Improved Method for Detection of Cystic Fibrosis Protein in Serum Using the LKB Multiphor Electrofocusing Apparatus

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Summary

We have developed an improved method for the detection of cystic fibrosis protein (CFP). The method employs the LKB Multiphor to electrofocus whole serum, instead of the apparatus used in previous studies. Two basic modifications were necessary: (*1*) a pH 2.5–10.0 gradient instead of a pH 5–10 gradient, and (*2*) constant power for focusing the serum proteins instead of constant voltage. The first modification ensured adequate dissolution of the CFP-IgG complexes (or other precursor complexes which may liberate CFP). The second modification ensured a linear gradient (between pH 3.8 and pH 9.2), excellent resolution in the pH 8–9 region, and the separation of CFP within 2 hr without overheating of the gel.

Electrofocusing with the LKB Multiphor permits the detection of CFP in as many as 24 serum samples per gel. Results obtained from the analysis of 31 cystic fibrosis, 28 obligate heterozygote carrier, and 28 normal control sera indicate that CFP can be reproducibly and accurately detected in sera using the LKB Multiphor.

Speculation

Strict adherence to our methods of serum sample collection, processing, storage, and the methods described for the detection of CFP using the LKB Multiphor electrofocusing apparatus may ensure the successful use of our technique to detect CFP and to determine individuals with the CF gene.

INTRODUCTION

Previous reports from our laboratory have shown that a cystic fibrosis protein (CFP) can be detected in sera from most CF patients (homozygotes for CF) and heterozygotes for CF by analytical isoelectric focusing (11,12). A standardized assay for the detection of CFP has been reported and the specificity of CFP for the CF gene has been established in two separate studies (9,11). Previously we characterized CFP as a cationic protein with an isoelectric point (pI) near pH 8.5 and a molecular weight of 3,500-10,000 which can be isolated concomitantly with serum IgG (10-12,15). The physicochemical properties of CFP resemble those established for the "CF factors" described by researchers utilizing biological assays for their detection (4). In fact, we have recently shown that CFP is found only in those serum fractions which harbor a CF-specific ciliary dyskinesia factor (CDF), thereby supporting our proposal (14) that CFP may be similar to a CF-CDF which we have isolated (15).

The standardized assay for CFP detection previously proposed as a useful method for the routine determination of heterozygotes for cystic fibrosis (11), was developed for use with the electrofocusing equipment described by Awdeh et al. (3,7). The electrofocusing apparatus unfortunately is not readily available since it is not commercially produced (8,13). Therefore, we indicated previously (8,10,13) that we were exploring the use of the LKB Multiphor electrofocusing apparatus for the detection of CFP and comparing the results with our results obtained using the apparatus of Awdeh et al. (3,7).

In the present report the complete methodology for the detection of CFP using the LKB Multiphor is described. A color photograph is provided to clarify the results obtained with our method.

MATERIALS AND METHODS

Collection and processing of samples

Venous blood from normal individuals, patient controls with bronchial asthma, CF homozygotes, and obligate heterozygotes for CF were collected and processed as described previously (8,11,13). The patients with bronchial asthma served as controls to recheck the specificity of CFP for the CF gene. All serum samples were stored frozen at -70°C until analyzed by electrofocusing. Informed consent was obtained from all subjects (or in the case of minors from their parents) prior to obtaining a blood sample.

Quantitation of IgG

The concentration of IgG in each serum sample was determined by single radial immunodiffusion as described previously (11).

Isoelectric focusing

Isoelectric focusing in thin-layer polyacrylamide gels utilizing the apparatus of Awdeh et al. (3,7) was performed in a pH 5-10 gradient exactly as previously described (11). Additional details of the technique and precautions concerning the method and sample collection and processing have recently been reported (8,13).

