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Relative Increase in IgG Antibodies to *Pseudomonas aeruginosa* 60-kDa GroEL in Prediabetic Patients with Cystic Fibrosis

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ABSTRACT

In recent years research has focused on a possible connection between bacterial infection and development of diabetes mellitus. In this study, serum antibody responses against bacterial antigens in diabetic and nondiabetic patients with cystic fibrosis (CF) were evaluated. The first part of the study included 252 CF patients of whom 46 (18%) had diabetes. This study showed that precipitating antibodies (precipitins) against *Pseudomonas aeruginosa* and other bacteria in crossed immunoelectrophoresis, and IgG antibodies against a 60-kD GroEL of *P. aeruginosa*, were highly variable and positively correlated with age. Patient material matched for age and sex showed no significant difference between diabetic and nondiabetic CF patients in precipitins or IgG antibodies to *P. aeruginosa* GroEL. Two longitudinal studies of 9 and 5 y using retrospectively selected sera from 29 prediabetic and 29 cross-matched nondiabetic CF patients were performed. As to precipitins against *P. aeruginosa*, we found no difference between the prediabetic and the nondiabetic group of patients during the study period. The study revealed, however, a significant increase of 24.6% ($p = 0.008$) of IgG antibodies against *P. aeruginosa* 60-kD GroEL, 3-12 mo before the onset of diabetes in patients with CF, compared with an overall increase of 5% to 6% per year in both groups during the observation period. This study shows that diabetes in CF appears after a peak of serum IgG antibodies against GroEL and indicates that development of diabetes in CF patients may not only be caused by a progressive fibrosis of the pancreatic tissue, but may be augmented by a short-term specific immunologic reaction, initially triggered by an ongoing and progressive pulmonary infection. **(*Pediatr Res* 49: 423–428, 2001)**

Abbreviations:
CF, cystic fibrosis
CFDM, diabetes in CF
CIE, crossed immunoelectrophoresis
Hsp, heat shock protein
IGT, impaired glucose tolerance
OGTT, oral glucose tolerance test
NGT, normal glucose tolerance
Precipitins, precipitating antibodies in CIE
IDDM, insulin-dependent type-1 diabetes mellitus

CF is the most common fatal autosomal recessive inherited disorder in the white population. The most important clinical feature is recurrent and chronic pulmonary infection, of which chronic infection with *Pseudomonas aeruginosa* is the most serious. In addition, insufficient pancreatic exocrine function and endocrine dysfunction are commonly associated with CF. Recent studies have described a high prevalence of impaired glucose tolerance (18%) and diabetes mellitus (24%) in CF patients (1). A variety of experimental data provides compelling evidence that bacterial 60–65 kD hsps may constitute a link between various infectious diseases and autoimmunity (2–7). Hsps of infecting microorganisms are known to constitute a major target of the innate and adaptive immune system. The bacterial 60–65 kD hsps are ubiquitous and have a very high interspecies sequence homology (8). The 60-kD GroEL of *P. aeruginosa*, previously designated common antigen (9), has a sequence homology of 52% with its human counterpart, hsp60 (8), originally designated P1 (10). Interestingly, various immunizations with peptides of the human hsp60 (p277) have been shown to induce diabetes or transient diabetes in various animal models and are linked to the appearance of hsp60-specific T cells (3–5).

The purpose of this study was to investigate in CF patients the possible association between bacterial infection and diabetes. This was accomplished by measuring the number of precipitins in CIE and the level of IgG antibodies specific for the 60-kD GroEL of *P. aeruginosa* in serum of prediabetic and nondiabetic patients with CF.

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Table 1. *Cross-sectional analysis of 252 CF patients analyzed for number of bacterial precipitins*

Variable	CFDM†	CF alone	All
No. of patients (%)	46 (18)	206 (82)	252 (100)
Age in years (mean, range)	26 (13–45)	15 (2–43)	17 (2–45)
Patient with chronic *P. aeruginosa* (%)	38 (83)	108 (52)	146 (58)
Precipitins‡ (range)			
P. aeruginosa	21.4 (0–46)*	10.8 (0–45)	12.7 (0–46)
S. aureus	1.3 (0–8)	1.5 (0–10)	1.4 (0–10)
H. influenzae	1.5 (0–9)	1.5 (0–15)	1.4 (0–15)
Others§	3.0 (0–46)*	1.0 (0–34)	1.4 (0–46)
Total precipitins	28.2 (0–96)*	15.5 (0–50)	17.8 (0–96)

* $p < 0.001$ compared with CF alone and all.

