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Crossed Immunoelectrophoretic Analysis of *Legionella pneumophila* Serogroup 1 Antigens

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By crossed immunoelectrophoresis, 85 different antigens were demonstrated in sonicated preparations of *Legionella pneumophila* serogroup 1 (Lp1). The precipitin patterns of 82 anodic-migrating antigens were numbered and were designated the Lp1 reference system. Eleven antigens were stable to boiling, and seven of these were shown to be surface antigens. One heat-stable surface antigen (antigen no. 61) was highly reactive with limulus amoebocyte lysates and formed a precipitin resembling lipopolysaccharide. Serum from an isolation confirmed case of Lp1 infection and serogroup-specific rabbit antiserum reacted specifically with antigen no. 61, which was designated the serogroup-specific antigen. Normal human and rabbit sera commonly had antibodies to antigen no. 66 of the Lp1 reference system. This antigen is antigenically related to the "common antigen" of *Pseudomonas aeruginosa*.

Difficulty in isolation of *Legionella* spp. from patients has caused heavy dependence on immunological techniques for diagnosis of legionellosis. The two most widely used diagnostic techniques are the indirect fluorescent antibody test for serum antibody (50) and the direct fluorescent antibody (9) test for staining *Legionella* spp. in clinical specimens. Performance of these tests has become complicated by the rapid increase in number of different antigenic types of *Legionella*. There have now been reported six serogroups of *Legionella pneumophila* (13, 33, 34), *Legionella* (*Fluoribacter*) *bozemanae* (5, 17), *L.* (*F.*) *dumoffi* (5, 6), *L.* (*F.*) *gormanii* (6, 36), *Legionella* (*Tatlockia*) *micdadei* (19, 20, 39) and two serogroups of *L. longbeachae* (4, 32) and *L. jordanis* (8).

Members of the *Legionellaceae* have been shown to have both serogroup-specific and common antigens (11, 12, 28, 42, 52, 53). Cross-reactions between *Legionella* antigens and those of other bacteria have also been reported (10, 30, 37). Identification and isolation of group- and type-specific antigens is a prerequisite for improvement of any type of immunological test for legionellosis.

Joly and Kenny (28) recently published an analysis of the antigens of *L. pneumophila* and *T. micdadei* by crossed immunoelectrophoresis (XIE) in an attempt to study the "total antigenic profile" of these organisms. Both group-specific and common antigens were demonstrated between six serogroups of *L. pneumophila* and *T. micdadei*. Probably for technical reasons, however, the study of Joly and Kenny only demonstrated 10 to 14 antigens in the five homologous antigen-antibody XIE patterns developed.

The purpose of this study was to develop an XIE reference system capable of demonstrating the maximal number of *L. pneumophila* antigens, to partially characterize the antigens, and to measure antibodies in serum from normal persons and patients with legionellosis. Subsequent studies will employ the *L. pneumophila* XIE reference system to identify those antigens which cross-react with other bacteria and those which are group- and type-specific for *L. pneumophila*.

MATERIALS AND METHODS

Antigen preparation. *L. pneumophila* serogroup 1 (Lp1), Philadelphia 1 strain (ATCC 33152), was used as the source of antigen. This strain was selected for study because of its historical precedence, because it is among the most thoroughly studied of the *Legionella* species and strains, and because of its frequency as the etiological agent of legionellosis (33). The method of antigen production previously described by Høiby and Axelsen (26) was adopted with minor modifications.

The bacteria were grown on charcoal yeast-extract agar (16) or buffered charcoal yeast-extract agar (38). Media were inoculated from a 48- to 72-h pure culture, using a cotton swab in a manner similar to that used when performing an antibiotic disk susceptibility test. A heavy, confluent bacterial lawn was produced in 72

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to 96 h at 35°C in humidified normal atmosphere. Contaminated cultures were easily detected and discarded.

L. pneumophila cells were harvested by gently scraping the growth from the agar plates, using a bent glass rod. The cells were suspended in a minimal volume of sterile distilled water and transferred to a centrifuge tube. After centrifugation at 9,200 × *g* for 10 min at 4°C, the supernatant was harvested, and the cell pellet was weighed. A portion of the supernatant was then added back to the cell pellet at a ratio of 2 ml/g (wet weight) of cells, and the cells were resuspended.

Bacterial disintegration was accomplished by sonication of the cell suspension three times for 45 s each time at 20,000 Khz/s, using a Rapidis 350, 19-mm probe with a 9.5-mm tip. The suspension was cooled with ice water during sonication and for 1-min periods between sonic treatments. Cell fragments were removed from the sonicate by centrifugation at 48,200 × *g* for 1 h at 4°C. The supernatant was filter sterilized by passage through 0.45-μm and 0.22-μm Millipore filters and stored in small portions at −18°C.

The antigen, designated Lp1-Ag, was used both for rabbit immunization and for XIE. Its colloid concentration varied between preparations from 11.2 to 32.0 mg of protein per ml (refractometry with human immunoglobulin as a standard). For all XIE experiments, a single batch of Lp1-Ag (21.3 mg/ml) was employed.

Antibody preparation. Antiserum to *L. pneumophila* was produced by immunization of 12 adult New Zealand White rabbits. Each rabbit was inoculated intracutaneously with 50 μl of Lp1-Ag in 50 μl of Freund incomplete adjuvant five times at 2-week intervals and monthly thereafter (18). One week after each monthly immunization, 40 ml of blood was drawn from the marginal ear vein of each rabbit. Antiserum harvested each month was pooled and tested for precipitating antibodies by XIE. After 4 months of immunizations, the antiserum produced maximal and stable numbers of precipitins by XIE. Antiserum from months 5 and 6 were combined forming pool no. 1 and antiserum from months 7, 8, and 9 formed pool no. 2. The immunoglobulins were isolated and purified according to the methods of Harboe and Ingild (18). This purified antibody was designated Lp1-Ab. Antibody from both pools of antisera produced similar XIE patterns with Lp1-Ag. Only the antibody concentration of the two pools differed.

Human and rabbit sera. Normal human sera were obtained from 17 laboratory personnel.

Acute and convalescent sera from an isolation-confirmed case of legionellosis (Lp1) were supplied by T. Marrie, Department of Microbiology, Dalhousie University, Halifax, Canada.

