INFECTION AND IMMUNITY, Oct. 1983, p. 88-98
0019-9567/83/100088-11$02.00/0
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Vol. 42, No. 1

Immunogenicity of *Pseudomonas aeruginosa* Outer Membrane Antigens Examined by Crossed Immunoelectrophoresis

J. S. LAM,[1] L. M. MUTHARIA,[2] R. E. W. HANCOCK,[2] N. HØIBY,[3] K. LAM,[1] LEIF BAEK,[3] AND J. W. COSTERTON[1]*

Department of Biology, University of Calgary, Calgary, Alberta, Canada, T2N 1N4[1]; Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1W5[2]; and State Serum Institute, Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark[3]

Received 8 June 1983/Accepted 5 July 1983

By crossed immunoelectrophoresis 36 different anode-migrating antigens were demonstrated in sonicated antigen preparations of *Pseudomonas aeruginosa*. We numbered these antigens to establish a reference precipitin pattern. Antigen no. 31 was identified as the lipopolysaccharide (LPS) antigen, because it was found to be responsible for the O-group specificity and because it reacted with anti-LPS monoclonal antibodies and with *Limulus* amoebocyte lysate. Purified outer membrane proteins F (porin), H_2, and I used as antigens formed precipitins with the reference antibodies, thus establishing their antigenicity. LPS that copurified with protein F and slightly contaminated protein H_2 was detectable as an extra precipitin (antigen no. 31). The use of monoclonal antibodies specific for smooth LPS and rough LPS revealed different antigenic determinants in the LPS molecule and suggested that antigen no. 5 could be the core region of the LPS which is equivalent to the rough LPS. Antibodies against these outer membrane antigens were detected in patients with chronic *P. aeruginosa* pneumonia and in patients with acute *P. aeruginosa* bacteremia. Antibodies with the same specificity were also found in rats chronically infected with *P. aeruginosa* 7 days postinfection. This demonstrates the surface accessibility and antigenic reactivity of outer membrane antigens.

Pseudomonas aeruginosa is an opportunistic pathogen. It poses a major threat to the lives of a certain population of patients including burn patients (37), cystic fibrosis patients (21), and cancer patients (3, 4, 38, 40). It has also become a major causative agent of nosocomial infections (4, 10, 39). The poor prognosis for such patients and the intrinsic resistance of this organism to antibiotics has led to an interest in immunoprophylaxis. Lipopolysaccharide (LPS) and outer membrane proteins are macromolecules on the surface of this organism and therefore are logical potential vaccine candidates. Most of the anti-*Pseudomonas* vaccines developed to date (36) are LPS based, and they have been shown to provide limited serotype-specific protection. Also, the endotoxic properties of the LPS molecule have argued against the use of such vaccines in debilitated burn patients and in cystic fibrosis patients. It is therefore important to study the immunogenicity of other surface components as alternative vaccines. This aspect has been studied in other organisms such as *Haemophilus influenzae* (19, 29, 31), *Neisseria* spp. (5, 45), *Shigella* spp. (2), and *Salmonella typhimurium* (26).

Previous studies from our group have shown that outer membrane proteins from different serotypes of *P. aeruginosa* are highly conserved (33) and that major outer membrane proteins interact with and mitogenically stimulate B lymphocytes (7). In this present paper, we employed the technique of crossed immunoelectrophoresis, which is known for its high resolution, specificity, sensitivity, and usefulness for membrane analysis (20, 35, 42), to study the immunogenicity of individual outer membrane components such as LPS, porin protein F, lipoprotein H_2, and lipoprotein I. We have also demonstrated the use of monoclonal antibodies in specific identification of antigens from a complex antigen-antibody precipitin reference pattern. This information was further used to show the immunogenicity of these outer membrane antigens in model *P. aeruginosa* infections of rats and in chronic and acute human pneumonias caused by *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PAO1 strain H103, of O-serogroup 5 according to the international antigen typing scheme (34), was used

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as a standard strain for the isolation of outer membrane proteins, antigen preparation, the immunization of rabbits and mice, and the pulmonary infection of rats. Strain AK1160, obtained from A. Kropinski (Queen's University, Kingston, Ontario), was a rough *P. aeruginosa* PAO derivative with defined LPS composition. It was used in this study for the production of rough LPS and the isolation of the corresponding monoclonal antibodies.

Generally, *P. aeruginosa* cultures were maintained on 1% (wt/vol) protease peptone no. 2 agar and used after growth in protease peptone no. 2 broth to an optical density at 660 nm of approximately 0.5 to 0.8. For antigen preparations, the bacteria were grown in Trüche agar (22), and for rat pulmonary infection studies, they were grown in modified Vogel and Bonner medium, which is known to enhance both exopolysaccharide production and microcolony formation (27).

Outer membranes were isolated by the one-step procedure previously described (16). The method of isolation of protein F (17) and proteins H_2 and I (7) was exactly as described before.

The method of LPS isolation will be described elsewhere (R. P. Darveau and R. E. W. Hancock, submitted for publication). Briefly, bacterial cells were broken in the French press and dissolved in 2% sodium dodecyl sulfate–10 mM EDTA–20 mM Tris-hydrochloride (pH 7.0) at room temperature before the digestion of protein and subsequent ethanol precipitation. The resultant LPS was obtained in high yield (60 to 80% of cellular LPS) and was protein and nucleic acid free as judged by absorbance at 260 and 280 nm and lipid free as judged by fatty acid analysis. Smooth LPS was obtained from strain H103, and rough LPS was obtained from strain AK1160 by the same method. Quantitation of the LPS was performed by assaying for the LPS-specific sugar, 2-keto-3-deoxyoctonate (35), assuming that this sugar was 4.3% by weight of LPS.

