

Occurrence of a Common Lipopolysaccharide Antigen in Standard and Clinical Strains of *Pseudomonas aeruginosa*

M. Y. C. LAM,¹ E. J. MCGROARTY,² A. M. KROPINSKI,³ L. A. MACDONALD,¹ S. S. PEDERSEN,⁴
N. HØIBY,⁴ AND J. S. LAM^{1*}

Department of Microbiology, University of Guelph, Guelph, Ontario, Canada N1G 2W1¹; Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824²; Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6³; and State Serum Institute, Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark⁴

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The lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* PAO1 contains two species of O polysaccharide termed A and B bands. The high-molecular-weight B-band LPS determines the O specificity of the bacterium, while the antigenically distinct A-band LPS consists of only shorter-chain polysaccharides. Seven hybridomas secreting A-band-specific monoclonal antibodies were produced and used to study the LPS of standard and clinical strains. Although A-band antibodies did not agglutinate any of the serotype strains presently in the International Antigenic Typing Scheme, Western immunoblots revealed that 11 of the 17 serotype strains possessed A-band LPS. In a group of 250 clinical isolates from patients with cystic fibrosis, 170 (68%) had A-band LPS on the basis of agglutination tests, but in silver-stained gels all were shown to be deficient in O-antigen-containing B band. Investigation of serial isolates from a single patient revealed a pattern of antigenic variation. During the course of the infection, serotypeable isolates became nontypeable, and the O antigen was replaced with A band as the major LPS antigen. These results suggest that A-band LPS may be the major LPS antigen in nontypeable clinical isolates and a common antigen among other *P. aeruginosa* strains.

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of gram-negative bacteria and has essential roles in the structure and function of the membrane (14). In addition, LPS is an important virulence factor (6) and therefore has a role in pathogenesis. Heterogeneity in the size of the LPS molecule has been reported in *Pseudomonas aeruginosa* (5) as well as in members of the family *Enterobacteriaceae* (9, 18, 20). This size heterogeneity has been attributed to variations in the number of repeating units in the O antigen. Aside from producing LPS molecules of different lengths, it also appears that some gram-negative bacteria are capable of producing LPS with chemically different polysaccharides linked to the core. These include *Bordetella pertussis* (4), *Salmonella typhimurium* (12), *Salmonella paratyphi* (12), and *P. aeruginosa* (5). In a study using *P. aeruginosa* PAC1, Koval and Meadow (13) proposed that there may be four types of LPS in a single strain: a rough LPS with an unsubstituted core polysaccharide, a smooth-rough LPS, and two long-chain LPS each with a different side chain. Recently, Rivera et al. (24) have demonstrated that *P. aeruginosa* PAO1 is capable of synthesizing more than one type of LPS. Analysis by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the PAO1 LPS consisted of two antigenically and chemically distinct molecules termed A- and B-band LPS, respectively (24). B band, generally composed of LPS with long-chain O antigen, is the LPS species responsible for the O specificity of the organism, while A band is composed of LPS molecules that only have short-chain polysaccharides. The two molecules are apparently antigenically different, since A-band LPS failed to react with O antigen (B-band)-specific monoclonal antibodies (MAbs).

For the purpose of studying the structure, function, and clinical significance of A-band LPS, we have produced seven hybridomas secreting A-band-specific MAbs. Using Western immunoblotting and A-band-specific MAbs, we present data that suggest that A-band LPS is a common antigen produced by most *P. aeruginosa* strains and may be the major LPS antigen in nontypeable (NT) clinical strains.