Isoelectric focusing in thin-layer polyacrylamide gel using the LKB Multiphor (Model 2117-411, LKB Produkter, Broma, Sweden) was performed by modification of the procedures described previously (8,11) for use of the apparatus of Awdeh et al. (3,7). The electrode solutions and gel composition were identical to those described (8) except that a gradient of pH 2.5-10.0 was used (2.0% W/V Ampholine carrier ampholytes, pH 2.5-4.0 and 3.5-10.0; LKB Produkter) instead of pH 5-10 (11). Preliminary experiments were run to compare the results obtained using the LKB Multiphor with pH 5-10 and 2.5-10.0 gradients. The use of a more acidic pH near the anode favored the demonstration of CFP. Prolonged exposure of the serum samples to an acidic pH and 4 M urea is also thought to favor the demonstration of CFP (8), possibly due to the dissolution of IgG-CFP complexes or the alteration of some other precursor that may liberate CFP. The apparatus of Awdeh et al. (3,7) requires 18-24 hr to focus (11,12), whereas the LKB Multiphor requires only 2 hr (see below). Since the samples are exposed to acidic conditions for only 2 hr, a more acidic pH may enhance liberation of CFP (8,13,15).

The gel was prefocused at 350 V (constant voltage) for 30 min. Samples were applied onto 6X8 mm pads of Whatman 3MM chromatography paper placed with the lower edge of the pad 5 mm from the anodal strip. The gel was focused at 35 W (constant power) for 1.5 hr by using an LKB Model 2103 power supply. The sample application pads were not removed until completion of the run.

A constant power source was found to be necessary, since to obtain adequate resolution of the protein bands at pH 8-9, voltages in excess of 1000 V must be used. Constant power control helps prevent localized overheating during the run. In addition, isoelectric focusing was performed at 4°C using a Lauda K2R controlled temperature circulating water unit (Brinkman Instruments).

All serum samples were analyzed using a volume of serum which contained 300 μg IgG, in accordance with our standardized protocol for CFP detection described previously (11).

Reading of the pH gradient, fixing, staining, and destaining of the gel were performed as described previously (11) with the following modification. Instead of a prior overnight fixation with 12% trichloroacetic acid (TCA) and 5% sulfosalicylic acid (SSA), the gel was fixed and stained simultaneously at 80°C for 60 min. The staining solution consisted of 135 g SSA, 450 g TCA, and 4.5 g Coomassie Blue R250 in 2790 ml water plus 1350 ml methanol. This procedure was used only for gels obtained from the LKB Multiphor.

RESULTS

Typical results obtained for the protein banding patterns of CF homozygote, heterozygote, normal healthy control, and normal patient control (patients with bronchial asthma) sera are shown in Fig. 1. The pH gradient as estimated with a surface pH electrode at 4°C is shown in Fig. 2. Analysis of 61 samples positive for CFP showed that this protein had a pI of 8.57 ± 0.02 (Table 1). This value agrees closely with the pI of 8.46 ± 0.05 previously reported using the apparatus of Awdeh et al. (3,7). CFP can be identified as the lower band of a band doublet focusing within the most cathodal centimeter of the stained serum protein pattern (about 7.8 cm from the bottom of the sample application pad, or 8.3 cm from the top of the anodal strip) (Fig. 1). Figure 3 shows an enlargement of the area containing CFP to clearly demonstrate its location.

In detecting CFP, confusion between distinct banding and diffuse background staining must be avoided. The normal control shown in Fig. 3 (the one on the left) appeared to contain a faint CFP band, but comparison of the sample with other CFP-positive samples allowed us to differentiate this normal control as being CFP-negative (Figs. 1 and 3). In addition, when a volume of serum containing 400 μg of IgG was run, this control remained negative, confirming that it was CFP-negative at 300 μg.

Figure 4 shows a color photograph of pH 2.5-10.0 gels containing 2 CF, 2 heterozygote and 3 normal control samples. The band doublet indicating CFP is marked by lines. A multitude of protein bands and excellent resolution in the pH 8-9 region were obtained by electrofocusing with our method. CFP is faint compared with many other serum protein bands on the gel. Attempts to provide adequate black and white photographs of gel sections containing CFP normally result in partial loss of protein patterns elsewhere (cf. Figs. 1 and 4). Therefore the present color photograph is also provided.