Immunogen preparation. For the immunization of rabbits, a culture grown for 12 h in modified Vogel and Bonner liquid medium from a 1% inoculum was subjected to Formalin killing by the standard method of Garvey et al. (15). The immunogens were stored at −70°C in 1-ml samples.

Antigen preparation. Antigens were prepared by the method of Høiby and Axelsen (22), with slight modifications. The bacteria were grown to a thick lawn on solid Trüche agar medium (there were no discernable differences between antigens prepared from either the Trüche agar or protease peptone no. 2 agar) for 18 h at 37°C. They were harvested by gently scraping off the plates with a bent glass rod and washed once in sterile distilled water. They were then centrifuged at 12,000 × *g* for 10 min at 4°C. The subsequent pellet was weighed to determine the wet cell weight. A portion of the supernatant was then added back to the cell pellet in the ratio of 5 ml/g of cells, and the cells were suspended. The suspended bacteria were disintegrated by sonication three times for 45 s at 20,000 kHz/s by using a Rapidis 350, 19-mm probe with a 9.5-mm tip. The specimens were cooled with ice water 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 filtered by passage through 0.45-μm and 0.22-μm membrane filters (Millipore Corp.) and stored in 100-μl samples at −20°C. The antigen thus prepared was designated PAO-1 antigen, has 16 μg of protein per ml (determined by refractometry with human immunoglobulin as a standard and by the method of Lowry et al. [31]), and was used for crossed immunoelectrophoresis.

Another pool of antigens used in this study was heterologous to *P. aeruginosa* strain PAO-1. It was the standard antigen prepared from *P. aeruginosa* strains from the Habs antigen typing scheme O-serogroups 3, 5, 6, 11, as described by Høiby and Axelsen (22).

Animals and human sera. Six New Zealand white female rabbits were used; their preimmune sera were collected and pooled to check for any preimmune nonspecific antibodies. They were then immunized intramuscularly with 0.5 ml of immunogen dispersed in Freund incomplete adjuvant (Difco Laboratories) (1:1 ratio) twice weekly for the first 2 weeks. The animals were then rested for 1 week and immunized again at the end of week 3. At the end of week 4, the animals were bled to provide the "early response sera." Thereafter, they were immunized once every 2 weeks until 8 weeks. To ensure a good supply of sera, the hyperimmune response of these rabbits was maintained by immunizing them once a month, followed by bleeding 1 week later. All blood was obtained from the marginal vein of the ear. The response of the rabbits was usually adequate after an 8-week immunization schedule, with a bacterial agglutination titer of 1/5,120 or higher. All the sera collected were pooled and stored at −20°C until use. These sera were used to develop a reference pattern of crossed immunoelectrophoresis of PAO1-Ag.

Rat sera were obtained from Sprague-Dawley rats chronically infected with *P. aeruginosa* PAO-1 incorporated into agar beads. The method of the artificial infection was exactly as reported in a previous study (27).

Two sources of human sera were used. One source was sera from patients with chronic *P. aeruginosa* pulmonary infections kindly supplied by L. E. Bryan (University of Calgary, Calgary). The second source was sera from leukemic patients with acute pneumonia and bacteremia caused by *P. aeruginosa*, kindly supplied by M. R. Moody (University of Maryland Cancer Center, Baltimore).

Immunoelectrophorectic methods. Crossed immunoelectrophoresis was performed with an intermediate gel for all studies. The intermediate gel contained either serum, antigen (for crossed-line immunoelectrophoresis) or saline (0.145 M) as a control. All immunoelectrophoresis techniques were performed according to published procedures (22, 25, 43) with slight modifications. Counterimmunoelectrophoresis was performed as described in Mackie et al. (32).

The following barbital buffer (pH 8.6) was used to suspend the agarose and as the electrode buffer: 4.48 g of (0.024 M) barbituric acid C IV (Fisher Scientific Co.), 8.86 g (0.073 M) of Tris-base (Sigma Chemical Co.), and 0.108 g (0.00035 M) of calcium lactate (Fisher). The pH was adjusted to 8.6 with a few drops of 1 N NaOH if necessary, and the volume was brought up to 1 liter. A 1% agarose (Seakem, Marine Colloid) with medium electroendosmosis ($-M_r$, 0.16 to 0.19) was used. It was dissolved by heating in the buffer and then poured onto the support surface, Gel Bond film (Marine Colloid), in an agarose/surface area

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ratio of 0.18 ml/cm^2. Electrophoresis was carried out on a water-cooled (10°C) flat-bed electrophoresis apparatus.

For crossed immunoelectrophoresis and crossed-line immunoelectrophoresis, the first-dimensional separation of antigens was performed at 10 V/cm until a bromphenol blue-labeled human albumin marker had migrated 26 mm. Second-dimension electrophoresis was performed at 3 V/cm for 18 h. The second-dimension gel contained 16.7 μl of antiserum per cm^2 unless otherwise stated. Antigens were incorporated into the intermediate gels at 8.3 or 16.7 μl/cm^2 for crossed-line immunoelectrophoresis. Sera used in the intermediate gel for specific antigen absorption were incorporated at 8.3, 16.7, or 33.3 μl/cm^2. After electrophoresis, the gels were washed, pressed, dried, stained, and destained as described by Weeke (43).

