Serum IgG and sputum IgA antibody to core lipopolysaccharide antigen from *Pseudomonas cepacia* in patients with cystic fibrosis

J. W. NELSON, S. L. BUTLER, P. H. BROWN*, A. P. GREENING* and J. R. W. GOVAN

Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG and *Respiratory Unit, Western General Hospital, Crewe Road, Edinburgh

Summary. The immunological response of cystic fibrosis (CF) patients to lipopolysaccharide (LPS) antigens of *Pseudomonas cepacia* was investigated. Enzyme-linked immunosorbent assays (ELISA) with either *P. cepacia* whole cells or extracted core LPS from a clinical isolate of *P. cepacia* as antigen were used to measure serum IgG and sputum IgA anti-*P. cepacia* antibodies. The ELISA with core LPS distinguished nine CF patients colonised by *P. cepacia* from nine age- and sex-matched non-colonised CF patients. The rate of increase of anti-*P. cepacia* IgG antibodies after bacteriologically proven *P. cepacia* colonisation varied in individual patients: in some patients the first isolation of *P. cepacia* was preceded or accompanied by a two-to-four-fold rise in anti-*P. cepacia* LPS IgG titres. Absorption studies and immunoblot analysis of serum from patients colonised with *P. cepacia* demonstrated that a significant component of the anti-*P. cepacia* core LPS antibodies was specific for *P. cepacia* and did not react with the core LPS of *P. aeruginosa*. Immunoblotting also illustrated that there may be a degree of core heterogeneity between different isolates of *P. cepacia*. Detection of *P. cepacia* LPS specific antibodies in serum (IgG) and sputum (IgA) from CF patients is recommended to assist the identification of *P. cepacia* colonisation in CF patients.

Introduction

*Pseudomonas cepacia* is a major pulmonary pathogen in patients with cystic fibrosis (CF). Originally considered a phytopathogen, the organism has been isolated with increasing frequency from patients with CF and is also recognised as an important aetiological agent in nosocomial infection. The possession of innate and inducible resistance to many anti-pseudomonal antibiotics and the ability to survive under conditions of minimal nutrition or in the presence of certain disinfectants contribute to the role of *P. cepacia* as a formidable nosocomial pathogen. Prevention and treatment of pulmonary infections due to *P. cepacia* present a major challenge. The association of *P. cepacia* with the CF lung is complex, and clinical sequelae include rapidly fatal deterioration of pulmonary function, long term colonisation accompanied by a slow decline in lung function, and chronic asymptomatic carriage.

Although the humoral response of CF patients to *P. aeruginosa* antigens has been investigated extensively, there are relatively few reports defining the response to *P. cepacia* antigens. The aim of this study was to investigate the antibody response of CF patients to *P. cepacia* whole-cell and extracted lipopolysaccharide (LPS) antigens. The extent to which antibodies cross-react with *P. cepacia* and *P. aeruginosa* core LPS was also analysed.

Materials and methods

Patients

Nine patients (five female and four male, mean age 21.6 years, range 16-27 years) attending the Edinburgh adult CF clinic, were identified as being persistently colonised by *P. cepacia* (PC+) by serial bacteriological sputum cultures (at least three consecutive samples positive). For each patient an age- and sex-matched CF control was selected from whom *P. cepacia* had never been isolated (PC-).

Serum and sputum samples

Serum samples were obtained from CF patients and from healthy blood donors at the Blood Transfusion Centre, Edinburgh; all were stored at -20°C. Sputum used for antibody analysis was centrifuged at 10000 g for 15 min and the supernate was stored at -70°C. Control sputum samples obtained from patients with chronic bronchitis were processed as for CF samples. Whenever possible, sputum and serum were obtained

Fig. 1. Serum IgG anti-*P. cepacia* core R-LPS antibodies in (A) nine CF patients persistently colonised with *P. cepacia* (titre range 3200–51200), (B) nine non-colonised CF patients (titre range 200–3200) and (C) nine healthy controls (titre range 200–800) measured by ELISA with core R-LPS as coating antigen; bars represent median values. Antibody titres for the PC+ CF patients were from serum samples obtained after colonisation with *P. cepacia* was confirmed by bacteriological culture.

from CF patients at the same time. Specimens from PC+ and PC− CF patients, including those with and without *P. aeruginosa* colonisation, were subjected to detailed immunological analysis; these patients were designated I–IX (see Results).

