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Indirect Immunofluorescence Test for Serodiagnosis of Legionnaires Disease: Evidence for Serogroup Diversity of Legionnaires Disease Bacterial Antigens and for Multiple Specificity of Human Antibodies

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Evidence obtained by others who used direct immunofluorescence staining to demonstrate serological differences among strains of Legionnaires disease bacterium prompted this study of parameters influencing the ability of the indirect immunofluorescence test to detect human antibodies to Legionnaires disease bacterium. A total of 25 Legionnaires disease bacterium strains, representing four serogroups, were used as immunofluorescence antigens to test selected human sera. The use of diethyl ether in preparing the antigens was discontinued when it was found that titers against ether-killed group 2 (Togus 1-like) antigens were impossible to determine. Instead, heat-killed suspensions of Legionnaires disease bacterium in 0.5% buffered normal chicken yolk sac were used to show the serogroup diversity of the strains and the serogroup specificity of the antibody response of some, but not all, patients with serological evidence of Legionnaires disease. These studies suggest that multiple antigens should be used in serological tests for Legionnaires disease. Furthermore, the fact that some sera contain antibodies that bind equally well to strains of all four serogroups implies that demonstration of a fourfold increase in titer of paired sera when tested with a single antigen should not be interpreted as evidence of infection with a strain of the same serogroup.

The most commonly used laboratory test for obtaining a retrospective diagnosis of Legionnaires disease (LD) is an indirect immunofluorescence (IF) method (3). Demonstration of a fourfold or greater increase in antibody titer to at least 128 during convalescence provides serological evidence of a recent infection with the LD bacterium (LDB). In the absence of a demonstrable fourfold seroconversion, a single or standing titer of at least 256 provides presumptive evidence of infection at an undetermined time (5). The usefulness of a presumptive interpretation is necessarily limited, although the greater the titer, the greater the index of suspicion in a case with clinical symptoms compatible with LD.

The indirect IF test described by McDade et al. uses a 5% suspension of LDB-infected chicken yolk sacs as antigen (3). As an alternative antigen for general distribution by the Center for Disease Control in kit form, an agar-grown, ether-killed Philadelphia 1 LDB strain was suspended in phosphate-buffered saline (PBS) containing normal chicken yolk sacs (NYS) at a concentration of 1%. Twofold dilutions of the sera to be tested were made in 10% NYS. Cell-bound antibodies were detected by staining them with fluorescein isothiocyanate-labeled rabbit antihuman immunoglobulin which was, in turn, visualized with fluorescence microscopy. Until recently, these were the reagents used to test all sera submitted to the Special Immunology Laboratory, Center for Disease Control, for LD serology.

Several recent discoveries about the immunological characteristics of the LDB, however, made it necessary to modify the indirect IF antigen and test procedure. For example, we recently described an LDB strain (Togus 1) that was antigenically distinct from previously recognized LDB, such as the Philadelphia 1 and Knoxville 1 isolates (4). Although certain antigens appeared to be common to all LDB strains, the ones presumably on the surface of the bacterial cell had serogroup specificity. Philadelphia 1- and Knoxville 1-like strains are now designated as LDB serogroup 1, and Togus 1-like strains are designated as serogroup 2 (R. M. McKinney, L. Thacker, P. P. Harris, K. R. Lewallen, G. A. Hebert, P. H. Edelstein, and B. M. Thomason, Ann. Intern. Med., in press). A third LDB strain (serogroup 3, Bloomington 2), sero-

logically distinct from serogroups 1 and 2 by direct IF tests (McKinney et al., in press), was isolated from a creek water tributary near a building associated epidemiologically with an outbreak of LD in Bloomington, Ind. (G. K. Morris, C. M. Patton, J. C. Feeley, S. E. Johnson, G. Gorman, W. T. Martin, P. Skaliy, G. F. Mallison, B. D. Politi, and D. C. Mackel, Ann. Intern. Med., in press). Subsequently, the Los Angeles 1 strain (serogroup 4), isolated from human lung tissue (2), was found to be nonreactive in the direct IF test with serogroup 1, 2, and 3 conjugates (McKinney et al., in press).

The fact that there are apparently several LDB serogroups raises the question of whether surface antigen-dependent serological tests, such as the indirect IF test as used currently, will ultimately be of practical value in demonstrating a seroconversion after infection with LDB. The answer will be influenced by the number of sera tested routinely, by whether demographic differences in serogroup prevalence are found, by how many serogroups are ultimately found, and by the extent to which the human antibody response to LDB varies.

The purpose of this study was to determine whether human sera can distinguish the four LDB serogroups differentiated so far by using fluorescein isothiocyanate-labeled rabbit antisera, to determine whether previously used methods for antigen preparation are satisfactory for all LDB serogroups, and to simplify the indirect IF test so that large numbers of diagnostic specimens can be handled expeditiously.

