

Role of LPS length in clearance rate of bacteria from the bloodstream in mice

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Strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis (CF) never spread systemically. This may be due to serum sensitivity since these strains are very sensitive to complement-mediated bactericidal activity. A serum-resistant mutant, *P. aeruginosa* TUM3 HSR, was obtained from serum-sensitive strain TUM3 from a CF patient in order to clarify the mechanism of failure of systemic spread. LPS profiles on silver-stained gels and immunological analysis revealed that a long O-polysaccharide side chain was overproduced on the LPS molecules of TUM3 HSR as compared with the LPS of TUM3. The clearance rate from the bloodstream in mice was compared in the two strains. The number of TUM3 bacteria in 1 ml of blood, 10 min after injection into the tail vein, significantly decreased from 1.7×10^8 to 3.7×10^5 c.f.u. ml⁻¹. In contrast, TUM3 HSR was not eliminated during the same period (decrease from 1.9×10^8 to 3.4×10^7 c.f.u. ml⁻¹). Interestingly, these isogenic strains were not killed by 40% murine serum, probably reflecting immaturity of the complement-mediated killing system in mice. These results pointed to a correlation between LPS structure and blood clearance rate in mice. This was confirmed by examining blood clearance kinetics using the smooth-LPS strain *Salmonella typhimurium* LT2 and LPS-deficient mutants derived from it. *S. typhimurium* LT2 resisted blood clearance while the LPS-deficient mutants were cleared rapidly. None of the *S. typhimurium* strains were killed by murine serum. The number of *P. aeruginosa* TUM3 and *S. typhimurium* LPS-deficient mutants trapped in the liver following injection into the peripheral circulation was greater than that of their counterparts. These results indicate that the long O-polysaccharide side chain of LPS may play a crucial role in evading phagocytosis by the reticuloendothelial system (RES), and therefore, may control the establishment of systemic infection by Gram-negative bacteria. The interaction between complement C3 on bacteria and C3 receptors on macrophages may also be involved in the trapping mechanism by the RES. It was expected that the level of C3 bound on the cell surface would be higher in *P. aeruginosa* TUM3 or the LPS-deficient mutants derived from *S. typhimurium* LT2. However, our flow-cytometric results demonstrated that the level of C3 was almost identical in isogenic strains.

Keywords: lipopolysaccharide, blood clearance, complement, *Pseudomonas aeruginosa*

INTRODUCTION

Patients with cystic fibrosis (CF) develop repetitive

Abbreviations: CF, cystic fibrosis; CR3, complement receptor type 3; FITC, fluorescein isothiocyanate; FHS, fresh human serum; FMS, fresh mouse serum; KDO, 2-keto-3-deoxyoctonic acid (3-deoxy-D-manno-2-octulosonic acid); RES, reticuloendothelial system.

infection caused by *Pseudomonas aeruginosa* during progression of the disease, but the infection is limited to the lung and does not spread to the blood. This is in contrast to several other classes of patients infected with *P. aeruginosa* that frequently develop severe systemic infections. It is still unclear why *P. aeruginosa* colonizing the lungs of CF patients never spreads systemically. Several lines of evidence suggest that serum sensitivity may play

an important role in this process. For example, isolates of *P. aeruginosa* from patients with CF are usually sensitive to human complement-mediated bactericidal systems (Thomassen & Demko, 1981). This is in contrast to isolates from patients with bacteraemia known to be resistant to serum bactericidal activity (Schiller & Hatch, 1983).

The long-chain O-polysaccharide portion of lipopolysaccharide (LPS) acts as a barrier for complement-mediated killing in Gram-negative bacteria, in addition being responsible for the O specificity (O antigen) that determines the serotype of a particular strain (Taylor, 1983; Palomar *et al.*, 1993). *P. aeruginosa* is capable of producing LPS with two different polysaccharides on the same LPS molecule (Hatano *et al.*, 1993), termed A- and B-bands, that have been identified in PAO1 (Rivera *et al.*, 1988; Rivera & McGroarty, 1989). The B-band is composed of LPS with a long O-polysaccharide side chain and is responsible for O specificity. In contrast, the A-band LPS has only a short neutral polysaccharide side chain, composed mainly of D-rhamnan (Arsenault *et al.*, 1991; Coyne *et al.*, 1994), which is a common antigen shared among *P. aeruginosa* strains (Lam *et al.*, 1989; Yokota *et al.*, 1990). Most isolates from CF patients have lost the high molecular mass polysaccharide B-band (Lam *et al.*, 1989). Therefore, these isolates are sensitive to human serum bactericidal activity.

