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Agglutinating Antibody Titers to Members of the Family *Legionellaceae* in Cystic Fibrosis Patients as a Result of Cross-Reacting Antibodies to *Pseudomonas aeruginosa*

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The objective of this study was to evaluate the prevalence and significance of antibody titers to organisms in the family *Legionellaceae* in 128 serum samples collected from cystic fibrosis patients at routine examinations. Antibody titers were determined for 10 antigenic types of *Legionellaceae*; *Legionella pneumophila* serogroups 1 to 6, *Fluoribacter* (*Legionella*) *bozemanae*, *Fluoribacter* (*Legionella*) *dumoffii*, *Fluoribacter* (*Legionella*) *gormanii*, and *Tatlockia* (*Legionella*) *micdadei*. The method of antibody titer determination was the microagglutination test. Elevated titers (≥1:64) to one or more antigens were found in 41.3% of cystic fibrosis patients but in only 9.7% of 103 normal control subjects ($P < 0.01$). Titers to 8 of the 10 antigens were directly correlated with the number of *Pseudomonas aeruginosa* precipitating antibodies in patient sera, as determined by crossed immunoelectrophoresis (correlation coefficients, ≥0.74). Cross-reactions between *P. aeruginosa* and *L. pneumophila* were substantiated by crossed immunoelectrophoresis of hyperimmune rabbit serum as well as patient sera against *P. aeruginosa* and *Legionellaceae* antigens. Monospecific antibody to the "common antigen" of *P. aeruginosa* was used to demonstrate the presence of this antigen in *L. pneumophila*. The presence of cross-reacting antibodies in cystic fibrosis patients chronically infected with *P. aeruginosa* emphasizes the need for cautious interpretation of antibody titers to members of the family *Legionellaceae*.

Bacteria in the family *Legionellaceae* have emerged in recent years as important opportunistic respiratory pathogens, particularly in immunocompromised persons. Children with cystic fibrosis (CF) have a marked susceptibility to bacterial infections of the respiratory tract. Petersen et al. (21) have reported that 76% of exacerbations of respiratory disease in 116 CF patients were associated with bacteria, most frequently *Pseudomonas aeruginosa*. Of the exacerbations of respiratory disease, 20% were associated with viruses, mycoplasma, or chlamydia, although bacteria were also present in most of these cases, and no etiology was established in 18% of the exacerbations. Studies by Katz and Holsclaw (16) and Efthimiou et al. (5a) have reported that CF patients have a higher than normal prevalence of antibody to *Legionella pneumophila*, using the indirect fluouescent antibody test, and suggested that CF patients acquire *Legionella* infections more often than normal persons. The purpose of the present study was to evaluate the prevelance and specificity of antibodies to members of the family *Legionellaceae* in serum samples from 128 CF patients by use of a different serological test and a broader range of antigens from *Legionellaceae* than in the previous studies.

MATERIALS AND METHODS

Serum specimens. Serum specimens from 128 CF patients were obtained during routine visits to the Danish Cystic Fibrosis Center, Copenhagen, Denmark, in 1980, regardless of the clinical history of the patient. Diagnosis of CF was established as previously described (12). Serum samples from 103 normal children and young adults were obtained from the serum bank of the State Serum Institute, Copenhagen, Denmark, for use as controls. The age and sex distributions of persons included in this study are shown in Table 1.