† Includes CF patients with diagnosed DM (capillary plasma glucose conc. ≥12.2 mM, determined by 2-h OGTT.

‡ Measured by CIE.

§ Includes *Burkholderia cepacia, Pseudomonas maltophilia, Pseudomonas stuzeri, Klebsiella pneumoniae, Enterobacter cloacae, Staphylococcus epidermidis, Serratia marcescens, Nocardia spp., Acinetobacter spp., Agrobacterium spp.*, and *Alcalignes spp.*

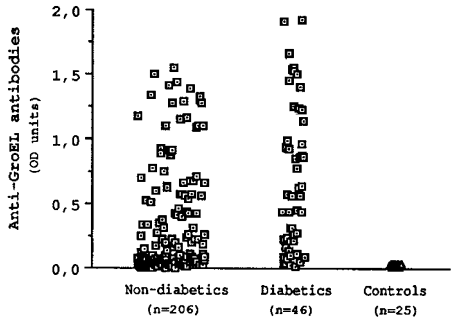

Fig. 1. Cross-sectional study of 252 patients with CF evaluated for IgG antibodies against 60-kD GroEL of *P. aeruginosa*. The ordinate indicates the level of GroEL-specific IgG antibodies in OD units determined by immunoblot scanning densitometry. The categories are nondiabetics (mean, 0.28 U; range, 0.002–1.928 U), diabetics (mean, 0.69 U; range, 0.013–1.912 U) and controls (healthy persons; mean, 0.02 U; range, 0.012–0.038 U).

METHODS

Patients. Danish CF patients attending the Cystic Fibrosis Center in Copenhagen were tested for number of precipitins against *P. aeruginosa* (normal range, 0–1), *Staphylococcus aureus* (normal range, 0–2), and *Haemophilus influenzae* (normal range, 0–1) using CIE, and if found by sputum culture also for precipitins against several other microorganisms as previously described (11, 12). Glucose tolerance was classified as normal (NGT) if the 2-h postload capillary plasma glucose concentration was ≤8.8 mM, impaired (IGT) if value was 8.9–12.1 mM, or diabetic (CFDM) if value was ≥12.2 mM using a 2-h OGTT according to the 1985 WHO recommendation (13). Serum samples were collected at weekly to 6-mo intervals depending on the patient's clinical condition. All patient sera collected were stored at −80°C until used. Collection of patient serum samples was approved by the patient or parent(s) with informed consent, and by the Ethical Committee of Copenhagen and Frederiksberg, Denmark. Chronic *P. aeruginosa* lung infection was defined as the presence of bacteria in sputum for 6 mo or an antibody response of ≥2 precipitins against *P. aeruginosa*.

Purification of* P. aeruginosa *60-kD GroEL. Recombinant GroEL of *P. aeruginosa* was purified as described elsewhere (8). Briefly, primers covering the coding sequence of the *P. aeruginosa* P1118 *groEL* gene were used in PCR, generating a product of approximately 1.7 kbp, which was ligated onto a *pET16b* expression vector. The ligated material was transformed into competent *Escherichia coli*, whereafter recombinant material was expressed and purified using a Ni^{2+} charged His-Bind resin column. The 60-kD GroEL protein was identified using a specific MAb to *P. aeruginosa* 60-kD GroEL, and stored at −20°C until used.

