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ORIGINAL ARTICLE

Discrepant serological assays for *Pneumococcus* in renal transplant recipients – a prospective study

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SUMMARY

Vaccine immunoprotection for *Streptococcus pneumoniae* is mediated by opsonizing antibodies targeting serotype-specific capsular polysaccharides. Quantitative antibody levels enzyme-linked immunosorbent assay (ELISA) and antibody-mediated opsonophagocytic assays (OPA) measure vaccine-induced protection; correlation of these assays in transplantation requires investigation. This study examines the laboratory assessment of antibody titers in vaccinated renal recipients. *Streptococcus pneumoniae* 19A is common in immunocompromised hosts and is represented in protein-conjugate vaccines (PCV) and polysaccharide vaccines (PSV). Antibodies to 19A in serial sera from 30 vaccinated renal transplant recipients were compared using ELISA and OPA assays. Subject titers were classified as protected or not by ELISA (>0.35 μg/ml) and OPA titer (>1:8).

Antibody titers analyzed using McNemar's test indicate that protection measured by the two assays are not the same ($P = 0.0078$); simple linear regression of within-subject geometric means of 19A enzyme-linked immunosorbent assay (ELISA) antibody levels versus 19A opsonophagocytic assays (OPA) titers demonstrates significant correlation between the two assays ($P < 0.001$).

Vaccination is increasingly important given increasing antimicrobial resistance worldwide. OPA and ELISA antibody assays do not correlate well using current values for protective immunity against the *Pneumococcus* in immunosuppressed transplant recipients. Future studies of vaccination in transplant recipients should evaluate protective antibody levels using both functional antibody assays and standard ELISA antibody titers. (ClinicalTrials.gov: NCT00307125).

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Introduction

Streptococcus pneumoniae is a major cause of community-acquired pulmonary infection with invasive disease occurring in up to 25% of immunologically normal individuals. Immunosuppressive medications and organ transplantation increase the risk for invasive pneumococcal disease (IPD) by up to 2.7-fold [1–6]. Increasing antimicrobial resistance among isolates of *S. pneumoniae* associated with invasive disease and pneumonia

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emphasizes the importance of vaccine immunoprotection, particularly for immunocompromised hosts [7–9]. Immunoprotection is largely mediated by opsonizing antibodies targeting bacterial serotype-specific capsular polysaccharides [10,11]. Quantitative antibody assays may detect both functional and nonfunctional antibodies; based on animal studies, nonopsonizing antibodies may have some role in seroprotection [12,13]. Discussions regarding the efficacy of pneumococcal vaccination focus largely on the relative merits of protein-conjugate vaccines (PCV) and polysaccharide vaccines (PSV) [2,14–19]. Despite widespread vaccination, recent studies detected vaccine strains in up to 11% of individuals with community-acquired invasive pneumococcal pneumonia, of which serotype 19A was most prevalent despite representation of this epitope in both PCV13 and PSV23 [20]. The incidence of invasive pneumococcal disease in immunocompromised individuals is up to 20-fold greater than in other adults with 50–64% of the isolates found among serotypes in PCV13; an additional 21% are caused by serotypes contained only in PSV23; some serotypes are in neither vaccine [1,21,22].

Data on vaccine efficacy from randomized trials in both normal and immunocompromised adults are inconsistent; comparisons between trials are hindered by variability in the techniques used to assess protective responses [23–32]. Bonten found that vaccine efficacy in normal adults in the Netherlands was 45% for vaccine strain nonbacteremic, noninvasive pneumococcal infections, and 75% for vaccine strain invasive disease [14,33]. Efficacy was lower in immunocompromised hosts (30% and 66.7%, respectively) [14]. Protection against strain 19A infections was not significantly different between placebo and vaccine groups. In renal transplant recipients, durability of antibody levels following either PCV7 or PPV23 was short-lived (often less than 2 months) and that neither vaccine type provided a significant advantage in the level or durability of response [34,35]. In liver transplant recipients, there were no differences in IgG levels or OPA titers between recipients of PPV23 or PCV7 [36]. Response in cardiac recipients was similarly muted [37]. In allogeneic stem cell transplant recipients, immunogenicity is poor with either vaccine [38].

