JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1987, p. 2080–2084
0095-1137/87/112080-05$02.00/0

Vol. 25, No. 11

Purified 60-Kilodalton *Legionella* Protein Antigen with *Legionella*-Specific and Nonspecific Epitopes

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Received 8 May 1987/Accepted 22 July 1987

In a previous study, all convalescent-phase sera from patients with culture-confirmed legionellosis reacted on immunoblots with a *Legionella* genus-wide 58-kilodalton (kDa) protein antigen (J. S. Sampson, B. B. Plikaytis, and H. W. Wilkinson, J. Clin. Microbiol. 23:92–99, 1986). The present study was done to immunologically characterize and determine the diagnostic relevance of this purified antigen. The antigen was precipitated from enriched cell extracts with ammonium sulfate and purified by high-pressure liquid chromatography. High-titered rabbit antiserum produced to the purified protein was used to show its presence on immunoblots in the 60-kDa range in 38 *Legionella* serogroups, representing 23 species, and in 39 non-*Legionella* bacteria. The antiserum was made specific for *Legionella* strains by sequential absorptions with *Bordetella pertussis*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* whole cells. Serum from legionellosis patients reacted with both specific and nonspecific epitopes. Results of indirect immunofluorescence experiments showed that neither specific nor nonspecific epitopes of the 60-kDa protein were surface exposed on *Legionella* cells and that cross-reactive epitopes were variably exposed on non-*Legionella* bacteria. The 60-kDa protein antigen should be useful in diagnostic tests for legionellosis if care is taken to expose cryptic epitopes and if the tests use or measure only the *Legionella*-specific epitopes.

Sampson et al. showed previously that all convalescent-phase sera from 15 patients with culture-proven *Legionella* pneumonia reacted with a 58-kilodalton (kDa) protein antigen that was present in *Legionella* strains representing seven species and 14 serogroups (9). This genuswide protein was the most reactive *Legionella* antigen detected on immunoblot analyses. In contrast, only 6 of the 15 convalescent-phase serum samples reacted with a 25-kDa antigen found only in *Legionella pneumophila* strains. If shown to be specific, the 58-kDa protein appears to be a prime candidate for developing diagnostic tests. In addition to its apparent sensitivity in detecting antibody responses to infection, the antigen could also be used to simplify serodiagnostic tests by replacing individual serogroup-specific antigens with a single genus-specific antigen. Furthermore, antisera to the genuswide antigen could be used to develop tests for the presence of *Legionella* cells and soluble antigen in clinical specimens and, perhaps, environmental specimens. Developing and performing tests with or to detect all 23 *Legionella* species and 38 serogroups have become impractical for most laboratories.

The purpose of this study was to determine the diagnostic relevance of the *Legionella* genuswide protein antigen, which was first purified as a 60-kDa protein and then used to produce antiserum. Specificity studies were carried out with a wide variety of *Legionella* and non-*Legionella* bacterial strains.

MATERIALS AND METHODS

Strains and human sera. Strains used in this study included the reference strains of 38 *Legionella* serogroups, divided among 23 species, and 39 gram-negative bacterial strains other than *Legionella*. The *Legionella* species included *L. anisa*, *L. bozemanii* (2 serogroups), *L. cherrii*, *L. dumoffii*, *L. erythra*, *L. feeleii* (2 serogroups), *L. gormanii*, *L. hackeliae* (2 serogroups), *L. israelensis*, *L. jamestowniensis*, *L. jordanis*, *L. longbeachae* (2 serogroups), *L. maceachernii*, *L. micdadei*, *L. oakridgensis*, *L. parisiensis*, *L. pneumophila* (12 serogroups), *L. rubrilucens*, *L. sainthelensi*, *L. santicrucis*, *L. spiritensis*, *L. steigerwaltii*, and *L. wadsworthii*. The non-*Legionella* bacteria included *Acinetobacter lwoffi*, *Alcaligenes faecalis*, *Bordetella bronchiseptica* (two strains), *Bordetella pertussis* (two strains), *Enterobacter aerogenes*, *Escherichia coli* (two strains), *Escherichia fergusonii*, *Escherichia hermannii*, *Haemophilus influenzae* (six strains; serotypes a, b, c, d, e, and f), *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kluyvera ascorbata*, *Neisseria meningitidis* (two strains; serogroups B and C), *Providencia rettgeri*, *Providencia stuartii*, *Pseudomonas acidovorans*, *Pseudomonas aeruginosa* (three strains), *Pseudomonas cepacia*, *Pseudomonas diminuta*, *Pseudomonas fluorescens*, *Pseudomonas maltophilia*, *Pseudomonas paucimobilis*, *Pseudomonas testosteroni*, *Serratia marcescens*, *Shigella flexneri*, *Shigella sonnei*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. The *Legionella* and non-*Legionella* strains were from the stock culture collection of the Immunology Laboratory, Centers for Disease Control. Human sera were from patients with culture-confirmed legionellosis.

