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Common Epitope on the Lipopolysaccharide of *Legionella pneumophila* Recognized by a Monoclonal Antibody

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Serogroup-specificity of *Legionella pneumophila* is related to lipopolysaccharide (LPS), and few cross-reactions between serogroups have been observed with rabbit or monkey antisera. C57BL/6 mice were sequentially immunized with crude outer membrane fractions of *L. pneumophila* serogroups 1, 5, and 7, *Legionella bozemanii*, and *Legionella micdadei*. Spleen cells from these mice were then fused with the Sp2-0/Ag14 mouse myeloma cell line. Outer membrane-rich fractions and LPS were prepared from *L. pneumophila* serogroups 1 to 8 and other *Legionella* and non-*Legionella* species. Immunoblots of these extracts were performed with monoclonal antibody obtained from these fusions. One of these monoclonal antibodies recognized an epitope common to all tested serogroups of *L. pneumophila* and attached to the major constituent of the outer membrane, LPS. This antibody did not react with other *Legionella* species and numerous gram-negative rods other than *Pseudomonas fluorescens* CDC93. This monoclonal antibody may be useful in preliminary identification of *L. pneumophila* as an alternative to direct fluorescent-antibody testing.

Legionella pneumophila was first characterized in 1977 and has been recognized as an important pulmonary pathogen (11, 24). A second clinical syndrome, Pontiac fever, is a nonpneumonic self-limited respiratory illness (15). Many species of the genus *Legionella* can cause legionellosis, but *L. pneumophila* accounts for 80% of all cases of Legionnaires disease (34).

At least 12 serogroups of *L. pneumophila* (3, 4, 10, 25–28, 36, 38) and 22 additional species (2, 5, 37) of legionellae have been identified from several sources. All have typical gram-negative ultrastructural morphology. As with other gram-negative bacteria, lipopolysaccharide (LPS) is a major constituent of the outer membrane of legionellae. Lipid analysis of crude cell envelope fractions and LPS from *L. pneumophila* have shown that branched-chain fatty acids predominate. In addition, hydroxy fatty acids, a common structural component of lipid A in other bacteria, are either present in low quantities or totally absent in legionellae (30). Upon mild acid hydrolysis, this LPS is split into lipid A and six or seven polysaccharide fractions (32). The banding pattern of *L. pneumophila* LPS determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) also differed from the usual pattern observed with other gram-negative bacteria (30).

In this study, we analyzed the outer membrane of *L. pneumophila* by producing monoclonal antibodies. With these antibodies, we tested outer membrane and LPS preparations of *L. pneumophila* serogroups 1 to 8 by Western blot (immunoblot). We also analyzed different serogroups of *L. pneumophila* by an enzyme-linked immunosorbent assay (ELISA), an immunofluorescence assay, and immunocytochemical labeling. One of the produced monoclonal antibodies (53-3D12) attached to an epitope common to the LPS from eight serogroups of *L. pneumophila* and not to other *Legionella* species or other gram-negative rods except *P. fluorescens* CDC93.

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MATERIALS AND METHODS

Organisms and culture conditions. The bacterial strains used in this study were obtained from the Centers for Disease Control (Atlanta, Ga.), the American Type Culture Collection (Rockville, Md.), Arnold Brown (William Jennings Bryan Dorn Veterans' Hospital, Columbia, S.C.), the Hôtel-Dieu de Québec (Quebec, Canada), or were isolated in our laboratory. These strains are listed in Table 1. The cells used for outer membrane preparation were cultured in a yeast extract broth containing 0.05% cysteine and 0.025% ferric PP_i. The cultures were incubated statically in flasks for 2 h at 37°C and then shaken on a Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 100 rpm for an additional 2 h and at 200 rpm for 24 h. Cells were streaked for purity on buffered charcoal-yeast extract medium (BCYE) (33) and blood agar plates. Cells for other experiments were grown for 48 to 72 h on BCYE agar plates at 37°C.

Production of monoclonal antibodies. Hybridomas were prepared by the method of Köhler and Milstein (23). Every other week, C57BL/6 mice were sequentially immunized with crude outer membrane fractions of *L. pneumophila* serogroups 1, 5, and 7, *Legionella bozemanii*,, and *Legionella micdadei* (intraperitoneal with Freund incomplete adjuvant). Four days before fusion of spleen cells with Sp2-0/Ag14 myeloma cells, the mice were reimmunized with an *L. pneumophila* serogroup 1 outer membrane preparation (intravenous injection). After 14 days, hybridoma supernatants were tested for the presence of antibodies against *L. pneumophila* serogroup 1, 5, or 7, *L. bozemanii*, and *L. micdadei* by an ELISA. Supernatants were also tested with other bacterial species (*Escherichia coli*, *Enterobacter cloacae*, *Hafnia alvei*, and *Pseudomonas aeruginosa*). Hybridomas that reacted with different serogroups and species of members of the family *Legionellaceae* but not with other bacterial species were cloned by limiting dilution. Further experiments were done with either hybridoma supernatants

