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Detection by Enzyme-Linked Immunosorbent Assays of Antibody Specific for *Pseudomonas* Proteases and Exotoxin A in Sera from Cystic Fibrosis Patients

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Enzyme-linked immunosorbent assays were developed to measure serum antibody specific for *Pseudomonas* elastase, alkaline protease, and exotoxin A. Antibody responses to each *Pseudomonas* antigen were measured in cystic fibrosis (CF) patients who were not colonized with *Pseudomonas aeruginosa*, in those who were colonized, in those who were chronically infected with this organism, and in control subjects. Antibody levels for each antigen in the colonized and infected CF patients were higher than levels in uncolonized CF patients or non-CF control subjects. The antibody responses to elastase were similar in patients of the colonized and infected groups. However, infected CF patients had significantly elevated levels of antibody to exotoxin A ($P < 0.01$) and alkaline protease ($P < 0.05$) when compared with patients simply colonized with *P. aeruginosa*. These findings confirm that *Pseudomonas* alkaline protease, elastase, and exotoxin A are produced by *Pseudomonas* strains which colonize and infect CF patients. As an adjunct to established procedures (X-ray, microbiological culture, etc.), the antitoxin and anti-protease enzyme-linked immunosorbent assays may be clinically useful tests for differentiating colonized CF patients from those who have more severe *Pseudomonas* pulmonary infections.

Part of the success of *Pseudomonas aeruginosa* as an opportunistic pathogen can be attributed to its potential for producing a variety of extracellular products. Several of these products contribute to *Pseudomonas* infections of the lung (7, 10, 16), the eye (8, 11, 12, 14), and burn wounds (1, 2, 9, 20, 22). Yet a unique microenvironment exists in the lungs of cystic fibrosis (CF) patients which favors the maintenance of mucoid variants of *P. aeruginosa* (5, 6) and the establishment of chronic infections (3, 4, 5, 17). The host and microbial factors which contribute to the unusual CF pulmonary microenvironment have not been identified, but many *Pseudomonas* exoproducts including alginate, exotoxin, proteases, hemolysin, and phospholipase are currently being considered as virulence factors which might promote colonization, persistence, or lung cytopathology.

Klinger and co-workers (13) detected by radioimmunoassay antibody against exotoxin A and *Pseudomonas* proteases in sera from CF patients and found that the titers of these antibodies inversely correlated with the clinical scores (Schwachman scores) of these CF patients. Hoiby and co-workers (21) showed that a number of *Pseudomonas*-specific precipitins were present in CF patient sera and sputa and that the number of precipitins increased as the *Pseudomonas* complications became more severe. Hoiby has not reported whether exotoxin A or *Pseudomonas* proteases elicit some of these precipitins.

The present study was designed to assess the efficacy of enzyme-linked immunosorbent assays (ELISAs) for measuring serum antibody specific for *Pseudomonas* exotoxin A, alkaline protease, and elastase, which may contribute either individually or collectively to *Pseudomonas* infections in the CF patient. At least one of these proteases is different from those studied by Klinger et al. (13) since it is devoid of elastolytic activity. Levels of antibodies to each exoproduct were compared in groups of CF patients who were uncolonized, colonized, or chronically infected with *P. aeruginosa*.

MATERIALS AND METHODS

Study subjects. All sera were obtained from volunteers attending the pulmonary clinics at the Children's Medical Center, Dayton, Ohio. Because CF patients are a heterogenous group with respect to their pulmonary complications, they were divided into several groups. CF patients were clinically evaluated based on

the following accepted criteria: sweat chloride tests, physical exam, case history with particular emphasis on pulmonary findings, chest roentgenogram, and microbiological culture reports.

Serum samples. Blood samples were obtained upon the consent of parents and patients and stored at 4°C. Within 24 h blood samples were separated by centrifugation in serum separator tubes and stored in small samples at −70°C. All of the samples carried only patient code numbers until the completion of the assays, when study group numbers were decoded.

Antigens. Alkaline protease and elastase purified from culture filtrates of *P. aeruginosa* were obtained from Nagase Ltd., Osaka, Japan. Both enzymes digest casein, but the alkaline protease possesses no elastolytic activity and is more active under slightly alkaline pH conditions (18, 19). Purified exotoxin was the kind gift of N. R. Baker (Ohio State University). All antigens were seen as single bands on sodium dodecyl sulfate-polyacrylamide gels (15).

