INFECTION AND IMMUNITY, May 1984, p. 508–513
0019-9567/84/050508-06$02.00/0

Vol. 44, No. 2

Role of Lipopolysaccharide in Virulence of *Pseudomonas aeruginosa*

S. J. CRYZ, JR.,[1]* T. L. PITT,[2] E. FÜRER,[1] AND R. GERMANIER[1]

Swiss Serum and Vaccine Institute, Berne, Switzerland,[1] and Division of Hospital Infection, Public Health Laboratory Service, Colindale, London, England[2]

Received 29 November 1983/Accepted 14 February 1984

The role of lipopolysaccharide (LPS) in the virulence of *Pseudomonas aeruginosa* was studied. The virulence of several *P. aeruginosa* strains for burned mice was found to be directly related to the dispersion of LPS into either the phenol or the water phase after extraction. Virulence decreased as the proportion of LPS recovered from the phenol phase increased. No similar correlation was observed when several other strain characteristics were investigated. This phenomenon was studied in greater detail by using the "smooth"-specific phage E79 to select mutants altered in LPS structure. One such mutant, PA220-R2, was extensively characterized. LPS isolated from PA220-R2 was found to be completely deficient in high-molecular-weight polysaccharide material. This alteration rendered the strain serum sensitive and dramatically changed the reaction with O-specific typing sera and sensitivity to typing phages. However, motility, toxin A and elastase production, and 22 metabolic functions remained unchanged. PA220-R2 was found to be comparatively nonvirulent, with a 50% lethal dose more than 1,000-fold higher than that of its parent for burned mice. This was due to the inability of PA220-R2 to establish an infection in burned skin.

Recent studies have shown that *Pseudomonas aeruginosa* virulence is multifactorial, involving both somatic and extracellular antigens (5, 12, 13, 25, 36, 41, 43). Numerous factors have been identified which are believed to contribute to virulence (13, 44). Insight into the role that several such virulence factors may play in the pathogenesis of *P. aeruginosa* infections has been obtained by the use of mutant strains deficient in only a single factor (4, 23, 25–27, 41). Most of the investigations have centered on toxic extracellular products. Alteration of the pathogenic potential of *P. aeruginosa* has been shown to be dependent not only upon the factor affected by the mutation but also upon the animal model employed. Therefore, specific toxin A-deficient mutants were found to be less virulent in a corneal infection model (25) and in a chronic lung model (41) but were as virulent as the parent strain in an acute lung model (5). Similar findings were reported when an isogenic elastase mutant was used (5, 25, 41).

The effect of loss or alteration of a somatic antigen on *P. aeruginosa* virulence has been studied only in regard to motility; a nonmotile mutant lacking flagella was far less virulent for burned mice than was its parent strain (23).

Several lines of evidence suggest that lipopolysaccharide (LPS) contributes substantially to *P. aeruginosa* virulence. These include (i) the toxic nature of *P. aeruginosa* LPS (29, 44); (ii) the fact that LPS may confer serum resistance (42); and (iii) the fact that antibody to LPS has been shown to be highly protective in experimental models (8–10, 30, 35). Although mutants altered in LPS structure have been reported for *P. aeruginosa* (19, 34), the effect that such mutations have on virulence is unknown.

In this study, we investigated the contribution of LPS to virulence by (i) examining the virulence of several *P. aeruginosa* strains in relation to LPS composition, and (ii) isolation and characterization of a mutant strain possessing an altered LPS structure.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and growth conditions. *P. aeruginosa* PA220 and PAO-1 were kindly provided by B. Wretlind, Karolinska Institute, Stockholm, Sweden. *P. aeruginosa* strains M-2, SBI-U, and SBI-2 were gifts from I. A. Holder, Shriners Burns Institute, Cincinnati, Ohio. *P. aeruginosa* strains IT-1, IT-2, IT-3, IT-4, IT-5, IT-6, and IT-7 were provided by M. Fisher, Parke, Davis & Co., Detroit, Mich. *P. aeruginosa* PA53 was a gift from B. H. Iglewski, University of Oregon Health Science Center, Portland, Oreg. *P. aeruginosa* 8505 was from the culture collection of the Public Health Laboratory Service, Colindale, London, England. The "smooth" LPS-specific bacteriophage E79 (16) was obtained from A. M. B. Kropinski, Queens University, Kingston, Ontario, Canada. Cultures for the isolation of LPS were grown at 37°C to stationary phase in Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% (vol/vol) glycerol. Cultures to be tested for toxin A production were grown in the deferrated TSB medium of Björn et al. (4) at 32°C with shaking for 16 h. Cultures to be tested for elastase production were grown in PTSB medium (26) at 37°C for 16 h with shaking. Phage typing plates consisted of 20 g of nutrient broth (Oxoid Ltd., Basingstoke, England), 5 g of NaCl, 7 g of Oxoid agar no. 1, and 400 μg of $CaCl_2$ per ml in 1 liter.