MATERIALS AND METHODS

Bacterial strains and sera from patients. *P. aeruginosa* serotype strains O1 to O17 of the International Antigenic Typing Scheme (IATS) and their growth conditions have been described in detail previously (17). Strain AK1401, an LPS-defective mutant of PAO1, was used to detect A-band-specific antibodies in sera from patients with cystic fibrosis (CF) (1). Clinical strains of *P. aeruginosa* isolated from patients with CF and sera from patients were obtained from the State Serum Institute, Rigshospitalet, Copenhagen, Denmark. These strains were typed by using both the *Pseudomonas* typing sera (Difco Laboratories, Detroit, Mich.) and a set of 17 serotype-specific MAbs produced in our laboratory (16, 17). A set of serial isolates, strains 49 to 58, was obtained from one patient with CF over a 1-month period, with samples being taken every 2 to 3 days. Serum samples from 179 patients with CF were analyzed in a dot blot assay. A group of 66 patients (mean age, 7.5 years) were not colonized with *P. aeruginosa*, while 113 patients (mean age, 15.9 years) had been chronically infected with *P. aeruginosa* for an average of 6.5 years (range, 1 to 15 years).

LPS isolation. LPS from *P. aeruginosa* PAO1 (IATS O5) was isolated by the method of Darveau and Hancock (7), followed by two chloroform-methanol extractions as described by Rivera et al. (24). LPS prepared by this method was used both for screening hybridomas and for column chromatography. For SDS-PAGE and Western immunoblot-

* Corresponding author.

ting, the LPS of standard serotype strains and clinical isolates was prepared by the method of Hitchcock and Brown (11). LPS for dot blot analysis was also prepared by the method of Hitchcock and Brown except that bromphenol blue was omitted from the lysing buffer.

A- and B-band LPS isolation. The separation of PAO1 LPS by gel filtration chromatography was performed as described previously (24). Briefly, approximately 30 mg of LPS was applied to a Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) column (64 cm by 25 mm) and run at room temperature using a deoxycholate buffer system. Fractions (5 ml) were collected at a flow rate of 8 ml/h and were assayed for the presence of amino sugars and 2-keto-3-deoxyoctulosonic acid (24). Pooled fractions were extensively dialyzed (using 12,000- to 14,000-molecular-weight cutoff membranes) against column buffer without deoxycholate at 37°C and then against distilled water at 4°C. The dialyzed A- and B-band fractions were then lyophilized and stored at 4°C until use.

SDS-PAGE. The discontinuous SDS-PAGE procedure of Hancock et al. (10) with 14% running gels was used. Gels were silver stained by the method of Hitchcock and Brown (11).

Mab production and characterization. To enhance the immunogenicity of the A-band antigen, A-band LPS was mixed with bovine serum albumin in a 2:1 ratio followed by sonication at 100 W for four 30-s intervals. The immunization schedule and method for MAb production were precisely as described by Lam et al. (17). Positive hybridoma clones were screened by an enzyme-linked immunosorbent assay as described previously (17), using microdilution plates (Immulon 2 U plates; Dynatech Laboratories, Inc., Alexandria, Va.) coated with 0.5 µg of PAO1 LPS per well. The isotypes of the MAbs were determined with an enzyme-linked immunosorbent assay method and a mouse typer kit (Bio-Rad Laboratories, Richmond, Calif.). The reactivities of the A-band-specific MAbs with standard serotype strains and clinical strains were examined by slide agglutination and Western immunoblotting (17). The immunoblotting method of Burnette (3) was used but with the following modifications. Nitrocellulose blots were blocked with 1.5% gelatin (Sigma Chemical Co., St. Louis, Mo.) followed by incubation with hybridoma culture supernatant containing A-band-specific MAbs. The blots were developed at room temperature, using a goat anti-mouse F(ab')₂ fragment-alkaline phosphatase-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) and a substrate consisting of 30 mg of Nitro Blue Tetrazolium and 15 mg of 5-bromo-4-chloro-3-indolyl phosphate toluidine in 100 ml of 0.1 M bicarbonate buffer (pH 9.8). The B-band-specific MAb, MF15-4, raised against unfractionated PAO1 LPS, and the set of serotype-specific MAbs have been described previously (17).