Antisera from rabbits immunized with *L. pneumophila* for the purpose of producing immunofluorescent antibody reagents were supplied by K. Lind, Mycoplasma Laboratory, State Serum Institute, Copenhagen, Denmark. The rabbits were immunized with heat-killed *L. pneumophila* serogroup 1, 2, 3, or 4 cells mixed with Freund incomplete adjuvant. Two immunizations were given with a 3-week interval, the first given intracutaneously and the second intramuscularly, and serum was collected 1 week after the second immunization. By immunofluorescent antibody test, the rabbit antiserum was serogroup-specific.

Immunoelectrophoretic methods. XIE was performed with intermediate gels for all studies. The intermediate gels contained either serum, antigen (crossed-line immunoelectrophoresis), or saline (0.154 M) as a control. These techniques have been described elsewhere in detail (2, 31, 49), and only the critical aspects of the procedures will be presented below.

Electrophoresis was performed in 1% agarose (1.5 mm deep) on glass slides (5 by 7 cm). Agarose (type LSM; Litex, Copenhagen, Denmark) with a relative endosmosis value (−Mr) of 0.17 ± 0.01 and 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, using 3 μl of Lp1-Ag and applying 10 V/cm until a bromphenol blue-labeled human albumin marker migrated 35 mm (ca. 45 min). Second-dimension electrophoresis was performed at 12°C, applying 1.5 V/cm for 18 h. Antibody was incorporated into the second-dimension gels at 19 μl/cm^2 for Lp1-Ab pool no. 1 and 4.8 μl/cm^2 for Lp1-Ab pool no. 2. Antigens migrating toward the cathode under the given conditions were demonstrated by reversing the polarity of electrophoresis in both directions. The pattern of precipitins formed by anodic-migrating Lp1 antigens with Lp1 antibodies in XIE with NaCl in the intermediate gel was designated with Lp1 reference system.

Antigens were incorporated into intermediate gels at 3.6, 14.3, or 21.4 μl/cm^2 for crossed-line immunoelectrophoresis. Human serum and rabbit antiserum were used in intermediate gels at 28.6 μl/cm^2.

Electrophoresis equipment and the procedures of washing, drying, and staining gels with Coomassie brilliant blue have been described previously (48). Staining for lipid- and polysaccharide-containing antigens was performed with oil red and periodic acid-Schiff stains, respectively (47).

Antigen heat stability experiment. Samples of Lp1-Ag were exposed to the following 11 temperature and time regimens: 50°C for 30 and 60 min; 60°C for 5, 10, or 30 min; 100°C for 5, 10, 15, or 60 min; and 121°C at 1 kg/cm^2 pressure for 15 or 60 min. Heat treatment of antigens at 50 to 100°C was performed in a water bath and with exposure to 121°C under pressure in an autoclave. The number of heat-stable antigens was demonstrated by XIE of the heat-treated antigens in the first dimension against Lp1-Ab. Identity of the predominant heat-stable antigens was determined by crossed-line immunoelectrophoresis, using the heated antigens in the intermediate gels of the Lp1 reference system.

Absorption studies with Lp1-Ab. *L. pneumophila* was cultured and harvested as described for Lp1-Ag. The cells were then heat killed in a boiling water bath for 10 min and centrifuged at 9,200 × *g* at 4°C for 10 min, and the pelleted cells were washed three times in 0.01 M phosphate-buffered saline, pH 6.4. The final bacterial cell pellet was suspended in phosphate-buffered saline at 50 mg/ml. Equal portions of the heat-killed cells and Lp1-Ab were mixed and incubated at 37°C for 1 h and then 4°C for 72 h. A final centrifugation was done (48,200 × *g* for 1 h), and the absorbed antibody was used in XIE at twice the normal concentration to compensate for dilution.

Antigen absorption by limulus amoebocyte lysate (LAL). Samples of Lp1-Ag were incubated with LAL in ratios of 1:1, 1:3, and 1:7 at 37°C for 1 h. Antigen diluted 1:1 with sterile distilled water and incubated under identical conditions served as a control. LAL formed a firm clot at all three antigen/LAL ratios. The gel was disrupted by agitation on a Vortex mixer, and 6, 12, and 20 μl of the three respective mixtures and 6 μl of control antigen were tested by XIE. Disappearance of antigen precipitin peaks in XIE patterns of LAL-treated antigens as compared with that of the control was interpreted as reaction of these antigens with LAL.

RESULTS

Lp1 reference system. Eighty-five antigens were reproducibly demonstrated in the Lp1 reference system. All but six of the antigens migrated toward the anode, and these were identified by number as shown in Fig. 1. Antigen no. 1 was an electrophoretically fast antigen, migrating 1.4 times farther than human albumin under the given conditions. Antigen no. 61 formed a precipitate which resembled in mobility and morphology the lipopolysaccharide seen in XIE analysis of other gram-negative bacteria (14, 21, 23, 26, 41). The most prominent precipitate stained by Coomassie brilliant blue was antigen no. 66. None of the anodic-migrating antigens were stained by the periodic acid-Schiff or oil red stains.

Heat stability of *L. pneumophila* antigens. Eleven antigens could be demonstrated in XIE after boiling Lp1-Ag for 10 min (Fig. 2B). Five of these heat-stable antigens could be identified with nos. 1, 37, 45, 61, and 66 by following the morphology and mobility changes of the antigens in XIE through successive levels of heating, and by crossed-line immunoelectrophoresis experiments. Antigen no. 1 changed morphology when heated to >60°C, developing a left shoulder on the precipitate (Fig. 2B). It was also the most heat-stable Lp1-Ag, withstanding autoclaving for periods up to 1 h. Crossed-line immunoelectrophoresis with boiled or autoclaved Lp1-Ag in the intermediate gels consistently removed antigen no. 1 from the precipitin pattern of the Lp1 reference system by in situ absorption of its homologous antibodies from the Lp1-Ab-containing gel.

The predominant antigen in boiled Lp1-Ag was antigen no. 37. This antigen, along with antigens no. 45 and 61, produced lines of identity in crossed-line immunoelectrophoresis experiments. A fourth line of identity could not be correlated to a specific antigen number (Fig. 2C).

