Characterisation of the external surfaces of *Pseudomonas aeruginosa* isolated from human blood and respiratory tract

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Summary. The surface structures of the cell envelopes of 16 clinical isolates of *Pseudomonas aeruginosa* were examined by electronmicroscopy with the new fixation technique of freeze-substitution. Two types of structures were observed among the organisms. In one group of strains, mostly isolated from blood, a dense fibrous layer c. 30 nm thick was found around the outer-membrane surface, whereas no such structure was observed in the other group of isolates, most of which were from sputum. Lipopolysaccharides extracted from the isolates with a dense fibrous layer were found by SDS-PAGE to have long O-polysaccharide chains, whereas strains without such a layer mostly had lipopolysaccharides that lacked high mol. wt. O-polysaccharide chains.

Introduction

Pseudomonas aeruginosa is a nosocomial pathogen that causes serious infections in compromised patients, especially in those with severe burns or wound injuries and immunological deficiency. In addition, this organism is a major cause of chronic infections in patients with cystic fibrosis.^{1,2} Isolates from patients with cystic fibrosis often produced a mucoid alginate,³ which may act as a barrier to the interaction of the bacteria with serum factors or phagocytes.⁴⁻⁶ In other types of infection it is conceivable that the *pseudomonas* may have protective barriers other than alginate that contribute to its survival. In particular, long O-polysaccharide chains of the lipopolysaccharide (LPS) may act as a shield. In this study we compared the length of the O-polysaccharide chains of LPS of isolates from blood and sputum, by electronmicroscopy and chemical methods.

Materials and methods

Bacterial strains and culture media

Sixteen clinical strains of *P. aeruginosa* isolated at Kyushu University Hospital were used; eight were from the blood of patients with haematopoietic disorders such as leukaemia and malignant lymphoma, and the other eight were from sputum samples of patients with chronic respiratory tract infections (table). NAC Selection Medium (Eiken Kagaku Co., Ltd, Tokyo, Japan) was used for the isolation of the

organisms. MacConkey agar (Eiken) supplemented with glycerol 5% was used for the demonstration of mucoid appearance and Brain Heart Infusion (BHI) broth or agar (Eiken) was used routinely for the maintenance or growth of bacteria.

Serotyping

Bacteria were serotyped according to the typing scheme of the Japanese *Pseudomonas aeruginosa* Society. The agglutination method was used with a set of 14 O-antisera (Denka Seiken Co., Ltd, Tokyo, Japan).

Electronmicroscopy

Thin sections of log-phase bacteria were made by freeze-substitution, as described previously.^{7,8} Sections were stained with uranyl acetate and lead citrate and examined with a JEM 2000EX electronmicroscope at 100 kV.

Immuno-electronmicroscopy

Before freeze-fixation, the bacteria were treated with O-antisera for the specific serotyping of *P. aeruginosa*, then washed with phosphate-buffered saline (PBS). Subsequently, antibodies that had bound to the bacterial LPS were labelled with protein A-colloidal gold by the method of Frens⁹ and Roth *et al.*¹⁰ and processed for electronmicroscopy as described above.

Extraction of LPS

LPS was extracted from the bacteria by the method of Preston and Penner.¹¹ Briefly, cells from a log-phase

Table. Characterisation of *P. aeruginosa* isolates and relationship between LPS pattern and serum sensitivity

Type of LPS*	Serotype†	Strain and source§	Serum sensitivity	Mucoid appearance
Type 1	A (O3)‡	B1	Resistant	—
		B8	Resistant	—
		B11	Resistant	—
		B5	Resistant	—
		B3	Resistant	—
		B7	Resistant	—
		S1	Resistant	+
		S12	Resistant	+
		B2	Resistant	—
		I (O1)	Resistant	—
Type 2	M (Homma 15, 17)	S2	Sensitive	+
		S3	Sensitive	—
Type 3	B (O2, 5, 16) G (O6)	S5	Sensitive	+
		S6	Sensitive	+
		S8	Sensitive	—
	H (O10)	S4	Sensitive	+

* See fig. 5

† Typing scheme of the Japanese *Pseudomonas aeruginosa* Society.

‡ (); International Antigenic Typing Scheme and Homma serotype corresponding to the Japanese Typing Scheme.

§ Isolates with prefix B were from blood, those with prefix S were from sputum.

culture were collected by centrifugation at 1000 *g* for 20 min then washed with BHI broth and resuspended in cold physiological saline to give an absorbance of 0.3 at 660 nm. The bacteria were collected by centrifugation from 3 ml of this suspension and resuspended in 400 μ l of a lytic buffer comprising glycerol 20%, 2-mercaptoethanol 5% and sodium dodecyl sulphate 4.6% in 0.125 M Tris-HCl buffer, pH 6.8, then heated at 100°C for 10 min. Afterwards, the suspension was cooled to room temperature and proteins were digested by adding 40 μ l of proteinase K (Wako Junyaku, Tokyo, Japan) 2.5 mg/ml. The mixture was allowed to stand for 60 min at 60°C. Finally, the enzyme was inactivated by boiling for 5 min and the remaining solution was used as a source of LPS for electrophoresis.

