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MINIREVIEWS

Use of Opsonophagocytosis for Serological Evaluation of Pneumococcal Vaccines

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Since 2000, when a pneumococcal conjugate vaccine (Prevnar) was introduced, pneumococcal infections in the United States among children have been dramatically reduced. The conjugate vaccine elicits antibodies to pneumococcal capsular polysaccharide, and these antibodies protect the host by opsonizing pneumococci and thus facilitating phagocytosis. The ability of a serum sample to opsonize bacteria can be measured by various in vitro opsonophagocytosis assays (OPAs), and OPAs have been shown to be the best functional correlate of protection in various studies. A minimum opsonic titer of 1:8 confers protection in a mouse model, is correlated with protection in infants vaccinated with pneumococcal conjugate vaccine, and was shown in infants to correspond to an immunoglobulin G (IgG) antibody concentration of 0.2 to 0.35 μg/ml (5). A Finnish study with an 11-valent pneumococcal conjugate vaccine conducted in the Philippines showed a good correlation between an opsonophagocytic killing assay and the IgG antibodies as measured by enzyme-linked immunosorbent assay (ELISA) (12), although the correlation may be limited in some populations, as described later in this review.

In the wake of the success of the conjugate vaccine (Prevnar; Wyeth Lederle Vaccines), new or improved pneumococcal vaccines for both children and adults are being actively developed. Future vaccine formulations may likely contain a higher number of capsular serotypes and may be formulated to also contain vaccines against other pathogens as part of a new combination vaccine. Evaluation of these vaccines would be heavily dependent on demonstrating that the new vaccines can also induce opsonic titers that are sufficient for protection. For these reasons, various forms of opsonization assays have been developed, and there have been significant technical improvements in pneumococcal antibody OPAs, such as the introduction of multiplexed OPAs. Thus, a workshop was held in Atlanta, Ga., on 5 June 2005 to discuss progress made for each methodology and discuss standardization of pneumococcal antibody opsonization assays. The workshop was supported by the National Institutes of Health, the Centers for Disease Control and Prevention, the World Health Organization, and the University of Alabama at Birmingham and was attended by various representatives from academia, industry, government agencies, and public reference laboratories. We present here a review of the current status of OPAs for anti-capsular polysaccharide antibodies as summarized during the workshop.

LESSONS LEARNED FROM THE USE OF CURRENT OPAs

Classical Killing-Type OPA

The classical OPA is an in vitro assay to determine the titers of sera that reduce the number of live bacteria by more than half due to opsonophagocytosis. Romero-Steiner et al. described such an assay for pneumococcal antibodies in 1997 using rabbit complement and HL-60 cells as phagocytes (15). A multilaboratory evaluation of the Romero-Steiner assay with 5 participating laboratories produced OPA titers with interlaboratory agreement of 0.8 (80%), and this suggested that the OPA can be standardized (14). The study resulted in the determination of median OPA titers for a panel of 24 sera (12 paired pre-postvaccination sera), which were obtained from adults receiving the 23-valent pneumococcal polysaccharide vaccine and are widely available for quality control purposes. Recent studies presented additional characterization of the assay parameters of the Romero-Steiner OPA (4) and an optical readout instead of the classic bacterial colony counting (1). This assay has been used extensively over the last 12 years and has effectively become the standard assay. Taken together, the Romero-Steiner assay should be useful in evaluation and validation of new OPAs.

Phagocytic Cell Lines

Although granulocytes from peripheral blood can be used as a source of phagocytes for opsonization assays, it is generally agreed that using cell lines as phagocytes is more convenient and reproducible. Promyelocytic leukemia cell lines can be induced to differentiate into granulocyte-like cells in response to various chemical treatments, and the differentiation can be monitored by the expression of surface antigens. Although many promyelocytic cell lines are available, the HL-60 and NB-4 cell lines have been used for OPAs. Comparatively, the NB-4 cell line contains the higher-affinity IgG receptor FcγII

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and HL-60 cells express the lower-affinity FcγII receptor. Nevertheless, the NB-4 cell line did not provide significant advantage over HL-60 cells, and the HL-60 cell line has been used extensively by the pneumococcal vaccine community for OPA. Experience has shown the importance of using HL-60 cells from one source because there are multiple sublines of HL-60 cells with different abilities to differentiate. The HL-60 line from the ATCC has been most consistent, but it is important for the vaccine testing community to standardize the source of the cell line. An extensive review on differentiation, standardization, and use of HL-60 for OPA has been recently published (3).