Antigen purification. Purification of the protein antigen is described completely in a separate publication (C. P. Pau, B. B. Plikaytis, G. M. Carlone, and I. M. Warner, submitted for publication). Briefly, *L. pneumophila* serogroup 1 (strain Philadelphia 1) cells were grown on BCYE (buffered charcoal-yeast extract agar) plates (Carr-Scarborough Microbiological Inc., Decatur, Ga.) at 37°C for 48 h. The growth was harvested and washed in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.2, and then the cells were disrupted with a French pressure cell. Cell debris was removed by centrifugation, and the supernatant fluid

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was recovered and centrifuged at 4°C for 2.5 h at 184,000 × *g*. The pellet was suspended in 10 mM HEPES, pH 7.2, with a hand-held homogenizer and centrifuged as before at 184,000 × *g*. The protein in the supernatant fluid was then precipitated with ammonium sulfate at 60% saturation. This enriched extract was further purified by high-pressure liquid chromatography on both size exclusion (Superose 6) and ion-exchange (Mono Q) columns (Pharmacia Inc., Piscataway, N.J.). The purified protein was stored at 4°C.

Antiserum production and absorption. Antiserum was produced by injecting young, adult New Zealand White rabbits with 0.5 ml of purified protein (0.15 mg of protein per ml of 20 mM HEPES, pH 7.5, in 0.38 M NaCl) mixed with an equal volume of Freund incomplete adjuvant. The protein was administered intracutaneously in 10 to 12 sites along the back of each rabbit. This procedure was repeated twice at 2-week intervals. Rabbits were bled 6 weeks after the first injection and once a week for 2 consecutive weeks afterwards. Preimmune sera were nonreactive, and immune sera reacted identically on Western immunoblots with representative *Legionella* and non-*Legionella* bacteria. Therefore, a single antiserum lot produced with the untreated purified protein was selected for subsequent experiments.

A portion of the antiserum was absorbed sequentially with each of three bacteria (indicated under Results) as follows. Cells were harvested in 2 ml of 0.01 M phosphate-buffered saline, pH 7.2 (PBS), per BCYE plate. The suspension was centrifuged, and after the supernatant fluid was decanted, the packed cells were mixed with a 1:10 dilution of antiserum in PBS at a ratio of 1:10 (cells to serum). The mixture was tumbled for 3 h at 4°C, the cells were packed by centrifugation in a microfuge (Brinkman Instruments, Inc., Westbury, N.Y.) at 4°C at 16,000 × *g* for 5 min, and the serum was transferred to a second tube of packed cells, which was mixed and tumbled as before. The same procedure was repeated for a third absorption with cells of the same strain, and then three absorptions each were carried out with the two remaining strains. After absorption with the last organism, the serum was separated from the cells by centrifugation and stored at 4°C.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by a discontinuous method, as described previously (8), with an 8% resolving gel, 0.75 mm thick, and 20 wells per gel. Protein concentrations of the samples were determined by the method of Markwell et al. (6), with bovine serum albumin as the standard. Wells were loaded with 2.4 or 0.6 μg of protein for whole-cell samples or purified protein samples, respectively. After completion of PAGE, gels were transferred to nitrocellulose for immunoblot analysis.

