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Factors Influencing the Reactivity of *Legionella* Antigens in Immunofluorescence Tests

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We examined several factors for their effects on the serological reactivity of *Legionella* antigens used for direct or indirect fluorescent-antibody tests. These factors included media, methods of killing, strain differences, and the nature of the reactivity with diverse human sera. The maximum serological reactivities were obtained with charcoal-yeast extract agar; the relative antigenicity of cells grown on a chemically defined medium could be fourfold less than those grown on the charcoal-yeast extract agar. Cells grown at 25°C showed only small antigenic differences from those grown at 35°C but had better morphological and staining characteristics. Cells killed by 1% Formalin or 37% Formalin vapors showed a 20% less relative antigenicity than those killed by heat, but their cell walls stained more clearly and they had fewer aberrations. As tested with several human sera, cells of Philadelphia 1 showed great variation in relative antigenicity with changes in media or methods of preparation; Bellingham 1 was quite stable under these same conditions. The data suggest that Bellingham 1 had serogroup 1-specific antigens, reactive with human sera, which were not present in Philadelphia 1.

Currently, two types of whole-cell antigens are distributed by the Biological Products Program, Center for Infectious Diseases (CID), Centers for Disease Control, Atlanta, Ga., for the diagnosis of legionellosis. One antigen is a Formalin-killed suspension used as a control antigen for the direct fluorescent-antibody (DFA) examination of clinical materials and isolated cultures (4). The second is a suspension of heat-killed cells in 0.5% normal egg yolk sac used for serological diagnosis of legionellosis by the indirect fluorescent-antibody (IFA) procedure (25). More recently, a third type of antigen was required to investigate a solid-phase immunofluorometric determination of antibody responses to the disease (2). The latter procedure required a lyophilized antigen having high serological reactivity, a morphologically homogeneous population of single small cells, and a stable antigenic composition resistant to repetitive washing.

Considerable variation has been observed in the serological reactivity of cells from various production lots. One strain, Philadelphia 1, completely lost reactivity in the IFA test when grown on a synthetic liquid medium. Because of these observations of the variability of antigenicity by this strain, we tested the effects of various media and cultural conditions on the serological reactivity of *Legionella* species in the DFA and IFA tests and by immunofluorometric assay (IFMA).

MATERIALS AND METHODS

Bacterial strains. The following strains were obtained from R. M. McKinney, Division of Bacterial Diseases, CID, Centers for Disease Control: *Legionella pneumophila* strains Philadelphia 1, Philadelphia 4, Bellingham 1, Knoxville 1 (all serogroup 1), Togus 1 (serogroup 2), Bloomington 2 (serogroup 3), Los Angeles 1 (serogroup 4), Dallas 1E, Dallas 2E, Cambridge 2 (all serogroup 5), Chicago 2, and Houston 2 (both serogroup 6), *L. micdadei* (Tatlock); *L. gormanii* (LS 13); *L. dumoffii* (New York 23); *L. bozemanii* (WIGA); *L. longbeachae* (Longbeach 4, serogroup 1); and *L. longbeachae* (Tucker 1, serogroup 2). The strains were transferred at 2-week intervals on charcoal-yeast extract (CYE) agar slants (8) and incubated at 35°C in air with 5% carbon dioxide.

The strain of Philadelphia 1, above, was designated strain Philadelphia 1 (R.B.) at approximately the 40th transfer after its receipt. Another strain of Philadelphia 1 (designated F.G.), obtained from W. B. Cherry, Division of Bacterial Diseases, CID, in 1979, was cultured on Feeley-Gorman (F-G) agar (9) and incubated as above; the cells were suspended in water and stored in ampoules at −70°C (18) during a 3-year period. A third strain of Philadelphia 1 (designated GPSp) and a strain of *L. pneumophila*, Vermont, were obtained from G. W. Gorman, Division of Bacterial Diseases, CID, as frozen guinea pig spleen from animals infected with egg yolk sac of the primary isolation from human patients. These were stored at −70°C.

Inocula. Inocula for diverse experiments were grown on CYE agar for 48 to 72 h; cells were washed from the agar with 1 ml of distilled water per 10 ml of media, and 0.1 ml was used to inoculate experimental

media. Inocula for Philadelphia 1 (F.G.) were taken directly from the frozen suspension; inocula of Philadelphia 1 GPSp and Vermont were grown for a single transfer on CYE slants before passage to CYE plates for production of cells.

