Longitudinal Study of Immune Response to *Pseudomonas aeruginosa* Antigens in Cystic Fibrosis

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Received 18 April 1983/Accepted 18 July 1983

During a 10-year period, the clinical states of 10 cystic fibrosis patients were evaluated on the basis of monthly measurement of lung function and weight; serum antibody titers to alkaline protease and elastase and the number of precipitins to *Pseudomonas aeruginosa* standard antigen were determined by radioimmunoassay and crossed immunoelectrophoresis. Alkaline protease and elastase concentrations of the *P. aeruginosa* strains from the patients were measured in vitro. The immune response increased in nearly all patients after the onset of chronic *P. aeruginosa* lung infection over years, suggesting unimpaired production of these antigens during *P. aeruginosa* lung infection, whereas the clinical states declined. The mean time for immune response was 15 months for alkaline protease, 11 months for elastase, and 6 months for standard antigen.

Several studies on sera of cystic fibrosis patients suffering from chronic *Pseudomonas aeruginosa* lung infections demonstrated the occurrence of specific humoral antibodies to the extracellular enzymes alkaline protease (AP) (10), elastase (Ela) (10, 20), exotoxin A (20), and other unidentified antigens of this pathogen (3, 4, 7, 17, 27) in the majority of studied patients. As suggested recently (10), antibodies to AP and Ela seem to protect cystic fibrosis patients from the direct pathogenic action of these enzymes, as demonstrated in several in vitro and in vivo studies (8, 13, 19, 34), since AP and Ela were only detected in bronchial secretions of these patients when antibodies to the enzymes were lacking. Thus, the question arises of how much time is afforded after onset of chronic *P. aeruginosa* lung infection in cystic fibrosis for antibody production and which time course antibody titers take. The purpose of the present study was to measure humoral antibody titers to AP and Ela and to determine the number of different precipitins to the standard antigen (St-Ag) of *P. aeruginosa* in a longitudinal study. The results were compared with the clinical states of the patients, since high antibody titers to *P. aeruginosa* antigens were correlated to a poorer clinical state of the patients (3, 7, 17, 20, 26).

**MATERIALS AND METHODS**

Subjects and sera. Multiple sera from 10 cystic fibrosis patients, 2 females and 8 males, aged 8 to 29 years, were obtained during the years 1972 to 1982 and were stored at −20°C. Informed consent was obtained from all patients or their parents. All patients were seen at monthly visits in the CF Clinic TG, Rigshospitalet, Copenhagen, Denmark. Diagnosis of cystic fibrosis was based on accepted criteria (33), including a typical history of cystic fibrosis with marked elevated sweat electrolyte levels in repeated tests and altered pulmonary function. During the chronic *P. aeruginosa* infection, the patients were treated with at least four 2-week courses of chemotherapy in the clinic each year. The additional therapeutic regime has been published previously (17).

*P. aeruginosa*. Sputa of cystic fibrosis patients were examined at the monthly visits in the CF Clinic TG during the years 1972 to 1982 for occurrence of *P. aeruginosa*. The beginning of chronic *P. aeruginosa* lung infection was defined as the point in time from which *P. aeruginosa* was found in every sputum sample for at least 6 months (16). At the beginning of chronic *P. aeruginosa* lung infection, the patients harbored mucoid or nonmucoid strains. For determination of AP and Ela of strains from patients, one colony of a pure blood agar culture was grown in Trypticase soy broth (BBL Microbiology Systems) as described previously (9). Bacterial growth was monitored by measuring the optical density at 578 nm. All strains had reached the stationary growth phase after 24 h and revealed optical densities between 7 and 9. After culture centrifugation, the concentration of AP and Ela in the culture supernatant fluids was determined by radioimmunoassay as described previously (9, 28).