Isolation of LPS mutants. PA220 was grown to mid-log phase in TSB medium (equivalent to 2×10^8 CFU/ml). To 0.9 ml of culture was added 10^8 PFU of phage E79, and the mixture was incubated for 30 min at 37°C. Aliquots (0.1 ml) were plated onto Trypticase soy agar plates, which were incubated overnight at 37°C. Several colonies were selected at random and used to inoculate 10 ml of TSB. After 6 h of incubation at 37°C, 0.1 ml was plated and allowed to dry. Approximately 2.5×10^6 PFU of phage in 25 μl was spotted onto each plate and incubated at 37°C overnight. Phage-

* Corresponding author.

resistant cultures were selected, and residual phage were removed by three successive platings of single colonies. One such phage-resistant strain, PA220-R2, was selected for further study.

Physiological and biochemical characterization. An API 20E test kit was used to monitor 22 metabolic functions. Motility was determined by the hanging drop method and by the use of Motility Test Medium (Difco).

Purification of toxin A and elastase. Toxin was purified as previously described (8). The 50% lethal dose (LD_{50}) upon intraperitoneal injection into 18- to 20-g outbred Swiss-Webster white mice was 0.24 μg. Elastase was purified from PAO-1 cell-free culture supernatants by the method of Morihara et al. (24). Purified elastase migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ($M_r \approx 39{,}000$).

Assays for toxin A, elastase, and alkaline protease. The adenosine 5'-diphosphate-ribose transferase assay was performed as previously described (15) for the detection and quantitation of toxin. Cultures were grown in deferrated TSB medium at 32°C for 16 h with shaking. Cell-free culture supernatants (10 μl) were assayed after treatment with urea-dithiothreitol (15), and quantitative assays were performed by comparing the enzymatic activity present in a culture supernatant with a standard curve simultaneously generated with purified toxin A. Qualitative assays were performed as described above. Cultures giving values less than four times greater than background were concentrated 10-fold against an Amicon UM-10 membrane (Amicon Corp., Danvers, Mass.) and reassayed. To determine whether a strain produced elastase, cultures were streaked onto PTSB-elastin agar plates (26), incubated for 48 h at 37°C, and observed for zones of clearing. Quantitation of elastase in cell-free supernatants (cultures grown in PTSB medium for 16 h at 37°C with shaking) was performed by measuring the release of ^{14}C-labeled elastin as previously described (3). This assay was capable of detecting at least 1 μg of elastase per ml. The capacity of a strain to produce alkaline protease was determined by using a modified Elek test with specific sheep anti-alkaline protease (7). A single precipitation band with all strains tested against this antiserum was observed.

Serological typing. Strains were serotyped by slide agglutination with suitably diluted antisera against the 17 O-type strains of the International Antigenic Typing Scheme (22). Cultures were grown on Trypticase soy agar plates (BBL) at 37°C for 18 h. Agglutination tests were performed as previously described (32).

Phage typing. The method of Asheshov (2) was used for phage typing. Briefly, 10 μl of phage suspension (10^4 PFU/ml) was applied to a phage-typing agar plate seeded with a 4-h culture grown in TSB. The plate was incubated overnight at 32°C, and lysis was recorded.

Serum sensitivity. An overnight culture grown in TSB (5×10^8 CFU/ml) was diluted 1:100 in Hanks salts solution, and 100 μl was added to an equal volume of fresh human serum. Controls contained identical amounts of diluted culture and Hanks salts solution. After incubation for 1 h at 37°C with shaking, appropriate dilutions were made and spread onto Trypticase soy agar plates. Plates were incubated overnight at 37°C. Serum-sensitive strains were defined as those for which the viable cell count after incubation was reduced by 100-fold or more. Serum-resistant strains gave a viable count similar to, or slightly greater than, the control.