Affinity absorption by *Limulus* amoebocyte lysate. PAO-1 antigen samples were incubated with *Limulus* amoebocyte lysate (prepared by L. Baek, State Serum Institute, Copenhagen) in ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 for 1 h at 37°C. Antigen diluted 1:1 with sterile and pyrogen-free water and incubated under identical conditions served as a control. *Limulus* amoebocyte lysate formed a firm clot at all antigen/lysate ratios. The clot in its gel form was disrupted by agitation on a Vortex mixer before being used as the antigen in crossed immunoelectrophoresis. Sample volumes of 4, 6, 8, 10, and 12 μl of the respective mixtures and 4 μl of control antigen mixture were tested by crossed immunoelectrophoresis. The disappearance of any antigen peaks in the crossed immunoelectrophoresis pattern as a result of treatment with *Limulus* amoebocyte lysate was interpreted as a reaction of these antigens with *Limulus* amoebocyte lysate.

Isolation of monoclonal antibodies and their use in intermediate gels of crossed immunoelectrophoresis. This procedure has been described in detail in one of our previous studies (18) and by Mutharia and Hancock (submitted for publication). Monoclonal antibodies obtained from ascites fluid of hybridomas secreting specific antibodies against protein F (MA4-4), smooth LPS (MA1-8), and rough LPS (MA3-8) were used and were incorporated into the intermediate gel of crossed immunoelectrophoresis preparations at 16.7 μl/cm^2.

RESULTS

PAO-1 antigen-antibody reference system. Thirty-six antigens that migrated toward the anode in the first dimension were routinely observed. They were numbered from 1 through 36 according to how rapidly each antigen moved away from the origin (Fig. 1). This pattern was repeatedly reproduced by using the mobility of the human albumin as a marker. Six other antigens were found; these antigens migrate toward the cathode.

Identification of LPS. Reagents have been incorporated into an intermediate gel to react with the electrophoretically separated antigens before they enter the second-dimension gel, and this has allowed us to identify some specific single antigens. Standard antigen, a heterologous pool of antigens from *P. aeruginosa* O-groups of 3, 5, 6 and 11 of the Habs typing scheme, was added to the intermediate gel for crossed-line immunoelectrophoresis. These antigens cross-reacted and formed precipitin lines of identity with all the PAO-1 antigens, except antigen no. 31 (Fig. 2B). This indicated that antigen no. 31 could be related to the O-group specificity of PAO-1. It should be noted that *P. aeruginosa* PAO-1 has a serotype classification of O-group 5 according to the international typing scheme, which corresponds to O-group 2 of the Habs typing scheme (23). When rabbit antibodies against outer membranes of *P. aeruginosa* strain PAO-1 were introduced into the intermediate gel they also formed a precipitin with antigen no. 31 and displaced it from the reference pattern (Fig. 2C). When purified LPS from PAO-1 was added to the intermediate gel for crossed-line immunoelectrophoresis, the identity of antigen no. 31 was clearly demonstrated (Fig. 2D). Antigen no. 31 was missing from the reference pattern, and a dark precipitin line that resembled the intensity of antigen no. 31 was formed in the intermediate gel. This dark precipitin line extended across the whole reference system. The LPS O-antigen-specific monoclonal antibody MA1-8 reacted with antigen no. 31 (Fig. 2E) such that it appeared much lower in the intermediate gel than its usual position in the reference pattern. However, a precipitin that may have been derived from antigen no. 5 was seen in the reference pattern and now formed a tail to antigen no. 31 such that the combined precipitins spanned the width of the gel. This particular tail-like precipitin was identical to the antigen reacting with LPS rough core-specific monoclonal antibody MA3-9 (Fig. 2F). In this case antigen no. 5 totally disappeared from the reference pattern.

Affinity absorption with *Limulus* amoebocyte lysate. At the lowest quantities of *Limulus* amoebocyte lysate added to the reaction mixture (1:1 ratio of PAO-1 antigen to *Limulus* amoebocyte lysate) the left part of antigen no. 31 disappeared (Fig. 3B). Antigens no. 5 and 6 were also shown to be highly reactive to *Limulus* amoebocyte lysate (Fig 3B). Two other antigens, 15 and 30, gradually decreased in the height of their precipitin peak with increasing amount of *Limulus* amoebocyte lysate added (Fig. 3C through F). At the highest amount of *Limulus* amoebocyte lysate added (Fig. 3F), the precipitin of antigen no. 15 was flattened to a line, and antigen no. 30 developed a "shoulder" on the left leg of its precipitin peak.

Analysis of outer membrane proteins, F, H, and I. The LPS that copurified with the purified protein F preparation formed a major precipitin peak in crossed immunoelectrophoresis (Fig. 4B). Overlapping double peak formation clearly

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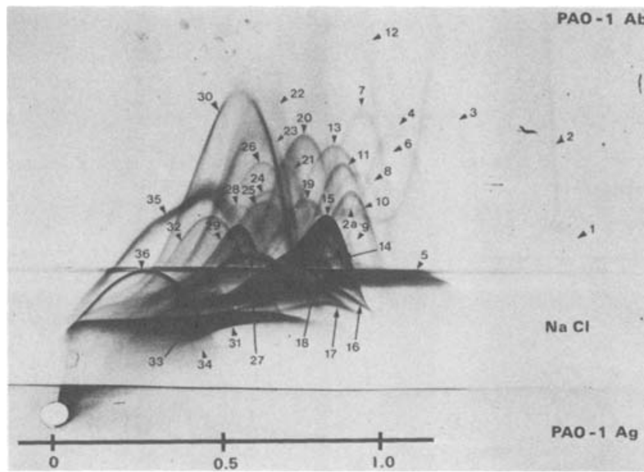