Antibodies to proteases and to the St-Ag of *P. aeruginosa*. Antibodies to AP and Ela in sera of cystic fibrosis patients were detected by radioimmunoassay as described previously (10). Briefly, the solid phase of flexible microtiter plates was covered with purified...
immunoglobulin G antibodies to AP or Ela, saturated with bovine serum albumin, and covered with purified AP or Ela. Test sera or control sera (pooled sera from healthy adults, healthy children, and 10 cystic fibrosis patients without _P. aeruginosa_ lung infection) were diluted 10^{-1} to 10^{-3} in phosphate-buffered saline (pH 7.2) and incubated for 12 h at 4°C. After washing, the plates were incubated again for 12 h at 4°C with ^{125}I-labeled IgG antibodies to AP or Ela.

Finally, the wells were washed, and the bound radioactivity of each well was measured. Samples with counts-per-minute values 75% of the maximal binding value of the tracer were considered to be positive for antibodies to AP or Ela. Control sera were found to be in the range of 90 to 100% of the maximal binding value of the tracer. The number of different serum precipitins to a preparation of water-soluble antigens of _P. aeruginosa_ (St-Ag) was determined by means of crossed immunoelectrophoresis (microtechnique) as described previously (14).

**Clinical state.** The clinical state of the patients was evaluated on the basis of the following parameters: peak expiratory flow rate, forced vital capacity, and body weight. Normal values of peak expiratory flow rate, forced vital capacity, and weight for age were derived from reference standards reported previously (17). Means for each year of these parameters in patients were expressed as percentages of predicted normal values. For each patient, the mean of these three values was then used as “score %” (Fig. 1).

**RESULTS**

With the exception of _P. aeruginosa_ strain no. 6, all strains produced AP and Ela in variable amounts (Table 1). The time for immune response to AP, Ela, and the St-Ag after onset of chronic _P. aeruginosa_ lung infection differed from patient to patient and from antigen to antigen. The means of the 10 patients were determined as 15 months for response to AP, 11 months for response to Ela, and 6 months for response to the St-Ag. Patient no. 6, harboring an Ela-negative strain, had no detectable antibody titer to Ela. The time for immune response to proteases did not correlate to the amount of in vitro protease production of the strains from the patients. The onset of _P. aeruginosa_ lung infection was independent of the age of the patients and, as is shown in the figures, was also independent of the clinical states as expressed by the score %.

After onset of chronic _P. aeruginosa_ lung infection, indicated by arrows in the figures, the clinical states of 9 of the 10 patients revealed declining curves (data of patients no. 4 and 5 are not shown). After specific antibody production to proteases and St-Ag had started, an increase of antibody titers and numbers of precipitins was seen in all patients. This increase was either fast (e.g., in patients no. 2 and 8) or slow (e.g., in patient no. 9). In sera from some of the patients, the increase in titers and antibodies paralleled well (no. 1, 2, 8, 9), whereas there was a discrepancy of the antibody response against one or more of the antigens in some patients (no. 3, 6, 7, 10). Titers and numbers of precipitins normally never declined after reaching a certain height, suggesting continuous production of antigens and thus continuous stimulation of the humoral immune system. Antibody titers up to 1:1,000 for AP and 1:720 for Ela were measured, and up to 49 different precipitins to St-Ag were detected in sera from patients.

**DISCUSSION**

_P. aeruginosa_, the predominant organism in cystic fibrosis (5, 15, 21), is virtually impossible to eliminate with therapy when lung infection has become chronic (4, 7, 11, 12, 24). This is associated with a change of nonmucoid to mucoid colony form (4, 6, 14), the occurrence of _P. aeruginosa_-specific antibodies (3, 4, 7, 10, 17, 20, 27), and a poorer clinical state of the patients (3, 7, 14, 17, 20). This situation is further substantiated in the present study, in which the clinical courses of 9 of the 10 patients studied—all harboring mucoid _P. aeruginosa_ strains during the time course of chronic lung infection—declined, despite intensive physical and chemotherapy (17), whereas antibody titers to the extracellular proteases AP and Ela and the number of precipitins to St-Ag of _P. aeruginosa_ occurred for the first time and then increased over the years.