Dot blot analysis of sera from patients. Dot blots were done as described by Rivera et al. (24) except that a rabbit anti-human immunoglobulin G-peroxidase-conjugated antibody (Dakopatts, Glostrup, Denmark) and a substrate consisting of 60 mg of 4-chloro-1-naphthol in 120 ml of Tris-buffered saline (20 mM Tris, 500 mM NaCl [pH 7.4]) with 16% methanol and 0.015% H₂O₂ were used.

RESULTS

Chemical analysis of PAO1 LPS and the elution profile from gel filtration chromatography have been previously reported (24). Pooled peak fractions and unfractionated

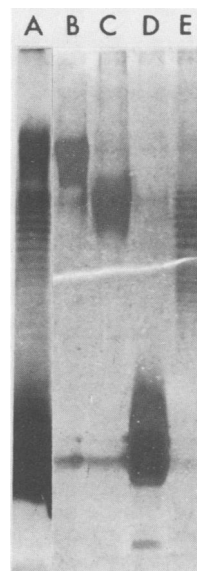


FIG. 1. Silver-stained SDS-PAGE gel of LPS from *P. aeruginosa* PAO1. Lanes: A, unfractionated LPS; B, longer-chain B-band LPS fraction; C, long-chain B-band LPS fraction; D, core-lipid A fraction; E, A-band LPS fraction. Approximately 10 µg of LPS was loaded per well. Note that A-band LPS lacks the high-molecular-weight bands.

PAO1 LPS were visualized on an SDS-PAGE silver-stained gel. Unfractionated LPS appeared as a ladderlike pattern of bands (Fig. 1, lane A) which are representative of LPS molecules containing increasing lengths of O repeating units (11). Separation by gel filtration showed that, in addition to showing variability in size, this LPS consisted of heterogeneous types. The three major amino sugar-containing peak fractions recovered representing B-band-type LPS consisted of two long-chain LPS fractions (Fig. 1, lanes B and C) and a core-lipid A fraction which may have one or two O repeats attached (Fig. 1, lane D). An O-specific MAb, MF15-4, raised against PAO1 O antigen reacted specifically with the long-chain B-band LPS fractions in Western immunoblots (Fig. 2, lane 4). A fourth minor peak corresponding to A-band LPS contained lower-molecular-weight O polysaccharides which did not react with MAb MF15-4.

The use of an A-band-bovine serum albumin mixture appeared to be an effective immunogen, since seven hybridomas secreting A-band-specific MAbs were isolated. These were designated N1C9, N1F10, N1C6, N1G7, N1F3, N1B6, and N1E7. All seven MAbs were immunoglobulin M antibodies and had reactivity with purified LPS in the enzyme-linked immunosorbent assay but failed to agglutinate PAO1 whole cells. Epitope specificity of the MAbs was determined by Western immunoblotting analysis. MAb N1C9 reacted with lower-molecular-weight bands of unfractionated LPS (Fig. 2, lane 1) and A-band LPS (Fig. 2, lane 2) but failed to react with B-band LPS. Since all other A-band MAbs showed similar reactivity to MAb N1C9, all subsequent analysis was done using this MAb. In contrast, MAb MF15-4 reacted with the full range of O-antigen ladders of unfractionated LPS and the high-molecular-weight B-band LPS (Fig. 2, lanes 3 and 4, respectively) but not with A-band-type LPS. These results confirmed that A- and B-band LPS are antigenically distinct molecules.