Immunoblots were done as described previously (9) with the following modifications. The overnight blocking buffer, TSEA, contained 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 20% fetal bovine serum. TSEA containing 0.3% Tween 20 was used for washing and diluting sera and conjugate. Human sera were diluted 1:500, unabsorbed rabbit serum was diluted 1:32,000, and absorbed rabbit serum (1:10 in PBS) was diluted 1:200. Anti-human polyvalent and anti-rabbit horseradish peroxidase conjugates (Sigma Chemical Co., St. Louis, Mo.) were diluted 1:1,000. Immunoblots were developed with 0.05% diaminobenzidine in TSEA with 0.1% H_2O_2.

Low- and high-molecular-weight protein standards (Bio-Rad Laboratories, Richmond, Calif.) were used in PAGE and immunoblotting experiments. Protein standards were visualized on the nitrocellulose with rabbit antisera to the protein standards (1). Molecular weights were calculated as described by Plikaytis et al. (7).

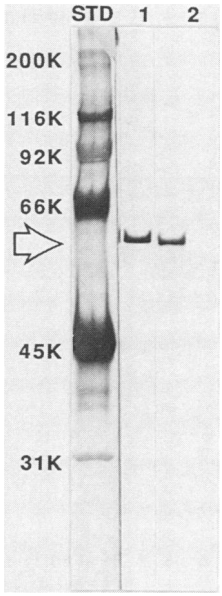

FIG. 1. Comparison of the immunologic reactivity of the untreated and urea-dissociated subunit of the 650-kDa purified *Legionella* protein. Lanes: STD, protein standards (in kilodaltons); 1, untreated purified protein; 2, urea-treated purified protein. Antigens were probed on immunoblots with convalescent-phase serum from a patient with culture-confirmed *L. pneumophila* serogroup 1 pneumonia. Both untreated and treated proteins resolved into single 60-kDa bands (arrow), and both showed serologic reactivity with the serum.

Indirect immunofluorescence assay. Antigen suspensions were prepared and fixed as described previously for direct immunofluorescence assays (13). The primary antibody was rabbit antiserum to the purified protein, diluted 1:100 in PBS for unabsorbed serum or 1:50 in PBS for the absorbed serum. The secondary antibody was anti-rabbit fluorescein isothiocyanate-labeled conjugate (Sigma), which was diluted 1:250 in PBS.

RESULTS

On SDS-PAGE after silver staining, the purified protein resolved into a single band of 60 kDa (not shown). However, the molecular size calculated after size-exclusion chromatography was 650 kDa. To resolve this discrepancy, from 1 to 10 M urea (final concentration) was added to the protein. Urea at concentrations greater than or equal to 5 M disassociated the 650-kDa aggregate into 63-kDa subunits (as calculated by size-exclusion chromatography), a finding similar to the SDS-PAGE results. Both the untreated and urea-treated purified proteins were immunologically reactive on immunoblots with human serum from a patient with culture-confirmed legionellosis (Fig. 1). Therefore, 5 M urea treatment dissociated the 650-kDa protein into immunologically reactive subunits which were detected as 60-kDa bands on immunoblots. For simplicity, the antigen will be referred to as the 60-kDa protein in subsequent experiments.

To determine whether the protein was specific for *Legionella*, rabbit antiserum prepared to the purified 60-kDa antigen was allowed to react on immunoblots with 38 *Legionella* serogroups, representing 23 species, and with 39 non-

