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Production of Monoclonal Antibodies to *Legionella pneumophila* Serogroups 1 and 6

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To better define the surface antigens of *Legionella pneumophila* for clinical and experimental purposes, we have produced monoclonal antibodies to *L. pneumophila* serogroups 1 and 6. Two hybridomas were produced in serogroup 1. One antibody, LP-I-17, recognized a serogroup-common antigen. The second antibody, LP-I-81, was specific for serogroup 1. This antibody was able to agglutinate bacterial cells belonging to the serogroup 1 reference strains, Philadelphia and Knoxville. Microagglutination assays of environmental and clinical isolates revealed a subgroup of serogroup 1 environmental isolates which were not agglutinated by LP-I-81. This subset of isolates was segregated to certain buildings in the medical complex. Immunodiffusion studies showed identity between the LP-I-81 antigen and the serogroup-specific antigen of serogroup 1 organisms. This antigen could be absorbed out of the serogroup 1 organism extract with LP-I-81-coated *Staphylococcus aureus*, leaving the serogroup-common antigens. Three hybridomas were produced to serogroup 6. All three produced antibodies which were serogroup 6 specific and agglutinated serogroup 6 bacteria.

Isolates of *Legionella pneumophila*, the etiological agents of Legionnaires disease, have been divided into seven serogroups based upon reactivity with antisera from rabbits immunized with the reference strains (1, 6, 16, 17). Additional antigenic complexity within the *Legionella* serogroups has been suggested by a number of investigators (3, 10, 22). Studies with absorbed antisera have demonstrated the presence of subgroups within serogroup 1 of *L. pneumophila* (3). The identification of such subgroups may be useful in studying outbreaks of Legionnaires disease (3).

To better define the serological relationship among *L. pneumophila* for taxonomic and epidemiological purposes and to develop tools for isolation and characterization of *Legionella* antigens, we have produced monoclonal antibodies to *L. pneumophila* serogroups 1 and 6. We report the production of the antibodies, their usefulness in identifying *Legionella* serogroups and in defining serogroup 1 subgroups, and their application to preliminary isolation of surface antigen.

MATERIALS AND METHODS

Bacteria and media. Strains of *L. pneumophila* serogroup 1 (Philadelphia-1, Knoxville-1), serogroup 2-Togus, serogroup 3-Bloomington, serogroup 4-Los Angeles, serogroup 5-Dallas, serogroup 6-Chicago, *L. gormanii*, and *L. dumoffii* were obtained from the Centers for Disease Control, Atlanta, Ga. (CDC). A second serogroup 6 isolate, from a Legionnaries disease patient at the University of Chicago, was provided by P. Arnow. The remaining isolates were from patients and from environmental sources in Columbus, Ohio. The organisms were grown on buffered charcoal yeast extract (7) and were stored at −80°C in 50% glycerol and 50% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Killed organisms for immunization were fixed overnight in saline containing 0.5% Formalin.

Production of monoclonal antibodies. The medium used for suspension of all cells was Dulbecco modified Eagle medium (K.C. Biological, Kansas City, Mo.) supplemented with 20% agamma horse serum (K.C. Biological), 2 mM glutamine, and 3.7 mg of sodium bicarbonate, 3.5 mg of glucose, 63 μg of penicillin, and 100 μg of streptomycin per ml. BALB/c mice were immunized intraperitoneally with a suspension of 0.25 ml of Formalin-killed Knoxville-1 organisms (McFarland number 3 opacity standard) in 0.01 M phosphate-buffered saline pH 7.2 (PBS) and 0.25 ml of incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). This immunization was repeated on day 10. On day 50, 0.1 ml of the diluted bacterial antigen was injected intravenously via a tail vein. Three days later, the mouse spleen was removed, and single cell suspensions in serum-free media were prepared by using a Dounce homogenizer. Mononuclear cells were separated from erythrocytes by using Lympholyte M (Ce-

darline Laboratories, London, Canada). SP 2/0 myeloma cells (1.5×10^7; reference 18) were fused with 10^8 spleen cells according to the method of McKearn (14). Hybridoma media were screened for anti-*Legionella* antibodies with an indirect immunofluorescence assay (21).

