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ARTICLE

Amount and Avidity of IgG Antibodies to *Pseudomonas Aeruginosa* Exotoxin A Antigen in Cystic Fibrosis Patients

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Amount and avidity of serum IgG antibodies to *Pseudomonas aeruginosa* exotoxin A in sera of 31 patients with cystic fibrosis (CF) was studied. Eight patients had *P. aeruginosa* isolated from the sputum on multiple occasions, while from 23 patients no *P. aeruginosa* was isolated. Amount of IgG antibodies to *P. aeruginosa* exotoxin A were significantly increased in the serum of patients with *P. aeruginosa* pulmonary colonization (p<0.0001). On the contrary, serum IgG avidity in the colonized and in the non-colonized CF patients was low (<10) and was statistically different when compared to the 30 age-matched healthy controls (p<0.0001). There was no change in IgG avidity in six chronically infected CF patients from whom we obtained serum samples after half a year period (p=0,55). (Pathology Oncology Research Vol 3, No 1, 26–29, 1997)

Key words: ELISA; avidity; cystic fibrosis; exotoxin A; *Pseudomonas aeruginosa*

Introduction

Bronchopulmonary infections due to *Pseudomonas aeruginosa* are a leading cause of morbidity and mortality among the CF patients.[2] The possibility of delaying chronic colonization is the reason for investigating the source of infection, including the possibility of nosocomial transmission. Genotyping,[17] serotyping[18] and phagetyping[13] have been used previously for identification of bacterial strains. It has been proved that the amount and avidity of the antibodies to exotoxin A in the sera of non-CF patients with mild to moderate infection rise significantly during the infection.[18] Although a rise in serum antibody titres to *P. aeruginosa* antigens are also widely observed in colonized CF patients, such antibodies do not appear to prevent or ameliorate the pulmonary infection.[5,14]

The antibodies to *P. aeruginosa* antigens in CF patients possess a reduced opsonic capacity.[4,15] On the other hand, it has been found, that CF serum can support the uptake of *P. aeruginosa* by polymorphonuclear leukocytes from healthy adults and that at least a subpopulation of naturally acquired antibodies possess functionally relevant traits.[2,4] Although in chronically colonized patients a marked antibody response is evident no increase in cell mediated immunity is detectable.[9] Furthermore, T cell cytotoxicity to an allogenic lymphocyte target has been found diminished in CF patients.[12]

Several methods based on ELISA for estimation of the avidity or affinity of antibodies have been developed and proved to be useful in clinical investigations.[6] Avidity is very important property of antibodies since it reflects the strength of binding between antigen and antibody as a function of their affinity and valency. Characterization of antibody avidity may help to predict antibody behaviour in experiments.[6]

The *P. aeruginosa* antigen that we use in this study, is an exoenzyme with the ADP ribosyl transferase activity. It is produced in 80-90% of all clinical strains and it is a significant virulence factor in *P. aeruginosa* infection.[21] It inhibits the protein synthesis in cultured mammalian cells and has an effect on non-specific defence mechanisms.[22] A pronounced systemic IgG response to exotoxin A in CF patients have been reported.[1] Since it was hypothesised that the excessive immune response could directly contribute to the tissue damage an CF patients,[20] we investigated the

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quality of the immune response by measuring the avidity of IgG antibodies. In this study we report that the avidity of serum anti-exotoxin A IgG in chronically colonized CF patients was low, but significantly different from the control group of healthy subjects.

Materials and Methods

Patients

Serum samples were obtained from 31 CF patients who attended the Pediatric Clinic of Ljubljana in 1991. Thirty serum samples from the healthy age-matched donors were used as controls.The mean age of the patients was 13,6 years and the mean age of the controls was 17,0 years. Diagnosis of the disease was verified in each case and was confirmed by laboratory data, including the elevated sweat chloride levels (more than 60 meq/liter) by the quantitative pilocarpine iontophoresis method.[10] At least one serum sample was obtained from all patients during the study. Two serum samples, taken six months apart, were available from 6 patients chronically colonized with *P. aeruginosa*. Eight patients, who were classified as colonized had *P. aeruginosa* isolated from the sputum or saliva samples at least once in the last 2 years before this study and on multiple occasions during the study. In the remaining 23 patients, who were not colonized, the isolation of *P. aeruginosa* was negative before and during the study.

Bacteriophage typing was performed with a standard set of bacteriophages as previously described[13] by Dr. Skalova (Skola Narodnog Zdravlja Andrija Stampar, Zagreb, Croatia; results not presented). However, this method is not sufficient to establish the similarity between the strains, since the surface of the bacteria is often changed due to the loss of the repeating polysaccharide chains in the lipopolysaccharides.