The results of electrofocusing 28 normal control sera, 28 heterozygote sera, 31 CF homozygote sera, and 8 sera from patients with bronchial asthma using the LKB Multiphor and our standard assay conditions are shown in Table 1. Three of the 36 controls (normal plus patient controls, Table 1) were CFP-positive, a frequency that does not differ significantly from the expected value of 2 in 40, suggesting as in previous studies that CFP is specifically diagnostic for a metabolic defect uniquely associated with the CF gene. Complete agreement was found between the results in Table 1 and those obtained using the apparatus of Awdeh et al. (3,7). Therefore, we feel that the LKB Multiphor can be used to screen sera for CFP, and that the bands shown in Figs. 1, 3, and 4 is analogous to the protein band described as CFP in previous publications. In addition, we have recently established the molecular weight range of CFP detected by the LKB Multiphor as 1,100-13,700 (15).

DISCUSSION

Since the publication of our original report (11) describing the methodology for a new diagnostic test to detect individuals who harbor the CF gene by analyzing whole serum using analytical electrofocusing, three attempts to reproduce our findings have been published (2,5,6).

Smith et al. (5) and Thomas et al. (6) claim to have analyzed CF homozygote and normal control sera using electrofocusing methods exactly analogous to ours (11,12); however, neither group used the apparatus of Awdeh et al. (3,7) to perform their studies. The investigators could not detect CFP or distinguish between CF homozygote and control sera.

In previous publications (8,13) we have indicated three probable reasons for their lack of success: (a) the apparatus used, (b) improper collection, processing, and storage of samples, and (c) the methodology used. Neither Smith et al. (5) nor Thomas et al. (6) presented photographic evidence that they ever reproduced the protein patterns shown previously (11), yet both groups went on to modify the technique extensively in attempts to increase the resolution. Their alterations, although resulting in serum protein patterns with better resolution outside the pH 8-9 region, failed to show obvious differences between CF homozygote and normal control sera. It is impossible for us to suggest exactly why these investigators were unable to reproduce our results, since their communications lack sufficient detail to permit a careful comparison of the data.

A few points should be made concerning the detection of CFP. First, localization of CFP in a gel based solely on recorded pI values should be avoided. Measurements of pH can differ between laboratories. Therefore, to detect CFP an investigator should evaluate the entire uppermost centimeter of the stained serum patterns rather than just the region at pH 8.5. Second, standardization of the assay using our procedure is essential for the detection of CFP in most CF homozygote and heterozygote samples (8,13; Table 1). Finally, extreme care in sample collection, processing, and storage must be exercised (8,11,13,14). The samples should be processed at 4°C from the time of removal of the blood to the time of serum storage at -70°C, and serum samples should not be left at 4°C upon thawing for analysis but should only be thawed immediately before electrofocusing.

In addition to Smith et al. (5) and Thomas et al. (6), Altland and co-workers (2) have attempted to detect CFP. Altland et al. (2) utilized our earlier methodology (12) (i.e., prior to standardization and development of improved methods for fixing and staining to reduce background). Although the intense background stain due to high variability within the IgG region (2) made it impossible to detect CFP, these investigators were able to demonstrate a low-molecular-weight component found only in CF homozygote and heterozygote sera by performing a second step of electrophoresis utilizing the serum protein fraction focusing at pH 8-9 (1,2). Whether the cationic protein described by Altland et al. (1,2) is our CFP is unknown at present; however, their findings add support to our claims that a protein with a molecular weight of 3,500-10,000 and a pI near pH 8.5 is found at a concentration high enough for detection in microliter quantities of sera from most homozygotes and heterozygotes for CF (10,11,15).

It is possible that normal control sera also contain small amounts of CFP; however, most normal control sera analyzed were CFP-negative when a volume of serum containing 300 μg of IgG [or more (11)] was used. In addition, the number of CFP-positive control sera found was not significantly different from an expected carrier frequency of 1 in 20 (ref. 16) in the Caucasian population [9 out of 110 and 2 out of 28 (refs. 10,11; Table 1)]. If normal sera do contain low levels of CFP, then the development of a radioimmunoassay (RIA) to detect CFP may allow us to quantitate it and establish cutoff levels defining normal and CF-genotypes. We are working to refine an RIA for this purpose.