MATERIALS AND METHODS

Sera. Sera from patients suspected of having LD were submitted to the Special Immunology Laboratory, Center for Disease Control, by state health laboratories for LD indirect IF testing. The following criteria were used to select sera to test with LDB strains of different serogroups. Sera from as many different geographic regions of the United States as possible were tested with antigens prepared from 25 LDB strains. In addition, all sera received from the state of Maine from May to October 1978 were tested with the serogroup 2 (Togus 1) strain. A total of 72 randomly selected sera from Indiana were tested with the serogroup 3 (Bloomington 2) strain. And finally, all sera received from the same hospital in California from which the Los Angeles 1 strain was isolated were tested with the serogroup 4 (Los Angeles 1) strain. A total of 245 sera were included in the study.

Organisms. A total of 25 LDB strains provided by Roger M. McKinney and William B. Cherry, Analytical Bacteriology Section, were each inoculated heavily on enriched Mueller-Hinton agar slants (1). The loosely capped slants were then incubated at 35°C in a candle extinction jar until good growth was present (3 to 5 days).

Preparation of LDB ether-killed antigens. The Philadelphia 1 (group 1) and Togus 1 (group 2) strains were used to evaluate the use of ether-killed antigens in the indirect IF test. Each loosely capped slant of bacterial growth (to which 2.0 ml of diethyl ether had been added) was allowed to sit at a 30° angle at ambient temperature for 5 min. The ether was then decanted and discarded, and residual ether was allowed to evaporate from the uncapped slant for 30 min. The bacterial growth was suspended in 2.9 ml of 0.01 M PBS, pH 7.2, centrifuged, and then resuspended in 2.0 ml of 0.5% buffered NYS. Enriched Mueller-Hinton agar plates were streaked heavily with the ether-killed antigens, incubated as described above, and observed for 10 days to determine whether bacterial growth occurred.

Preparation of LDB heat-killed antigens. Each of the 25 LDB strains was grown on an enriched Mueller-Hinton agar slant and handled as follows. Organisms from each slant were suspended in 2.0 ml of sterile distilled water and transferred to a screw-capped test tube. The tube was placed in a boiling water bath for 15 min, and a Mueller-Hinton agar plate was streaked to test for sterility as described above. The bacteria were then centrifuged, and the packed cells were resuspended in 2.0 ml of distilled water. Working dilutions of the concentrated suspension were made in 0.5% buffered NYS. The range of antigen dilutions found to be optimal in the IF test (that is, the dilution that contained approximately 500 bacterial cells per microscopic field at a magnification of ×315) was 1:20 to 1:80, with most strains being dispersed optimally at a dilution of 1:50.

Indirect IF test. Each well of a microscope slide (25- by 75-mm glass slide with 12 staggered wells 5 mm in diameter, between acetone-resistant coating; Cel-Line Associates, Inc., Minotola, N.J.) was covered with the thoroughly mixed antigen suspension, excess liquid was removed with a Pasteur pipette and the slide was allowed to air-dry at room temperature. Antigen-bearing slides were fixed in acetone for 15 min at room temperature, again were allowed to air-dry, and then were used to test sera as follows. The serum to be tested was diluted 1:16 in buffered 3% NYS by placing 0.01 ml of the serum in a test tube containing 0.15 ml of the diluent. Subsequent doubling dilutions were made with 0.05 ml of PBS in rigid polystyrene U-bottomed microtitration trays (Linbro Scientific Co.) on a Cooke automatic diluter (Cooke Engineering Co., Alexandria, Va.). Each serum dilution was placed on an antigen-bearing slide well. Slides were incubated at 37°C in a moist chamber for 30 min, rinsed quickly with PBS, and placed in a PBS bath for 10 min. After they were removed from the bath, slides were blotted dry, and 20 μl of fluorescein isothiocyanate-labeled antihuman immunoglobulin were placed on each well. Slides were incubated, rinsed, and dried as before and mounted with cover slips placed on several drops of 0.5 M carbonate-buffered glycerol (1 part of 0.5 M carbonate-bicarbonate buffer, pH 9.0, to 9 parts of glycerol).

Slides were read with a Leitz Dialux 20 fluorescence microscope equipped with an HBO-100 mercury incident light source, the I-cube filter system (2 × KP490, 1-mm GG455 excitation filters, TK510 dichroic beam splitting mirror, and K515 suppression filter), a 40×

dry objective, and 6.3× eyepieces. The titer of each serum was expressed as the reciprocal of the highest dilution which produced 1+ fluorescence staining of at least 50% of the LDB cells estimated per microscopic field.