ELISA. Specific IgG antibodies to *P. aeruginosa* 60-kD GroEL were measured by ELISA. Flat-bottom microdilution plates (96-well, Maxisorb, NUNC, Roskilde, Denmark) were coated with recombinant *P. aeruginosa* GroEL (2.0 μg protein/well) in PBS, pH 7.2, and incubated overnight. Nonspecific binding was blocked with diluting buffer [PBS, 0.1% (vol/vol) Tween-20, and 1.5% (wt/vol) NaCl, pH 7.2] for 1 h. Sera in dilutions of 1:1,000 to 1:100,000 were applied and incubated for 2 h. The plates were washed three times in PBS-Tween buffer (PBS, 0.1% Tween-20) and further incubated for 1 h with a 1:2000 dilution of peroxidase-conjugated rabbit anti-human IgG (P214, Dako A/S, Glostrup, Denmark) in diluting buffer. The plates were washed six times in PBS-Tween buffer and finally developed with 5 mM *o*-phenylenediamide (S2000, Dako A/S, Denmark) in phosphate-citrate buffer (35 mM citric acid, 67 mM Na_2HPO_4, pH 5.0) with 0.0125% (vol/vol) hydrogen peroxide (Merck, Darmstadt, Germany). The reaction was developed in the dark for 20 min, terminated by the addition of 1 M H_2SO_4, and measured at OD_{492nm} using an automatic plate reader (EIA Reader 2550, Bio-Rad Laboratories, Hercules, CA, U.S.A.). All reactions were performed in 100-μL volumes and at room temperature. Antibody levels expressed in units were calculated as the mean of OD values in triplicate assays given as ratio between sample and standard sera. The ratio was converted to units using a high titered anti-*P. aeruginosa* GroEL CF serum as standard assigned 10 U/mL. The day to day and the interplate variation were determined by testing 10 CF sera in triplicate in ELISA. The day to day variation for the *P. aeruginosa* IgG GroEL assay was 14% (95% CI, ±18%), and 4% (95% CI, ±6%) for the interplate variation. The results for each patient were normalized by calculating the mean of all antibody results obtained during the observation period (mean = 100%). Each observation was expressed as a percentage of the mean value for each patient.

SDS-PAGE, Western blotting, and immunostaining. The SDS-PAGE, Western blotting, and immunostaining were performed essentially as previously described (8).

Laser scanning densitometry. The precipitating bands in immunostaining assays were measured by laser scanning densitometry (diapositive slide, Fuji Velvia, Fujifilm, Copenhagen, Denmark). Photographic dias of immunoblots were scanned with a helium-neon laser scanner (UltroScanXL, LKB 2222, Bromma, Sweden), and the geometric peak area of intensity (OD_{633nm}) was integrated automatically. The calculation was $\int AU \times mm$, where AU is absorption (abs) − $abs_{baseline}$ in units, baseline determined by the 16 lowest points

