Variable cross-reactivity of *Pseudomonas aeruginosa* lipopolysaccharide-core-specific monoclonal antibodies and its possible relationship with serotype

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The core region of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) was analysed by four LPS-core-specific human monoclonal antibodies (mAbs; FK-2E7, MH-4H7, OM-1D6 and NM-3G8). Reactivity of these mAbs to about 180 *P. aeruginosa* strains was tested. FK-2E7 bound to strains of Homma serotype E and I at a frequency of about 90%, to strains of serotype M at about 50%, and to strains of serotype A and G at about 30%. MH-4H7 bound to *P. aeruginosa* strains of serotype A, F, G, H, K and M at a high frequency (45–87%), but did not bind to any strains of serotype B, C, E and I. OM-1D6 and NM-3G8 bound to *P. aeruginosa* strains in a nearly serotype-specific manner. OM-1D6 reacted with all strains of serotype G so far tested, and a few strains of serotype M. Furthermore, L-rhamnose in the LPS core of serotype G was an immunodominant sugar recognized by OM-1D6 as an epitope. NM-3G8 bound to only a few strains of serotype B and M. The variable reactivity of these mAbs suggests that antigenic heterogeneity of the LPS core is somewhat related with (O-polysaccharide-based) serotype. Among these mAbs, MH-4H7 and OM-1D6 showed a high level of protective activity against *P. aeruginosa* in an experimental infection model using normal mice. *In vivo* protective activity was shown to be closely related to *in vitro* binding activity to whole cells as determined by agglutination and flow cytometry, but not ELISA.

Introduction

Pseudomonas aeruginosa is a major pathogen of opportunistic infections. *P. aeruginosa* lipopolysaccharide (LPS), as well as that of the *Enterobacteriaceae*, consists of three regions, namely O-polysaccharide, core oligosaccharide and lipid A (Wilkinson, 1983). These regions are distinguished by chemical constituents and antigenic properties. It is well known that O-antigenic specificity, namely O-serotype, is determined by the structure of the O-polysaccharide and the endotoxic principle is expressed by lipid A (Luderitz *et al.*, 1982).

In enterobacterial LPS, the core region is divided into two parts, the outer core and inner core. Generally, the inner core region contains characteristic sugars, 3-deoxy-octulosonic acid and heptose (Luderitz *et al.*, 1982;

Rietschel *et al.*, 1985). The immunological properties, as well as the chemical constituents of the inner core region are expected to be common to all Gram-negative bacteria, including *P. aeruginosa* (Brade & Galanos, 1983; Brade & Brade, 1985). The outer core region exists between the O-polysaccharide and the inner core, and consists of about ten sugar residues. In *P. aeruginosa*, the outer core region commonly contains L-rhamnose, D-glucose, D-galactosamine and L-alanine, but the molar ratio of these constituents seems to be heterogeneous between strains (Wilkinson, 1983). However, the detailed chemical structure of this region has been reported only for strain NCTC1999 (Drewry *et al.*, 1975), and PAC1R and its defective mutants (Rowe & Meadow, 1983), and their proposed partial structures were different from each other. Thus, the extent of the heterogeneity of the LPS core region is still obscure.

Our recent study (Yokota *et al.*, 1989) demonstrated that human monoclonal antibodies (mAbs) MH-4H7 and KN-2B11, recognizing the LPS outer core region,

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Abbreviations: LPS, lipopolysaccharide; mAb, monoclonal antibody.

bound to *P. aeruginosa* strains of particular serotypes, namely A, F, G, H, K and M (classification of Homma, 1976), at a high frequency. Hence, we supposed that the heterogeneity of the outer core region of *P. aeruginosa* LPS might be related to the O-serotype. However, this result arose from only one epitope, recognized by MH-4H7 and KN-2B11. In the present paper, we further characterized this heterogeneity by using four distinct types of LPS-core-specific human mAbs, including MH-4H7. Furthermore, biological activity of these mAbs was determined by *in vitro* and *in vivo* methods.

Methods

Bacterial strains. Homma serotype standard strains of *P. aeruginosa* (IID number) (Homma, 1976) were obtained from the Institute of Medical Science, University of Tokyo, Tokyo, Japan. The international antigenic typing scheme (Liu *et al.*, 1983) and Fisher serotype (Fisher *et al.*, 1969) standard strains (ATCC number) were obtained from the American Type Culture Collection, Rockville, Maryland, USA. *P. aeruginosa* PAC1, PAC1R (Habs serotype 3) and their LPS-defective mutant strains (Rowe & Meadow, 1983) were a kind gift from P. M. Meadow, University College of London, London, UK. Clinical isolates of *P. aeruginosa* (SP number) were stocked in our laboratory, and serotyping was carried out by the slide agglutination method with a serotype grouping kit, Mei-assay (Meiji Seika Kaisha).