When we examined the reactivity of the A-band MAb with LPS from all 17 *P. aeruginosa* serotype strains, 11 of the 17

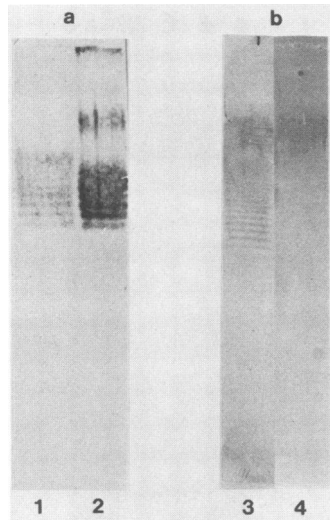


FIG. 2. Western immunoblot of unfractionated and fractionated LPS from *P. aeruginosa* reacted with A-band-specific MAb N1C9 (a) and O5-specific MAb MF15-4 (b). Lanes: 1 and 3, unfractionated PAO1 LPS (20 μ g); 2, A-band LPS (10 μ g); 4, B-band LPS (10 μ g).

showed reactivity in Western immunoblots (Fig. 3). Serotype strains O7, O12, O13, O14, O15, and O16 were not reactive with the MABs and thus appear to lack A-band-type LPS. The reactivity of many serotype strains in Western blots is of interest because none of the serotype strains reacted with the A-band MAB in agglutination tests. The lack of agglutination reactions may be due to masking of the shorter-chain A-band LPS by the longer-chain B-band LPS molecules.

In a search for an A-band-producing reference strain, we examined a number of LPS-defective mutants and found strain AK1401 to be an A-band producer that was deficient in B-band expression. This reference strain was isolated previously (1), and preliminary data indicate that AK1401 is analogous to the *S. typhimurium rfc* mutants which are

unable to polymerize O polysaccharides and thus lack reactivity with O-specific MABs (data not shown). Strain AK1401, however, did react with the A-band MAB in agglutination tests and Western blots (Fig. 4, lane C). Silver-stained gels revealed that the AK1401 LPS lacks the high-molecular-weight bands that are generally seen in the PAO1 strain (Fig. 4, lanes A and B). In addition, the spacing between these bands was similar to that of the pooled A-band fraction (Fig. 1, lane E). Once established as an A-band reference strain, AK1401 was used to detect A-band antibody response in sera from patients with CF in subsequent experiments.

The occurrence of A-band LPS in *P. aeruginosa* strains isolated from patients with CF was examined by slide

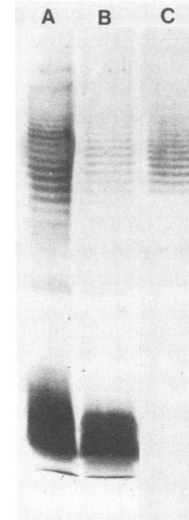


FIG. 4. Silver-stained SDS-PAGE gel of LPS from *P. aeruginosa* PAO1 and AK1401 (lanes A and B) and Western immunoblot (lane C) of AK1401 LPS reacted with A-band-specific MAb N1C9. Lanes: A, PAO1 LPS (20 μ g); B and C, AK1401 LPS (15 μ g).

