Specific IgG2 Antibodies to *Pseudomonas aeruginosa* Lipid A and Lipopolysaccharide Are Early Markers of Chronic Infection in Patients with Cystic Fibrosis

**Summary:** The IgG subclass antibody response to the two parts of *Pseudomonas aeruginosa* lipopolysaccharide; endotoxic lipid A and the O-polysaccharide, were investigated in a retrospective longitudinal study involving 16 patients with cystic fibrosis and chronic *P. aeruginosa* lung infection. The purpose of the study was to see if any of the IgG subclasses of either specificity could be used as prognostic markers in the development and subsequent course of the lung disease. IgG2 anti-lipid A, IgG3 anti-lipid A, and IgG2 anti-polysaccharide showed a significant positive correlation with deteriorating pulmonary function already before chronic *P. aeruginosa* lung infection was diagnosed as well as in subsequent years. The findings suggest antigenic exposure of the patient before chronic infection is detected by routine sputum examinations, and further support our previous findings of a critical role of the IgG subclass response in modulating the course of inflammatory lung damage in these patients.


**Introduction**

Chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis (CF) runs a variable course in individual patients. Some patients show only slight progression in pulmonary damage during many years of chronic infection, while others display a progressive inflammatory lung destruction. The deterioration of pulmonary function has been related to high levels of antibodies to *P. aeruginosa* antigens [1]. The distribution of IgG antibodies also plays a role and a predominant IgG2 and IgG3 response to *P. aeruginosa* antigens is associated with poor prognosis [2]. In these patients *P. aeruginosa* lipopolysaccharide (LPS) has been shown to be a major antibody inducing antigen [3–5], and LPS has also been identified as an antigen in immune complexes isolated from sputum from CF patients [6].

The O-polysaccharide of the LPS on the bacterial cell surface is not toxic by itself, but it renders the toxic lipid part of the molecule soluble, thereby facilitating its biological interaction with host cells. The IgG anti-LPS antibodies have been shown to be of all four IgG subclasses [4, 7], whereas little is known about the IgG subclass pattern of the IgG anti-lipid A antibodies in CF patients. However, the extensive IgG anti-LPS response measured in CF patients seems not only to fail in eradicating the bacteria and the LPS, but may mediate inflammatory pulmonary destruction. Since IgG subclasses vary in their ability to bind to Fcγ-receptors on leukocytes, to induce release of free oxygen radicals, to promote opsono-phagocytosis, and to induce complement activation, individual differences in the IgG subclass antibody pattern has been suggested to account for differences in the course of the infection [2, 7].

An early prognostic marker of the severity of lung disease in CF patients is needed as a guide for the intensity of treatment of the individual patient, e.g. with antibiotics, steroid hormones, or maybe even pulmonary transplantation. In this study we have (i) followed the IgG subclass antibody response to the two parts of *P. aeruginosa* LPS, endotoxic lipid A and the O-polysaccharide, during the course of chronic infection in 16 CF patients, and (ii) inves-

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tigated if a specific IgG subclass pattern could be used as predictor for development of inflammatory destruction of the lungs in CF patients.

Patients and Methods

Patients and healthy controls: The diagnosis of CF was established on the basis of abnormal sweat electrolytes and clinical features. All patients were monitored by monthly visits, including sputum bacteriology, at the Danish CF Center in Copenhagen. Sixteen patients with chronic P. aeruginosa infection were selected at random for inclusion in the study. The mean age at the beginning of the study was nine years (range, 1 to 15 years) and at the end 17 years (range, 10 to 24 years). Multiple serum samples from the 16 CF patients were obtained at intervals of 6 months to 1 year. The samples covered the preinfection period (CF -P) and the early (1–4 years of infection, CF +P) and late phases of chronic P. aeruginosa infection (> 6 years of infection, CF +P). Six serum samples from each patient were assayed covering a period of 7–15 years. The pulmonary function of the patient was evaluated at each monthly visit by measuring forced vital capacity (FVC) and forced expiratory volume in 1 sec (FEV1) with an electronic spirometer (Spirotron; Dräger). All values were expressed as percentage of expected values according to height and sex [8].

Fourteen patients with chronic P. aeruginosa infection but without CF (non CF +P) [10 patients with paraplegia and chronic or intermittent P. aeruginosa urinary tract infection, three patients with primary ciliary dyskinesia and chronic P. aeruginosa lung infection, and one patient with P. aeruginosa osteomyelitis]), ten healthy children and ten healthy adults were included in the study as controls.

