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Detection of the Cystic Fibrosis Protein by Isoelectric Focusing of Serum and Plasma

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Summary

We used the isoelectric focusing method developed by Wilson to analyze serum from individuals homozygous or heterozygous for cystic fibrosis. The presence of cystic fibrosis protein (CFP) was found in 37 out of 52 homozygous and 24 out of 34 heterozygous patients, which leads to a frequency of 71% for both families. Five out of 24 controls were found positive. The same study, performed on 26 plasma samples collected from the same patients, demonstrated that the detection of CFP is possible in plasma as well as in serum. Our results confirm the presence of a protein "marker" of CF in serum, but also underlines the lack of sensitivity of the isoelectric focusing technique to be used for diagnosis.

Abbreviations

CFP, cystic fibrosis protein
IEF, isoelectric focusing

Cystic fibrosis, characterized by a generalized metabolic disorder, is one of the most prevalent genetic diseases in the Caucasian population. An incidence of 1 in 2000 live births has been reported (3), and approximately 1 in 20 of the population is a carrier of the gene or heterozygote (8).

In 1973, Wilson *et al.* (8) detected a "cystic fibrosis factor" now designated "cystic fibrosis protein" in serum using IEF in thin layer polyacrylamide gels. Other studies from this laboratory (9, 11) characterized this CFP as having a pI of 8.4, a molecular weight between 3500–10,000, and a close association with IgG in serum. The importance of these findings prompted different laboratories to reproduce this IEF technique but with widely varying success (2, 5, 7). Recently, Nevin *et al.* (4) confirmed the CFP frequency reported by Wilson using the same IEF technique. Because of several unsuccessful reports, another set of experiments to confirm the presence of this protein in serum was necessary. Furthermore, the protein had never been characterized in plasma. Only Wilson recommended that samples obtained from heparinized or citrated blood not be used (9); therefore, it is of interest to study the presence of CFP in plasma and serum from the same patients for a further understanding of the nature of CFP, and to eliminate the possibility of the origin of CFP by proteolysis during blood clotting.

MATERIALS AND METHODS

Material. Sera from 52 CF patients were supplied by J. P. Chazalette (Giens) and R. Gilly (Lyon). Diagnosis of the disease was established by clinical criteria and an abnormal sweat test. Sera from 34 obligate heterozygotes and 24 controls were studied in parallel.

Preparation of sera and plasma. All sera were carefully collected as described by Wilson *et al.* (9). After collection, all samples of venous blood were immediately cooled at 4°C and allowed to clot in glass tubes for 4–6 h. The samples were then centrifuged at 1700 *g* for 10 min at 4°C and the serum was transferred by aliquots into plastic tubes, then frozen and stored at −75°C.

Plasma (5 ml) from the same patients (12 CF patients, nine heterozygotes, and five controls) were collected in tubes containing 25 μl heparin (50,000 IU) and immediately centrifuged at 400 *g* for 5 min, then at 3000 *g* for 10 min at 4°C. After centrifugation, plasma samples were stored as described for serum.

IEF. IEF was performed in thin layer polyacrylamide gels

using the pH gradient 5–10, recommended by Wilson in his first publication (9) and prepared with the following mixture of Ampholine carrier ampholytes: 1.5 ml pH 5–8, 1 ml pH 8–9.5, 0.25 ml pH 7–9, and 0.25 ml pH 9–11. To prepare the gel this mixture was added to 7.4 ml stock solution of acrylamide (25 g/100 ml), 8 ml bisacrylamide (1.0 g/100 ml), 15 g cristallised urea, and distilled water up to 50 ml final volume. After deaeration under reduced pressure for 4–5 min with mild agitation 0.83 ml of 1% ammonium persulfate, 3.3 ml of 0.004% riboflavine, and 0.06 ml of TEMED were added to induce polymerization. This mixture results in a gel with T = 4% and C = 4% (13) containing 4.9 M urea and 2.2% Ampholine-carrier ampholytes.

After polymerization for one night, the glass moulding plate was removed, duplicate samples were applied, and electrofocusing was carried out for 2 h at 25 W constant power using the LKB Multiphor. The electrolytes used were 1 M PO_4H_3 for the anode and 1 M NaOH or 5% TEMED for the cathode. Subsequently, the gel was simultaneously fixed and stained in a solution of 45 g trichloracetic acid, 13.5 g sulphosalicylic acid, 0.45 g Coomasie Blue R in 135 ml methanol, and 280 ml distilled water. The staining was carrried out in boiling water for 15 min. After cooling to room temperature in the staining solution, the destaining of the gel was performed with the mixture methanol/water/acetic acid (6:13:1, v/v).

