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NOTES

Purification and Seroreactivity of Pneumococcal Surface Adhesin A (PsaA)

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Pneumococcal surface adhesin A (PsaA) is a 37-kDa common protein antigen of *Streptococcus pneumoniae*. In the present study, the protein was purified so that its immunoreactivity could be determined. PsaA was released and purified from cells by lysis in the presence of *n*-laurylsarcosine; this was followed by ammonium sulfate precipitation and subsequent preparative isoelectric focusing. A capture antibody enzyme-linked immunosorbent assay was used to determine the immunoreactivity of purified PsaA. The assay had a 67% sensitivity for sera from patients with bacteremic pneumococcal pneumonia. A specificity of 97% was estimated on the basis of a lack of reactivity with sera from patients with pneumonia caused by other organisms. PsaA is a potential vaccine candidate and may be useful as an antigen in a diagnostic assay for pneumococcal disease.

Streptococcus pneumoniae continues to be an important cause of morbidity and mortality among very young, elderly, and immunocompromised individuals in the United States and many other countries (14, 17). The pneumococcal capsular polysaccharide vaccine currently used for *S. pneumoniae* infection comprises 23 of the 84 type polysaccharides and is not recommended for use in children under 2 years of age because many of the polysaccharides included do not stimulate an adequate immune response in this age group (4). With 84 capsular serotypes of *S. pneumoniae*, a common protein might better serve as a vaccine, either alone or as an oligosaccharide-protein conjugate. Recent efforts have been directed toward this goal, with the investigation of several different proteins as candidates (8, 12).

In previous reports, we described monoclonal antibodies that recognized a 37-kDa protein in all strains of *S. pneumoniae* tested ($n = 25$), including all serologic types represented in the 23-valent commercially available vaccine (9). The gene encoding this protein was cloned and sequenced, and the protein was designated pneumococcal surface adhesin A (PsaA) (10). In this report, we describe the purification of PsaA and an enzyme-linked immunosorbent assay (ELISA) for measuring the 37-kDa immunoreactive antibody in sera from patients with pneumococcal disease. PsaA is a possible vaccine candidate and shows promise as being useful in the diagnosis of pneumococcal disease.

S. pneumoniae type 22F (provided by R. Facklam, Centers for Disease Control and Prevention, Atlanta, Ga.) was used as the source for purified protein. This type strain was a clinical blood isolate and was grown on Trypticase soy agar plates supplemented with 5% defibrinated sheep blood for 16 h at 37°C and was subsequently inoculated into 2 liters of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). After 16 h at 37°C, the broth cultures were harvested by centrifugation at 2,600 × *g* for 20 min. The pellet was washed with 0.01 M phosphate-buffered saline (PBS) and was lysed by the method of Anderson et al. (1), with some modifications. Briefly, the cells were suspended in lysis buffer (50 mM Tris [pH 8.0], 1 mM EDTA) containing 7% sucrose. They were made soluble by adding phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), EDTA, and *n*-lauroylsarcosine to make final concentrations of 1 mM, 30 mM, and 1.3%, respectively. This was followed by incubation at 37°C for 15 min. Contaminating nucleic acids were removed by the addition of RNase and DNase (Sigma Chemical Co., St. Louis, Mo.) solutions to the suspension (final concentration, 10 μg of each enzyme per ml), and the mixture was again incubated at 37°C for 1 h.

The suspension was dialyzed (molecular weight cutoff, 10,000) against several changes of deionized water to remove the lysing reagents, and the proteins were precipitated from the cell lysate with ammonium sulfate at 40% saturation at 4°C for 1 h. This suspension was centrifuged at 15,000 × *g* for 30 min at 4°C to obtain the pellet, which was washed in 2 volumes of 40% ammonium sulfate and centrifuged again at 15,000 × *g* for 30 min. This final pellet, which contained most of the cellular PsaA, was dissolved in approximately 3 volumes of deionized water and was then dialyzed against water to remove the ammonium sulfate. During dialysis, a precipitate formed. Following a final dialysis against 150 mM glycine buffer (pH 10) to solubilize insoluble components resulting from dialysis, the protein solution was centrifuged first at 12,000 × *g* at 4°C for 20 min to remove minute, visible particles and then at 105,000 × *g* for 1 h at 4°C. A small pellet was discarded and the supernatant was analyzed for PsaA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis as described previously (11).