Titer Estimation Methods

Experience gained in the process of standardizing the pneumococcal antibody ELISA showed that standardizing the data calculation method is as important as standardizing reagents and assay methods (13). To minimize variability in the assay results, one should either select one calculation method as the standard method or establish a performance-based criterion with defined levels of tolerable variation. A preliminary study suggests that a four-parameter logistic curve-fitting method can be used to fit opsonization assay data. A computer program can produce a continuous titer determination using the curve-fitting method and thus calculate the titer producing 50% of the maximum number of bacterial colonies used as the end point for the OPA titer. Although a wide range of killing percentages can be chosen as the endpoint, the 50% killing yields the most robust way to determine the titer, since this portion of the killing curve varies less than the upper or lower portions of the curve.

EXPERIENCE FROM CLINICAL STUDIES USING DIFFERENT TYPES OF OPSONIZATION ASSAYS

Mainly because of the labor requirements of the standard OPA, other OPAs were developed. A study compared four different OPA methodologies: (i) the killing-type OPA method determined the titer by measuring the number of surviving bacteria, (ii) two phagocytosis-type OPA methods measured the phagocytosis of fluorescent bacteria by flow cytometry, and (iii) one method measured the uptake of radiolabeled bacteria (16). The killing-type OPA was found to be more sensitive than the two fluorescent bacterium phagocytosis methods. The National Public Health Institute (KTL) in Finland has used the killing-type OPA for the majority of immunogenicity assays, with manual counting of bacterial colonies. Recently, KTL began evaluating the use of a flow cytometric OPA.

KTL has used both OPA and ELISA to measure pneumococcal conjugate vaccine immunogenicity in various clinical trials, two of which were presented. The killing-type OPA was used for the FinOM trial (7), and the flow cytometric uptake OPA was used to analyze sera from vaccinated human immunodeficiency virus (HIV)-infected or noninfected infants in South Africa (9). When the ELISA/OPA ratios were obtained, the ratios differed between serotypes in the FinOM trial, being low for serotype 6B with good protection and high for serotype 19F with poor protection against acute otitis media. At the population level, the correlation with protection was better for the ELISA/OPA ratio than for ELISA. In the South African study, the ELISA/OPA ratios were lower for non-HIV-infected than for HIV-infected individuals, despite similar antipneumococcal antibody concentrations. This suggests that the HIV-infected population had many persons with dysfunctional antibodies (i.e., high ELISA/OPA ratios). These findings support the need to use OPA in measuring the immunogenicity of pneumococcal vaccines.

MULTIPLEXED OPAs DEVELOPED TO INCREASE THROUGHPUT

With increasing needs for OPAs, multiplexed OPAs were developed using two entirely different approaches. One approach is to measure phagocytosis by flow cytometry. Flow cytometry-based phagocytosis assays use fluorescently labeled bacteria or latex particles (10). Recently, a multiplexed phagocytosis assay for three different serotypes was developed using three different types of latex beads, which were coated with different polysaccharides. Since the polysaccharide labeling could be standardized, this approach should have less analytical variability. Compared to multiplexed killing-type OPA, multiplexed phagocytic OPA would be more technically difficult but would not use infectious targets and would be more automated. Also, reagents for multiplexed phagocytic OPA will be commercially available.