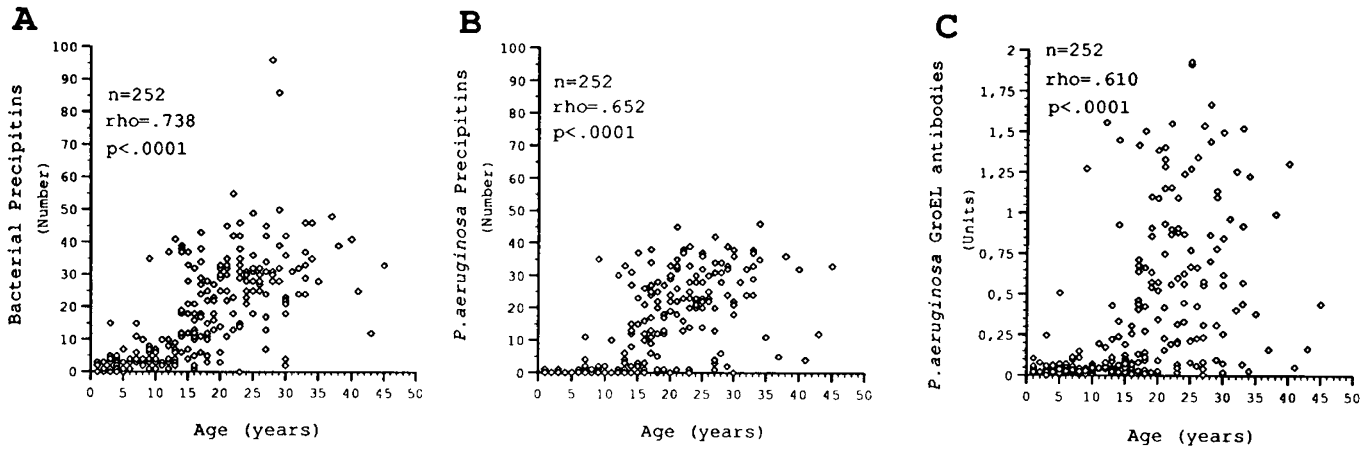

Fig. 2. Cross-sectional analysis showing correlation of total number of bacterial precipitins by CIE (*A*), *P. aeruginosa* precipitins by CIE (*B*), and number of IgG antibodies against the 60-kD GroEL of *P. aeruginosa* by immunostaining (*C*) with age of patients with CF.

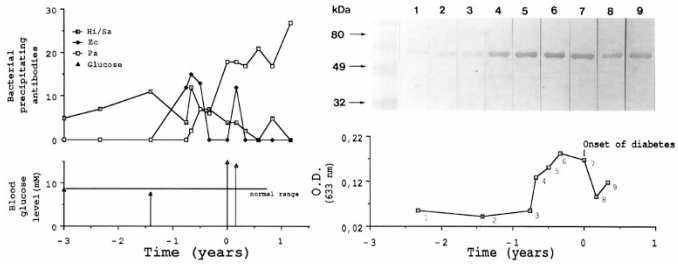

Fig. 3. An 18-year-old girl with CF who developed CFDM at an age of 15 y was evaluated for number of bacterial precipitins by CIE (*Top left*), glucose tolerance by OGTT (*Bottom left*), and IgG antibodies to *P. aeruginosa* 60-kD GroEL by immunostaining (*Top right*) for a 31-mo period during development of CFDM. *Lane 1,* sample drawn May 17, 1989; *lane 2,* sample drawn April 4, 1990; *lane 3,* sample drawn November 28, 1990; *lane 4,* sample drawn January 9, 1991; *lane 5,* sample drawn March 6, 1991; *lane 6,* sample drawn April 22, 1991; *lane 7,* sample drawn August 21, 1991; *lane 8,* sample drawn November 1, 1991; and *lane 9,* sample drawn January 3, 1992. The patient developed CFDM diagnosed on August 25, 1991, with a 2-h blood glucose level of 14.7 mM. An immunoblot scanning assay (*Bottom right*) revealed an increase of 69.4% of GroEL-specific IgG antibodies in a 5-mo period (from November 28, 1990, to April 22, 1991), 9 mo before the onset of CFDM. Legends indicate precipitins against *H. influenzae* and *S. aureus* (Hi/Sa), *E. coli* (Ec), and *P. aeruginosa* (Pa).

in the scan, and mm is the peak width at baseline. The peak area was presented in OD units.

Statistics. The following statistical methods were used: Mann-Whitney *U* test, Spearman rank correlation, Page test, Wilcoxon signed rank test, and linear regression analysis, with a 5% level of significance. The programs StatView (StatView ver 4.51, Abacus Concepts, Berkeley, CA, U.S.A.), for statistics and CricketGraph (CricketGraph III ver 1.5.3, Computer Associates International, Islandia, NY, U.S.A.), for graphic presentation were used.

RESULTS

Cross-sectional study. The first part of the study included 252 patients with CF, in which 146 (58%) had chronic *P. aeruginosa* pulmonary infection and 46 patients (18%) had diabetes (CFDM). The mean age at onset of CFDM was 20.6 y (range, 3–40 y). Two patients (4%) exhibited CFDM before the age of 10, and 34 patients (74%) were >15 y old at the time of onset of CFDM. Of the 39 patients (15%) who developed IGT, 4 (10%) progressed to CFDM and 23 (59%) reverted to NGT, whereas 12 (31%) manifested IGT a second time. The CFDM group of patients had been chronically infected with *P. aeruginosa* for 8.7 y (range, 0–23.9 y) before onset of CFDM. The CFDM group of patients had a significantly higher number of bacterial precipitins and of precipitins against *P. aeruginosa* than the nondiabetic group of patients (Table 1). CFDM patients also show a variable and high level of IgG antibodies to the 60-kD GroEL of *P. aeruginosa* compared with the nondiabetic group and with healthy persons evaluated by immunostaining (Fig. 1). However, the number of bacterial precipitins and the level of IgG antibodies to the 60-kD GroEL of *P. aeruginosa* are correlated with age as shown in Figure 2. Consistently, the difference in precipitins between the diabetic group and the nondiabetic group is related to the difference in the mean age (26 *versus* 15 y) between these groups (Table 1). This was confirmed by a test that showed no difference in number of bacterial precipitins or in GroEL-specific IgG antibodies between diabetic *versus* nondiabetic CF patients cross-matched for age and sex (Table 2).