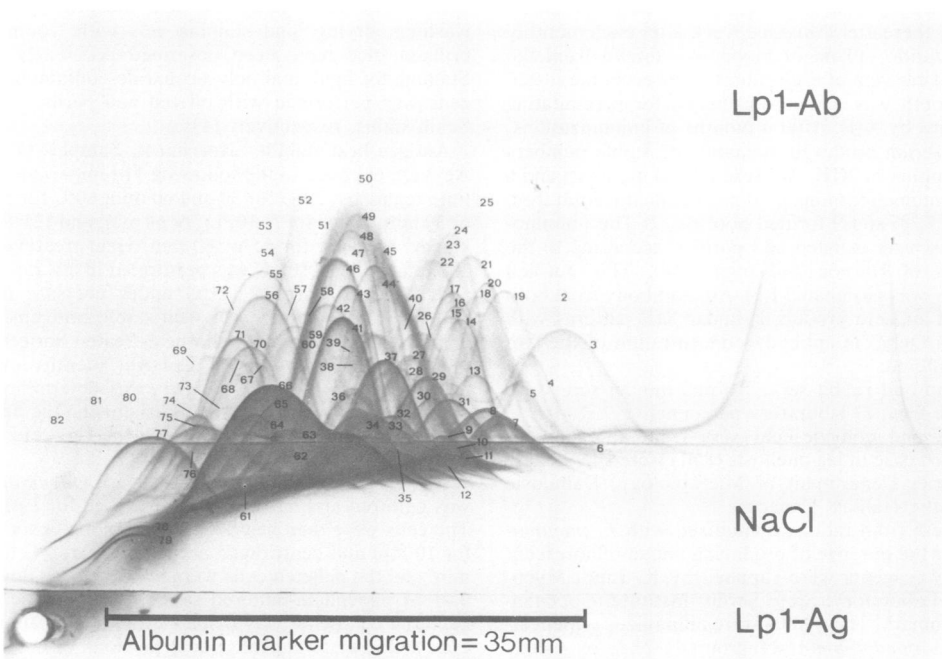

FIG. 1. Lp1 antigen-antibody reference system. XIE with intermediate gel of 3 μl of Lp1-Ag against Lp1-Ab (pool no. 1, 19 μl/cm^2). Saline is in the intermediate gel. First-dimension electrophoresis: anode to the right. Second-dimension electrophoresis: anode to the top. Horizontal bar represents migration of human albumin in first dimension. The gel was stained with Coomassie brilliant blue.

Antigen no. 66 produced an unusual reaction in crossed-line immunoelectrophoresis experiments. The right leg of antigen no. 66 in the reference system formed a clear line of identity with the boiled Lp1-Ag, whereas the left leg of the precipitin formed a line of nonidentity (Fig. 2D).

Antibody absorption studies. Antibodies to antigens no. 1, 37, 45, and 61 were absorbed from Lp1-Ab by boiled and washed Lp1 cells. (Fig. 3A and B). The absorbed Lp1-Ab precipitated 4 of the 11 heat-stable antigens including antigen no. 66 (Fig. 3C and D). Antigens no. 1, 37, and 61 were among the seven heat-stable antigens not precipitated by absorbed Lp1-Ab, providing presumptive evidence that they were surface antigens.

Reaction of Lp1-Ag with LAL. The endotoxic activity of Lp1-Ag (21.3 mg of protein per ml) was 0.16 mg of *Escherichia coli* equivalent units (standard, *E. coli* O26:B6 W Difco) per ml, according to the methods of Reinhold and Fine (40). XIE of the clot formed by reaction of Lp1-Ag with LAL revealed that antigens no. 61 and 66 disappeared from the precipitin patterns of all Lp1-Ag–LAL mixtures tested, thus indicating that they were highly reactive with LAL. Antigens no. 1, 3, 4, 36, 75, 78, and 80 were distinctly nonreactive with LAL, exhibiting no significant changes in precipitin peak height or morphology. Several other antigens gradually disappeared or changed in precipitin morphology when Lp1-Ag was reacted with LAL at decreasing Lp1-Ag/LAL ratios.

Antibodies in human and rabbit sera. Precipitating antibodies to antigen no. 66 were found in 6 of 17 (35%) normal persons tested and in preimmune rabbit sera. Sera from normal persons occasionally (2 of 17) also formed a weak precipitin with an *L. pneumophila* antigen in the same position as antigen no. 1, but not showing reaction of identity with it (Fig. 4B).

Sera from an isolation-confirmed human case of Lp1 infection, taken 10 and 44 days after admission to the hospital, demonstrated weak precipitin reactions to seven antigens of the Lp1 reference system in both samples. The precipitating antibody response to antigen no. 61, however, changed significantly between the two serum samples, being negligible on day 10 postadmission and very strong on day 44 postadmission. Antigen no. 61 was precipitated by antisera against serogroup 1, but not by antisera against serogroup 2, 3, or 4 (Fig. 4C).

DISCUSSION

The Lp1 reference system described in this communication demonstrated the largest number of *L. pneumophila* antigens reported to date. The precipitin pattern of 82 anodic-migrating antigens was very complex, similar to the results obtained with other gram-negative bacterial species by the same methods (7, 14, 21, 23, 26, 41, 43, 44), and more closely approximates a demonstration of the total antigenic profile suggested by Joly and Kenney (28).

Several differences in techniques resulted in over six times as many antigens being demonstrated in the present XIE analysis of *L. pneumophila* compared with a similar study by Joly and Kenny (28). It is important to discuss the XIE technique to explain the differing results between the two studies and to emphasize the technical details critical to the XIE procedure.

Differences in the methods of antigen preparation account for some discrepancies. The antigens employed by Joly and Kenny for immunization of rabbits were formalin-killed whole cells, whereas those used for XIE were obtained by homogenization of live bacteria and were mixed with sodium deoxycholate. In the present study, the same type of antigen, derived by sonication of live bacteria, was used for both immunization and XIE. Formalin treatment of antigens has been shown to alter or destroy some antigens, rendering them not detectable by XIE (22). Furthermore, any difference between the antigen used for immunization and that used for serological testing may result in incomplete detection of all antigens or antibodies in complex antigen-antibody systems.

