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Identification of a Species-Specific Antigen in *Legionella pneumophila* by a Monoclonal Antibody

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A species-specific antigen in *Legionella pneumophila* was identified by a monoclonal antibody in enzyme-linked immunosorbent and immunofluorescence assays of serogroups 1 through 8. The species-specific antigen was heat stable, and the molecular weight of the major band was 29,000 by immunoblot analysis. In direct immunofluorescence assays, the antigen was cryptic or only partly exposed on the surface of the cells but was effectively exposed by treating the cells with detergent and EDTA. The monoclonal antibody was utilized in direct immunofluorescence assays to specifically identify multiple cultures of *L. pneumophila* serogroups.

Legionnaires disease, first characterized in 1977, is of clinical interest as an acute human illness with a high mortality rate (5, 13, 24). In most patients, the infection is manifested as pneumonia; however, a second clinical syndrome, Pontiac fever, is a nonpneumonic, mild, and self-limited respiratory illness (14, 20). Although many species of the genus *Legionella* can cause legionellosis, the etiological agent of Legionnaires disease is *Legionella pneumophila*, a gram-negative bacterium which consists of at least eight serogroups (2, 3, 10, 25, 26). Laboratory diagnosis of the disease is presently demonstrated by a fourfold increase in serum indirect fluorescent antibody titer, isolation and culture of *Legionella* spp. from clinical specimens, or direct fluorescent antibody examination of clinical specimens for the presence of organisms.

The direct fluorescent antibody test is the most rapid means of diagnosis of Legionnaires disease in laboratories, but there are several associated problems. Multiple antibody reagents are required to identify all presently recognized serogroups of *L. pneumophila*. In addition, cross-reactions have been reported with strains of *Pseudomonas fluorescens*, *Pseudomonas alcaligenes*, *Bacteroides fragilis*, and the *Flavobacterium-Xanthomonas* group when conjugates of polyclonal antibodies are used for diagnosis (8, 27).

Serological techniques utilizing polyclonal antisera have been used to identify serogroup-common antigens of *L. pneumophila* (7, 19). In addition, monoclonal antibodies directed against *L. pneumophila* antigens have been previously described (17, 18, 28). Most of the monoclonal antibodies reported have been serogroup specific. Optimally, the most useful diagnostic antibody would be one that reacts with a common antigen present on all isolates of *L. pneumophila* but is absent from closely related bacteria. In this report, we describe and characterize a species-specific antigen which is detected by a monoclonal antibody. We have utilized the antibody to detect the bacterium in immunofluorescence assays on clinical isolates of *L. pneumophila* grown in culture.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were received from the American Type Culture Collection, Rockville, Md., Centers for Disease Control, Atlanta, Ga., or Harborview Medical Center, Seattle, Wash., and are listed in Table 1. *Legionella* microorganisms were grown on charcoal-yeast extract agar for 2 to 7 days or, in some cases, in defined medium broth (12, 29). Harvested cells used as immunogens were either fixed in 0.5% Formalin or sonicated on ice at 175 W with a Braun-sonic 1510 (B. Braun, Melsungen, Federal Republic of Germany) for a total of 7.5 min.

Production of monoclonal antibodies. Hybrid cell lines producing monoclonal antibodies were prepared by the method of Kohler and Milstein (21), with previously described modifications (16, 31). Spleen cells for the fusion were obtained from a BALB/c mouse that was immunized with *L. pneumophila* serogroups 1 through 6. Methods for propagation and stabilization of cloned cell lines and for ascites production have been previously described (16, 31).

Antibody assays. Anti-*L. pneumophila* antibodies were detected by enzyme-linked immunosorbent assay (ELISA) on 96-well plates (Linbro Tissue Culture Multi-Well Plate; Flow Laboratories, McLean, Va.) coated with individual serogroups (1 through 6) of *L. pneumophila* or *Pseudomonas aeruginosa* (used as a negative control). Bacterial suspensions were added to the plates and centrifuged at 740 × *g*. Supernatants were aspirated, and the bacteria were fixed to plates by the addition of 95% ethanol. The plates were then blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS pH 7.2) for 1 h at 37°C, washed with 0.05% Tween 20 in PBS (PBS-Tween 20); and then with deionized water, and stored dry at 4°C until use. Plates were incubated with culture fluids (50 μl per well) at 37°C for 45 min and then washed three times in PBS-Tween 20. Protein A-peroxidase (1:3,000 dilution in PBS-Tween 20; Zymed Laboratories, Inc., South San Francisco, Calif.) was added (50 μl per well), and the plates were incubated for 30 min at room temperature and washed as above. Substrate (0.05 M citric acid–0.1 M dibasic sodium phosphate buffer [pH 5.0] containing 7 mg of *o*-phenylenediamine and 25 μl of 30% H_2O_2 per 50 ml) was added, and the plates were incubated for 30 min at room temperature in the dark. The reaction was stopped with 3 N H_2SO_4, and the colorimetric reactions were quantified with a Microelisa Auto Reader (MR580; Dynatech Laboratories, Inc., Alexandria, Va.).