TABLE 1. ELISA with different strains of *L. pneumophila*, members of the *Legionellaceae*, and other bacterial species with monoclonal antibody 53-3D12

Strain (serogroup)	No. positive/ no. tested[a]
Legionella	
L. pneumophila	
1	80/80
2	10/10
3	10/10
4	10/10
5	10/10
6	10/10
7	2/2
8	10/10
L. bozemanii	
1	0/1
2	0/1
L. longbeachae	
1	0/1
2	0/1
L. micdadei	0/8
L. wadsworthii	0/1
L. feelei	0/1
L. gormanii	0/1
L. jordanis	0/1
L. sainthelensis	0/1
L. oakridgensis	0/1
L. dumoffii	0/7
L. anisa	0/1
L. parisiensis	0/1
L. hackleia	0/1
L. rubrilucens	0/1
L. spiritensis	0/1
L. erythra	0/1
L. jamestowniensis	0/1
Legionella-like organisms	
684	1/1
687	1/1
U7W	1/1
U8W	1/1
MICU-B	1/1
Other bacteria	
Acinetobacter sp.	0/1
Proteus rettgeri	0/1
Serratia marcescens	0/3
Pseudomonas aeruginosa	0/9
Escherichia coli	0/38
Hafnia alvei	0/1
Staphylococcus aureus	9/13[b]
Klebsiella pneumoniae	0/9
Proteus mirabilis	0/8
Streptococcus viridans	0/2
Rothia dentocariosa	0/1
Streptococcus pyogenes	0/4
Klebsiella oxytoca	0/5
Enterococcus sp.	0/6
Citrobacter freundii	0/1
Morganella morganii	0/2
Enterobacter cloacae	0/1
Enterobacter sp.	0/1
Staphylococcus epidermidis	0/1
Neisseria gonorrhoeae	0/1
Pseudomonas fluorescens CDC93	1/1
Pseudomonas fluorescens EB	0/1
Pseudomonas alcaligenes ABB50	0/1
Flavobacterium-Xanthomonas strain CDC65	0/1

[a] Only the optical densities three times higher than those of negative controls were kept as positive results.

[b] Owing to nonimmune binding to protein A (see text for explanation).

or purified antibody obtained from ascites fluids. Pristane-primed CB6F1 mice were used for ascites fluid production.

ELISA. The capture antigens were whole cells fixed with 1% (vol/vol) Formalin in 0.85% (wt/vol) NaCl. Flexible polyvinylchloride plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.) were coated overnight at 37°C with 0.05 ml of antigen (0.05 mg/ml) in phosphate-buffered saline (PBS) (50 mM, pH 7.4). The supernatants (0.05 ml) were then added and incubated for 1 h at room temperature. The plates were washed with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T; J. T. Baker Chemical Co., Phillipsburg, N.J.), and 0.05 ml of peroxidase-conjugated rabbit anti-mouse immunoglobulins diluted 1:250 in PBS (DAKO-immunoglobulins a/s, Copenhagen, Denmark) was added and incubated for 1 h at room temperature. The plates were washed again with PBS-T and revealed with 0.1 ml of 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (0.4 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in phosphate-citrate buffer (50 mM, pH 5.0) with 0.005 μl of 30% hydrogen peroxide per ml. The optical densities were read (415 nm) after 30 min with a Titertek Multiskan MC (Flow Laboratories, Irvine, Scotland).

Preparation of outer membranes. Outer membranes of *L. pneumophila* and other species of members of the *Legionellaceae* were obtained by extraction of whole cells with sodium N-lauroyl sarcosinate (sarcosyl; Sigma) by the procedure described by Butler et al. (6). Cells from 24-h-old yeast extract broth were centrifuged and suspended in Tris hydrochloride (50 mM, pH 7.2) (Sigma) to achieve an optical density of 0.2 (660 nm). The cells were centrifuged again, and the pellet was resuspended in Tris hydrochloride (50 mM, pH 7.2) containing 2% (wt/vol) sarcosyl. After incubating for 1 h at 37°C, DNA was sheared by ultrasonic vibration (30 s at 50% of the sonicator maximum power; Sonic 300 dismembrator; Artek Systems Corp., Farmingdale, N.Y.) and lysozyme (1 mg/ml; Sigma) was added. The outer membranes were pelleted by ultracentrifugation at 100,000 × *g* for 30 min and washed three times in Tris-hydrochloride (50 mM, pH 7.2) containing 2% (wt/vol) sarcosyl. The crude outer membrane preparations were stored at −70°C.