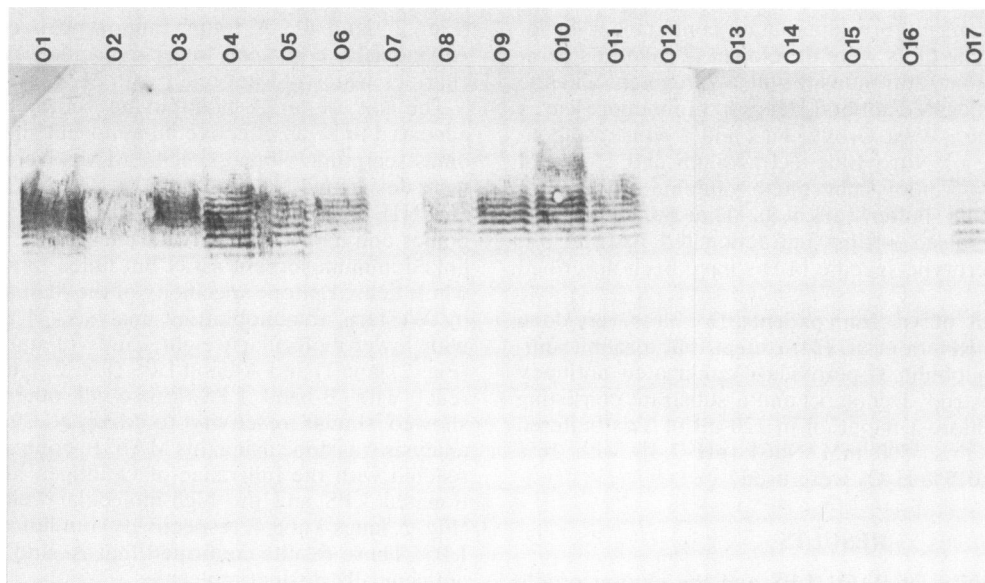


FIG. 3. Western immunoblot of LPS from *P. aeruginosa* serotype strains O1 to O17 reacted with A-band-specific MAb N1C9. Approximately 20 μ g of LPS was loaded per well.

TABLE 1. Serotyping of CF isolates with MABs against serotypes O1 to O17 (16, 17) and MAB N1C9

Serogroup ^a	No. of isolates (%)	No. of isolates which tested ^b :	
		A ⁺	A ⁻
O3	148 (59.2)	118	30
O6	14 (5.6)	0	14
O9	5 (2)	1	4
O11	6 (2.4)	4	2
O1	6 (2.4)	2	4
NT	69 (27.6)	45	24
AA	2 (0.8)	ND	ND

^a Serogroups are defined by agglutination reactions of isolates with O-specific MABs.

^b A⁺, Positive agglutination with the A-band LPS-specific MAB N1C9; A⁻, no agglutination with the A-band MAB; ND, not done.

agglutination tests (Table 1). Among the 250 isolates examined, 179 were serotypeable with our set of MABs, and 71 were NT. Of the NT group, 69 strains were nonagglutinating and 2 were autoagglutinating. The unusually high number of isolates found to belong to serogroup O3 is probably due to the fact that many of these isolates were obtained by serial isolation from several patients. The percentage of NT strains, 28.4%, is relatively low compared with other serotyping studies which used polyclonal antisera. Among the entire set of CF strains examined, 170 of the 250 isolates (68%) showed a positive agglutination with the A-band-specific MAB N1C9. Within the group of O3 isolates, 118 of 148 (79%) agglutinated strongly with MAB N1C9. This may indicate that these O3-agglutinating strains are sufficiently deficient in B-band LPS expression to allow A-band LPS to be exposed on the cell surface and available for interaction with MAB N1C9. Among the NT nonagglutinating strains, 45 of 69 (65%) agglutinated with MAB N1C9, thus confirming that A band is their major LPS antigen. Those that did not agglutinate with MAB N1C9 probably possess the rough LPS chemotype which is completely devoid of any O side chain sugars. When the LPS of one of these latter strains, isolate 189, was analyzed by SDS-PAGE, no ladderlike bands could be observed and only one heavily staining band representing lipid A-core polysaccharide was observed at the dye front (data not shown).

Further studies were performed with serial isolates of *P. aeruginosa* from a single patient with CF. Variation in the LPS banding pattern as revealed by SDS-PAGE was observed in serial isolate strains 49 through 58. Two early isolates, strains 49 and 50, and one later isolate, strain 58, were shown to be of serotype O9 by slide agglutination with an O9-specific MAB, MF94-1 (not previously reported), while all subsequent strains were NT and nonagglutinable. Strains 50 and 57 had banding profiles similar to that of the standard IATS O9 strain in silver-stained gels (Fig. 5). All other isolates appeared to have similar LPS banding profiles but one that was distinctly different from that of serotype O9. Despite the observable differences in LPS banding profile, the LPS from all serial isolates of this patient reacted with an O9-specific MAB, MF94-1, in Western immunoblotting (Fig. 6a). Although the MAB reacted differently with the LPS of the O9 and NT strains, the detection of reactivity indicates that the strains contain O9-reactive epitopes and are probably related. A-band LPS appeared to be a common antigen, since all serial isolates reacted with the A-band MAB N1C9 in Western blots (Fig. 6b) and all except strain 49 agglutinated with MAB N1C9. Serum samples from this same patient obtained yearly over a 13-year period appeared

