Production and characterisation of mouse monoclonal antibodies reactive with the lipopolysaccharide core of *Pseudomonas aeruginosa*

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Summary. Monoclonal antibodies (MAbs) to the core antigen region of lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* were produced from mice immunised with whole cells of heat-killed rough mutants of *Pseudomonas aeruginosa* expressing partial or complete core LPS. MAbs were screened in an enzyme-linked immunosorbent assay (ELISA) against three different antigen cocktails: S-form LPS from three *P. aeruginosa* strains, R-form LPS from six *P. aeruginosa* strains and, as a negative control, R-form LPS from *Salmonella typhimurium* and *Escherichia coli*. Selected MAbs were subsequently screened against a range of extracted LPS and whole cells from both reference strains and clinical isolates of *P. aeruginosa*. The antibodies were also screened in ELISA against whole-cell antigens from other *Pseudomonas* spp. as well as strains of *Haemophilus influenzae*, *Neisseria subflava* and *Staphylococcus aureus*. Five MAbs reacting with the core component of *P. aeruginosa* LPS were finally selected. Two of these, MAbs 360.7 and 304.1.4, were particularly reactive in immunoblots against unsubstituted core LPS, including that from O-antigenic serotypes of *P. aeruginosa*. The MAbs also reacted with some of the other *Pseudomonas* spp., but not with *P. cepacia* or *Xanthomonas (Pseudomonas) maltophilia*. Cross-reactivity with whole cells from other bacterial species was minimal or not observed. Reactivity of MAbs with some *Staph. aureus* strains was observed, and binding to the protein A component was implicated. The reactivity of the MAbs was investigated further by flow cytometry and immunogold electronmicroscopy. The suitability of the MAbs for an immunological assay for detection of *P. aeruginosa* in respiratory secretions from CF patients is discussed.

Introduction

Pseudomonas aeruginosa is an opportunist pathogen *par excellence*, responsible for a diverse range of infections in patients compromised by chemotherapy, injury, surgical procedures or underlying disease, and by the inherited disease cystic fibrosis (CF). Pulmonary colonisation of patients with CF by *P. aeruginosa* can be diagnosed by bacteriological culture and detection of pseudomonas-specific antibodies.^{1–3} However, in the early stages of asymptomatic colonisation such techniques may be unhelpful.

There has been increasing interest in the development of immunoassays which can detect infective agents directly in clinical specimens and be adapted to the rapid screening of a large number of specimens.^{4–6} The development of an immunoassay based on a monoclonal antibody (MAb) for the early detection of *P. aeruginosa* antigens in saliva and sputum specimens

from CF patients was considered a potentially useful diagnostic technique.

P. aeruginosa is serologically heterogeneous and contains at least 20 different heat-stable O-antigens.⁷ The aim of the current study was to produce and characterise MAbs specific for *P. aeruginosa* and then assess their suitability for the detection of any of the distinct serotypes which may colonise individual CF patients. *P. aeruginosa* produces several intra- and extra-cellular antigens. Amongst these, lipopolysaccharide (LPS) is highly immunogenic and readily released from bacterial cells.^{8,9} Therefore, the common core region of LPS was considered to be an appropriate antigen for the production of anti-*P. aeruginosa* MAbs for use in an immunoassay to detect *P. aeruginosa* in clinical specimens from CF patients.