Agglutinating antibodies to members of the family *Legionellaceae*. The microagglutination (MA) test of Farshy et al. (6) was used with minor modifications as described in a previous report from our laboratory (3). The initial serum dilution used in this study was 1:2 instead of 1:4, 1:8, or 1:10 as described in other reports (7, 18, 23). Antigens for each strain of *Legionella*, *Fluoribacter*, and *Tatlockia* were grown on buffered charcoal-yeast extract agar (20). Carbol-fuchsin (final concentration, 0.002%) was used instead of safranin to increase the visibility of the agglutination reaction. All serum samples were tested against the six serogroups (SG) of *L. pneumophila* (SG 1, Philadelphia 1 strain; SG 2, Togus 1 strain; SG 3, Bloomington 2 strain; SG 4, Los Angeles 1 strain; SG 5, Dallas 1E strain; SG 6, Chicago 2 strain) and *Fluoribacter* (*Legionella*) *bozemanae* Wiga, *Fluoribacter* (*Legionella*) *dumoffii* NY-23, *Fluoribacter* (*Legionella*) *gormanii* LA 12-13, and *Tatlockia* (*Legionella*) *micdadei* Tatlock.

Precipitating antibodies to *P. aeruginosa*. All CF patient serum samples were tested for the number of precipitins against sonicated whole-cell antigens of *P. aeruginosa* by crossed immunoelectrophoresis (XIE) as described by Høiby and Axelsen (12). Serum samples from normal persons in this study were not tested for *P. aeruginosa* precipitins since previous studies have documented that no precipitating antibodies to *P. aeruginosa* occur in the sera of children up to the age of puberty, and only one precipitin was found in only 6% of adult sera (8, 11, 15).

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TABLE 1. Age and sex distribution of CF patients and normal persons included in the study

Patient	No. with the following ages (yr)				Median age[a]
	<5	6–10	11–20	20–30	
CF					
Male	14	22	21	9	10
Female	27	12	18	5	8
Normal					
Male	6	5	31	14	12
Female	7	8	14	18	14

[a] Total median age for CF patients was 9 years, and the total median age for normal subjects was 13 years.

Immunoelectrophoretic methods for analysis of cross-reactions between *P. aeruginosa* and *L. pneumophila*. XIE was performed with intermediate gels as described previously (1, 26). Intermediate gels contained either saline (0.154 M), patient serum, or purified rabbit immunoglobulins. Rabbit immunoglobulins specific for the "common antigen" of *P. aeruginosa* were obtained from a previous study reported from our laboratory (25). The antigen-antibody XIE reference system employed for *P. aeruginosa* was described previously by Høiby (9). An analogous XIE reference system for *L. pneumophila* SG 1 developed in our laboratory was also used (2).

Electrophoresis was performed in 1% agarose (depth, 1.5 mm) on glass slides (5 by 7 cm). Litex type LSM agarose (Litex, Copenhagen, Denmark), with a relative endosmosis value of 0.17 ± 0.01 and a gelling temperature of 35 to 37°C, was employed. Barbital buffer (pH 8.6; ionic strength, 0.02) was used for agarose preparation and as the running buffer during all electrophoretic procedures. First-dimension electrophoresis was performed at 12°C, applying 10 V/cm until a bromphenol blue-labeled human albumin marker migrated 35 mm (approximately 45 min). Second-dimension electrophoresis was performed at 12°C, applying 1.5 V/cm for 18 h. Patient serum or purified rabbit immunoglobulins were incorporated into the intermediate gels at 18.6 μl/cm^2. The agarose gels were stained with Coomassie brilliant blue.

Statistical methods. CF patients were grouped by the number of *Pseudomonas* precipitins in their sera. Geometric mean titers to each antigen for each CF patient group were calculated and plotted. Correlation coefficients between mean numbers of *Pseudomonas* precipitins and mean *Legionellaceae* titers were calculated by standard methods (24). The Student *t* test was used for comparison of means (24).

RESULTS

The distribution of antibody titers to 10 antigenic types of *Legionellaceae* in CF patients and normal persons is shown in Table 2.

MA titers of ≥1:64 were considered positive, which is consistent with the criterion set by other workers with the MA test (6, 18). Positive MA titers to one or more of the *Legionellaceae* antigens were found in 41.3% of the CF patient sera, but in only 9.7% of normal human sera from persons of the same age ($P < 0.01$). The highest rate of positive titers in CF patients occurred with antigens from *L. pneumophila* SG 2 (7.2%), *F. bozemanae* (35.6%), *F. dumoffii* (10.3%), and *T. micdadei* (3.2%).