Preparation of LPS. LPS extracts were prepared by proteinase K (Sigma) digestion of whole-cell lysates by the method of Hitchcock and Brown (21). Bacteria were grown on BCYE agar plates, harvested after 48 to 72 h of incubation at 37°C, scraped off the plate with a glass rod, and suspended in distilled water. After centrifugation for 2 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Rexdale, Ontario, Canada), the pellet was suspended in 1 ml of lysing buffer containing 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 62.5 mM Tris (pH 6.8). The cells were broken by adding 0.5 ml of glass beads (Sigma) and shaking them for 2.5 min with a test tube mixer (Vortex-Genie; Allied, Fischer Scientific, New York, N.Y) at the maximum power. The solution was centrifuged as above, and 10 μl of a fresh solution of proteinase K at a concentration of 2.5 mg/ml in lysing buffer was added to the supernatants. The lysates were incubated at 60°C for 1 h and then centrifuged for 3 min. Supernatants were finally heated at 85°C for 30 min before a second digestion with proteinase K. The latter preparation was again centrifuged for 3 min, and the supernatants were retained for analysis.

Periodate oxidation. The LPS extracts (10 mg) were oxidized in the dark at 4°C for 6 days with 10 ml of 0.05 M $NaIO_4$. The excess of $NaIO_4$ was destroyed by incubation with 80 μl of 50% ethylene glycol for 2 h at room temperature. Samples were dialyzed four times against distilled

water. The oxidized preparations were allowed to react with the monoclonal antibody by ELISA and Western blot.

Indirect microimmunofluorescence assay. The indirect microimmunofluorescence technique described by Wang and Grayston (41) was used in this study. Strains were cultured on BCYE agar for 48 to 72 h, scraped off the plates with a glass rod, and suspended in filter-sterilized 1% (vol/vol) Formalin in 0.85% (wt/vol) NaCl to achieve a turbidity equivalent to a McFarland no. 3 standard. A 1:10 dilution was then made in normal chicken yolk sac, and the bacteria were placed on microscope slides that contained multiple wells. The slides were heat fixed and placed in acetone for 15 min at room temperature. Bacteria were incubated with monoclonal antibody 53-3D12 for 30 min at 37°C in a humidified chamber, rinsed twice in PBS, and reincubated as above with fluorescein-labeled rabbit antibody to mouse immunoglobulin G (DAKO). After being mounted, the slides were examined with an epi-illumination fluorescence microscope. One set of slides containing the fixed antigens was also treated by submerging them for 20 min in PBS (150 mM, pH 8) containing 1% (vol/vol) Triton X-100 (J. T. Baker Chemical Co.) and 100 mM EDTA (Sigma).

SDS-PAGE. SDS-PAGE was performed in a 12.5% acrylamide slab gel. Gels were silver stained (Bio-Rad Laboratories, Richmond, Calif.), and commercial low-molecular-weight standards (Pharmacia, Uppsala, Sweden) were used in each experiment (40).

Western blot. Transfer of antigens was done by the procedure of Towbin et al. (39) at a constant current of 0.8 A for 1 h (Transfor; Bio-Rad). The blotting buffer contained 192 mM glycine and 25 mM Tris hydrochloride (pH 8.5). Monoclonal antibodies were probed as follows. Nitrocellulose membranes were soaked in PBS–1% (wt/vol) gelatin for 1 h. Strips or whole blots were incubated overnight in ascites fluid diluted 1:250 in PBS–0.1% (wt/vol) gelatin. The blots were washed extensively in PBS and incubated 1 h in peroxidase-labeled anti-mouse immunoglobulin G. The blots were again extensively washed and revealed with 0.03% (wt/vol) 3,3'-diaminobenzidine (Sigma) in Tris buffer (50 mM, pH 7.5) with 25 μl of 30% hydrogen peroxide.

Preparation of bacteria for ultrastructural studies. Actively growing colonies of *L. pneumophila* serogroup 1 were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 for 2 h at room temperature. Samples were thereafter dehydrated in an ethanol series and finally embedded in Epon 812. In some instances, postfixation with 1% osmium tetroxide in sodium cacodylate buffer was done before dehydration and embedding. Ultrathin sections collected on nickel grids were further processed for immunocytochemical labeling.

Immunocytochemical labeling. The postembedding indirect protein A-gold technique developed by Bendayan (1) was used in this study. Gold particles (BDH Chemicals, Toronto, Ontario, Canada) of approximatly 5-nm diameter were prepared by the method of Frens (12). The pH of this gold suspension was adjusted to 5.9. The minimal amount of protein A (Sigma) needed to stabilize 10 ml of this suspension was estimated to be 100 μg. The pellet recovered after centrifugation at 60,000 × *g* was suspended in 0.5 ml of PBS (pH 7.4) containing 0.02% polyethylene glycol 20,000 (Fisher Scientific Co., Pittsburgh, Pa.).