Longitudinal studies. In a prediabetic CF patient, from whom sera were collected in a 5-y period during onset of CFDM, the samples were evaluated for blood glucose tolerance, number of bacterial precipitins, and level of IgG antibodies against the 60-kD GroEL of *P. aeruginosa* (Fig. 3). This revealed an increase of the GroEL-specific IgG antibodies of 69.4% before onset of CFDM. During the same period, the patient exhibited chronic *P. aeruginosa* infection as indicated.

A study including 29 prediabetic and nondiabetic CF patients matched in pairs for age, sex, and chronic *P. aeruginosa* pulmonary infection was performed (Table 3). Patient sera were retrospectively selected from a collection of sera from all CF patients and included 673 serum samples, of which 420 were prediabetic samples, and 676 samples were included in the control group. This study included evaluation of the number of precipitins against *P. aeruginosa* in a 9-y period, initiated 6 y before the onset of CFDM for each patient (Fig. 4, *A* and *B*). At entry into the study (−6 y), no significant difference

Table 2. *Cross-matched diabetic* vs. *nondiabetic CF patients matched for age and sex*

Variable	CFDM	CF alone	*p* value‖
No. of patients (M/F)	34 (17/17)	34 (17/17)	
Age in years (median, range)	25 (13–45)	25 (13–43)	0.859
Glucose tolerance† (median, range)	14.3 (12–21)*	7.3 (5–9)**	<0.001
No. of bacterial precipitins‡ (median, range)	27 (0–96)	26 (1–49)	0.659
No. of *P. aeruginosa* precipitins‡ (median, range)	22 (0–42)	21 (0–38)	0.636
Specific *P. aeruginosa* GroEL Ab§ (median, range)	0.629 (0.01–1.90)	0.381 (0.01–1.44)	0.082

* Three observations by fasting (morning) glucose tolerance (mean, 10.6; range, 10–11).

** Twelve observations by fasting (morning) glucose tolerance (mean, 5.; range, 4–8) and glucose tolerance is performed within the same year as the corresponding CFDM observation.

† Determined by 2-h OGTT (capillary plasma glucose concentration in mM).

‡ Determined by CIE.

§ Determined by ELISA (units).

‖ Mann-Whitney *U* test.

Table 3. *Clinical data of diabetic* vs. *nondiabetic CF patients cross-matched for age, sex, and precipitins against* P. aeruginosa

Variable	CFDM	CF alone	*p* value§
Number of patients (M/F)	29 (17/12)	29 (17/12)	
Age in years (median, range)	21.0 (13–40)	19.5 (13–38)	0.655
At the time of diagnosis of diabetes			
Glucose tolerance† (median, range)14.5 (12–21)	6.8 (4–10)	<0.001	
at entry of study (−6 y)			
Number of patients with *P. aeruginosa* infection‡			
No colonization	8	8	
Intermittent colonization	6	6	
Chronic infection	15	15	
Number of *P. aeruginosa* precipitins (median, range)	16.0 (0–45)	18.0 (0–39)	0.930

* Two observations by fasting (morning) glucose tolerance (mean, 5.6; range, 5–6), and glucose tolerance is performed within the same year as the corresponding CFDM observation.

† Determined by 2-h OGTT (capillary plasma glucose concentration in mM).