Geometric mean MA titers were calculated for the controls and for four groups of CF patients based on the number of serum *P. aeruginosa* precipitins. A total of 56, 26, 17, and 27 CF patients had 0 to 1, 2 to 10, 11 to 20, and >20 *P. aeruginosa* precipitins, respectively. Mean MA titers for each group are depicted in Fig. 1. Mean MA titers to 5 of 10 *Legionellaceae* antigens were significantly higher than normal in CF patients with ≥11 *P. aeruginosa* precipitins ($P < 0.01$). The magnitude of the titers was directly correlated to the number of *P. aeruginosa* precipitins for 8 of 10 antigens (correlation coefficients, ≥0.74). Correlation coefficients between the mean MA titers to *F. dumoffii* or *T. micdadei* and the mean numbers of *P. aeruginosa* precipitins for each group of CF patients was 1.00.

Antibody titers to *F. bozemanae* were elevated most dramatically in CF patients, with all *P. aeruginosa* precipitin groups having mean MA titers higher than normal ($P < 0.01$). The correlation coefficient between MA titers and *P. aeruginosa* precipitins for this antigen was 0.91. The group of CF patients with >20 *P. aeruginosa* precipitins had a mean MA titer to *F. bozemanae* equal to eight times that of the normal controls (mean MA titer of 92.7 versus 20.3). Two CF patients with 31 and 37 *P. aeruginosa* precipitins had MA titers to *F. bozemanae* of 1:1,024.

Cross-reaction between *P. aeruginosa* and *L. pneumophila* was substantiated by XIE of the *L. pneumophila* reference system with CF patient sera or purified immunoglobulins to *P. aeruginosa* in the intermediate gels (Fig. 2A, C, and E). Figure 2C illustrates that a CF patient with precipitating antibodies to >20 *P. aeruginosa* antigens also precipitated antigen 66 of *L. pneumophila*. Purified immunoglobulins from rabbits immunized to *P. aeruginosa* reacted similarly, precipitating antigen 66 plus at least five additional *L. pneumophila* antigens (Fig. 2E). Precipitation of antigen 66 by heterologous antibody caused a separation of the precipitin arch into two components, indicating some heterogeneity of this antigen as previously described (2).

Reciprocal cross-reactions were evaluated by use of the *P. aeruginosa* XIE reference system with CF patient serum or

TABLE 2. Distribution of MA titers against *Legionella* species, *Fluoribacter* species, and *Tatlockia* species in sera from CF patients and normal persons

Antigen and serogroup in the following subjects:		% with a titer of:					
		≤8	16	32	64	128	≥256
CF (n = 128)							
L. pneumophila	SG1	98.4	0	1.6	0	0	0
L. pneumophila	SG2	55.6	20.6	16.6	5.6	1.6	0
L. pneumophila	SG3	87.3	7.1	4.8	0	0.8	0
L. pneumophila	SG4	98.4	1.6	0	0	0	0
L. pneumophila	SG5	94.4	4.8	0.8	0	0	0
L. pneumophila	SG6	95.2	3.2	1.6	0	0	0
F. bozemanae		23.0	17.6	23.8	23.0	6.3	6.3
F. dumoffii		61.2	19.8	8.7	6.3	2.4	1.6
F. gormanii		99.2	0.8	0	0	0	0
T. micdadei		80.1	11.9	4.8	2.4	0.8	0
Normal (n = 103)							
L. pneumophila	SG1	96.8	0	3.2	0	0	0
L. pneumophila	SG2	58.1	30.6	9.7	1.6	0	0
L. pneumophila	SG3	98.4	1.6	0	0	0	0
L. pneumophila	SG4	100.0	0	0	0	0	0
L. pneumophila	SG5	95.2	4.8	0	0	0	0
L. pneumophila	SG6	91.9	4.9	1.6	1.6	0	0
F. bozemanae		64.5	19.4	12.9	3.2	0	0
F. dumoffii		69.4	19.4	4.8	3.2	3.2	0
F. gormanii		100.0	0	0	0	0	0
T. micdadei		98.4	1.6	0	0	0	0