ELISA. The procedures used in the determination of the working conjugate dilution and the optimum antigen-coating concentration for the indirect microtiter ELISA were essentially those of Voller et al. (23). Polystyrene microtiter plates were used as the carrier surface.

The optimum coating concentration of each antigen was determined as follows. Wells of microtiter plates were filled with 0.2-ml volumes of antigen dilutions prepared in 0.05 M carbonate buffer (coating buffer), pH 9.6; dilutions ranged from 0.02 to 66.6 μg of protein per ml. The plates were sealed and incubated overnight in a humidity chamber at 4°C and then were washed six times with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20. A positive control serum was obtained from a CF patient with a prolonged history of pulmonary infections with *P. aeruginosa*. A negative control serum was obtained from a healthy, age-matched control with no history of CF, respiratory problems, or *P. aeruginosa* infections. Dilutions of control sera (1:50, 1:100, and 1:500) prepared in phosphate-buffered saline–Tween 20 were added in 0.2-ml volumes to microtiter wells coated with each different concentration of antigen. Each sample was run in triplicate. After a 2-h incubation period, all of the wells were washed extensively as described above. The working dilution (1:100) of conjugate (alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G; M. A. Bioproducts, Walkersville, Md.; lot no. 3-9081) was prepared in phosphate-buffered saline–Tween 20 and added to all wells in 0.2-ml volumes. Plates were incubated at room temperature for 3 h and then were washed again. The substrate, *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, Mo.) prepared in 10% diethanolamine buffer, was added to each well in 0.2-ml volumes. After a 30-min incubation at room temperature, the reaction was stopped with the addition of 0.1 ml of 1.5 M NaOH. Absorbance values for the contents of each well were measured at 405 nm with a Gilford Stasar II spectrophotometer.

The optimum antigen concentration for coating microtiter plates gave an absorbance value of approximately 1.0 with the positive control serum and an absorbance value of less than 0.200 with the negative control serum. Optimum coating concentrations of the elastase, alkaline protease, and exotoxin A were 33, 1, and 0.5 μg/ml, respectively. Routinely, patient and control sera were diluted 1:100 when measuring anti-alkaline protease or anti-toxin and 1:50 when measuring anti-elastase. Tests on patient sera were run, using the standard method of Voller et al. (23) for indirect microtiter ELISAs, and were essentially similar to the methods described above, with the optimum coating concentrations and standard serum dilutions determined for each antigen.

Statistical analysis. Statistical evaluations were performed by the Student *t* test. Differences were considered significant if they exceeded the 0.05 probability level.

RESULTS

The subject groups included in the present study were as follows. Group 1 consisted of CF patients who had no evidence of pulmonary disease and who had negative *Pseudomonas* cultures. CF patients who were colonized with *P. aeruginosa*, as determined by routine culture, but who showed no evidence of pulmonary disease were in group 2 (colonized group). In group 3 were CF patients with positive *Pseudomonas* cultures and evidence for chronic suppurative bronchitis as determined by productive cough and respiratory symptoms with or without X-ray findings suggestive of chronic respiratory disease (infected group). These are clearly arbitrary groupings devised for the purpose of subdividing the CF patients who had positive *Pseudomonas* cultures into those who were simply colonized (without any signs of chronic bronchitis) from those who were persistently infected with *P. aeruginosa*. Physicians managing the study subjects placed them into groups based on the criteria indicated above. Code numbers were given to each subject, and group numbers were not revealed until all of the ELISA studies were completed. Group 4 was comprised of non-CF patients with various respiratory disorders. Group 5, containing healthy, age-matched individuals, served as the control group.