‡ No colonization (<1 precipitin), intermittent colonization (≥1, ≤15 precipitins) and chronic infection (>15 precipitins) determined by CIE.

§ Mann-Whitney *U* test.

was found in the median number of *P. aeruginosa* precipitins between the prediabetic group and the matched nondiabetic group ($p = 0.9304$). The number of *P. aeruginosa* precipitins increased in both groups during the 9-y period ($p < 0.0001$); serial Mann-Whitney *U* tests between each observation in the prediabetic group with the corresponding observation of the nondiabetic group did not reveal any significant difference.

Sera collected from the same 29 prediabetic CF patients as above were evaluated by ELISA for IgG antibodies to the 60-kD GroEL of *P. aeruginosa* in a 5-y observation period, initiated 3 y before the onset of CFDM. This study included 320 serum samples, of which 167 were prediabetic samples, and 349 samples were included in the control group. The level of GroEL-specific IgG antibodies varied in these patients from 0.18 U (range, 0.12–0.22 U) in a low-titer patient to 86.91 U (range, 49.22–187.08 U) in a high-titer patient at onset of CFDM (not shown). In addition, the level of IgG antibodies to *P. aeruginosa* GroEL was found to vary (up to 100-fold) within a given patient during the observation period. The IgG antibodies to *P. aeruginosa* GroEL were calculated as a percentage of the mean (normalization), as shown in Figure 4, *C* and *D*. This revealed for the prediabetic group a median increase of 5.4%/y during the observation period ($\rho = 0.787$; $p = 0.0001$) and a relative and significant increase of 24.6% of GroEL-specific IgG antibodies during a 9-mo period (from −1 to −0.25 y) before onset of CFDM ($p = 0.0084$). The cross-matched nondiabetic CF patients were followed in the same observation period and showed a median level of GroEL-specific IgG antibodies of 4.0 U (range, 0.6–105.3 U) at the matched time of onset of CFDM in the prediabetic group of patients (not shown). There was no significant difference between the prediabetic group and the cross-matched group 1 y before, at onset, and 1 y after the time of onset of CFDM ($p > 0.3$). The IgG antibody level to *P. aeruginosa* GroEL was normalized in the matched nondiabetic group of patients (Fig. 4*D*). An increase of 6.2%/y was found during the observation period ($\rho = 0.831$; $p = 0.0001$), and this group showed no significant increase in GroEL-specific antibodies at any time during the observation period.

DISCUSSION

In CF patients, chronic lung infections caused by *P. aeruginosa* are difficult to treat and impossible to eradicate because of ineffective clearance by the CF lungs and development of antibiotic resistance in the bacteria (14). Despite development of antibiotic resistance, aggressive therapy using specific antibiotics is beneficial to the patient. By use of aggressive therapy for the last two decades, the CF Center in Copenhagen improved the CF survival rate markedly (15). However, any therapy depends on a good method for diagnosis. In addition to conventional bacterial culture and microscopy, diagnosis of