Overall, flow cytometric phagocytosis OPA titers were higher with granulocytes than with HL-60 cells. To improve the quality of the HL-60 cells used in the flow cytometric assays, various HL-60 cell culture conditions were investigated. A useful parameter of phagocyte quality was the proportion of cells in the S, G_2, and M phases of the cell cycle. When the HL-60 cells have well differentiated into phagocytes, the cells in S, G_2, and M phases were less than 3% of the total cells. To determine the OPA titer, the number of viable HL-60 granulocytes containing fluorescent bacteria or beads was obtained, and the OPA titer was defined as the dilution of a test serum with 50% or more of the maximal uptake that was observed for the test serum. The titer was not calculated in comparison to the complement controls. Titers could be determined using various computer programs, including Atractors and FlowJo as well as the program Statlia, which is a percent parameter curve-fitting software.

The other form of a multiplexed OPA was developed based on a killing-type OPA. The killing-type OPA is an accepted reference method but has problems due to tediousness in bacterial colony counting and slow assay speed. To increase the assay speed, two improvements have been made. One approach was to develop a multiplexed killing-type OPA, and the other was to automate counting of pneumococcal colonies. Automation was achieved by coloring pneumococcal colonies red with 2,3,5,-triphenyltetrazolium chloride and then using an image analysis instrument to count the red colonies. With this approach, the number of pneumococci in 96 reaction wells can be determined in 2 or 3 min. The killing-type OPA was then multiplexed by using antibiotic-resistant target strains of pneumococci (6, 11). In addition to increasing the analytical speed, the multiplexed killing-type OPA provides an additional important advantage, reducing the amount of serum required for the assays.

The fourfold multiplexed killing-type OPA can be performed using pneumococcal strains resistant to four clinically irrelevant antibiotics: optochin, streptomycin, spectinomycin, and trimethoprim. Under development is a panel of antibiotic-resistant target strains that would be useful in evaluating 11- or 13-valent conjugate vaccines. The panel would be made available to the scientific community for multiplexed killing-type OPA. The distribution information will be posted at a website (www.vaccine.uab.edu). The assay uses the standard Romero-Steiner OPA protocol with a small modification. To maintain an effector-to-target cell ratio close to the original, the inoculum of each bacterial strain was reduced twofold (from 1,000 CFU/well to 500 CFU/well) and twofold-more aliquots (10 μl instead of 5 μl) are plated. With this change, the effector-to-target cell ratio is 200:1 instead of 400:1. A preliminary study shows that the fourfold multiplexed killing-type OPA still retained the overall performance of the assay.

Based on the same principle, another laboratory has developed a sevenfold multiplexed OPA (2). The seven strains represent the serotypes included in the currently available 7-valent conjugate vaccine. This method uses seven antibiotic-resistant pneumococcal strains that are either from clinical cases or are laboratory derived. The assay conditions are different from those of the Romero-Steiner assay conditions. It uses 30 μl of serum sample, 15 μl of a mixture of 7 pneumococcal strains (3,000 bacteria/serotype, 2×10^5 bacteria/ml), 15 μl of complement, and 20 μl of 1.4×10^6 HL-60 cells. The OPA titer was determined as the reciprocal of the serum dilution killing 90% or more. The results compared well with conventionally determined OPA titers. The assay, however, has not been fully standardized nor validated. Nevertheless, this study has demonstrated the adaptability and flexibility of an antibiotic-based, multiplexed, killing-type OPA.

INDUSTRY EXPERIENCES

Sanofi-Pasteur described steps necessary to bring an OPA to good laboratory practices/good manufacturing practices level in a vaccine manufacturing laboratory. The validation steps require establishment of the pass/fail criteria for the limit of detection, limit of quantitation, precision, linearity, specificity, and accuracy. Although a standard reference serum is not available for OPA, accuracy can be estimated by performing spike/recovery studies across the range of the assay with multiple samples and operators; these data also support assessment of linearity. The validation requires a minimum of 60 stratified samples, covering the range of the assay, to be tested in triplicate by three operators on three separate days to assess precision. The major parameters for tolerance within the assay, definition of the valid assay data, and out-of-specification assay results and how to resolve them should be established in a prevalidation phase to qualify the assay and be applicable to a formal validation protocol. Quality control samples need to be monitored, and a trend analysis needs to be performed. Incident tracking also has to be documented. The primary parameters affecting assay utilization and performance are standardization of target bacteria, cell line banking and distribution, reference/calibration reagent, and performance range controls. The most favorable assay should be the classical OPA killing assay, as it has already been validated. If a new OPA technology is developed, the assay should be qualified, validated, and used to develop bridging data before it can be used to produce clinical data useful for vaccine registration. Additional information useful for assay validations can be found at two websites http://www.fda.gov/cder/guidance/4252fnl.pdf and http://www.fda.gov/cder/guidance/ichq2a.pdf.