Isolation and purification of LPS. Cell wall fragments were produced by disrupting bacterial cells in a Dyno-Mill (W. Bachofen AG, Basel, Switzerland) as follows. To 70 g (wet weight) of bacterial cells was added 250 g of glass beads (0.1 to 0.2 mm diameter), and the volume was adjusted to 140 ml with buffer (0.05 M Tris–0.3 M NaCl, pH 9). Cells were processed for three 2-min cycles with cooling. The glass beads were removed by filtration through a G-1 glass filter. Cell wall fragments were sedimented by centrifugation at $18{,}000 \times g$ for 40 min at 22°C, and LPS was extracted by the hot phenol-water method of Westphal et al. (39). Both phases were exhaustively dialyzed against water to remove the phenol and then subjected to three rounds of ultracentrifugation at $100{,}000 \times g$ for 3 h. LPS preparations were then lyophilized. These final preparations contained approximately 1% nucleic acids. The protein content for most preparations was < 2% (<1 to 5%). The proportion of LPS found in either the phenol or water phase was calculated by comparing the dry weight values obtained after lyophilization. For analytical studies, PA220 and PA220-R2 LPSs were further processed as follows. LPS was suspended in phosphate-buffered saline (pH 7.4) (5 to 10 mg/ml). Ribonuclease and deoxyribonuclease (Boehringer Mannheim, Mannheim, West Germany) were added to a final concentration of 20 μg/ml, and the mixture was incubated at 37°C for 3 h. Pronase (100 μg/ml) was then added and there was a further incubation for 24 h at 22°C. This material was ultracentrifuged three times at $100{,}000 \times g$ for 3 h, and the sediment was lyophilized.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (21). A 7.5 to 15% linear gradient acrylamide gel (2 by 140 by 140 mm) was used, and 3.75 μg of LPS in SDS-solubilizing buffer was applied per well. The gel was run at 35 mA until the tracking dye was approximately 1 cm from the bottom. Preparation of the LPS sample before electrophoresis and silver staining of the gel was by the method of Tsai and Frasch (38).

Analysis of LPS amino sugar composition. The amino sugars of PA220 and PA220-R2 LPS were analyzed by gas-liquid chromatography (14). LPS samples (10 mg) were hydrolyzed in 6 ml of 6 M HCl for 3 h at 100°C in a sealed ampoule. Amino sugars were converted to their aminodeoxyalditol acetates. Analysis was performed with a Perkin-Elmer 881 gas chromatograph equipped with a flame-ionization detector. A column of 3% ECNSS-M on Gas-Chrom Q (2 mm × 2 m) was employed. Known standards consisted of derivatized glucosamine and galactosamine.

Sephadex G-50 chromatography. Analysis of polysaccharide derived from LPS by liquid chromatography was performed as follows. LPS (10 mg) was hydrolyzed in 3 ml of 1% acetic acid for 90 min at 100°C in a sealed ampoule. Lipid A was removed by centrifugation, and the supernatant was applied to a Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2 by 67 cm). The column was eluted with pyridine acetate buffer (pH 5.4) at a flow rate of 35 ml/h. Fractions (2.3 ml) were collected, and carbohydrate was determined by the phenol-sulfuric acid method (10).

Quantitative determination of glucose and galactose. Quantitation of glucose and galactose was performed by a glucose-fructose UV test and a lactose-galactose UV test kit, respectively (Boehringer Mannheim).

Burn wound sepsis model. The murine burn wound model of Stieritz and Holder (37) was used with slight modification as previously described (8). Animals were observed for a minimum of 5 days postchallenge.

Quantitation of bacteria in blood and tissues. Bacterial counts (expressed as CFU) in the skin (at the challenge site), liver, and blood were determined at various times postchallenge as previously described (8).

TABLE 1. Characteristics of *P. aeruginosa* isolates

Bacterial strain	Virulence (LD_{50})[a]	LPS dispersion (%)[b]		Serum sensitivity[c]	Motility[d]	Exoproduct synthesis[e]		
		Water	Phenol			Toxin A	Alkaline protease	Elastase
PA220	$<2 \times 10^1$	79	21	R	+	+	+	+
M-2	$<2 \times 10^1$	87	13	R	+	+	+	+
IT-7	$<2 \times 10^1$	96	4	R	+	+	+	+
8505	$<2.5 \times 10^1$	>99	<1	R	+	−	+	+
IT-6	$<2.5 \times 10^1$	96	4	R	+	+	+	+
IT-5	7.5×10^1	77	23	R	+ (w)	+	+	+
IT-3	6×10^3	56	44	R	+ (w)	+	−	+
PA53	1.4×10^4	44	56	R	+	+	+	+
SBI-2	2.1×10^5	4	96	R	+	+	+	+
SBI-U	3.1×10^5	15	85	R	+	+	+	+
IT-4	1.8×10^6	19	81	R	+ (w)	+	+	+
IT-2	3.5×10^6	1	99	R	+	+	−	+
IT-1	4.1×10^6	<1	>99	R	+	−	+	+

[a] LD_{50} was determined in a murine burn wound model. Groups of six animals were used.
[b] Percent LPS (dry weight) found in the water or phenol phase after extraction of LPS from cell wall fragments.
[c] Normal human serum was used. R, Resistant; S, sensitive.
[d] w, Weakly motile; motility observed only by hanging drop method.
[e] +, Exoproduct synthesized; −, exoproduct not synthesized. See the text for assay systems.