Electrophoresis of LPS

Extracted LPS (12.5 μ l) was subjected to SDS-PAGE on the gel system of Laemmli,¹² then visualised by silver staining with the method of Tsai *et al.*¹³

Immunoblotting of LPS with O-specific antiserum

LPS molecules in the SDS-PAGE gels were transferred to nitrocellulose sheets (0.45 μ m pore size; Schleicher and Schuell Ltd, Dassel, Germany). The transfer buffer, described previously by Towbin *et al.*,¹⁴ comprised 25 mM Tris-HCl, 192 mM glycine and methanol 20% v/v. Electrophoresis was continued for 18 h at 30 V and 0°C. After transfer of the proteins, the nitrocellulose sheets were immersed in PBS containing Tween 20 0.05% (PBS-Tween) for 20 min and then in PBS-Tween containing bovine serum albumin (Wako)

0.05% for 30 min to quench non-specific binding of antibody. The sheet was then immersed for 90 min at room temperature in PBS-Tween containing specific anti-O antisera (Denka) that has been diluted *c.* 50-fold. Afterwards, the sheets were washed with PBS-Tween and the reactions of the antibody with the LPS were visualised by adding peroxidase-conjugated goat anti-rabbit IgG (Cappel, Cooper Biomedical, Inc., Malvern, USA), used at a dilution of 1 in 750, and its substrate 4-chloro-1-naphthol (Wako) and hydrogen peroxide. Details of the method were as described by Preston and Penner.¹¹

Serum bactericidal activity

Serum from the venous blood of 10 healthy volunteers was separated, pooled and stored at -70°C until required.

To test the serum resistance of the bacteria, equal volumes of the pooled normal human sera (PNHS) and log-phase bacteria (1×10^8 cfu/ml) were mixed in PBS. After incubation at 37°C for 60 min, 100- μ l amounts of the mixtures were serially diluted in PBS and 250- μ l amounts of the dilutions were spread on BHI agar for colony counts.

Results

Surface structure of *P. aeruginosa* isolates

Preparations of the 16 isolates were freeze-fixed and examined by electronmicroscopy. All eight organisms isolated from the blood of patients with haematopoietic disorders exhibited a layer of thin fibres on the outer surface of the outer membrane (figs. 1 and 3a).

Fig. 1. Electronmicrograph of a cell of strain B8 (serotype A, LPS Type 1), prepared by the freeze substitution method. Arrows indicate the fibrous layer of the outer membrane around the cell. Bar = 500 nm.

Fig. 2. Electronmicrograph of a cell of strain S3 (serotype M, LPS Type 2) prepared by the freeze-substitution method. Bar = 500 nm.

The thickness of this layer was *c.* 30 nm. A layer of this type was found in only two of the isolates from the sputum of patients with chronic respiratory tract infection and was absent from the remaining six (figs. 2, 3b and 3c).

Immuno-electronmicroscopy with anti-O serum

To ascertain whether the layer detected was the polysaccharide region of LPS or a unique capsular structure, the commercially available anti-O sera to serotypes A (corresponding to O3 of the International Antigenic Typing Scheme,¹⁵ IATS) and M (unlisted in IATS; Homma serotype¹⁶ 15, 17) were used to label the fibrous layer for immuno-electronmicroscopy. Strains B8 and S3 were tested as these were of serotypes A and M, respectively. The reaction sites for the antiserum on the bacterial surface were visualised with a probe of protein A-colloidal gold. As shown in fig.

4a, many colloidal gold particles were found to associate with the fibrous surface layer on the outer membrane of isolate B8, whereas antiserum for serotype M reacted directly on the outer-membrane surface of strain S3 (fig. 4b). These observations suggested that the fibrous structure comprised the O-polysaccharide chains of LPS, and further experiments aimed to identify the length of this material.

Electrophoretic analysis of LPS from clinical isolates

Profiles of the molecular size of the LPS from the 16 clinical isolates of *P. aeruginosa* were analysed by SDS-PAGE (fig. 5). Ten isolates showed extensive ladders of high mol. wt O-polysaccharide material (fig. 5, lanes 1–3), whereas two did not show any ladder pattern (lanes 4 and 5) and seemed to be rough-type organisms. The remaining four isolates showed ladders only in the intermediate molecular size range

Fig. 3. High magnification view of the surfaces of *P. aeruginosa* cells: **a**, strain B11 (serotype B, LPS Type 1); **b**, strain S2 (serotype M, LPS Type 2); **c**, strain S5 (serotype B, LPS Type 3). Arrows indicate the fibrous layer present in strain B11 and absent from the other organisms. Bars = 100 nm.