purified immunoglobulins to *L. pneumophila* in the intermediate gels (Fig. 2B, D, and F). Figure 2D shows an example of precipitation of >20 *P. aeruginosa* antigens by serum from a CF patient chronically infected with *P. aeruginosa*. Figure 2F demonstrates that purified rabbit immunoglobulins against *L. pneumophila* precipitated six *P. aeruginosa* antigens with the most prominent precipitin being that designated as antigen 10 in the *P. aeruginosa* XIE reference system (9).

L. pneumophila antigen 66 and *P. aeruginosa* antigen 10 have been previously shown to cross-react with a wide range of gram-negative bacteria and have been therefore designated common antigens (4, 14). Figure 3A and B demonstrate the precipitation of both *L. pneumophila* antigen 66 and *P. aeruginosa* antigen 10 in the intermediate gels by antibody to the common antigen of *P. aeruginosa* developed by Sompolinsky et al. (25). This antibody preparation precipitated *L. pneumophila* antigen 66 as a single arch with a curiously shaped right leg. We believe this indicates that although there is some physiochemical heterogeneity of this antigen, there are also antigenic determinants in common to both the left and right legs of the precipitin arch.

DISCUSSION

The MA test was simple, rapid, and easy to perform, which thus facilitated the testing of a large number of serum samples against 10 *Legionellaceae* antigens. MA titers in the control population of normal children and young adults were similar to those described for *L. pneumophila* SG 1 to 4 in adults by other investigators (3, 7, 18, 22). MA titers to *L. pneumophila* SG 5 and 6, the three *Fluoribacter* species, and *T. micdadei* have not been reported previously. Our results indicate that there are important differences in the prevalence of antibodies to the different antigenic types of *Legionellaceae* in the normal population and that elevated antibody titers ocur more frequently to *F. bozemanae* and *F. dumoffii* than to the other eight antigens tested.

Cross-reactions between *Legionella* species and other bacteria have been previously reported by use of immunofluorescence techniques (5, 19, 27, 28). Wilkinson et al. have demonstrated that these cross-reacting antibody titers could be distinguished from specific antibody titers by absorption with an extract from *Escherichia coli* (27). Using the MA test, Klein has observed that serum samples with elevated titers to *Pseudomonas pseudomallei* had a significantly greater incidence of *L. pneumophila* antibody titers ≥1:32 as compared with sera with negative titers for *P. pseudomallei*, and he has suggested that these two organisms share one or more cross-reactive antigens (17).

Naturally occurring antibodies to members of the family *Legionellaceae* in normal populations may be the result of antibodies directed against cross-reactive antigens such as the common antigen of *P. aeruginosa*. Høiby et al. (14) have demonstrated that there was considerable variation between persons in their response to exposure to the common antigen. This was postulated to be due to genetic differences in the immune response or an immunological tolerance phenomenon.

The organism which most frequently infects CF patients is *P. aeruginosa* (21). As *P. aeruginosa* infections persist or reoccur, CF patients produce antibodies to a steadily increasing number of *P. aeruginosa* antigens (13). Over 70% of

