A New Multiantigen Immunoassay for the Quantification of IgG Antibodies to Capsular Polysaccharides of *Streptococcus pneumoniae*

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A new nitrocellulose-based solid-phase multiantigen immunoassay (MAIA) for the detection of serum antibodies to *Streptococcus pneumoniae* capsular polysaccharides (PPSs) is presented. Evaluation with human sera showed that the MAIA test is reproducible, sensitive, and specific. It correlated well with a conventional ELISA method. The multiantigen strip system allowed quantification of antibodies against several PPS serotypes simultaneously and with a minimal amount of serum specimen. The presented solid-phase immunoassay for the quantification of anti-PPS antibodies seems to be a superior and attractive alternative to currently used ELISA tests and offers possibilities for standardization.

Streptococcus pneumoniae is a predominant causative agent for life-threatening invasive diseases, such as meningitis, pneumonia, and sepsis [1, 2]. Recent emergence of antibiotic-resistant strains stresses the need for safe and efficacious vaccines [3].

To evaluate the efficacy of existing and newly produced polysaccharide vaccines against *S. pneumoniae,* valid and reproducible methods for the measurement of serotype-specific antibodies are needed [4]. Currently used ELISA techniques have some shortcomings. Their reproducibility is limited by poor and inconsistent binding of capsular polysaccharide (PPS) to plastic surfaces [4, 5]. Contamination of PPS preparations with cell wall polysaccharides of *S. pneumoniae* may lead to nonspecific binding of antibodies [6]. Also, ELISAs require separate tests for each serotype investigated. Standardization of the ELISA for the comparability of results within and between laboratories has not been possible so far [4]. Despite claims that RIA is not affected by the presence of antibody to CPS [7, 8], several investigators have used a variety of approaches in vitro [4–6] and in experimental animals [6, 9] to show that such antibody is detected. However, those methods involve handling of radioactivity and also pose problems with standardization [4].

A novel multiantigen immunoassay (MAIA) has been used to detect antibodies against protein and lipid antigens derived from *Mycobacterium tuberculosis* cells (Roth F, Riniker F, Burkart T, unpublished data). This nitrocellulose-based, rapid, sensitive, solid-phase immunoassay system offers a wide application potential. Here we describe the adaptation of this technique to quantify IgG serum antibodies against PPSs of various *S. pneumoniae* serotypes.

Material and Methods

Serum samples. The donor pool was 100 sera from healthy adult blood donors of unknown pneumococcal vaccination status and was obtained from the Blood Donor Center (St. Gallen, Switzerland).

A standard serum pool (Food and Drug Administration [FDA] lot 89-SF) consisting of sera from 17 healthy adults vaccinated with the 23-valent pneumococcal polysaccharide vaccine (vaccinated pool) was provided by C. Frasch (Laboratory of Bacterial Polysaccharides, Center for Biologics Evaluation and Research, FDA, Bethesda, MD). The vaccinated pool has IgG levels defined for PPS serotypes 19F (13 μg/mL), 14 (27.8 μg/mL), 6B (16.9 μg/mL), 1 (6.3 μg/mL), 9V (6.9 μg/mL), 4 (4.1 μg/mL), and 18C (4.5 μg/mL).

For further evaluation of the test, 101 consecutive sera from umbilical cord blood (cord sera) submitted to the diagnostic division of our institute between 1991 and 1993 were used. Sera from 11 patients vaccinated because of splenectomy were examined. Paired sera taken before (range, 2 weeks to 30 months) and after (range, 2 weeks to 3 years) vaccination were available from 6 patients, and single sera after vaccination (range, 1–13 months) from 5 additional patients. In addition, antibodies were measured in a patient with invasive infection due to *S. pneumoniae* serotype 9N. All sera were stored at −20°C.

Antigens. PPSs of serotypes 19F, 14, 6B, 1, 3, 7F, 9N, 9V, 4, and 18C (Danish designation) were obtained from the American Type Culture Collection (Rockville, MD). These serotypes are contained in the currently used 23-valent polysaccharide vaccine and are among the most prevalent serotypes circulating in Switzerland [10]. Cell wall polysaccharide preparations (CPS) were purchased from Statens Seruminstitut (Copenhagen).