Statistical analysis. The LD_{50} was calculated by the method of Reed and Muench (33).

RESULTS

Initial studies investigating the virulence of several *P. aeruginosa* strains for burned mice demonstrated a direct correlation between virulence and the partitioning of extracted LPS into either the phenol or the water phase (Table 1). LPSs with a high ratio of hydrophilic O-polysaccharide to hydrophobic lipid A were found in the water phase, whereas LPSs with a short O-side chain or none were recovered from the phenol phase. LPS isolated from highly virulent strains (PA220, M-2, IT-7, 8505, IT-6, and IT-5; $LD_{50} < 10^2$ was found predominantly in the water phase. Strains of intermediate virulence (IT-3 and PA53; LD_{50}, 6×10^3 and 1.4×10^4) possessed LPSs that partitioned almost equally between the two phases. The majority of LPSs from strains displaying low virulence (SBI-2, SBI-U, IT-4, IT-2, and IT1; $LD_{50} > 10^5$) were recovered from the phenol phase. Attempts to correlate virulence with other strain traits gave no similarly clear-cut results. All strains analyzed were resistant to the killing effects of normal human serum. Similarly, all strains were motile. The majority of strains produced toxin A. Of the two strains deficient in toxin A production, one (8505) was highly virulent ($LD_{50} < 2.5 \times 10^1$), whereas the other was comparatively nonvirulent (LD_{50}, 4.1×10^6). All strains produced alkaline protease, and most synthesized elastase. Therefore, the relative virulence of a given strain of *P. aeruginosa* for burned mice appeared closely associated with the length of the O-polysaccharide side chain.

It has been noted in earlier studies that analysis of *P. aeruginosa* virulence by the use of genetically unrelated strains in an effort to identify a factor of critical importance may have limited value (25). Therefore, mutants of PA220 resistant to an LPS-specific phage (E79) were selected. Several such mutant strains were isolated, and one, designated PA220-R2, was chosen for further study. Some characteristics of PA220-R2 and its parent strain are shown in Table 2. Whereas the majority of LPS isolated from PA220 partitioned into the water phase, that from PA220-R2 was found predominantly in the phenol phase. The mutation in PA220-R2 also affected several other strain characteristics, including (i) agglutination pattern with O-specific typing sera; (ii) loss of sensitivity to several typing phages; and (iii) loss of serum resistance. Motility was unaltered, as was the ability to produce toxin A and elastase. Furthermore, there were no distinguishable differences when 22 metabolic functions were analyzed by an API system. These results are consistent with characteristics expected of a *P. aeruginosa* strain possessing an altered (rough) LPS.

Next, LPSs from parent and mutant strains were analyzed to define the structural alteration present in PA220-R2 LPS. Intact mutant LPS contained substantially less glucose and rhamnose than did PA220 LPS (63 and 35% of parenteral levels, respectively). Additionally, analysis of amino sugar content by gas-liquid chromatography showed the presence of six compounds in PA220 LPS which were present only in trace quantities when PA220-R2 LPS was analyzed (data not shown). Since glucose, rhamnose, and amino sugars are major constituents of serotype 6 LPS, their marked reduction in PA220-R2 LPS suggests that this strain possesses an

TABLE 2. Characteristics of *P. aeruginosa* PA220 and PA220-R2[a]

Strain	LPS dispersion (%)		Reactions with O-antisera	Bacteriophage sensitivity	Serum sensitivity	Motility	Metabolic functions	Exoproduct synthesis (μg/ml)	
	Water	Phenol						Toxin A	Elastase
PA220	79	21	6	7, 16, 44, F8, 109, 119x, 1214, Col. 11	Resistant	+	−	0.147	24.4
PA220-R2	18	82	6, 9, 10, 13	109	Sensitive	+	Unaltered	0.183	22.2

[a] For assay methods, see the text.