Preparation of LPS. *P. aeruginosa* was cultured in heart infusion broth (Nissui Pharmaceuticals) to late exponential-phase. LPS was extracted from bacterial cells by Triton X-100 according to the method of Uchida & Mizushima (1987). The resulting LPS fraction was treated with 50 µg proteinase K ml⁻¹ (Sigma) at 60 °C for 1 h, and then LPS was recovered by centrifugation (100000 g, 90 min) and ethanol precipitation. Protein and nucleic acid contents were less than 0.3% in these preparations.

Human mAbs. Human-mouse heterohybridoma cell lines producing human mAbs were established basically as previously described (Yokota *et al.*, 1989). Human peripheral blood lymphocytes were prepared from heparinized blood of a healthy adult donor with a high antibody titre for *P. aeruginosa* immunogens. The lymphocytes were stimulated *in vitro* by culturing with 0.1% pokeweed mitogen (Gibco) and 0.0002% formalin-killed *P. aeruginosa* cells for 6 d, and then fused with a human-mouse heteromyeloma using polyethylene glycol. Specific antibody production was screened by ELISA using plates coated with whole *P. aeruginosa* cells of some serotype standard strains (A, B, E, G, I and M).

Four LPS-core-specific human mAbs, MH-4H7, FK-2E7, OM-1D6 and NM-3G8, and one lipid A-specific human mAb, NM-3D7, were obtained. FK-2E7 was IgM κ , and the other human mAbs were IgM λ . The parental heteromyeloma cell line was a triple hybridoma (3HL3-6J-C5) for establishing hybridoma OM-1D6, and a heterohybridoma (SHM-D33) (Teng *et al.*, 1983) was used to establish the other mAb-producing hybridoma cell lines.

The hybridoma was adapted to a serum-free medium, Celgros H (Sumitomo Pharmaceuticals). IgM was purified by gel chromatography on a column of Superose 6 (Pharmacia LKB) in Dulbecco's PBS from a serum-free culture supernatant.

ELISA. The methods for ELISA and preparation of bacterial-cell-coated plates were 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 anti-

body (Kirkegaard & Perry Laboratories Inc.) and sodium *p*-nitrophenyl-phosphate were used as secondary antibody and substrate, respectively. Binding activity was expressed as A_{405} .

For competitive ELISA, the antibody solution and competitor solution were mixed, preincubated for 37 °C for 1 h, and then subjected to ELISA.

SDS-PAGE and Western blotting. Each LPS preparation (5 µg), all of which were treated with proteinase K, was subjected to SDS-PAGE using a 10–20% (w/v) polyacrylamide gradient gel (Daiichi Chemicals) according to the method of Laemmli (1970). LPS was visualized by silver staining according to the method of Tsai & Frasch (1982). Western blotting was carried out as described previously (Yokota *et al.*, 1989). Briefly, LPS on the gels was transferred to a PVDF filter (Millipore). The filter was blocked with 2% (w/v) casein in PBS, and subsequently incubated with mAb (1 µg ml⁻¹) and alkaline-phosphatase conjugated goat anti-human IgM antibody (1000-fold dilution). Specific binding was detected with sodium 5-bromo-4-chloro-3-indoylphosphate (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-MgCl₂.

Flow cytometry. Flow cytometry was carried out basically according to the method of Evans *et al.* (1990). *P. aeruginosa* cells were cultured in heart infusion broth to late exponential-phase, washed with PBS and fixed with 1% (v/v) formaldehyde at 25 °C for 1 h. Washed cells were resuspended in PBS to an A_{600} of 0.5 (approximately 1×10^8 c.f.u. ml⁻¹). The suspension (0.5 ml) was mixed with a 5 µg mAb ml⁻¹ solution (0.5 ml), and then incubated at 37 °C for 1 h. The resulting samples were washed twice with PBS, suspended in 0.5 ml fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM antibodies (Tago) 100-fold diluted with PBS, and incubated at 37 °C for 1 h. Each sample was prepared in triplicate. Flow cytometry was performed with FACScan (Becton-Dickinson), equipped with a 15 mW argon ion laser. Stained bacterial cells were suspended in PBS at a concentration of about 10^6 c.f.u. ml⁻¹, and 20000 stained cells were totally evaluated in each analysis. Bacterial aggregates and background noise were excluded with the use of gates on the light scatter signals.