Samples. Twenty five microliters of each serum were applied to the gel. Each sample contained 200–400 μg of IgG determined by single radial immunodiffusion using Tri-partigen IgG plates (Behring).

RESULTS

Analysis of sera. Figure 1 represents an electrofocusing plate containing different serum from CF patients, heterozygote carriers for CF, and normal control subjects. The CF protein band, the most basic protein of serum, is clearly visible in samples from CF patients, especially in samples CF2, CF5, CF6, and CF7 (shown with arrow). In samples 1, 3, and 4 the band is faint and more diffuse. In the two samples of normal serum the band is completely absent and the same pattern is obtained with two samples from heterozygotes (Hz3–Hz4). For Hz1 and Hz2 the result is less evident. This figure shows a classical pattern of IEF of serum and points out the difficulty in evaluating the presence of CFP. Figure 2 shows an enlarged area of the CFP band after

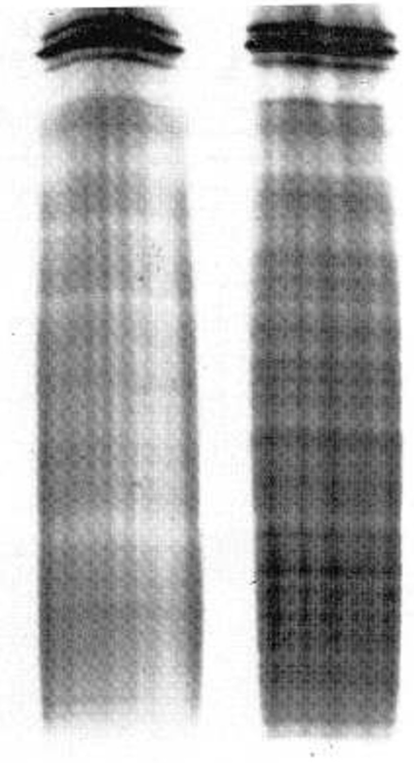

Fig. 2. Enlargement of the isoelectric focusing pattern of serum in the basic protein area.

