Purification, Partial Characterization, and Seroreactivity of a Genuswide 60-Kilodalton *Legionella* Protein Antigen

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A genuswide protein antigen extracted from *Legionella pneumophila* serogroup 1 (strain Philadelphia 1) cells was enriched by differential pelleting and ammonium sulfate precipitation and subsequently purified with a combination of high-performance size-exclusion and ion-exchange chromatography. The protein has an apparent molecular weight of 650,000 before and 63,000 after urea (5 M) treatment, as determined by size-exclusion chromatography. These proteins resolved to a single band of 60,000 after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The urea-treated protein had an isoelectric point of 5.8. This purified 60-kilodalton protein reacted with a convalescent-phase serum sample from a patient with legionellosis and rabbit immune sera prepared against each of 23 *Legionella* species. The 60-kilodalton protein may be useful in developing diagnostic tests for legionellosis.

Legionella pneumophila serogroup 1 is the most common cause of *Legionella* pneumonia in the United States (9). Since this organism was first isolated in 1977 (3), 23 *Legionella* species containing 39 serogroups have been recognized (12). Serologic methods are routinely used for *Legionella* antigen detection and species identification, since the closely related phenotypic characteristics and slow growth rates of the organisms have precluded the use of classic biochemical methods for identification and since the use of genetic probes requires further evaluation (13). Testing each *Legionella*-like isolate with 39 different antisera, however, is beyond the capabilities of most laboratories. Testing each strain with a single antiserum to a genuswide antigen would be preferable.

Using immunoblot analysis, Sampson et al. (10) showed that a protein with a molecular weight of approximately 58,000 was present in all 14 *Legionella* strains examined. This common protein antigen was highly reactive with sera from 15 patients with culture-confirmed *L. pneumophila* serogroup 1 disease and might be useful in developing diagnostic tests for legionellosis.

In this study, we developed a procedure for extracting the *Legionella* 58-kilodalton (kDa) protein antigen by differential pelleting and ammonium sulfate precipitation. Then we purified the protein by using a combination of high-performance size-exclusion and ion-exchange chromatography. The protein was partially characterized by determining the optimal extraction conditions, apparent molecular weight, isoelectric point, and immunoreactivity with rabbit antiserum against 23 *Legionella* species and with sera from a patient with culture-confirmed *L. pneumophila* serogroup 1 pneumonia.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. pneumophila* serogroup 1 (strain Philadelphia 1) was obtained from the stock culture collection (Immunology Laboratory, Division of Bacterial Diseases, Centers for Disease Control, Atlanta,

Ga.) and grown on *N*-(2-acetamido)-2-aminoethanesulfonic acid-buffered charcoal-yeast extract (BCYE) agar plates (Carr-Scarborough Microbiologicals, Decatur, Ga.) in sealed polyethylene bags at 37°C for 48 h.

Protein extraction and enrichment. Bacterial cells were harvested from each BCYE plate into 2 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, Mo.) buffer (pH 7.2). They were washed once with 300 ml of HEPES buffer and pelleted by centrifugation at $6,000 \times g$ for 15 min at 4°C. The supernatant fluid was decanted, and the cell pellet was resuspended in another 300 ml of HEPES buffer and filtered through gauze to remove medium particles. The cells were pelleted by centrifugation as described above (approximately 9 g [wet weight] of cells was obtained from 60 plates), resuspended in 30 ml of HEPES buffer, and passaged three times through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 16,000 lb/in². After the first passage, 0.3 mg each of DNase and RNase (Sigma) was added. Unbroken cells and debris were removed by centrifugation at $20,000 \times g$ for 30 min at 4°C after the lysate volume was increased to 120 ml with HEPES buffer. The supernatant fluid was centrifuged at $184,000 \times g$ for 2.5 h at 4°C with a Beckman L8-80M centrifuge and a Ti70 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant fluid was decanted, and the pellet was layered with 2 ml of 10 mM HEPES buffer (pH 7.2). The pellet was allowed to sit overnight at 4°C, after which it was resuspended in a 10-ml glass tissue homogenizer with a Teflon pestle while on ice. The resuspended pellet volume was increased to 180 ml with HEPES buffer. The suspension was shaken on ice for 30 min and then centrifuged at $184,000 \times g$ for 2.5 h at 4°C. The pellet was discarded. The total protein in the supernatant fluid was 70 mg as determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Saturated ammonium sulfate (final concentration, 60% of saturation) was added to the supernatant fluid, and the suspension was held overnight at 4°C. The proteins (50 mg) were pelleted by centrifugation at $4,500 \times g$ for 20 min at 4°C and redissolved in 15 ml of 20 mM HEPES buffer (pH 7.5). This

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preparation, which was highly enriched for the *Legionella* protein antigen, was further purified by high-performance liquid chromatography (HPLC).