Protective activity against *P. aeruginosa* infection. The experimental infection model using normal mice was as described previously (Terashima *et al.*, 1991). Four-week old male ICR mice (Japan SLC Inc.) (22–24 g body wt.) were used. Briefly, a cell suspension (0.2 ml) of each bacterial strain containing 5% (w/v) mucin (Difco) was injected intraperitoneally into ten mice per group. One hour later, 0.2 ml mAb in PBS was administered intraperitoneally at a dosage of 0.1 or 1.0 µg per mouse. As a control group, BSA (10 µg per mouse) was administered instead of mAb. The mice were observed for 6 d. The lethal dosage of bacteria causing death in 50% mice (LD₅₀) was calculated by probit analysis. The significance of difference from the control group was indicated by non-overlapping 95% confidence intervals.

Other materials and methods. Agglutination activity using viable bacterial cells was determined as described previously (Terashima *et al.*, 1991). Other materials and methods were the same as those described in previous papers (Yokota *et al.*, 1989, 1990).

Results

Binding to various serotypes of P. aeruginosa in ELISA

The binding profile of mAbs was determined by ELISA using whole bacterial cells as a solid antigen (Table 1). MH-4H7 (μ , λ) and FK-2E7 (μ , κ) cross-reacted to strains

Table 1. *Binding profile of anti-core mAbs to P. aeruginosa standard strains and clinical isolates*

Whole cells of each strain were fixed to ELISA plates with glutaraldehyde. An A_{405} value of >0.15 in ELISA (mean value from triplicate experiments) at a concentration of $1\ \mu\text{g}$ antibody ml^{-1} was considered to be binding-positive.

Homma serotype	No. of strains tested	No. of strains giving positive binding			
		FK-2E7	MH-4H7	OM-1D6	NM-3G8
A	21	7	15	0	0
B	28	0	0	0	6
C	8	0	0	0	0
E	24	22	0	0	0
F	7	1	6	0	0
G	23	8	20	23	0
H	16	0	10	0	0
I	23	21	0	0	0
K	4	0	3	0	0
M	22	10	10	4	2
NT	6	2	1	1	1

belonging to some serotypes. FK-2E7 bound to strains of serotype E and I at a frequency of about 90%, serotype M at about 50%, serotype A and G at about 30%. MH-4H7 bound to strains of serotype A, F, G, H and K with a high frequency (63–87%), to serotype M at about 50%, but did not react with any strains of other serotypes. OM-1D6 (μ , λ) bound to 100% of serotype G strains tested and a few strains of serotype M, but did not bind to strains of any other serotypes. NM-3G8 (μ , λ) bound to only a few strains of serotype B and M.

Western blotting analysis

LPS preparations were subjected to SDS-PAGE and Western blotting. All four mAbs specifically bound to a relatively low-molecular-mass population of each LPS preparation which was isolated from a binding-positive strain in ELISA (Fig. 1). Namely, MH-4H7 stained low-molecular-mass LPSs derived from serotype A and G, but not serotype B (Fig. 1c). FK-2E7 specifically stained LPSs derived from IID1130 (serotype E) and IID1010 (serotype I) (data not shown), as well as LPS derived from serotype A and G as shown in Fig. 1(b). OM-1D6 specifically stained serotype G LPS (Fig. 1d). NM-3G8 only stained LPS derived from IID1002 (serotype B), and gave a much weaker reaction in the immunoblot assay than the other mAbs (Fig. 1e).

Binding to LPS-defective mutants

Binding activity of anti-core mAbs to LPS-defective mutants derived from *P. aeruginosa* PAC1 and PAC1R (Rowe & Meadow, 1983), of which LPS core structures

had been well defined, was determined by ELISA using whole cells as a solid antigen (Table 2). First, we determined the serotype of these strains according to Homma's classification by agglutination testing using mAbs (Mei-assay). Whereas the smooth type strains were classified into serotype A, serologically related to Habs serotype 3, the semirough and rough strains derived from PAC1R were classified into serotype M. Human anti-lipid A mAb NM-3D7 was used as a control in this study. NM-3D7 specifically bound to the lipid A portion of LPS derived from *Salmonella* and *Escherichia coli*, as well as *P. aeruginosa* (data not shown). The mAb NM-3D7 bound to rough strains more strongly than to smooth strains. FK-2E7 reacted more strongly with rough-natured strains, PAC608 and PAC557, but reacted weakly with deep rough strains PAC556 and PAC611. In contrast, MH-4H7 did not react at all to these deep rough strains PAC556 and PAC611, as previously reported (Yokota *et al.*, 1989). PAC556 is reported to lack only the L-rhamnose residue of the LPS outer core region compared with PAC557 (Rowe & Meadow, 1983). The other anti-core mAbs, OM-1D6 and NM-3G8, were not observed to bind to a series of strains, including a parental strain of serotype A, at all.