Media. The media tested were CYE agar, buffered CYE agar (17), F-G agar, chemically defined *L. pneumophila* (CDLp) broth (18) with or without agar or Norit charcoal, chemically defined broth with 2-(*N*-morpholino)-ethanesulfonic acid buffer and adjusted metals (CDM) (20), and chemically defined broth with choline and rhamnose (Ristroph) (21). To determine the effects of media and methods of killing on antigen production, cells of the first 18 strains cultured on CYE slants were transferred, in duplicate, to 125-ml Erlenmeyer flasks containing 25 ml of each synthetic broth and incubated with shaking at 35°C for 72 h. Three passages were made in each of the synthetic broths. Transfers were then made to CYE slants, to F-G slants, or from CYE slants to F-G slants since several cultures failed to grow when they were transferred from synthetic broth to F-G media. Samples of cell suspensions from each culture were made and killed by heating in a boiling-water bath for 15 min or by suspension in 1% Formalin in phosphate-buffered saline (0.01 M, pH 7.2) for 24 h at 5°C. Killed cells were washed twice with buffered saline and then adjusted to an optical density (OD) of 0.1 (Beckman model B spectrophotometer, 660 nm, 1.8 cm) for evaluation of relative antigenicity.

Antisera and control antigens. Antisera for use in the direct immunofluorescence test were prepared in rabbits against the respective serogroup or species, using a schedule and procedure described previously (15). Strain Knoxville 1 was used as the vaccine strain for *L. pneumophila* serogroup 1. Immunoglobulin was isolated by precipitation with 50% ammonium sulfate and was purified by DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) chromatography and elution with 0.1 M (pH 8.4) Tris-hydrochloride–0.11 M NaCl buffer. The fluorescein isothiocyanate (FITC) conjugates of the isolated immunoglobulins were adjusted to contain 10 mg of immunoglobulin per ml; the fluorescein/protein ratio of the conjugate was approximately 25 μg of fluorescein per mg of protein. A human serum (A) was obtained from a patient infected with *Legionella* as diagnosed by seroconversion (64 to 1,024) with the IFA test, using Philadelphia 1 antigen. An FITC conjugate of this serum was prepared as described above. Human serum B was obtained from a patient with legionellosis diagnosed by seroconversion (1,024 to 4,096), using Philadelphia 1 antigen; this case was from a Vermont outbreak (3). Human serum 001 was from a patient with legionellosis diagnosed by the isolation of strain Detroit 1 from lung tissue obtained by needle biopsy (15). Human sera to serogroups 2, 3, and 4 were reference sera obtained from the Biological Products Program. Rabbit antihuman immunoglobulin conjugate and control antigens for the DFA and IFA tests were those of the Biological Products Program, CID, Centers for Disease Control.

Procedures for killing cells. Cells were brought to 10% (vol/vol) in distilled water. To kill with Formalin, we added 37% Formalin to the suspension to bring the final concentration of Formalin to 1%, and the suspension was incubated at 5°C for 24 h. Cells were also killed by placing the suspension in a boiling-water bath for 15 min or by adding ether (25). For comparison of cells killed by heat, 1% Formalin, or 37% Formalin vapors, 12 CYE agar plates inoculated with Philadelphia 1 (F.G.) frozen inoculum were incubated at 25°C for 5 days. Cells of nine plates were pooled in distilled water to give a 10% suspension. Cells of one sample were heat killed, cells of a second were washed three times with distilled water and then heat killed, and the cells of a third sample were killed in 1% Formalin. The lids of the three remaining plates were raised and held partially open with masking tape; the plates were then exposed in a closed jar to the vapors of 37% Formalin for 48 h at room temperature. The cells were removed and washed three times with distilled water. The four lots of cells were adjusted to an OD of 1.00 (1cm, 660 nm), and the suspensions were diluted 1/20 for use in the IFMA.

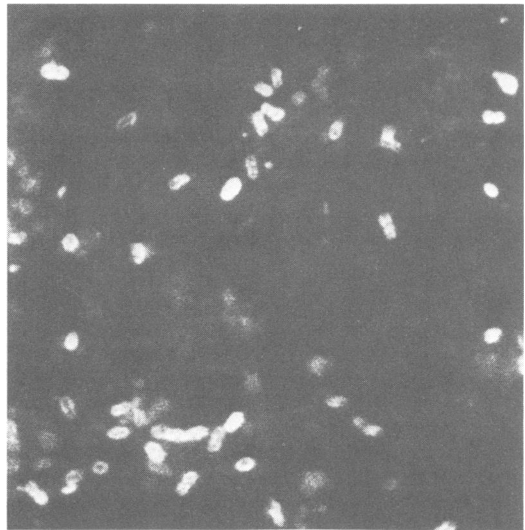

FIG. 1. Cells of *L. pneumophila* strain Philadelphia 1 (F.G.) grown on CYE agar; IFA test. Cells were standard lyophilized preparations. ×1,000.