**Bacteriological analysis of sputum**

Bacteriological examination was performed on sputum which had been homogenised in sputalysin (Calbiochem, La Jolla, CA, USA). After appropriate dilutions, sputum was cultured quantitatively on blood agar, horse digest agar, *Pseudomonas* Isolation Agar (Difco), and *P. cepacia* Selective Medium (Mast Laboratories, Bootle). Bacteria cultured on *P. cepacia* selective medium were identified biochemically by the API 20 NE system (API System, La Balme les Grottes, France).

**Bacterial strains**

*P. cepacia* reference strains J1780 (serotype 1), J1680 (serotype 2), J1705 (serotype 3), J1774 (serotype 4), J1479 (serotype 5), J1772 (serotype 6), J1690 (serotype 7), J1687 (serotype 8), J1758 (serotype 9) and J1745 (non-tybable) were obtained from the Centers for Communicable Disease Control (Atlanta, GA, USA). The serotype was based on the serotyping scheme proposed by Heidt *et al.*[^15^] *P. cepacia* strains J1359 (non-tybable), SBC21 (non-tybable), SBC42 and SBC29 were isolated from the sputum of CF patients. *P. cepacia* SBC8 was an environmental strain isolated from a plant. Serotyping sera were unavailable for the typing of strains SBC42, SBC29 and SBC8, but these were confirmed as distinct strains by ribotyping[^14^]. *P. aeruginosa* R-mutant PAC608, defective in the production of LPS, was obtained from Professor P. M. Meadow (University College, London).

**Preparation of LPS**

The aqueous phenol, chloroform, petroleum ether method of Galanos *et al.*[^17^] incorporating the diethyl ether precipitation of LPS described by Qureshi *et al.*[^19^] (as described by Hancock and Poxton[^18^]) was used to prepare LPS from *P. cepacia* strains expressing R-form LPS. Briefly, LPS was extracted from washed, freeze-dried bacteria from an overnight culture, washed and purified by centrifugation at 100000 g for 4 h and freeze-dried. The proteinase K digestion method[^10^] was also used to prepare LPS for analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

**SDS-PAGE and immunoblotting**

LPS was separated on polyacrylamide 14% gels with the buffer system of Laemmli[^21^] (except that
Fig. 2. Sputum IgA anti-\textit{P. cepacia} core R-LPS antibodies in (A) nine CF patients persistently colonised with \textit{P. cepacia} (titre range 200–6400), (B) nine non-colonised CF patients (titre range 25–100) and (C) nine chronic bronchitics (titre range 25–400) measured by ELISA with core R-LPS as coating antigen; bars represent median values. Antibody titres for the PC + CF patients were from sputum samples obtained after colonisation with \textit{P. cepacia} was confirmed by bacteriological culture.

Enzyme-linked immunosorbent assay (ELISA)

Antigens used for coating microtitre plates were: (a) \textit{P. cepacia} whole cells, serotypes 1–9 and a non-typable strain; and (b) extracted LPS from a rough isolate, \textit{P. cepacia} J1359. For the whole-cell assay, bacteria were grown overnight in nutrient broth containing yeast extract 0-5% w/v (NYB), harvested and washed twice with PBS. Cells were resuspended to a density of $10^7$ cells/ml in carbonate-bicarbonate coating buffer (pH 9.6) and 100 µl was added to the wells of polystyrene “Polysorb” microtitration plates (Nunc, Roskilde, Denmark). Plates were centrifuged at 1365 g for 5 min to sediment bacteria on to the wells. In the case of the LPS antigen, extracted LPS was complexed with polymyxin as described by Scott and Barclay\textsuperscript{24} and used at a final concentration of 10 ng/ml. LPS-polymyxin complexes were diluted in coating buffer and added to microtitration plates at 100 µl/well. All plates were coated overnight at room temperature and washed four times with wash buffer (PBS, pH 7-2, containing Tween 20 0-05% w/v and sodium azide 0-02% w/v). All plates were then post-coated with post-coat buffer consisting of 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 $-20^\circ$C until used.

