were grown in suspension to $\leq 1 \times 10^6$ cells/ml at 37°C in a 5% CO_2 atmosphere in the upright position. Undifferentiated cells were divided once a week (starting inoculum, 2×10^5 cells/ml) and were fed once a week by decantation and the addition of fresh medium at room temperature (20% of the old medium was kept in the bottom of the flask). Undifferentiated cells grown to a cell density of $\sim 5 \times 10^5$ to 7×10^5 cells/ml were used for differentiation. Differentiation was carried out in cultures with a 200-ml volume (T-150) of RPMI 1640 medium containing 1% L-glutamine, 10% FBS, 100 mM *N*,*N*-dimethylformamide (DMF; 99.8% purity; Fisher Scientific, Fair Lawn, N.J.), and no antibiotics. DMF (1,552 μl) was added to a 45-ml aliquot of culture medium, and the components were mixed and added to 150 ml of medium in the T-150 flask. The pellet of centrifuged ($160 \times g$ for 10 min at room temperature), undifferentiated cells was resuspended in 5 ml of fresh medium and the mixture was added aseptically to the medium containing DMF. Flasks to be differentiated were inoculated at a cell density of 2×10^5 cells/ml, as judged by viable counts with 0.4% trypan blue exclusion. Cultures were incubated in the slanted position at 37°C in a 5% CO_2 atmosphere with the caps loose for 8 to 9 days. Differentiation medium was not replaced during the incubation period. Granulocytic differentiation was determined by visual examination of the tissue culture flasks with an inverted microscope (×10 objective; Leitz, Rockleigh, N.J.), by microscopic examination of Giemsa-stained smears, and by using the nitroblue tetrazolium test for the qualitative measurement of superoxide anion production (36).

Opsonophagocytic assay with HL-60 cells. Differentiated HL-60 cells were used in the opsonophagocytic assay at an effector/target cell ratio of 400/1. Differentiated cells were harvested by centrifugation ($160 \times g$ for 10 min at room temperature). The volume of differentiated cell culture required per microtiter plate varied according to the viable cell count of the culture. This was determined by dividing the number of cells needed per well (4×10^5) by the cell count (number of cells per milliliter) and then multiplying by 100 wells. This volume was centrifuged as described above, and the supernatant was discarded, removing any excess medium. The cell pellet was resuspended in Hanks' buffer without Ca^{2+} and Mg^{2+} (Life Technologies) by using 5 ml per 50 ml of centrifuged cell culture. Resuspended cells were kept at 37°C in a 5% CO_2 atmosphere until 10 min before using them in the functional assay. At this point, the cell suspension was centrifuged as described above, and the supernatant was discarded. The cell pellet for one assay plate was gently resuspended in opsonophagocytosis buffer (4 ml of Hanks' buffer with Ca^{2+} and Mg^{2+} [Life Technologies] and 0.1% gelatin) and used immediately in the functional assay. The total number of cells per well was 4×10^5 in a 40-μl volume.

For the functional assay, 20 μl of each serum sample was aliquoted into each well (round bottom) in the first row of the microtiter plate (Costar, Cambridge, Mass.). Samples were serially diluted (twofold) in 10 μl of opsonophagocytosis buffer for a total of 8 dilutions (1:8 to 1:1,024) with the aid of a multichannel pipet. All samples were run in duplicate. Sandoglobulin was used as a reference preparation with known opsonophagocytic titers for each serotype tested. Once all serum samples were diluted, 20 μl of bacterial suspension appropriately diluted (~1,000 CFU) in opsonophagocytosis buffer was added to each well. The bacterial suspension was prepared by dilution of frozen stocks, without regrowing the bacteria to the mid-logarithmic phase. The assay plate was allowed to incubate at 37°C in a 5% CO_2 atmosphere for 15 min. Following this incubation

period, 10 μl of complement source (sterile baby rabbit [age, 3 to 4 weeks] serum; Pel-Freez, Brown Deer, Wis.) was added to each well. Rabbit serum was kept frozen at −70°C in 1-ml aliquots until used. Immediately after the addition of complement, 40 μl of washed differentiated HL-60 cells (4×10^5 cells) was added to each well. The assay plate was incubated at 37°C for 45 min with horizontal shaking (220 rpm) in room air to promote the phagocytic process. A 5-μl aliquot from each well was plated (by using a multichannel pipette) onto solid medium prepared from Todd-Hewitt broth with 0.5% yeast extract and 1% agar. Aliquots were allowed to air dry, and culture plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. Viable colony counts were performed after an ~18-h incubation period to avoid overgrowth of the colonies. Colony counts per aliquot were ~60 CFU (1:16 dilution of the well volume containing 1,000 CFU). Complement control wells included all the test reagents except antibodies to pneumococci. A viable count of the initial number of bacteria added per well at time zero (T_0) was included in each run. Complement control counts and T_0 counts were expected to be close to each other (±20%). A complement source control (with heat-inactivated complement) was performed for all serotypes tested to determine the presence of pneumococcal antibodies in the baby rabbit serum.