Both serotype specific antibody levels (ELISA) and functional, serotype-specific antibody-mediated OPA are used to measure vaccine-induced protection [31,39,40]. In normal hosts, data from clinical trials demonstrate correspondence between capsular antipolysaccharide IgG and antibacterial OPA responses [39,41]. Antibody concentrations measured by the standardized World Health Organization (WHO) ELISA assays in the range 0.20–0.35 mg/l correlated with OPA titers of 1:8, which appeared to predict protective efficacy [31]. The OPA assay is designed to assess the ability of functional antibody (from heat-inactivated human serum) to bind pneumococcal bacteria in the presence of a functional complement source (baby rabbit serum) facilitating bacterial engulfment and death by phagocytic human cell line (differentiated HL-60 cells). The OPA assay is complex, and quantitative response values cannot be compared between serotypes. In adults, the correlation of ELISA IgG assays with the production of functional antibodies has not been investigated [32]. Studies of OPA titers in solid organ transplant recipients are complicated by prophylactic antimicrobial agents including trimethoprim-sulfamethoxazole (TMP-SMZ) targeting *Pneumocystis jirovecii* but with broad antibacterial activity including many strains of *S. pneumoniae* [42].

The Clinical Trials in Organ Transplantation (CTOT) and pediatric CTOT (CTOT-C) are research consortia sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) to conduct clinical trials and associated mechanistic studies to improve outcomes in organ transplantation. Given increasing rates of antimicrobial resistance and variable strategies for antibacterial prophylaxis after transplantation, this study was designed to assess approaches to laboratory assessment of antibody testing in previously vaccinated immunocompromised hosts. We hypothesized that OPA titers provide a distinct assessment of functional antipneumococcal antibodies in immunocompromised transplant recipients when compared with ELISA titers for the same subjects and might provide valuable data in future studies of vaccination in solid organ recipients.

Materials and methods

Study design

Patient samples were derived from 30 adult transplant recipients of renal allografts from living or deceased donors transplanted within 3 and 36 months of study entry. Each provided at least three blood samples at various times from 6 to 48 months following transplantation. Sera were derived from Clinical Trials of Organ Transplantation CTOT02 observational study that were frozen and shipped to our site (ClinicalTrials.gov Identifier: NCT00307125). All participants were known to have been vaccinated within 5 years prior to

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study entry, but the specific vaccine received and the timing of vaccination were not recorded. The study protocol was approved by the Human Studies Committee of Massachusetts General Hospital. Informed consent was obtained from study participants. Immunosuppression was based on site-specific protocols.

Enzyme-linked immunosorbent assay

The ELISA was developed in the WHO Pneumococcal Serology Reference Laboratories by Drs. Moon H. Nahm and David Goldblatt and is available on line (http://www.vaccine.uab.edu/ELISAProtocol(007sp).pdf). Antibody concentrations measured by the standardized ELISA assays above 0.35 μg/ml were considered "protective." [43,44] Pneumococcal cell wall polysaccharides and serotype 19A pneumococcal polysaccharide and goat anti-human IgG-AP conjugated antibody were generously provided by Robert L. Burton and Moon H. Nahm of the University of Alabama, Birmingham. Serum concentrations of anti-19A IgG were determined by validated assay and expressed as micrograms per milliliter (μg/ml). ELISA absorbance data were recorded using a Bio-Tek uQuant ELISA reader. Pneumococcal quantifying reference serum 007sp was kindly provided by Dr. Mustafa Akkoyunlu at the CBER, U.S. Food and Drug Administration. ELISA analysis software was developed at the CDC [45]; custom pneumococcal ELISA Excel templates were kindly provided by Robert L. Burton. All assays were performed in duplicate for each time point and were averaged for statistical analysis.