Sections of *L. pneumophila* serogroup 1 cells were incubated on a drop of PBS containing 1% bovine serum albumin for 10 min and then transferred onto a drop of monoclonal antibody diluted 1:10 in PBS–1% bovine serum albumin for 60 min at room temperature in a moist chamber. After being rinsed with PBS, sections were incubated on a drop of the protein A-gold complex diluted 1:20 in PBS-polyethylene glycol at pH 7.4. The sections were then washed with PBS, rinsed with distilled water, air dried, and contrasted with uranyl acetate and lead citrate.

RESULTS

Isolation of hybrid cell line producing monoclonal antibody against *L. pneumophila*. The monoclonal antibodies obtained from these fusions were first tested by ELISA. Of the 50 monoclonal antibodies that were produced in these experiments, 16 reacted with two or more serogroups of *L. pneumophila* (serogroups 1, 5, and 7). Of these 16 antibodies, 3 also reacted with *E. coli*, *E. cloacae*, *H. alvei*, and *P. aeruginosa*. Only one antibody reacted with *L. pneumophila* serogroups 1, 5, and 7 but not with the other gram-negative bacteria. This monoclonal antibody (53-3D12) was kept for further analysis.

ELISA for *L. pneumophila*, nonpneumophila legionellae, and other bacterial species. Several serogroups of *L. pneumophila*, nonpneumophila legionellae, and other bacterial species were tested. Antibody 53-3D12 reacted with all tested *L. pneumophila* isolates (Table 1). However, U7W, U8W, and MICU-B isolates, described by Garrity et al. (14) as being antigenically related to *L. pneumophila* serogroup 5 but genetically distinct, also reacted with monoclonal antibody 53-3D12. Also described by Garrity et al. (14), environmental strains 684 and 687 are antigenically and genetically related to *L. pneumophila* serogroup 5; these two strains also reacted with monoclonal antibody 53-3D12. No cross-reaction was observed when nonpneumophila legionellae were tested. Among nonlegionella species only *P. fluorescens* CDC93 (28) and *Staphylococcus aureus* did react with this monoclonal antibody.

Periodate oxidation. When LPS was allowed to react with periodate before the ELISA or before electrophoresis, reactivity was abolished. These findings suggest that LPS contains a common epitope for all eight tested serogroups of *L. pneumophila* and that this epitope is located on the carbohydrate moiety of LPS. Digestion of these proteins with proteinase K abolished this staining, whereas a ladderlike pattern similar to one obtained with LPS did appear.

Indirect microimmunofluorescence assay. The specificity of monoclonal antibody 53-3D12 was further evaluated by an indirect immunofluorescence assay (Table 2). Formalin-killed cells of *L. pneumophila* diluted in normal chicken yolk sac and then fixed to slides with acetone were moderately stained, whereas no fluorescence could be detected with other species of legionellae or nonlegionellae except for the *P. fluorescens* CDC93 strain. When *L. pneumophila* was treated with Triton X-100–EDTA, fluorescence disappeared. The epitope recognized by monoclonal antibody 53-3D12 was thus shown to be exposed on the cell surface and to be sensitive to treatment with Triton X-100–EDTA. Acetone or Formalin fixation did not alter the antigenic reactivity of the epitope recognized by this monoclonal antibody.

SDS-PAGE and Western blot. SDS-PAGE of *L. pneumophila* serogroup 1 to 8 LPS preparations are shown in Fig. 1. The LPS pattern visualized by silver staining revealed numerous distinct bands that were regularly spaced. Outer membrane proteins and LPS of *L. pneumophila* serogroups 1 to 8 were electrophoretically transferred to nitrocellulose and stained with monoclonal antibody 53-3D12 (Fig. 2 and 3, respectively). Ladderlike patterns were observed only with

TABLE 2. Indirect microimmunofluorescence assay with different strains of *L. pneumophila*, members of the *Legionellaceae*, and other bacterial species with monoclonal antibody 53-3D12

Strain (serogroup)	Fluorescence[a]	
	Before treatment	After treatment
L. pneumophila		
1	++	−
2	++	−
3	++	−
4	++	−
5	++	−
6	++	−
7	++	−
8	++	−
L. bozemanii		
1	−	−
2	−	−
L. longbeachae		
1	−	−
2	−	−
L. micdadei	−	−
L. wadsworthii	−	−
L. feelei	−	−
L. gormanii	−	−
L. jordanis	−	−
L. sainthelensis	−	−
L. oakridgensis	−	−
L. dumoffii	−	−
P. fluorescens CDC93	++	−
E. coli	−	−
E. cloacae	−	−
P. aeruginosa	−	−
H. alvei	−	−

[a] Staining intensity: −, no fluorescence; +, minimum fluorescence; ++, medium fluorescence; +++, high fluorescence. Antigens after treatment with 1% (vol/vol) Triton X-100 plus 100 mM (wt/vol) EDTA.

L. pneumophila serogroups 1 to 5, 7, and 8. Western blots of LPS prepared from other *Legionella* species were not stained with monoclonal antibody 53-3D12. The large number of bands observed in the outer membrane protein preparations is most probably due to the fact that a crude extraction procedure was used and that these proteins are contaminated with LPS.