Competition of monosaccharide with mAb binding

Competition experiments with monosaccharide were performed by ELISA using LPS-coated plates. All mAbs bound to SP10067 (serotype M) and IID1020 (serotype G), except that NM-3G8 did not react to IID 1020. L-Rhamnose and methyl-L-rhamnoside (the preparation contained 90% α -anomer) inhibited the binding of both

Fig. 1. SDS-PAGE and Western blotting analysis of *P. aeruginosa* LPS preparations with human mAbs. The LPS preparations, which were treated with proteinase K, were derived from *P. aeruginosa* IID1001 (serotype A; lane 1), IID1020 (serotype G; lane 2) and IID1002 (serotype B; lane 3). To each lane, 5 µg sample was applied. Silver staining pattern of each LPS preparation (a) and immunoblots with (b) FK-2E7, (c) MH-4H7, (d) OM-1D6 and (e) NM-3G8 are shown.

Table 2. Binding of anti-LPS-core mAbs and anti-lipid A mAb to *P. aeruginosa* PAC1R and its LPS-defective mutant strains

Binding activity was determined by ELISA using plates coated with whole cells of each strain and expressed as mean A_{405} values from triplicate experiments.

Strain	Chemotype*	Serotype	Binding activity (A_{405})				
			FK-2E7	MH-4H7	OM-1D6	NM-3G8	NM-3D7
PAC1R	S	A	0.15	0.25	0	0	0.02
PAC609	S	A	0.18	0.28	0	0	0.05
PAC608	SR	M	1.48	1.11	0	0	0.13
PAC557	R	M	1.62	1.13	0	0	0.49
PAC556	R	M	0.38	0.02	0	0	0.74
PAC611	R	M	0.20	0.04	0	0	0.85

* S, smooth; SR, semirough; R, rough.

OM-1D6 and MH-4H7 to LPS derived from both IID1020 (serotype G) and SP10067 (serotype M). Other monosaccharides, such as L-fucose, D-fucose, D-quinovose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine

and N-acetylneuraminic acid, did not inhibit the binding of OM-1D6 and MH-4H7 even at concentrations of 500 mM. A much lower concentration of L-rhamnose and methyl-L-rhamnoside inhibited the binding of OM-1D6 compared with MH-4H7 (Fig. 2). Inhibition of the

Table 3. Comparison in binding activity of anti-LPS core mAbs to whole bacterial cells determined by ELISA, agglutination and flow cytometry

mAb	Strain	Serotype	ELISA (A_{405})	Agglutinating activity ($\mu\text{g ml}^{-1}$)*	Flow cytometry (percentage positive fluorescence†)
FK-2E7	SP6783	A	0	>25	2.0
	SP9759	E	1.72	>25	10.2
	SP10046	I	1.68	>25	22.3
	SP6788	G	1.76	>25	18.2
	SP10067	M	1.98	>25	60.0
	SP6764	M	2.10	>25	26.0
MH-4H7	IID5018	M	0	>25	3.1
	SP6783	A	1.10	0.47	64.9
	SP6788	G	2.44	0.94	74.9
	SP9792	G	1.98	0.94	74.9
	SP10067	M	2.19	0.12	71.9
	SP6764	M	2.24	15	59.3
OM-1D6	IID5018	M	0	25	1.5
	SP6783	A	0	>25	0.9
	SP6788	G	2.20	0.47	97.6
	SP9792	G	1.85	0.94	95.2
	SP10067	M	2.10	0.12	92.9
	SP6764	M	0.23	>25	5.9
NM-3G8	IID5018	M	0	>25	1.5
	SP10067	M	0.13	>25	31.4
	IID1002	B	0.26	>25	9.6

* The value represents the minimum concentration of mAb required for visible agglutination.

† Value represents the percentage of bacterial cells giving a positive fluorescence intensity over the background level at the level of sensitivity shown in Fig. 2.