We previously compared the bactericidal activity of human and murine (ICR, male, 4 weeks) serum against *P. aeruginosa* strains from CF patients (Ohno *et al.*, 1992). Most strains were sensitive to human serum; however, all strains were unexpectedly resistant to murine serum. Interestingly, in spite of their resistance to murine serum, the human-serum-sensitive strains were rapidly eliminated from the bloodstream after injection into the tail vein of mice, whereas the human-serum-resistant strains were not eliminated (unpublished data). Thus, it was considered that mechanisms other than serum sensitivity might play a role in blood clearance, at least in mice. On the other hand, phagocytosis by the reticuloendothelial system (RES) is an important factor involved in blood clearance as well as the serum bactericidal system. Complement component C3, bound on the bacterial surface, particularly LPS, plays an important role in phagocytosis by binding to C3 receptors such as CR1 or CR3 on phagocytic cells. It is also known that the long O-polysaccharide chains of LPS may prevent binding of C3 to LPS (Taylor *et al.*, 1983; Schiller & Joiner, 1986; Arsenault *et al.*, 1991; Merino *et al.*, 1992). Thus, the relationship between the length of the LPS side chain and binding ability of C3 may explain the difference in blood clearance of strains with or without a long polysaccharide chain.

To explore these issues, we isolated a serum-resistant mutant of *P. aeruginosa* strain TUM3. In this derivative the biosynthesis of long O-polysaccharide chains on the LPS molecule was increased. Using these isogenic strains, in addition to *Salmonella typhimurium* LT2 and its LPS-deficient mutants, we investigated the role of LPS length and phagocytosis in blood clearance in mice.

METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. The mucoid *P. aeruginosa* strain TUM3, isolated from the sputum of a CF patient, was kindly provided by Dr M. I. Marks, Miller Children's Hospital, Long Beach, CA, USA. *P. aeruginosa* TUM3 HSR was isolated from TUM3 as a serum-resistant mutant using the method of Shiller *et al.* (1984). Briefly, *P. aeruginosa* was incubated at 35 °C for 18 h in brain-heart infusion (BHI, Difco) broth containing fresh human serum (FHS) in increasing concentrations (0.31–40%; by the twofold dilution method). Cells grown in the broth, including FHS of the highest concentration, were transferred to the next set and the same procedure was repeated three times. Finally, cells grown on a medium including 40% FHS were selected as serum-resistant mutants. Serotyping was carried out by the slide agglutination method with a serotype grouping kit (Mei-assay; Meiji Seika). *S. typhimurium* LT2 and its LPS-deficient mutants (Lindberg & Hellerqvist, 1971; Roantree *et al.*, 1977) were kindly provided by Professor T. Sawai, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan. Unless otherwise stated, organisms were grown at 35 °C on BHI agar.

Laboratory animals. SPF male ICR mice (Japan Charles River), weighing 19–21 g, were used for blood clearance assays and serum bactericidal microassays.

Blood clearance of bacteria in mice. Colonies grown on a BHI agar plate for 18 h at 35 °C were suspended in saline and adjusted to 10^8 c.f.u. ml⁻¹. Five mice were used in each group; they were injected in the tail vein with 0.2 ml of the bacterial suspension. Following anaesthesia of the animal, 30 µl blood was obtained from the retro-orbital plexus using disposable heparinized capillary tubes, 10 and 30 min after injection, and mixed with 270 µl saline. At the same time, the liver was removed aseptically, and a volume of saline equal to its weight was added before homogenization in a Teflon homogenizer. The blood suspension and homogenized liver were diluted in saline using the serial tenfold dilution method. One hundred microlitres of each dilution was plated on a modified Drigalski agar (Eiken Chemical). After overnight culture at 35 °C, bacterial counts were calculated in c.f.u. (ml blood)⁻¹ or in c.f.u. (g liver)⁻¹. The control values were calculated by estimating the total blood volume as 8% (v/w) of the body weight of each mouse used (Leunk & Moon, 1982). The ratio of bacteria in the liver to that in the blood was defined as the phagocytosis index of the RES.

Complement killing microassay. Fresh serum was obtained from healthy volunteers and from mice. Colonies grown for 18–24 h on a BHI agar plate were suspended in phosphate-buffered saline (PBS) and washed three times with PBS. The bacterial concentration was diluted in PBS to a final value of about 10^4 or 10^8 c.f.u. ml⁻¹. One hundred microlitres of bacterial suspension was mixed with the same volume of 20% FHS or 80% fresh mouse serum (FMS). The mixture was incubated at 37 °C for 2 h, and diluted to 10^{-1} to 10^{-3} . The percentage of surviving bacteria was determined by plating 100 µl of the original and diluted samples onto BHI agar plates. The number of c.f.u. ml⁻¹ was determined after overnight incubation at 35 °C and compared with that of control, containing PBS in place of FHS and FMS.

