Isolation of the “Cystic Fibrosis Protein” from Serum
Catherine Barthe,1 Jacqueline Carrera,2 Catherine Figarella,1 and Odette Guy-Crotte1

“Cystic fibrosis protein” (CFP), a minor serum protein marker of the cystic fibrosis allele, was isolated from serum of patients with cystic fibrosis by use of the “FPLC” high-resolution chromatography system and preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis. CFP currently is characterized by its isoelectric point (8.4) on isoelectric focusing. However, after the first purification steps, we identified the protein, which was present in a very low concentration, by the immunosorbent assay of Hayward et al. (J Immunol Methods 1986;91:117–22), by virtue of its immunological relationship with the “CF antigen,” a protein characterized in granulocytes by these same authors [Nature (London) 1985;315:513–5]. CFP, a protein of low molecular mass, about 14 kDa, appears to be strongly associated with IgG in serum. Using the same procedure with control serum permits us to assume that CFP normally is present in serum in trace amounts.

Cystic fibrosis (CF) is the most common genetic disease affecting the lifespan of white people throughout the world (about 1 in 2700).3 Despite the recent localization of the CF locus on chromosome 7 (1–3) and the findings of a decreased epithelial chloride (Cl−) permeability in several CF organs systems (4–6), both the basic gene defect and its protein product remain to be identified.

Many groups of investigators have tried to characterize abnormal proteins in serum of patients with CF. In two independent studies (7, 8), we confirmed by isoelectric focusing the presence of cystic fibrosis protein (CFP), first described by Wilson et al. (9) as a doublet protein of pI 8.4, in 70% of sera and plasmas from individuals who were homozygous or heterozygous for CF. Two years ago, Van Heyningen et al. (10) found in granulocytes a protein immunologically indistinguishable from CFP; they called it “CF antigen.” This new tissue source has permitted the immunopurification of “CF antigen” from granulocytes, leading to the sequencing of a DNA by Dorin et al. (11), who localized the gene encoding for “CF antigen” on chromosome 1, a localization different from that of the CF locus (2).

Recently, Odink et al. (12) cloned two calcium-binding proteins from human blood leukocytes, MRP-8 and MRP-14, both of which are expressed by infiltrate macrophages in subjects with rheumatoid arthritis. These two proteins were later identified as the light chain (MRP-8) and the heavy chain (MRP-14) of the leukocyte L1 complex (13). Interestingly, MRP-8 turned out to be identical to the “CF antigen” cloned by Dorin et al., but specific enzyme immunoassays of the two proteins, MRP-8 and MRP-14, failed to demonstrate the presence of MRP-8 in most samples of serum and plasma from carriers and noncarriers of CF gene. In contrast, MRP-14 was present in all sera and plasmas tested, but in greater concentrations in plasmas from CF carriers (14). These data show that the “CF antigen” cloned by Dorin et al. (11) differs from CFP, underlining the necessity to isolate CFP from serum to determine more precisely its molecular identity despite the difficulties in purifying it from this biological fluid.

Here we present a method for isolating CFP from serum by FPLC (Fast Protein Liquid Chromatography) and electrophoresis. After the first purification step, CFP is present at too low a concentration to be characterized by isoelectric focusing; therefore, we used the immunoassay of Hayward et al. (15), in which two monoclonal antibodies directed against “CF antigen” from granulocytes and CFP from serum are used.

Materials and Methods

Preparation of sera. Sera from 24 CF patients were supplied by J. P. Chazalette (Giena). Diagnosis of the disease was established by clinical criteria and abnormal results for the sweat test of Schales and Schales (16). In addition, seven control sera were collected from healthy volunteer adults with no family history of CF.

Specimens of venous blood were collected into disposable glass centrifuge tubes and kept at 4 °C on crushed ice for 4 to 6 h, then centrifuged (1700 × g, 10 min, 4 °C). The sera were then apportioned into plastic tubes and frozen at −75 °C.