Fig. 2. Competition of L-rhamnose and methyl-L-rhamnoside with the binding of mAbs to *P. aeruginosa* SP10067 LPS in ELISA. OM-1D6 (● and ▲) or MH-4H7 (○ and △) ($1 \mu\text{g ml}^{-1}$ each) were incubated with various concentrations of L-rhamnose (● and ○) or methyl-L-rhamnoside (containing 90% α -anomer; ▲ and △), and then applied to ELISA using the plate coated with LPS derived from SP10067 (serotype M). The results are expressed as a percentage of the A_{405} reading compared with that without competitor. Mean values from triplicate experiments are shown. The 100% A_{405} values were 1.02 (MH-4H7) and 0.82 (OM-1D6).

binding of MH-4H7 by L-rhamnose was weaker than by methyl-L-rhamnoside. In contrast, FK-2E7 and NM-3G8 were not observed to compete in binding to LPS derived from IID1020 (in the case of FK-2E7) and

SP10067 with any monosaccharides mentioned above, even at high concentrations of 500 mM.

Cross reaction to other Gram-negative bacterial LPSs

Cross-reaction of the anti-core mAbs to LPSs derived from various Gram-negative bacteria other than *P. aeruginosa* was tested by ELISA using LPS as a solid antigen. No cross reaction to any other bacterial LPSs tested was observed, except the reaction of MH-4H7 to *E. coli* O26:B6 LPS as described previously (Yokota *et al.*, 1990). Furthermore, anti-core mAbs were not observed to bind in ELISA to lipid A derived from *E. coli* J5, *Salmonella minnesota* R595 and *P. aeruginosa* IID1020.

Binding activity of mAb to bacterial cells in flow cytometry and agglutination activity

We compared the binding activity in ELISA of each mAb to whole bacterial cells with the binding activity in flow cytometry and agglutination activity (Table 3 and Fig. 3). High agglutination activity was observed for MH-4H7 and OM-1D6, but not for FK-2E7 and NM-3G8 (Table 3). In flow cytometry analysis, MH-4H7 and OM-1D6 gave relatively high intensities of fluorescence

Fig. 3. Flow cytometry analysis of the binding of human anti-LPS-core mAbs, FK-2E7 (a), MH-4H7 (b) and OM-1D6 (c), to *P. aeruginosa*. Green fluorescence intensity of the mAbs with whole cells of *P. aeruginosa* is expressed as a log scale on the horizontal axis. The number of cells giving an intensity of green fluorescence is indicated on the vertical axis. The name of each strain is shown at the top of the traces and the serotype of each strain used is shown in parentheses.

Table 4. Protective activity of human mAbs against *P. aeruginosa* infection in experimental mouse model

mAb	Dose administered (μ g per mouse)	Strain challenged	Serotype	LD ₅₀ (c.f.u.)		Protection* (-fold)
				Control	mAb-treated	
MH-4H7	0.1	SP6788	G	3.0×10^4	3.2×10^5	11†
	0.1	SP9792	G	5.0×10^5	1.6×10^7	32†
	1.0	SP6783	A	4.0×10^5	3.2×10^6	8.0†
	1.0	SP7514	H	1.5×10^6	6.6×10^6	4.4†
OM-1D6	0.1	SP6788	G	3.6×10^4	$> 3.4 \times 10^5$	> 9.4 †
	0.1	SP9792	G	2.6×10^5	6.3×10^6	24†
FK-2E7	1.0	SP9759	E	1.2×10^5	1.4×10^5	1.2
	1.0	SP10046	I	1.2×10^5	1.1×10^5	0.9
	1.0	SP6788	G	2.3×10^4	1.8×10^4	0.8

* LD₅₀ (mAb-treated)/LD₅₀ (control).

† Significantly different ($P < 0.05$) from control group.

against both smooth and rough (serotype M) strains in parallel with the binding activity observed in ELISA (Fig. 3*b, c*). OM-1D6 gave a relatively higher intensity of fluorescence than MH-4H7. On the other hand, FK-2E7 did not exhibit a high intensity fluorescence with strains to which it bound at high frequency in ELISA, except with SP10067 (serotype M) [Fig. 3 (*a*) and Table 3]. In the case of MH-4H7 and FK-2E7, apparently non-staining populations were observed when the level of sensitivity was as used in the experiments of Fig. 3 and Table 3. However, we observed that almost all populations showed significant fluorescence for ELISA-positive strains measured in conditions of higher sensitivity but not for ELISA-negative strains (data not shown).