The method of antibody production used in the present study also differed from that of Joly and Kenny (28). In the latter study, rabbits were immunized with a higher dose of antigen, with the first dose mixed with Freund incomplete adjuvant and injected intramuscularly and subsequent booster injections given intravenously without adjuvant. The duration of these immunizations was only 6 weeks, after which the rabbit antiserum was harvested. By contrast, the present study followed the immunization methods described by Harboe and Ingild (18), involving a prolonged schedule (minimum, 5 months) of low-dose, intracutaneous injections with Freund incomplete adjuvant. Although the immunization schedule of Joly and Kenny may produce a more group-specific antiserum (35), it was not suitable for producing precipitating antibodies to all antigens in a complex antigen mixture. Several studies have demonstrated that the prolonged duration and intracutaneous route of immunization are particularly efficient for production of high-avidity precipitating antibodies in rabbits for use in XIE analysis of complex antigens (1, 14, 18, 21, 23, 26). Use of antiserum pooled from 12 rabbits was also important in the present study since this minimized the large individual variations seen in the immune response of rab-

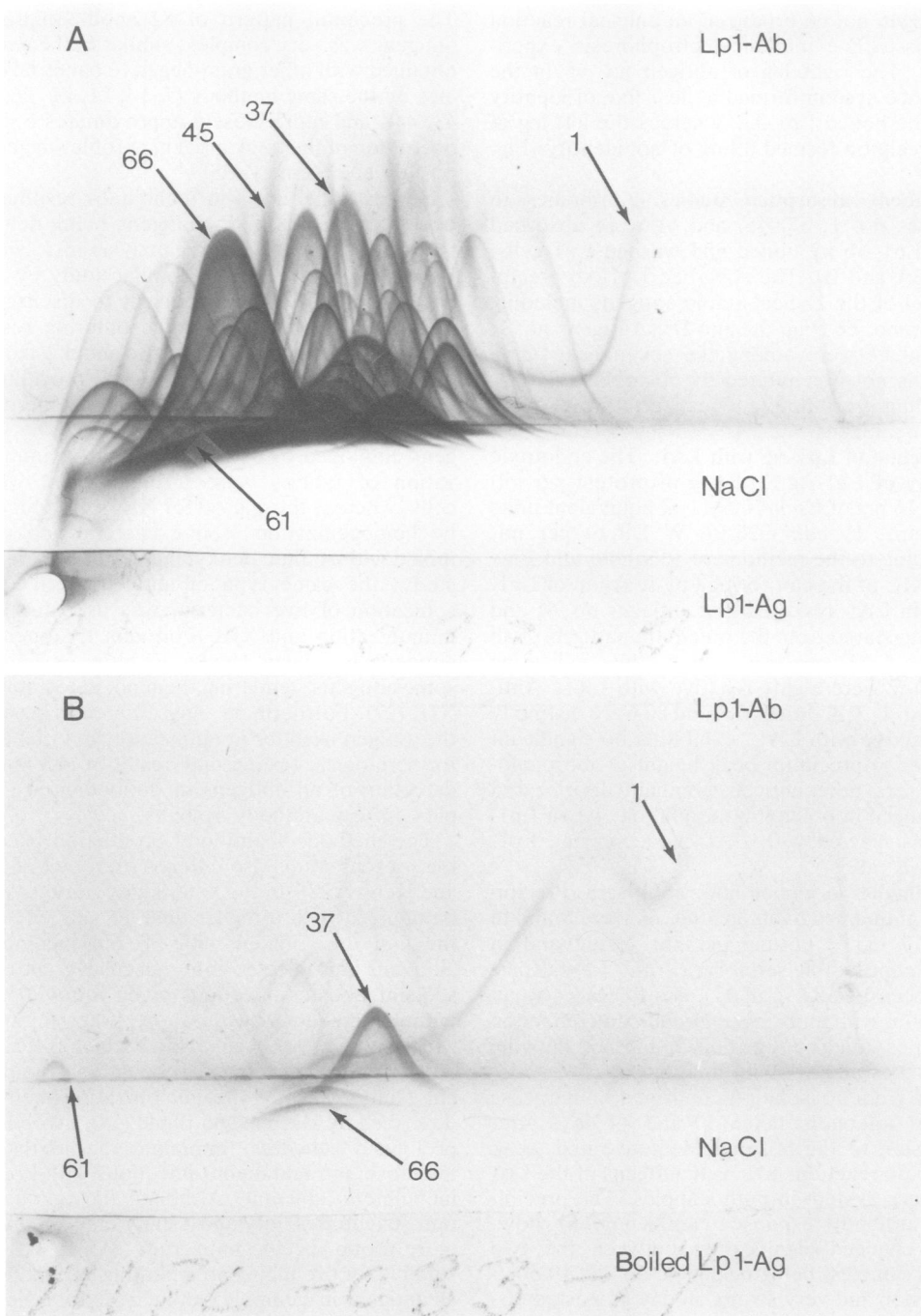

FIG. 2. XIE and crossed-line immunoelectrophoresis of heat-stable *L. pneumophila* antigens. A through D, Lp1-Ab (pool no. 2) at 4.8 μl/cm^2. (A) Lp1 reference system; 3 μl of Lp1-Ag against Lp1-Ab with saline in the intermediate gel (control). Five selected antigens are identified by number. (B) Boiled (100°C for 10 min) Lp1-Ag (3 μl) against Lp1-Ab with saline in the intermediate gel. Eleven heat-stable antigens are demonstrated; 4 are identified by number. (C) Lp1-Ag (3 μl) against Lp1-Ab with 25 μl of boiled Lp1-Ag in the intermediate gel. Antigen no. 1 is missing owing to in situ absorption of homologous antibodies. Antigens no. 37 and 45 show lines of identity. (D) Lp1-Ag (3 μl) against Lp1-Ab with 150 μl of boiled Lp1-Ag in the intermediate gel. Antigens no. 1, 37, and 45 are missing owing to in situ absorption of homologous antibodies. Antigen 61 shows a line of identity. Antigen 66 shows a line of identity with the right leg of the precipitin but not with the left leg.