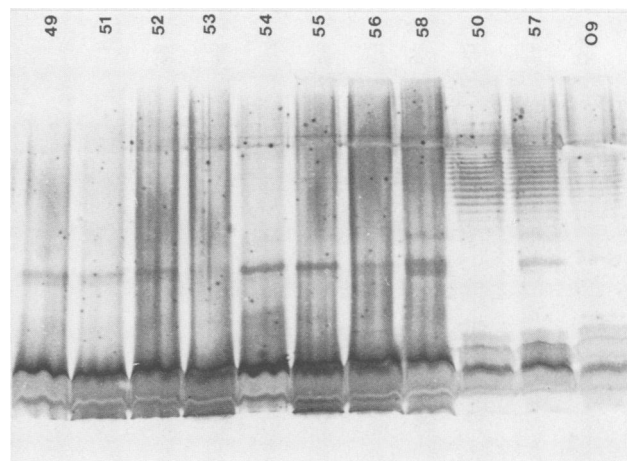


FIG. 5. Silver-stained SDS-PAGE gel of LPS from serial isolates of *P. aeruginosa* obtained every 2 to 3 days over a 1-month period from a patient with CF. The strains analyzed were: 49 (O9/A⁻), 51 (NT/A⁺), 52 (NT/A⁺), 53 (NT/A⁺), 54 (NT/A⁺), 55 (NT/A⁺), 56 (NT/A⁺), 58 (O9/A⁺), 50 (O9/A⁺), 57 (NT/A⁺), and IATS O9. Approximately 20 μ g of LPS was loaded per well. The designations in parentheses are as follows: O9, strain typeable with O9-specific MAB MF94-1; NT, nontypeable; A⁺, agglutinable with A-specific MAB N1C9; and A⁻, nonagglutinable with MAB N1C9.

to contain A-band-specific antibodies since all sera agglutinated strongly with strain AK1401. Sera from three other patients with CF were also shown to contain A-band antibodies. Serum samples which contained two or more precipitin bands against standard antigen (whole-cell sonic extracts of serotypes O1 to O17) in crossed immunoelectrophoresis analysis (21) were found to agglutinate whole cells of strain AK1401 (data not shown). These data suggest that A-band polysaccharides are frequently present on the cell surface of clinical strains and can stimulate a humoral response in patients with CF.

Dot blot results of the interaction of sera from patients with CF with LPS of strain AK1401 revealed a highly significant difference ($P < 0.0001$) between the infected and noninfected groups of patients with respect to the reactivities of their sera with A-band LPS (Table 2). Analysis of variance showed that patients with positive dot blot results had significantly lower pulmonary function, higher numbers of precipitating antibodies against *P. aeruginosa*, increased duration of *P. aeruginosa* infection, and increased age than those with negative tests.

DISCUSSION

The LPS of *P. aeruginosa* PAO1 was shown to consist of two different types of LPS molecules termed A and B bands. The ability to produce heterogeneous forms of LPS in *P. aeruginosa* was first proposed by Chester and Meadow (5), who provided evidence that strain PAC1 could make two types of side chain designated H1 and H2 polysaccharide. These molecules were differentiated by their elution profiles in column chromatography. With the development of more sophisticated methods of LPS analysis, such as silver staining of SDS-PAGE-separated LPS and Western immunoblotting with specific antibodies, we demonstrated that the two LPS species of PAO1 have unique migration properties in gels and are antigenically distinct. B-band LPS is responsible for the serotype specificity of an organism, and thus each