FPLC chromatofocusing. Samples of these sera were submitted independently to chromatofocusing, performed at room temperature with the FPLC system (Pharmacia-LKB, Les Ulis, France) on a Mono P column (Mono P column bound with tertiary and quaternary amines) equilibrated in a 75 mmol/L Tris buffer adjusted to pH 9.3 with acetic acid. Proteins were eluted with a 100 mL/L solution of “Polybuffer 96” (Pharmacia; a mixture of amphoteric buffering substances in the range pH 9–6) adjusted to pH 6.0 with acetic acid. All buffers were filtered and de-aerated before use. Serum samples were diluted in the equilibration buffer to give a protein concentration of about 10 mg/mL, then filtered through a 0.22-μm (pore size) sterile disposable filter, and 500 μL of the filtrate was applied to the column. For the separation we used the elution buffer at a flow rate of 1 mL/min for 30 min. Absorbance was measured at 290 nm with a UV-1 detector (Pharmacia) and recorded by a linear recorder set at 1.0 A full scale.

FPLC gel filtration. For gel filtration with the Pharmacia FPLC system we used a Superose 12 column (HR, 10/30) with an optimal separation range of 1 to 300 kDa, equilibrated in 0.15 mol/L ammonium bicarbonate buffer, pH 7.8, or in this same buffer plus 5 mol/L urea, pH 7.8. A pool of four “fractions containing CFP,” previously isolated by chromatofocusing the CF sera, was dissolved in 160 μL of distilled water and diluted with 200 μL of the chromatography buffer. We applied 200 μL of this solution to the...
column and performed the separation by using a flow rate of 0.35 mL/min for 100 min. Detection was at 280 nm, as for chromatofocusing. We collected 0.7-mL fractions. We repeated the gel-filtration procedure with a pool of chromatofocusing fractions isolated from control serum.

The column was calibrated with aldolase (158 kDa), IgG (150 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsigen A (25 kDa), ribonuclease A (13.5 kDa) (all from Combithek, Boehringer, Mannheim, F.R.G.), and aprotinin (6 kDa) (a gift from Bayer AG, Leverkusen, F.R.G.) in the ammonium bicarbonate/urea buffer.

Desalting of FPLC chromatographic fractions. FPLC chromatographic fractions were submitted to treatments for desalting and concentration that differed depending on the following step. For isoelectric focusing, sandwich-type assay, and gel filtration, salts were removed by filtration on Sephadex G-25 (PD10 columns from Pharmacia) equilibrated in distilled water. For electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS), precipitation with 100 g/L trichloracetic acid was performed. Vacuum evaporation in a "Speed Vac" concentrator (Savant) was used to concentrate desalted proteins or eliminate traces of acid.

Isoelectric focusing. For this we used thin-layer polyacrylamide gels in the presence of 5 mol/L urea and a pH gradient of 5 to 10, as recommended by Wilson et al. (9), with some slight modifications of the method set up by Grataroli et al. (7), as described before (8).

SDS–PAGE. Polyacrylamide gel electrophoresis was performed in slab gel (120 × 80 × 1.5 mm), with use of the system 75 to 150 g/L in the presence of 1 g/L SDS according to the technique of Laemmli (17), under nonreducing conditions, at room temperature. Before electrophoresis, dried samples were dissolved in 10 µL of distilled water and 10 µL of sample buffer (pH 6.8) containing 30 g of SDS, 100 mL of glycerol, and 65 mmol of Tris hydrochloride per liter. For silver staining we used a Bio-Rad Labs. kit.

Elution of proteins from SDS–PAGE. After SDS–PAGE and staining of control strips, the corresponding bands of protein were eluted from excised gel by overnight incubation with isotonic saline. We dialyzed the eluate against isotonic saline for 24 h to remove traces of SDS, then concentrated it by dialysis against polyethylene glycol (300 g/L). The eluted proteins were concentrated by evaporation under reduced pressure.

Estimation of the protein stained after SDS–PAGE. To estimate how much purified CFP was obtained, we compared the intensity of the stained band with that of a series of known concentrations of a pure protein (lysozyme from chicken, a gift from C. Kopeyan, Hôpital Nord, Marseille, France) having a molecular mass (14 kDa) close to that of CFP. After SDS–PAGE followed by staining with Coomassie Blue, we measured the density of the bands with a laser densitometer (Ultrascans 2202; LKB) and a recording integrator (Model 2220; LKB).