Materials and methods

Bacterial strains

P. aeruginosa R-mutants defective in the production

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of LPS (PAC556, PAC557, PAC605, PAC608, PAC609 and PAC611) were obtained from Professor P. M. Meadow, University College, London.¹⁰ Standard serotype strains of *P. aeruginosa* were obtained from Dr R. J. Jones, University of Liverpool. The standard genetic strain *P. aeruginosa* PAO1 was also included in the study. Strains of *P. acidovorans* NCIB 9681, *P. fluorescens* NCIB 10525, *P. mendocina* NCIB 10541, *P. pseudoalkaligenes* NCIB 9946, *P. putida* NCIB 1007, *P. stutzeri* NCIB 9040 and *P. testosteroni* NCIB 8893 were supplied by the National Collection of Industrial Bacteria. *P. cepacia* strains J1685, J1695, J1705, J1715, J1725, J1735, J1765 and J1775 were obtained from the Center for Communicable Disease Control, Atlanta, GA, USA.¹¹ Bacteria denoted by the prefix JN were isolated from sputum of patients with CF and included strains of *P. aeruginosa*, *P. cepacia*, *Xanthomonas (Pseudomonas) maltophilia*, *Haemophilus influenzae*, *H. parainfluenzae*, *Neisseria subflava*, *Moraxella (Branhamella) catarrhalis* and *Staphylococcus aureus*. Enterobacterial strains used as controls included Ra mutants *Escherichia coli* R1 and *Salmonella typhimurium* 1542, and a strain of *Proteus mirabilis*.

Immunisation procedure and production of MAbs

P. aeruginosa was grown in nutrient broth containing yeast extract 0.5% w/v (NYB), washed twice with phosphate-buffered saline (PBS, pH 7.2) and resuspended to a density of 10^8 cells/ml. Five female BALB/c mice were immunised by intravenous (i.v.) injection with 10^7 heat-killed (100 °C for 5 min) cells. Each mouse received 0.1 ml of cells from *P. aeruginosa* PAC608 (full core mutant) on days 1, 2, 3, 8, 9 and 10 followed by cells from *P. aeruginosa* PAC605 (partial core mutant) on days 22, 23 and 24. Blood was taken from the tail vein of each mouse on days 1 (pre-inoculation), 20, 31 and 38, and the antibody response was measured by ELISA (see below). A mouse with serum showing a strong, cross-reactive antibody response to *P. aeruginosa* LPS antigens was selected for fusion. The mouse was given a booster injection of 0.1 ml of heat-killed cells of PAC608 (10^7 cells), i.v., and was killed 3 days later. Mouse spleen cells were fused with cells of the NS-1 plasmacytoma cell line¹² by the method of Oi and Herzenberg.¹³ Hybridomas producing anti-*P. aeruginosa* LPS antibodies were screened by ELISA (see below). Antibody-positive hybrids were cloned by limiting dilution.

Preparation of LPS

Extraction of LPS from bacterial strains expressing the S-form LPS phenotype was based on the aqueous phenol method of Westphal and Luderitz.¹⁴ The aqueous phenol, chloroform, petroleum ether method of Galanos *et al.*,¹⁵ incorporating the diethyl ether precipitation of LPS described by Qureshi *et al.*¹⁶ (as described by Hancock and Poxton¹⁷), was used to

prepare LPS from bacteria expressing the R-form LPS.

Characterisation of MAbs

Isotype determination. The class and subclass of MAbs in culture supernates were determined by a mouse MAb isotyping kit (RPN29: Amersham International plc).

Enzyme-linked immunosorbent assay (ELISA). An ELISA was used (a) to detect the antibody response of mice to vaccination; (b) to screen hybridomas for the production of anti-*P. aeruginosa* antibody; and (c) to test the reactivity of MAbs against other bacterial strains. A primary ELISA screen consisting of three different LPS cocktails was used for an initial assessment of the reactivity of antibodies in sera from mouse-tail samples and in hybridoma supernates. Extracted LPS antigens were complexed with polymyxin as described by Scott and Barclay¹⁸ and antigen cocktails were formed by the addition of equimolar proportions of individual complexes. Cocktail-A consisted of O-antigenic S-form LPS from three serotype strains of *P. aeruginosa* (O1, O2 and O11); cocktail-B consisted of core R-LPS from six LPS R-mutants (PAC556, PAC557, PAC605, PAC608, PAC609 and PAC611); cocktail-C (negative control) consisted of LPS from *S. typhimurium* 1542 and *E. coli* R1. Hybridomas producing a strong antibody response directed to one or both of the *P. aeruginosa* LPS cocktails were selected and the antibodies were subsequently tested in a secondary ELISA consisting of the individual LPS antigens contained in the LPS cocktails of the primary screen. Hybridomas producing antibodies of interest were further screened in ELISA against a comprehensive range of antigens which included additional LPS-polymyxin complexes, individual pseudomonas vaccine antigens PVA-1-16 which comprise the polyvalent pseudomonas extract vaccine (PEV),¹⁹ and whole-cell bacterial antigens. All antigens for ELISA studies were diluted in carbonate-bicarbonate coating buffer (pH 9.6) and added to the wells of polystyrene microplate 8-well strips (Nunc, 'polysorb') at 100 µl/well. LPS-polymyxin complexes were diluted 1 in 50 in coating buffer and vaccine antigens were coated at 1 µg/ml. Bacterial whole cells (grown overnight in NYB and washed twice with PBS) were resuspended to a density of 10^7 cells/ml in coating buffer and added to the microtitration plate wells, and the plates were centrifuged at 1400 g for 5 min to sediment bacteria to the wells. After washing with wash buffer (PBS containing Tween 20 0.05% v/v and sodium azide 0.02% w/v), all plates were post-coated with post-coat buffer (PBS containing bovine serum albumin, BSA, 5% w/v) at 100 µl/well. After being washed four times with wash buffer, plates were stored at -20 °C until use.