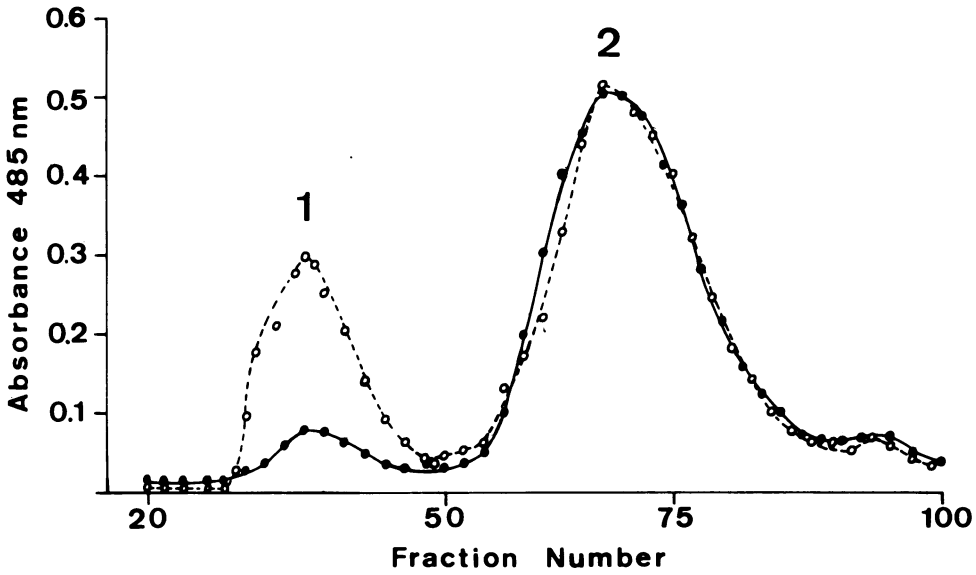

FIG. 1. Chromatography of lipid A-free polysaccharides on Sephadex G-50. Polysaccharide was derived from PA220 LPS (○) and PA220-R2 LPS (●).

LPS lacking at least a portion of the repeating O-polysaccharide side chain.

This theory was explored by further characterization of PA220-R2 LPS. Chromatography of lipid A-free polysaccharide over Sephadex G-50 showed that PA220-R2 was deficient in the high-molecular-weight peak (Fig. 1, peak 1). Peak 2, which corresponds to "core" LPS, was nearly identical for both parent and mutant LPSs. SDS-PAGE confirmed that PA220-R2 LPS lacked the high-molecular-weight polysaccharide bands observed with the parent strain (Fig. 2, arrow). These results indicated that PA220-R2 was markedly deficient in O-polysaccharide side chains.

The virulence of PA220-R2 was compared with that of its parent strain in a burn wound sepsis model (Table 3). The LD_{50} for PA220-R2 was at least 1,000-fold higher than that for its parent in two independent determinations. The virulence of PA220-R2 for burned CBA mice was also tested, and the LD_{50} was found to be more than 1,000-fold greater than that for its parent strain (courtesy of B. Wretlind, Karolinska Institute, Stockholm, Sweden). A low challenge dose (10^2) of PA220 was capable of rapid multiplication in burned skin leading to fatal sepsis (Fig. 3). In contrast, an equivalent amount of PA220-R2 was unable to establish an infection. The challenge bacteria were cleared by 19 h postchallenge (earliest time point taken).

DISCUSSION

Demonstration of the protective capacity of anti-LPS antibody in experimental infections (8, 9, 11, 30, 35) and promising results from clinical trials (1) have prompted numerous studies on the structure and function of *P. aeruginosa* LPS (6, 14, 17, 31, 34, 40). Mutants possessing an altered LPS have proved to be useful tools for such investigations (18–20, 34). However, the use of LPS mutants to evaluate the role of LPS in the pathogenesis of *P. aeruginosa* infections is limited to a single report (18). In the present study, a "smooth" LPS-specific bacteriophage was used to isolate mutants with altered LPS structure. This approach for isolating mutants is particularly attractive for virulence studies since alterations to LPS only would be expected.

The physical state of the core and of the O-polysaccharide side chain was found to greatly influence virulence, with strains possessing complete core and side chain being far more virulent than those with an abbreviated structure. Side chain length did not appear to influence virulence via serum resistance since all natural isolates evaluated were serum resistant. A more likely explanation is that an increased amount of LPS would allow the strains to escape from

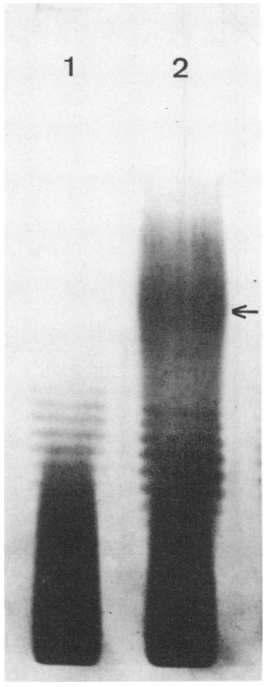

FIG. 2. SDS-PAGE of PA220 and PA220-R2 LPS. Lane 1, PA220-R2 LPS; lane 2, PA220 LPS.