Immunocytochemical localization of antigens. Treatment of ultrathin sections from glutaraldehyde-fixed material with monoclonal antibody 53-3D12 and gold-complexed protein A resulted in a strong surface labeling. In contrast, postfixation with osmium tetroxide was found to greatly reduce the labeling intensity. Thus, specimens fixed only with glutaraldehyde were selected for further immunocytochemical studies. Examination of the labeling pattern revealed that gold particles were mainly distributed over the outer membrane, whereas cytoplasmic membrane was not significantly labeled (Fig. 4A). Typical septa resulting from bacterial division were also specifically labeled (Fig. 4A). Cytoplasmic structures were devoid of labeling. A more detailed observation showed that gold particles were located preferentially over both sides of the outer membrane (Fig. 4B). The slight variations in gold particle localization over the outer membrane are due to the plane of sectioning. All controls were negative (Fig. 4C).

DISCUSSION

Of the different monoclonal antibodies that were produced in this study, only one reacted with all eight tested serogroups of *L. pneumophila* but not with the other *Legionella* species or the other gram-negative rods that were initially tested. Further studies with this antibody showed that it reacts with the bacterial LPS.

In previous studies, Gosting et al. (16) produced a monoclonal antibody that reacts with a 29-kilodalton antigen that is common for different serogroups of *L. pneumophila*. Nolte and Colin (29) have demonstrated that this monoclonal antibody is directed against the major outer membrane protein of *L. pneumophila*. The antibody produced by Gosting et al. (16) poorly stained whole cells of *L. pneumophila* when tested by indirect immunofluorescence. However, after treatment of whole cells with Triton X-100–EDTA, a strong fluorescence could be observed. Our monoclonal antibody stained whole cells of *L. pneumophila* fairly well (2+ reaction). However, when these same cells were treated with Triton X-100–EDTA, fluorescence completely disappeared, thus suggesting that the epitopes recognized by these two antibodies were different. In addition, these results suggested that the antigen recognized by monoclonal antibody 53-3D12 was on the bacterial surface. This localization was further demonstrated by immunocytochemical staining with protein A-gold (Fig. 4). Interestingly, specific staining could be observed inside and outside the outer membrane and on the septa of dividing bacteria.

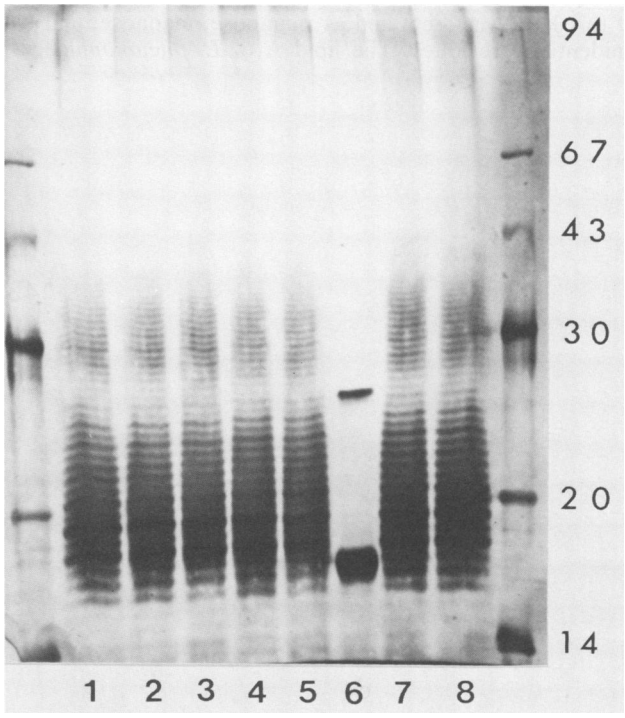

FIG. 1. SDS-PAGE (12.5% polyacrylamide) of LPS preparations of *L. pneumophila* serogroups 1 through 8. The gel was stained with silver nitrate. Lanes 1, serogroup 1 (Philadelphia 1 strain); 2, serogroup 2 (Togus 1 strain); 3, serogroup 3 (Bloomington 2 strain); 4, serogroup 4 (Los Angeles 1 strain); 5, serogroup 5 (Dallas 1E strain); 6, serogroup 6 (Chicago 2 strain); 7, serogroup 7 (Chicago 8 strain); 8, serogroup 8 (Concord 3 strain). Molecular weight standards are phosphorylase *b*, 94,000 (94); bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; and alpha-lactalbumin, 14,000.