Serum and sputum samples were serially diluted in dilution buffer and added to coated microtitration plates at 100 µl/well in triplicate. Antibody diluent consisted of PBS (pH 7-2) containing Tween 20 0-05% v/v, BSA 0-5% w/v, polyethylene glycol 6000 4% w/v and sodium azide 0-002% w/v. After incubation at 37°C for 90 min, plates were washed four times with wash buffer. Conjugates, including alkaline phosphatase conjugated to anti-human IgG or anti-human IgA (Sigma) were diluted 1 in 1000 in dilution buffer and added at 100 µl/well. Plates were incubated for a further 90 min at 37°C and then washed and rinsed before addition of alkaline phosphatase substrate (Sigma) at 100 µl/well. After incubation for 30 min at room temperature, the optical density (OD) of wells was read at 405 nm in a Titertek Multiscan plate reader (Flow Laboratories Ltd, Irvine). Final results were expressed as the titre giving an OD > 0-1 after
Fig. 3. Longitudinal study of serum IgG anti-\textit{P. cepacia} LPS antibodies in eight CF patients measured by ELISA with core R-LPS as coating antigen. Patients I (a, ---) III (a, ---) IV (a, --) and V (b, ---) were colonised by \textit{P. cepacia} only; patients II (a, +), VI (b, +) and VII (b, +) were colonised by both \textit{P. cepacia} and \textit{P. aeruginosa}; patient VIII (b, ---) was colonised by \textit{P. aeruginosa} only. The arrows indicate the time when \textit{P. cepacia} was first isolated from sputum from each patient.

subtraction of the OD of negative controls (wells coated only with post-coat buffer) for each sample. Analytical variation of the ELISA, including intra-plate and day-to-day (inter-plate) variations, was performed with six serum samples (three determinations for each), two each with low, medium and high ELISA titres, and the coefficient of variation was calculated. The intra- and inter-plate variations of the ELISA were 5.5\% and 13.9\%, respectively.

Absorption studies

Serum from CF patients was serially absorbed with whole cells of \textit{P. cepacia} J1359 and \textit{P. aeruginosa} PAC608. Overnight cultures of bacteria grown in NYB were harvested by centrifugation, washed twice in PBS and resuspended to a density of $10^8$ cells/ml. Bacterial suspensions (1 ml) were placed in Eppendorf tubes and cells were harvested by centrifugation with a microcentrifuge. Bacteria were resuspended in serum diluted 1 in 200 in dilution buffer, incubated for 15 min at room temperature and re-centrifuged. The supernatant was then added to another pellet of cells and the process was repeated. This step was repeated three times for each absorbing bacterial strain. Serum anti-\textit{P. cepacia} and anti-\textit{P. aeruginosa} core LPS antibodies were assayed by ELISA and immunoblotting as described above.

Statistical analysis

Data were not normally distributed and statistical analysis was by Wilcoxon signed rank tests.

Results

Whole cell ELISA

IgG antibodies directed to \textit{P. cepacia} whole-cell antigens were detected in serum from both PC+ and PC− CF patients. A response to each of the serotype strains was evident although the difference in antibody titres between PC+ and PC− patients was significant.
Fig. 4. Immunoblots of *P. cepacia* LPS antigens with serum from two CF patients colonised with *P. cepacia* and analysed for IgG anti-LPS antibodies; (a) serum from patient III, (b) serum from patient VII. The *P. cepacia* LPS antigens preparations were: lane 1, J1780 (serotype 1); 2, J1680 (serotype 2); 3, J1774 (serotype 4); 4, J1690 (serotype 7); 5, J1758 (serotype 9); 6, J1359 (non-typable); 7, SBC21 (non-typable); 8, SBC42; 9, SBC29; 10, SBC8. (c) Silver stained PAGE of isolated core LPS from *P. cepacia* J1359.

Fig. 5. Immunoblot of *P. cepacia* LPS antigens with sputum from CF patient III colonised with *P. cepacia* and analysed for IgA anti-LPS antibodies. *P. cepacia* LPS antigens were as in fig. 4.
only in the case of serotype 5 and the non-typable whole cells (p < 0.05).

Core R-LPS ELISA

Serum IgG and sputum IgA antibodies. The ELISA employing extracted LPS from an isolate expressing core R-LPS was better than the whole-cell ELISA in discriminating between PC+ and PC− patients (fig. 1). In nine CF patients persistently sputum-positive for *P. cepacia*, IgG titres were significantly higher (median 12800, range 3200–51200) than in nine age- and sex-matched CF PC− controls (median 400, range 200–3200) (p < 0.01 Wilcoxon signed rank test), and in nine healthy blood donors (median 400, range 200–800). There was no significant difference in the IgG titres between the PC− CF patients and the healthy blood donors. Seven of nine PC− CF controls were colonised by *P. aeruginosa* and six of nine PC+ CF patients were colonised by *P. aeruginosa*.