Opsonophagocytic titers were the reciprocal of the serum dilution with ≥50% killing compared with the growth in the complement control wells. Occasionally, sera with high titers needed to be retested at higher initial dilutions than 1:8 to determine the opsonophagocytic titer. Serum samples with titers of <8 were reported as a titer of 4 for purposes of data analysis.

PBL opsonophagocytic assay. The PBL opsonophagocytic assay used was described previously (13). PBLs (99% neutrophils) were purified from heparinized human blood by gradient centrifugation in 6% dextran (molecular weight, 500,000; Sigma Chemical Co., St. Louis, Mo.) in 0.9% NaCl (13). Viable counts were compared with those of the complement control to determine the opsonophagocytic titer (≥50% killing). A fourfold dilution scheme (four dilutions; 1:8 to 1:512) instead of a twofold scheme (eight dilutions; 1:8 to 1:1,024) was performed with the serum samples so that 10^6 neutrophils could be added to each test well. The effector:target cell ratio used was 500:1. Bacterial suspensions were obtained by dilution of frozen stocks previously prepared in Hanks' buffer with 15% glycerol. Serum from a single donor was used per assay day. A total of 17 different donors were required to complete the study. All sera were assayed in duplicate.

Receptor analysis. The presence of the receptors CR1, CR3, FcγRI, FcγRII, and FcγRIII and the CD15 marker was determined by flow cytometric analysis of differentiated HL-60 cells and purified PBLs from human donors. A FACSCalibur (Becton Dickinson, San Jose, Calif.) was used to detect fluorescein-labeled cells that were stained with mouse monoclonal antibodies conjugated to fluorescein isothiocyanate according to the manufacturer's instructions. Monoclonal antibodies against FcγRI, FcγRII, and FcγRIII were purchased from Medarex, Inc. (Annandale, N.J.). Antibodies to CR1 (CD35) and CR3 (CD11b) were from Biosource International (Camarillo, Calif.). Antibodies to Leu-M1 (CD15) and mouse isotype controls (IgG1 and IgG2a) were purchased from Becton Dickinson.

Statistical analysis. The opsonophagocytic titers and ELISA IgG levels (in micrograms per milliliter) were converted to a $\log_2$ base and were analyzed for a linear correlation by the Pearson's product moment correlation coefficient. The level of significant correlation was set at $P < 0.05$. Significant differences between two groups of data were determined by the t test with a set value of P of <0.05. When the distribution of the data was not normal, the Mann-Whitney rank sum test was used instead.

RESULTS

Differentiated HL-60 cells were used to measure the opsonophagocytic activities of infant and adult sera before and after vaccination with pneumococcal vaccines. In 8 to 9 days, DMF-induced HL-60 cells were 90 to 95% differentiated into granulocytes (44% were myelocytes and metamyelocytes, and 53% were bands and polymorphonuclear-like). Differentiated cells were used as phagocytes, with an average yield of 4×10^5 cells/ml. These culturable phagocytes were found to be as effective and specific as PBLs for the measurement of the opsonophagocytic activity in sera against seven serotypes or serogroups of *S. pneumoniae*. The reproducibility of the opsonophagocytic assay with differentiated HL-60 effector cells was found to be within 1 dilution of the median for most serotypes and serogroups. The coefficients of variance with a panel of quality control sera ($n = 4$) were determined by testing each serum sample three to seven times against each serotype or serogroup (6B, 14, 19F, and 23F). The mean ± standard deviation coefficient of variance values for each serotype or serogroup were as follows: 13% ± 4.2% for type 6B, 7.5% ± 6.1% for group 14, 16.8% ± 2.7% for type 19F, and 8% ± 5.4% for type 23F. No reduction in bacterial viability was observed when only bacteria and HL-60 cells were present. To accept a run, the bacterial counts in complement control wells had to be within ± 20% of the bacterial counts at T_0. Bacterial counts at T_0 were ~60 to 80 CFU. This assay measured the complement-dependent opsonophagocytic activity in human serum. In the absence of complement, the opsonophagocytic activity with PBLs and differentiated HL-60 cells was significantly reduced ($P = 0.01$). When a panel of six immune serum samples was tested for functional activity by using heat-inactivated complement, the median titer for serotype 6B was reduced from 256 to 16. These results were in agreement with those in a previous report of a study of complement-dependent phagocytosis for serogroup 14 (21).