To further characterize the epitope identified by monoclonal antibody 53-3D12, we purified LPS from *L. pneumophila* and prepared outer membrane-rich fractions of *L. pneumophila* serogroups 1 to 8 and other species of *Legionellaceae*. After separation of these purified antigens by SDS-PAGE and transfer to nitrocellulose sheets, we could demonstrate that antibody 53-3D12 reacts with the LPS preparation of all tested serogroups of *L. pneumophila* but not with that of other species of *Legionellaceae*. The staining pattern of the immunoblots realized with monoclonal antibody 53-3D12 was strikingly similar to that demonstrated by Otten et al. (32) for *L. pneumophila* LPS. In addition, Otten et al. (32) have demonstrated that periodate oxidation of LPS substantially reduces or abolishes antigenic activity. These results are similar to ours. Migration of preformed carbohydrate from the cytoplasmic membrane to the outer membrane may explain the internal labeling observed in immunocytochemical studies. Recently, Caldwell and Hitchcock (7) have described a monoclonal antibody that reacts with LPS isolated from 15 *Chlamydia trachomatis* serovars and seven *Chlamydia psittaci* strains isolated from five different animal species. The epitope recognized by this monoclonal antibody was heat resistant (100°C) and was destroyed by sodium metaperiodate treatment. These results are strikingly similar to those we report here with another intracellular pathogen, *L. pneumophila*.

LPSs of *L. pneumophila* serogroups 1 to 4 have recently been studied (32). The composition of the polysaccharide chain of *L. pneumophila* serogroups 1 to 4 is unusual in that it does not contain heptose. The carbohydrate composition of this LPS was identified as rhamnose, mannose, and two unidentified residues. The lipid A of *L. pneumophila* also seems to be different from that of other gram-negative rods since it did not comigrate with LPS preparations of *Salmonella minnesota* on SDS-PAGE (32). The major differences in the composition of LPS isolated from all four tested serogroups are the molar ratios of the carbohydrate residues and the presence of galactose in serogroup 4 LPS. One of the unidentified carbohydrates is present in equal amounts in each tested serogroup. In this study, LPS bands of similar intensity were observed with LPS preparations from seven of the eight tested serogroups. This suggests that one of these unidentified carbohydrates is the epitope recognized by monoclonal antibody 53-3D12. The sole exception, *L. pneumophila* serogroup 6, does not have a smooth-type LPS like the other tested serogroups. For this serogroup, the ladderlike pattern observed with all other serogroups was replaced by two separate bands in the 29- and 45-kilodalton areas, respectively. These correspond to the molecular sizes of the major outer membrane proteins found in numerous other studies.

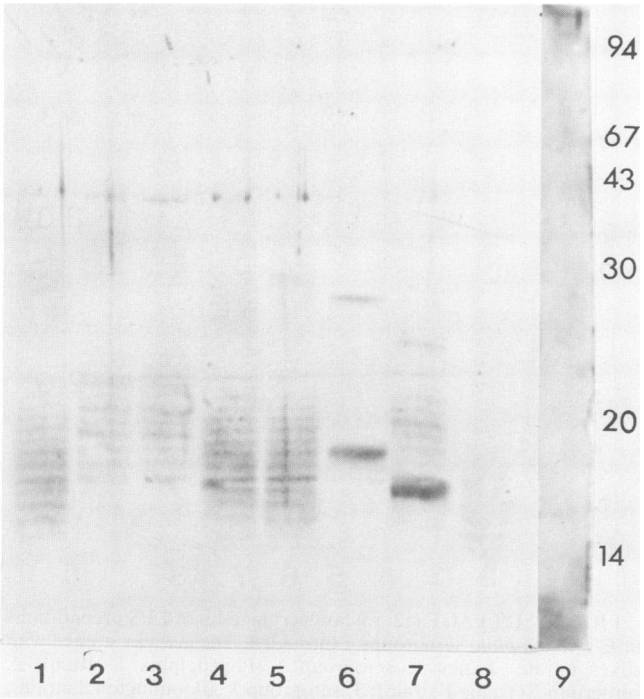

FIG. 2. Western blot of LPS preparations of *L. pneumophila* serogroups 1 to 8 with monoclonal antibody 53-3D12. Number below each lane indicates the serogroup; lane 9 is a negative control with a monoclonal antibody against *L. micdadei*. Bacterial strains and molecular weight standards used in this experiment are identical to those described in the legend to Fig. 1.