The nine PC+ CF patients also had significantly higher titres of sputum IgA antibodies reactive with core LPS of *P. cepacia* (median 1600, range 200–6400) than did the nine PC− patients (median 50, range 25–100) and nine chronic bronchitics (median 50, range 25–400) (p < 0.01 Wilcoxon signed rank test); there was no significant difference between the titres of the non-colonised CF patients and the chronic bronchitics (fig. 2). None of the chronic bronchitic patients was colonised by *P. cepacia*.

Longitudinal analysis of serum IgG and sputum IgA. Longitudinal analysis of CF serum samples indicated that the rate of increase in the levels of anti-*P. cepacia* IgG antibodies after bacteriological diagnosis of *P. cepacia* colonisation varied from patient to patient; in some cases (e.g., patients III, IV, VI and VII) the first isolation of *P. cepacia* was preceded or accompanied by a two-to-four-fold rise in anti-*P. cepacia* R-LPS IgG titre (fig. 3).

Longitudinal analysis of sputum IgA titres from five CF patients, four of whom were colonised by *P. cepacia*, demonstrated the variable IgA antibody
response to core R-LPS of \textit{P. cepacia}. During a 10-week study period, the sputum IgA antibody titre ranged from 400 to 6400 for two of the PC+ CF patients, and from 800 to 3200 for a third PC+ patient. In a fourth PC+ patient, the IgA antibody titre was maintained at 100–200, which was similar to that of the PC− patient included in the study. There was no correlation between the sputum IgA antibody titre and the number of \textit{P. cepacia} isolated by bacteriological culture from sputum, but there was a tendency for IgA levels to rise during hospitalisation for pulmonary exacerbations.

\textbf{Immunoblot analyses}

Isolated core LPS from \textit{P. cepacia} J1359 is shown in the silver-stained PAGE gel (fig. 4c). Reactivity of serum from two PC+ CF patients against \textit{P. cepacia} LPS antigens is shown in the Western blots (fig. 4a and b). In both cases, a positive reactive band corresponding to the low mol. wt core LPS of most but not all of the \textit{P. cepacia} isolates was observed: reactivity with nine and six of the 10 core LPS antigens was observed with serum from patients III and VII, respectively (fig. 4a and b). In addition, a response corresponding to higher mol. wt O-antigen subunits of LPS from strains SBC42, SBC29 and SBC8 was noted. The serum used for the preparation shown in fig. 4a was obtained from a CF patient colonised with strain J1359 (ribotype A); the serum for the preparation shown in fig. 4b was obtained from a CF patient colonised with strains J1359 (ribotype A) and SBC42 (ribotype C). Immunoblot analysis of serum from two PC− CF patients, one of whom was colonised with \textit{P. aeruginosa}, did not produce a visible response to any of the \textit{P. cepacia} LPS antigens used.

Sputum from the same PC+ CF patient whose serum was used for fig. 4a was used for immunoblot
analysis of IgA antibodies reactive with *P. cepacia* LPS antigens (fig. 5). A strong response to the core LPS of strain J1359 and a weak response to the O-antigen subunits of strain SBC8 was observed.

**Absorption studies**

Serum from CF patients colonised with *P. cepacia* or *P. aeruginosa*, or both or neither, was absorbed with whole cells from *P. cepacia* strain J1359 and *P. aeruginosa* strain PAC608 and analysed by ELISA (fig. 6) and immunoblotting (fig. 7). With serum from PC+ CF (patients I–VII, fig. 6) antibodies reactive with *P. cepacia* core LPS were substantially removed after absorption with the corresponding *P. cepacia* whole cells, but not after absorption with *P. aeruginosa* whole cells. Serum from patients colonised with *P. aeruginosa* (patients II, VI, VII and VIII, fig. 6) had a relatively high antibody titre to *P. aeruginosa* core LPS; these antibodies were removed by absorption with *P. aeruginosa* whole cells but not by absorption with *P. cepacia* whole cells. These absorption studies demonstrated that a significant component of the anti-*P. cepacia* core LPS antibodies was specific for *P. cepacia* and not reactive with core LPS from *P. aeruginosa*; e.g., patient I, colonised by *P. cepacia* but not by *P. aeruginosa*, had a serum IgG titre of 51,200 against *P. cepacia* core LPS and a titre of 200 against *P. aeruginosa* core LPS. The antibodies against *P. cepacia* core LPS were removed by absorption with *P. aeruginosa* whole cells as shown by ELISA (fig. 6) and immunoblotting (fig. 7). Conversely, serum from a CF patient colonised by *P. aeruginosa* but not by *P. cepacia* (patient VIII) reacted with *P. aeruginosa* core LPS but not with *P. cepacia* core LPS.