FIG. 1. PAO-1 antigen (Ag)-antibody (Ab) reference system. Crossed immunoelectrophoresis with an intermediate gel of 2 μl (32 μg of protein) of PAO-1 antigen against PAO-1 antibody (16.7 μl/cm^2). Saline was added to the intermediate gel at 33.33 μl/cm^2. The anode is to the right and the top of the gel. The horizontal bar from 0 to 1.0 represents migration of human albumin in the first-dimension electrophoresis. The gel was stained with Coomassie brilliant blue.

indicated the close association of these macromolecules. A precipitin that formed a tail to the LPS antigen no. 31 (Fig. 4B) closely resembled the one (probably antigen no. 5) that reacted with monoclonal antibody MA3-8 (Fig. 2F). Two other minor precipitins at the bottom of the major peaks were also seen (Fig. 4B) and presumably represent contaminants present in low levels in the purified protein F preparation. Crossed-line immunoelectrophoresis with the purified protein F added to the intermediate gel (Fig. 4C) confirmed the identity of the dark precipitin in Fig. 4B as antigen no. 31. Other than the LPS associated with the purified protein F, this outer membrane protein did not form a line of identity with any antigens of the reference pattern (Fig. 4A and C). Monoclonal antibody MA4-4, found to have specificity against protein F, reacted to form a single precipitin peak with protein F (Fig. 4D).

Crossed immunoelectrophoresis of purified protein I produced two major and three very minor precipitin peaks. This was indicative of either the presence of more than one antigenic determinant or of a slight contamination (Fig. 5B). Antigen a in Fig. 5B had a relative mobility (R_f) of 0.46 as compared with the human albumin marker. This R_f value was similar to that of antigen no. 32; however, on subsequent crossed line immunoelectrophoresis of protein I, antigen no. 32 remained unchanged (Fig. 5C), which eliminated such a possibility of identity. Antigen b in Fig. 5B resembled antigen no. 31 in its position and in the intensity of its Coomassie blue staining. However, their nonidentity was also quite obvious (Fig. 5C) in that antigen no. 31 remained unchanged. Presumably anti-protein I antibodies represent a minor component of antibody to whole PAO-1, in agreement with data suggesting that the protein is not surface located (Mutharia and Hancock, unpublished data).

Crossed immunoelectrophoresis of protein H_2 produced two precipitins (Fig. 5D). The darkly stained one resembled antigen no. 31; upon subsequent crossed-line immunoelectrophoresis with protein H_2 in the intermediate gel, it was proved to be LPS. Antigen c in Fig. 5D has a relative mobility (R_f value) of 0.85, which is the same as that of antigen no. 10 in the reference pattern, but antigen no. 10 was unchanged during crossed-line immunoelectrophoresis of protein H_2 (data not shown).

Immunogenicity of protein F in patients and animal models with *P. aeruginosa* infections. A total of 220 sera from 33 cystic fibrosis patients with chronic *P. aeruginosa* infections were shown to contain precipitating antibodies that recognized many of the *P. aeruginosa* antigens (Fig. 6A). Ninety percent of these sera formed 10 or more precipitins with PAO-1 antigen or standard antigen, and 10% of these sera formed 5 to 9 precipitins. Invariably, these were precipitating antibodies against protein F (Fig. 6B), regardless of whether the sera form more or less precipitin with PAO-1 antigen. As a control, 10 sera from eight healthy cystic fibrosis patients which had no detectable response against PAO-1 antigens were also studied. Anti-protein F antibodies could not be detected in these latter sera.

In the acute cases, antibodies against only one or two antigens of *P. aeruginosa*, including no.

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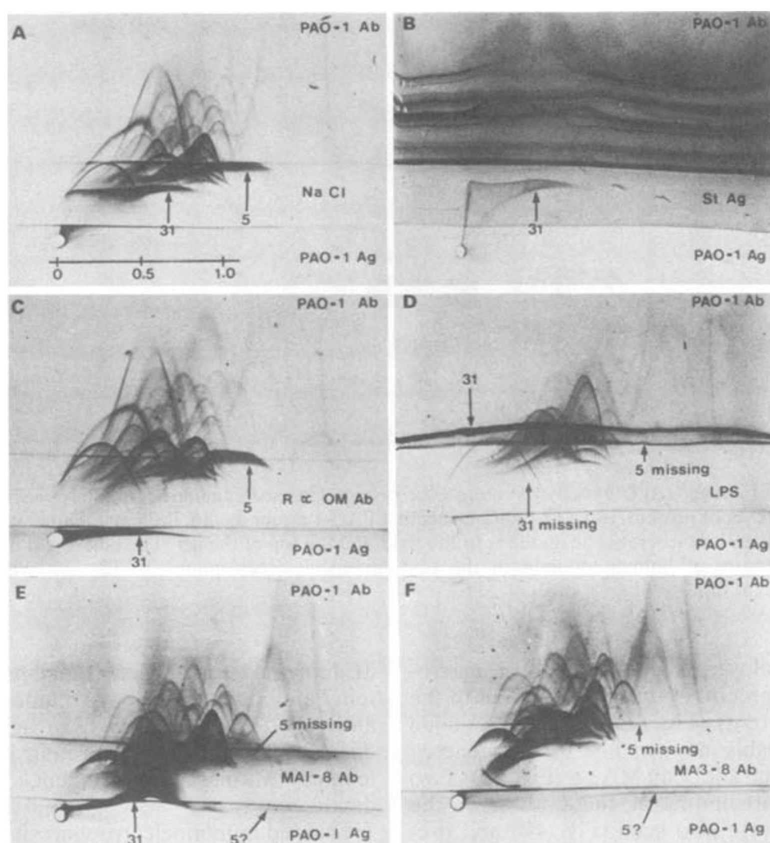