Determination of C3 binding to the surface of bacteria. Bacterial colonies grown on a BHI plate were suspended in PBS and washed three times with the same buffer. The suspension was adjusted to OD₅₅₀ 0.4 using a Beckman DU 640 spectrophotometer and mixed with an equal volume of FMS. After incubation at 37 °C for 30 min, cells were pelleted by brief

Table 1. Bacterial strains

Strain	Revelant characteristics*	Source or reference
<i>P. aeruginosa</i>		
TUM3	Mucoid, serotype G, short-polysaccharide chain LPS, serum sensitive	Cystic fibrosis
TUM3 HSR	Mucoid, serotype G, long-polysaccharide chain LPS, serum resistant	Derivative of TUM3
<i>S. typhimurium</i>		
LT2	Autotroph (S)	Lindberg & Hellerqvist (1971)
SL1034	<i>rfe-465</i> (SR)	Roantree <i>et al.</i> (1977)
TV119	<i>rfa-430</i> (Ra)	Roantree <i>et al.</i> (1977)
SL733	<i>rfaK</i> (Rb1)	Roantree <i>et al.</i> (1977)
TV160	<i>metA22 bryB2 galT411 rfa-418</i> (Rb2)	Lindberg & Hellerqvist (1971)
TV148	<i>rfa-433</i> (Rb3)	Lindberg & Hellerqvist (1971)
LT2M1	<i>galE</i> (Rc)	Roantree <i>et al.</i> (1977)
SL1004	<i>rfa-430 rfaG571</i> (Rd1)	Roantree <i>et al.</i> (1977)
TA2168	<i>bisC3076 galE506 rfa-1009</i> (Re)	Lindberg & Hellerqvist (1971)

*Serotyping of *P. aeruginosa* was performed using the Mey-assay monoclonal typing system (Meiji Seika). Structure of LPS (*S. typhimurium*): S, smooth LPS; SR, O-antigen with only one repeating unit; Ra, outer core without O-antigen; Rb1, outer core without GlcNAc; Rb2, outer core without GlcNAc-Glc; Rb3, outer core without GlcNAc-Glc-Gal; Rc, outer core without GlcNAc-Glc-Gal-Gal; Rd1, inner core only, without outer core; Re, inner core without triheptose (deep rough LPS).

Table 2. Sensitivities to human serum of *P. aeruginosa* TUM3 and TUM3 HSR, and *S. typhimurium* LT2 and the LPS isogenic mutants

Strain	Sensitivity to 10 % human serum (survival ratio, %*) with an inoculum of:			Sensitivity to 40 % murine serum (survival ratio, %*) with an inoculum of:	
	10 ⁴	10 ⁶	10 ⁸	10 ⁴	10 ⁶
<i>P. aeruginosa</i>					
TUM3	< 0.03	0.0006 (0.0002)	0.048 (0.002)	294 (48.4)	NT
TUM3 HSR	182 (96)	367 (44)	125 (16)	266 (61.9)	NT
<i>S. typhimurium</i> †					
LT2 (S)	0.14 (0.02)	18.2 (2.8)	61.8 (3.4)	NT	770 (286)
SL1034 (SR)	< 0.01	0.0008 (0.0006)	0.025 (0.008)	NT	NT
TV119 (Ra)	0.02 (0.01)	0.08 (0.12)	0.02 (0.001)	NT	NT
SL733 (Rb1)	< 0.01	0.02 (0.02)	0.095 (0.091)	NT	NT
TV160 (Rb2)	0.025 (0.017)	0.028 (0.046)	0.082 (0.029)	NT	NT
TV148 (Rb3)	0.10 (0.14)	1.97 (0.51)	0.048 (0.009)	NT	NT
LT2M1 (Rc)	< 0.01	< 0.0005	0.27 (0.033)	NT	NT
SL1004 (Rd1)	0.01 (0.005)	< 0.0006	0.025 (0.0002)	NT	NT
TA2168 (Re)	< 0.01	< 0.0006	0.00007 (0.00006)	NT	77.1 (25.3)

*Survival ratio (%) for the different inoculum sizes shown was expressed as the ratio of survived cells to inoculum cell numbers after 2 h incubation. The figures in parentheses are standard deviations of three experiments. NT, Not tested.