Of 550 wells seeded with the fusion products, 350 showed hybridomas. Two hybridoma cell lines produced anti-*Legionella* antibodies. These were cloned by limiting dilution according to the method of McKearn (13). Stable cell lines were frozen, and ascites were produced as described previously (15).

A similar protocol was used for production of serogroup 6 monoclonal antibodies, except that the mice were immunized intraperitoneally on days 1 and 10 and intravenously on day 21. Because of a lower yield of viable spleen cells, only 220 wells were seeded, and 60 grew hybridomas. Three of the cell lines produced antibody to *Legionella* serogroup 6.

Immunofluorescent assays. Direct fluorescence assays for *Legionella* serotyping were performed according to the method of Cherry et al. (5), with reagents obtained from Biological Products Division, CDC. Indirect fluorescence assays (IFA) were performed as outlined for human anti-*Legionella* antibody (21), with the modification that 10 μl of hybridoma supernatant and 10 μl of a 1 to 20 dilution of fluorescein-labeled antimouse immunoglobulin G (IgG) and IgM (MA bioproducts, Walkersville, Md.) were substituted in place of the corresponding human reagents.

Microagglutination and reverse passive agglutination assays. Microagglutination assays were performed by mixing 40 μl of hybridoma media and either 40 μl of Formalin-fixed *Legionella* organisms provided by the CDC (for IFA) or bacteria from a single 1-mm colony suspended in 40 μl of PBS. Visible clumping was evident with positive reactions.

Reverse passive agglutination was used for detection of soluble *Legionella* antigens according to a modification of the method of Tang et al. (19). Formalin-fixed *Staphylococcus aureus* organisms (Bethesda Research Laboratories, Gaithersburg, Md.) were coated with monoclonal antibody by incubation of 100 μl of 10% (vol/vol) bacterial suspension with 1.5 ml of hybridoma media (from cell cultures containing 10^6 cells per ml) for 1 h at 25°C. The coated bacteria were centrifuged in PBS and resuspended in 0.5 ml of PBS to make a 2% suspension. The agglutination assays were performed by mixing 10 μl of coated *S. aureus* organisms and 50 μl of solubilized *Legionella* antigen. The mixture was gently rocked for 5 min and then was read in indirect light against a dark background.

Antigen preparation. Soluble *Legionella* antigens were prepared by gently scraping the bacteria from a 100-mm petri dish of buffered charcoal yeast extract after 3 days of growth. This quantity of bacteria was suspended in 1.0 ml of PBS and then was autoclaved for 30 min. The suspension was mixed well and the organisms were removed by centrifugation at 10,000 × *g* for 2 min. The protein content of the soluble antigen preparations was estimated by the method of Bradford (2), modified by the use of a commercial reagent (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used as the standard.

Immunodiffusion. Ouchterlony double immunodiffusion was performed in 1% agarose (Bethesda Research Laboratories) made in PBS. Melted agarose (5 ml) was layered onto a microscope slide. Wells were made with a gel punch. A 10-μl sample of ascites fluid, antisera, or antigen was added to the wells, and the slides were incubated for 48 h at room temperature. The rabbit antiserum was prepared against Formalin-killed *L. pneumophila* serogroup 1 Knoxville strain according to the method of Cherry and McKinney (4).

RESULTS

Isolation of monoclonal antibodies to *L. pneumophila*. Fusion of the myeloma SP 2/0 cells and spleen cells from a mouse immunized with *L. pneumophila* serogroup 1 Knoxville strain yielded two hybridoma cell lines which produced *Legionella* antibody. The immunization schedule used for serogroup 6 Chicago strain resulted in three hybridomas secreting anti-*Legionella* antibody. The percentage of antibody-positive wells per well of growing cells was higher with the serogroup 6 immunization scheme.

The two serogroup 1 hybridomas were frozen, and after subsequent thawing, the majority of the subclones continued to produce *Legionella* antibody.