Isoelectric focusing was used to separate *S. pneumoniae* proteins and to obtain purified PsaA. This was done with the Rotofor isoelectric focusing apparatus (Bio-Rad Laboratories, Richmond, Calif.) (2, 3, 15, 16). The supernatant described above was dialyzed against 100 volumes of water to decrease

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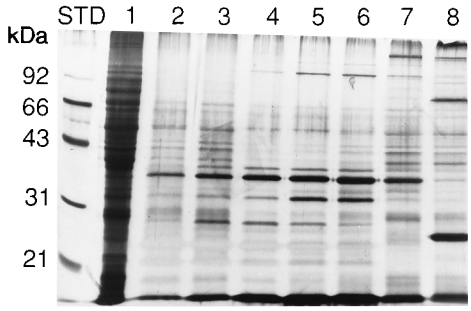

FIG. 1. SDS-PAGE analysis of fractions collected from the first fractionation of the cell lysate on the Rotofor apparatus. Aliquots of the fractions were electrophoresed and silver stained. Lane 1, *S. pneumoniae* cell lysate; lanes 2 through 8, fractions 1 through 7, respectively. Molecular size standards (STD) appear on the left.

the amount of glycine present, and the solution was then brought to 3 M urea, 10% (vol/vol) glycerol, 1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, and 2% Bio-Lyte ampholytes (pH 3 to 10; Bio-Rad) in a total volume of 55 ml for fractionation on the Rotofor apparatus.

Focusing of the proteins was carried out for 5.5 h with cooling at 12 W of constant power with a 2,000-V limit. Twenty fractions (2.5 ml each) were collected, separated by SDS-PAGE, and silver stained (11) to determine the locations of the proteins. The PsaA focused into fractions 1 to 6 and could be seen in a silver-stained gel (Fig. 1). The pH range of these fractions was from 3.6 to 5.6. Fractions containing PsaA were conclusively identified by dot blot analysis as described by Sampson et al. (11) with the following modifications. A casein-thimerosal buffer (6) was used both for blocking and for all washes as well as for dilution of the primary antibody (monoclonal antibody designated 1E7A3D7C2) and the goat anti-mouse immunoglobulin–horseradish peroxidase conjugate (Bio-Rad Laboratories). The fractions containing PsaA were pooled and fractionated a second time, and the purified PsaA protein was identified by SDS-PAGE and silver staining. A purified product showing a single band in the silver-stained gel was found in fractions 18 to 20, as shown in Fig. 2. These fractions, when assayed for immunoreactivity, were found to be

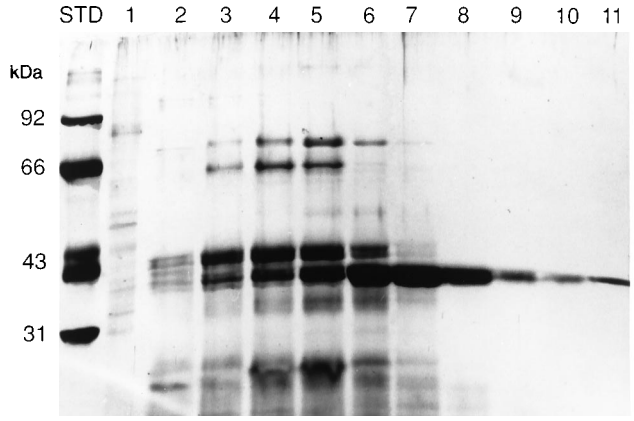

FIG. 2. SDS-PAGE analysis of fractions collected from the second Rotofor fractionation of the positive fractions from the initial fractionation. Aliquots of the fractions were electrophoresed and silver stained. Lane 1, pooled positive fractions (fractions 1 to 6) from the first fractionation; lanes 2 through 10, fractions 12 through 20, respectively; lane 11, purified PsaA. Molecular size standards (STD) appear on the left.

TABLE 1. Reactivities of paired sera from patients with pneumococcal pneumonia and other pneumonias against PsaA

Disease category	No. of paired serum specimens tested	No. (%) positive
Pneumococcal pneumonia	30	20 (67)
Chlamydial pneumonia	10	0 (0)
Mycoplasma pneumonia	10	0 (0)
Legionellosis	10	1 (10)
Healthy	30	0 (0)

positive; they were pooled, brought to 1 M NaCl, and dialyzed against PBS. Silver staining of the combined fractions revealed a single protein band (Fig. 2, lane 11). Sodium azide (0.02%) was added to prevent contamination, and the solution was stored at −70°C. The yield of PsaA, as quantitated by the bicinchoninic acid method (13) (BCA Protein Assay Reagent; Pierce Chemical Company, Rockford, Ill.), was approximately 200 μg of protein per g (wet weight) of whole pneumococcal cells. Bovine serum albumin (BSA) was used as the standard in calibration curves.