†Structure of LPS (see Table 1) shown in parentheses.

centrifugation in a microcentrifuge, washed three times, and suspended in PBS. Fluorescein-conjugated goat IgG to mouse complement C3 (Organon Teknika) was added to the sus-

pension at a ratio of 1:200 and incubated at room temperature for 30 min with gentle shaking. Free IgG was completely removed by washing with PBS and the intensity of fluorescence

per 10000 bacterial cells in suspension was determined by flow cytometry (Becton Dickinson) using Consort 30 software.

LPS profile. LPS was isolated and purified according to the method of Darveau & Hancock (1983). LPS bands were visualized by silver staining after SDS-PAGE in a 12.5% (w/v) polyacrylamide gel.

Monoclonal antibodies. Monoclonal antibodies TK-5B12 (IgG), OM-1D6 and TS-1B2, were used. TK-5B12 (human IgG2), OM-1D6 (human IgM) and TS-1B2 (human IgM) recognized the serotype G O-polysaccharide, the serotype G core region and the *P. aeruginosa* common antigen, D-rhamnan (A-band LPS), respectively. The hybridomas producing these mAbs were prepared by cell fusion of pokeweed nitrogen-stimulated human peripheral blood lymphocytes from healthy adult volunteers and human-mouse heteromyeloma SHM-D33 (ATCC CRL 1668) using polyethylene glycol (Yokota *et al.*, 1989). The hybridomas were cultured in serum-free medium Celgros H (Sumitomo Pharmaceuticals). The mAbs were purified from the concentrated culture supernatant by gel filtration chromatography on Superose 6 (Pharmacia) for IgM, and by Protein A-Cellulofine (Seikagaku Kogyo) chromatography for IgG.

Immunoblotting. Western blotting was carried out as described previously (Yokota *et al.*, 1989). Briefly, after SDS-PAGE, LPS on gel was transferred to a PVDF membrane with a semi-dry electroblotting apparatus. The membrane was blocked with 2% (w/v) casein in PBS, and subsequently incubated with mAb ($1 \mu\text{g ml}^{-1}$) and alkaline-phosphatase-conjugated goat anti-human IgG antibody (1000-fold dilution). Specific binding was detected with sodium 5-bromo-4-chloro-3-indoyl phosphate (0.15 mg ml^{-1}) and nitroblue tetrazolium chloride (0.3 mg ml^{-1}) in 100 mM sodium carbonate buffer (pH 9.8) containing 1 mM MgCl_2 .

ELISA. The methods for ELISA and preparation of bacterial-cell-coated plates have been described previously (Yokota *et al.*, 1989). LPS-coated plates were prepared according to the method of Pollack *et al.* (1987). Alkaline-phosphatase-conjugated goat anti-human IgM antibody (Kirkegaard & Perry Laboratories) and sodium *p*-nitrophenyl phosphate were used as secondary antibody and substrate, respectively. Binding activity was expressed as A_{405} .

Statistical analysis. The one-factor ANOVA test was used to determine statistical significance of differences in blood clearance between *P. aeruginosa* TUM3 and TUM3 HSR or *S. typhimurium* LT2 and the LPS mutants.

RESULTS

Complement killing microassay

The results of the complement killing microassay are summarized in Table 2. Over 99.9% of *P. aeruginosa* TUM3 was killed by 10% FHS in inoculum sizes of 10^4 – 10^8 c.f.u. ml^{-1} . TUM3 HSR completely resisted killing by 10% FHS. *S. typhimurium* LT2 (smooth LPS) was also resistant to 10% FHS. However, the degree of resistance was significantly influenced by inoculum size. LPS mutants derived from *S. typhimurium* LT2 were all sensitive to 10% FHS at all inoculum levels, with the highest sensitivity observed for the Re mutant TA2168. FMS did not kill any strain even when the serum concentration was increased to 90% (data not shown).