For direct immunofluorescence assays, antibodies were purified from ascites fluid with protein A-Sepharose and conjugated to fluorescein-isothiocyanate by procedures previously described (11, 15). Antigen slides were prepared by

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TABLE 1. Reactions of microorganisms tested in direct immunofluorescence assays with monoclonal antibody LP3IIG2

Organism	No. positive/no. tested
L. pneumophila	
Serogroup 1	50/50
Serogroup 2	5/5
Serogroup 3	3/3
Serogroup 4	3/3
Serogroup 5	7/7
Serogroup 6	6/6
Serogroup 7	2/2
Serogroup 8	1/1
Serogroup 9	1/1
Legionella sp., non-pneumophila	
L. micdadei	0/6
L. oakridgensis	0/1
L. jordanis	0/1
L. longbeachae	0/6
L. sainthelensi	0/2
L. bozemanii	0/3
L. dumoffii	0/3
L. wadsworthii	0/1
L. gormanii	0/1
Streptococcus pneumoniae	0/15
S. pyogenes	0/15
S. viridans group sp.	0/21
Haemophilus influenzae	0/20
H. parainfluenzae	0/5
Neisseria sicca	0/1
N. mucosa	0/2
N. gonorrhoeae	0/3
Klebsiella pneumoniae	0/16
Staphylococcus aureus	2/5[a]
S. epidermidis	0/3
Flavobacterium sp.	0/2
Pseudomonas fluorescens	0/2
P. maltophilia	0/1
P. alcaligenes	0/1
P. aeruginosa	0/15
Bacteroides fragilis	0/2
B. melaninogenicus	0/1
Escherichia coli	0/15
Salmonella sp.	0/3
Enterobacter sp.	0/1
Serratia sp.	0/1
Proteus sp.	0/5
Lactobacillus sp.	0/13
Peptostreptococcus sp.	0/1

[a] Due to nonimmune binding to protein A.

adding 1% Formalin-killed organisms to 10-well microscope slides and allowing them to air dry on a 37°C slide warmer. The slides were immersed in 1% Triton X-100 in 0.15 M PBS (pH 8.5) with 100 mM EDTA solution for 20 min, washed in deionized water, and dried. Twenty-five microliters of antibody solution (25 μg of antibody per ml in 0.05% PBS-Tween 20 with 1 mg of bovine serum albumin per ml, 0.01% NaN_3, and Evans blue; optical density at 620 nm of 1.6) was added per well, and the slides were incubated for 30 min at 37°C. The slides were washed in 0.15 M PBS and deionized water. Cover slips were mounted with 9 parts glycerol to 1 part 0.1 M Tris (pH 8.5), and the slides were examined with an epiillumination fluorescence microscope and ×63 oil objective.

Biochemical procedures. Monoclonal antibodies were evaluated by immunoblot methods, with modifications to previously published procedures (1, 4, 22, 30, 32). Briefly, solubilized antigen preparations of *L. pneumophila* serogroups 1 through 8, *Legionella bozemanii*, *Legionella micdadei*, *Legionella dumoffii*, *Legionella jordanis*, *Legionella longbeachae* serogroups 1 and 2, and *P. fluorescens* were made by incubating concentrated live cultures in a solubilization buffer (0.3% sodium deoxycholate, 0.02 M 2-mercaptoethanol, 30% glycerol, 0.03 M Tris-hydrochloride [pH 7.6]) overnight at 4°C or by sonicating them in 2% (wt/vol) sodium deoxycholate. The antigen preparations were mixed with sample buffer (2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 10% glycerol in 0.06 M Tris-hydrochloride [pH 6.8], final concentrations), heated at 100°C for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis in 14% slab gels. Antigens in the gel were then transferred to nitrocellulose membranes (NCMs) by electrophoresis for 2 h at 27 V in 25 mM sodium phosphate buffer (pH 7.0). After transfer, the NCM was blocked in PBS-Tween 20 for 1 h at room temperature. The NCM was incubated with antibody diluted in PBS-Tween 20 for 1 h, washed with PBS-Tween 20, and then incubated with protein A-horseradish peroxidase (1:2,000 dilution in PBS-Tween 20) for 1 h at room temperature. The NCM was washed and then immersed in horseradish peroxidase color development solution (Bio-Rad Laboratories, Richmond, Calif.) for 20 min, and the reaction was stopped by immersion in deionized water. The molecular weights of the antigens were determined by including markers (prestained protein molecular weight standards for SDS-polyacrylamide gel electrophoresis; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in the SDS-polyacrylamide gel electrophoresis. The relative migrations versus the logarithms of the molecular weights of the standards were evaluated by linear regression to derive molecular weight values for *L. pneumophila* antigens.