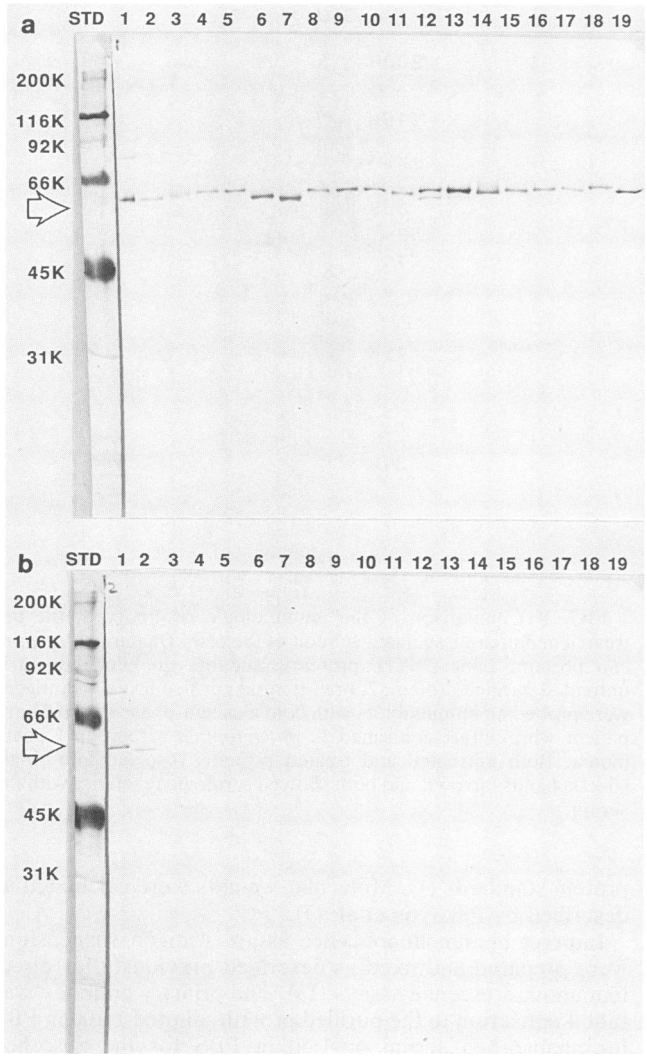

FIG. 2. Presence of protein antigen in the 60-kDa range among non-*Legionella* bacteria as shown by immunoblot analysis. Blots were probed with unabsorbed rabbit anti-60-kDa protein serum (a) or the same serum absorbed with three non-*Legionella* bacterial strains (b). Lanes: STD, protein standards (in kilodaltons); 1 and 2, positive control strains of *L. pneumophila* serogroup 1 and *L. longbeachae* serogroup 1, respectively; 3, *Escherichia hermanii*; 4, *Escherichia fergusonii*; 5, *Kluyvera ascorbata*; 6, *Yersinia enterocolitica*; 7, *Yersinia pseudotuberculosis*; 8, *Shigella flexneri*; 9, *Providencia rettgeri*; 10, *Providencia stuartii*; 11, *Shigella sonnei*; 12 and 13, *Pseudomonas aeruginosa*; 14, *Serratia marcescens*; 15, *Enterobacter aerogenes*; 16, *Klebsiella oxytoca*; 17, *Klebsiella pneumoniae*; 18, *Pseudomonas maltophilia*; 19, *Alcaligenes faecalis*. Unabsorbed antiserum revealed an antigen in the 60-kDa range (arrows) in all strains tested (a), whereas the absorbed antiserum reacted only with the two *Legionella* control strains (b).

Legionella gram-negative bacteria. Unabsorbed antiserum reacted with a protein in the 60-kDa range among all bacteria tested, including the non-*Legionella* strains. Immunoblots of 17 of the 38 non-*Legionella* strains tested are shown in Fig. 2a; two *Legionella* species were included as positive controls. Sequential absorptions of the antiserum with *Bordetella pertussis*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* removed all cross-reactivity with the non-*Legionella* bacteria (Fig. 2b). The absorbed antiserum retained its reactivity with 38 serogroups representing 23 species of *Legionella* (Fig. 3). Therefore, the mosaic of epitopes within the 60-kDa protein includes those common to all the gram-negative bacteria tested, in addition to one or more epitopes specific for the *Legionella* genus. Absorption of the rabbit antiserum with the purified 60-kDa protein blocked the reactivity of the serum on immunoblots with whole cells of *L. pneumophila* serogroup 1 and *P. aeruginosa*, showing that reactivity was the result of antigen-antibody complexes. Also shown by the rabbit antiserum probe (Fig. 2 and 3), but not by convalescent-phase human serum (Fig. 1) (9) was an 80-kDa *L. pneumophila*-specific antigen and several minor bands in some preparations. Whether these proteins also dissociated from the 650-kDa aggregate or were minor contaminants of the purified antigen