NM-3G8 gave a very low intensity of fluorescence against serotype B strains, whereas it gave a relatively high intensity of fluorescence against serotype M strains, as did FK-2E7 (Table 3).

Protective activity against experimental infection in mice

In vivo protective activity of the human mAb (IgM) was assessed in an experimental infection model using normal mice (Table 4). MH-4H7 and OM-1D6 showed highly protective activity against infections with *P. aeruginosa* strains, which the mAbs bound to and caused agglutination *in vitro*. In contrast, FK-2E7 did not show any significant *in vivo* protective activity against

strains to which the mAb bound *in vitro*. As mentioned before, FK-2E7 did not cause agglutination of the strains used.

Discussion

Four different types of human mAbs recognizing the *P. aeruginosa* LPS core region were prepared. The sites recognized by these mAbs were deduced to be in the core region from the following. (i) In Western blot analysis (Fig. 1), all the mAb preferentially reacted with a low-molecular-mass population of LPS preparations. (ii) The mAbs did not bind to the lipid A preparation in ELISA (data not shown). (iii) In the case of FK-2E7 and MH-4H7, the mAbs bound strongly to rough mutants PAC608 and PAC557 at higher rate than a parental strain PAC1R (Table 2). The mAbs bound weakly to PAC556 and PAC611, the core region of which was defective in part. In addition, cross reactivity to Gram-negative bacterial LPS was not observed with the exception of the cross-reaction of MH-4H7 to *E. coli* O26:B6 LPS, as reported previously (Yokota *et al.*, 1990).

The core region, especially the outer core region, had been thought to be characteristic of, and common to *P. aeruginosa*. D-Galactosamine, L-rhamnose, D-glucose and L-alanine are reported to be common constituents of the outer core (Wilkinson, 1983). However, a few researchers have pointed out that antibodies directed to the LPS core cross-react with different serotype strains of *P. aeruginosa* (Hancock *et al.*, 1983; Fomsgaard *et al.*, 1988; Yokota *et al.*, 1989), and the cross-reactivity is likely to be caused by the heterogeneity of the LPS core region. A systematic study on LPS core antigens of *P. aeruginosa* has not been carried out, because a detailed structural study on the LPS core of each strain is thought to be very laborious. In this study, we tried to analyse the LPS core region of *P. aeruginosa* by using four human mAbs and about 180 *P. aeruginosa* strains including serotype standard strains and clinical isolates.

The mAbs FK-2E7 and MH-4H7 cross-reacted with strains belonging to some serotypes (Table 1). The epitope is likely to exist in some, but not all, of *P. aeruginosa* strains, and it might be distributed in a serotype-dependent manner. The epitope recognized by MH-4H7 was shared by almost all strains belonging to serotype A, F, G, H, K and M. We have demonstrated that the L-rhamnose residue in the core region acts as an epitope to some extent, this being deduced from the results of a binding experiment with a series of LPS-defective mutants of PAC1R (Table 2) and a competition experiment with monosaccharide (Fig. 2). On the other hand, the epitope recognized by FK-2E7 was found on

about 90% of serotype E and I strains, about 30% of serotype A and G strains, and about 50% of serotype M strains. The binding profile of FK-2E7 to *P. aeruginosa* strains was different from MH-4H7, and L-rhamnose, as well as methyl-L-rhamnoside, did not compete with the binding of FK-2E7, in contrast to MH-4H7 even though the binding experiments using a series of LPS-defective mutants (Table 2) suggested that the epitope for FK-2E7 is located in the neighbourhood of that for MH-4H7, at least on *P. aeruginosa* PAC1R LPS. Two mAbs, OM-1D6 and NM-3G8, bound to *P. aeruginosa* strains in a nearly serotype-specific manner (Table 1). Thus, a characteristic epitope recognized by OM-1D6 was shared with 100% of serotype G strains tested, and OM-1D6 was shown to strongly react to the whole cells from the results of flow cytometry analysis (Fig. 3) and *in vivo* protective activity (Table 4). Furthermore, a competition experiment with monosaccharide (Fig. 2) suggests that L-rhamnose might be greatly involved as an epitope for OM-1D6. In other words, L-rhamnose seemed to be an immunodominant sugar in the LPS core region of serotype G strains. Some serotype B and M strains had an epitope recognized by NM-3G8, but other serotype strains did not. A line of results suggest that the heterogeneity of the LPS core of *P. aeruginosa* is related to (O-polysaccharide-based) serotype.