Antigens were further evaluated by susceptibility to heat and degradation by proteinase K. One percent of Formalin-killed suspensions of *L. pneumophila* serogroup 1 (strain Philadelphia 1) and serogroup 4 (strain Los Angeles 1) were diluted in 0.015 M carbonate–0.035 M bicarbonate buffer (pH 9.6) to 45 μg of protein per ml. The cell suspensions were immersed in a boiling water bath for 10 min and then coated on 96-well plates overnight at 4°C. An ELISA was performed as described above. Solubilized antigen preparations of *L. pneumophila* serogroups 2 and 4 were diluted to 50 μg of protein per ml in the carbonate-bicarbonate buffer and divided into two samples. Proteinase K (Merck & Co., Inc., Rahway, N.J.) was added at the rate of 20 μg of proteinase K per 50 μg of bacterial protein. The solutions were incubated in a 37°C water bath, and samples were removed at 30, 60, 90, and 120 min. The samples were coated on 96-well plates overnight at 4°C, fixed with 95% ethanol, and blocked with 1% bovine serum albumin. The antigens were then assayed by ELISA.

RESULTS

Isolation of a hybrid cell line producing anti-*L. pneumophila* monoclonal antibody. Hybridizations were performed between NS-1 myeloma cells and the lymphocytes from a BALB/c mouse immunized with *L. pneumophila* serogroups

1 through 6. Culture fluids from hybrid cells were tested for anti-*L. pneumophila* antibodies by an ELISA utilizing replicate plating techniques with individual serogroup antigen preparations. From one fusion, 17 hybrids were produced, which secreted monoclonal antibodies reacting specifically with various serogroups of *L. pneumophila*. Sixteen of these antibodies were either specific for individual serogroups of *L. pneumophila* or demonstrated limited cross-reactivity among two or three serogroups (data not shown). However, one cell line produced a monoclonal antibody designated LP3IIG2 which was reactive with all eight serogroups of *L. pneumophila*. This hybrid cell line was subsequently cloned by limiting dilution until phenotypically stable.

Characterization of the serogroup common antigen. Monoclonal antibody LP3IIG2 was examined by immunoblot analysis on soluble antigen preparations of *L. pneumophila* serogroups 1 through 8, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, *L. jordanis*, *L. longbeachae* serogroups 1 and 2, and *P. fluorescens*. Antigens were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to NCM. The NCMs were incubated with normal mouse serum, *L. pneumophila*-immune mouse serum, or monoclonal antibody LP3IIG2, and antigen-antibody reactions were detected by protein A-horseradish peroxidase. The monoclonal antibody recognized a specific antigen consisting of a major band present in all eight serogroups of *L. pneumophila* (representative serogroups are shown in Fig. 1). This antigen was not detected in non-pneumophila *Legionella* spp. or *P. fluorescens* organisms. The molecular weight of the major band, estimated by comparison with the relative migration of protein standards, was 29,000. The monoclonal antibody also reacted with other minor bands, but these reactions varied in intensity, depending on the serogroup tested and the method of antigen extraction.

The serogroup-common antigen detected by LP3IIG2 was tested for degradation by proteinase K by incubation with the enzyme, coating on 96-well plates, and ELISA. There was a substantial decrease (0.45 to 0.03) in the optical density after incubation with the enzyme, which suggested that the antigen was a protein. The antigen was also evaluated for heat stability by boiling for 10 min in carbonate-bicarbonate buffer (pH 9.6). Treatment of serogroup 4 whole bacteria in this fashion before coating ELISA plates increased the signal by 2.7 to 3.0 times compared with the unheated control when reacted with LP3IIG2.