FIG. 2. Identification of LPS from PAO-1 antigen (Ag) by crossed immunoelectrophoresis with specific antibodies (Ab) added to the intermediate gel and crossed-line immunoelectrophoresis with other antigens added to the intermediate gel PAO-1 antigen (2 μl) and PAO-1 antibody (16.7 μl/cm^2) were used. Materials added to the intermediate gels were as follows. (A) Saline, as control. (B) Standard antigens, prepared from *P. aeruginosa* with O-groups 3, 5, 6, and 11. This is a crossed-line immunoelectrophoresis; all of the cross-reacting antigens form lines of identity, except antigen no. 31. (C) Serum from rabbits immunized with outer membrane of PAO-1 (R α OM); note that antigen no. 31 has dropped out from the precipitin pattern. (D) purified LPS of PAO-1. This is also a crossed-line immunoelectrophoresis. Note that it has formed a line and that antigen no. 31 is missing. (E) Monoclonal antibody (MA1-8) against the smooth LPS of PAO-1; antigen no. 31 clearly has dropped away from the reference pattern. (F) Monoclonal antibody (MA3-8) against the rough LPS of strain AK1160. Antigen no. 31 seems to be unaffected, but a new precipitin line is formed (arrow), and antigen no. 5 is missing from the precipitin pattern.

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31, could be observed a few days after the initial onset of *P. aeruginosa* bacteremia (Fig. 6C). In 29 sera of four patients within the first 7 days of acute infections, precipitating antibodies against protein F were not detected. In contrast (Fig. 6D), precipitating antibodies were formed against protein F by one of the patients approximately one month after the initial *P. aeruginosa* bacteremia. This showed that protein F was exposed to the host defense system and was immunogenic, in that it elicited antibody production.

In one of our previous studies (27), we were able to use the rat model of chronic pulmonary infection developed by Cash et al. (6) to study the mode of growth of *P. aeruginosa* in rat lungs. Taking advantage of this useful system, we were able to monitor the immune response to various *P. aeruginosa* antigens. The series of crossed immunoelectrophoresis studies of protein F against these rat sera (Fig. 7A through G) was an effective titration of the production of anti-protein F antibodies from as early as 7 days and sustained until 56 days. The precipitin formed resembled that formed in reaction with monoclonal antibody MA4-4 (Fig. 7H).

Immunogenicity of protein H_2 and I in *P. aeruginosa* systems. Counterimmunoelectrophoresis was performed with proteins H_2 and I as antigens. Antibodies that precipitated anti-

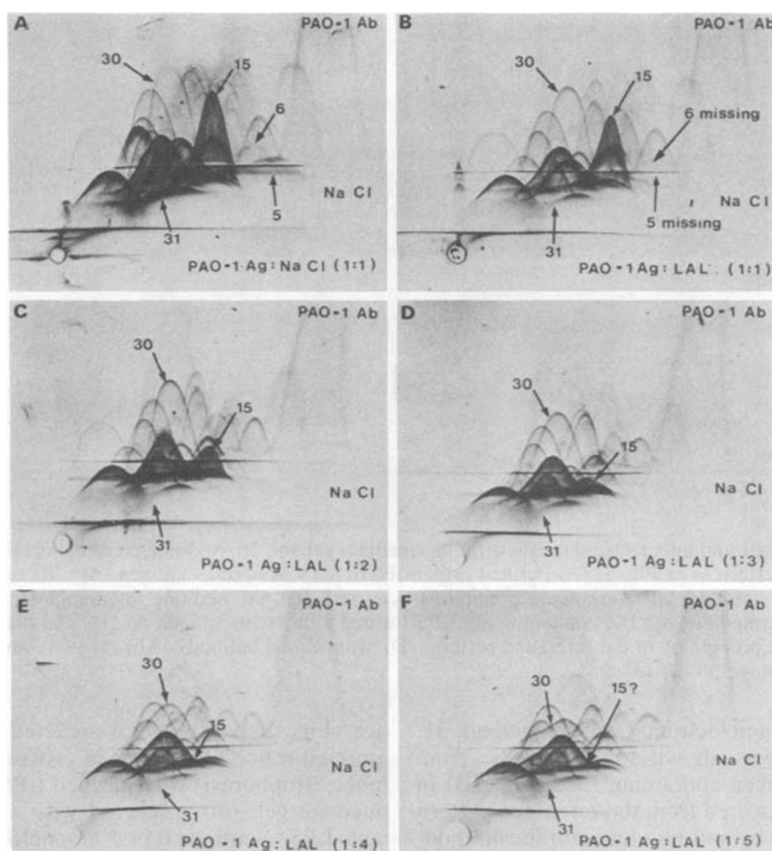

FIG. 3. Absorption of PAO-1 antigen (Ag) with *Limulus* amoebocyte lysate. Crossed immunoelectrophoresis with intermediate gel of the PAO-1 antigen after 1 h of incubation at 37°C with *Limulus* amoebocyte lysate. (A) PAO-1 antigen–saline (1:1), as control, 4μl. (B) PAO-1 antigen–*Limulus* amoebocyte lysate (1:1), 4 μl; notice that antigens no. 5 and 6 are missing and antigens no. 31, 30 and 15 are diminished. (C) PAO-1 antigen–*Limulus* amoebocyte lysate (1:2), 6 μl; peak height of antigens no. 30 and 15 has decreased further. (D) PAO-1 antigen–*Limulus* amoebocyte lysate (1:3), 8 μl; the left leg of antigen no. 31 is missing. (E) PAO-1 antigen–*Limulus* amoebocyte lysate (1:4), 10 μl antigen no. 15 has diminished to a small hump. (F) PAO-1 antigen–*Limulus* amoebocyte lysate (1:5), 12μl; a shoulder has formed to the left leg of peak 30.

gens present in protein H_2 and I were found in patients with chronic *P. aeruginosa* infections, whereas antibodies were not detected in the first 7 days in acutely infected patients. Even though it varied from patient to patient, 14 days of acute infection was about the average time before the detection of antibodies toward these two proteins in patients' sera. In rats with artificial lung infections, antibodies to these proteins were formed as early as 7 days postinfection.