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by a discontinuous method with an 8% resolving gel (8), and proteins were stained with silver nitrate (4). Molecular weights were calculated by the method of Plikaytis et al. (7). Some HPLC fractions were screened for proteins with the Phastsystem (Pharmacia, Inc., Piscataway, N.J.) by using SDS-polyacrylamide gels with a 10 to 15% gradient and the separation method and silver staining conditions suggested by the manufacturer.

Immunoblot analysis. Immunoblot analysis was carried out as described previously (10), with the following modifications. TSEA (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA) and TSEA-Tw (TSEA plus 0.3% Tween 20) buffer were used for washing, and overnight treatment with TSEA containing 20% fetal bovine serum was used for blocking nonspecific protein binding sites. Rabbit immune serum was diluted 1:500, and goat anti-rabbit immunoglobulin-horse-radish peroxidase conjugate (Sigma) was diluted 1:1,000 in TSEA-Tw. Standards were visualized by anti-standard rabbit sera as described by Carlone et al. (2). Antisera prepared with formalinized whole-cell vaccines of the type strains of 23 *Legionella* species (12) were from the Immunology Laboratory of the Centers for Disease Control.

Dot blot analysis. *L. pneumophila* serogroup 1 (strain Philadelphia 1) whole-cell lysate and purified proteins used in dot blot analysis were prepared as described by Sampson et al. (10), except that the dye in the sample buffer was omitted. Each antigen (2 μ l) was absorbed as a dot onto a pretreated 0.45- μ m-pore-size nitrocellulose sheet with an imprinted grid (Schleicher & Schuell, Inc., Keene, N.H.). The sheet was dried at 37°C for 15 min and then blocked at room temperature for 15 min with 2 ml of 20% fetal bovine serum in phosphate-buffered saline (PBS) (pH 7.2) containing 0.3% Tween 20 (PBS-Tw). The blocking buffer was removed by washing, and an acute- or convalescent-phase serum sample from a patient with culture-confirmed *L. pneumophila* serogroup 1 pneumonia (2 ml, 1:200 dilution in PBS-Tw) was added and incubated at room temperature for 2 h with shaking. The sheet was washed three times with 2 ml of PBS-Tw for 5 min and then treated with 2 ml (1:1,000 dilution) of rabbit anti-human immunoglobulin-horseradish peroxidase conjugate (Sigma) at room temperature for 2 h with shaking. The sheet was then washed two times with 2 ml of PBS-Tw and once with 2 ml of PBS, each time for 5 min. Dot blots were developed with a mixture of 0.05% 3,3'-diaminobenzidine (Sigma) and 0.1% hydrogen peroxide in PBS. The substrates were removed by washing, and the nitrocellulose sheet was air dried.

Isoelectric focusing. Isoelectric focusing was performed with an LKB 2117 Multiphor II electrophoresis unit (LKB Instruments, Inc., Bromma, Sweden) by the method described in the LKB 1804 instruction booklet for Ampholine PAGplates. LKB Ampholine PAGplates (pH 3.5 to 9.5) were prefocused at 1,000 V for 30 min at 4°C, and then 15 μ l (2 μ g) of each sample was loaded and focused at 1,000 V for 45 min. The gel was fixed in a solution of methanol, trichloroacetic acid, and sulfosalicylic acid (30%:10%:3.5%) for 1 h, washed four times for 30 min each with a solution of methanol and trichloroacetic acid (30%:12%), and then stained with silver nitrate (4). The isoelectric point (pI) of the protein was determined with a pI calibration kit (Pharmacia).