Primary antibody (sera from mouse tail samples diluted 1 in 100, or supernate from hybridomas diluted 1 in 5) was added (100 µl) to each well. Antibody diluent was PBS with Tween 20 0.05% v/v, BSA,

Fig. 1. Reactivity in ELISA of anti-LPS MAb against *P. aeruginosa* whole cells of standard serotype strains O1–O16, R-LPS mutants, PAC605–PAC557, the standard genetic strain PAO1 and clinical isolates from patients with CF, J1385–JN62.

0.5% w/v, polyethylene glycol 6000 4% w/v and sodium azide 0.02% w/v. After incubation at 37 °C for 90 min, plates were washed four times with wash buffer. Urease-conjugated anti-mouse Ig (Sera Lab) was diluted 1 in 500 in diluent, and then 100 µl was added to each well. The plates were incubated for a further 90 min at 37 °C. Thereafter they were washed and rinsed, and 100 µl of urease substrate (Sera Lab) was added to each well. Finally, the plates were incubated for 60 min at room temperature and the optical density (OD) of wells was read at 590 nm in a Titertek Multiscan. Results were expressed after subtraction of the corresponding OD of negative control wells (coated only with BSA) for each antibody sample.

Immunoblotting. LPS was prepared by the proteinase K method of Hitchcock and Brown²⁰ and separated on polyacrylamide 14% gels with the buffer system of Laemmli²¹ but without sodium dodecyl sulphate in the stacking and separating gel buffers.

Separated antigens were transferred to nitrocellulose membranes (Schleicher and Schuell, pore size 0.2 µm) by the method of Towbin *et al.*²² and processed by the method of Hancock and Poxton.¹⁷ Antigens were probed with hybridoma culture supernate diluted 1 in 5 for 3 h at room temperature and the immune complexes were detected with anti-mouse Ig horseradish peroxidase conjugate (ICN Biomedicals) and HRP colour reagent (BioRad).

Flow cytometry. The binding of MAb (hybridoma supernates) to whole bacteria was analysed by the flow cytometry method described by Nelson *et al.*²³

Immunogold electronmicroscopy. *P. aeruginosa* strains were grown overnight in NYB, and harvested by centrifugation at 10000 *g* for 10 min. Cells for thin sections and the subsequent immunoassay were prepared as described by Hancock and Poxton.¹⁷ MAb supernates and the anti-mouse IgG immunogold conjugate (10 nm particle size, Sigma) were diluted 1 in 10 in antibody diluent which was the same as for the

Table 1. Cross-reactivity in ELISA of anti-LPS MAbs against bacterial whole cells