Opsonophagocytic assay

The single serotype pneumococcal OPA was developed by Moon H. Nahm and Robert L. Burton in the WHO Bacterial Respiratory Pathogen and Pneumococcal Serology Reference Laboratories, Departments of Pathology and Microbiology at the University of Alabama at Birmingham (http://www.vaccine.uab.edu/UAB-MOPA.pdf). The target strain of *Pneumococcus* was kindly provided by Stephen I. Pelton (http://www.vaccine.uab.edu/UAB-MOPA.pdf) and Amy Silverio of the Pediatric Infectious Disease Division of the Boston University Medical Center. Functional serum antibacterial OPA titers were measured using a 19A-specific validated assay. Titers were defined as the interpolated reciprocal serum dilution that resulted in complement-mediated killing of 50% of assay bacteria.

Preliminary studies demonstrated wide variation in pneumococcal killing *in vitro* between samples derived from single patients attributable to TMP-SMZ prophylaxis during the first 6–12 months after transplantation. As a result, all data are reported from studies performed using a TMP-SMZ-resistant 19A pneumococcal strain. A *S. pneumoniae* serotype 19A isolate resistant to TMP-SMZ was selected as the target strain based on clinical data indicating that multiple patients received anti-*Pneumocystis* prophylaxis at various times during the trial. These patients' sera killed target organisms on solid media in the absence of antibiotic, complement or target cells. Concentrations measured by the OPA assays above 0.35 μg/ml were considered "protective." All assays were performed in duplicate for each time point and were averaged for statistical analysis.

Statistical analysis

Antibody titers were classified as protective for each assay if two or more of their respective values were protective, and the classifications were compared by McNemar's test. Quantitative comparisons of the two assays were performed by simple linear regression and correlation on the within-subject geometric means of the respective assay values ($n = 30$). Two-tailed P-values less than 0.05 were considered statistically significant. All statistical analyzes were performed in JMP PRO Version 12 (SAS Institute, Cary, NC, USA).

Results

Opsonophagocytic assays and antibody levels for individuals were generally stable over times up to 4 years after transplantation based on multiple determinations using both techniques. All 30 patients were considered protected against invasive pneumococcal infection based on WHO criteria for ELISA antibody levels in at least two of three determinations with antibody concentrations greater than 0.35 μg/ml (mean 19A antibody 3.5862 ± 0.512 SEM) [31,32]. By contrast, only 22 patients achieved OPA levels considered to be protective (mean opsonic index 189.155 ± 73.642 SE). Classifications of titers as protective versus not protective by each assay are shown in Table 1. McNemar's test ($P = 0.0078$) indicates that the two classifications are distinct. The simple linear regression of the within-subject geometric means of 19A antibody versus 19A OPA is shown in Fig. 1. The Pearson correlation between the two assays of $r = 0.58$ is significant ($P = 0.0008$), as is the Spearman correlation of $r = 0.52$ ($P = 0.003$),

Table 1. For each subject number of sera protective by 19A OPA vs. number protective by 19A antibody.

	Number protective by OPA					
	Count	0	1	2	3	Total
Number protective by antibody	0	0	0	0	0	0
	1	0	0	0	0	0
	2	1	0	1	1	3
	3	6	1	4	16	27
	Total	7	1	5	17	30

Shaded cells represent subjects with 2 or 3 protective values out of 3 total by each measure. McNemar's test comparing numbers of subjects protective vs. non-protective is significant ($P = 0.0078$).

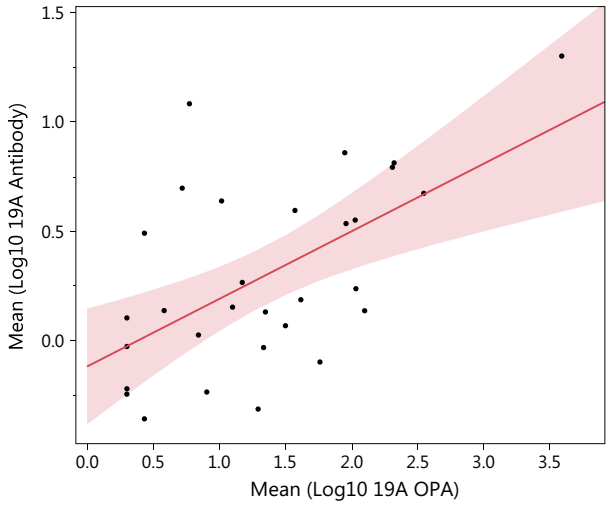

Figure 1 Linear regression of mean $\log_{10}$ 19A antibody on mean $\log_{10}$ 19A opsonophagocytic assays (OPA) within each subject. Solid line is the simple linear regression line and the shaded area represents the 95% confidence limits about the fitted line. Correlation = 0.58 ($P < 0.001$).