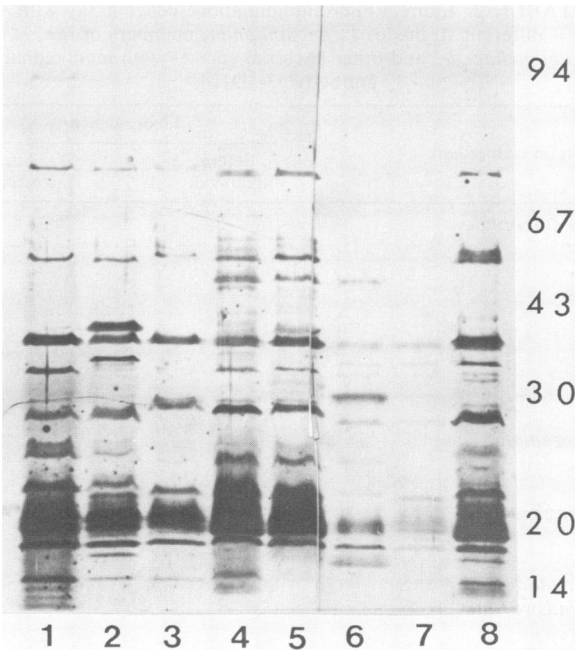

FIG. 3. Western blot of outer membrane fractions of *L. pneumophila* serogroups 1 to 8. Number below each lane indicates the serogroup. Bacterial strains and molecular weight standards used in this experiment are identical to those described in the legend to Fig. 1.

Antibody 53-3D12 also reacted with the 29-kilodalton outer membrane protein of *L. pneumophila*. However, this protein is known to be tighly bound to LPS (19), and immunoreactivity of this antigen with our monoclonal antibody is thus not surprising. Different studies on the characterization of major outer membrane proteins have demonstrated that major outer membrane proteins are stably associated with LPS (20). In *Neisseria gonorrhoeae*, this complex is resistant to dissociation in SDS at high temperature (100°C). Recently, Gulig et al. (17) have described four monoclonal antibodies that were initially identified as being specific for the 39-kilodalton major outer membrane protein of *Haemophilus influenzae* type b. Upon further analysis, these same monoclonal antibodies were demonstrated to react with *Haemophilus influenzae* type b LPS (18). In the latter study, coprecipitation of this major outer membrane