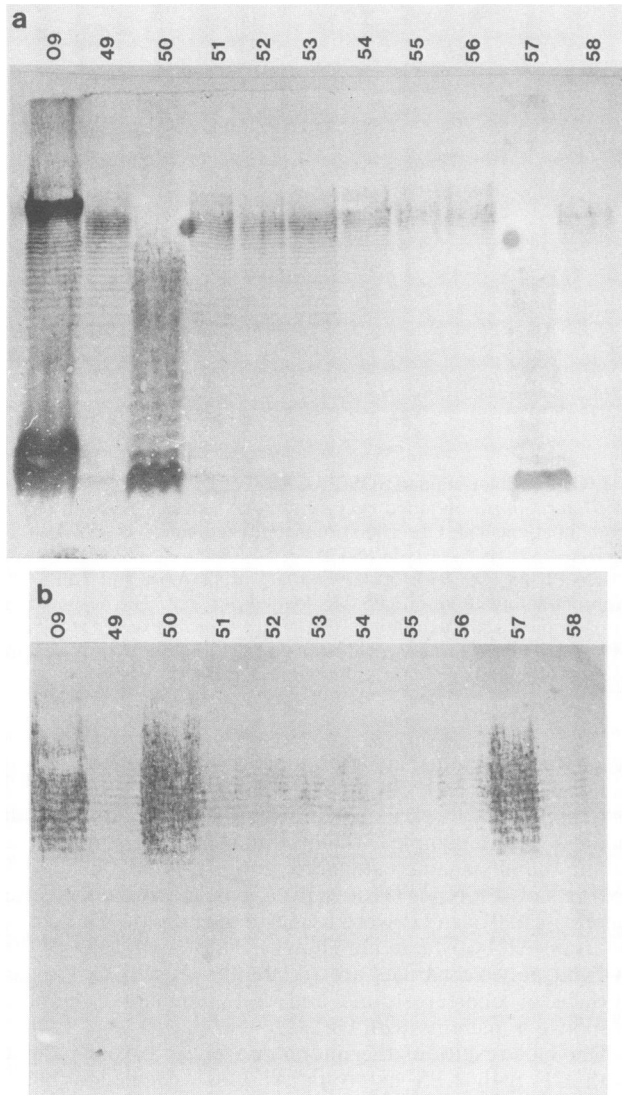


FIG. 6. Western immunoblots of LPS from serial isolates of *P. aeruginosa* from a patient with CF reacted with O9-specific MAb MF94-1 (a) and A-band-specific MAb N1C9 (b). The strains analyzed are identical to those described in the legend to Fig. 5. Approximately 20 μ g of LPS was loaded per well.

serotype has a unique type of B-band LPS on its surface. As demonstrated by both chromatography and SDS-PAGE results, A-band LPS consists of shorter-chain polysaccharides. This A-band polysaccharide appears to be a frequently occurring antigen in *P. aeruginosa* serotype strains, as it was detected in 11 of the 17 standard serotype strains. The reactivity of A-band MAbs with LPS in Western immunoblots and the lack of reactivity with standard serotype strains in whole-cell agglutination tests suggest that on the cell surface long-chain B-band molecules may be masking shorter-chain A-band LPS, thereby making the A-band antigens inaccessible to antibodies. Sawada et al. (25) have reported the production of a MAb, E87, that is also reactive against a common polysaccharide antigen. MAb E87 appeared to react with a lower-molecular-weight rhamnose-rich polysaccharide which may be similar to A-band LPS. Preliminary experiments using Western immunoblotting of A- and B-band LPS with MAb E87 revealed that this MAb reacted

TABLE 2. Correlation between dot blot reactivity and status of infection of 179 patients with CF

Status of infection by <i>P. aeruginosa</i>	No. of patients with dot blot reaction ^a :			
	Negative	Intermediate	Positive	Total
Infected	17	16	80	113
Noninfected	29	15	22	66
Total	46	31	102	179

^a Reactivity of sera from patients with LPS from strain AK1401, an A-band producer which does not express O-specific B-band LPS. Negative, No reaction; intermediate, weak reaction; positive, strong reaction. Relevant positive and negative controls were done.

with A-band LPS and not B band (E. J. McGroarty, unpublished results). However, it will not be known whether our MAbs have the same epitope specificity as E87 until further analysis is done, since their results were obtained using enzyme-linked immunosorbent assay, a technique which does not analyze epitope specificity as precisely as immunoblotting.