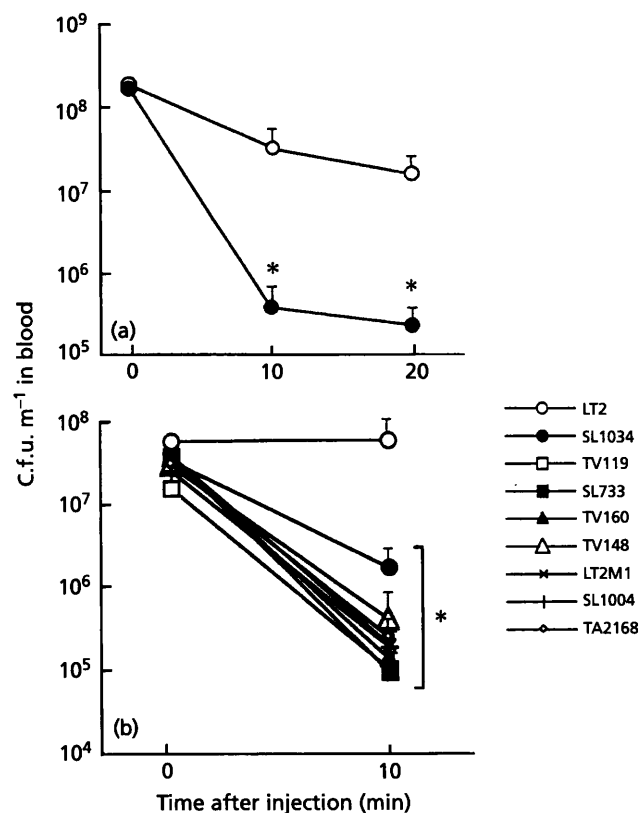


Fig. 1. Blood clearance rate in mice of (a) *P. aeruginosa* TUM3 (●) and TUM3 HSR (○), and (b) *S. typhimurium* LT2 and the LPS mutants (see figure for symbols). The experiments were repeated three times. A representative experiment is shown. Data shown are the mean and standard deviation from three animals. The differences between TUM3 and TUM3 HSR at time points of 10 min and 30 min, and between *S. typhimurium* LT2 and the LPS mutants were statistically significant at $P < 0.01$ (ANOVA).

Blood clearance

Fig. 1(a, b) shows the blood clearance rates in mice of different bacterial strains. *P. aeruginosa* TUM3 and *S. typhimurium* TA2168 were rapidly cleared from the blood, with the bacterial count diminishing 10 min after injection to 0.21% and 0.06% of the initial inoculum, respectively. The survival ratios of *P. aeruginosa* TUM3 HSR and *S. typhimurium* LT2 10 min after injection, 17.8% and 97.6%, respectively, were significantly higher than those of their isogenic strains.

The bacterial numbers trapped in the liver 10 min after injection are shown in Fig. 2 as the phagocytic index. The phagocytic indices of *P. aeruginosa* TUM3 and the *S. typhimurium* LPS mutants were 200–10000-fold higher than those of *P. aeruginosa* TUM3 HSR and *S. typhimurium* LT2.

LPS profile

Silver-stained LPS profiles of *P. aeruginosa* TUM3 and TUM3 HSR, and *S. typhimurium* LT2 and TA2168, are shown in Fig. 3. High molecular mass LPS species were

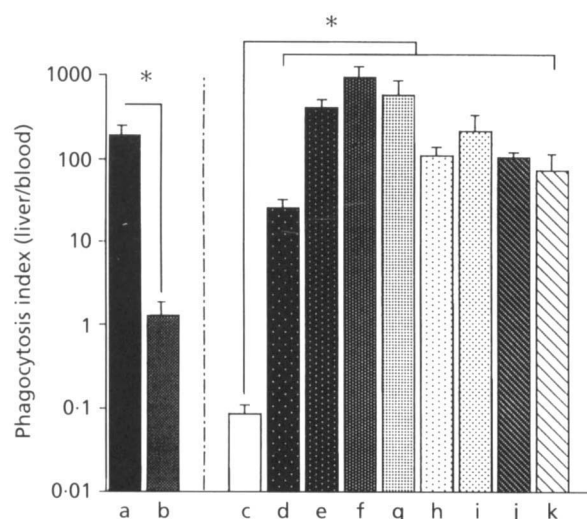


Fig. 2. Ratio of bacteria trapped in the liver to bacteria in the blood, 10 min after bacterial injection: a, *P. aeruginosa* TUM3; b, TUM3 HSR; c, *S. typhimurium* LT2; d, SL1034; e, TV119; f, SL733; g, TV160; h, TV148; i, LT2M1; j, SL1004; k, TA2168. Blood clearance and trapping in the liver were determined at the same time. Livers were removed immediately after blood was sampled. The experiments were repeated three times. A representative experiment is shown. Data shown are the mean and standard deviation from three animals. The differences between *P. aeruginosa* TUM3 and TUM3 HSR, and between LT2 and the LPS mutants, were statistically significant at $P < 0.01$ (ANOVA).