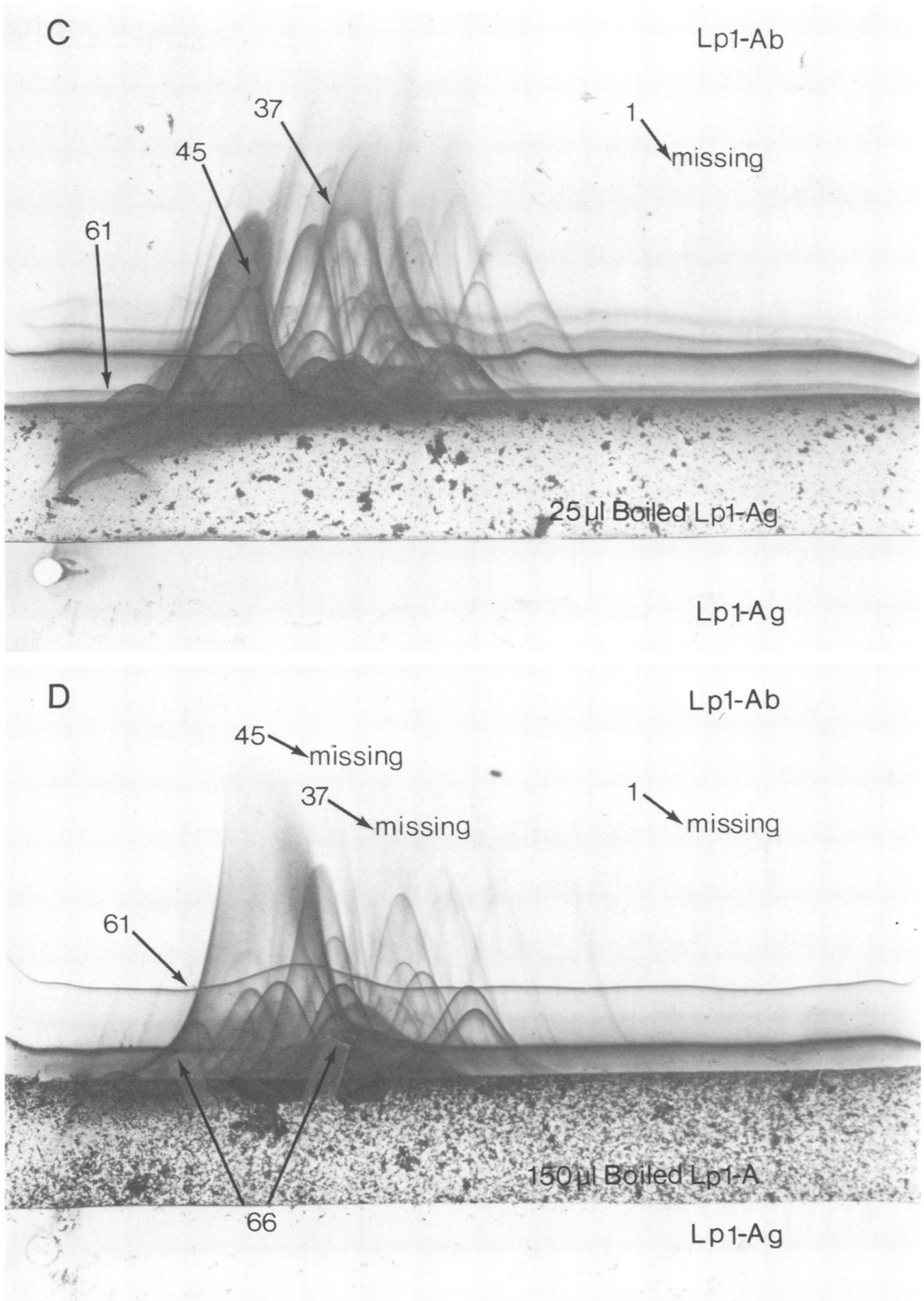

FIG. 2—*Continued*

bits by Joly and Kenny (28) as well as others (3, 18, 25).

Background staining was significantly reduced and the visibility of faintly staining precipitates was markedly enhanced by use of purified antibody in the present study. Use of whole antiserum in XIE gels, as in the Joly and Kenny study, results in dense background staining due to serum lipoproteins causing fewer immunoprecipitates to be distinguishable (18). In addition,