Binding of monoclonal antibodies to Formalin-fixed *Legionella* organisms. The binding of the monoclonal antibodies to the six serogroups of *L. pneumophila* and four other *Legionella* species was evaluated by using Formalin-fixed organisms provided by the CDC for IFA testing (Table 1). Monoclonal antibody LP-I-81 was serogroup 1 specific. Monoclonal antibodies LP-VI-2, LP-VI-5, and LP-VI-6 were all serogroup 6 specific. None of these antibodies bound to other *L. pneumophila* serogroups or to *L. bozemanii, L. dumoffii, L. gormanii*, or *L. micdadei*. LP-I-17 exhibited binding to all six *L. pneumophila* serogroups, but the fluorescence was less intense than that seen with LP-I-81 or the direct fluorescence assay reagent (fluorescein-linked antisera) provided by the CDC, particularly for serogroups 2 to 6. The filamentous bacterial forms of the *Legionella* appeared more prominent with this antibody. LP-I-17 also bound to less than 1% of the bacteria present on the *L. gormanii* and *L. dumoffii* smears. Neither LP-I-17 nor LP-I-81 recognized surface antigens of a variety of clinical isolates of non-*Legionella* species, including *Pseudomonas aeruginosa* (two isolates), other *Pseudomonas* species (eight isolates), *Flavobacterium species* (four isolates), *Bacteroides fragilis* (three isolates), and one isolate each of *Enterobacter aerogenes, Proteus mirabilis, Escherichia coli, Serratia marcescens, Providentia stuartii, Klebsiella pneumoniae, Citrobacter diversans, S. aureus*, and *Streptococcus faecalis*.

Microagglutination of clinical and environmental isolates. *L. pneumophila* isolates were also tested for LP-I-81 binding by a microagglutina-

TABLE 1. Indirect fluorescent-antibody assay of *Legionellaceae* with monoclonal antibodies

Legionella species	Monoclonal antibody fluorescence[a]				
	LP-I-17	LP-I-81	LP-VI-2	LP-VI-5	LP-VI-6
L. pneumophila					
SG-1	+2	+4	–	–	–
SG-2	+1	–	–	–	–
SG-3	+1	–	–	–	–
SG-4	+1	–	–	–	–
SG-5	+1	–	–	–	–
SG-6	+1	–	+4	+4	+4
L. bozemanii	–	–	–	–	–
L. dumoffii	–[b]	–	–	–	–
L. gormanii	–[b]	–	–	–	–
L. micdadei	–	–	–	–	–

[a] Positive fluorescence was rated from +1 (faint) to +4 (very bright).
[b] Few organisms (less than 1%) were fluorescent.

tion assay. LP-I-81 agglutinated only serogroup 1 *L. pneumophila*. Clinical and environmental isolates from Columbus, Ohio, which had been typed as serogroup 1 by direct fluorescence assay were also tested for microagglutination by LP-I-81 (Table 2). The two *L. pneumophila* serogroup 1 strains obtained from the CDC, Philadelphia-1 and Knoxville-1, agglutinated in the presence of LP-I-81 media. Fourteen of the 15 *L. pneumophila* isolates from University Hospitals patients with Legionnaires disease were also agglutinated. Serogroup 1 environmental isolates from several sites in Columbus varied in agglutination pattern, depending upon the source of the isolate. Two buildings in the University Hospitals medical complex, RH and UPH, consistently yielded organisms which failed to be agglutinated by LP-I-81. Another two buildings in the medical center, UH and MH, and two of three other Columbus hospitals yielded organisms which agglutinated with LP-I-81. All of the above-mentioned isolates bound the LP-I-17 antibody as determined by IFA, but none agglutinated in the related hybridoma media.

TABLE 2. Microagglutination of *L. pneumophila* serogroup 1 organisms by LP-I-81

Isolate origin	Agglutination
Philadelphia-1	+
Knoxville-1	+
University Hospitals	
LD patients[a]	+[b]
Building RH	–
Building UPH	–
Building UH	+
Building MH	+
Community Hospital 1	+
Community Hospital 2	+
Community Hospital 3	–

[a] LD, Legionnaires disease.
[b] Positive in 14 of 15 patients.

The serogroup 6 monoclonal antibodies were also tested for microagglutination capability. All three monoclonal antibodies, LP-VI-2, LP-VI-5, and LP-VI-6, would agglutinate only serogroup 6 organisms. Two strains of *L. pneumophila* serogroup 6 available for testing agglutinated in the presence of all three monoclonal antibodies. Again, LP-I-17 bound to both serogroup 6 isolates, as determined by IFA, but did not agglutinate either organism.