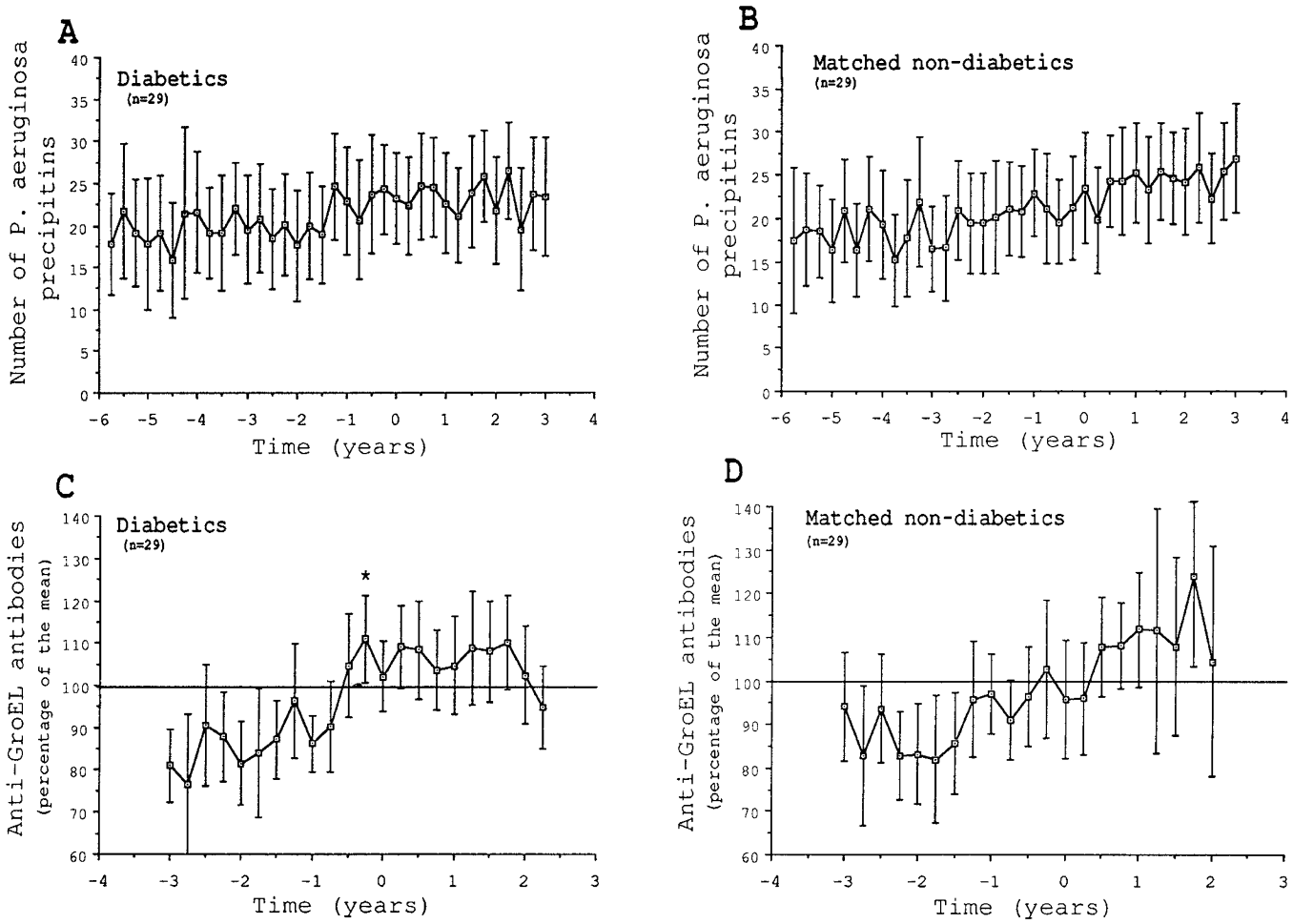

Fig. 4. Longitudinal study of prediabetic (*A* and *C*) and cross-matched nondiabetic (*B* and *D*) CF patients, matched in pairs for age, sex, and chronic *P. aeruginosa* infection. Serum samples were evaluated for number of *P. aeruginosa* precipitins during development of CFDM (*A* and *B*), and for GroEL-specific IgG antibodies determined by ELISA and calculated as the percentage of the mean (*C* and *D*). Time = 0 indicates the onset of CFDM in the prediabetic group of patients. Error bars represent 95% CI. *$p = 0.0084$ compared with −1 y.

chronic pulmonary infection is made by measuring the number of precipitins against bacterial pathogens by CIE. CIE along with immunostaining and ELISA have been used in this study to measure precipitins against various bacteria plus IgG antibodies against the 60-kD GroEL of *P. aeruginosa*. In the cross-sectional study, major increase in precipitins, bacterial as well as *P. aeruginosa*-specific precipitins, is observed at an age of 14–15 y (Fig. 2, *A* and *B*). Interestingly, CFDM is diagnosed in the majority of patients at an age of 20 y. It is possible that CFDM is caused by an infection; however, we failed to find any correlation between precipitins and CFDM when using age- and sex-matched patients (Table 2).