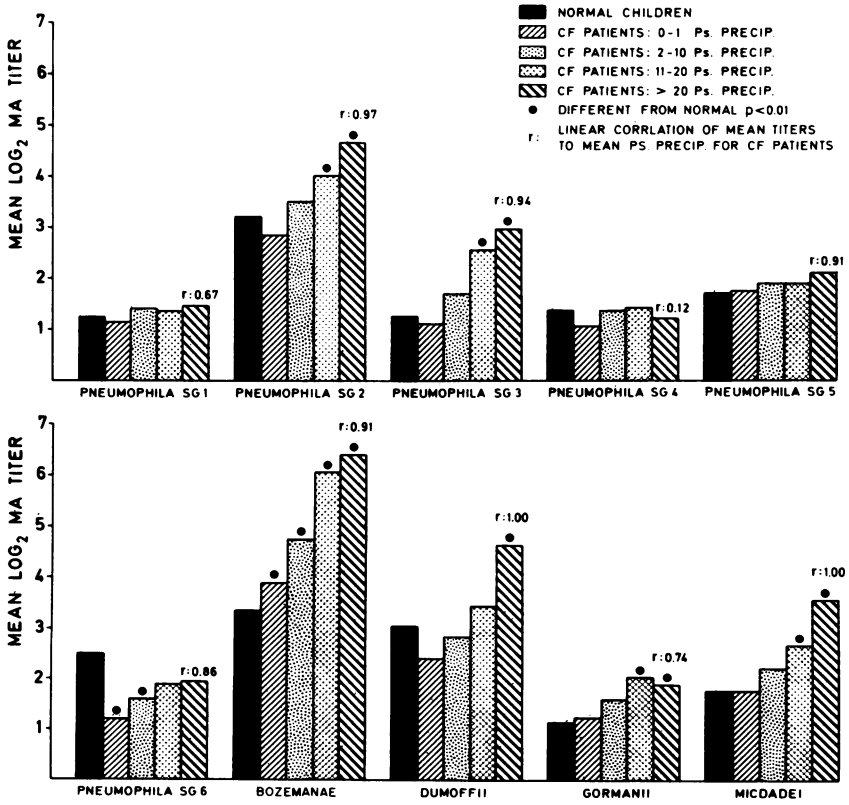

FIG. 1. Mean antibody titers to members of the family *Legionellaceae* in normal subjects and CF patients grouped by the number of *P. aeruginosa* precipitins (Ps. precip.) in their serum samples.