indicating that the correlation is robust to the influence of any of the more extreme observations. Thus, while the respective assay values are correlated, the current classification of antibody titers as protective or not by the two assays is not the same.

Discussion

The rate of pneumococcal antimicrobial resistance is increased in immunocompromised hosts and with broad antimicrobial use, increasing the importance of effective vaccination. Among the risk factors for resistance is prophylactic antimicrobial use including TMP-SMZ. Since PCV7 was introduced, the seven serotypes included in that vaccine have largely been replaced in community-acquired infections by serotypes (e.g., 19A, 15A, 23A, 35B, 6C) carrying high and/or increasing levels of antimicrobial nonsusceptibility. Studies with PCV7 in HIV-infected children showed that immunoglobulin G (IgG) levels measured by ELISA correlated poorly with functional antibodies measured by flow cytometric OPA and appeared to vary with serotype and time points studied [32,46]. Correlations between antibody concentration and OPA titer in patients with AIDS and renal transplant recipients have been poor [34,47,48]. Our preliminary data using OPA indicated that the routine use of TMP-SMZ in transplant recipients confounded interpretation of data in that residual drug was present in many but not all clinical samples. Thus, the OPA assay required modification using *S. pneumoniae* strains carrying common vaccine epitopes and resistance *in vitro* to TMP-SMZ. Serotype 19A is present in both the PCV13 and PPSV23 vaccines and is among the serotypes increasingly represented in pneumococcal pneumonia worldwide and has been associated with septic shock during pneumococcal pneumonia [20–22]. Despite uniform vaccination, this study was limited to examination of residual antibody levels by the unavailability of data on the timing of vaccination and the specific vaccine used. We selected a common target serotype represented in both vaccines and for which a TMP-SMZ-resistant strain was available. Other serotypes were not investigated, and therefore, the correlation between OPA and ELISA for those serotypes is not known. For individuals, data became internally consistent when the 19A strain was used in place of a series of clinical isolates.

In this study, while assay values are correlated for individuals, the current classification of transplant recipients' antibody titers as protective or not by these two assays should not be considered to be the same. This suggests that evaluation of protective antibody levels in transplant recipients utilize functional assays as well as ELISA measurements. Surprisingly, once vaccinated, serum antibody levels in renal transplant recipients are better maintained over time than expected despite data that acute vaccine-induced responses wane rapidly. Such observations may be serotype specific and cannot predict vaccine responses required to provide protection against the broader range of pneumococcal serotypes. These data are consistent with vaccination studies in the elderly who demonstrate reduced vaccine responses. In such studies, mean IgG concentrations were preserved up to 10-year postvaccination with 23-valent pneumococcal polysaccharide vaccine, while there was a significant reduction in the IgG antibody avidity of postvaccination measured by OPA [35,49]. Future vaccine trials in immunocompromised hosts should include

measurement of both total and functional antibody levels and confirmation of protection in patients and animal models [50]. New vaccines for such populations may require additional bacterial targets and consideration of the role of nonopsonic antibodies in protection. Vaccine trials in immunocompromised hosts should be encouraged to assess antibody assays associated with clinical protection as well as indirect measures such as functional and absolute type-specific antibody levels. New approaches to vaccination in highly susceptible populations should be considered.

Authorship

JAF: designed the study and assay development, contributed to data analysis and manuscript preparation (no conflicts). DNI: contributed to data analysis and manuscript preparation (no conflicts). RAW: performed the assays, contributed to data analysis and manuscript preparation (no conflicts).

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Conflict of interest

The authors have declared no conflicts of interest.

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