SUMMARY

To alleviate some of the problems encountered by other investigators in detecting CFP and to provide a more rapid assay for its detection, we adapted our previously described technique (11) for use with the LKB Multiphor. Our results suggest that the LKB Multiphor can be used to detect CFP and therefore screen sera for the CF genes rapidly in a single step. Fifty-eight of 59 homozygote and heterozygote sera were CFP-positive as compared with 2 of 28 normal control sera. Only 1 of 8 patients with bronchial asthma included as patient controls was CFP-positive, suggesting as in other studies that CFP is a specific marker for CF (9,11).

Two modifications were required for detection of CFP using the LKB Multiphor: (a) a pH 2.5-10.0 gradient instead of a pH 5-10 gradient, and (b) the use of constant power for focusing to ensure that adequate resolution and a linear pH gradient (between pH 3.8-9.2) are obtained in only 2 hr without overheating of the gel. The complete methodology of the technique is described. Extreme care in sample collection, processing, and storage should be exercised, and the method should be followed exactly as stated to assure accurate and reproducible results.

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TABLE 1

Serum Positivity for Cystic Fibrosis Protein (CFP) as Determined by Electrofocusing with the LKB Multiphor

Serum type	No. tested	Donor age (yr)		IgG level (mg/100ml)		Sex		CFP present[a]		
		Mean ± S.D.	Range	Mean ± S.D.	Range	M	F	+	±	-
Normal control	28	29 ± 8	12-48	1034 ± 269	601-1650	11	17	2	0	26
CF heterozygote	28	34 ± 6	20-57	962 ± 145	600-2000	9	19	25	2	1
CF homozygote	31	12 ± 4	2.5-25	1298 ± 518	550-3600	19	12	29	2	0
Asthma control[b]	8	22 ± 8	10-32	981 ± 282	660-1500	5	3	1	0	7

[a] +, CFP band present; ±, CFP band present but faint; -, CFP band absent. Analysis of serum sample containing 300 µg IgG.

[b] Normal patients with bronchial asthma.

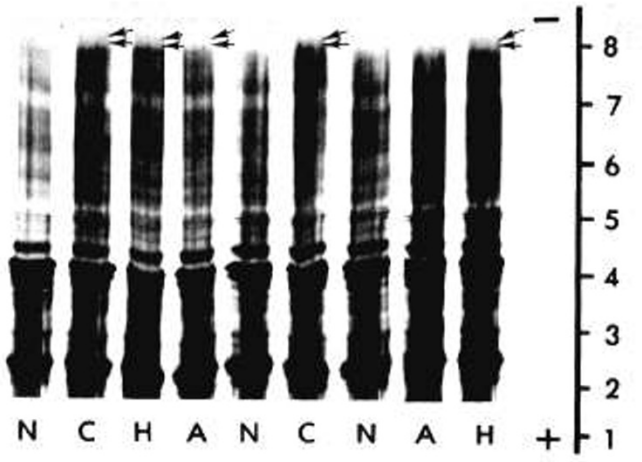

Fig. 1. Results of isoelectric focusing of whole serum in a pH 2.5-10.0 gradient. N, normal control; C, CF homozygote; A, patient with bronchial asthma; H, obligate heterozygote. All serum samples contained 300 μg IgG. The scale on the right indicates the distance in centimeters from the bottom of the sample application pad (0.5 cm from the top of the anodal strip). The arrows indicate the position of a band doublet found in CFP-positive samples. The lower band is CFP. The circle at the bottom of the fourth sample from the left is an artifact. The figure shown is a composite of one gel with seven samples and another (far right) with two samples.