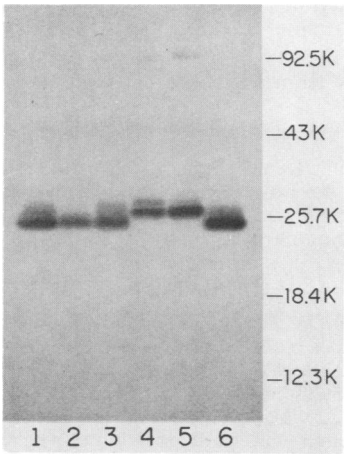

FIG. 1. Immunoblot of *L. pneumophila* serogroups 1 to 6 with monoclonal antibody LP3IIG2. Lanes: 1, serogroup 1 (Philadelphia-1 strain); 2, serogroup 2 (Togus-1 strain); 3, serogroup 3 (Bloomington-2 strain); 4, serogroup 4 (Los Angeles-1 strain); 5, serogroup 5 (Dallas-1E strain); and 6, serogroup 6 (Chicago-2 strain).

Immunofluorescence assays on culture isolates. The monoclonal antibody was conjugated to fluorescein and tested in direct immunofluorescence assays on bacteria grown in culture. In these assays, it was necessary to treat the bacteria with a detergent-EDTA solution to obtain bright surface staining of the bacterial cells. Assays on untreated cells or those fixed with Formalin or ethanol showed incomplete staining of the bacteria. It therefore appeared that the antigen recognized by the monoclonal antibody was cryptic or only partially exposed at the cell surface. With the direct immunofluorescence assay and detergent treatment, the antibody was evaluated on smears of bacteria after growth in culture. Representative staining of *L. pneumophila* as a positive control and *P. fluorescens* as a negative control is shown in Fig. 2. The antibody was examined on multiple culture isolates of each serogroup of *L. pneumophila*, non-pneumophila *Legionella* spp., and unrelated microorganisms. This study included the bacteria known to be cross-reactive with polyclonal antisera to *Legionella* spp., such as *P. fluorescens* (CDC 93), *B. fragilis*, and the *Flavobacterium-Xanthomonas* group. All strains of *L. pneumophila* were positive, and there was no evidence of specific immune cross-reaction to non-pneumophila *Legionella* or non-*Legionella* bacteria. There was staining of some strains of *Staphylococcus aureus* due to nonimmune binding of the mouse immunoglobulin to protein A (for a review, see reference 23). The results are summarized in Table 1.

DISCUSSION

Serotypic diversity of *L. pneumophila* has expanded as increased numbers of isolates are obtained from clinical and environmental sources. Currently, eight serogroups are recognized, but this number will probably continue to increase. Already, an additional serogroup 9 has recently been proposed (P. H. Edelstein, W. F. Bibb, G. W. Gorman, W. L. Thacker, D. J. Brenner, H. W. Wilkinson, C. W. Moss, R. S. Buddington, C. J. Dunn, P. J. Roos, and P. L. Meenhorst, Ann. Intern. Med., in press). This poses a serious disadvantage to the direct serological diagnosis of the infection with polyclonal serogroup-specific reagents. Not only does the multiplicity of serogroups create difficulties in the production of a polyvalent serum, but there is a lag time between the recognition of a new serogroup and production of the appropriate serogroup-specific antiserum.

A far more attractive approach is to develop diagnostic reagents against species-specific antigens. Studies have been performed to analyze these common antigens in multiple serogroups of *L. pneumophila* and a variety of *Legionella* species. Analysis by crossed immunoelectropheresis of *L. pneumophila* (7, 19) has revealed numerous serogroup-common antigens (85 to 87% of total antigens). Of the *L. pneumophila* serogroup 1 reference system antigens (51 total), 39 to 53% cross-reacted with *L. bozemanii*, *L. dumoffii*, *L. gormanii*, and *L. micdadei*, and up to six antigens were observed to cross-react with non-*Legionella* organisms (7). For species-specific antigens to be useful for the production of diagnostic reagents, they must be structurally located so that they are accessible to antibodies and are represented in sufficient density to provide an adequate signal in selected assays.