HPLC. Purification procedures were carried out on ana-

lytical columns by using the Beckman model 344 gradient HPLC system equipped with a model 165 variable wavelength detector (Beckman, Berkeley, Calif.). Protein elution profiles were monitored at 280 nm. Proteins were first separated isocratically by size-exclusion chromatography on a Superose 6 HR 10/30 column (Pharmacia) as follows. Aliquots (0.25 ml) of the enriched protein preparation were injected into the column and eluted with 20 mM HEPES buffer (pH 7.5) containing 150 mM NaCl at a flow rate of 0.4 ml/min. Selected peak fractions were collected and analyzed by SDS-PAGE to detect the *Legionella* protein antigen. Once it was detected, the fractions were pooled, and the protein antigen was finally purified on a Mono Q HR 5/5 ion-exchange column (Pharmacia) as follows. Aliquots (0.5 ml) of the pooled fractions were loaded onto the column equilibrated with 20 mM HEPES buffer (pH 7.5) (buffer A) and eluted at a flow rate of 0.5 ml/min, first with a linear gradient from 0 to 25% buffer B (buffer A plus 1 M NaCl) in 1 min. They were then eluted isocratically with 25% buffer B for 8 min and with 38% buffer B for 9 min, followed by a linear gradient from 38 to 100% buffer B for 3 min, and finally, isocratically with 100% buffer B for 9 min. Peak fractions were collected and again analyzed for the *Legionella* protein antigen by SDS-PAGE. Peak fractions containing the protein antigen of interest were pooled and were used immediately or stored at 4°C.

RESULTS AND DISCUSSION

We have described a procedure for initial enrichment and subsequent purification by HPLC of a 60-kDa protein antigen from *L. pneumophila* serogroup 1 (strain Philadelphia 1) and, after partial characterization, showed that this protein has genuswide seroreactivity.

The purification procedure is rapid and nondenaturing and does not require detergent for protein solubility. A total of 2.5 mg of pure protein antigen was obtained from approximately 9 g (wet weight) of cells and represented approximately 5% of the total protein (50 mg) precipitated with ammonium sulfate from the antigen-enriched supernatant fluid. Three separate protein extracts with similar protein recovery were prepared and analyzed. Protein purity was ensured by rechromatography, SDS-PAGE, and isoelectric focusing.

An enrichment procedure was used after preliminary experiments showed that at pH 7.2 in 10 mM HEPES buffer, most of the *Legionella* protein antigen was in the pellet (Fig. 1A). An incremental increase of buffer ionic strength with the addition of up to 0.5 M KCl (which increases hydrophobic interactions) had no effect on the protein antigen sedimentation (data not shown). When higher buffer pH values (7.4 to 7.8) were used, progressively less protein antigen sedimented in the pellet. When the pellet was resuspended in 10 mM HEPES buffer (pH 7.2) and recentrifuged, however, most of the protein antigen was detected in the supernatant fluid (Fig. 1B). The protein antigen could be selectively sedimented by changing conditions of pH and ionic strength. The soluble protein antigen was concentrated by ammonium sulfate precipitation; all detectable protein antigen was precipitated at 60% saturation. The pellet was resuspended in 20 mM HEPES buffer (pH 7.5), and the *Legionella* protein antigen was purified by HPLC.

Most of the protein antigen eluted from the size-exclusion column at 31.6 min, as confirmed by SDS-PAGE (Fig. 2). The eluate containing the antigen was subjected to ion-exchange chromatography. A step gradient was used to

FIG. 1. SDS-PAGE and silver staining of *L. pneumophila* serogroup 1 (strain Philadelphia 1) whole-cell proteins during the enrichment procedure. Shown are protein profiles of the supernatant fluid (lane 1) and pellet (lane 2) after the first (A) and second (B) centrifugation at $184,000 \times g$ for 2.5 h in 10 mM HEPES buffer (pH 7.2). Molecular weight standards (in thousands) appear on the left. Arrows indicate the 60-kDa protein antigen.

completely resolve the antigen from coeluting proteins in size-exclusion chromatography. The protein was eluted from the column at 16.3 min with 0.38 M NaCl in 20 mM HEPES buffer (pH 7.5) (38% buffer B) and represented approximately 80% of the total integrated area under the curve for the eluted proteins (Fig. 3).