ELISA

Exotoxin A for this study was kindly donated by Dr. Dzirzanowska (Children's Memorial Hospital, Warsaw). It was purified from the PA103/pDF 191,8-202 mutant of *P. aeruginosa* which was a kind gift from Dr. Iglewski (University of Rochester, Rochester, N.Y.) by the method of Iglewski and Sadoff.[11]. The trypticase soy broth (Difco) was treated with Chelex-100 (Bio-Rad) and dialysed against deionised water. This dialysate (TSB-DC) was enriched with 0,05 M monosodium glutamate and 1% glycerol. The overnight culture in TSB-DC (2 ml) was used to inoculate 1 liter of TSB-DC into a 5 liter Erlenmeyer flasks. The flasks were incubated on the reciprocating shakers at 32 °C for twenty hours. Purification of exotoxin A was performed at 5 °C. Culture supernatants were diluted 4 fold with deionized water, DEAE-Sephacel (Pharmacia) was added and suspension was stirred gently for 1 hour. DEAE-Sephacel was filtered with Buchner funel and introduced into the column. Step gradient of 0,05, 0,1 and 0,2 M NaCl in 0,01 M Tris-HCl buffer, pH 8,1 was used. Exotoxin A was eluted at 0,2 M NaCl concentration, dialyzed overnight against 0,01 M Tris-HCl buffer, concentrated with PM-30 membrane (Amicon) and stored at -70 °C. Material containing exotoxin A was purified by chromatography on Sephadex C 200 using 0,01 M Tris-HCl buffer as eluent. Fractions containing exotoxin A were adjusted to pH 7,1 with 0,1 M HCl and applied to the column of hydroxylapatite (Bio-Rad) equilibrated with 0,005 M sodium phosphate in 0,05 M NaCl, pH 7,0. Exotoxin A was eluted at 0,04 M phosphate concentration, dialysed against PBS, concentrated with PM-30 membrane (Amicon) and frozen at -70 °C in small portions. The exotoxin A preparation showed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis a single band at approximately 65000 Da. Sera were tested as described previously.[8] Polystirene plates (Plastomed, Warsaw) were coated with antigen by incubation at 37 °C for 18 h. Each well was incubated with 200 µl of coating antigen in 0,05 M carbonate buffer, pH 9,6. Exotoxin A was used at a concentration of 2 µg/ml. Excess antigen was removed and the plates were washed three times with phosphate buffered saline (PBS)-T (0.05% Tween 20 in PBS, pH 7,4) and 150 µl of the appropriate dilution was dispensed into the well. A fourfold serum dilution scheme from 1:10 to 1:40,960 was used. After 3 h incubation at 37 °C the plates were washed three times with PBS-T. The horseradish peroxidase labeled goat anti-human immunoglobulin G (IgG) (Heintel, Austria) was added and incubated for 1 h at 37 °C.

After the washing, 150 µl of freshly prepared solution of a o-phenylenediamine HCl (0.34 mg/ml) and H_2O_2 (0.01% in a 0.1 M phosphate citrate buffer, pH 5,4) was added to each well and incubated for 30 min at 37 °C. The enzyme reaction was stopped by adding 75 ml of H_2SO_4 (Merck). The reaction product was measured by reading the A_{490} in a Minireader II (Dynatech Laboratories Inc.). The specificity of ELISA was described previously.[8]

Estimation of avidities and amounts of antibodies were based on the protocol described by Gripenberg and Gripenberg[6] and were performed by using a computer program. Quantitative differences in antibodies are reflected by the shape of a dose-response curve. A method based on the approximation of the absorbance value by a polynomial:

$$P(X) = A_1X + A_2X^2$$

where 1/x is the dilution factor; was used to characterize the dose response curves in the ELISA. The parameters used are: $E = a_1$ and $A = a_1^2/a_2$. E is defined as the effective amount of antibodies and A, which represents the function of the reaction constant between antibody and antigen, is avidity. The avidity values higher than 10 were considered high.[18]

Statistical Analysis

Arithmetic mean A for antibody avidities and logarithms of arithmetic mean E for antibody amounts were determined. Spearman rank correlation coefficient was estimated for the comparison of E and A values separately in a control group and both CF patient groups. The E and A parameters of all three study groups were compared by Kruskal-Wallis test. For the comparison of paired sera in six chronically colonized CF patients a Student's test was used.

Results

The mean antibody amounts of anti-exotoxin A in CF patients with isolated *P. aeruginosa* were significantly higher (p<0,0001) compared to the control group and a group of patients who were not chronically infected (*Table 1*). Also, the mean antibody amounts were higher (p<0,003) in the CF patients without isolated *P. aeruginosa* then in the control group (*Table 1*).

The mean avidity of anti-exotoxin A IgG antibodies in CF patients (with and without isolated P. *aeruginosa*) were very low (<10) but were significantly higher than in the control group of healthy subjects (*Table 1*, p<0,0001). There was no difference in the mean avidities between the noninfected patients and the control group (p>0,9).

The statistical correlation between the ranks of A and E was evident in the control group (*Table 2*, p<0,001) and in the CF patients without the isolated bacteria (*Table 2*, p<0,005).