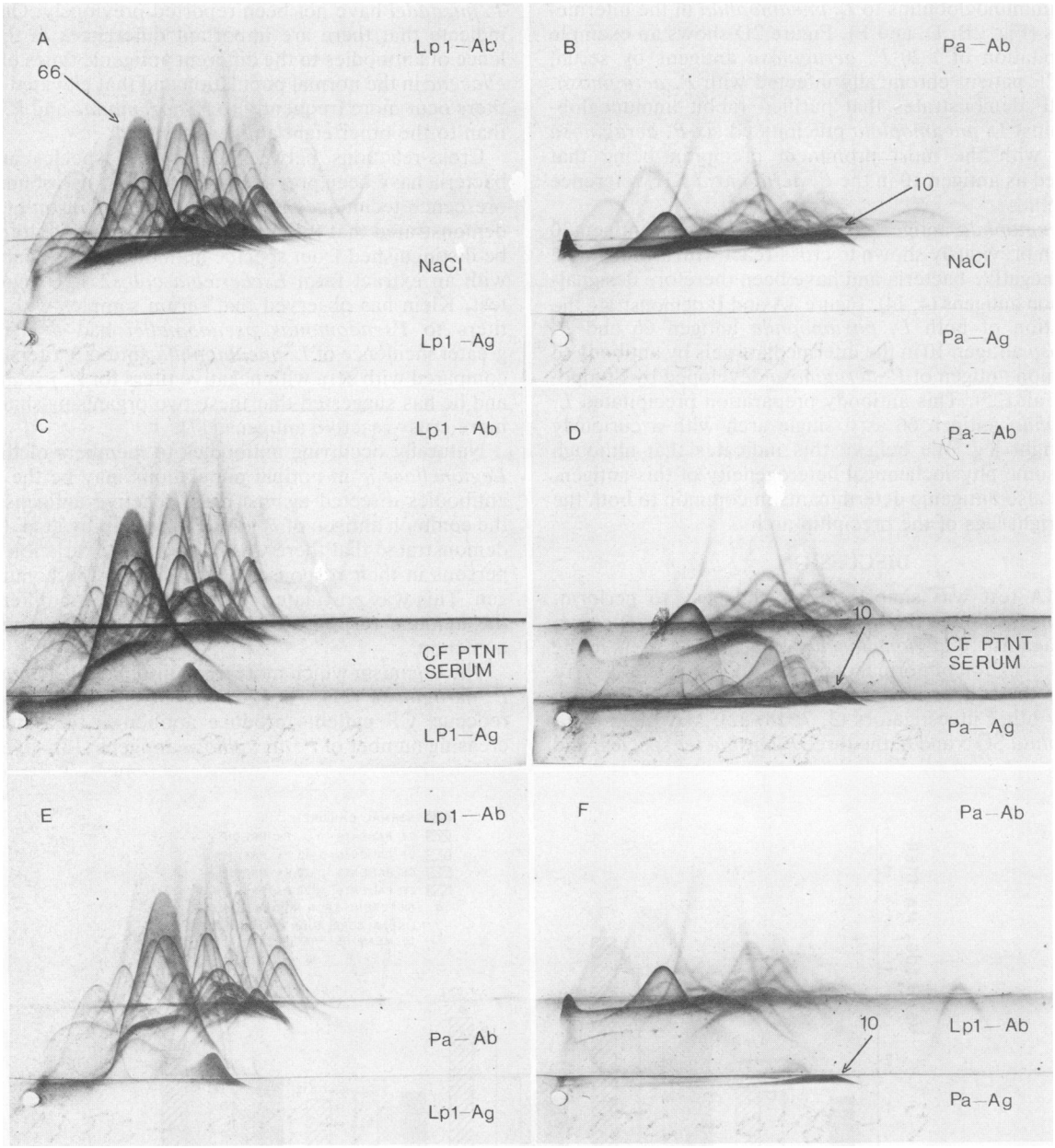

FIG. 2. XIE of *L. pneumophila* and *P. aeruginosa*. (A, C, and E) *L. pneumophila* SG 1 antigen (Lp1-Ag) (3 μl) and corresponding purified rabbit immunoglobulins at 4.8 μl/cm^2 (Lp1-Ab). (B, D and F) *P. aeruginosa* antigen (Pa-Ag) (1 μl) and corresponding purified rabbit immunoglobulins at 14.3 μl/cm^2 (Pa-Ab). (A and B) NaCl (100 μl) in the intermediate gels. The control reference patterns have been described and numbered previously for *L. pneumophila* SG 1 (2) and *P. aeruginosa* (9). (C and D) CF patient (ptnt) serum (100 μl) in the intermediate gels. CF patient antibodies precipitated antigens of both *L. pneumophila* and *P. aeruginosa* in the intermediate gels, but far more precipitins were present to *P. aeruginosa* than to *L. pneumophila* in the CF patient serum. (E) *P. aeruginosa* antibody (200 μl) in the intermediate gel. Polyspecific *P. aeruginosa* reference antibody precipitated seven *L. pneumophila* antigens in the intermediate gel as evidenced by extended precipitin legs. The most prominent of these cross-reacting antigens was that labeled 66. (F) *L. pneumophila* SG 1 antibody (200 μl) in the intermediate gel. The reciprocal cross-reaction of that in (E) is illustrated with *L. pneumophila* reference antibody in the intermediate gel. Antigen 10 is strongly precipitated at the base of the intermediate gel, and six other antigens show extended precipitin legs (not all weak cross-reactions may be evident in the photo reproduction of this gel).

the CF patients chronically infected with *P. aeruginosa* produce antibodies to the common antigen of this organism (14). This common antigen cross-reacts with most gram-negative bacteria (10). In the present study, we demonstrated that the common antigen also cross-reacts with *L. pneumophila* SG 1. Previously, we have reported that at least five additional antigens of *P. aeruginosa* cross-react with *L. pneumophila* as well (4).