The same patient population was used to study antibody responses to exotoxin A, alkaline protease, and elastase. It is important to note that antibody levels in the control subjects (groups 4 and 5) and in the CF subjects with no signs or history of *Pseudomonas* colonization or infection (group 1) are minimal (absorbance of 0.200 or less) for each of the antigens studied. Because *Pseudomonas* organisms are ubiquitous in nature, base-line levels resulting from possible environmental exposure to this organism needed to be established. Figure 1 shows the serum antibody levels specific for *Pseudomonas* elastase in the five subject groups. Both the colonized (group 2) and the infected (group 3) CF patients had higher serum levels of anti-elastase than the uncolonized CF patients (group 1) or control subjects (groups 4 and 5) did. The

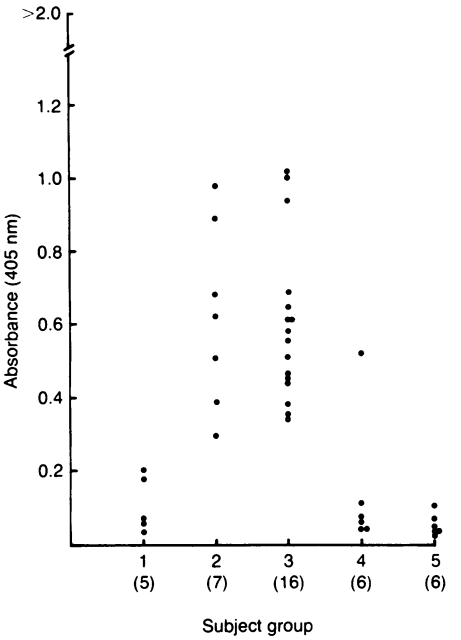

FIG. 1. Serum antibody specific for *Pseudomonas* elastase as detected by ELISA. Figures in parentheses below group numbers indicate the number of subjects in each group. Absorbance values of less than 0.200 are considered to be the base line in these assay systems (Fig. 1 through 3).

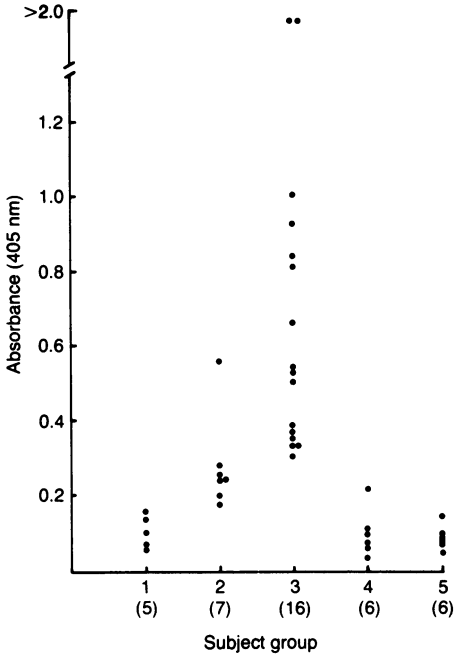

FIG. 2. Serum antibody specific for *Pseudomonas* alkaline protease as detected by ELISA.

anti-elastase levels of patients in the colonized group were not significantly different from those of infected patients. It was noted that one patient in group 4 had fairly high levels of anti-elastase. Although this patient had no charted history of *Pseudomonas* infections, the individual had Down's syndrome and suffered multiple episodes of pneumonia with various etiologies.

Both colonized and infected CF groups had elevated serum anti-alkaline protease levels as compared with any of the other three groups (Fig. 2). However, patients in the infected group also had significantly higher serum levels of anti-alkaline protease than those in the colonized CF group ($P < 0.05$).

Absorbance readings indicating the presence of antitoxin (Fig. 3) were generally much higher than positive readings in the anti-elastase or anti-alkaline protease ELISA systems. Clearly, the infected patients (group 3) had levels of antitoxin much higher than those of the colonized patients ($P < 0.01$) or the subjects in any of the other three groups. Again, one subject in control group 4 had notably elevated levels of antitoxin antibody; this is the same control subject whose anti-elastase level was elevated. One patient in group 1 (uncolonized) and two patients in group 2 (colonized) had antitoxin levels higher than those of the remaining subjects in their respective groups and may represent patients advancing to the next group or patients colonized (group 2) with strains producing high levels of toxin in vivo.

DISCUSSION

The potential for use of these ELISAs as clinical tools to help differentiate between colonized and infected CF patients is evident. These ELISAs are readily adaptable to clinical laboratories since they avoid the use of radiolabels, are relatively inexpensive, and require very small amounts of patient serum.