No such correlation could be proved in the group of chronically colonized CF patients (*Table 2*, p>0,05). The paired sera from 6 chronically infected CF patients, taken after half a year period, did not show any significant difference in mean A differences (*Table 3*, p=0,56) and mean E differences (*Table 3*, p=0,137).

Table 1. Avidity and amount of anti-exotoxin A antibody in CF patients and in controls

Group	*n*	*Mean avidity*	*Log of mean amount*
1	8	5.23 ± 1,08[a]	2,43 ± 0,12[c]
2	23	3,20 ± 1,36[b]	1,31 ± 0,24[d]
3	30	3,22 ± 0,63	1,15 ± 0,03

[a]p<0,0001 (versus group 2 and 3);
[b]p<0,9 (versus group 3);
[c]p<0,0001 (versus group 2 and 3);
[d] p<0,003 (versus group 3)
group 1 – CF patients with isolated P. aeruginosa;
group 2 – CF patients without isolated P. aeruginosa;
group 3 – control group
n – number patients/individuals in the given group;
p – probability; ± standard error

Table 2. Spearman correlation coefficients (rs) for A and E of IgG antibodies to exotoxin A in CF patients and in controls

	n	*rs*	*p*
control group	30	0,67	<0,001
group 1	23	0,58	<0,005
group 2	8	0,28	>0,05

group 1 – CF patients without isolated P. aeruginosa
group 2 – CF patients with isolated P. aeruginosa

Discussion

In the previous studies of the immune response to *P. aeruginosa* antigens it has been shown that serum titres to LPS, alginate and exotoxin A were elevated in chronically colonized CF patients.[1,5,14] Such antibodies, though, do not seem to be protective. Moreover, direct and indirect evidence of induction of immunologic damage by *P. aeruginosa* has been reported in CF patients.[20] Several laboratories have found also a positive correlation between severe disease and elevated anti-*P. aeruginosa* antibody titres.[16,19] The precise reason why such antibodies are not protective is not known. One of the explanations is that heat stable opsonins in CF patients, which were investigated for their ability to promote phagocytosis of ^{35}S-labeled *P. aerurginosa* by human polymorphonuclear neutrophils, possess a greatly reduced opsonic capacity, compared to the healthy population.[4] As neutralizing antibodies to the toxins of *P. aeruginosa* are produced in chronically infected patients, the tissue damage produced by such toxins could be also explained by the immune complex formation.[4,9]

Table 3. The mean difference in anti-exotoxin A IgG amounts (E) and mean difference in avidities (A) in paired sera of six chronically colonized CF patients

	x_1	x_2	*d*	*p*
E	448,5	364,6	83,92	0,137
A	5,1	5,2	0,083	0,56

x_1 – arithmetic mean values of E and A in the samples obtained at the beginning of the study;
x_2 – arithmetic mean values of E and A in the samples obtained after half year
d – mean difference in A and E in the paired sera;
p – probability

In this study we have examined the amounts and the avidities of serum IgG antibodies to exotoxin A in CF patients. Significant differences in the IgG antibody amounts were found between the CF patients and the control group. Mean amounts of IgG antibodies in both non-

Table 4. **Age distribution**

Years	*Patients*	*Controls*
0-5	6	3
5-10	14	4
10-15	4	2
15-20	4	16
20-30	3	5
Total	31	30

colonized CF patients and the control group were significantly lower (11 times and 16 times, respectively) than in chronicallly colonized CF patients. These results are in agreement with findings reported.[16,19]

It was reported that the patients with different severity of P. *aeruginosa* wound infections respond with high amounts and high avidities (A>10) of anti-exotoxin A antibodies.[16] On the contrary, the mean avidities in our study were low (A<10) in chronically infected CF patients, although significantly different from noninfected CF patients and the control group of healthy subjects (p<0,0001). However, no rise in mean avidity was noticed in the paired serum samples of six chronically colonized CF patients after a six months period and no significant changes in IgG amounts were observed during this period.

Our study confirms the previous report that CF patients appear to produce low-affinity non-opsonic antibodies after an early exposure to *P. aeruginosa.* It was observed that although chronically colonized patients had elevated levels of total anti-LPS antibodies, these antibodies possessed affinities at least 900 fold less than vaccine-induced antibodies. The reason was attributed to slow maturation of high-affinity antibodies in chronically infected patients.[2] Another explanation suggests that the long-term antigen exposure via the lungs in CF patients causes an antigen specific suppression of the IgG response, which is evident by a reduction in affinity.[3]

In summary, we conclude that the amounts and the avidities of anti-exotoxin A antibodies proved to be useful in differentiation of infected and noninfected CF patients. The persistance of mean low-avidity IgG antibodies in CF patient chronically colonized with *P. aeruginosa* indicates one of the reasons for functionally less effective antibodies in chronically colonized CF patients. In the fiuture the immunoglobulins in CF patients should be investigated on the molecular level.

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