DISCUSSION

The PAO-1 antigen-antibody reference system described in this study contained 36 precipitins (Fig. 1). Even though this number was less than the 55 precipitins reported by Høiby and Axelsen (22), there were no major differences in the antibody produced against the more prominent antigens of *P. aeruginosa*. We were able to demonstrate this point by mixing antibodies from the two systems and by interchangeably using the two types of antigens in either of the reference systems (data not shown). However, one reason for these discrepancies could be the nature of the immunogens, since Formalin-fixed cells were used in this study rather than whole cell sonicates (22). Thus, the bacterial antigens being presented to the humoral immune system will be those preserved on the bacterial cell surface (15), thereby giving us a different reference pattern with emphasis on antibody production to those surface antigens most directly presented to the host. Both reference systems were adequate as base-line references to investigate antibody production to particular antigens.

From the full spectrum of antigens displayed in the PAO-1 reference system, the first outer

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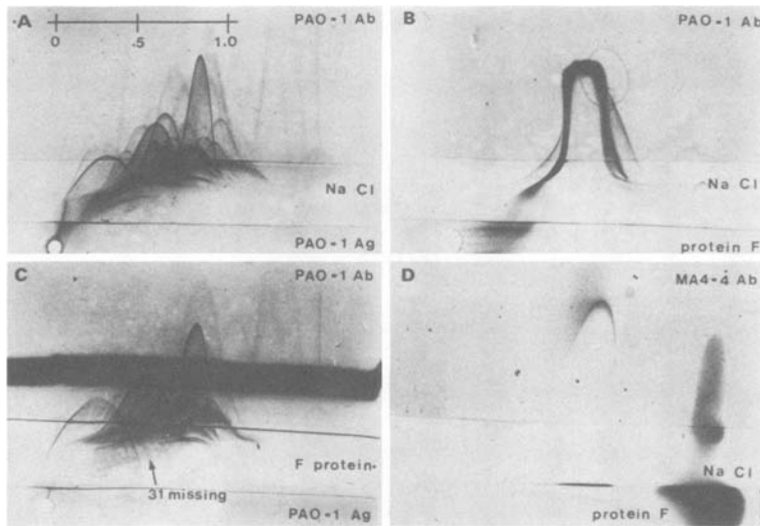

FIG. 4. Crossed immunoelectrophoresis with intermediate gel and crossed-line electrophoresis of protein F. (A) Reference pattern as in Fig. 1. (B) Purified protein F (16 μl) was used as antigen (Ag). Its tight association with LPS was obvious, and overlapping precipitins occurred. (C) Crossed-line immunoelectrophoresis with protein F in intermediate gel; the contaminating LPS formed a line with antigen no. 31, and protein F did not identify with the precipitins in the reference pattern. (D) Monoclonal antibody (Ab) (MA4-4) was added to the second-dimension gel at 8.4 μl/cm^2.

membrane antigen identified was antigen no. 31. This antigen was shown to be specific from smooth, O-antigen-containing LPS (Fig. 2B) in that (i) it disappeared from the reference pattern when absorbed by anti-outer membrane antibodies (Fig. 2C), (ii) it cross-reacted to form the precipitin line of identity in crossed-line immunoelectrophoresis with purified LPS in the intermediate gel, (iii) it reacted with a homologous anti-LPS O-antigen type 5 monoclonal antibody,

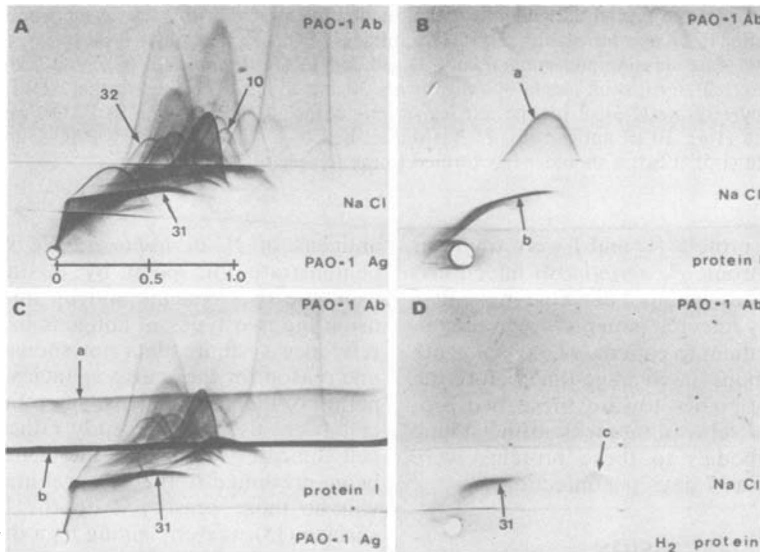

FIG. 5. Crossed immunoelectrophoresis with intermediate gel and crossed-line immunoelectrophoresis of proteins I and H_2. (A) PAO-1 antigen (Ag) and PAO-1 antibody (Ab) reference system. (B) protein I (16 μl) was used as antigen; note that there were four overlapping precipitins. (C)Crossed-line immunoelectrophoresis with protein I in the intermediate gel (8.33 μl/cm^2). A precipitin line was formed, but it did not identify with any precipitin of the reference system, because there was no disappearance of any antigen from the reference pattern. (D) Protein H_2 (8 μl) used as the antigen; note that this purified protein also contained a small amount of contaminating LPS.