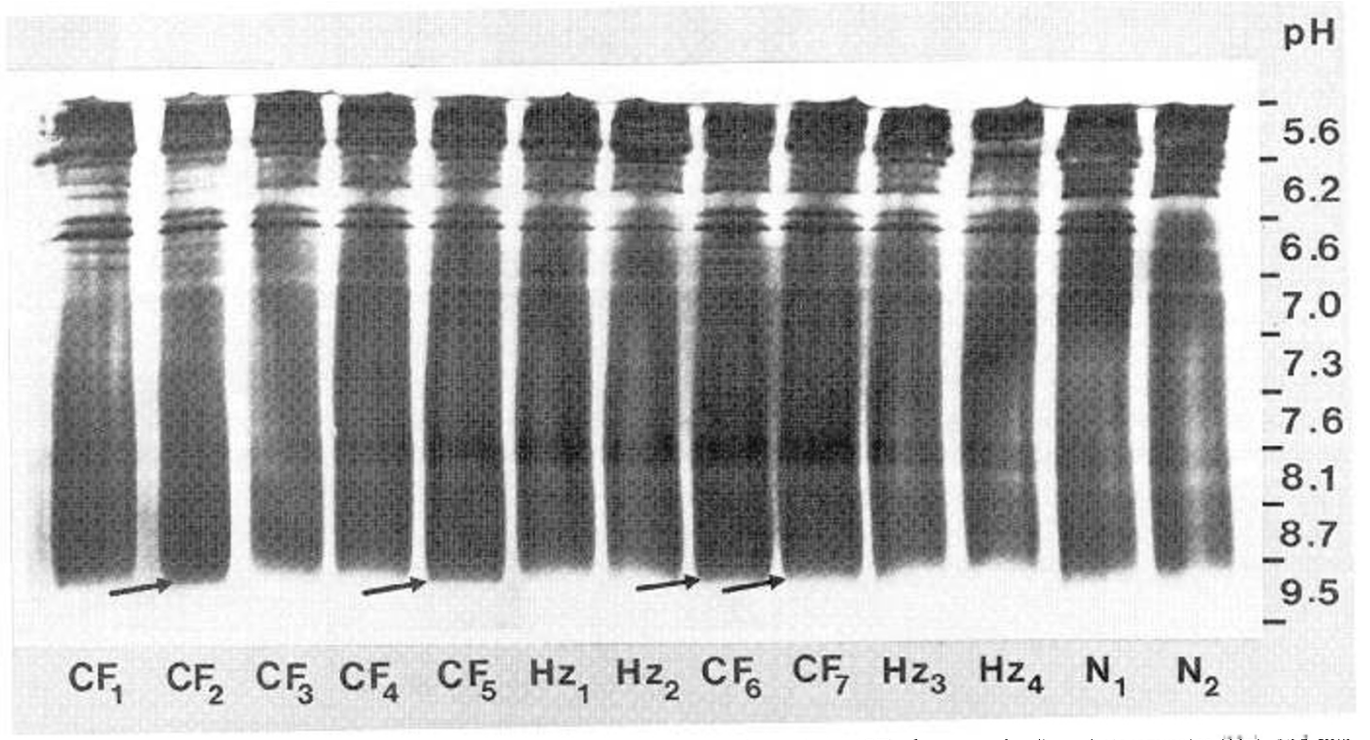

Fig. 1. Isoelectric focusing pattern of serum proteins. Seven samples from CF patients (CF), four samples from heterozygotes (Hz), and two controls (N) are represented. The pH gradient is shown to the side.

electrofocusing of sera from one CF patient and one normal control. On this CF sample, the additional protein band is evident. But, because the results were not always obvious, each sample was assayed at least three times and more when doubtful results were obtained. In most serum samples the amount of IgG was 300 μg as recommended by Wilson (9), but the analysis of results shows that above this minimal amount the visualization of the CFP band is not modified. The results of the CFP detection in 101 sera are summarized in Table 1. They are significantly different ($2P \leq 0.001$ by χ^2 test) between CF homozygotes or heterozygotes and normal controls.

Analysis of plasma. The electrofocusing analysis of eight different plasma is presented in Figure 3. Some samples (CF4, CF6, CF7, Hz3) came from the same patients as did the serum and are presented in Figure 1. The same protein band is present in most CF plasma and in some heterozygote plasma. Table 2 summarizes the IEF results of 26 patients whose plasma and serum have been studied. Only three patients gave opposite results in serum and plasma. Different tests used (sign test, exact probability of Fisher) did not indicate any significant difference between results obtained with serum and with plasma; therefore, the CF protein can be characterized in plasma as well as in serum, but the difficulty remains the same.

Table 1. *Serum positivity for cystic fibrosis protein as determined by isoelectric focusing*

Patients (n)	+	−	% (+)
Normal controls (24)	5	19	20
CF heterozygotes (34)	24	10	71
CF homozygotes (52)	37	15	71

DISCUSSION

Our results confirm the presence of a peculiar protein in the sera of CF patients and obligate heterozygotes; however, we did not find the same CFP frequency as that reported by Wilson (10) and by Nevin (4). The frequencies in homozygotes (71% instead of more than 90%) and heterozygotes (71% instead of 85%) are lower, and alternatively the frequency in controls is higher (20% instead of 8%). This last result can be explained by the small number of control subjects studied and also by the presence of at least one positive control with a known bronchiectasis. By contrast we think that our results obtained with the sera of CF patients and obligate heterozygotes are probably due to the lack