In this study, the antitoxin levels were generally much higher for all of the CF groups assayed than the anti-protease levels were. This observation points to a potential artifact of the anti-protease systems. Since plates are coated with enzymatically active elastase and alkaline protease, these enzymes could degrade serum components (such as immunoglobulin) during the incubation period with patient sera. If so, anti-protease antibodies which bind to the adsorbed antigen (and subsequently the conjugate) might not be representative of the total level of specific antibody in the sera of each patient. However, this would not alter relative levels in different patient groups because the degree of degradation would be the same in all sera assayed simultaneously. In effect, the actual CF patient antibody responses to the protease antigens may be greater than represented by these data. Alter-

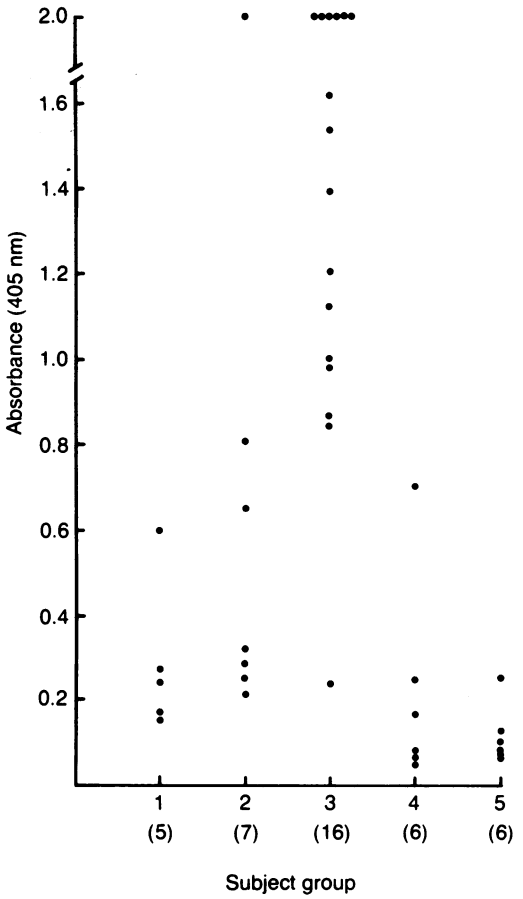

FIG. 3. Serum antibody specific for *Pseudomonas* exotoxin A as detected by ELISA.

natively, it is possible that exotoxin A is simply a better immunogen than either protease.

Our data confirm some of the findings of Klinger et al. (13), who first suggested that exotoxin A and proteases are produced in vivo during CF patient infections. These investigators reported an inverse correlation between antibody titers to exotoxin A or proteases and the patients' clinical status. In Klinger's study, patients were evaluated based on Schwachman scores, which take into consideration certain factors which are unrelated to infectious complications (e.g., nutritional status). In the present study, the separation of patients into different groups was based primarily on the degree of *Pseudomonas* pulmonary complications. However, this distinction may be a minor consideration since both studies showed that the patients with the most severe complications had the highest titers of antitoxin in their sera. We also found this to be true for the patient antibody responses to alkaline protease, which was distinct from the proteases described by Klinger since it is not elastolytic. However, a similar correlation was not found in our study with respect to antibody titers against *Pseudomonas* elastase. Results of tests using the anti-elastase ELISA indicated that colonized and chronically infected patients have comparable levels of antibody, which were elevated by comparison with other CF (uncolonized) and non-CF control groups.

There are several possible explanations for the differential antibody responses to these *Pseudomonas* antigens. Infected patients could have a more chronic exposure to alkaline protease and exotoxin A than to elastase. Changes develop in the pulmonary milieu as CF symptoms progress and may stimulate the infecting strains of *Pseudomonas* to produce more alkaline protease and exotoxin A than they produced during colonization. Alternatively, CF patients can be simultaneously colonized with several different strains of *P. aeruginosa*, and those capable of establishing chronic infections may produce greater levels of alkaline protease and exotoxin A. Based on data presented here, one can only speculate about when and where each of these *Pseudomonas* exoproducts could contribute to the disease process. Determination of whether exotoxin A and alkaline protease are important in the transition between the colonized and infected stages or in later stages of the chronic infection would require a long-term study of several patients as their CF disease and *Pseudomonas* complications progress.

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