A search for a different method to evaluate the immune response in CF patients was performed. This led to the evaluation of serum IgG antibody response against a 60-kD GroEL of *P. aeruginosa* in prediabetic CF patients. This hsp of the hsp60 family is 52% identical to the homologous human hsp60 (8), and high levels of antibodies to hsp60 have previously been seen in CF patients (16). Because of the homology between bacterial GroELs and human hsp60, the hsp60 antibody detected is most likely caused by ongoing pulmonary infections in these patients.

The finding in this study of a relative increase in the IgG antibody response to the 60-kD GroEL of *P. aeruginosa* a few months before onset of CFDM (Fig. 4*C*) could be a result of activated B cells in response to the 60-kD GroEL of *P. aeruginosa* or in response to other microbial GroEL proteins produced in the course of microbial infections. Thus, a microbial infection may precede and perhaps be a cause of the progressive destruction or down-regulation of pancreatic β cells in CF.

Early studies show that the number of islets of Langerhans seems to be normal in CFDM (17), but later studies have established by immunocytochemical techniques that the density and number of islets in CFDM are reduced (18). A relative preservation of β cells in CFDM may explain the absence of ketoacidosis in the disease (19) and explain the mild nature of CFDM. Several mechanisms may apply to the development of CFDM. The most prevalent theory is that CFDM is caused by a slow, year-long fibrosis of the exocrine pancreatic tissue with a subsequent strangulation of the islets of Langerhans and thereby reduced number of β cells (20). The second event may be an autoimmune destruction or down-regulation of pancreatic β cells, a similar mechanism found in autoimmune IDDM.

However, CFDM and IDDM are different in many ways. The prevalence of CFDM increases with age, which is not the case in IDDM (21), CFDM shows no ketoacidosis as is found in IDDM, and progression toward diabetes in CFDM is considered mild compared with IDDM. Given the range in age at which diabetes develops in CF (3–40 y), several mechanisms may apply to CFDM, strangulation as well as autoimmune related. Against CFDM being autoimmune related is the conflicting evidence concerning the presence of islet cell antibodies in CF. This marker has been found to be present in 15% of patients with CF compared with a prevalence of 0.5% in a normal population (22). However, others have either not found islet cell antibodies present in CF (23) or only found them in 3% compared with a frequency of 2% to 3% in healthy controls, which was not different for diabetic *versus* nondiabetic CF patients (24). In a recent study, a group of 28 CF patients were followed in a 10-y period; 12 developed diabetes and were found to be more frequently linked with the genotype Δ508 as compared with the nondiabetic patients, whereas the latter group was linked with the genotype N1303 K (25). This study suggests that the Δ508 mutation may induce susceptibility to CFDM, whereas the N1303 K mutation may give protection. In an earlier study, however, including 215 CF patients, of which 211 had the Δ508 mutation, we found no difference between patients who were homozygous or heterozygous for the Δ508 mutation, and endocrine pancreatic function was normal in 72.5%, impaired in 12.3%, and diabetic in 15.2% of the patients, with no difference between CF patients who were homozygous (21). This study indicates that there is no correlation between the Δ508 mutation and development of CFDM. The finding, however, that CFDM may be related to the genotype HLA DQB1 (26) and the finding in this study of an increase in the 60-kD GroEL antibody in prediabetic CF patients indicate an immunologic complicity.

Development of IGT is common in CF, although most of the cases will revert to NGT (1). Some patients, however, acquire IGT repeatedly and subsequently develop CFDM (1). Interestingly, Elias *et al.* (4, 5, 27) found the hsp-induced diabetes to be transient in the nonobese diabetic mouse model (4, 27) as well as in standard mice using the peptide p277 of hsp60 (5). Development of IGT in CF might therefore be a result of an autoimmune-related β-cell destruction or down-regulation, depleting the β-cell mass to the extent of impairing tolerance without necessarily progressing to CFDM. CFDM could well be related to the experimental hsp-induced diabetes by sharing an early environmental event such as a microbial infection, acting in genetically susceptible individuals. The inducible and protective role of hsp-reactive T cells on diabetes in mouse models further substantiates the possible role of bacterial 60-kD GroELs on CFDM. Extensive studies need to be performed to determine the possible impact of infection on diabetes in CF.

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