Elevated antibody titers to *Legionellaceae* in CF patients found by the MA test in this study correlated directly with the number of *P. aeruginosa* precipitins in the CF patient sera. This evidence, coupled with the documented cross-

reactivity of the common antigen of *P. aeruginosa* with an antigen of *L. pneumophila*, leads us to conclude that the high prevalence of antibody titers to *Legionellaceae* in CF patients found in this study and reported by other investigators may be false-positive results caused by cross-reacting antigens.

The effect of the cross-reactions between *P. aeruginosa* and *L. pneumophila* on MA titers to the various antigenic types of *Legionellaceae* differed. Titers to *F. bozemanae* were most significantly affected. This observation is possibly due to expression of different cross-reactive antigens on each of the *Legionellaceae*, differences in exposure of certain antigens, like the common antigen on the surface of the cells, or both.

High antibody titers to *Legionellaceae* in CF patients may, in fact, be due to actual infections by members of the family *Legionellaceae*. However, the problem cross-reactivity between the antigens of *L. pneumophila* and *P. aeruginosa* hampers the specificity of the current serological tests for *Legionellaceae* in this unique population of patients. Definition of the infection rate of members of the family *Legionellaceae* in CF patients rests on the development of more specific serological tests, rigorous attempts to isolate the organism, or both.

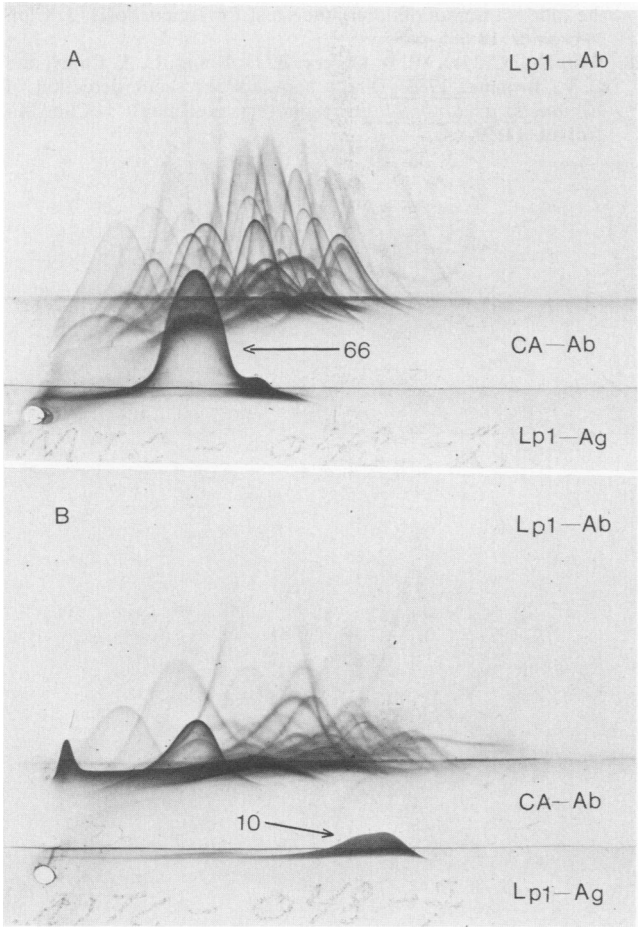

FIG. 3. XIE of *L. pneumophila* and *P. aeruginosa* with 200 μl of monospecific antibody to the common antigen of *P. aeruginosa* in the intermediate gels. (Technical details are as described in the legend to Fig. 1.) Only one antigen was precipitated in the intermediate gel of each reference system by monospecific antibody to the common antigen (CA-Ab). The precipitin reactions were strong. Peak precipitin peaks were identified based on comparison with the control reference gels as shown in Fig. 2A and B. Lp1-Ab, Purified rabbit immunoglobulins; Lp1-Ag, *L. pneumophila* SG 1 antigen.

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