TABLE 2. Competitive inhibition of opsonophagocytic activity with type-specific polysaccharide

Serotype or serogroup[a]	Opsonophagocytic activity (GMT) with the following concn of type-specific Ps (μg/ml) added[b]:		% Inhibition[c]
	0	100	
4	338	8	98
6B	128	16	88
9V	111	8	93
14	111	8	93
18C	158	8	95
19F	69	8	88
23F	97	8	92

[a] Serogroup or serotype of *S. pneumoniae*. The strains used are indicated in Table 1. The concentration of type-specific polysaccharide (Ps) added to the initial serum sample for competitive inhibition is indicated.

[b] GMT of the opsonophagocytic titers from six reference serum samples. A dilution of 1:8 was the lowest dilution tested for all sera.

[c] Percent inhibition of opsonophagocytic activity after addition of type-specific polysaccharide. All but one serum sample were reduced by >90%; the titer of one serum sample was reduced by 87% with serotype 6B.

Competitive inhibition. The inhibitory effect of type-specific polysaccharide (serogroups or serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was demonstrated with a panel of six immune serum samples. Table 2 shows the complete inhibition of opsonophagocytic activity in the presence of type-specific polysaccharide for all types (1:8 lowest dilution tested). Only one serum sample with antibodies against type 6B did not have a complete inhibition of the opsonophagocytic activity. This caused the increase seen in the geometric mean titer (GMT) for type 6B (Table 2). This experiment demonstrated that the opsonophagocytic activity measured in human sera is type specific and measures functional antibodies directed against the polysaccharide pneumococcal antigens present in the vaccine. However, since the polysaccharide preparations that are commercially available are not absolutely pure (protein content, ≤1%), antibodies to other components present in these preparations may also elicit opsonophagocytic activity.

Similarly, the competitive inhibition of CPS was analyzed with a panel of nine serum samples against serotype 6B. No significant difference in the opsonophagocytic titers was found after CPS absorption ($P = 0.56$). All opsonophagocytic titers were either the same or one dilution apart. The ELISA IgG antibody concentrations were determined for the same panel of sera with and without CPS absorbant. As expected, there was a significant difference in the IgG concentrations measured under both conditions ($P = 0.002$). This experiment

TABLE 3. Correlation of opsonophagocytic assays comparing HL-60 cells with PBLs as effectors

Serogroup or serotype[a]	*r* value[b]	Slope	Mean ± SD opsonophagocytic activity (GMT) (n = 55)[c]	
			HL-60 cells	PBLs
4	0.58	0.73	315 ± 5	200 ± 7
6B	0.90	0.90	104 ± 7	91 ± 7
9V	0.91	1.03	45 ± 5	42 ± 6
14	0.85	0.90	158 ± 6	208 ± 7
18C	0.90	1.12	147 ± 4	119 ± 6
19F	0.87	1.01	111 ± 4	294 ± 5
23F	0.84	0.93	94 ± 7	60 ± 8

[a] The *S. pneumoniae* serogroup or serotype tested. The reference strains used for this study are indicated in Table 1.

[b] Pearson's product moment correlation coefficient. There was a linear correlation between the $\log_2$ opsonophagocytic titers obtained with the differentiated HL-60 cell and the PBL assays for 55 serum samples from vaccinees who received the 14-valent polysaccharide, the 23-valent polysaccharide, or the fourth dose of a 7-valent polysaccharide-protein conjugate pneumococcal vaccine. Both assays had a high correlation between opsonic titers ($P < 0.01$).

[c] GMT ± standard deviation for 55 serum samples. Opsonophagocytic GMTs of prevaccination sera (6 of 55) ranged from 8 to 64; data for all serotypes and effector cells are included.

TABLE 4. Correlation of opsonophagocytic assays with HL-60 cells and PBLs with IgG antibody concentrations

Serogroup or serotype[a]	PBLs		HL-60 cells		GM ELISA IgG concn (μg/ml [range])[b]
	r[c]	Slope	*r*	Slope	
4	0.56	0.63	0.61	0.55	2.6 (0.01–85.0)
6B	0.47	0.66	0.60	0.86	4.6 (0.2–85.0)
9V	0.61	1.02	0.67	1.00	3.7 (0.5–45.0)
14	0.90	1.01	0.90	0.96	9.2 (0.1–239.0)
18C	0.71	0.79	0.61	0.55	4.0 (0.02–85.0)
19F	0.31	0.36	0.39	0.39	7.5 (0.1–85.0)
23F	0.62	0.95	0.57	0.79	3.0 (0.2–34.0)

[a] *S. pneumoniae* serogroup or serotype tested. The reference strains used for this study are indicated in Table 1.