TABLE 3. Virulence of *P. aeruginosa* PA220 and PA220-R2 for burned mice

Strain	LD_{50}[a]	
	Expt 1	Expt 2
PA220	$<1.5 \times 10^{1}$[b]	1.4×10^{1}
PA220-R2	$>1.9 \times 10^{4}$[c]	2.5×10^{5}

[a] Groups of six mice were used.
[b] Lowest challenge dose tested. Greater than 50% of animals died.
[c] Highest challenge dose tested. All animals survived.

normal host defense systems, of which phagocytosis appears to be the most critical for *P. aeruginosa* (43).

The resistance of PA220-R2 to phage E79 was due to a deficiency in O-side chain material which carries the phage receptor site (16). this alteration not only affected the agglutination and phage sensitivity pattern of PA220-R2 but also rendered it serum sensitive. This finding would indicate that LPS O-side chain polysaccharide acts to mask the complement binding site that initiates cell lysis. This site is represented by the core-lipid A region of LPS or a membrane protein or both.

PA220-R2 was comparatively nonvirulent for burned mice, with an LD_{50} more than 1,000-fold that for its parent strains. Although the serum-sensitive characteristic of PA220-R2 would be expected to limit its survival in the bloodstream, it was somewhat surprising to find that it was unable to establish an infection in burned skin. Serum resistance, which was lost due to the lack of O-side chain, undoubtedly contributed to the reduced virulence. However, loss of serum resistance in itself is probably not the only reason for decreased virulence since earlier studies have shown that a serum-sensitive strain can be virulent for burned mice and is capable of invading the bloodstream (28). The absence of O-side chain would also be expected to decrease the ability of PA220-R2 to evade other normal host-defense systems, particularly phagocytosis, thereby lowering virulence.

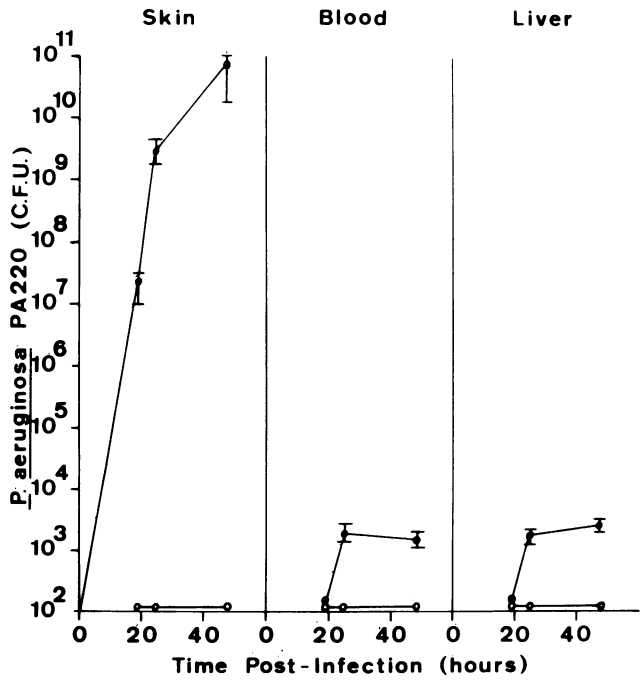

FIG. 3. Quantitation of bacteria in the skin (per gram wet weight at the challenge site), blood (per milliliter), and liver (per liver) ± standard error of the mean after challenge with 10^2 organisms of PA220 (●) or PA220-R2 (○). Groups of three mice were sacrificed at each time point.

The present study demonstrates the critical role of LPS in the virulence of *P. aeruginosa*. Although a deficiency in the O-side chain of LPS was found to markedly reduce virulence, only a single mutant strain of this phenotype was employed. Additional studies involving stepwise mutants lacking in O-side chain should allow for a more precise assessment of this LPS moiety in relation to virulence. Furthermore, such studies could be expanded to include other regions of the LPS molecule. One distinct possibility would be the use of mutants altered in the core region, similar to those described by Rowe and Meadow (34). In this manner, structural or biochemical lesions induced in the various domains of the LPS molecule could be correlated to changes in virulence.

ACKNOWLEDGMENTS

Excellent technical assistance was provided by E. Kohler, J. Schlup, D. Pavlovic, and C. Elaaeieida. We thank M. Maag and M. Radosavljevic for providing fermentor-grown cultures and V. Rupp for help in preparation of this manuscript.