An ELISA with the purified PsaA as capture antigen was used to determine the seroreactivity of patients with culture-confirmed *S. pneumoniae* infections (pneumonias). Briefly, U-bottom microtiter plates (Costar, Cambridge, Mass.) were coated with 50 μl of protein (1.25 μg/ml) in 0.1 M carbonate buffer (pH 9.6) and were kept at 4°C for 16 h. The plates were emptied and then blocked for 1 h at 37°C with 100 μl of 0.5% nonfat milk in PBS (pH 7.2). The plates were washed five times with 0.9% NaCl containing 0.05% Tween 20. Optimum serum and conjugate dilutions were established by checkerboard titration prior to specimen testing. Human sera were diluted 1:800 in 0.5% gelatin–0.05% Tween 20–0.02% NaN_3 in PBS (pH 7.2), 50 μl was added to the plates, and the plates were incubated for 1 h at 37°C. A 50-μl aliquot of normal pooled sera was added to each plate as a negative control. The plates were washed as described above, and 50 μl of goat anti-human immunoglobulin alkaline phosphatase conjugate (Southern Biotechnology Associates, Inc., Birmingham, Ala.) diluted 1:4,000 in 2% BSA–10% normal rabbit serum–0.3% Tween 20–0.02% merthiolate in PBS (pH 7.2) was added to each well, and the plates were incubated for 3 h at 37°C. The plates were again washed five times, 50 μl of substrate (*p*-nitrophenyl phosphate [2 mg/ml] in 0.05 M carbonate buffer [pH 9.8]) was added to each well, and the plates were incubated for 1 h at 37°C. The reaction was stopped by adding 50 μl of 5 N NaOH, and the optical density (OD) was read on an ELISA reader (Dynatech Laboratories, Alexandria, Va.) at 410 nm. The OD of each test specimen was divided by the OD of a control serum specimen to correct for plate-to-plate variation. This value was designated the OD ratio (ODR).

The results were interpreted by comparing the fold increase in the ODR values between acute- and convalescent-phase sera. A normal baseline was established by dividing the value for the second specimen by that for the first specimen of paired sera from non-ill persons from whom blood samples were obtained twice within 30 days. These persons were controls in a vaccine study and did not receive vaccine at any time. The normal mean and standard deviation were calculated to be 1.03 and 0.28, respectively. A positive cutoff ODR value for paired sera was set at 1.4 standard deviations above the normal mean value, or 1.42. A total of 90 paired serum specimens were assayed: 30 serum specimens from persons with pneumococcal bacteremia; 10 *Chlamydia pneumoniae*-positive, 10 *Mycoplasma pneumoniae*-positive, and 10 *Legionella pneumophila*-positive (all determined by standard serological tests) serum

specimens; and 30 serum specimens from healthy subjects (Table 1). By using the above-stated criteria for a positive specimen, of 30 pneumococcal bacteremic pairs of serum specimens, 20 were considered positive, yielding a sensitivity of 67%. The specificity of the ELISA was calculated to be 97% on the basis of one false-positive result from 30 patients with *C. pneumoniae*, *L. pneumophila*, and *M. pneumoniae* infections.

We have described a simplified two-step method for the purification of PsaA involving ammonium sulfate precipitation and then preparative isoelectric focusing. This procedure yields purified protein with a minimum of steps and manipulations in the protocol and does not involve the use of high concentrations of denaturing chemicals. Thus, the protein retains its immunoreactivity throughout the purification process, and refolding procedures are not necessary. Because in native pneumococci PsaA is a minor protein and only a small amount of actual protein is produced (evident by silver stain; Fig. 1, lane 1), purification from native pneumococci is not an ideal route. Nevertheless, this procedure should serve as a source of protein for evaluating the immunologic and physical characteristics of PsaA.

We then used purified PsaA to ascertain the serologic responses of patients with *S. pneumoniae* pneumonia to this protein. A significant number of patients showed an increase in antibody when acute-phase sera were compared with convalescent-phase sera, yielding a sensitivity of 67% with a specificity of 97%. This suggests that the PsaA ELISA may be useful in the diagnosis of pneumococcal disease. Further investigations on well-documented, appropriately timed specimens are needed to verify these preliminary results.

The etiologic diagnosis of pneumonia in patients remains a challenge because of the lack of sensitive diagnostic methods. Most methods have focused on a few, limited *S. pneumoniae* components: C polysaccharide, capsular polysaccharide, and pneumolysin (5, 7). With the development of a method that can be used to purify PsaA, we can now further examine the possibility of using it in diagnostic tests. The ELISA developed in the present study is a simple and rapid test and may be improved by combining PsaA with other antigens. In addition, since our original goal was to search for common protein antigens that might serve as candidate vaccines, especially for children, we can now begin to determine the suitability of PsaA as a vaccine.

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