Wyeth described validation parameters that were performed in the laboratory using the classical OPA killing assay (4). The assay was validated in terms of consistency in results, usage of peripheral blood granulocytes, and usage of differentiated HL-60 cells. The minimum requirements on HL-60 cells were cell viability of 65% or greater, increased CD35 expression (≥55%) with concomitant decrease (≤15%) in CD71 expression (marker for proliferation), and low binding (<35% of cells) of annexin 5, an apoptosis marker.

Merck presented a recently reported killing-type OPA with an automated colony-counting step (8). The OPA is performed in a 96-well plate format. At the end of the phagocytosis step, a 10-μl aliquot of the OPA reaction mixture is transferred into 100 μl of H_2O in Millipore filtration plates to lyse the phagocytes. The liquid in the plates is then aspirated by vacuum, and the plates are placed in a plastic bag for overnight incubation. The small bacterial colonies are stained with 100 μl of Coomassie blue and counted with an enzyme-linked immunospot reader. The counting variability was 14.3% in manual counts but only 1.7% in automated counts. Results obtained with Millipore plates were comparable to those with the conventional agar plate method.

GlaxoSmithKline Biologicals (GSK) adapted the Romero-Steiner methodology to an automated colony counting by using an enzyme-linked immunospot reader. GSK modified the original Romero-Steiner method slightly by using agar overlays instead of plating the reaction mixture on an agar surface. The coefficient of variance was <30% for reproducibility and <25% for repeatability, the deviation from linearity was <30%, the coefficient of variance at the end of a daily run (last few plates in a daily measurement) was <30%, and the recovery percentage was between 76% and 108%. Because the throughput of the GSK OPA is high, GSK feels that the primary assay for measuring vaccine immunogenicity can be an OPA instead of an ELISA.

GSK used this OPA in an acute otitis media (AOM) clinical trial studying their 11-valent PD-conjugate vaccine, where PD is the recombinant nonlipidated form of protein D from *Haemophilus influenzae*. The vaccine prevented 33.6% of all AOM, 57.6% of AOM due to pneumococci, and 35.6% of AOM due to hemophilus (R. Prymula, P. Peeters, V. Chrobok, P. Kriz, E. Novakova, I. Kohl, P. Lommel, J. Poolman, J.-P. Prieels, and L. Schuerman, Abstr. Eur. Soc. Ped. Infect. Dis., abstr. 28, 2005). When ELISA was performed on sera from young children with and without 22F adsorptions (17), the 22F adsorption influenced the results for the samples containing antibody levels of <1.0 μg/ml. The WHO-proposed threshold of 0.35 μg/ml (non-22F ELISA) corresponds to 0.20 μg/ml with 22F ELISA. The concordance analysis for a study where *n* is 1,772 indicated that, for a titer of 8 in OPA, an antibody level of 0.2 μg/ml to 0.3 μg/ml (22F ELISA) is sufficient. Efficacy of the conjugate vaccine Prevnar against invasive pneumococcal disease (IPD), as measured by case control studies, corresponded to an antibody level of 0.2 μg/ml by ELISA and a titer of 1:8 by OPA for