Determination of antigenicity and RA. Antigenicity or antigenic reactivity was determined by the DFA procedure (4), the IFA procedure (24), and two types of IFMA procedures for antigen, indirect (I-IFMA) and direct (D-IFMA) (2, 22; L. Pine and R. F. Benson, Curr. Microbiol., in press). Slides from DFA and IFA tests were examined by microscope at ×1,000 magnification. Brightness of cells obtained with the DFA procedure, using FITC conjugates, were recorded subjectively from negative to 4+. The IFA analyses, using human sera B and 001, were recorded similarly, but the sera were serially diluted and the endpoint titers, taken as 1+ fluorescence (25), were determined. The I-IFMA and D-IFMA procedures were used to determine relative antigenicity (RA), i.e., the RA of one antigen as compared with another. The I-IFMA procedure was identical to that used for evaluation of human legionellosis sera (2) except the logarithm (ln) of fluorescence (*y* value) versus ln antibody dilution (*x* value) was used instead of a logit conversion (Pine and Benson, in press). The D-IFMA data were treated similarly. The slope of the line segment subjectively

TABLE 1. IFA titration of a human serum by diverse whole-cell antigens of *L. pneumophila*, serogroup 1

Strain	Medium[a]	Relative fluorescence at reciprocal antibody dilution[b] of:									
		16	32	64	128	256	512	1,024	2,048	4,096	8,192
Philadelphia-1	CYE	4	3	3	2	2	2	1	1	±	−
	Ristroph	1	1	1	1	1	1	1	−	−	−
	CDLp	4	3	3	2	2	2	1	±	−	−
Knoxville-1	Ristroph	2	1	1	1	±	±	−	−	−	−
	CDLp	4	3	3	2	1	2	2	±	−	−
	CDM	4	4	4	3	3	3	2	1	1	±
Bellingham-1	Ristroph	4	4	3	3	3	3	2	±	−	−
	CDLp	3	3	2	2	2	2	1	1	±	−
	CDM	4	4	4	3	3	3	1	1	1	1

[a] See text for composition of media.

[b] Underlined values of antibody dilution were taken as the endpoint titer; serum was B. Cells were heat killed.

determined to be in the linear portion of the curve for each sample was determined from the least-squares fit to that segment. For computation of the RA (10), the slopes of the standard and test samples were compared (t-test) and, when shown not to be significantly different, combined to form a common slope for the parallel line analysis. RA was calculated as the antilogarithm of the distance between the parallel lines on the $\ln x$ axis for any value of $\ln y$. Inference about RA, therefore, was restricted to those dilutions corresponding to the linear portions of the curves that were used in the analysis. RA values are given as decimal parts of one antigen as compared with the standard antigen given a value of 1.00.

Preparation of standard lyophilized *Legionella* antigen. Standard inocula of 0.1 ml of cell suspension were streaked over the surface of CYE agar plates, and the plates were incubated at 25°C in candle jars until it appeared that maximum growth had been attained (7 to 12 days). The plates were then removed, and the lids were raised slightly with masking tape; the plates were then placed in sealed jars containing an approximately 0.25 in. (ca. 0.64-cm) level of 37% Formalin and incubated at room temperature for 48 h. The plates were removed, the cells were removed from the surface with distilled water, and the cells were washed three times with distilled water by centrifugation at 2,700 × *g*. Cells were resuspended in 0.01 M phosphate-buffered saline (pH 8.0) containing 0.25% bovine serum albumin and 0.1% sodium azide, adjusting to an OD of 1.0 (660 nm, 1.0 cm). The suspension was then distributed in 1.0-ml volumes per 3-ml vial and lyophilized.

Absorption of serum. Eight vials of Philadelphia 1 and Bellingham 1 were rehydrated with 1.0 ml of distilled water each. All of the vials of each antigen were pooled and centrifuged at 10,000 × *g* for 30 min, the supernatant fluid was discarded, and 0.8 ml of serum 001 was added to each pellet (0.1 ml). The mixed suspensions were incubated at 37°C for 3 h and overnight at 4°C. The suspensions were centrifuged at 10,000 × *g* for 45 min; the supernatant fluid was removed and filtered through a 0.45-μm filter (Millipore Corp., Bedford, Mass.). Absorbed and unabsorbed sera were tested against strains Philadelphia 1 and Bellingham 1.

Additional procedures. Photography was done by J. D. Howard, Laboratory Training and Consultation Division, Laboratory Improvement Program, Centers for Disease Control. Photographs of comparative fluorescence were prepared by using the identical light exposure for all antigens except Philadelphia 1, for which an increased exposure was required to demonstrate the cells, as given below.

Protein was determined with the Folin phenol reagent (14); growth characteristics on agar or in liquid media were determined with a Beckman model B spectrophotometer, as described earlier (18).