Enzyme-linked immunosorbent assay (ELISA) of the "CF antigen." We performed two-site sandwich-type assay for the "CF antigen," using two monoclonal antibodies and reagents kindly supplied by C. Hayward and D. Brock and using plates according to the method of Hayward et al. (15). The monoclonal antibody used to coat the polyvinyl chloride plates was raised in mice immunized with the excised, homogenized precipitin peak obtained on "rocket" immunoelectrophoresis, corresponding to the antigen from CF serum (18). The rocket immunoelectrophoresis was performed with polyclonal antibodies reactive with the CFP that had been excised from isoelectric focusing gel. The biotinylated monoclonal antibody was obtained after immunization of mice with a granulocyte lysate from patients with chronic myeloid leukemia (15).

Before the assay, different treatments were applied to samples according to the isolation procedure of the samples. Desalted and dried fractions obtained after chromatofocusing were dissolved in isotonic saline to have approximately the same protein concentration in all fractions; the dilutions we used were 100-, 500-, and 1000-fold. Gel-filtration fractions (except peak 2) were dissolved in 25 µL of isotonic saline and diluted 10-, 25-, 50-, and 100-fold. Material corresponding to peak 2 was dissolved in 300 µL of isotonic saline to have the same absorbance at 280 nm as for chromatofocusing fractions, then diluted 50-, 100-, 250-, and 500-fold. Proteins eluted from SDS–PAGE were dissolved in 25 µL of isotonic saline and diluted 10-, 25-, 50-, and 100-fold.

Results

Chromatofocusing of serum. In chromatofocusing of serum, we used a pH gradient of 9.3 to 6.0, because the isoelectric point of CFP is near 8.4. A major peak was eluted between pH 8.6 and 8.1 (Figure 1). Analysis of the different fractions by isoelectric focusing indicated the presence of CFP in the first fractions of chromatography, corresponding to the major protein peak (Figure 2). These “fractions containing CFP,” analyzed by electrophoresis on SDS–polyacrylamide gel (data not shown), contained low-molecular-mass proteins, ~14 kDa, and also a family of proteins corresponding to serum immunoglobulins, ~150 kDa.
kDa. Testing by immunoelectrophoresis produced a line of precipitation with anti-IgG but not with anti-IgM, demonstrating that these fractions contained essentially immunoglobulins G. When the same chromatography was performed with control serum, we obtained the same elution pattern but detected no CFP by isoelectric focusing of the different fractions.

Gel filtration of "fractions containing CFP." "Fractions containing CFP" isolated by chromatofocusing were submitted to gel filtration. When we used the ammonium bicarbonate buffer (0.15 mol/L, pH 7.8) for this, proteins were eluted in only one peak (data not shown). When urea, 5 mol/L, was added to the buffer, four peaks were eluted (Figure 3). The protein concentration was greatest in peak 2, with peaks 1, 3, and 4 being detected only when the sensitivity was increased by 20-fold. From the calibration of Superose 12, determined under the same conditions, peak 2 corresponded to the elution of IgG (150 kDa), whereas peak 3 corresponded to the elution of protein of low molecular mass such as aprotinin (6 kDa). The first minor peak of protein (peak 1) was eluted in the position of a protein of 900 kDa. From the results of SDS–PAGE and this calibration, it could be a polymerized form of immunoglobulins. This would be in agreement with the behavior of pure immunoglobulins, which, when applied to the same filtration column, were eluted in the position of peaks 1 and 2. Peak 4 was an artifact created by the presence of urea in the chromatography buffer. Only peak 2 was subjected to isoelectric focusing, because peaks 1 and 3 contained insufficient proteins. However, there was no evidence for CFP in peak 2. We then treated chromatographic fractions by SDS–PAGE. Figure 4 depicts the detection of proteins after silver staining. Peaks 1 and 2 contained mostly high-molecular-mass proteins corresponding to IgG. As expected, peak 3 contained a protein of low molecular mass but, surprisingly, any IgG not completely dissociated from CFP during filtration was also present in peak 3.