Both OM-1D6 and NM-3G8, which reacted in a nearly serotype-specific manner, with serotype G and B, respectively, also reacted with some strains of serotype M. Serotype M strains were rough-natured, due to defective or decreased amounts of O-polysaccharides (unpublished results). Homma (1976) also reported that the standard strain of serotype M was rough. Antigenic target(s) of serotype M have not been clarified yet. In this study, we also observed that rough and semirough strains were classified as serotype M according to the serotype grouping kit (Table 2). Thus, serotype M does not seem to be based upon the nature of the O-polysaccharide antigen. The results indicated that binding specificity of OM-1D6 and NM-3G8 was able to be regarded as serotype-specific.

Human mAbs directed against some common epitopes in the LPS core region are one of the candidates for antibody therapy against *P. aeruginosa* infections and will be used as an alternative to, or in combination with anti-O-polysaccharide mAbs. Two mAbs, MH-4H7 and OM-1D6, showed a high level of protective activity against *P. aeruginosa* infections in a normal mouse infection model (Table 4). We have recently reported details of *in vivo* protective activity of MH-4H7 (Terasima *et al.*, 1991). Among these LPS-core-specific human mAbs, we compared *in vivo* protective activity with *in vitro* binding activity determined by different methods. Flow cytometry has recently been used for

analysing bacterial cell surface antigens (Klebba *et al.*, 1990; Evans *et al.*, 1990; Nelson *et al.*, 1990). Applying flow cytometry to our system (Fig. 2), OM-1D6 and MH-4H7, which showed a potent protective activity, were shown to give relatively higher fluorescence than FK-2E7 and NM-3G8. In the case of FK-2E7 and MH-4H7, the non-staining subpopulations were observed with a level of sensitivity as shown in Fig. 3 and Table 3. This observation is not likely to be due to the existence of a true non-staining subpopulation within an ELISA-positive strain, because in measurement with a higher level of sensitivity, almost all bacterial populations showed a significant degree of fluorescence by staining with the mAbs MH-4H7 and FK-2E7. However, ELISA-negative strains did not show any significant fluorescence. The *in vivo* protective activity was shown to correlate well with *in vitro* binding activity as determined by flow cytometry (Tables 3 and 4), and agglutination, but not by binding in ELISA. In ELISA, a significant difference of binding activity among mAbs was not observed in antibody dilution curve experiments (data not shown). Nelson *et al.* (1990) pointed out that ELISA has tended to over-emphasize the binding of anti-core mAbs to whole bacterial cells in contrast to flow cytometry. In our study, a similar phenomenon was observed. The physical state of bacteria in a cell suspension was probably different from that on a solid surface. Thus, ELISA does not seem to be a suitable method for evaluating true binding activity, which would predict *in vivo* activity, among different antibodies.

A line of evidence suggested that the epitopes in the LPS core recognized by MH-4H7 and OM-1D6 were likely to be exposed on the cell surface to a greater extent than those for FK-2E7 and NM-3G8. In conclusion, the heterogeneity of the LPS core region of *P. aeruginosa* might be related to (O-polysaccharide-based) serotype, and some epitopes in the LPS core would be valuable and useful as protective antigens.