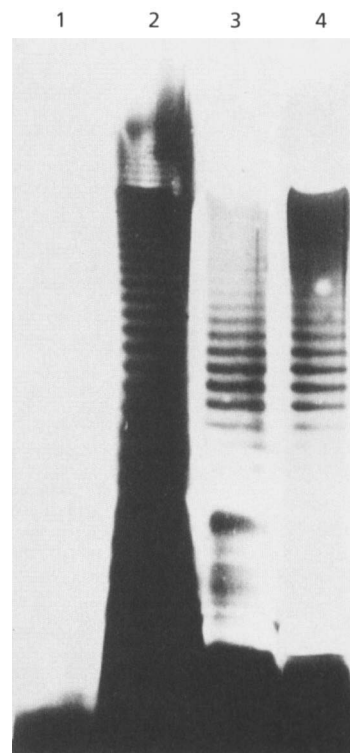


Fig. 3. Silver-stained SDS-PAGE (12.5% gel) of LPS from *P. aeruginosa* TUM3 and TUM3 HSR, and *S. typhimurium* LT2 and LPS-deficient mutant TA2168. Lanes: 1, *S. typhimurium* TA2168; 2, *S. typhimurium* LT2; 3, *P. aeruginosa* TUM3; 4, *P. aeruginosa* TUM3 HSR.

expressed to a much greater extent in TUM3 HSR compared with the parent strain TUM3. The LPS profile of *S. typhimurium* LT2 and TA2168 showed typical bands of smooth and rough type (Re) LPS.

Western blotting analysis

Anti-serotype G O-polysaccharide mAb TK-5B12 specifically stained LPS derived from TUM3 as well as TUM3 HSR. However, the reaction was stronger in LPS of TUM3 HSR (Fig. 4a). Anti-D-rhamnan mAb TS-1B2 specifically stained the position of A-band on the LPS molecules. The intensity of reaction was similar in TUM3 LPS and the TUM3 HSR LPS (Fig. 4b). Anti-serotype G core mAb OM-1D6 strongly stained LPS core region of TUM3 compared with that of TUM3 HSR (Fig. 4c).

Binding of mAbs to *P. aeruginosa* TUM3 and TUM3 HSR cells

The binding of TK-5B12 to TUM3 HSR was stronger than that to TUM3 (Fig. 5a). OM-1D6 strongly bound to TUM3, but showed little binding to TUM3 HSR (Fig. 5c). TS-1B2 bound to both TUM3 and TUM3 HSR with approximately similar intensity (Fig. 5b).

Determination of complement C3 binding to the surface of bacteria

The results from flow cytometry quantified the specific binding of an FITC-labelled anti-C3 mAb to the cell

surface of each bacterial strain. The level of C3 bound to the bacterial surface of the serum-sensitive strain was similar to that of the serum-resistant strains in both *P. aeruginosa* and *S. typhimurium* (data not shown).

DISCUSSION

The complement-mediated serum bactericidal system is an important factor limiting the invasion of the host by organisms colonizing the mucous membranes. Various surface antigens, such as LPS with long O-polysaccharide chains, outer-membrane proteins (Heffernan *et al.*, 1992) and capsules (Glynn & Howard, 1970) render bacterial cells resistant to complement-mediated bactericidal killing. The profiles on silver-stained gels of LPS purified from the serum-sensitive *P. aeruginosa* TUM3 and its resistant derivative, TUM3 HSR, showed that a high molecular mass band was more strongly stained in TUM3 HSR, which was also confirmed by the results of Western blotting analysis and ELISA by anti-serotype G O-polysaccharide mAb TK-5B12. The level of A-band (D-rhamnan) on the LPS molecules from strain TUM3 HSR was approximately equal to that from strain TUM3. Interestingly, anti-serotype G core mAb OM-1D6 bound to the cell surface of strain TUM3 but not to that of strain TUM3 HSR although it specifically stained the core region on LPS molecules from both TUM3 and TUM3 HSR. These observations suggest that the long O-

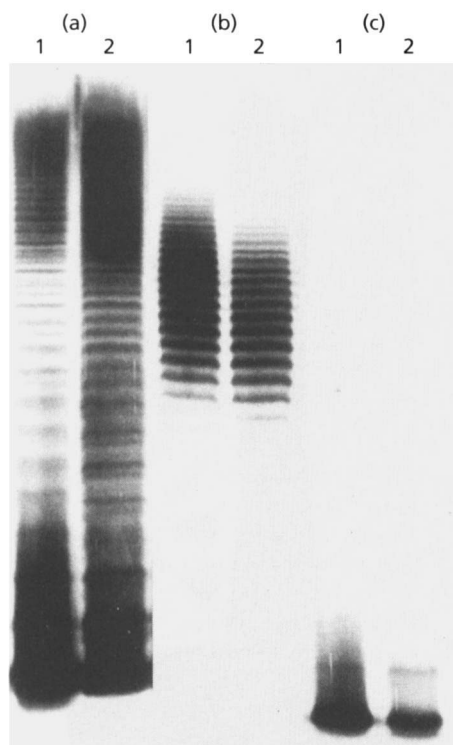


Fig. 4. Western blotting analysis of the LPS from *P. aeruginosa* TUM3 and TUM3 HSR with human mAbs. LPS preparations, which were treated with proteinase K, were derived from *P. aeruginosa* TUM3 (lanes 1) and TUM3 HSR (lanes 2). Immunoblots with (a) TK-5B12 (anti-serotype G O-polysaccharide mAb), (b) TS-1B2 (anti-D-rhamnan mAb) and (c) OM-1D6 (anti-serotype G core region mAb).