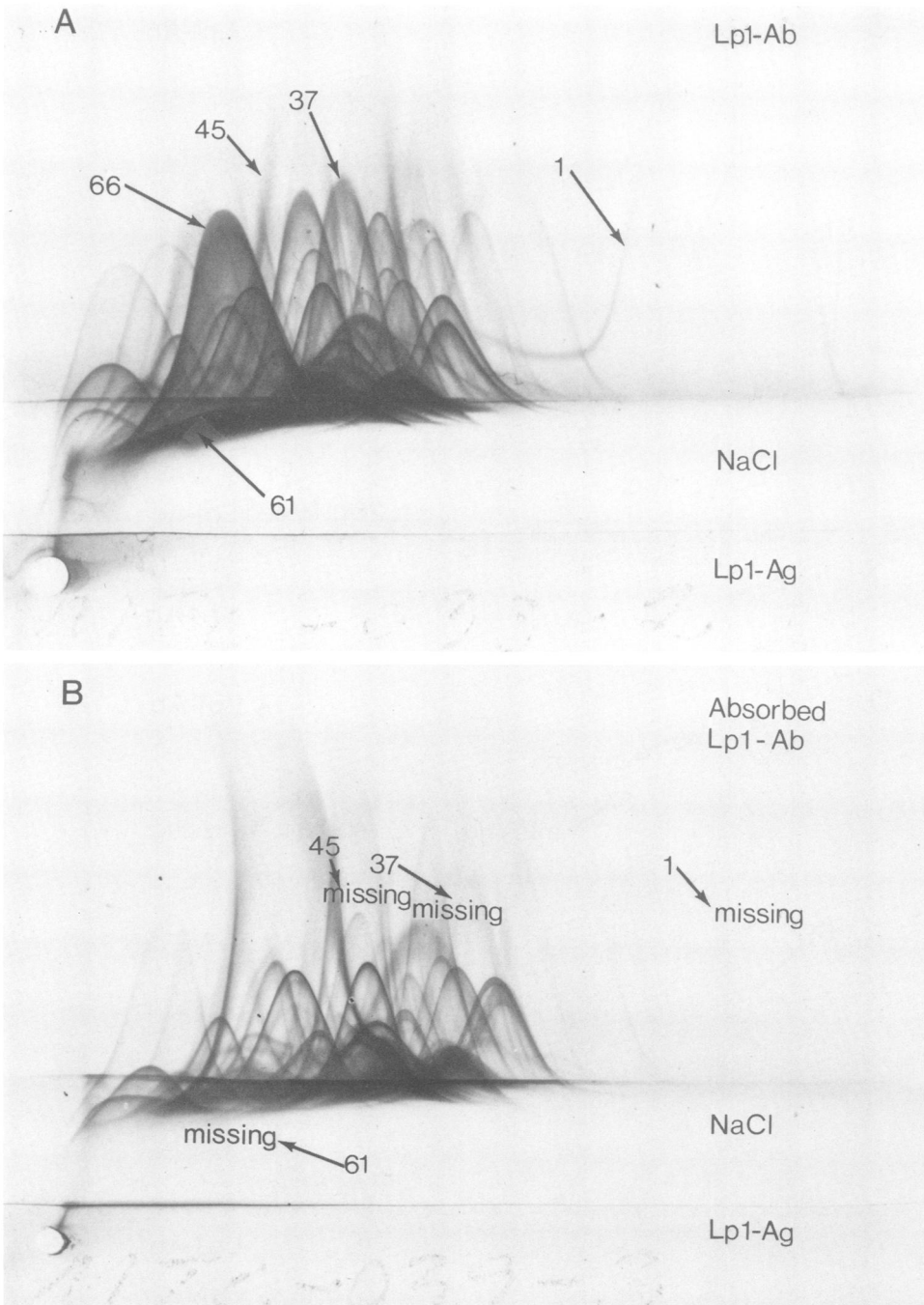

FIG. 3. XIE with nonabsorbed or absorbed Lp1-Ab (absorbed with boiled whole Lp1 cells) and nonheated or boiled Lp1-Ag. (A) Lp1 reference system control, as in Fig. 2A. (B) Lp1-Ag (3 μl) against absorbed Lp1-Ab (9.6 μl/cm^2) with saline in the intermediate gel. Antibodies to antigens no. 1, 37, 45, and 61 were absorbed out (precipitins missing compared with reference system). (C) Boiled Lp1-Ag (3 μl) against Lp1-Ab with saline in the intermediate gel, as in Fig. 2B. (D) Same as (C) but with absorbed Lp1-Ab in the intermediate gel. Antigen no. 66 plus three others were precipitated by absorbed Lp1-Ab in the intermediate gel, indicating that they are not surface antigens. Antigens no. 1 and 61 were unaffected by absorbed Lp1-Ab. The precipitin morphology of antigen no. 37 was altered slightly (inward feet reaction) indicating a remaining low titer of antibodies against antigen no. 37. These three antigens are therefore probably surface antigens.

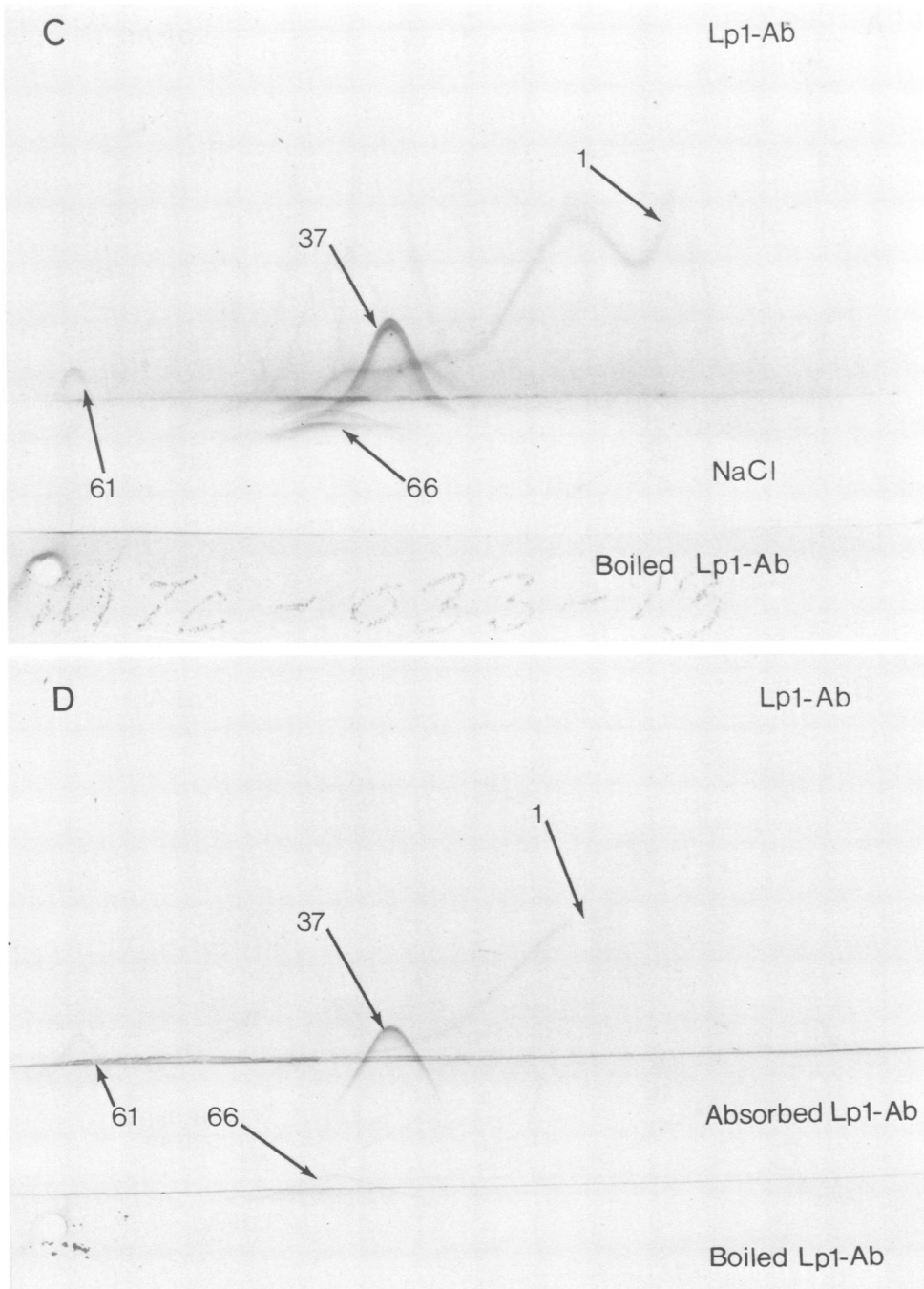

FIG. 3—*Continued*

the routine use of intermediate gels during XIE made differentiation and quantification of precipitins with low peak heights much easier.