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References

- BRADE, H. & GALANOS, C. (1983). Common lipopolysaccharide specificity: new type of antigen residing in the inner core region of S- and R-form lipopolysaccharides from different families of Gram-negative bacteria. *Infection and Immunity* **42**, 250–256.
- BRADE, L. & BRADE, H. (1985). A 28000-dalton protein of normal mouse serum binds specifically to the inner core region of bacterial lipopolysaccharide. *Infection and Immunity* **50**, 687–694.
- DREWRY, D. T., SYMES, K. C., GRAY, G. W. & WILKINSON, S. G. (1975). Studies of polysaccharide fractions from the lipopolysaccharide of *Pseudomonas aeruginosa* NCTC 1999. *Biochemical Journal* **149**, 93–106.
- EVANS, M. E., POLLACK, M., HARDEGEN, N. J., KOLES, N. L., GUELDE, G. & CHIA, J. K. S. (1990). Fluorescence-activated cell sorter analysis of binding by lipopolysaccharide-specific monoclonal antibodies to gram-negative bacteria. *Journal of Infectious Diseases* **162**, 148–155.
- FISHER, M. W., DEVLIN, H. B. & GNABASIK, F. J. (1969). New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. *Journal of Bacteriology* **98**, 835–836.
- FOMSGAARD, A., CONRAD, R. S., GALANOS, C., SHAND, G. H. & HOIBY, N. (1988). Comparative immunochemistry of lipopolysaccharides from typable and polyagglutinable *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis. *Journal of Clinical Microbiology* **26**, 821–826.
- HANCOCK, R. E. W., MUTHARIA, L. M., CHAN, L., DARVEAU, R. P., SPEERT, D. P. & PIER, G. B. (1983). *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infection and Immunity* **42**, 170–177.
- HOMMA, J. Y. (1976). A new antigenic schema and live-cell slide-agglutination procedure for the infrasubspecific serologic classification of *Pseudomonas aeruginosa*. *Japanese Journal of Experimental Medicine* **46**, 329–336.
- KLEBBA, P. E., BENSON, S. A., BALA, S., ABDULLAH, T., REID, J., SINGH, S. P. & NIKAI, H. (1990). Determinants of OmpF porin antigenicity and structure. *Journal of Biological Chemistry* **265**, 6800–6810.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LIU, P. V., MATSUMOTO, H., KUSAMA, H. & BERGAN, T. (1983). Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. *International Journal of Systematic Bacteriology* **33**, 256–264.
- LUDERITZ, O., FREUDENBERG, M. A., GALANOS, C., LEHMANN, V., RIETSCHEL, E. T. & SHAW, D. H. (1982). Lipopolysaccharides of gram-negative bacteria. *Current Topics in Membranes and Transport* **17**, 79–151.
- NELSON, D., NEILL, W. & POXTON, I. R. (1990). A comparison of immunoblotting, flow cytometry and ELISA to monitor the binding of anti-lipopolysaccharide monoclonal antibodies. *Journal of Immunological Methods* **133**, 227–233.
- POLLACK, M., RAUBITSCHKE, A. A. & LARRICK, J. W. (1987). Human monoclonal antibodies that recognize conserved epitopes in the core-lipid A region of lipopolysaccharides. *Journal of Clinical Investigation* **79**, 1421–1430.
- RIETSCHEL, E. T., BRADE, H., BRADE, L., KACA, W., KAWAHARA, K., LINDER, B., LUDERITZ, O., TOMITA, T., SCHADE, U., SEYDEL, U. & ZAHNINGER, U. (1985). Newer aspects of the chemical structure and biological activity of bacterial endotoxins. *Progress in Clinical and Biological Research* **189**, 31–50.
- ROWE, P. S. N. & MEADOW, P. M. (1983). Structure of the core oligosaccharide from the lipopolysaccharide of *Pseudomonas aeruginosa* PAC1R and its defective mutants. *European Journal of Biochemistry* **132**, 329–337.
- TENG, N. N. H., LAM, K. S., RIERA, F. C. & KAPLAN, H. S. (1983). Construction and testing of mouse-human heteromyelomas for human monoclonal antibody production. *Proceedings of the National Academy of Sciences of the United States of America* **50**, 499–506.
- TERASHIMA, M., UEZUMI, I., TOMIO, T., KATO, M., IRIE, K., OKUDA, T., YOKOTA, S. & NOGUCHI, H. (1991). Protective human monoclonal antibody directed to the outer core region of *Pseudomonas aeruginosa* lipopolysaccharide. *Infection and Immunity* **59**, 1–6.
- TSUI, C.-M. & FRASCH, C. E. (1982). A sensitive stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- UCHIDA, K. & MIZUSHIMA, S. (1987). A simple method for isolation of lipopolysaccharides from *Pseudomonas aeruginosa* and some other bacterial strains. *Agricultural and Biological Chemistry* **51**, 3107–3114.
- WILKINSON, S. G. (1983). Composition and structure of lipopolysaccharides from *Pseudomonas aeruginosa*. *Reviews of Infectious Diseases* **5**, S941–S949.
- YOKOTA, S., OCHI, H., OHTSUKA, H., KATO, M. & NOGUCHI, H. (1989). Heterogeneity of the L-rhamnose residues in the outer core of *Pseudomonas aeruginosa* lipopolysaccharide, characterized by human monoclonal antibodies. *Infection and Immunity* **57**, 1691–1696.
- YOKOTA, S., KAYA, S. & NOGUCHI, H. (1990). Antigenic epitope in *Pseudomonas aeruginosa* lipopolysaccharide immunologically cross reactive with *Escherichia coli* O26 lipopolysaccharide. *FEMS Microbiology Letters* **68**, 245–248.