Bacterial species tested	Number of strains tested	Percentage antibody binding relative to <i>P. aeruginosa</i> *				
		304.14	360.7	61.32	73.5	334.4
<i>P. aeruginosa</i>	21	100	100	100	100	100
<i>P. mendocina</i>	2	56	89	48	100	44
<i>P. fluorescens</i>	3	78	84	47	69	65
<i>P. pseudoalkaligenes</i>	1	56	66	13	95	87
<i>P. acidovorans</i>	1	83	85	0	35	85
<i>P. putida</i>	4	86	73	64	100	100
<i>P. stutzeri</i>	1	77	87	30	86	78
<i>P. testosteroni</i>	1	0	0	0	93	0
<i>P. cepacia</i>	10	0	0	0	0	0
<i>X. maltophilia</i>	7	0	7	12	0	0
<i>H. influenzae</i>	5	0	0	0	0	0
<i>H. parainfluenzae</i>	2	0	6	5	0	0
<i>N. subflava</i>	2	0	0	0	0	0
<i>M.(B.) catarrhalis</i>	2	0	0	0	0	0
<i>Staph. aureus</i>	4	32	29	33	50	38
<i>E. coli</i> R1	1	0	0	0	0	0
<i>S. typhimurium</i> 1542	1	0	0	0	0	0
<i>Pr. mirabilis</i>	1	0	0	0	0	0

* Percentage antibody binding values represent the mean OD₅₉₀ relative to the mean OD₅₉₀ calculated for *P. aeruginosa*.

ELISA studies. Sections were examined before and after staining with aqueous uranyl acetate, in a Hitachi 12A electronmicroscope at 75 kV.

Protein A assay. The ELISA for detection of protein A was performed as described by Warnes *et al.*²⁴

Results

A total of 530 hybridoma supernates were screened in ELISA; 42 hybridomas produced an ELISA OD of > 0.5 in the initial screening against the *P. aeruginosa* R- and S-LPS cocktails. Antibody-positive hybrids were cloned and five hybridomas that produced specific antibody were characterised further.

Isotype determination

One MAb (73.5) was classified as IgG₁, two MAbs (304.1.4 and 360.7) as IgG_{2b} and two MAbs as IgG₃ (61.32 and 334.4).

Reaction in ELISA

The five MAbs selected for study produced a positive response against all the *P. aeruginosa* whole-cell antigens used in the screening (fig. 1). A strong reaction (ELISA OD > 1.0) against most of the *P. aeruginosa* whole-cell antigens was particularly marked for MAbs 304.1.4 and 360.7. The MAbs also showed reactions with whole cells from other *Pseudomonas* spp. (table I). All five MAbs reacted with the whole cells of *P. fluorescens*, *P. mendocina*, *P. pseudoalkaligenes*, *P. putida* and *P. stutzeri*. All the MAbs except 61.3.2 produced a positive reaction with *P. acidovorans*, but only MAb 73.5 produced a detectable reaction with *P. testosteroni*. MAb 304.1.4 failed to show a detectable reaction with any of the *P.*

cepacia or *X. maltophilia* strains, whereas MAbs 73.5 and 334.4 produced weak reactions with individual *X. maltophilia* strains. MAbs 360.7 and 61.3.2 also failed to produce a positive reaction with any of the *P. cepacia* strains, although weak reactions were obtained with some of the *X. maltophilia* strains. None of the MAbs reacted with whole cells of *H. influenzae*, *H. parainfluenzae*, *E. coli* R1, *S. typhimurium* 1542, *Pr. mirabilis*, *N. subflava* and *M.(B.) catarrhalis*. Positive reactions were observed with *Staph. aureus* strains JN91 and JN92 which were subsequently identified as strong producers (> 25 ng/ml) of protein A.

Confirmation of the cross-reactive nature of the five MAbs with all serotypes of *P. aeruginosa* were also provided by the positive response of each MAb to the cell-wall vaccine antigens (PVA), serotypes O1–O16 (fig. 2). The strongest reactions were again observed with MAbs 304.1.4 and 360.7. The MAbs also reacted with each of the extracted *P. aeruginosa* LPS antigens. In ELISA, the response of the five MAbs to core R-LPS from the PAC mutants tended to be lower towards the shortest core structure PAC605 and the complete core structures PAC557 and PAC608. The antibodies did not react with the enterobacterial R-LPS antigens from *E. coli* R1 and *S. typhimurium* 1542.