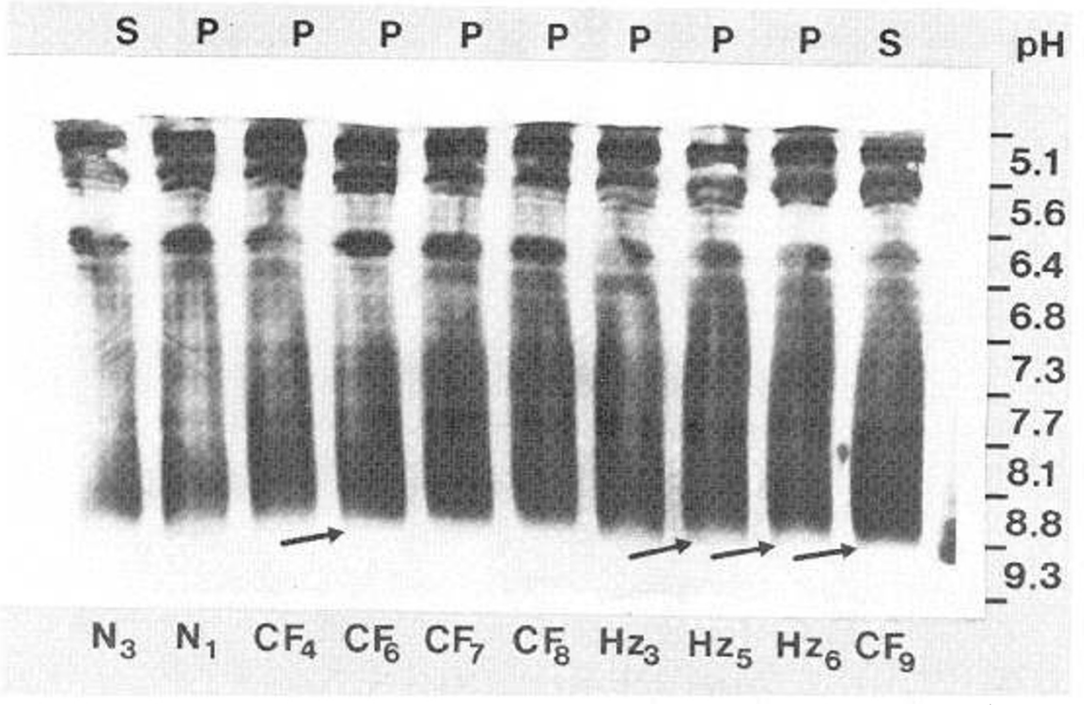

Fig. 3. Isoelectric focusing pattern of plasma proteins, P. Four samples from CF patients (CF), three samples from heterozygotes (Hz) and one control (N) are represented. Two serum samples, S, (N3 and CF9) are given for comparison.

Table 2. *Comparison of the positivity for cystic fibrosis protein between serum and plasma*

CF homozygotes			CF heterozygotes			Normal controls		
Sample	Plasma	Serum	Sample	Plasma	Serum	Sample	Plasma	Serum
1	+	−	1	+	+	1	−	−
2	+	+	2	+	+	2	+	+
3	+	−	3	−	+	3	−	−
4	+	+	4	−	−	4	−	+
5	+	+	5	+	+	5	−	−
6	−	−	6	+	+			
7	+	+	7	+	+			
8	+	+	8	+	+			
9	+	+	9	−	−			
10	+	+						
11	+	+						
12	+	+						

of sensitivity of the IEF technique as suggested by others. We also tried the modified technique of Wilson *et al.* (10) with a pH 2.5–10.0 gradient instead of a pH 5–10 gradient and with a prefocus 30 min at 300 volts, but the detection of the CFP band was not improved.

Recently, this CFP specific material, isolated from polyacrylamide gel after electrofocusing, has been used with success by different groups to develop a quantitative immunoassay. Manson and Brock (1, 2) have raised antisera in guinea pigs and Wilson in mice (12). Both antisera allowed a quantitative distinction between CF homozygotes, heterozygotes, and controls. Even if there are still a number of unsolved problems such as blood collection and antiserum supply (1), their data, which shows a difference in levels of CFP strongly argue that this protein may be a marker of cystic fibrosis. Our characterization of CFP in plasma as well as in serum demonstrates that this low molecular weight protein is present in blood before blood clotting and could permit a better standardization of blood collection, which looks critical for its evaluation.

In conclusion, we agree with many workers that the IEF technique is too delicate and not sufficiently sensitive to be used for diagnosis. This technique remains, however, the first step in identifying the CFP which seems to be closely associated with the CF gene and whose characterization would allow future investigation of the basic defect of the disease.

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Changes in the Growth-Promoting Activity of Human Milk during Lactation

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Summary

We measured the concentrations of protein, insulin, and epidermal growth factor (EGF) in human milk from mothers delivering at term. Samples were obtained from d 1 (colostrum) to d 42 after birth. Human colostrum contains very high concentrations of protein [83.7 ± 7.4 g/l (SEM)], insulin (3.75 ± 0.88 nM), and EGF (53.9 ± 6.9 nM). Similar concentrations have been measured in prebirth milk. Insulin, EGF, and protein in milk decline rapidly during the first few days of lactation but remain constant thereafter. Although the concentrations of insulin and EGF in mature milk are only 10% of those in colostrum, they are considerably higher than in serum. We also showed that human milk has a growth-promoting activity in cultured cells, causing a stimulation of protein synthesis in L6 myoblasts and 3T3-L1 fibroblasts and an increase in DNA synthesis in L6 cells and T47D breast cancer cells. This mitogenic activity declines as lactation progresses, with a similar time-course to the fall in insulin and EGF; however, the cell lines used here are not responsive to either of these two growth factors in the range of concentrations found in milk. This indicates that human milk also contains high concentrations of additional, unidentified growth factors. The occurrence of high concentrations of growth factors in milk suggests that they may be important for the proliferation and differentiation of infant tissues.

Abbreviations

EGF, epidermal growth factor
PBS, phosphate buffered saline