[b] GM, geometric mean for 55 serum samples. The range of minimum and maximum concentration is given in parentheses. The geometric mean ELISA IgG concentrations of prevaccination sera (6 of 55) ranged from 0.2 μg/ml for serogroup 4 to 2.5 μg/ml for serotype 19F.

[c] Pearson's product moment correlation coefficient. Linear correlation between $\log_2$ opsonophagocytic titer and $\log_2$ IgG level as determined by ELISA ($n = 55$). All serotypes had a high correlation of opsonophagocytic activity and ELISA IgG levels ($P < 0.01$).

confirmed the need for a CPS absorption step in ELISA, but indicated that there was no need to perform this step in opsonophagocytic assays.

Effector cells. The opsonophagocytic assays with either differentiated HL-60 cells or PBLs as effector cells were in good agreement ($P < 0.01$ for all serotypes; median $r = 0.87$; range, 0.58 to 0.91). Individual *r* values and slopes for the correlations between opsonophagocytic titers are given in Table 3. Serotype 9V had the highest correlation ($r = 0.91$), followed by serotypes 6B, 18C, 19F, 14, and 23F (range, 0.90 to 0.84). The lowest correlation was found with serogroup 4 ($r = 0.58$). This serogroup gave the shallowest slope (0.73), whereas the remainder of the serogroups and serotypes had slopes ranging from 0.9 to 1.1. The *r* value for serogroup 4 increased to 0.83 (slope = 0.86) when the viable counts were performed on Levinthal's medium instead of Todd-Hewitt broth with 0.5% yeast extract and 1% agar. The viable counts in the complement control were closer to those in the T_0 control when Levinthal's medium was used with strain 0215-95.

Correlation of opsonophagocytic titers with ELISA IgG antibody levels. Although there was variability among the seven serotypes tested, the correlation coefficients between ELISA IgG levels and the opsonophagocytic assays with either type of effector cells were similar (Table 4). When HL-60 cells were used as the effector cells the median *r* was 0.61 (range, 0.39 to 0.90; $P < 0.01$) for all types. Similarly, the opsonophagocytic assay with PBLs had a median *r* of 0.61 (range, 0.31 to 0.91; $P < 0.01$) for all types. Table 4 gives the *r* values from the linear correlation between the opsonophagocytic assays (PBLs and HL-60 cells) and the IgG antibody levels for the individual serotypes. Serotype 19F had the lowest correlation between IgG antibody levels and opsonophagocytic activity with both PBLs and HL-60 cells (*r* for HL-60 cells = 0.39; *r* for PBLs = 0.31), whereas serotype 14 gave the highest correlation (*r* for HL-60 cells = 0.90; *r* for PBLs = 0.91). Five of the 55 serum samples tested against serotype 19F had elevated ELISA IgG values (5.7 to 85 μg/ml); however, no opsonophagocytic activity was detected with either type of effector cell (titers, between 4 and 8 for functional antibody). In general, except for the *r* values for serotypes 18C and 23F, the *r* values were slightly higher when HL-60 cells were used. The *r* values and slopes for serogroups and serotypes 4, 9V, 14, and 19F were almost identical, regardless of the type of effector cell being used.

Correlation of Sandoglobulin opsonophagocytic activity and IgG antibody levels by using HL-60 cells. The opsonophagocytic activities of Sandoglobulin (reference IgG preparation) highly correlated with the IgG antibody concentrations against each serotype tested. Figure 1 gives a graphic representation of seven duplicate tests in which Sandoglobulin was serially diluted and the opsonophagocytic activity against serotype 6B was determined. The IgG antibody concentration at the 50% kill intercept was calculated to be 0.06 μg/ml. Although the *r* value was 1.0, the slope was 6.9, indicating a greater change in