Antigens critical to the serodiagnosis of legionellosis by the microagglutination (15) or immunofluorescent antibody techniques (52) in many laboratories are those antigens on the surface of bacteria killed by heating. In laboratories using *Legionellaceae* antigens produced by other methods of killing the organism, such

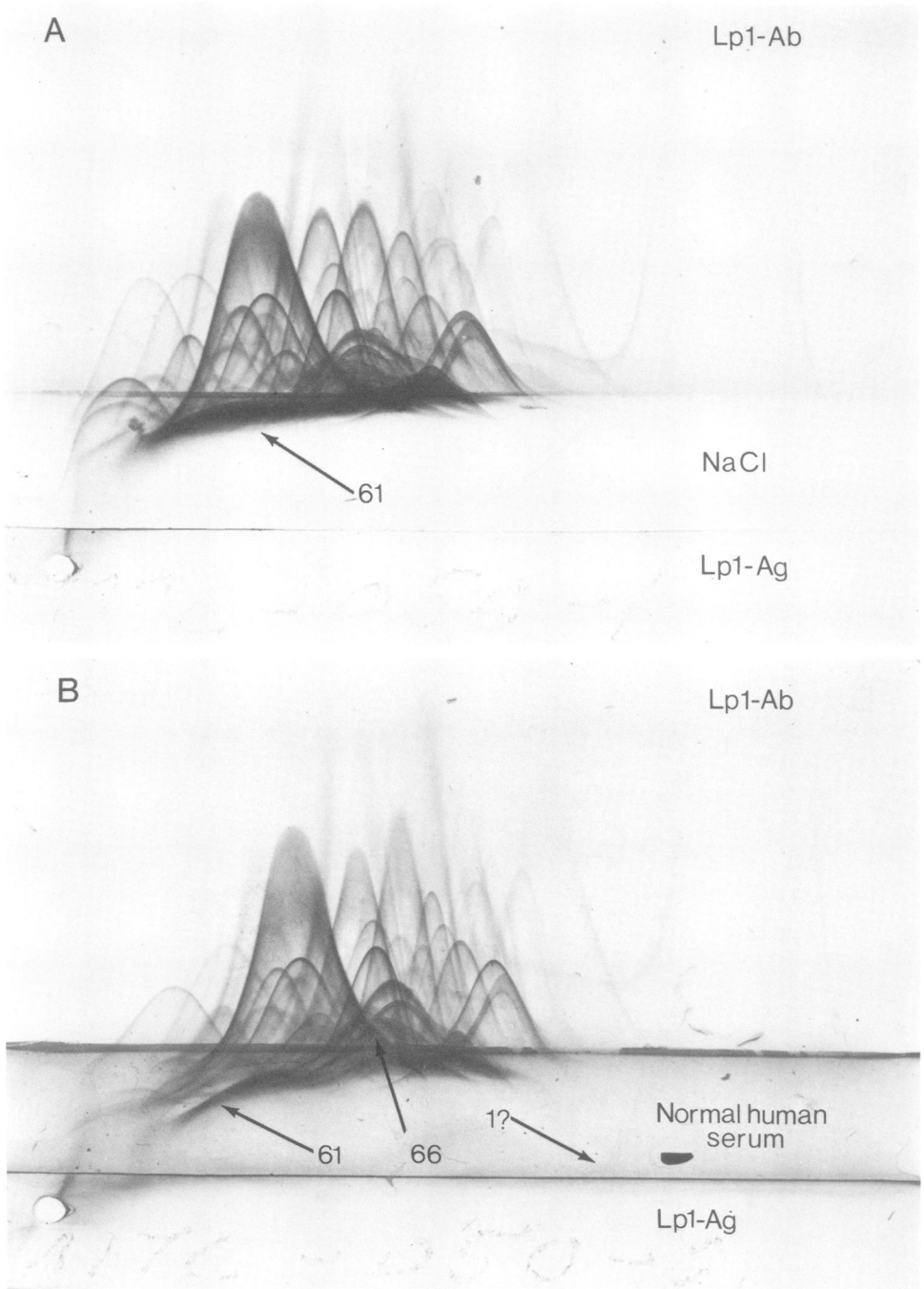

FIG. 4. XIE of the Lp1 reference system with human or rabbit serum in the intermediate gels. (A) Control with saline in the intermediate gel, as in Fig. 2A. (B) 200 μl of normal human serum in the intermediate gel. Low titers of antibody to antigen no. 66 (inward feet reaction) and to an antigen in the position of antigen no. 1 were present. No change occurred in antigen no. 61. (C) 200 μl of rabbit antiserum specific for Lp1 in the intermediate gel. Only antigen no. 61 was precipitated in the intermediate gel. (D) 200 μl of convalescent serum from a patient with an isolation-confirmed case of Lp1 infection in the intermediate gel. A high titer of antibody to antigen no. 61 and low titers to six other antigens were present.