Immunoblotting

The immunoblot of MAb 360.7 is shown in fig. 3. A positive reactive band corresponding to the low-M_r core glycolipid of the *P. aeruginosa* LPS preparations was observed. The apparent absence of bands corresponding to the high-M_r LPS of serotype strains indicated that the MAbs were unable to recognise core LPS substituted with O-antigenic side chains. However, a very faint ladder pattern corresponding to the high-M_r LPS of serotypes O4 and O6 (tracks 4 and 6

Fig. 2. Reactivity in ELISA of anti-LPS MAbs against *P. aeruginosa* S-LPS O-serotype vaccine components (PVA-1-16), extracted LPS antigens (LPS complexed with polymyxin) including S-LPS (O1-O11) and R-LPS (PAC605-PAC557), LPS from two *P. aeruginosa* strains (JN61 and JN62) from patients with CF, and LPS from two Enterobacteriaceae strains (negative controls).

respectively) was observed with MAb 360.7. The ladder patterns are barely visible on the photograph shown. The immunoblots obtained with MAbs 61.3.2, 73.5 and 334.4 were all weaker than the immunoblots obtained with MAbs 304.1.4 and 360.7. However, a positive reaction with the core components of most of the different LPS preparations was observed (data not shown).

Flow cytometry on whole bacteria

The binding of MAbs 304.1.4, 360.7 and 334.4 to whole *P. aeruginosa* cells was analysed by flow cytometry. The highest percentage values of bacteria exhibiting positive fluorescence were observed for the LPS R-mutants that expressed core or partial core structures. The MAbs also reacted with *P. aeruginosa* whole cells expressing O-antigen LPS. No reactivity was observed with whole cells of *P. cepacia* or the enterobacterial R-mutants *E. coli* R1 and *S. typhimurium* 1542 (table II).

Immunogold electronmicroscopy

Immunogold electronmicroscopy was also used to assess the binding of MAbs to *P. aeruginosa* cells processed for thin-sections. Fig. 4a and b shows the binding of MAb 360.7 to *P. aeruginosa* PAC611, which expresses only a partial core LPS, and to *P. aeruginosa* serotype O1. There was negligible binding of the MAbs to cells of *P. cepacia* (data not shown).

Discussion

The aim of this investigation was to prepare MAbs suitable for an immunoassay for the detection of non-mucoid strains of *P. aeruginosa*. MAbs recognising *P. aeruginosa* core LPS unsubstituted by O-serotype antigens were isolated and characterised. The MAbs were shown to be cross-reactive with core LPS antigens belonging to a variety of reference and clinical *P. aeruginosa* strains. The pan-reactive core-specific response of the MAbs, particularly MAbs 304.1.4 and

Fig. 3. Immunoblot of proteinase K whole-cell digests of 20 *P. aeruginosa* strains separated by SDS-PAGE followed by transfer to nitrocellulose paper and probed with MAb 360.7 (supernate diluted 1 in 5). The immunoblot profiles against *P. aeruginosa* serotype strains O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, O13, O15, and *P. aeruginosa* LPS R-mutants PAC611, PAC605, PAC609, PAC557 and PAC608 are shown in tracks 1–20 respectively.

Table II. Reactivity in flow cytometry of anti-LPS MABs against whole bacterial cells.

Strain tested	Percentage reactivity* of MAB		
	304.1.4	360.7	334.4
LPS-defective mutants			
<i>P. aeruginosa</i> PAC605	71	80	75
<i>P. aeruginosa</i> PAC611	20	50	18
<i>P. aeruginosa</i> PAC556	40	73	40
<i>P. aeruginosa</i> PAC609	13	64	52
<i>P. aeruginosa</i> PAC608	24	87	60
<i>P. aeruginosa</i> PAC557	21	39	40
Serotype strains			
<i>P. aeruginosa</i> O1	28	31	27
<i>P. aeruginosa</i> O2	30	45	31
<i>P. aeruginosa</i> O6	30	52	28
<i>P. aeruginosa</i> O9	37	51	41
<i>P. aeruginosa</i> O11	32	31	29
Negative controls			
<i>P. cepacia</i> JN93	0	0	0
<i>E. coli</i> R1	0	0	0
<i>S. typhimurium</i> 1542	0	0	0

* Values represent percentage of bacteria exhibiting positive fluorescence above background levels.