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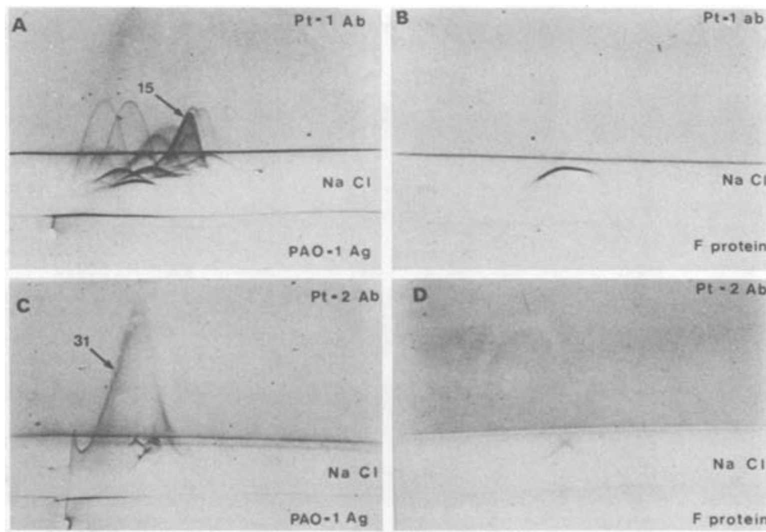

FIG. 6. Immunogenicity of protein F in patients with either chronic or acute *P. aeruginosa* infections. Antigens (Ag) used were PAO-1 antigens and protein F, and saline (NaCl) was added to the intermediate gels. Patient sera were incorporated into the second-dimension gel as follows. (A) Serum from a chronic patient (pt-1 Ab); about 20 precipitins were formed with PAO-1 antigens. (B) Serum from a chronic patient (pt-1 Ab); note that a precipitin was formed with protein F. (C) Serum from an acute patient (pt-2 Ab); note that there were high-titer antibodies (Ab) against antigen no. 31. (D) Serum from an acute patient (pt-2 Ab); note that there was a positive precipitin reaction with protein F.

and (iv) it reacted chemically with *Limulus* amoebocyte lysate. As a point of interest the mobility and morphology of antigen no. 31 in the reference pattern resembled the LPS of other gram-negative bacteria in crossed immunoelectrophoresis analyses (8, 11, 44).

It was most intriguing to see how monoclonal antibodies MA1-8 (LPS O-antigen specific) and MA3-8 (LPS rough core specific) reacted with their respective antigen, the LPS molecule (Fig. 2E and F). The appearance of a "tail" to the precipitin of antigen no. 31 when monoclonal antibody MA1-8 was added to the intermediate gel occurred, whereas antigen no. 5 disappeared. This same tail-like precipitin was also formed when MA3-8, an anti-LPS rough core monoclonal antibody, was used. MA3-8, however, appeared to neither recognize nor react with the heavily stained part of antigen no. 31. The specificity of monoclonal antibodies was therefore capable of demonstrating the existence of the multiple antigenic determinants in the LPS molecule. Antigen no. 5 is probably the core region of the LPS, which is equivalent to the antigenic determinant found in rough LPS. The characteristic heavy staining of antigen no. 31 by Coomassie brilliant blue stain, which specifically stains proteins, suggested that this precipitin possibly represented high-titer antibodies reacting with a protein antigen. Thus, this heavily stained part of antigen no. 31 closely resembled the description of the protein moeity (OEP) tightly associated with endotoxin, as described by Homma and co-workers (1, 24). Its lack of interaction with MA3-8 could be interpreted as the masking of rough core determinants by protein associated with the LPS.

Thus far, the most sensitive method for the detection or quantitation of endotoxin has been the *Limulus* amoebocyte lysate test (14, 29). This test is based on the fact that endotoxin is capable of causing coagulation of a clottable protein, coagulogen, present in the lysate of the amoebocyte (the blood cell of the horseshoe crab, *Limulus polyphemus*). Thus, when we mixed the PAO-1 antigen with *Limulus* amoebocyte lysate, antigen no. 31 reacted strongly and lost the left part of its precipitate. Again, this provided us with further evidence that antigen no. 31 is an endotoxin (or LPS) complex, probably consisting of several antigenic determinants as well as the lipid A portion that causes gelling of the *Limulus* amoebocyte lysate. It was also interesting to see four other antigens that reacted with *Limulus* amoebocyte lysate. Two of these, antigens no. 5 and 6 were highly reactive, in partial agreement with other experiments suggesting antigen no. 5 might be another form of LPS. Antigens no. 15 and 30 reacted less strongly with a gradual change of the precipitin morphology (Fig. 3B through F).