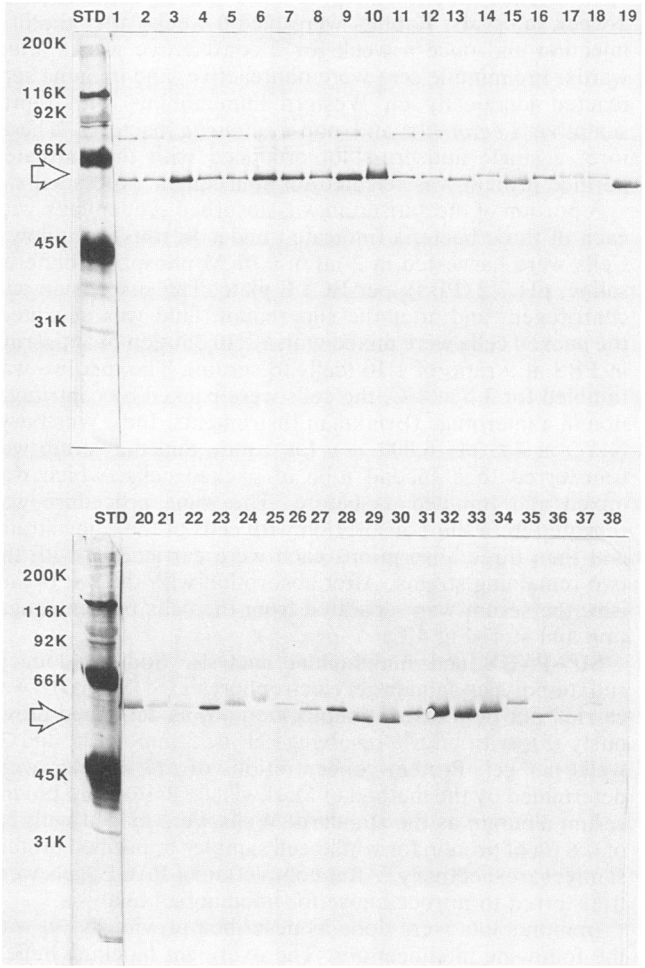

FIG. 3. Presence of *Legionella*-specific epitope(s) on the genuswide *Legionella* 60-kDa antigen among all published *Legionella* species and serogroups, as shown by immunoblot analysis with absorbed rabbit anti-60-kDa protein serum. Lanes: STD, protein standards (in kilodaltons); 1 to 12, strains representing *L. pneumophila* serogroups 1 to 12, respectively; 13, *L. dumoffii*; 14, *L. gormanii*; 15, *L. jamestowniensis*; 16, *L. cherrii*; 17, *L. rubrilucens*; 18, *L. erythra*; 19, *L. israelensis*; 20 and 21, *L. bozemanii* serogroups 1 and 2, respectively; 22 and 23, *L. longbeachae* serogroups 1 and 2, respectively; 24, *L. anisa*; 25, *L. maceachernii*; 26, *L. oakridgensis*; 27, *L. wadsworthii*; 28, *L. jordanis*; 29 and 30, *L. feeleii* serogroups 1 and 2, respectively; 31, *L. sainthelensi*; 32, *L. santicrucis*; 33, *L. steigerwaltii*; 34, *L. parisiensis*; 35, *L. spiritensis*; 36 and 37, *L. hackeliae* serogroups 1 and 2, respectively; 38, *L. micdadei*. Estimated molecular sizes ranged from 57 to 62 kDa.