ELISA. IgG antibodies to PPSs of serotypes 14 and 19F were measured in a modified ELISA system as described by Musher et al. [11]. Briefly, 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated for 5 h at 37°C with 110 μL of a solution of 10 μg of PPS or CPS/mL in PBS, pH 7.4, per well. Excess antigen was removed by 5 washes with PBS. All sera diluted 1:50 with PBS–0.05% Tween 20 (PBS-Tween) were preadsorbed with 10 μg of CPS/mL for 30 min. Preadsorbed vaccinated pool was used in seven 2-fold dilutions starting at 1:400. Patient sera were analyzed at a dilution of 1:200. Preadsorbed sera (100 μL) were incubated for 120 min, followed by 5 washes with PBS-Tween. Then, 100 μL of monoclonal mouse anti-human IgG–HRPO conjugate (Hybridoma Reagent Laboratory, Baltimore) diluted 1:2000 in PBS-Tween was added and incubated for 120 min. After 10 washes (as above) 100 μL of OPD substrate (0.4 mg of

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o-phenylenediamine and 4 μL of 3% H_2O_2/mL in 0.1 *M* citric acid–phosphate buffer, pH 5.0) was added and reacted in the dark. The enzyme reaction was stopped after 15 min with 100 μL of 2 *M* H_2SO_4 per well.

All incubations were done at room temperature (RT). For all buffers and dilutions, endotoxin-free, sterile, double-distilled H_2O was used. Optical densities (OD) were measured at 490 nm (Thermo Max; Molecular Devices, Menlo Park, CA). Background signals were obtained from wells with PBS-Tween instead of serum. Preadsorption efficacy was controlled by testing the vaccinated pool on CPS-coated wells. Logarithmically transformed OD values of the vaccinated pool dilutions were used to define a standard curve. Sera with net OD values outside the range from 0.1 to 0.9 were retested using other serum dilutions.

MAIA. IgG antibodies to PPS of serotypes 19F, 14, 6B, 1, 3, 7F, 9N, 9V, 4, and 18C were tested using a modified MAIA system (Roth F, Riniker F, Burkart T, unpublished data) [11]. PPS antigen (100 μg/mL in 0.9% [wt/vol] NaCl) was sprayed as parallel bands (1 μL/cm each) on a nitrocellulose membrane (BA 85, 0.45 μm pore size; Schleicher & Schuell, Feldbach, Switzerland) with an applicator (Linomat III; Camag, Muttenz, Switzerland). Air-dried sheets were cut into 3-mm strips (Accutran; Schleicher & Schuell) and stored dry in the dark at RT.

All incubations were performed in 8-channel transfer plates (Inotech, Wohlen, Switzerland) at RT under moderate agitation. To block nonspecific binding, the strips were incubated with 1 mL of 0.5% (wt/vol) casein (Sigma, St.Louis) in TRIS-buffered saline (Cas-TBS), pH 7.4, for 30 min. In the meantime, 1 mL of serum sample and controls diluted 1:100 with Cas-TBS were preadsorbed with 0.1 μg of CPS for 30 min. The strips were exposed for 60 min to the preadsorbed sera (dilution 1:100). After 3 washes with 1 mL of Cas-TBS for 10 min, strips were incubated with 50 ng/mL alkaline phosphatase–conjugated anti-human IgG (Sigma) for 90 min, followed by 3 wash steps. Incubation with 5-bromo-4-chloroindoxylphosphate and nitroblue tetrazolium led to the generation of a colored precipitate (figure 1) [13].

The insoluble dye product was quantified at 546 nm with a densitometer (CD 60; Desaga, Heidelberg, Germany). Net OD values were obtained after subtraction of the background (blank) values on the film (performing the assay with PBS instead of serum never gave higher background signals). Specific IgG levels of the sera in micrograms per milliliter were calculated, with reference to the IgG values of the vaccinated pool.

To study the adsorption of antigen to nitrocellulose, PPS 14 and PPS 19F were labeled with ^{125}I (120 μCi/100 μL; 4.44×10^6 Bq) using a commercial radioiodination kit (Bio-Rad, Richmond, CA) [14]. Antigen retention was monitored over the entire assay, except for the color reaction step, using blood donor aliquots and nonconjugated anti-human IgG. Adherent ^{125}I-labeled antigen was measured by exposing nitrocellulose strips for 16 h to x-ray film and quantifying signals by densitometry at 546 nm.

Specificity of the MAIA was evaluated by antibody inhibition. After preadsorption with CPS, the vaccinated pool was incubated for 1 h with 2 μg/μL serotype-specific polysaccharides.