Other reagents. An LDB group 1, Philadelphia 1 antigen was kindly supplied by Richard George and Knox Harrell, Biological Products Division, Center for Disease Control. This antigen had been grown on agar, killed with ether, suspended in 1% buffered NYS, and distributed for general use in kit form. It will hereafter be called kit antigen. The above investigators also supplied the fluorescein isothiocyanate-labeled anti-human immunoglobulin, prepared in rabbits and hereafter called the conjugate, and a 10% buffered NYS suspension, dilutions of which were used to dilute both the test sera and the antigens.

RESULTS

As expected, results of indirect IF staining of the ether-killed kit antigen and the ether-killed experimental group 1, Philadelphia 1 antigen were comparable. Because no standard existed for a group 2, Togus 1 antigen, however, we compared the ether-killed to a live Togus 1 antigen in both 0.5 and 1.0% buffered NYS. The titer of a control serum which had a titer of 8,192 against the live antigen was impossible to measure because of the excessive green film on the microscopic field and particulate staining of the bacteria when the ether-treated antigen was used. These results suggested that the surface antigenic determinants in the Togus 1 strain were extracted with or degraded by the ether used to kill the organisms. For this reason, we explored the feasibility of using heat-killed antigens.

Heat-killed antigens of group 1 (Philadelphia 1) and group 2 (Togus 1) were next compared in the indirect IF with group 1 kit antigen and group 2 live antigen, respectively. No differences in titers of the respective control sera were observed. Furthermore, enriched Mueller-Hinton agar plates, inoculated heavily with the concentrated heat-treated antigens, were sterile after 10 days of incubation, whereas several colonies were observed on similar plates inoculated with the ether-treated organisms. Thereafter, all experiments were done with heat-killed antigens suspended in 0.5% buffered NYS.

Heat-killed antigens of 25 LDB strains and over 100 sera were used to find different IF staining patterns, representative examples of which are shown in Table 1. Serum A was from a patient whose titer against Philadelphia 1 (group 1) antigen increased from 32 to 2,048 in 2 months. Although this convalescent-phase serum has the same high titer against the Los Angeles 1 (group 4) antigen, a titer of only 128 was obtained with the Togus 1 (group 2) and Bloomington 2 (group 3) antigens. In contrast, serum B was from a patient described previously (4), whose fourfold seroconversion measured by a Philadelphia 1 antigen had suggested that he had LD. This serum, however, had a titer against the group 2 (Togus 1) antigen that was at least 16-fold higher than were those against groups 1, 3, or 4. Serum C was from a patient whose titer was 2,048 to 4,096 against the group 1 (or group 4) antigen during months 1, 2, and 3 after onset of illness. This serum was included in Table 1 because its titer against the group 3 antigen was the highest of the sera tested. A serum with group 3 specificity has not yet been found. Serum D was taken from a patient 3 weeks after onset of illness, and although insignificant titers were obtained with serogroups 1, 2, and 3, a titer of 512 was demonstrated with the group 4 antigen. These data suggest that the human antibody response to LD is at least partly serogroup specific. That it may also be nonspecific for serogroup is suggested by the results of testing serum E. This serum, obtained 3 weeks after onset, had a titer of 256 with all four antigens, which was a fourfold increase over the titer of 64 found with all antigens in the acute-phase serum of this terminally ill patient.

TABLE 1. *Diverse specificity of the human antibody response to LD as measured by antigens prepared from strains of LDB serogroups 1 through 4 in the indirect IF test*

LDB antigen		Titer of convalescent-phase serum:				
Serogroup	Strain	A	B	C	D	E
1	Philadelphia 1	2,048	256	4,096	64	256
2	Togus 1	128	4,096	256	32	256
3	Bloomington 2	128	256	512	32	256
4	Los Angeles 1	2,048	256	2,048	512	256

To determine whether the same serogroup patterns among the 25 LDB strains which McKinney et al. (Ann. Intern. Med., in press) obtained with rabbit antisera could be demonstrated with human sera, we compared the staining intensities of 10 control sera against the 25 heat-killed strains. The IF staining patterns of most of the isolates were identical to that observed with the Philadelphia 1 antigen and are therefore listed in Table 2 as serogroup 1. Three isolates had a serogroup 2 staining pattern. So far, serogroups 3 and 4 contain only one strain each. These results are in complete agreement with those of McKinney et al. (Ann. Intern. Med., in press) and suggest that multiple antigens may be necessary in the IF test.

A comparison of IF titers obtained with the

ether-killed (kit) and heat-killed group 1, Philadelphia 1 antigens (Table 3) with 127 sera showed that 99.2% of the titers agreed within one doubling dilution. The only discrepant titers were measured in one serum which had a titer of 512 against the heat-killed antigen and a titer of 64 against the ether-killed antigen. End points were more easily determined with the heat-killed antigen because of its uniformly stained cell surface, in contrast to the spotty fluorescence observed on the surface of the ether-treated antigen. For these reasons and because ether-treated group 2 (Togus 1) antigens are not suitable IF antigens, we now routinely use heat-killed antigens for LD serology.