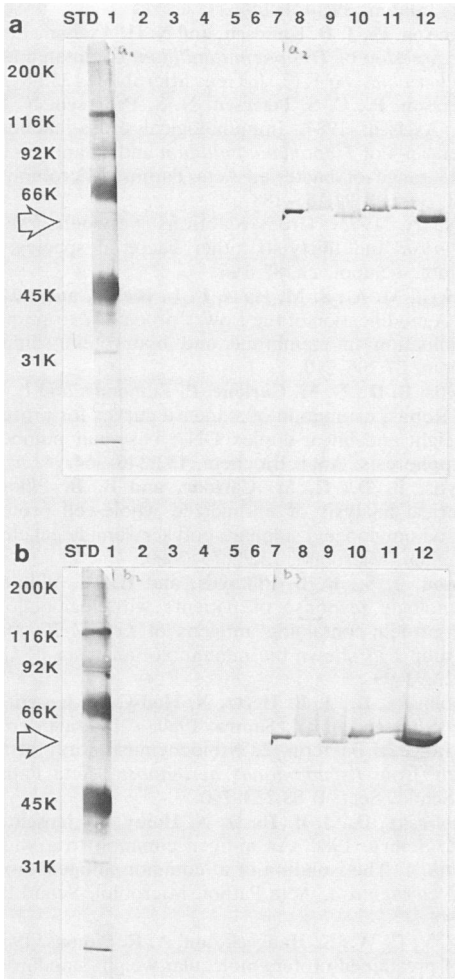

FIG. 4. Immunologic response of patients with legionellosis to gram-negative organism-wide epitopes of the 60-kDa protein as shown by immunoblot analysis. Blots were probed with acute-phase (lanes 1 to 6) or convalescent-phase (lanes 7 to 12) serum samples from patients with culture-confirmed *L. pneumophila* serogroup 1 (a) or *L. pneumophila* serogroup 6 (b) pneumonia. Lanes: STD, protein standards (in kilodaltons); 1 and 7, 60-kDa purified protein; 2 and 8, *L. pneumophila* serogroup 1 cells; 3 and 9, *L. dumoffii* cells; 4 and 10, *Pseudomonas aeruginosa* cells; 5 and 11, *Klebsiella pneumoniae* cells; 6 and 12, *Bordetella pertussis* cells. Both convalescent sera reacted with the purified 60-kDa protein and with the 60-kDa antigen in both *Legionella* and non-*Legionella* strains.

was not determined since they were detected only on immunoblots.

As shown above, unabsorbed rabbit antiserum reacted with a 60-kDa protein common to both *Legionella* and non-*Legionella* strains. To determine whether the human antibody response is also to gram-negative organism-wide epitopes on the protein, acute and convalescent sera from patients with culture-documented *L. pneumophila* serogroup 1 and serogroup 6 pneumonia were allowed to react on immunoblots with the purified 60-kDa protein and with strains representing two *Legionella* species and three non-*Legionella* bacteria. Both convalescent sera reacted with all the antigens, whereas their acute-phase sera were nonreactive (Fig. 4). This illustrates the fact that serologic tests must exclude cross-reactive epitopes of the 60-kDa protein to be specific for *Legionella*.

Diagnostic tests utilizing whole cells rely on the presence of exposed surface antigens. Whether the 60-kDa protein antigen is surface exposed was investigated by an indirect immunofluorescence assay with unabsorbed and absorbed rabbit antiserum to the 60-kDa protein. No fluorescence was observed on whole-cell antigens of any of the 23 *Legionella* species. Therefore, neither nonspecific nor specific epitopes of the 60-kDa protein are accessible to antibody on the cell surface.

DISCUSSION

In this study, we immunologically characterized a purified genuswide *Legionella* protein antigen shown previously to be the most reactive *Legionella* protein with sera from 15 patients with legionellosis (9). The antigen was extracted as an aggregated protein of 650 kDa that subsequently dissociated to an immunoreactive 63-kDa (calculated by size-exclusion chromatography) antigen when treated with 5 M urea. Both the 650-kDa aggregate and the 63-kDa subunit resolved into a 60-kDa band on SDS-PAGE. The 60-kDa protein corresponds to the 58-kDa protein that we described previously from experiments with prestained protein standards (9). Migration distances of prestained standards are known to vary because of the attached dye (12).