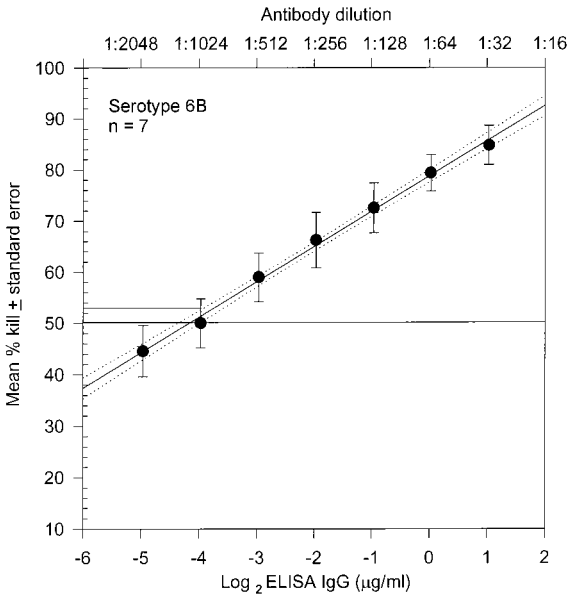

FIG. 1. Sandoglobulin (reference preparation) killing curve for *S. pneumoniae* serotype 6B versus ELISA IgG concentrations. ($r = 1.0$; slope = 6.9; 95% confidence intervals at 50% killing = 50 to 53%, and ELISA at 50% killing = 0.06 μg/ml). The ELISA IgG antibody concentration of undiluted Sandoglobulin was 65.8 μg/ml. The linear regression (——) is based on the mean for seven independent assays with serotype 6B (reference strains are indicated in Table 1). Confidence intervals (– – –) were set at 95%.

TABLE 5. IgG antibody concentrations for Sandoglobulin reference preparation giving 50% killing of *S. pneumoniae*

Serogroup or serotype	IgG antibody concn (μg/ml), undiluted[a]	IgG antibody concn (μg/ml) at 50% killing[b]	% Range of opsonic activity at 50% killing[c]	*r* value (*n*)[d]	Slope
4	23.6	0.04	48–52	1.00 (4)	8.6
6B	65.8	0.06	50–53	1.00 (7)	6.9
9V	38.5	0.15	45–55	0.98 (3)	11.8
14	72.3	0.03	45–55	0.97 (6)	6.2
18C	34.9	0.06	50–57	0.98 (3)	8.2
19F	97.0	0.17	37–63	0.94 (3)	11.4
23F	32.8	0.02	41–59	0.96 (5)	8.8

[a] ELISA IgG concentrations for undiluted Sandoglobulin at 6%.
[b] ELISA IgG concentrations for diluted Sandoglobulin giving 50% killing of the serogroup or serotypes tested.
[c] The 95% confidence intervals were calculated at 50% killing.
[d] Pearson's product moment correlation coefficient. Linear correlation between $\log_2$ opsonophagocytic titer and $\log_2$ IgG value of Sandoglobulin at various concentrations. All serotypes had a high correlation of opsonophagocytic activity and ELISA IgG concentrations ($P < 0.01$). *n* indicates the number of Sandoglobulin opsonophagocytic killing curves analyzed.

opsonophagocytic activity per ELISA unit. Similar correlation coefficients and slopes were obtained for serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Table 5 summarizes these values for all seven serotypes. However, variability in slopes and IgG values at the 50% kill intercept was found among serotypes. Serotypes 9V and 19F had an average IgG antibody concentration of ~0.16 μg/ml at the 50% kill intercept and the highest slopes of all seven serogroups and serotypes (11.4 and 11.8). The other five serotypes had lower IgG values at the 50% kill intercept (range, 0.02 to 0.06 μg/ml) and their slopes were also reduced (range, 6.2 to 8.8).

Since ELISA IgG concentrations were determined by using the 89-SF reference serum standard, it was of interest to compare the opsonophagocytic activity of this reference serum sample and that of the Sandoglobulin IgG preparation, which was used as a reference in the opsonophagocytic assay described previously. Table 6 gives the opsonophagocytic titers and IgG concentrations for the 89-SF reference serum and Sandoglobulin against seven serotypes. The IgG values reported for the 89-SF reference serum have been published by Quataert et al. (29). Although the correlations between opsonophagocytic activity and IgG concentrations within each serogroup or serotype were significant ($P < 0.01$), as shown in Tables 3 and 4, the overall correlations for all seven types were low ($P > 0.1$) for both 89-SF and Sandoglobulin (*r* values, 0.34 and 0.41, respectively).

Interassay variability of HL-60 opsonophagocytic assay. The variability among assays performed on separate days for the HL-60 assay was determined by using Sandoglobulin as the reference material. Figure 2 presents the median titer and degree of variability for the seven serotypes tested. Serogroup or serotype 9V, 14, 18C, 19F, and 23F opsonophagocytic titers were within 1 dilution of each other. Although serogroup 4 and serotype 6B gave a higher degree of variability, 75% of the titers obtained were within 1 dilution of the median titer and approximately 25% were within 2 dilutions of the median titer.