LITERATURE CITED

1. **Alexander, T. W., and M. Fisher.** 1974. Immunization against *Pseudomonas* in infection after thermal injury. J. Infect. Dis. **130**(Suppl.):152–158.
2. **Asheshov, E. H.** 1974. An assessment of the methods used for typing strains of *Pseudomonas aeruginosa*, p. 9–23. *In* A. Arseni (ed.), Proceedings of the Sixth National Congress of Bacteriology. Leontiadi Medical Editions, Athens.
3. **Bielefeld, D. R., R. M. Senior, and S. Y. Yu.** 1975. A new method for determination of elastolytic activity using (^{14}C)-labeled elastin and its application to leukocyte elastase. Biochem. Biophys. Res. Commun. **67**:1553–1559.
4. **Björn, M. J., B. H. Iglewski, S. K. Ives, J. C. Sadoff, and M. L. Vasil.** 1978. Effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa* PA-103. Infect. Immun. **19**:785–791.
5. **Blackwood, L. L., R. M. Stone, B. H. Iglewski, and J. E. Pennington.** 1983. Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. Infect. Immun. **39**:198–201.
6. **Chester, I. R., P. M. Meadow, and T. L. Pitt.** 1973. The relationship between the O-antigenic lipopolysaccharides and serological specificity in strains of *Pseudomonas aeruginosa* of different O-serotypes. J. Gen. Microbiol. **78**:305–318.
7. **Cryz, S. J., Jr., R. L. Friedman, and B. H. Iglewski.** 1980. Isolation and characterization of a *Pseudomonas aeruginosa* mutant producing a nontoxic immunologically cross-reactive (CRM) toxin A protein. Proc. Natl. Acad. Sci. U.S.A. **77**:7199–7203.
8. **Cryz, S. J., Jr., E. Fürer, and R. Germanier.** 1983. Protection against *Pseudomonas aeruginosa* infection in a murine burn wound sepsis model by passive transfer of antitoxin A, antielastase, and antilipopolysaccharide. Infect. Immun. **3**:1072–1079.
9. **Cryz, S. J., Jr., E. Fürer, and R. Germanier.** 1983. Passive protection against *Pseudomonas aeruginosa* infection in an experimental leukopenic mouse model. Infect. Immun. **40**:659–664.
10. **Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
11. **Fisher, M. W.** 1977. A polyvalent human γ-globulin immune to *Pseudomonas aeruginosa*: passive protection of mice against