Detection of soluble *Legionella* antigens with LP-I-81. Soluble *L. pneumophila* serogroup 1 antigens could be detected by reverse passive agglutination assays, using fixed *S. aureus* organisms coated with LP-I-81 antibody. The LP-I-81-coated *S. aureus* organisms would agglutinate only in the presence of serogroup 1 antigens (Fig. 1). As little as 100 ng of protein in the soluble antigen could be detected with this assay.

The LP-I-81 antibody appears to be an IgG, based on its binding to *S. aureus* organisms and formation of a precipitin band in immunodiffusion with antisera to mouse IgG but not mouse IgM. The LP-I-17 antibody behaved similarly and also appears to be an IgG.

Immunodiffusion. *L. pneumophila* antigen extracts were also analyzed by immunodiffusion (Fig. 2). The soluble serogroup 1 antigens reacted with serogroup 1 rabbit antisera to produce two precipitin lines. The line closer to the LP-I-81 antigen well showed identity with the sharper single line present against LP-I-81. This identity was more definitive in other immunodiffusion studies in which only three wells, containing LP-I-81, rabbit antisera, and antigen were used. The precipitin line closer to the antisera well was contiguous with a less prominent line present between the antisera and the other serogroup

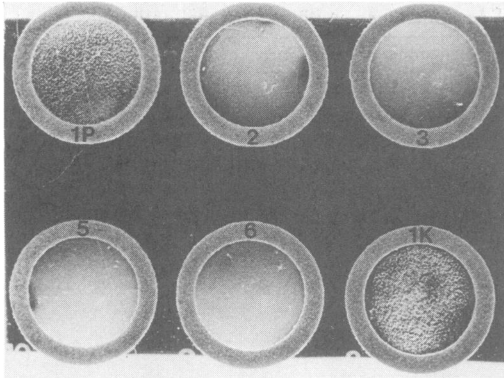

FIG. 1. Serogroup 1-specific agglutination of LP-I-81-coated *S. aureus* organisms by solubilized *Legionella* antigens. (1P) *L. pneumophila* serogroup 1 Philadelphia strain antigen; (2) serogroup 2; (3) serogroup 3; (5) serogroup 5; (6) serogroup 6; (1K) serogroup 1 Knoxville strain.

antigens. This line represents a serogroup-common antigen. Solubilized serogroup 1 antigens, which were repeatedly absorbed with LP-I-81-coated *S. aureus* organisms until the *L. pneumophila* antigen preparation no longer agglutinated the antibody-coated bacteria, were also tested by immunodiffusion. The precipitin band which had shown identity with the LP-I-81 antigen was selectively removed, whereas the serogroup-common antigen remained present in the absorbed serogroup 1 extract. LP-I-81 did not produce precipitin lines with the extracts of serogroup 2, 3, 5, or 6. LP-I-17 did not produce precipitin lines with any antigen preparation (data not shown).

DISCUSSION

This report describes the production of monoclonal antibodies directed against surface antigens of *L. pneumophila*. These antibodies were capable of recognizing either serogroup-specific or serogroup-common antigens. In addition, the monoclonal antibody binding activity also defined subsets of serogroup 1. One antibody was used in reverse passive agglutination assays to detect soluble *Legionella* surface antigens.

The major advantage of monoclonal antibodies over conventional antisera for diagnostic evaluations is the nearly limitless supply of uniform reagents which are available because of the ease of storage and monoclonal nature of hybridoma cells. Standard *Legionella* antisera are produced by immunization of animals with a single serogroup of killed *L. pneumophila* organisms. The resultant antisera are directed against a major serogroup-specific antigen and, to a variably lesser extent, against serogroup-common antigens. Pooling of various serogroup-specific antisera produced in animals allows an effective polyvalent antiserum (20). The development of the monoclonal antibodies LP-I-81 and LP-I-17 allows a definite separation between serogroup-specific and serogroup-common antibodies. Diagnostic application of LP-I-17, the monoclonal antibody which recognizes a serogroup-common antigen, may simplify screening of clinical specimens, particularly in areas in which several serogroups are known to cause Legionnaires disease. Coupling a fluorescent label to the LP-I-17 antibody should further facilitate use of this antibody in diagnostic evaluations. Because the monoclonal antibodies recognize antigens on Formalin-fixed organisms, retrospective analysis of fixed pathology specimens from previous suspected cases may be possible. However, before the monoclonal antibodies can be used for widespread clinical testing, our observations will require confirmation with additional *Legionella* isolates.