In addition to being immunoreactive, the 60-kDa urea-treated or untreated purified protein was immunogenic, as shown by its ability to elicit high-titered rabbit antiserum (1:32,000). On immunoblots this antiserum showed that the protein was present in all published *Legionella* species and serogroups, with estimated sizes of 57 to 62 kDa. A protein in the 60-kDa range with shared epitopes was also found in 39 other gram-negative bacteria; however, antisera absorbed sequentially with three non-*Legionella* strains reacted only with *Legionella* strains, indicating that the 60-kDa protein contains *Legionella*-specific epitopes. Absorption studies also suggested that the cross-reactive epitopes are not always surface exposed. Absorption of 60-kDa rabbit antiserum with *Acinetobacter lwoffi*, *Escherichia coli*, and *Yersinia enterocolitica* failed to remove the cross-reactivity (data not shown), whereas absorption with *Bordetella pertussis*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* did remove all cross-reactions with the non-*Legionella* bacteria. However, all three strains were required for complete absorption. When only one or two of the strains were used, various patterns of immunoreactivity were observed. Each strain removed all reactivity with itself and only partial reactivity with the other strains examined. These observations suggest that there are multiple cross-reactive epitopes shared variably by gram-negative bacteria or that there are differences in surface exposure among various strains and therefore differing accessibility of antigen to antibody. Neither cross-reactive nor specific epitopes were exposed on the cell surface of *Legionella* species, as shown by the failure of 60-kDa antiserum to bind to the cells in indirect immunofluorescence assays.

The 60-kDa cross-reactive *Legionella* antigen described above may well be the same or similar to the common antigen (CA) described previously for a variety of gram-negative bacteria. Hoiby et al. found a CA in *Pseudomonas aeruginosa* that cross-reacted with 36 other bacterial species (5). Sompolinsky et al. purified this *Pseudomonas aeruginosa* CA which, by gel filtration, was a 665-kDa aggregate composed of 62-kDa polypeptide subunits (10, 11). Hindersson et al. showed that *Treponema pallidum* and *Treponema phagedenis* contain a 60-kDa CA with *Treponema*-specific

epitopes and expanded the list of bacteria with a CA to 56 (3, 4). Collins et al. described immunoelectrophoretic studies in which a *Legionella* antigen similar to the CA of *Pseudomonas aeruginosa* cross-reacted with 18 non-*Legionella* bacteria (2). Our results expand these findings and show that the *Legionella* CA also contains genus-specific epitopes.

These studies indicate that the *Legionella* genuswide 60-kDa protein could be used to develop diagnostic tests if the tests do not depend on surface exposure of the antigen or if the *Legionella*-specific cryptic epitopes could be exposed. Preliminary studies showed that whole cells solubilized by heating in SDS-PAGE sample buffer could be dot-blotted onto a nitrocellulose membrane and probed for the 60-kDa protein with absorbed antiserum. This could be the basis for rapid detection of *Legionella* in cultures. Another application would be to use absorbed antiserum or a *Legionella* epitope-specific monoclonal antibody as the capture antibody in a test designed to detect soluble antigen in body fluids of patients with legionellosis if they excrete the immunoreactive 60-kDa protein. A third application is to use *Legionella*-specific peptides in tests of antibody responses to infection. In this regard, we are initiating studies to map peptide epitopes of the 60-kDa protein antigen and then to synthesize immunogenic *Legionella* peptides. In all applications, the test must be carefully evaluated to ensure that only *Legionella*-specific antigens or antibodies to *Legionella*-specific epitopes are detected.

ACKNOWLEDGMENTS

We thank Ray Simons for photography and Joan H. Nagel for secretarial assistance.

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