When corresponding fractions isolated by chromatofocusing of control serum were submitted to the same gel filtration, four peaks again were separated. As analyzed by SDS–PAGE (data not shown), the same proteins were found in each corresponding peak, but the low-molecular-mass proteins in peak 3 were much less concentrated than in peak 3 from CF serum, even though the protein concentration of the applied samples was the same.

Immunoreactivity against "CF antigen" in chromatographic fractions and SDS–PAGE protein bands. We tested the eluted chromatographic fractions by ELISA, using the two monoclonal antibodies raised against "CF antigen" and CFP (15). As shown in Figure 1, the first fractions on chromatofocusing, corresponding to the "fractions containing CFP" detected by isoelectric focusing, contained immunoreactive material. Compared with the total immunoreactivity present in the unfractionated serum sample, this peak contained 70% of the immunoreactive antigen. Figure 1 also shows the results of chromatofocusing the fractions from control serum. The values found for the major peak are much lower than was the case for the CF serum, the immunoreactivity representing <7% of the maximal value obtained with CF serum. Because of their low protein

---

**Fig. 3.** Gel filtration of "fractions containing CFP" in the presence of urea 5 mol/L.

We loaded the column with "fractions containing CFP" pooled from four chromatofocusing. Proteins were measured by the absorbance at 280 nm (UV) with absorbance unit full scale set at 0.1 for peaks 1, 3, and 4 and at 2.0 for peak 2. Immunoreactivity against CF antigen (C) was determined by ELISA performed on the peaks pooled from three columns. Fraction volume, 0.7 mL.

**Fig. 4.** SDS–PAGE pattern of fractions eluted from gel filtration after silver staining.

Each fraction was precipitated with trichloroacetic acid and electrophoresed. Standards (Std): albumin (66 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.6 kDa), and lysozyme (14.3 kDa). a, b, c: 5, 10, and 20 μL of the top of peak 2 (see Fig. 3). Tubes 62 to 130 are chromatographic fractions of peaks 3 and 4.
concentration, we had to pool the fractions corresponding to peaks 1 and 3 from three identical columns before we could test the gel-filtration fractions of the CF sera by ELISA. The most immunoreactivity was found in peak 3 and corresponded to 14% of the antigen present in the "fraction containing CFP." Peak 1 also contained immunoreactivity, but it corresponded to only 7% of the antigen loaded onto the column (Figure 3). By contrast, peak 2, which contained the major part of the protein loaded onto the column, contained no immunoreactivity. When we assayed the same fractions of control serum at the same protein concentration as that of CF fractions, we observed no immunoreactivity in peaks 1, 2, or 3.

We also performed the immunosorbent assay on the high- and low-molecular-mass protein bands eluted from polyacrylamide gel after electrophoresis of peaks 1 and 3 of the gel-filtration procedure (Figure 4). We found CF immunoreactivity in the two high-molecular-mass bands of IgG in peaks 1 and 3, and in the low-molecular-mass protein in peak 3, in a ratio of 1/3/5 (the immunoreactivity detected in the low-molecular-mass protein band of peak 3 represented 50% of the antigen present in peak 3). Thus, as expected, most of the immunoreactivity was in the protein band of low molecular mass corresponding to purified CFP. However, some immunoreactivity clearly remains bound to IgG.

Elution of purified CFP and test of homogeneity. The immunosassay clearly indicated that the low-molecular-mass protein in peak 3 corresponded to CFP. Therefore, for the last step of purification, we simply eluted this protein band from SDS–PAGE of peak 3. As shown in Figure 5, the eluted CFP migrated as a single protein, at 13.5 kDa. The purified material amounted to about 10 μg of protein.