polysaccharide molecules were overproduced in this derivative, and therefore, anti-core mAb OM-1D6 was not capable of accessing the core LPS region on the cell surface of strain TUM3 HSR. Furthermore, no change was observed in the outer-membrane proteins on TUM3 HSR compared with the serum-sensitive parent strain TUM3 (data not shown).

Most *P. aeruginosa* strains isolated from patients with CF are sensitive to the serum bactericidal system. This is due to a deficiency of long O-polysaccharides on their LPS molecules. However, human-serum-sensitive strain TUM3 possessed serotype G (O6) O-antigen. Dasgupta *et al.* (1994) recently compared the serum sensitivity of a serotype O5 strain with that of a serotype O6 strain. Whereas the serotype O5 strain was completely resistant to the human serum bactericidal system, the serotype O6 strain was partially resistant. These investigators demonstrated that the B-band LPS molecules of the O5 strain formed an extensive fibrosis coat on the outer leaflet of the outer membrane, while the B-band LPS of the O6 strain could be discerned only as patches of fibrotic material on the bacterial surface, and suggested that the distribution of LPS molecules bearing the long O-antigen side chains influences the serum sensitivity. In the present study, the profiles on silver staining, Western blotting analysis and

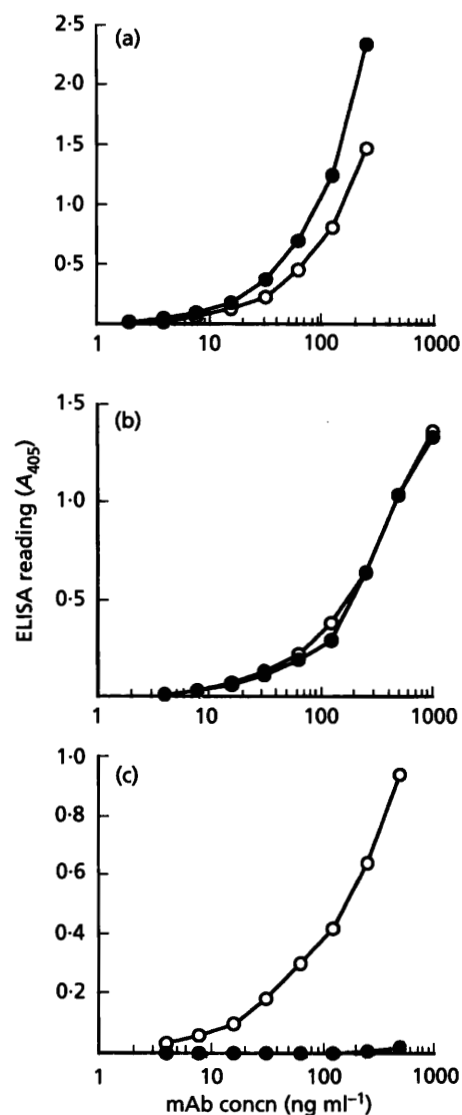


Fig. 5. Binding of (a) anti-serotype G O-polysaccharide mAb TK-5B12, (b) anti-D-rhamnan mAb TS-1B2 and (c) anti-serotype G core region mAb OM-1D6 to *P. aeruginosa* TUM3 (○) and TUM3 HSR (●). Binding activity was determined by ELISA using plates coated with whole cells of each strain and expressed as A_{405} .

ELISA demonstrated a poor expression of long O-polysaccharide molecules on the LPS from strain TUM3. Therefore, a major determinant of serum resistance in TUM3 HSR appeared to be an overproduction of a long O-polysaccharide side chain on the LPS molecule.

Comparison of the blood clearance rate in mice indicated that strain TUM3 was eliminated more rapidly than strain TUM3 HSR. However, the murine serum used in this study was not capable of killing strain TUM3, even when the serum concentration was 90% (data not shown). Furthermore, murine serum could not kill either *S. typhimurium* LT2 or LPS-deficient mutants of this strain. *S. typhimurium* LPS-deficient mutants were also more rapidly eliminated from the blood of mice compared with

strain LT2. Inoue (1972) has described the mouse complement system as immature, due to negligible serum bactericidal activity compared with nonhuman primates and humans. These results point to the existence of mechanisms other than serum killing that limit the spread into the bloodstream of bacteria lacking or having decreased long O-polysaccharide on their LPS molecules.