Microagglutination of organisms with serogroup-specific hybridoma media provides a simple method for serogrouping *L. pneumophila* isolates. The finding of a subset of environmental isolates of *L. pneumophila* serogroup 1 bacteria which do not microagglutinate in the presence of LP-I-81 necessitates caution in the exclusive use of monoclonal antibodies for serogrouping purposes. Nevertheless, microagglutination with hybridoma media may still find use for screening purposes.

Of potential importance in understanding the epidemiology of nosocomial Legionnaires dis-

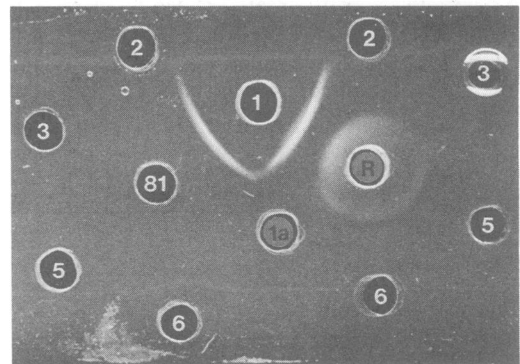

FIG. 2. Double immunodiffusion showing serogroup specificity of LP-I-81 and the absorption of the LP-I-81 antigen from the solubilized antigens of *L. pneumophila* serogroup 1. Wells: 1, serogroup 1 antigens; 2, serogroup 2; 3, serogroup 3; 5, serogroup 5; 6, serogroup 6; 1a serogroup 1 antigens previously absorbed with LP-I-81-coated *S. aureus* organisms; 81, LP-I-81 ascites; R, rabbit anti-Knoxville sera.

ease is the agglutination of a subset of serogroup 1 organisms of LP-I-81 and the segregation of this subset of bacteria to certain buildings in the medical complex. Further epidemiological investigation is under way to define the relationship among cases of hospital-acquired Legionnaries disease, patient locations within the medical complex, the presence of *Legionella* spp. in the potable water, and the antigenic nature of the isolates from the patients and their immediate environment.

The ultrastructure of *Legionella* organisms appears similar to that of other gram-negative organisms (8). The outer membrane contains the surface antigens. The biochemical composition of these antigens remains largely undefined. A number of investigators have prepared serogroup-specific and serogroup-common antigens from organism extracts (9, 11, 12, 22). The apparent molecular weight and chemical composition of the various antigen preparations have not been uniform, and interrelationships are unclear. Monoclonal antibodies may assist in the specific isolation and characterization of *Legionella* antigens. The agglutination of the LP-I-81 antibody-coated *S. aureus* in the presence of small amounts of antigen will provide a simple and sensitive means of monitoring purification of this serogroup 1-specific component. In addition, the coated *S. aureus* selectively absorbed the LP-I-81 antigen from an outer membrane extract. Once freed from the antibody, the LP-I-81 antigen could be further characterized.

Immunodiffusion studies also confirmed other investigations which demonstrated both serogroup-specific and serogroup-common antigens in organism extracts (22). Serogroup-common antigens were shown to be present by the precipitin line between the serogroup 2, 3, 5, and 6 antigens and the antiserum to serogroup 1. LP-I-81 produced a single precipitin line with serogroup 1 extracts and no bands with extracts from other serogroups. This further confirmed the serogroup-specific nature of this monoclonal antibody and clearly identified a serogroup 1-specific antigen precipitin line.

In conclusion, the availability of monoclonal *Legionella* antibodies will aid in understanding the epidemiology of Legionnaires disease and the antigenic composition of *L. pneumophila*. Antibodies which precisely define serogroup subsets could also help elucidate the peculiar environmental colonization of *Legionella* spp. The employment of monoclonal antibodies which recognize serogroup-specific and serogroup-common antigens may also make diagnostic tests for Legionnaires disease more widely available. Finally, monoclonal antibodies will facilitate the isolation of *Legionella* antigens and allow further biochemical characterization.

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