ELISA for IgG1, 2, 3 and 4 anti-lipid A, and anti-polysaccharide antibodies: We have recently described the antigens and coating procedure for the antigen specific ELISAs [5, 7, 9]. Smooth-form LPS was extracted by the hot phenol-water method [10] from P. aeruginosa 1118 Habs O group 3 (O:3) isolated from the sputum of a CF patient with chronic pulmonary infection. The O:3 strain was chosen because all patients included in this study had been infected with strains reacting with anti-O:3 typing sera. Also, O:3 is the most common serotype among CF patients in Denmark [11], and the sera of all CF patients examined so far have reacted with the P. aeruginosa 1118 O:3 LPS. Free lipid A was prepared from P. aeruginosa 18S rough-form LPS by acetic hydrolysis as described previously [12] and used for coating of ELISA plates. When using the smooth-form LPS as coating antigen in the ELISA, absorption experiments indicated that the main antigenic determinant exposed was located in the O chain of the molecule [5]. Serial dilutions of a pool of sera from nine CF-patients with chronic P. aeruginosa lung infection for more than 5 years were included to create a standard curve for each plate. Peroxidase-conjugated monoclonal antibodies to human IgG1, IgG2, IgG3, and IgG4 (Janssen Biochimica, Belgium) were used as second antibodies at dilutions of 1:10,000 (anti-IgG1), 1:100 (anti-IgG2), 1:1,000 (anti-IgG3), and 1:100 (anti-IgG4) for the anti-lipid A antibodies respectively and at dilutions of 1:5,000 (anti-IgG1), 1:1,000 (anti-IgG2), 1:1,000 (anti-IgG3), and 1:500 (anti-IgG4) in the anti-polysaccharide ELISAs. Incubation volumes were 100 µl per well, incubation time 1 h on a shaker at room temperature, and the incubation steps were separated by four washings in 0.15 M NaCl-0.1% Tween-20. Enzyme substrate (14 mg of o-phenylenediamine in 18 ml of distilled water plus 7.5 µl of hydrogen peroxide) 30% (vol/vol) was added (100 µl per well), and the enzyme reaction was stopped after 30 min by the addition of 100 µl of 2.5 M sulfuric acid per well. The optical density of the wells was measured at 492 nm with a microtest plate photometer (2550 enzyme immunoassay reader; Bio-Rad Laboratories). Anti-lipid A and anti-polysaccharide titres are expressed as the reciprocal dilution of serum corresponding to an optical density of 0.500 at 492 nm, which represents the steepest slope of the dilution curve [4].

Immune complex formation and antibody detection: Immune complexes were made in vitro using whole serum from the CF patients as the antibody source and P. aeruginosa O:3 LPS as the antigen according to reference [13]. To investigate which IgG subclasses were involved in the immune complex reaction and whether the IgG subclass pattern changed during the course of the infection, three serum samples from each patient, representing the pre-infection period and the early and late phases of the infection were examined. Sera form healthy controls were also used for immune complex formation experiments. The IgG subclasses involved in the immune complex formation were determined using a dot blot assay as previously described [14]. The intensity of the colour reaction in the dot blot was scored as: 0 = no reaction, 1 = visible reaction, and 2 = strong reaction.

Statistics: The Mann-Whitney U test for nonparametric unpaired data was used for comparison of ELISA titres for the different groups of patients and healthy controls investigated. For correlation of pulmonary function and ELISA titres we used Spearman rank sum correlation test. The Fourfold Table Test was used for comparison of IgG subclasses in immune complex formation between the different groups of patients. The level of significance was 5% (two-tailed).

Results

Table 1 shows the level of IgG1–4 response to P. aeruginosa lipid A and polysaccharide LPS in sera from healthy controls, in serum from patients with chronic P. aeruginosa infection but without CF, and in serum from patients with CF before and during chronic infection with P. aeruginosa. All healthy controls had measurable anti-P. aeruginosa lipid A antibodies involving all four IgG subclasses, and most also to polysaccharide. There was a significantly higher level of IgG2 anti-lipid A antibodies in the healthy adults compared to the healthy children. This was also the case for anti-polysaccharide antibodies. In contrast, there was no significant difference between healthy children and adults in the levels of specific IgG1, IgG3, and IgG4 anti-lipid A, and anti-polysaccharide antibodies.

Chronically infected patients without CF had a significantly higher level of IgG1 anti-lipid A compared to healthy controls (Table 1). In contrast, there was a significantly higher level of IgG2, IgG3, and IgG4 anti-polysaccharide antibodies in the group of infected non-CF patients as compared to healthy controls, whereas the higher level of IgG1 anti-polysaccharide antibodies in the patients was not significant.

Already before the onset of chronic infection the group of CF patients had a significantly higher level of IgG2- and IgG4 anti-lipid A antibodies compared to healthy children (Table 1). This was in contrast to the almost identical levels of IgG1–4 anti-polysaccharide antibodies in the two groups.