Continuously increasing antibody titers and numbers of precipitins suggest a permanent antigen production in the lungs of the patients. This production, despite the presence of specific antibodies which were also detected in bronchial secretions of these patients (10), may have its cause in the mucoid character of the strains which are seen predominantly in cystic fibrosis as compared with other _P. aeruginosa_ infections (15). Mucoid strains produce a slime layer of polyuronic acids (23) in which the pathogen exists in microcolonies (22). Several studies (1, 30, 31), with only one exception (2), suggest that mucoid coating contributes to the persistence of _P. aeruginosa_ in the lungs of patients with cystic fibrosis. The access of _P. aeruginosa_-specific antibodies to the cells may be hindered by the slime layer (25); thus, extracellular protease production can go on continuously.

Longtime stimulation of specific antibodies to proteases and other _P. aeruginosa_ antigens may deteriorate the clinical courses of cystic fibrosis patients due to continuous immune complex formation, a factor which is believed to be important for tissue damage in this disease (18, 29). Furthermore, _P. aeruginosa_-specific antibodies may in some cases inhibit phagocytosis of antigens by alveolar macrophages (32) and
FIG. 1. Longitudinal study (1972 to 1982) of antibody titers to AP, Ela, and numbers of precipitins to the St-Ag of P. aeruginosa in sera of eight cystic fibrosis patients, as determined by radioimmunoassay and crossed immunoelectrophoresis. The clinical states of the patients were evaluated on the basis of peak expiratory flow rate, forced vital capacity, and weight. The three parameters were expressed as percentages of normal values; these were summarized, and the means are given as "score %." Onset of chronic P. aeruginosa lung infection is indicated by the arrow. The patient number is indicated in the left upper corner of each panel. Symbols: ▲-▲, AP; ■-■, Ela; □-□, precipitins; ○. . . . . , score %.
thus contribute to a poorer clinical state in cystic fibrosis.

In contrast to the other patients, patient no. 3 showed increases in clinical score and antibody titers to *P. aeruginosa* antigens. This might be explained by successful chemotherapy treatment. It is noteworthy that all 10 investigated patients were treated with several courses of chemotherapy (17) during *P. aeruginosa* lung infections; therefore, the full natural history of cystic fibrosis with concomitant *P. aeruginosa* lung infection was not seen in any of the patients. Therefore, correlations between clinical states and antibody titers to *P. aeruginosa* antigens are sometimes difficult to interpret.

Since even low antibody titers to proteases neutralize proteases (10), the direct pathogenic action of the proteases may be limited to the beginning of chronic lung infection, when antibodies are lacking. The ability of AP to cleave human IgA, and of Ela to cleave human IgG, IgA, and secretory IgA, was demonstrated in an in vitro study (8), and lung elastin degradation by Ela was shown in an animal model (34). For detection of serum antibodies to AP, Ela, and other *P. aeruginosa* antigens after onset of chronic lung infection, time intervals from zero to 54 months were determined. Since a period of 8 to 10 days is generally sufficient for immune response after invasive infection, the considerably longer intervals found in the majority of patients may reflect the importance of the secretory immune system and other defense mechanisms to prevent invasion of *P. aeruginosa* antigens at an early stage of infection. It would be interesting to investigate the time course of appearance of antibodies to *P. aeruginosa* in saliva or bronchial secretions. After the local defense system is overwhelmed, active invasion by *P. aeruginosa* enzymes may take place. Interestingly, Ela antibodies were seen faster than AP antibodies, suggesting a greater proteolytic activity of the former, a fact which correlates with the enhanced cleavage of human immunoglobulins in vitro (8) by Ela. Intervals for other, mostly unidentified antigens of *P. aeruginosa* are even shorter; an attractive candidate among them might be exotoxin A.

Further longitudinal studies involving other toxins of *P. aeruginosa* are necessary to gain more insight into the pathogenicity of *P. aeruginosa* in this disease.

**LITERATURE CITED**