TABLE 3. *Comparison of indirect IF titers of 127 human sera obtained with ether-killed or with heat-killed LDB group 1 (Philadelphia 1) antigens*

Titer with ether-killed antigen	No. of sera having titer with heat-killed antigen of:				
	≤64	128	256	512	≥1,024
≤64	63[a]	13		1	
128	5	19			
256		2	8	3	
512			1	3	
≥1,024				1	8[a]

[a] Titers of all sera agreed within one doubling dilution.

DISCUSSION

A total of 25 LDB strains, including two environmental isolates, were used as antigens in the indirect IF test for LD in an effort to demonstrate the serogroup diversity found previously in direct IF tests with conjugates prepared from rabbit antisera. In complete agreement, 20 strains belonging in serogroup 1 (Philadelphia 1-like), 3 in serogroup 2 (Togus 1-like), and 1 each in serogroups 3 (Bloomington 2) and 4 (Los Angeles 1) were found. Sera from patients with serological evidence of LD were found that preferentially bound antigens of serogroups 1, 2, or 4. No sera were found among those tested that preferentially bound the environmental isolate representing serogroup 3, but one serum with a titer of 4,096 when measured with the serogroup 1 antigen had a titer of 512 with the serogroup 3 antigen. The absence of specificity for serogroups 1 through 4 in some sera was documented in sera from a patient who, before he died, had titers of 256 against antigens of all four serogroups, in contrast to the 64 titer of his acute-phase serum. These data suggest that the immunological response to LD can be against antigenic determinants that are specific for and characteristic of each serogroup, that it can also be against LDB antigenic determinants that are common to all strains of serogroups 1 through 4, or that it can be multiple, with the proportion of antibodies specific for each antigenic determinant being an individual characteristic.

TABLE 2. *Serogroup specificity by indirect IF of 25 strains of LDB*

Serogroup	Strain designations
1	Philadelphia 1, 2, 3, and 4; Flint 1 and 2; Detroit 1 and 2; Albuquerque 1; Allentown 1; Berkeley 1, Birmingham 1; Burlington 1; Miami Beach 1; Olda 1; Bellingham 1; Rochester 1; Knoxville 1; Pontiac 1; Bloomington 1[a]
2	Togus 1; Atlanta 1 and 2
3	Bloomington 2[a]
4	Los Angeles 1

[a] Environmental isolates: strain 1 from cooling tower water, strain 2 from creek water. The remaining strains were clinical isolates.

The significance of these findings for a laboratory which uses indirect IF tests for LD routinely is that, as the number of antigens in the test increase, the number of sera to be tested must decrease proportionately within the same time-resource constraints. Currently, we are testing sera with heat-killed serogroup 1 (Philadelphia 1) antigen. This method of killing was found to be far superior to the use of diethyl ether, which apparently extracted some of the Philadelphia 1 antigen and all of the detectable Togus 1 antigen. These observations are not surprising in view of the high lipid content of a purified antigen of the Knoxville 1 strain (K. H. Wong, W. O. Schalla, R. J. Arko, J. C. Ballard, and J. C. Feeley, Ann. Intern. Med., in press). Ether could have extracted antigenic determinants or, alternatively, could have disrupted the integrity of the lipid-containing cell envelope. A distinct advantage to using heat-killed antigens of the Philadelphia 1 strain is the more readily distinguishable end point obtained because of a more uniformly stained cell surface. Reducing the concentration of NYS in the antigen to 0.5% also contributed to the ease of reading. Although it would be most desirable to eliminate the NYS completely, concentrations lower than 0.5% allowed the LDB to aggregate, which made it difficult to determine end points. The amount of NYS used as serum diluent could also be reduced (to 3%) in the initial 1:16 dilution, with subsequent doubling dilutions made with an automatic diluter in PBS. The NYS concentration could not be lower than 2.5% because apparently nonspecific factors in some sera created a green fluorescent film over the microscopic field. A constituent of NYS may absorb these as yet undefined factors.

The practicality of using the indirect IF test for routine diagnostic LD serology can only be determined ultimately when the prevalence of antibodies of different serogroup specificity among patients with LD is known. It is to be hoped that the number of antigens required to measure seroconversion to LD will be limited. Studies now in progress indicate that a polyvalent antigen containing heat-killed strains of serogroups 1 through 4 can be used to select sera to be titrated against monovalent antigens.

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ADDENDUM

The name proposed for LDB by Don J. Brenner and Joseph E. McDade at the International Symposium on Legionnaires' Disease, 14 November 1978, Center for Disease Control, Atlanta, Ga., was *Legionella pneumophila* (prototype strain, Philadelphia 1).

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