360.7, supports the idea of the presence of a common antigenic structure in the core region of *P. aeruginosa* LPS. The inner core region of *P. aeruginosa* LPS is believed to be homogeneous and also common to most gram-negative bacteria, whereas the outer core region is considered to be heterogeneous^{25, 26} and different from that of other gram-negative genera.²⁷ Failure of the MABs to react with LPS from *E. coli* R1 or *S. typhimurium* 1542, both of which express an Ra core LPS chemotype (complete core), or with whole cells of enterobacteria demonstrate that the MABs generated recognise core epitopes in *P. aeruginosa* LPS which are not present in these selected strains of Enterobacteriaceae.

Failure of the MABs to react with the ladder pattern

of core LPS substituted with O-antigen, as shown by the immunoblot analysis, suggested that the epitope recognised was present at the distal end of the unsubstituted core oligosaccharide which became unavailable when O-polysaccharide was linked. The fact that the MABs showed strong reactions in ELISA and flow cytometry with *P. aeruginosa* whole cells expressing S-LPS antigen indicated that there was sufficient unsubstituted core glycolipid to allow binding by the MABs. This may be a reflection of the low capping frequency of *P. aeruginosa* LPS (the percentage of core LPS constituents covered with O-antigen) leaving a large percentage of core determinants exposed at the cell surface.^{25, 27, 28} In contrast, however, this low level of covering with O-polymer

Fig. 4. Electronmicrographs of thin sections from cells of *P. aeruginosa* serotype O1 (a) and *P. aeruginosa* PAC611 (b) prepared by reaction with MAb 360.7, followed by anti-mouse IgG-gold conjugate. Bars, 1 μ m.

was able to inhibit the binding of the rough-core specific MAbs characterised by Sadoff *et al.*²⁹ MAbs 304.1.4, 360.7 and 334.4 produced a reaction in ELISA, immunoblotting and flow cytometry with all the core mutants which indicated binding to a common epitope.

The MAbs investigated in this study were shown to react with various forms of *P. aeruginosa* LPS, including extracted LPS preparations, cell-wall extracts and whole bacterial cells, including those immobilised on microtitration-plate wells and those prepared for flow cytometry.

Cross-reactivity of the MAbs with whole cells representing a number of other *Pseudomonas* spp. indicates that these species contain core LPS similar in composition and structure to *P. aeruginosa*. Alternatively, their LPS may be structurally dissimilar, but serologically cross-reactive with that of *P. aeruginosa*. The absence of a significant response to (formerly *Pseudomonas*) *X. maltophilia* confirms the observations of Neal and Wilkinson³⁰ who reported similarities between the LPS of *P. maltophilia* and that of some *Xanthomonas* spp. The lack of a significant response to *P. cepacia* may also reflect some of the reported differences in the structure of *P. cepacia* LPS.³¹

MAbs against *P. aeruginosa* core LPS and reactive with some²⁶ or all serotypes have been reported previously.³² The MAbs described in this study are LPS-specific, pan-reactive with all *P. aeruginosa* regardless of serotype and, therefore, potentially useful for the detection of any of the serotype strains which

may colonise individual CF patients. Recent studies have focused on the incorporation of the MAbs into a rapid immunological assay for the early diagnosis of asymptomatic colonisation of the CF respiratory tract. False positive reaction caused by the detection of *H. influenzae*, *X. maltophilia*, *P. cepacia*, *Neisseria* spp. and *M.(B.) catarrhalis*, any of which may be found in CF respiratory secretions, are unlikely, given the fact that the MAbs failed to react significantly with whole cells of these organisms. However, the MAbs demonstrated reactivity with strong protein A-producing *Staph. aureus* cells which was probably caused by the interaction of protein A with the Fc portion of antibodies. Since *Staph. aureus* is commonly found in respiratory secretions of patients with CF, any false positive reactions may have to be eliminated from an immunoassay for the detection of *P. aeruginosa* by pre-incubation with an Ig of a different non-cross-reactive species.

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