Results

Overall retention of ^{125}I-labeled polysaccharides on nitrocellulose was 53% for PPS 14 and 40% for PPS 19F. The major loss (96% for PPS 14 and 85% for PPS 19F) occurred during the first incubation, the blocking step (data not shown). Dilution curves of the vaccinated pool for IgG antibodies against PPS serotypes 19F, 14, 6B, 1, 9V, 4, and 18C were close to linear for all serotypes over a broad antibody dilution range (correlation coefficients, .91–.96). Sensitivity was at least 0.1 μg of IgG/mL for all serotypes. Specificity was excellent, as demonstrated by antibody inhibition with serotype-specific PPS (figure 1A).

IgG antibodies to 10 different PPS serotypes were measured in 101 cord sera. As expected, the mean IgG titers of cord sera were considerably lower than those in the vaccinated pool and slightly but consistently lower than those in the donor pool (data not shown).

Reproducibility of the MAIA was excellent. For example, coefficients of variation for up to six separate assays, performed on the same or different days, on the donor pool were 1.73% for PPS 19F, 2.26% for PPS 14, 2.18% for PPS 6B, 0.43% for PPS 1, 2.75% for PPS 9V, 1.41% for PPS 4, and 1.53% for PPS 18C.

MAIA was compared to ELISA for serotypes 14 and 19F (figure 2). IgG levels obtained from both tests for the 101 cord sera correlated well (serotype 14: $r = .70$; serotype 19F: $r = .68$). The slope of the curve was 1.09 for serotype 14 and 1.12 for serotype 19F. However, the ELISA gave slightly but consistently higher IgG values than the MAIA. This discrepancy was accentuated in the very low and very high antibody level ranges.

Figure 1B demonstrates the antibody response to pneumococcal vaccination (patients 1–11). Antibody titers rose by $\geq$2-fold against five to nine serotypes within 2 weeks to 3 months (figure 1B, patients 1–5). A $\geq$2-fold decrease of the antibody titer against serotypes 3 and 18C was observed in patient 6 at 3 years after vaccination. Antibody levels in patients 7–11 were comparable to those in the vaccinated pool and higher than those in the donor pool for most serotypes. Patient 14 mounted a specific antibody response to serotype 9N upon invasive infection.

Discussion

Poor binding of polysaccharides to polystyrene affects the performance of the most commonly used ELISA [4, 5]. The excellent reproducibility of the MAIA suggests that nitrocellulose might have better adsorption qualities for polysaccharides than plastic surfaces. Loss of PPS occurred only during the first incubation step of the MAIA (probably due to nonadsorbed antigen). Similar studies on the amount of bound PPS during the ELISA cannot be performed as yet, and therefore a comparison is not possible. The generation of insoluble reaction products in the MAIA might have further contributed to enhanced reproducibility.

Results obtained by MAIA correlated well with those from ELISA. However, the ELISA measured slightly but consistently higher antibody concentrations. The reason for this discrepancy is not clear, but the remarkably linear dilution curves