Strain variability studies. We attempted to determine whether the use of a variety of strains from the same serotype would have an effect on the opsonophagocytic titers of selected reference sera. The results of these studies are given in Fig. 3. The opsonophagocytic titers obtained with strains belonging to serogroup 4 and serotype 6B presented the highest degree of variability. The serum titers obtained with strains belonging to these serotypes varied 1 to 3 dilutions from the median titer. Such a degree of variability was not observed with strains of serogroups or serotypes 9V, 14, 18C, 19F, and 23F, in which variation from the median titer was only 1 to 2 dilutions.

Source of complement. We compared human serum and newborn rabbit serum as a source of complement in the HL-60

TABLE 6. Opsonophagocytic titers of reference serum 89-SF and Sandoglobulin reference preparation against serotypes or serogroups of *S. pneumoniae*[a]

Serotype or serogroup[a]	Opsonophagocytic titer (IgG antibody concn [μg/ml])[b]	
	89-SF	Sandoglobulin
4	128 (4.1)	512 (23.6)
6B	2,048 (16.9)	2.048 (65.8)
9V	128 (6.9)	256 (38.5)
14	256 (27.8)	2,048 (72.3)
18C	128 (4.5)	512 (34.9)
19F	32 (13.0)	512 (97.0)
23F	256 (8.1)	1,024 (32.8)

[a] Serogroup or serotype of *S. pneumoniae*. The strain used is indicated in Table 1.
[b] IgG antibody concentrations reported by Quataert et al. (29).

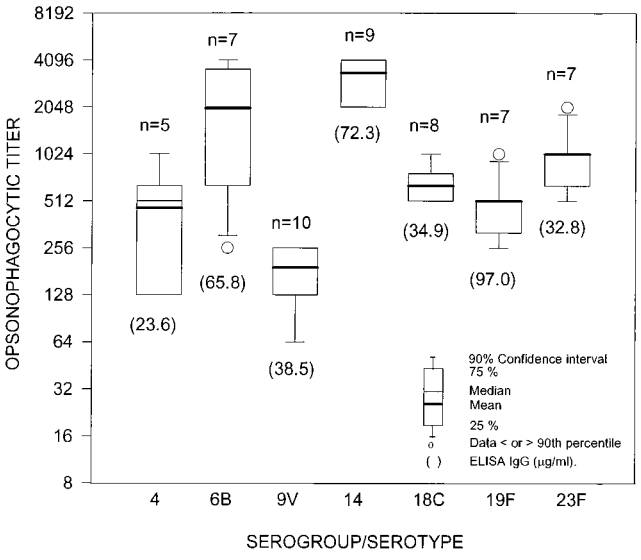

FIG. 2. Opsonophagocytic titers for the Sandoglobulin reference preparation against seven serogroups or serotypes of *S. pneumoniae*. The number of independent assays (*n*) performed with each serogroup or serotype is given above each box plot.

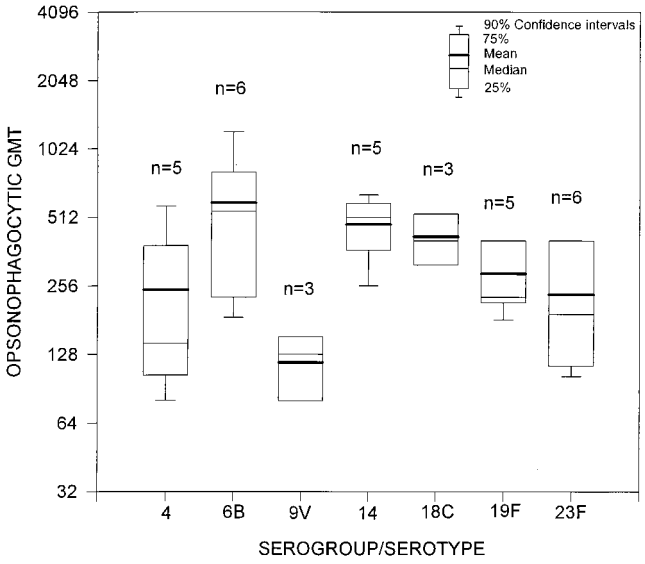

FIG. 3. Opsonophagocytic GMTs from five reference serum samples and the Sandoglobulin reference preparation against various strains of *S. pneumoniae*. The number of strains tested (*n*) is given above each box plot. Individual strains are listed in Table 1.

opsonophagocytic assay using five reference serum samples (Sandoglobulin included) and six serotypes (6B, 9V, 14, 18C, 19F, and 23F). The GMTs obtained under both conditions were ≤1.5 dilutions apart. There was no significant difference between the results obtained with the two complement sources ($P = 0.79$).