In this report, we describe a species-specific antigen

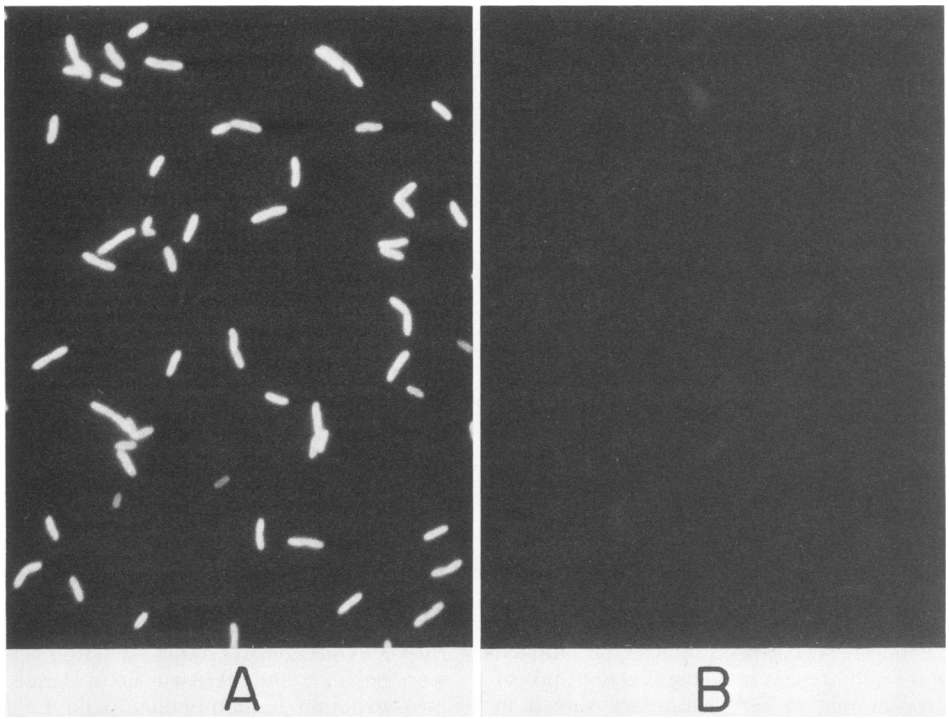

FIG. 2. Direct fluorescent antibody assay with monoclonal antibody LP3IIG2 on bacterial smears. (A) *L. pneumophila* serogroup 1 (Houston-1 strain); (B) *P. fluorescens* (CDC 93 strain).

which is detected by a monoclonal antibody in ELISA and immunofluorescence assays. Further characterization by immunoblot analysis showed that the antigen had a molecular size of 29,000 daltons and was present in *L. pneumophila* serogroups 1 through 8 but not in non-pneumophila *Legionella* spp. or unrelated microorganisms. In immunoblots, additional satellite bands of various intensities were observed in most of the serogroups. This could be due to proteolytic or heat degradation of the major antigen, incomplete dissociation of the antigen from other cellular components, in vivo modifications, or a second antigen with shared antigenic determinants. Experiments are in progress to evaluate these possibilities. Studies also showed that the antigen was sensitive to proteinase K and resistant to heat, which suggested that it was a heat-stable protein. Other investigators have reported a major outer membrane protein of molecular weight 29,000 that was present in multiple serogroups of *L. pneumophila* (9). In addition, it was reported that a species-specific surface antigen of *L. pneumophila* was stable to heat (6).

The determinant to which the antibody reacts on the species-specific antigen is only partly exposed at the surface of the cell in direct immunofluorescence assays. Treatment of the cells with detergent and EDTA was necessary to completely expose the antigen to antibody. Immersion of cells in a boiling water bath also exposed the antigen, but this treatment resulted in variable staining patterns in fluorescence assays. The antigen on bacterial cells could be exposed by the detergent-EDTA treatment whether they were suspended in water or 1% Formalin and was unaffected by subsequent fixation with 95% ethanol.

The species-specific antigen identified by the monoclonal antibody was present in all *L. pneumophila* isolates tested, including the recent isolate of serogroup 9. Therefore, this antibody is a potential diagnostic reagent for *L. pneumophila* with a number of advantages over polyclonal antisera. The antibody is species-reactive and stains all serogroups of *L. pneumophila*. Each newly described serogroup will need to be tested, but it may no longer be necessary to produce additional polyvalent sera for diagnostic purposes as new serogroups are discovered. The antibody does not cross-react with *Pseudomonas* or *Bacteroides* strains which have been shown to react with the polyclonal reagents. Because the antibody is monoclonal, there is minimal nonspecific staining in the specimens. This may be especially helpful in assays on clinical specimens taken directly from patients, such as sputum and lung tissue. In preliminary tests on three touch imprints from infected lung tissue, the antibody specifically detected the *L. pneumophila* organism with minimal background staining of the specimens. Further studies are in progress to define the sensitivity and specificity of the antibody for the diagnosis of *L. pneumophila* in specimens taken directly from patients.

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