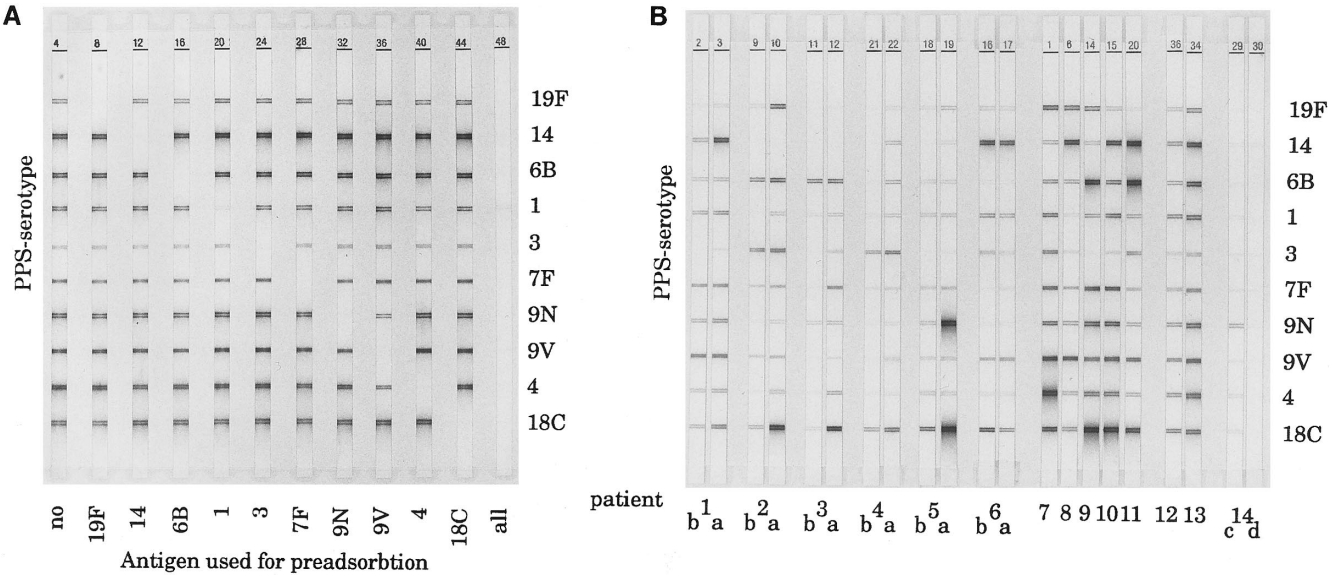

Figure 1. Specificity of MAIA assay in antibody inhibition experiments (**A**) and antibody response to pneumococcal vaccination or invasive infection (**B**). **B**, Lanes 1–6, patients before (b) and after (a) vaccination; 7–11, patients after vaccination; 12, donor pool; 13, vaccinated pool (FDA lot 89-SF); 14, patient at time of infection (c) with serotype 9N and 6 months later (d).

over a broad range of antibody levels for various serotypes of *S. pneumoniae* indicate that MAIA is a sensitive assay. Differences between the two tests might also be at least partly due to shortcomings of the ELISA.

Contamination of PPS preparations with CPS of *S. pneumoniae* necessitate the preadsorption of sera for ELISA [6]. This extends also to MAIA. Recently, Nahm et al. [8] claimed that a modified Farr assay was less affected by CPS contaminations and more specific than ELISA. However, the Farr test involves radioactivity. Also, the study by Nahm et al. [8] demonstrated that preadsorption of sera for ELISA led to good correlation with results from the modified Farr assay [8].

The new MAIA has important advantages that existing tests do not share. First, the principle of multiantigen-carrying strips allows determination of antibody levels to various *S. pneumoniae* serotypes simultaneously and with a minimal amount of serum. For example, 1 person can test a large number of sera against 12 serotypes in 1 day. Second, in our laboratory, the antigen-coated nitrocellulose strips have been stable over at least 1 year. Prepared nitrocellulose strips could be distributed by one center. This would obviate a special infrastructure (e.g., the Linomat applicator for uniform coating of the polysaccharide) in individual laboratories and it would save time. Most importantly, it would enhance comparison of results within and between laboratories.

In conclusion, MAIA seems to have potential as a rapid, sensitive, specific assay for the quantification of IgG antibodies against PPS of *S. pneumoniae.* The principle of this assay offers a greater possibility of standardization than do commonly used tests.

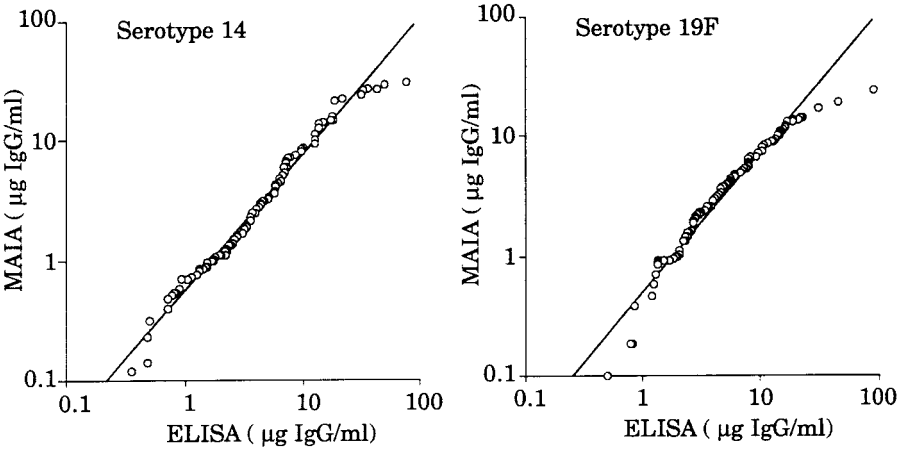

Figure 2. Comparison of MAIA and ELISA for IgG concentrations of antibodies to *S. pneumoniae* serotypes 14 and 19F in 101 umbilical cord sera.

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