Receptor analysis of effector cells. Complement-dependent opsonophagocytosis depends on the presence of the appropriate cell receptors on the effector cell. Differentiated HL-60 cells have been shown to express the markers of mature neutrophils when differentiated into granulocytes (3, 15). It was important to demonstrate the presence of these receptors under the differentiation conditions used in this particular assay. In general, differentiated HL-60 cells presented the same markers as PBLs isolated from human donors (Table 7). Primarily, FcγII, CR1, and CD15 were found in similar amounts in both types of cell populations. The number of cells staining for FcγI, FcγIII, and CR3 were lower in differentiated HL-60 cells than in PBLs. However, the phagocytic activity in the opsonophagocytic assay described here was not affected, and similar levels of phagocytosis were obtained with either type of effector cell (Table 4). Passage number and the FBS concentration (10 or 20%) in the differentiation medium did not have an effect on the expression of the surface receptors analyzed (data not shown).

TABLE 7. Receptor analysis of differentiated HL-60 cells and PBLs from a human donor

Cell type	% FITC-labeled cells[a]					
	FcγI	FcγII	FcγIII	CR1	CR3	CD15
HL-60	19.6	76.8	28.5	72.2	36.5	99.3
PBL	24.0	96.0	91.0	95.0	96.0	94.0

[a] Percent positively stained cells after the appropriate isotype controls were performed. FITC, fluorescein isothiocyanate.

DISCUSSION

Opsonophagocytosis is the primary mechanism of defense in the host against pneumococcal disease; therefore, an opsonophagocytic assay that measures functional antibody may represent a better surrogate of in vivo protection than the commonly used ELISA or radioimmunoassay. Although functional antibodies can be measured by using animal models (20, 31), these methods are costly and are not amenable to testing large numbers of samples in immunogenicity studies. Measurement of type-specific antibodies is made difficult by the presence of anti-CPS antibodies (mainly IgG2), which are considered nonprotective (19, 25). Antibodies against CPS can represent the majority of the signal measured by ELISA, due to the contaminating CPS present in the polysaccharide preparations. However, the modified Farr assay (radioactive antigen-binding assay) has been shown to be more specific than ELISA in the measurement of type-specific antibodies (23). In the Farr assay, there was no difference in the assay results before or after CPS absorption, indicating that in this assay, as in opsonophagocytosis, the interference by CPS antibodies is minimal. Vióarsson et al. (40) previously reported the lack of interference by CPS absorption in the opsonophagocytic activity against *S. pneumoniae* serogroup 23. Anti-CPS antibodies have been found to be protective in mice, but their protective role in humans has not been clearly established (22). Previous opsonophagocytic assays have been performed by a variety of methods with PBLs isolated from human volunteers; however, a standardized assay is not available for evaluating new and developing vaccines.

The method described here is based on the determination of viable counts as an indicator of opsonophagocytic activity in a given serum sample. It also offers an alternative to the use of PBLs as effector cells and allows for the parameter determination necessary in the development of a standardized assay. Assay components such as reference and quality control sera, complement sources, bacterial strains, effector cells, interassay variability, and reproducibility have been analyzed in order to develop a standardized opsonophagocytic assay with differentiated HL-60 cells. A standard assay would reduce interassay and interlaboratory variability during the evaluation and comparison of vaccines.

The HL-60 cell line is a continuous cell line that can be grown in suspension and differentiated into multiple cell lineages (4, 8, 10). The granulocytic differentiation with polar organic compounds, such as DMF (9), generates polymorphonuclear-like cells that possess the cell receptors necessary for an effective phagocytic function, primarily FcγII, CR1, and CR3. Although the differentiated HL-60 cell population had lower numbers of cells staining for CR3 than PBLs, in the current protocol the effector:target cell ratio used was 400:1, providing an excess of effector cells. Differences in opsonophagocytic activity between HL-60 cells and PBLs could only be noticed when lower ratios, i.e., 100:1, were used, (data not shown). HL-60 cells can be induced into phagocytes according to laboratory needs, eliminating the need for human PBL donors. An average of 30 samples can be analyzed per day by a single technician. The differentiated cells give consistently high cell yields, while eliminating the variability that can be observed among human donors (27, 32, 39). The interassay variability found in the opsonophagocytic assay with HL-60 cells was low: 1 to 2 dilutions from the Sandoglobulin median titers against all the serogroups or serotypes tested. Possible disadvantages of culturable effector cells include the need for tissue culture, planning of cell inductions and assays, presence of antibiotics in the serum, and limitations on passage numbers

for differentiations. Passages 22 to 35 were used in this protocol; after passage 35, yields of differentiated cells were low ($<2 \times 10^5$ cells/ml). Current differentiation protocols have shown that increasing the concentration of FBS to 20% in the differentiation medium generates higher yields (4×10^6 to 6×10^5 cells/ml) in cultures at a high passage number. With the addition of 20% FBS, the differentiation time was decreased to only 5 days. A serum sample control containing only test serum, bacteria, and Hanks' buffer is recommended to determine the presence of antibiotics or any other inhibitory substances, especially when testing sera of unknown clinical status.