RESULTS

General observations. Initially, our basic criterion for evaluating a *Legionella* antigen was its relative ability to titrate standard sera to a reproducible endpoint in the IFA test. However, diverse reactions were observed with the IFA test. First, it was immediately obvious that many lots of cells were not homogeneous in their staining reactivities and that it was possible for fluorescent brightness of the cells in any given field to be rated from a 4+ to a negative (Fig. 1). Second, it was apparent from the titration of a serum that certain populations of the cells would lose their 4+ fluorescence in a stepwise manner to an endpoint titer, whereas another antigen preparation would start at a lower, 2+ or 1+, fluorescence but would maintain this level of staining throughout most of the dilution series to give the same titer as its more brilliant counterpart (Table 1). In some cases, marked differences in serum titer were actual reflections of losses of antigenicity by certain lots of cells that stained poorly, if at all (Fig. 2). In all cases it was difficult to quantitate the antigenic differences by the IFA procedure, whereas the I-IFMA test

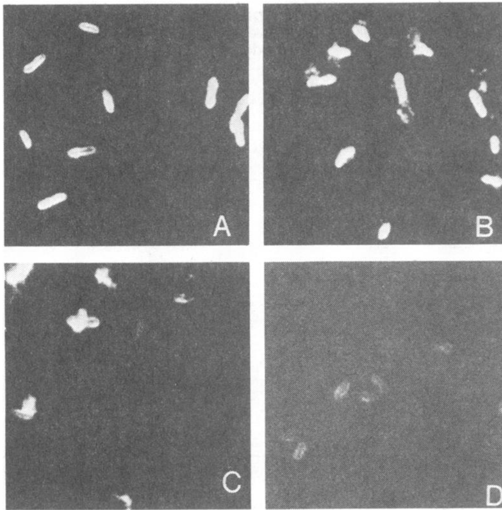

FIG. 2. Comparative IFA serological reactivity of whole-cell antigens of *L. pneumophila*, serogroup 1 (×1,000). All photographs were taken and developed under identical conditions except (D), which required additional exposure for development of the print. (A) Strain Bellingham 1, grown on CDM broth, killed with 1% Formalin; (B) strain Bellingham 1, grown on the CDM broth, heat killed; (C) strain Bellingham 1, grown on CYE agar, heat killed; (D) strain Philadelphia 1 (F.G.), grown on CDM broth, heat killed.

provided a quantitative result. The I-IFMA test permits a precise measure of the minimum plateau fluorescence and the maximum plateau fluorescence, calculation of the RA, and, in some cases, antigenic differences as shown by differences of the slope of the response curves.

Although the IFMA procedure permitted an evaluation of several quantitative aspects of the antigenic reactivity, it still measured the average fluorescence of the cell population. It did not evaluate the morphological aspects of homogeneity of the cell population necessary for good IFA antigens. Thus, all products were first examined by the DFA and IFA procedures and then by I-IFMA and D-IFMA procedures for particular preparations.

Effects of media, cultural conditions, and growth phase on antigenicity of *Legionella* strains. When transferred sequentially through the diverse media (see Materials and Methods), all cultures grew equally well. No one medium seriously affected the DFA straining of the various strains consistently. All rabbit FITC conjugates were adjusted to the maximum dilution, which gave 4+ staining of the reference antigens (15). Several cultures (Philadelphia 1, Togus 1, Chicago 2, Houston 2, Dallas 2E, and Dallas 1E) showed sporadic decreases in staining reactivity when grown on synthetic liquid media. The original antigenicity was regained when the strain was transferred to CYE agar.

Due to limited human sera, only *L. pneumophila* serogroups 1 through 4 were tested by the IFA test. Serious losses in the ability to titrate the test serum were observed in several cultures grown on synthetic liquid media, but the results were sporadic and no definitive effect of any one synthetic broth was noted (Table 1). On one or more of the synthetic broths, strains Knoxville 1, Philadelphia 1 (Fig. 2), Togus 1, Bloomington 2, and Los Angeles 1 showed moderately to severely decreased staining by IFA, although the cells continued to show 3+ and 4+ staining in the DFA test. The variability in the IFA staining observed for Philadelphia 1 on chemically defined broths was not observed with Bellingham 1 (Fig. 2; Table 1).

Effects of different agar media, phase of growth, and temperature of incubation on the RA of Philadelphia 1 were determined (Fig. 3 and 4). Each set of plates harvested at the times shown (Fig. 3) were killed with 1% Formalin and analyzed by the I-IFMA test (Fig. 4; Table 2). Cells grown on the CYE agar had an RA two to four times that obtained on the CDLp agar, with or without activated charcoal (Table 2, experiment 5). As judged by a decreased slope and lower maximum fluorescence, cells grown on F-G agar showed less antigenicity than CYE-grown cells (Fig. 4; Table 2). Examination of the cells at different phases of growth on each of the different media showed no major fluctuations of RA, nor was there any suggestion that any particular phase of growth on any of the media had cells of a markedly different antigenicity (Table 2). In general, cells entering the stationary phase appeared to have maximum antigenicity.