lethal infection. J. Infect. Dis. **136**(Suppl.):181–185.
12. **Homma, J. Y.** 1977. Progress in the study of *Pseudomonas aeruginosa* with emphasis on its pathogenicity. J. Jpn. Med. Assoc. **78**:275–286.
13. **Homma, J. Y.** 1980. Roles of exoenzymes and exotoin in the pathogenicity of *Pseudomonas aeruginosa* and the development of a new vaccine. Jpn. J. Exp. Med. **50**:149–165.
14. **Horton, D., G. Rodemeyer, and T. H. Haskell.** 1977. Analytical characterization of lipopolysaccharide antigens from seven strains of *Pseudomonas aeruginosa*. Carbohydr. Res. **55**:35–47.
15. **Iglewski, B. H., and J. C. Sadoff.** 1979. Toxin inhibitors of protein synthesis: production, purification and assay of *Pseudomonas aeruginosa* toxin A. Methods Enzymol. **60**:780–793.
16. **Jarrell, K., and A. M. Kropinski.** 1977. Identification of the cell wall receptor for bacteriophage E79 in *Pseudomonas aeruginosa* strain PAO. J. Virol. **23**:461–466.
17. **Jarrell, K. F., and A. M. Kropinski.** 1981. *Pseudomonas aeruginosa* bacteriophage ϕPLS27-lipopolysaccharide interactions. J. Virol. **40**:411–420.
18. **Kropinski, A. M. B., and J. S. Chadwick.** 1975. The pathogenicity of rough strains of *Pseudomonas aeruginosa* for *Galleria mellonella*. Can. J. Microbiol. **21**:2084–2088.
19. **Kropinski, A. M. B., L. Chan, and F. H. Milazzo.** 1978. Susceptibility of lipopolysaccharide-defective mutants of *Pseudomonas aeruginosa* PAO to dyes, detergents, and antibiotics. Antimicrob. Agents Chemother. **13**:494–499.
20. **Kropinski, A. M. B., L. Chan, and F. H. Milazzo.** 1979. The extraction and analysis of lipopolysaccharides from *Pseudomonas aeruginosa* strain PAO and three rough mutants. Can. J. Microbiol. **25**:390–398.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan.** 1983. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **33**:256–264.
23. **Montie, T. C., D. Doyle-Huntzinger, R. C. Craven, and I. A. Holder.** 1982. Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned-mouse model. Infect. Immun. **38**:1296–1298.
24. **Morihara, K., H. Tsuzuki, T. Oka, H. Inoue, and M. Ebato.** 1965. *Pseudomonas aeruginosa* elastase. J. Biol. Chem. **240**:3295–3304.
25. **Ohman, D. E., R. P. Burns, and B. H. Iglewski.** 1980. Corneal infections in mice with toxin A and elastase mutants of *Pseudomonas aeruginosa*. J. Infect. Dis. **142**:547–555.
26. **Ohman, D. E., S. J. Cryz, and B. H. Iglewski.** 1980. Isolation and characterization of a *Pseudomonas aeruginosa* mutant PAO that produces altered elastase. J. Bacteriol. **142**:836–842.
27. **Ohman, D. E., J. C. Sadoff, and B. H. Iglewski.** 1980. Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect. Immun. **28**:899–908.
28. **Pavlovskis, O. R., M. Pollack, L. T. Callahan III, and B. H. Iglewski.** 1977. Passive protection by antitoxin in experimental *Pseudomonas aeruginosa* burn infections. Infect. Immun. **18**:596–602.
29. **Pennington, J. E., H. Y. Reynolds, R. E. Wood, R. A. Robinson, and A. S. Levine.** 1975. Use of a *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med. **58**:629–636.
30. **Pier, G. B., H. F. Sidberry, and J. C. Sadoff.** 1978. Protective immunity reduced in mice by immunization with high-molecular-weight polysaccharide from *Pseudomonas aeruginosa*. Infect. Immun. **22**:919–925.
31. **Pier, G. B., H. F. Sidberry, S. Zolyomi, and J. C. Sadoff.** 1978. Isolation and characterization of a high-molecular-weight polysaccharide from the slime of *Pseudomonas aeruginosa*. Infect. Immun. **22**:908–918.
32. **Pitt, T. L., and Y. J. Erdman.** 1978. The specificity of agglutination reactions of *Pseudomonas aeruginosa* with O antisera. J. Med. Microbiol. **11**:15–23.
33. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
34. **Rowe, P. S. N., and P. M. Meadow.** 1983. Structure of the core oligosaccharide from the lipopolysaccharide of *Pseudomonas aeruginosa* PAC1R and its defective mutants. Eur. J. Biochem. **132**:329–337.
35. **Sadoff, J. C., S. L. Futrovsky, H. F. Sidberry, B. H. Iglewski, and R. C. Seid, Jr.** 1982. Detoxified lipopolysaccharide-protein conjugates, p. 346–354. *In* L. Weinstein and B. N. Fields (ed.), Seminars in infectious disease. Thieme-Stratton Inc., New York.
36. **Snell, K., I. A. Holder, S. A. Leppla, and C. B. Saelinger.** 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with *Pseudomonas aeruginosa*. Infect. Immun. **19**:839–845.
37. **Stieritz, D. D., and I. A. Holder.** 1975. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: description of a burned mouse model. J. Infect. Dis. **131**:688–691.
38. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
39. **Westphal, O., O. Lüderitz, and F. Bister.** 1952. Ueber die Extraktion von Bakterien mit Phenol-Wasser. Z. Naturforsch. **7**:148–155.
40. **Wilkinson, S. G., and L. Galbraith.** 1975. Studies of lipopolysaccharide from *Pseudomonas aeruginosa*. Eur. J. Biochem. **52**:331–343.
41. **Woods, D. E., S. J. Cryz, R. L. Friedman, and B. H. Iglewski.** 1982. Contribution of toxin A and elastase to virulence of *Pseudomonas aeruginosa* in chronic lung infections of rats. Infect. Immun. **36**:1223–1228.
42. **Young, L. S.** 1972. Human immunity to *Pseudomonas aeruginosa*. II. Relationship between heat-stable opsonins and type-specific lipopolysaccharides. J. Infect. Dis. **126**:277–287.
43. **Young, L. S.** 1980. The role of exotoxins in the pathogenesis of *Pseudomonas aeruginosa* infections. J. Infect. Dis. **142**:626–630.
44. **Young, L. S., R. D. Meyer, and D. Armstrong.** 1973. *Pseudomonas aeruginosa* vaccine in cancer patients. Ann. Intern. Med. **79**:518–527.