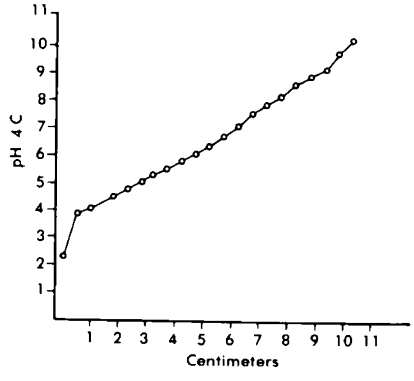

Fig. 2. Results of pH measurements at 4°C from anode to cathode on the surface of a polyacrylamide gel similar to the one shown in Figure 1. Abscissa: centimeters from the anode.

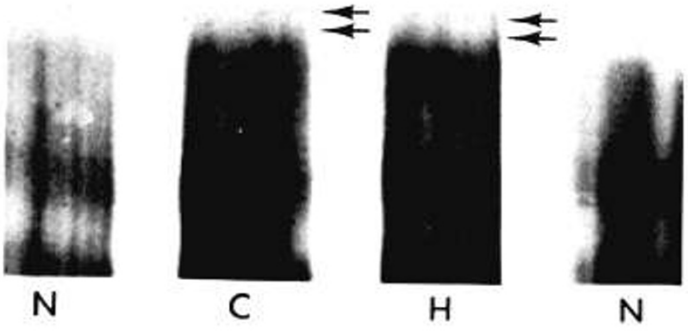

Fig. 3. Enlargement of part of the most cathodal region of the polyacrylamide gel shown in Figure 1. Samples shown (N,C,H,N) are the first three and the fifth samples in Figure 1 (left to right). Arrows clearly depict two distinct bands found in CF homozygotes and heterozygotes but not in the normal controls. The lower band is CFP. Although the two N samples appear lighter, the other N and A samples shown in Figure 1 are as dark as the C and H samples, yet fail to show CFP.

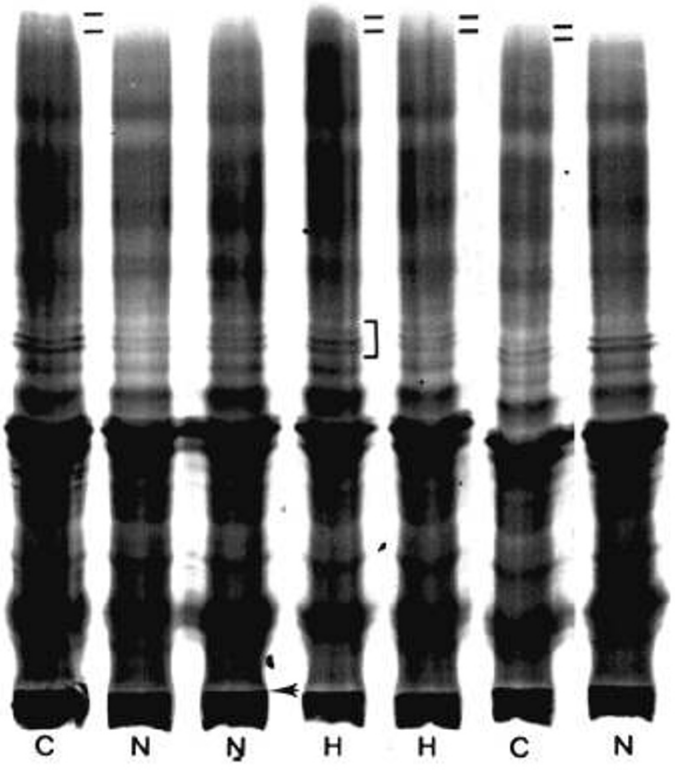

Fig. 4. Color photograph of gels showing the complete serum protein patterns obtained for 2 CF homozygote, 2 heterozygote, and 3 normal control samples after electrofocusing in a pH 2.5-10.0 gradient. Note that a distinct doublet (lines) is observed in all homozygote and heterozygote samples (CFP is the lower line). The normal samples do not have a distinct band doublet but do have diffuse background banding. All samples contained 300 μg of IgG. The lower bracket encloses 3 bands. These can be used to facilitate comparison of the color print with the black and white gel in Figure 1 and to compare the different samples run. Arrow at bottom indicates the top of the indentation left by the sample application pad.

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