Recently, a CYE agar buffered with *N*-(2-acetamido)-2-aminoethanesulfonic acid was described (17). It has been our experience, and that of others (5), that buffered medium gives a much more rapid growth of most strains than CYE agar; these two media were compared for antigen production. The RA ratios (buffered CYE/CYE) obtained with serum 001 and standard antigens of Philadelphia 1, Philadelphia 4, Bellingham 1, and Knoxville 1 were 0.90, 1.04, 1.52, and 1.02, respectively, indicating no major differences in RA. Both media are therefore recommended for antigen production.

There was no clear demonstration in these experiments (Table 2, experiment 1) or in ancillary IFA tests that cells grown at either 25 or 35°C had differences in antigenicity. However, at 35°C, the CDLp broth and, to a lesser degree, the F-G agar caused abundant filament formation by several strains; fewer filaments were formed on CYE agar. However, remarkably

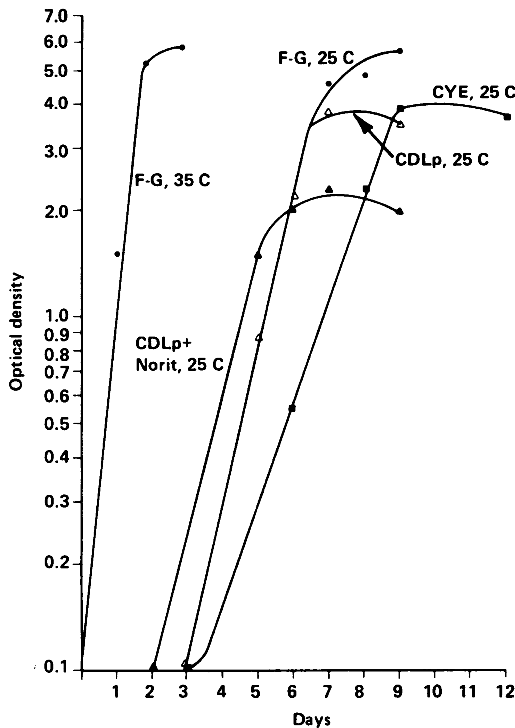

FIG. 3. Comparative growth of *L. pneumophila* Philadelphia 1 (F.G.) on different agar media at 25 or 35°C. CYE agar was prepared with 1.5% agar, whereas the CDLp agar, with or without Norit, contained 1% ion agar. Three or four plates were harvested at each time, and the cells from each plate were suspended in 10 ml of distilled water. The average OD (660 nm, 1.8 cm) is given for each point.

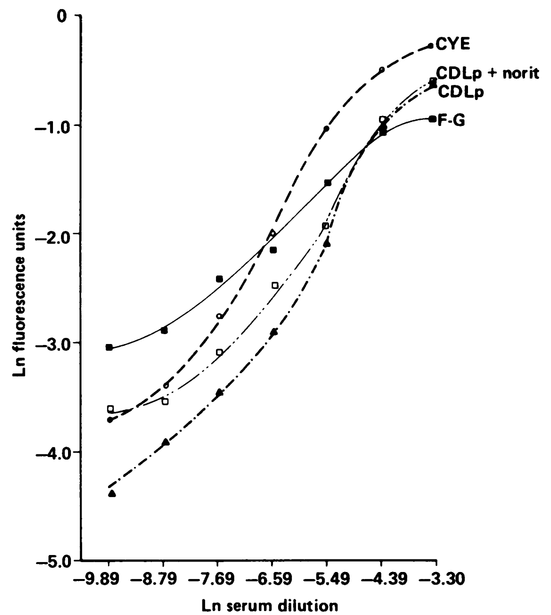

FIG. 4. Relative I-IFMA fluorescence of *L. pneumophila* cells Philadelphia 1 (F.G.), grown on different media at 25°C. Cell suspensions were those of Fig. 3 and Table 2 (experiment 5 and experiment 1, 9 days).

homogeneous suspensions of small single or double cells or both were obtained at 25°C with all media.

All antigen preparations were adjusted to an OD of 1.00 before RA determinations, although we suspected that a closer correlation might exist between RA and protein content. However, determinations of protein relative to RA showed no obvious correlations, although some compared products differed by as much as 40% in their relative protein content (Table 2, experiments 1 and 5).

The effects of growth in air, in air plus carbon dioxide, and in candle jars on the antigenicity of Philadelphia 1 cells were determined with the IFA test. No differences in antigenicity were observed under any particular atmospheric condition.

On the basis of the above results, we used CYE agar at 25°C for experiments relative to the production of a standard lyophilized antigen; growth in a candle jar was chosen arbitrarily.