The purified protein antigen (0.19 mg/ml) was then treated with urea (final concentration of 0, 1, 3, 4, 5, and 10 M) for 15 min on ice and then rechromatographed on the same size-exclusion column previously used. With 0 to 3 M urea,

FIG. 2. Size-exclusion chromatography elution profile of enriched protein extract (after ammonium sulfate precipitation) and silver-stained SDS-PAGE profiles of corresponding peak fractions (inset, lanes 1 to 7). Chromatographic conditions are described in the text. Most of the protein antigen eluted at 31.6 min (peak 2), with an estimated molecular weight of 650,000. This protein had a molecular weight of 60,000 after SDS-PAGE (lane 2); arrow indicates 60-kDa protein antigen. Molecular weight standards (in thousands) appear on the left.

FIG. 3. Ion-exchange chromatographic elution profile of size-exclusion peak fraction 2 (Fig. 2) containing the *Legionella* protein antigen. Chromatographic conditions are described in the text. The protein eluted at 16.3 min with 20 mM HEPES (pH 7.5) containing 0.38 M NaCl (38% buffer B) by using a step gradient as shown. After SDS-PAGE and silver staining, the protein antigen had an estimated molecular weight of 60,000 (lane 2) as indicated by the arrow. Molecular weight standards (in thousands) appear in lane 1.

a single peak at 31.6 min was obtained that corresponded to a molecular weight of 650,000 (Fig. 4); with 4 M urea, a second peak of molecular weight 63,000 appeared at 40.6 min; with 5 and 10 M urea, only the peak at 40.6 min remained. In addition, prolonged incubation (ca. 3 h) with 4

FIG. 4. Size-exclusion rechromatography of the purified protein antigen with and without treatment with urea. Chromatographic conditions were the same as for Fig. 2. A 650-kDa protein eluted at 31.6 min with 0, 1, and 3 M urea; a 650- and a 63-kDa protein eluted at 31.6 and 40.6 min, respectively, with 4 M urea; and a 63-kDa protein alone eluted at 40.6 min with 5 and 10 M urea. The total integrated area under the curves for each chromatogram was essentially the same.

M urea resulted in a single peak eluting at 40.6 min (data not shown). The total integrated area under the curves was essentially the same with or without urea treatment. The urea (5 M)-treated and -untreated purified protein maintained immunoreactivity when stored at -20°C , whereas reduced reactivity was observed with the urea-treated protein upon prolonged storage at 4°C .

Both the 650- and 63-kDa protein antigen resolved as a single band of molecular weight 60,000 on SDS-PAGE. This 60-kDa protein and the 58-kDa antigen described by Sampson et al. (10) are the same. Sampson et al. (10) calculated molecular weights with prestained standards, a procedure known to give molecular weight estimations slightly different from those of analyses with unstained standards (11), which were used in this study.

These results suggest that the 650-kDa protein is an aggregate of 60-kDa subunits. To investigate this possibility, we incubated the protein with 50 mM dithiothreitol (Sigma) for 45 min at 37°C to reduce potential subunit disulfide linkages. This treatment had no effect on protein elution time on rechromatography. Thus, the 650-kDa protein antigen may be found as an aggregate of 10 to 11 noncovalently bonded subunits. Note that the protein markers used to calibrate the size-exclusion chromatographic column for molecular weight estimation are globular, however, and the shape of the purified protein, either before or after urea treatment, is unknown. Therefore, estimates of both the 650-kDa molecular mass and the 10- to 11-subunit aggregate rely on the assumption that the protein antigen is globular. It is also possible that aggregation is an artifact of purification and that, therefore, it does not resemble the native structure.

The purified 650-kDa protein antigen was further characterized physiochemically. This antigen was not contaminated with lipopolysaccharide or lipid, as shown by negative tests for 3-deoxy-D-mannooctulosonic acid (1) and fatty acids (5), respectively. The pI of the 650-kDa protein could not be determined by isoelectric focusing, since the estimated molecular weight of the protein aggregate exceeds the exclusion limit of the 5% polyacrylamide gel. After silver staining, relatively little of the 650-kDa protein was observed in the gel or at the origin of application. A single, well-resolved band (pI = 5.8), however, was observed for the urea-treated protein antigen (Fig. 5), indicating that the aggregate subunits (63 kDa by size-exclusion chromatography) could enter the gel.