**Discussion**

Antibodies reactive with *P. cepacia* whole cells and extracted core R-LPS were demonstrated in serum and sputum from patients with CF. A core LPS preparation was used as a coating antigen because (a) LPS represents a more defined antigenic preparation than a system based on whole cells; (b) available evidence suggests antigenic cross-reactivity between some outer-membrane protein antigens of *P. cepacia* and *P. aeruginosa*; (c) core LPS is believed to be a relatively conserved component of LPS and, therefore, provides a suitable antigen for the detection of an antibody response against all serotypes of *P. cepacia*; and (d) many strains of *P. cepacia* isolated from CF patients produce rough LPS (i.e., lacking O-specific side-chains) (S. Butler, unpublished observations).

The ELISA system based on core R-LPS from a CF clinical isolate of *P. cepacia* clearly differentiated between serum samples from PC+ and PC− CF patients. However, figs. 1 and 2 showed that a range of serum IgG and sputum IgA antibody titres were obtained with serum from both PC+ and PC− CF patients and it is possible that overlap between the two groups could occur. Thus, detection of a rising antibody titre between two or more serum samples would appear desirable. Indeed, in the longitudinal studies a rise in the level of anti-*P. cepacia* IgG antibodies was demonstrated in some patients before or accompanying the first isolation of *P. cepacia*. An ELISA based on core R-LPS from *P. aeruginosa* has also been reported to be useful for the diagnosis of chronic *P. aeruginosa* infection in CF.  

Measurement of anti-*P. cepacia* antibodies to identify *P. cepacia* colonisation may prove useful. Although the value of selective media for *P. cepacia* is well recognised, problems may still be encountered, including growth of *Xanthomonas malophilia* and *P. acidovorans*, and the fact that some clinical isolates of *P. cepacia*, on initial isolation from sputum, may take up to 7 days to grow. In view of the considerable concern relating to pulmonary colonisation of CF patients by *P. cepacia*, the ability to identify *P. cepacia* colonisation early and accurately is important, especially in clinics where segregation of colonised and non-colonised patients is practised.

Recent studies have demonstrated the presence of IgG antibodies to outer-membrane antigens of *P. cepacia* in serum from CF patients colonised with *P. cepacia* or *P. aeruginosa* or both. These authors concluded that some *P. cepacia* outer-membrane components may be related antigenically to those of *P. aeruginosa*. Similarly, in our study, serum antibodies reactive with *P. cepacia* whole cells were found in CF patients colonised with *P. cepacia* or *P. aeruginosa* or both. In terms of LPS antigens however, the Western blotting and absorption studies demonstrated that a significant proportion of the antibodies that reacted with core LPS of *P. cepacia* did not react with core LPS of *P. aeruginosa*. These observations indicate differences in the structure and composition of core LPS between *P. aeruginosa* and *P. cepacia*, confirming previous findings, including structural differences (e.g., the lack of phosphorus in the core LPS of *P. cepacia* and the inability of a monoclonal antibody reactive with *P. aeruginosa* core LPS to react with *P. cepacia*). Furthermore, there is unlikely to be cross-reactivity with core LPS of *X. malophilia* because of the structural differences reported by Neal and Wilkinson. There may be some core heterogeneity between different isolates of *P. cepacia* because Western blotting demonstrated that serum from patients colonised with *P. cepacia* produced a band reactive with some but not all *P. cepacia* core LPS preparations.

Although anti-core IgA antibodies were demonstrated in sputum from chronically colonised patients, the levels of these antibodies may have been underestimated because of the possibility of immune-complex formation and fragmentation of antibody by elastases derived from neutrophils or *P. aeruginosa*. Our data suggest that, in CF patients colonised by
References