Effects of killing procedures on RA. For use as antigens, the cells are generally killed with heat (25) or Formalin (11, 13) and then washed one or more times. In several experiments, we compared the relative reactivity of cells in the IFA test when killed by heat, different concentrations of Formalin, Formalin vapors, or diethyl ether. Ether-killed cells showed high background fluorescence and many morphological aberrations such as that reported previously (25). Experiments comparing heat-killed versus Formalin-killed cells showed no marked differences in reactivity in the IFA or I-IFMA test. In an I-IFMA test comparing heat-killed cells, cells washed three times and heat killed, cells killed with 1% Formalin, and those killed with 37% Formalin vapors, the RA values were 1.24, 1.26, 0.92, and 1.00, respectively. However, cells killed by 1% Formalin or by Formalin vapors showed a more clearly defined cell without surface debris or irregularities (Fig. 2). Washing either heat-killed or Formalin-killed cells with distilled water did not alter the relative fluorescence of the cells, although large amounts of background staining material previously observed in the IFA test were removed. In these experiments, little differences were seen in the relative reactivity of live cells that were heat fixed on the slide, heat-killed cells, cells killed by 1% Formalin, or cells killed by Formalin vapors. However, in one experiment with the F.G., R.B., and GPSp strains of Philadelphia 1, large numbers of flagella were seen on those

TABLE 2. Effect of temperature, growth phase, and agar medium on the RA of *L. pneumophila* strain Philadelphia 1 (F.G.)[a]

Expt	Medium[b]	Temp (°C)	Days grown	Protein (mg/ml)[c]	Fluorescence maximum	Slope (LB, UB)[d]	RA[e]
1	F-G	35	1.0	320	0.396	0.419 (0.357–0.481)	0.80
			1.8	335	0.410	0.492 (0.460–0.525)	1.17
			2.8	392	0.414	0.474 (0.440–0.507)	1.00*
	F-G	25	7.0	240	0.374	0.464 (0.384–0.544)	1.38
			8.0	238	0.397	0.437 (0.407–0.467)	1.61
			9.0	268	0.411	0.394 (0.369–0.418)	1.41
2	CYE	25	6.0	262	0.543	0.636 (0.591–0.682)	0.75
			7.0	267	0.789	0.692 (0.654–0.730)	1.00*
			9.0	260	0.759	0.694 (0.646–0.741)	0.68
			10.0	280	0.746	0.730 (0.685–0.776)	0.77
3	CDLp	25	5.0	275	0.410	0.837 (0.732–0.942)	0.99
			6.0	280	0.564	0.757 (0.696–0.818)	1.00*
			7.0	295	0.549	0.718 (0.626–0.811)	0.89
			9.0	295	0.473	0.750 (0.713–0.786)	0.77
4	CDLp + Norit	25	5.0	295	0.570	0.644 (0.577–0.710)	1.08
			6.0	295	0.564	0.631 (0.566–0.696)	1.00*
			7.0	270	0.563	0.541 (0.467–0.615)	1.03
			9.0	257	0.539	0.588 (0.546–0.631)	1.04
5	CYE	25	7.0	267	0.789	0.713 (0.664–0.762)	1.00*
	CDLp		6.0	280	0.546	0.753 (0.693–0.813)	0.37
	CDLp + Norit		6.0	295	0.564	0.626 (0.556–0.696)	0.43

[a] Culture history and basic fluorometric data are given in Fig. 3 and 4. Cells were killed with 1% Formalin.
[b] See text for composition of media.
[c] Protein per milliliter of bacterial suspension (OD = 1.0).
[d] LB, UB, Lower and upper 95% confidence bounds of the slope. Experiments 1 and 2 were done at threefold dilution of 1/81 to 1/6,561 dilutions of serum 001; dilutions for experiments 3 to 5 were made at 1/81 to 1/2,187, inclusively. Cells killed with 1% Formalin were washed three times with water, adjusted to an OD of 1.0 (1.0 cm, 660 nm), and diluted 1/24. Coefficients of correlation for all experiments ranged from 0.961 to 0.998.
[e] Standard sample has asterisk.

cells of the GPSp strain that were killed by Formalin vapors. No other preparations showed flagella. The flagella were maintained, although in decreasing numbers, through three wash cycles. Because of the potential of Formalin vapors to fix surface antigens in situ, this procedure was the one used routinely to prepare lyophilized cells for use in the I-IFMA procedure.