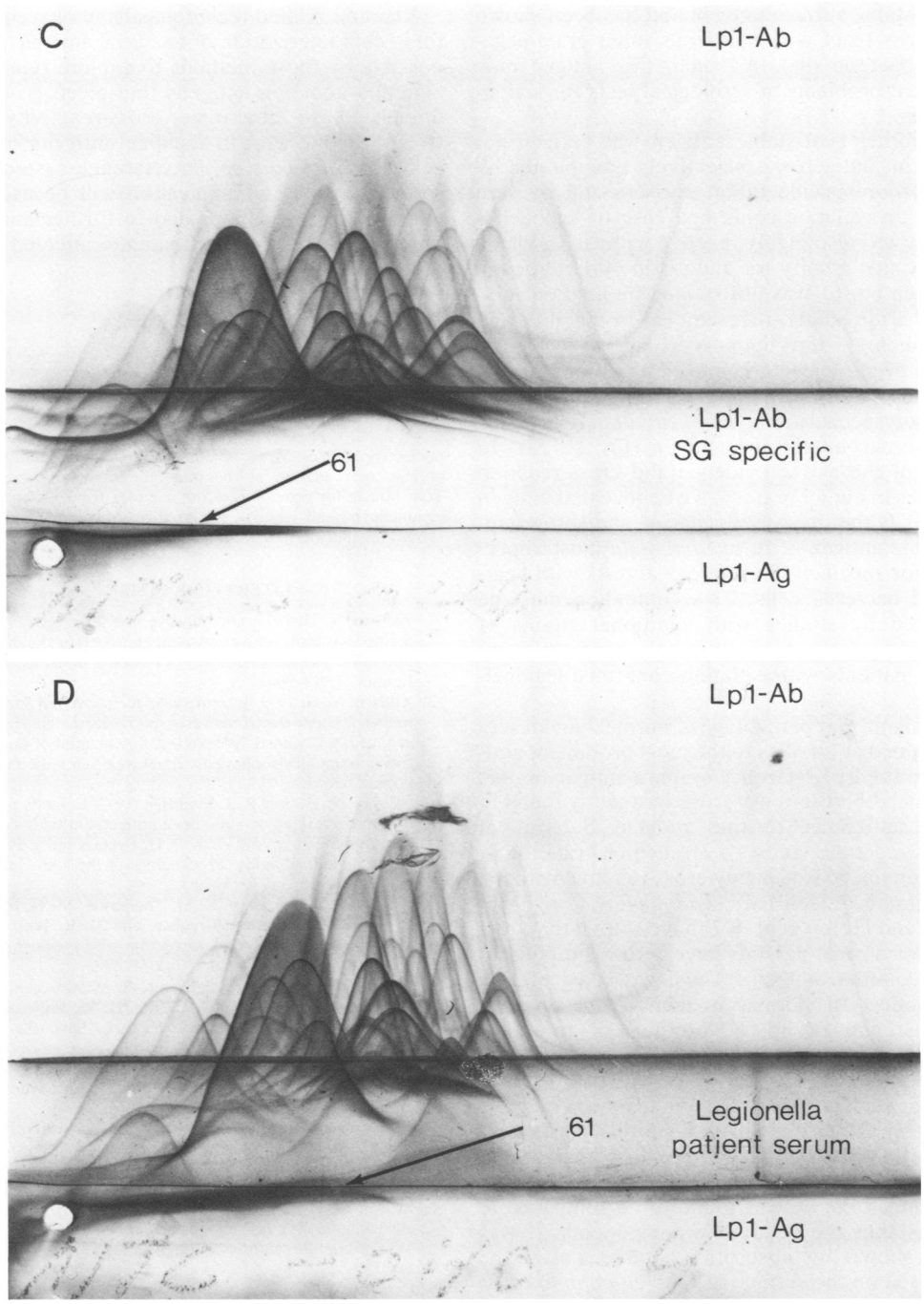

FIG. 4—*Continued*

as formalin, the surface-borne antigens involved in the serological tests may be different. Whereas heat-killed bacteria appear to retain at least one serogroup-specific antigen, they also retain cross-reactive antigens (10, 30, 51). Boiling of sonicated *L. pneumophila* antigens reduced the number of anodic-migrating antigens from 81 to 11. Autoclaving reduced this number further. Of the 11 antigens stable to boiling, 7 were shown to be surface antigens. Antigen no. 1 was the most

heat-stable surface antigen and has been shown to cross-react with numerous other gram-negative bacteria (9a). As such, this antigen may present problems in serological tests for legionellosis.

Another heat-stable antigen was antigen no. 61. This antigen was selectively precipitated by serogroup-specific rabbit antisera and by sera from an isolation-confirmed case of legionellosis. It was also highly reactive with LAL, which has a high affinity for endotoxin (46). Although antigen no. 61 was not stained by lipid or polysaccharide stains, it resembled in mobility and morphology the lipopolysaccharide of other gram-negative bacteria in XIE analyses (14, 41). Studies by other investigators suggest that the lipopolysaccharide of *L. pneumophila* contains the serogroup-specific antigen (12, 28, 53). In light of the above evidence and cross-reaction studies in our laboratory, we believe that antigen no. 61 is the lipopolysaccharide and serogroup-specific antigen of *L. pneumophila* most important for specificity in serological tests with heat-killed bacterial cells. This contention must be verified by studies with additional strains of serogroup 1 organisms and with sera from many more patients with isolation-confirmed legionellosis.

A significant percentage of normal human sera contained antibodies to the most prominent antigen in the Lp1 reference system, antigen no. 66. Normal rabbit sera were also frequently found to have antibodies to this antigen. Subsequent cross-reaction studies (9a) demonstrated that antigen no. 66 was highly cross-reactive with the "common antigen" of *Pseudomonas aeruginosa* (24), and Høiby et al. (27) have shown that over 50% of normal persons have serum antibody to this common antigen. Thus, we consider that antibodies in normal human serum directed against antigen no. 66 of the Lp1 reference system are the result of exposure of persons to the common antigen of *P. aeruginosa* or other similar such antigens common to a wide range of bacteria. It has been postulated that these natural antibodies are induced by the normal mucosal flora (27).

Antigen no. 66 was found to be composed of heat-stable and heat-labile components. It was also shown by absorption studies not to be exposed on the surface of *L. pneumophila* cells. If antigen no. 66 is similar to the common antigen of *P. aeruginosa*, as indicated above, characterization of it as an internal antigen is consistent with the findings of Sompolinsky et al. (45) and Kaijser (29). As an internal antigen, the cross-reactivity of antigen no. 66 may not present a problem for serological diagnosis of legionellosis if well-washed whole cell test antigens are employed.

XIE and related techniques have proven useful in characterization of bacterial antigens. It is possible by these methods to explore type-specific and common antigens in a given group of organisms, to determine cross-reactivity between genera, and to localize antigens in the cell. The *L. pneumophila* reference system described in this communication will be used to explore these questions and to further analyze the immune response of patients infected with this organism.

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