serotypes 4, 6B, 9V, 14, 18C, and 23F (5). For serotype 19F, OPA seropositivity (86%) was a better predictor for IPD efficacy than ELISA (99%). This implies that some sera with ELISA seropositivity are OPA negative. An OPA titer of 8 was achieved with 0.22 μg/ml for serotype 19F compared to 0.003 μg/ml for serotype 6B and 0.002 μg/ml for serotype 23F. For serotype 6B, postconjugate sera of <0.2 μg/ml were generally found to be OPA positive. The significant serotype 6A IPD efficacy (87%) is reflected by OPA seropositivity (74%). The findings with respect to serotypes 6A, 6B, and 19F further indicate the importance of using OPA in clinical trials of pneumococcal vaccines. GSK is currently evaluating the relevance of ELISA and OPA measurements with respect to a correlation with AOM efficacy. Preliminary results indicate OPA to be the more relevant laboratory endpoint than ELISA antibody concentrations.

GENERAL CONSENSUS

The outcomes of the workshop can be summarized as follows. OPA is important, since there may not be additional efficacy trials for newer vaccines in children, and ELISA results may have limited relevance in older adults, who often have nonfunctional anti-capsular polysaccharide antibodies. The use of OPA as the primary analysis of vaccine efficacy is encouraged in studies in the elderly.

The killing-type OPA may be preferred at this time over the phagocytosis-type OPA because the killing assay is more biologically relevant and there is more information regarding the performance of the killing-type OPA as well as the validation parameters needed. Killing-type rather than phagocytosis-type OPAs may be preferred for vaccine evaluations. The killing-type OPA that was originally published in 1997 by Romero-Steiner et al. (15) and is posted on the internet (www.vaccine.uab.edu) should be the reference assay and can be used to validate other OPAs. Additional information will have to be provided if additional cell lines are used, such as the NB-4 cells, especially in terms of standardization.

Multiplexed assays are important because there is an increasing demand for testing antibody responses to many different antigens with small serum samples from young children. Also, multiplexing should facilitate throughput, especially if more than one vaccine is being evaluated. An assay using a set of four serotypes appears to be a good approach for multiplexing.

Classically, the term "titer" implies discrete values. Now that the colony counts can be automatically determined, they can be interfaced with the data analysis programs, and data interpolation is simple. Thus, efforts should be made to determine continuous OPA titers. Perhaps, one should not use the term "titer" but rather use a new term such as "interpolated endpoints." Also, it is important to analyze data from clinical studies in terms of the percent seroconversion using the two thresholds that we have available thus far, an OPA titer of >8 and an ELISA result of >0.2 μg/ml (or, more conservatively, of >0.5 μg/ml) IgG-specific antibody.

Since the OPA technology has now been adopted and implemented by diverse types of institutions, it is clear that the OPA can be performed with the reproducibility and high throughput required for vaccine evaluation. In view of this, there will be increased demands for OPA to be used to measure the functionality of the antibodies in clinical trials.

There was an agreement regarding the need of a single source for HL-60 cells and of a standardized panel of target pneumococcal strains. Many attendees agreed that the fourfold multiplex killing assay is a viable method. In addition, there was a call to provide a chat room on a website (www.vaccine.uab.edu) to facilitate discussion on OPA-related topics. The website currently has the presentations from the meeting and other information useful for OPA. Some participants suggested formation of a steering group to facilitate the adoption of a standard OPA protocol.

MEETING SUMMARY

- A website (www.vaccine.uab.edu) has information on OPA protocols and obtaining reagents.
- OPA can now be performed with reproducibility and high throughput.
- OPA may be used as the primary assay to measure the immunogenicity of pneumococcal vaccines.
- HL-60 cells that have been differentiated in vitro are adequate as phagocytes for OPA.
- Killing-type OPA is preferred over the phagocytosis-type OPA.
- A killing-type multiplexed OPA has been developed using antibiotic-sensitive bacteria.
- HL-60 cells from ATCC should be used.

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G.C. provided the meeting's introduction, S.R.-S. described the classical killing-type OPA, R.A.F. described phagocytic cells, M.H.N. described killing-type multiplexed OPA, and C.E.F. summarized the meeting.

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