Lyophilization of whole-cell antigens. Initial tests with Formalin-killed cells (Philadelphia 1) and cells killed with Formalin vapors showed that these cells, when lyophilized in water or phosphate-buffered saline, could not be reconstituted with distilled water, 0.1% glycine, or 0.015% Tween 20 to give a good suspension; large flat sheets of cells were formed that did not give homogeneous suspensions even with strong agitation. On the basis of the results of Redway and Lepage (19), we tested suspending media of 4% mannitol, inositol, glycerol, and soluble starch as media for lyophilization, but without success. Colored products were formed with inositol and mannitol, and the antigenic reactivity (IFA test) was reduced in the presence of glycerol, inositol, and starch. Additional tests with 0.05 and 0.25% bovine serum albumin, 0.2% glycine, or glutamic acid as the suspending medium for lyophilization gave cells that were readily brought into suspension with water. However, small reductions in brilliance (IFA test) were observed in all lyophilized preparations. A suspending medium of 0.25% bovine serum albumin with 0.1% NaN_3, in phosphate-buffered saline (0.01 M, pH 8.0) was chosen for freeze-drying standard antigens. With this suspending medium we have observed no loss in antigenicity of freeze-dried preparations stored for 1 to 2 years at 5°C or in cells reconstituted with water and stored at 5°C for several weeks.

Strain differences exposed by diverse sera. During these studies it rapidly became apparent that variation of RA with changes in procedure was primarily strain dependent. Whereas Philadel-

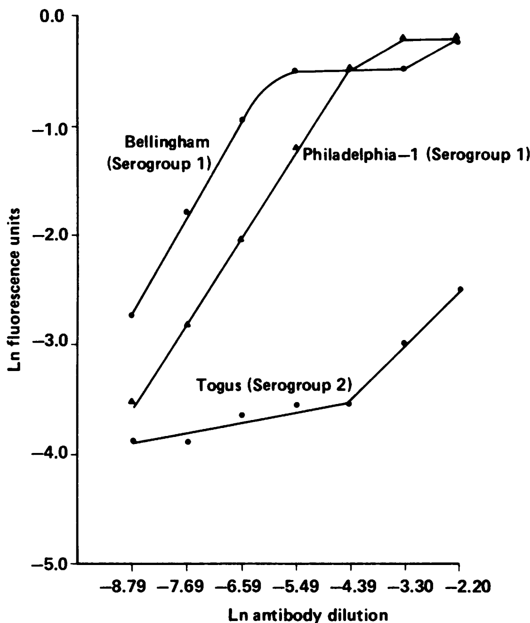

FIG. 5. Response curves of *L. pneumophila* antigens, serogroups 1 and 2 (I-IFMA test), to human serum 001.

phia 1 showed marked variation with changes in media and growth conditions, Bellingham 1 did not (Fig. 2; Table 1). These results were obtained with sera 001, A, and B. Although serum 001 came from a patient infected with Detroit 1, a Bellingham-like organism (15), this association was not true for the other sera.

The relative antigenicity of strains Philadelphia 1 (F.G.) and Bellingham 1 were compared further, using FITC-conjugated rabbit immunoglobulin and conjugated and nonconjugated human sera in the DFA, D-IFMA, IFA, and I-IFMA tests. Philadelphia 1, as described (15), reacted much more strongly than Bellingham 1 (D-IFMA) with the conjugated rabbit immunoglobulin (RA = 17). However, with human serum 001, A, or B, the RA of Bellingham 1 to Philadelphia 1 ranged from 3.72 to 4.38; serogroup 2 antigen was essentially unreactive with these human sera at dilutions >1/27 (Fig. 5). Although absorption of sera 001 and B (data not shown) by Bellingham 1 greatly depleted these sera of reactive antibody, absorption with Philadelphia 1 left antibody highly reactive for Bellingham 1 (RA = 19.24) (Table 3).

DISCUSSION

Although we observed small reductions in brilliance with the IFA test after the cells were lyophilized, the antigens Philadelphia 1 (F.G. and R.B.) were comparable to the *Legionella* inventory antigen of the Biological Products Program. For production of a standard antigen, our results emphasized the need to choose a strain that was consistent in its responses to production procedures. The Formalin-killed, lyophilized cells showed no surface or morphological aberrations when reconstituted with water and were antigenically stable in suspension for several weeks at 5°C. Cells of *Legionella* grown on the CYE agar for 9 to 12 days at 25°C were small, of a homogeneous population of single or double cells, and without filaments. All of these characteristics were basically essential for the I-IFMA test. As stated earlier, the I-IFMA test used for serum analysis eliminates the use of the egg yolk sac membrane, permits a precise quantitative fluorescent readout, automatically records an average fluorescence of the suspended cell population, and permits a precise calculation of antibody potency (2, 22; Pine and Benson, in press). Its potential for use with a pooled antigen remains to be explored (25).