Genuswide seroreactivity of the purified *L. pneumophila* serogroup 1 (strain Philadelphia 1) 60-kDa protein was shown by immunoblot analysis with rabbit immune sera prepared against whole cells of each of the 23 type strains of *Legionella* (Fig. 6). Since all 23 immune sera reacted with the purified 60-kDa protein at a serum dilution of 1:500, we did not attempt to optimize the staining intensity. Prebleed sera from hyperimmunized rabbits were nonreactive with the 60-kDa protein antigen, implying that preexisting antibodies to *Legionella* spp. were not present or detectable in these rabbits by immunoblot analysis. Although the 60-kDa protein is neither the major nor the immunodominant protein found in *Legionella* species, detectable antibody to this protein is apparently produced by hyperimmunization with all species of the genus *Legionella*.

Seroreactivity with convalescent-phase serum from a patient with culture-confirmed *Legionella* pneumonia was observed by dot blot analysis of the urea-treated (5 M) and non-urea-treated purified 60-kDa protein antigen (Fig. 7). In contrast to results obtained with the acute-phase serum sample from this patient, both protein preparations showed

FIG. 5. Isoelectric focusing and silver staining of urea-treated and size-exclusion rechromatographed 60-kDa protein antigen. Protein standards are in lane 1; the protein antigen is in lane 2.

strong reactivity with the convalescent-phase serum sample. These results agree with those of Sampson et al. (10), who showed, with whole-cell lysates, that antibodies to the 60-kDa protein are produced during *Legionella* infection. Further studies showed that the purified 60-kDa protein

FIG. 6. Immunoblot analysis showing genuswide seroreactivity of *L. pneumophila* serogroup 1 (strain Philadelphia 1) purified 60-kDa protein antigen (lanes 1 to 25) with rabbit immune sera prepared against the type strain of each of 23 *Legionella* species (lanes 3 to 25). Nonreactive buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA [pH 7.5]) is shown in lane 1, and a representative prebleed serum control is shown in lane 2. Rabbit immune sera used to probe for the 60-kDa protein antigen were prepared against whole cells of the following strains: *L. pneumophila* serogroup 1 (lane 3); *L. bozemanii* serogroup 1 (lane 4); *L. dumoffii* (lane 5); *L. gormanii* (lane 6); *L. micdadei* (lane 7); *L. longbeachae* serogroup 1 (lane 8); *L. jordanis* (lane 9); *L. oakridgensis* (lane 10); *L. feeleeii* serogroup 1 (lane 11); *L. wadsworthii* (lane 12); *L. anisa* (lane 13); *L. cherrii* (lane 14); *L. spiritensis* (lane 15); *L. saintelensi* (lane 16); *L. hackeliae* serogroup 1 (lane 17); *L. maceachernii* (lane 18); *L. jamestowniensis* (lane 19); *L. santacrucis* (lane 20); *L. steigerwaltii* (lane 21); *L. parisiensis* (lane 22); *L. rubrilucens* (lane 23); *L. erythra* (lane 24); and *L. israelensis* (lane 25). The molecular weights (MW; in thousands) of protein standards are indicated on the left.

FIG. 7. Dot blot analysis showing seroreactivity of a whole-cell lysate of *L. pneumophila* serogroup 1 (strain Philadelphia 1) (blot 1) and a non-urea-treated (blot 2) and 5 M urea-treated purified 60-kDa protein (blot 3) with an acute-phase (A) and convalescent-phase (B) human serum sample from a patient with culture-confirmed *L. pneumophila* serogroup 1 pneumonia. Total proteins used per dot are 0.8, 0.2, and 0.1 µg for whole-cell lysate, non-urea-treated, and urea-treated purified 60-kDa protein, respectively.

contains both *Legionella*-specific epitopes and epitopes common to a wide range of bacteria (6) and that the *Legionella*-specific antibodies to the 60-kDa protein can be prepared. Therefore, the 60-kDa protein may be useful in developing a diagnostic test for legionellosis that is not dependent on serogroup- or species-specific antigens.

The 60-kDa protein is a well-conserved antigen of unknown function and an as yet undefined cellular location. Further studies are in progress to structurally characterize the 60-kDa protein, define its cellular location, and determine the amino acid composition and sequence.

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