Differentiated HL-60 cells achieved similar levels of opsonophagocytic activity as PBLs with most of the serotypes tested. A high effector:target cell ratio was used in the opsonophagocytic assays with both types of effector cells to maximize the phagocytosis of all opsonized bacteria. This required the use of large numbers of cells per well (4×10^5 differentiated HL-60 cells and 1×10^6 PBLs). Although the effector: target cell ratio used was reversed from that used in previously reported opsonophagocytic assays, this high ratio has been shown to be bactericidal in PBL opsonophagocytic assays by Esposito et al. (11) for serogroup 3 *S. pneumoniae* and by Gray (13) for *Haemophilus influenzae*. Serogroup 4 was the only serotype with a low correlation coefficient ($r = 0.58$). It is possible that the viability of strain 0215-95 is lower in Todd-Hewitt broth with 0.5% yeast extract and 1% agar than in Levinthal's medium. The r value increased to 0.83 when the viable counts were performed in Levinthal's medium. However, other strains of serogroup 4 demonstrated consistent bacterial counts for complement controls in relation to those for T_0 controls. This allowed for the determination of opsonophagocytic titers for serogroup 4, with no difference between these two controls. The differences in opsonophagocytic activity in serogroup 4 may also be related to strain variability. With the exception of serogroup 4 and serotype 6B, in which a higher degree of variability was observed, strain diversity was not a major variable in the determination of functional activity by opsonophagocytosis. However, the strains used in this study were recent clinical isolates, were highly encapsulated, and had undergone a limited number of in vitro passages. When a rough strain (R36A) was used in the opsonophagocytic assay with HL-60 cells, it was not possible to determine an opsonophagocytic titer because of the elevated degree of killing observed in the complement control wells (data not shown).

Similar correlation coefficients between the opsonophagocytic activity and ELISA IgG concentrations were obtained for each serotype, regardless of the type of effector cell used in the opsonophagocytic assays. There was a marked contrast between the correlation coefficients for serotype 19F (r for HL-60 cells = 0.39; r for PBLs = 0.31) and serogroup 14 (r for HL-60 cells = 0.90; r for PBLs = 0.91) for the 55 serum samples tested. These differences were not observed with the Sandoglobulin quality control. Sandoglobulin had high correlation coefficients between opsonophagocytic activity (percent killing) and various concentrations of IgG (median $r = 0.98$; range, 0.96 to 1.0). These results may reflect the differences among serum samples. Previous studies have demonstrated that the majority of the IgG antibody response to the 23-valent pneumococcal polysaccharide vaccine as measured by ELISA is of the IgG2 subclass (17, 21). A detailed study of IgG subclasses and immunoglobulin classes (IgM and IgA) in samples with high opsonophagocytic titers and low ELISA IgG concentrations would aid in the determination of actual differences between ELISA antibody levels and opsonophagocytic activity. Differences in antibody affinity and avidity may explain observed differences between total binding antibody and functional antibody measurements. Several of these aspects are under investigation.

Human serum can be substituted for newborn rabbit serum in the assay without significantly altering the opsonophagocytic titers of reference sera. However, careful screening of potential donors was necessary to ensure the absence of type-specific antibodies in the complement source. In addition, several donors were required so that we could test several serotypes. In this study, three complement donors were required to test six different serotypes.

The opsonophagocytic assay with differentiated HL-60 cells used as effector cells can be used for the determination of the type-specific functional antibody response elicited by *S. pneumoniae* vaccines. A multilaboratory study that will enable the validation of this assay is being organized. Once validated, this assay could potentially be used in the evaluation of the immunogenicities of current and future vaccines, as well as various vaccine formulations. The correlation of a standard opsonophagocytic assay with other methods of measurement of the immune response, i.e., ELISA, Farr assay, passive protection studies in animal models, and avidity and affinity determinations, among others, may aid in the establishment of a surrogate of protection against *S. pneumoniae* in different target populations.

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