Although a wide variety of media and cultural conditions have been used for growth of *Legionella*, the effects of media on the production of antigen that reacts with fluorescein conjugates are not clearly defined. In general, chemically defined liquid media lead to poorer antigen production by Philadelphia 1 and sporadic losses in other strains. Initial results obtained with cells grown on F-G and CYE agars led to the impression that growth on F-G agar, although poorer than that on CYE agar, gave cells of greater IFA brilliance. This impression, however, was not supported by several tests that used the I-IFMA procedure, and in the range of serum dilutions where the responses were linear, CYE-grown cells were superior. The three chemically defined media differed radically in their relative amounts of amino acids, trace element composition, and presence or absence of vitamins. Growth was equally good in all of these media. Although sporadic losses of antigen occurred, there was no suggestion that a compound of any one medium regulated production of the IFA reacting antigen; little variation among the diverse strains was observed during growth through several transfers in the three chemically defined media and back to the complex organic media.

We considered the possibility that loss of antigen by Philadelphia 1, when grown on chemically defined media, was due to curing of a plasmid-mediating gene. However, at present, plasmids have not been observed in this strain (1). Strain Bellingham 1, however, did not show fluctuation of surface antigens and appeared to maintain one or more serogroup-specific antigens not observed on Philadelphia 1 with human sera. Other workers have reported large antigenic differences among strains of the same sero-

TABLE 3. Differences in RA of strains Bellingham and Philadelphia 1 with human convalescent serum (I-IFMA test)

Expt	Serum	Serum dilution range[a]	Serum treatment	Antigen tested	Fluorescence maximum	Slope (LB, UB)[b]	RA[c]
1	001	1/729 to 1/2,187	None	Bellingham	0.847	0.822 (0.584–1.061)	3.72
				Philadelphia-1	0.806	0.656 (0.593–0.718)	1.00*
2	001	1/9 to 1/1,944	Bellingham	Bellingham	0.594	0.895 (0.881–0.909)	ND
			absorbed	Philadelphia-1	0.329	0.806 (0.768–0.844)	ND
3	001	1/9 to 1/1,944	Philadelphia	Bellingham	1.622	0.860 (0.764–0.956)	19.24
			absorbed	Philadelphia-1	0.256	0.736 (0.690–0.781)	1.00*

[a] The values are those dilutions used to determine RA. All sera were diluted 1/3 or 1/6 in the range of 1/9 to 1/6,561, inclusively.

[b] LB, UB, Lower and upper 95% confidence bounds of the slope. The antigen dilutions for experiments 1, 2, and 3 were 1/24, 1/10, and 1/10, respectivelv.

[c] Standard sample has asterisk. When confidence bounds of the slopes did not overlap, RA could not be determined (ND).

group as distinguished by sera of immunized rabbits (15) or sera of infected guinea pigs (16).

Ether-, Formalin-, and heat-killed cells have been used for the IFA test (11, 12, 24); however, ether cannot be used to prepare Togus 1 antigen (25). Ether- and heat-killed cells clearly showed physical disruption of the antigenically reactive cell's surface; this effect either was not present or was much less with cells killed by Formalin vapors (Fig. 2). Lattimer and Cepil (13) have claimed that 2% Formalin-killed *L. pneumophila* cells gave a more sensitive and specific test antigen when used with normal egg yolk sac. Recently, Wilkinson and Brake (23) have shown that comparison of heat-killed and Formalin (10%)-killed cells as test antigens gave results that were interpreted the same for 96% of 60 paired sera if the cutoff level used to interpret a positive test for the formalinized antigen was lowered one twofold dilution factor. Johnson et al. (12) have demonstrated that simple washing removes soluble antigen that is serogroup specific in its inhibition of microagglutination tests and in its opsonic activities. This antigen is associated with the cell wall (7). Elliott and Johnson (6) have demonstrated that flagella, produced by cells grown on CYE agar, are serologically reactive but are not serogroup specific. Although Formalin treatment of the cells would possibly prevent loss of the water-soluble component, it would also fix the cross-reacting flagella. Washing cells directly from the plate into 1% Formalin might also bond extraneous interfering antigens of the medium to the cells; unfortunately, none of the chemically defined media qualified as a substitute for the CYE agar. Killing the cells with Formalin vapors eliminated certain of these considerations, and this procedure gave us our most suitable lyophilized preparations of Philadelphia 1 (2). However, this strain (F.G.) showed no flagella, whose presence could have increased nonspecific staining and necessitated dilution of serum beyond a useful range. The strain (GPSp) that did show flagella by this procedure was poorly antigenic in the I-IFMA test. Our data suggest that the nonflagellated laboratory strain may have greater relative antigenicity with serum from human patients than the flagellated or nonflagellated strain.

Testing of sera A and 001 in the I-IFMA procedure and six additional human sera (unpublished data) with Philadelphia 1 and Bellingham 1 showed that Bellingham 1 was more reactive than Philadelphia 1 for seven of the sera. Absorption of sera A and 001 with Philadelphia 1 and Bellingham 1 suggest that Bellingham 1 contains a serogroup 1 immunogen reactive in human infections that is only minimally present on Philadelphia 1. These results emphasize the differences between using immune serum of rabbits and human convalescent serum for evaluation of the antigen.

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