A-band LPS also appears to be a common antigen among clinical isolates. In a group of 250 clinical strains isolated from patients with CF, 68% agglutinated with A-band-specific MAbs. We propose that this is an underestimated value because agglutination tests would not detect B-band-containing strains which possess sufficient O antigen to block antibody accessibility to A-band LPS.

Penketh et al. (22) have reported that *P. aeruginosa* isolates from patients with CF with chronic bronchopulmonary infections show a loss of O-serotype reactions and express a new somatic antigen called polyagglutinating antigen on their surface. Further characterization of this antigen has not been reported; however, it may be related to the A-band antigen reported here.

On many NT clinical isolates (65%), A-band LPS appears to be a major LPS antigen. NT isolates are thus designated because they do not react with any of the 17 O-specific MAbs and therefore cannot be placed into any serotype. We have previously shown in silver-stained SDS-PAGE gels that the LPS of many NT strains are deficient in the high-molecular-weight B-band antigens (15a). O-antigen deficiency in NT isolates has also been reported by several researchers (10, 19, 23). Because serotyping is a reflection of differences in the O antigen of the LPS molecule, the absence of high-molecular-weight B bands could explain the nontypeability of these clinical isolates. Our investigation of strains obtained from a single patient with CF at different times following infection revealed an interesting pattern of antigenic variation in which earlier isolates were serotypeable and possessed both A- and B-band LPS, while subsequent isolates from the same patient were found to be NT with O-specific MAbs but were reactive with A-band MAbs in agglutination tests. All isolates from this patient appeared to be related since the isolated LPS of all strains showed reactivity with the O-specific MAb, MF94-1. The NT isolates may have originated from serotypeable strains which during the infection altered the expression of their LPS antigens. Because there is an unusually high percentage (60 to 80%) of NT isolates from CF sources (10, 22, 23) and because many of these NT strains appear to be deficient in high-molecular-weight LPS (10, 15a), it is of considerable interest to find that A band becomes the major LPS antigen when there is a deficiency in B-band LPS. As suggested by Pitt et al. (23), antigenic variation in *P. aeruginosa* isolates from patients with CF may be induced by a selective

pressure of the immune system of the host against the LPS of the initial infecting strain. Presumably, the appearance of a variant strain with altered LPS antigens would provide the organism with some survival advantage. The ability of the environment to influence surface antigen properties has been observed previously (2). Cell surface macromolecules of gram-negative bacteria undergo variation at different growth temperatures and when specific nutrients are limiting (8, 15). Phenotypic variation in turn affects the susceptibility of the organism to host defenses, antibiotics, and disinfectants. The exact influence that the A-band antigen has on bacterial surface properties remains to be determined. In particular, the influence of A-band LPS on surface charge distribution and the chemical nature of the A-band sugars warrant further investigation.

A-band LPS is a unique type of LPS that is antigenically different from the O-specific B-band LPS. It is present in many standard serotype strains as well as in many clinical isolates and therefore appears to be a common *P. aeruginosa* antigen. In addition, A-band LPS is the major LPS antigen in many O-antigen-deficient NT isolates and appears to induce a humoral response in patients with CF infected by various serotype strains of *P. aeruginosa*. Furthermore, the appearance of A-band-specific antibodies in sera from patients can be correlated with decreased lung function and increased duration of *P. aeruginosa* infection. These data demonstrate the potential of using A-band polysaccharide as a vaccine candidate.

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