Discussion

The FPLC system allowed us to isolate from serum a fraction containing partly purified CFP. For the final purification of CFP we used preparative SDS–PAGE followed by elution of the protein from the gel. In summary, this first reported method of CFP purification from serum includes four steps: chromatofocusing of serum, gel filtration of "fractions containing CFP," SDS–PAGE of the peak containing immunoreactivity, and elution of the low-molecular-mass protein from polyacrylamide gel. The final product (roughly 10 μg) contained 5% of the immunoreactivity present in the starting material. This low recovery underlines the difficulty of purifying this minor protein in a fluid as complex as serum.

During all purification steps, CFP appeared to be strongly associated with IgG. In chromatofocusing these proteins co-eluted, and during gel filtration only the presence of 5 mol/L urea allowed dissociation. However, despite the use of urea, some undissociated complex was eluted in peak 1 and, surprisingly, in peak 3, which contained low-Mr protein. Moreover, after SDS–PAGE, positive immunoreactivity was still present in the high-Mr protein bands eluted from the gel. CFP migrates like a protein of 13.5 kDa. A protein of the same low molecular mass was found by Getliffe et al. (19), who isolated it by chromatofocusing serum in a different pH gradient (10.5 to 7.0) and 4 mol of urea per liter. Their fraction containing CFP was eluted at pH 9.1, and by SDS–PAGE it showed traces of a low-Mr protein of 12 kDa. An additional protein of 52 kDa was considered to be a polymeric form of CFP, but we never characterized this with our purification steps. In a recent paper (8), we showed that possession of the CF allele was correlated with the expression of a serum protein of 12 kDa (P12) in parallel with the expression of CFP. The same molecular mass found for CFP in the present study is an additional argument for the existence of a strong homology between these two proteins, CFP and P12, if not molecular identity. Control serum showed some immunoreactivity in the first chromatofocusing fractions, but it was so low that further characterization in subsequent purification steps was impossible. These results confirm and extend the previous hypothesis suggesting that CFP is normally present in serum in trace quantities but is in higher concentrations in subjects possessing the CF allele (20).

The structural characterization of CFP and its relationship with MRP-14, the protein found in rheumatoid arthritis that may be the antigen tracking with the CF allele (14), would be the next important steps to elucidate the role of these proteins. The most interesting data concerning these two proteins, CFP and MRP-14, are their increased concentrations in serum and plasma from heterozygotes, who are devoid of any clinical signs of CF (8, 14). We have recently shown (21) that the concentration of lactoferrin in plasma or serum, which supposedly reflects granulocyte activation, is significantly higher in individuals who are homozygous for CF than in heterozygous and control subjects, even in plasma samples from homozygotes with a normal number of neutrophils (2 to 6 g/L). But even though the CFP concentration was closely related to the increase of lactoferrin concentration in CF patients, there was no difference in lactoferrin concentration between sera with or without CFP in heterozygotes whose lactoferrin concentration was similar to that of controls (21). These results are in accordance with those of Hayward et al. (22), who measured "CF antigen," lactoferrin, and C-reactive protein (an indicator of acute inflammation) in sera from CF carriers and controls and found that the concentration of "CF antigen" was greater in CF heterozygotes than in healthy controls, whereas concentrations of lactoferrin and C-reactive protein in CF heterozygotes were in the normal range. Therefore, if the increased concentrations of CFP and (or) MRP-14 in CF patients are attributable to two components (one being the increase of granulocyte turnover found in active inflammation), the increases observed in heterozygous subjects are mainly related to a CF genespecific component, which remains to be identified.

Fig. 5. SDS–PAGE of purified CFP
Standards (Std): reference proteins (see legend of Fig. 4)
We thank J. P. Chazalette and C. Galabert for their clinical collaboration and fruitful discussion, and all donors of blood used in this work. D. Brock and C. Hayward are gratefully acknowledged for their gift of monoclonal antibodies and all reagents necessary to perform the immunosorbent assay. We thank C. Gianfilippo and E. Rubio for their skillful technical assistance, and A. Martinez (Pharmacia) for his help and advice regarding FPLC chromatography. This work was supported by a fellowship to C. Barthe from the Association Française de Lutte contre la Muco-vidose (AFLM) and by a "Contrat Jeune Formation" INSERM 88-09.

References