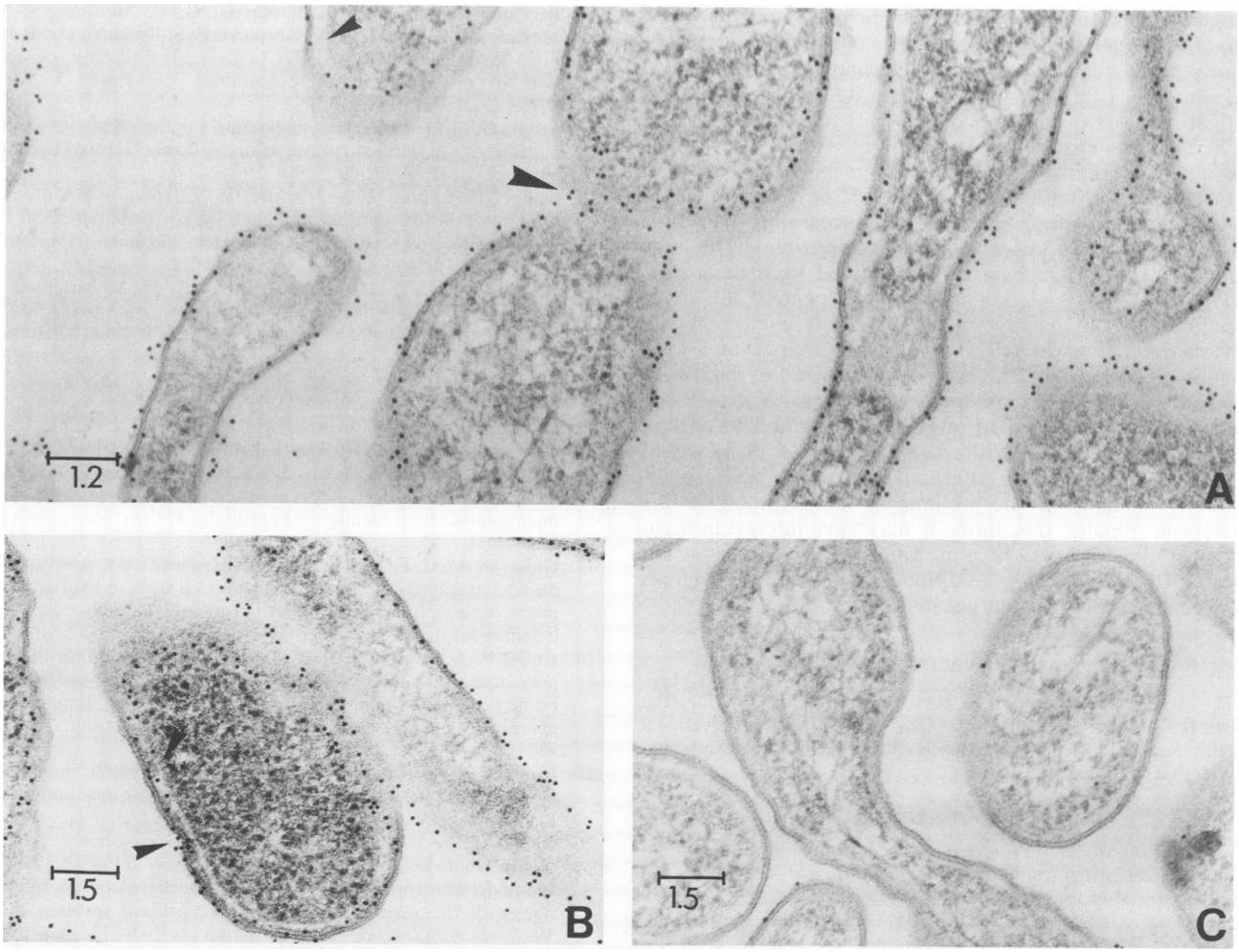

FIG. 4. Immunocytochemical labeling of *L. pneumophila* serogroup 1 Philadelphia 1 strain with monoclonal antibody 53-3D12. (A) Large arrowhead indicates staining of septum of dividing bacterium; small arrowhead shows lack of staining of cytoplasmic membrane (×81,000). (B) Arrowhead indicates staining on both sides of the outer membrane (×67,000). (C) Negative control with a monoclonal antibody specific to *L. pneumophila* serogroup 2 (×67,000) (22). Specificity of the labeling was further assessed through the following controls: incubation with preimmune mouse serum instead of monoclonal antibody followed by treatment with the protein A-gold complex; incubation with monoclonal antibody 53-3D12 previously adsorbed with *L. pneumophila* serogroup 1 followed by incubation with the protein A-gold complex; omission of the monoclonal antibody followed by incubation with protein A-gold complex. All these controls were also negative. Bars, 10^{-1} μm.

protein with LPS was clearly demonstrated. Our results thus confirm a strong association between LPS and the major outer membrane protein of another bacterial species.

Monoclonal antibody 53-3D12 reacted with more than 100 clinical and environmental isolates of *L. pneumophila*. In contrast, of the 112 clinical strains of gram-negative and gram-positive bacteria that were tested by ELISA, only 10 presented a cross-reaction. Of these 10 strains, 9 were *S. aureus*, a bacterial species that is known to nonspecifically bind immunoglobulin G though protein A. However, when these strains of *S. aureus* were preincubated with normal rabbit serum, monoclonal antibody 53-3D12 did not react with these bacteria. This suggests that this cross-reaction was due to nonspecific binding of immunoglobulin G to protein A. Of the gram-negative rods, only the CDC93 strains of *P. fluorescens* reacted with monoclonal antibody 53-3D12. This reaction is not suprising since Orrison et al. (31) have demonstrated the strong antigenic relatedness between this strain of *P. fluorescens* and *L. pneumophila* serogroup 1. Other strains of nonlegionellae described by Orrison et al. (31) as giving cross-reactions with anti-*L. pneumophila* rabbit antiserum did not react with monoclonal antibody 53-3D12. Finally, this monoclonal antibody did react with some strains of legionellae that are genetically distinct from *L. pneumophila*. In our ELISA, the MICU-B, U7W, and U8W *Legionella* strains described by Garrity et al. (14) reacted strongly with monoclonal antibody 53-3D12. The DNA homology of these strains with *L. pneumophila* is just below the 70% level at 64°C and between 15 and 42% under more stringent conditions (75°C). Whether these strains should be classified as *L. pneumophila* still has not been resolved. However, our results do suggest that they share an epitope that is common to all tested strains of *L. pneumophila* but not to other *Legionella* species.

In prior studies, LPS has always been thought to be the serogroup-specific antigen (8, 9, 13), and Gabay and Horwitz (13) have demonstrated that the majority of the antibody produced by patients infected with *L. pneumophila* reacts with the LPS. Although some level of antigenic cross-reactivity has always been observed between the different serogroups of *L. pneumophila*, these cross-reactions were attributed to the outer membrane proteins or cytoplasmic

antigens that were shown to be common to *L. pneumophila* (16) or to the genus (6, 35). The results presented in the study clearly show that an antigenic determinant present on *L. pneumophila* LPS is common to all tested serogroups. This antigen is most probably a minor epitope on the bacterial LPS. The immunization procedure that was used for hydridoma production probably accounts for its identification.

In conclusion, we produced a monoclonal antibody, 53-3D12, reacting with *L. pneumophila* serogroups 1 to 8 by ELISA, immunofluorescence, and Western blot. The antigenic determinant recognized is removed by a detergent-EDTA treatment and is a major constituent of the outer membrane of *L. pneumophila*, LPS. These experiments demonstrated that *L. pneumophila* serogroup 1 to 8 LPS contains a species-specific epitope recognized by monoclonal antibody 53-3D12. Preliminary results suggest that this antibody when coupled to latex beads produces a strong agglutination reaction with *L. pneumophila*. This could represent an interesting alternative to direct fluorescent-antibody staining for the preliminary identification of this bacterium. Finally, this antibody may have interest in taxonomic studies and may be a useful antigenic marker for the species *L. pneumophila*. Additional studies are in progress to evaluate these different possibilities.

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