Fig. 4 Immuno-electronmicroscopy of the outer-membrane surface of: **a**, *P. aeruginosa* B8 (serotype A, LPS Type 1); **b**, *P. aeruginosa* S3 (serotype M, LPS Type 2). Note the gold probes attached to the fibrous layer of strain B8 but directly to the outer-membrane surface of strain S3. Bars = 100 nm.

Fig. 5. Electrophoretic profiles of LPS of strains of *P. aeruginosa*. Lane 1, strain B7, serotype G; 2, strain B8, serotype A; 3, strain B11, serotype B; 4, strain S2, serotype M; 5, strain S3, serotype M; 6, strain S5, serotype B; 7, strain S6, serotype G. HMW-LPS: high mol. wt region of LPS.

Fig. 6. Immunoblotting of electrophoresed LPS with anti-O antisera. Isolates belonging to serotypes A, B and M were selected. Their LPS was electrophoresed and then immunoblotted with anti-A, -B and -M antisera. Lane 1 in each zone was silver-stained and lane 2 was immunoblotted. **A**, strain B8 (serotype A, LPS Type 1); **b**, strain B11 (serotype B, LPS Type 1); **C**, strain S3 (serotype M, LPS Type 2); **D**, strain S5 (serotype B, LPS Type 3).

(lanes 6 and 7). We termed these three electrophoretic patterns Types 1, 2 and 3, respectively. The relationship between LPS pattern and sources of the bacteria is presented in the table. All the isolates from blood belonged to Type 1 and most respiratory tract isolates belonged to Types 2 and 3.

Immunoblotting of ladder patterns of LPS with O-antisera

To confirm that the ladder pattern seen in SDS-PAGE reflected heterogeneity in the size of the O-polysaccharide chains, immunoblots were prepared with anti-O antisera specific to the particular O-serotypes of the isolates (fig. 6). With the Type 1 organisms (fig. 6, panels A and B) the antibodies reacted with ladders in the high mol. wt range. Only rapidly-migrating bands were stained in the LPS from the Type 2 group (panel C) and only the ladder in the intermediate mol. wt range was stained in the LPS from the Type 3 isolates (panel D).

Fig. 7. Serum sensitivity test of the isolates assessed in PNHS, with exposure at 37°C for 60 min. The data are sorted according to the LPS profile of the isolates: □ Type 1; ■ Type 2; ▨ Type 3. Columns represent mean and bars are SD in two experiments.

Serum sensitivity

The relationship between the length of O-polysaccharide chains of the LPS and serum sensitivity was tested for all 16 strains and results are presented in fig. 7 and the table. Type 1 strains were serum-resistant, whereas organisms belonging to the other two Types were serum-sensitive. Conversely, O-serotype was not related to serum sensitivity, as strains of serotype B (IATS O2, O5, O16) and G (IATS O6) were found in both LPS types (table). In serum bactericidal assays in PNHS 25% the survival of Type 1 strains averaged nearly 90% (fig. 7), whereas survival of Type 2 and 3 strains was substantially lower.

Colonial appearance

None of the blood isolates gave mucoid colonies but most of the sputum isolates did yield such colonies (table).

Discussion

In this study the fine structures of the outer surfaces of clinical isolates of *P. aeruginosa* were examined by freeze-substitution and by electrophoresis of LPS. Isolates from blood had a fibrous layer on their surface but this was absent from most respiratory tract isolates. Previous authors have claimed that bacteria isolated from blood or the infection source of purulent

diseases often have capsular surface layers,¹⁷⁻²⁰ and the fibrous structure of our strains superficially appeared much like a capsule. However, the experiments described in this study supported the view that the layer was not a capsule but was composed of long O-polysaccharide chains of the LPS. Evidence for this view came from the observation by immunoelectronmicroscopy that the layer reacted with specific anti O-sera for homologous serotypes, and that the isolates with the fibrous layer gave an extensive ladder pattern in LPS electrophoresis. The size (30 nm) of the O-polysaccharide chains of *P. aeruginosa* observed in this study is consistent with that observed by Lam *et al.*,^{21,22} but greater than that (15 nm) observed for *Klebsiella pneumoniae*.²³

Most of the *P. aeruginosa* strains isolated from sputum produced mucoid material. Unlike the O-polysaccharide chains, the mucoid material is not firmly anchored on the outer membrane and can be removed easily from the bacterial surface by mechanical washing. In respiratory tract infection the mucoid material may cover the bacteria and act as a barrier to serum killing activity. On the other hand, the blood-stream isolates lacked mucoid material and may have been protected by their long O-polysaccharide LPS chains acting as a barrier to host defence factors.

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