Crossed immunoelectrophoresis of the purified outer membrane proteins F, H_2, and I with the reference antibody system of PAO-1 helped

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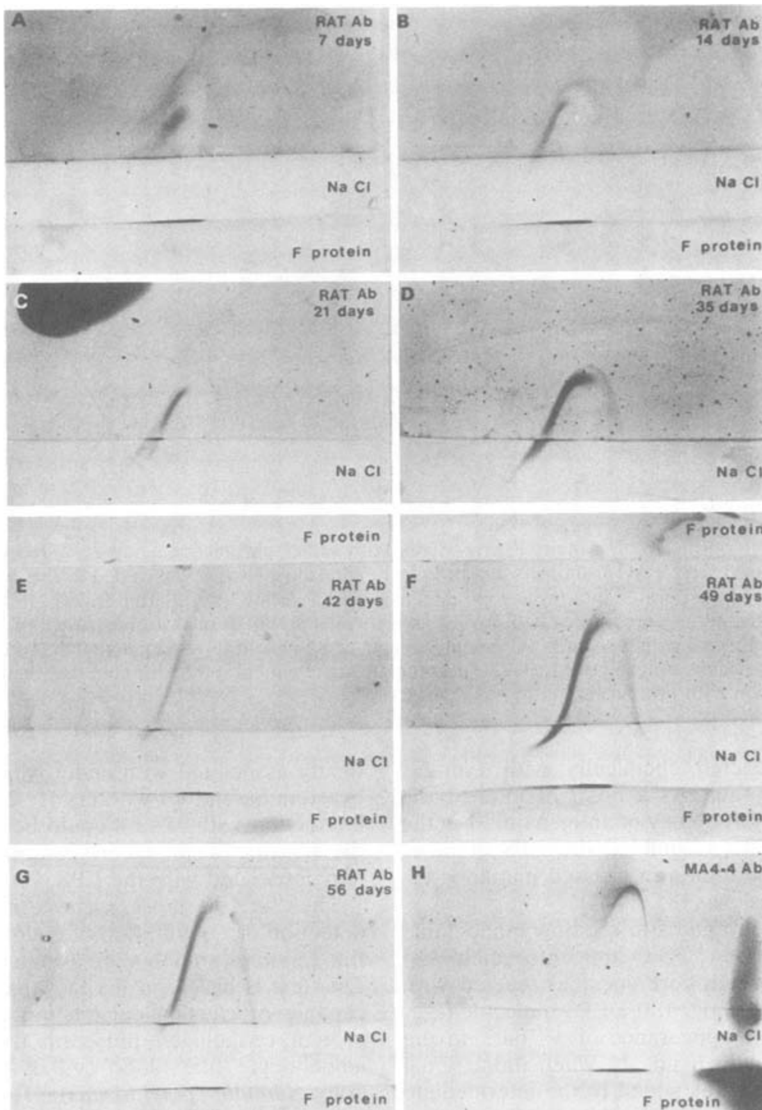

FIG. 7. Immunogenicity of protein F in rats with chronic *P. aeruginosa* infections. Crossed immunoelectrophoresis of protein F (8 μl) against sera of rats collected at the following regular intervals postinfection: (A) 7 days, (B) 14 days, (C) 21 days, (D) 35 days, (E) 42 days, (F) 49 days, (G) 56 days. (H) For comparison, monoclonal antibody (MA4-4) against protein F was used in this second-dimension gel.

to confirm their immunogenicity. Precipitin formation was indicative that they elicited antibody response and were presented to the host's humoral immune system perhaps after a measure of antigen processing after phagocytosis of the immunogens by macrophages. However, they could not be identified in the reference PAO-1 antigen system. By performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we were able to demonstrate that these antigens were present in the PAO-1 antigen preparation in extremely low quantities (data not shown). The small amount was probably too low to be detected in the cross immunoelectrophoresis system. When the quantities of these proteins were enriched by purification, they were easily recognizable by the antibodies raised against them (Fig. 4B, 5B, and 5D).

Patients harboring *P. aeruginosa* chronically were known to have high levels of antibody response to most of the antigens of this organism (21), and Fernandes et al. (12) reported the detection of antibodies produced to two cell envelope proteins with apparent molecular weights of 58,500 and 37,500. In this study, we found antibodies to all three of the outer mem-

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brane proteins F, H_2, and I in 220 sera from patients chronically infected with *P. aeruginosa*. In the chronic rat lung infection model, antibody response toward the three outer membrane proteins studied was detected at 7 days postinfection, in agreement with the data on chronically and acutely infected patients.

The frequency of *P. aeruginosa* infections was reported as high among acute leukemic patients (13). This high frequency might be related to the deficiency in neutrophils (44). Crowe et al. (9), in a recent study, were able to detect antibodies against *P. aeruginosa* exoproducts like exotoxin A and proteases. In this study, we detected antibodies, in this type of patient, to the proteins F, H_2, and I.

In conclusion, we have used affinity absorption with *Limulus* amoebocyte lysate and the exquisite specificity of monoclonal antibodies to identify the LPS antigen in immunogenic preparations of *P. aeruginosa* and to detect anti-LPS antibodies formed in response to injection of these immunogens or in response to animal model and human infections. We have shown that crossed immunoelectrophoresis can be used as an immunochemical tool to check the absolute purity and comparative antigenicity of bacterial cell surface macromolecules, and we have demonstrated that outer membrane proteins, F, H_2, and I are immunogenic in animals and patients with both chronic and acute *P. aeruginosa* infections.

ACKNOWLEDGMENTS

We received financial assistance from the Canadian Cystic Fibrosis Foundation and the Alberta Heritage Foundation for Medical Research. J.S.L. has an AHFMR studentship, and L.M.M. has a Commonwealth Scholarship.

We thank Joyce Nelligan and Sheila Costerton for the photographic work and Val Preuter and Lee Watkins for typing the manuscript.

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