On the other hand, the number of *P. aeruginosa* TUM3 and *S. typhimurium* LT2 LPS-deficient mutants trapped in the liver 10 min after injection was higher than that of their respective counterparts. This finding indicates that the loss or decrease of long O-polysaccharide molecule renders the bacteria sensitive to serum bactericidal systems and also facilitates trapping by the RES. Consequently, the systemic spread of these strains becomes significantly limited.

An inhibitor of the synthesis of KDO heptose, which constitutes the inner core of the LPS, enhances the rate of bacterial clearance from mouse blood (Hammond, 1992), further indicating that bacteria lacking long O-side chains are readily cleared from the bloodstream. In the present study, however, experiments using smooth-LPS-type *S. typhimurium* LT2 and a series of LPS-deficient mutants with gradually diminishing length of O- or core-polysaccharide side chain revealed that rapid elimination from the bloodstream occurred even with strain SL1034 [*rfe* (SR) mutant], which has an O-side chain of only one repeating unit, but the longest LPS among the mutant strains studied. This result demonstrated that the presence of more than at least one repeating unit of the O-polysaccharide was important in protecting against early clearance from the bloodstream. It is, of course, clear that the structure of LPS side chain in *P. aeruginosa* is different from that of *S. typhimurium*. The serum-resistant property of *S. typhimurium* LT2 was significantly less than that of *P. aeruginosa* TUM3 HSR, suggesting that the difference in the chemical structure of the polysaccharide side chain may also influence the sensitivity to serum bactericidal systems (Grossmann & Leive, 1984). It is, however, not established whether such differences in structure play a part in the clearance rate from the bloodstream.

The initial stage of bacterial phagocytosis involves the attachment of bacteria to complement receptors, such as CR1 and integrin $\alpha_{\text{mac}}\beta_2$ (CR3) (Speert, 1993). These receptors recognize complement fragments C3b and iC3b, respectively. C3 attaches both to the Fc portion of antibody, and to LPS. These complement receptors can also bind directly to LPS on nonopsonized bacteria (Wright & Jong, 1986). If the level of binding of C3 components to bacterial cell surfaces is higher in strains with short O-side chains on their LPS compared with strains with long O-side chains on their LPS, it would be expected that the LPS mutants would be cleared more easily from the bloodstream. However, flow-cytometry results demonstrated that murine C3 binding to bacterial cells was almost equal among the isogenic sets: *P. aeruginosa* TUM3 and TUM3 HSR, and *S. typhimurium* LT2 and *rfa* (Re) mutant TA2168, with the shortest LPS molecule. These results are inconsistent with those

reported by Schiller & Joiner (1986) demonstrating that the *P. aeruginosa* mucoid strain 144M with a short-side-chain LPS was able to bind C3 twice as much as *P. aeruginosa* mucoid strain 144M SR (derived from 144M) with long O-side chains. These investigators suggested that the binding site of C3 to LPS was hindered by long O-side chains on the 144M SR LPS. On the other hand, Joiner *et al.* (1982a, b) reported that twice as many molecules of radiolabelled C3 were bound per cell of an *S. minnesota* smooth LPS strain compared with a mutant strain having only core-LPS. They also reported that the membrane attack complex formed more rapidly on LPS smooth strains, but the binding of C3 to the long O-side chains was unstable and C3 was released easily from the LPS chain.

Based on these results, another mechanism could also be considered. The LPS-binding protein (LBP), that exists in a concentration of 5–10 μg per ml of blood (Mathison *et al.*, 1993), binds with a high affinity to LPS via lipid A (Schumann *et al.*, 1990). The LPS–LBP complex in turn binds to CD14 expressed on the surface of phagocytic cells. Since macrophages tend to bind LPS–LBP complexes rather than free LPS, it is possible that LPS with short side chains or only the core region bind better to LBP. Such a scenario is possible since lipid A, a target of LBP, is exposed in strains with a short-side-chain LPS. Furthermore, phagocytic cells express low levels of CD14 and CR3, but the level increases substantially in response to LPS (Weingarten *et al.*, 1993). Thus, it is important to examine the expression level of CD14 and CR3 following exposure to LPS isolated from each strain. On the other hand, we must consider that receptors on macrophages directly bind to LPS. Furthermore, these receptors preferentially bind to short-side-chain LPS rather than to smooth LPS. Current studies are attempting to define these potential mechanisms further.

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