Letter to the Editor: Is Cystic Fibrosis Protein a Diagnostic Marker for Individuals Who Harbor the Defective Gene?

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It has been almost 5 years since the publication of our original report describing a "cystic fibrosis factor" (now designated "cystic fibrosis protein" or CFP) detected in the serum of *most* homozygotes and heterozygotes for cystic fibrosis (CF) when analyzed using isoelectric focusing in thin layer polyacrylamide gels (20). Based on our results from 24 positive samples, the CFP was initially characterized as a protein with an isoelectric point (pI) of 8.41 ± 0.10 (20). Later, we developed rigidly standardized conditions for the analysis of sera by electrofocusing, including improved methods for fixing and staining the gel and conditions for the collection, processing, and storage of the sera, and reported a revised pI of 8.46 ± 0.05 for CFP (17, 19). Since the CFP band was identified in sera from more than 90% of 85 individuals homozygous and 66 heterozygous for the CF gene but not in sera from 92% of 110 normal control subjects when tested under standardized and controlled conditions, the CFP was felt to be a marker useful for the detection of the majority of individuals who harbor the CF gene (17, 19). (CF is probably a heterogeneous syndrome, one subgroup of which may be negative for CFP.) Subsequent studies determined the specificity of the CFP for CF by examining sera from individuals with other diseases (over 25 different diseases were represented) (14, 19). With the possible exception of individuals afflicted with certain forms of leukemia (monomyelocytic or monocytic leukemia), the CFP was determined to be a marker specific for cystic fibrosis (19).

As indicated by James Scholey and his coworkers (4), the existence of CFP and its possible use as a marker for the detection of the CF gene have been topics of considerable controversy. Much of the controversy arose from reports published by investigators who failed to detect a unique protein band with a pI between pH 8.4 and 8.5 in sera from individuals known to be homozygotes or obligate heterozygotes for the CF gene (6-8). Those reports stated either that the methods used were analogous to those used in our laboratory (19) or that they were improvements over our method (6-8). Nonetheless, neither group of investigators could detect CFP.

We have always maintained that problems concerning detection of CFP in serum can arise from three different sources: 1) differences in the apparatus used for electrofocusing; 2) slight or subtle differences in the collection, processing, and storage of the serum samples; and 3) changes in the methodology used for electrofocusing (11, 22).

In response to earlier reports concerning attempts to detect CFP in serum (6, 7), we provided further details concerning several aspects of our original electrofocusing technique and emphasized that extreme care in sample collection, processing, and storage must be exercised. Since the results of most of our earlier work (14, 17, 19, 20) were obtained with a custom-built electrofocusing apparatus, we also adapted our method for use with the LKB Multiphor electrofocusing apparatus (10, 22). In the later report (10), we speculated that strict adherence to our methods of serum sample collection, processing, and storage (19) and the methods described for the detection of CFP using the LKB Multiphor

electrofocusing apparatus (10, 11, 22) would be necessary to ensure the successful use of our technique to detect CFP and to identify individuals with the CF gene.

The recent report by Scholey *et al.* (4) confirms that CFP is present in the majority of CF sera and that it can be detected by our methods (11, 19, 22). Before discussing whether the CFP can be used as a diagnostic marker for cystic fibrosis, we would like to examine more closely some of the requirements of the method which seemed to have been critical for the detection of CFP by Scholey *et al.* (the band discussed is only assumed to be CFP since no photograph documenting their results was provided) (4). Table 1 summarizes the key points and relevant comments concerning sample collection, processing, and storage; the electrofocusing method; and fixing, staining, destaining, and viewing of the gel. The information in Table 1 is provided only to supplement the details and requirements of our technique for use with the apparatus of Awdeh *et al.* (2) and the LKB Multiphor as stated in previous communications (10, 11, 19, 22), and should not be construed as superseding our previous reports (10, 11, 19, 22), unless specifically indicated in the "Comments" column. When our methods are compared with those of Scholey *et al.* (4) and other investigators (6-8), several items seem particularly noteworthy. 1) Clearly, the procedures employed for serum sample collection, processing, and storage are critical and should not be varied as some investigators have done (8). 2) The composition of the gel seems to be extremely important, whereas the apparatus used for electrofocusing does not. 3) The cathode solution must consist of an alkenyldiamine, whereas the nature of the acid solution used at the anode is less critical. 4) The background staining of the gel can make detection of CFP by visual inspection of even trans-illuminated gels difficult. In our earlier work (20) we made note of this fact, and in our later communication (19) we indicated that the gels should be viewed with a colored filter. Neither Altland *et al.* (1), Smith *et al.* (6), nor Thomas *et al.* (7, 8) indicated that they used a colored filter or x-ray viewing box to inspect the stained gels, although they did indicate that the background staining made it difficult to detect the CFP band (1, 7, 8). As Scholey *et al.* (4) point out, the use of a colored filter is essential for the detection of the CFP. 5) The method used to fix and stain the gel is also critical. CFP is a low molecular weight protein (mol wt 3,500-10,000) which could possibly polymerize but still is readily lost from the gel unless it is fixed using trichloroacetic acid (TCA) and sulfosalicylic acid (SSA) at 4° before staining (19) or concurrently fixed and stained at 80° employing both TCA and SSA (10). The temperature and concentration of the acids are both important variables. Both acids should be used if urea is in the gel.

We would like to point out that Scholey and his coworkers adhered closely to most of our guidelines during their attempts to detect CFP. They were successful in localizing what appears to be the CFP band, which was not detected in any of their 16 control sera tested and was seen in 7 out of 10 CF sera. The percentage of CFP positive control sera found by Scholey *et al.* (4) agrees well

Table 1. Comparison of methods used by Wilson *et al.* (10, 11, 17, 19, 22) and by Scholey *et al.* (4) for detection of CFP in serum by isoelectric focusing

	Wilson <i>et al.</i>	Scholey <i>et al.</i>	Comments
Serum samples			
Collection and processing	Blood clotted 4–6 hr at 4°	Identical method	Improper handling of sera could lead to decreased concentration of CFP (22)
Storage	Frozen in aliquots at –70° (19)	Identical	Same as above
Standardization of the volume used for focusing	Amount of IgG determined by single radial immunodiffusion	Identical	Possible source of error if IgG concentration is actually lower than determined
Amount of serum used for focusing	Volume of sample containing 300 µg IgG	Identical	If sample contains less than 300 µg could yield false negative result
Isoelectric focusing reagents and equipment			
Apparatus	LKB Multiphor or apparatus of Awdeh <i>et al.</i> (10, 19)	Brinkmann-Desaga	May indicate that apparatus used is not critical
Gel composition	5%T–3% C containing 4 M urea (19)	Identical	This gel composition favors demonstration of CFP. It retards the movement of larger macroglobulins including CFP-IgG or other CFP-macroglobulin complexes. Acid pH at the anode and 4 M urea favor dissolution of complexes and release of CFP. Probable reason why CFP focuses as a single band (10, 11)
pH gradient	2% (w/v) Ampholine carrier ampholyte, pH 2.5–10 with LKB Multiphor, pH 5–10 with apparatus of Awdeh <i>et al.</i> (2)	2% (w/v) Ampholine carrier ampholyte, pH 3.5–10	Possible incomplete dissolution of CFP-macroglobulin complexes can occur, dependent on voltage and time allowed for focusing relative to pH at node (10, 11, 22)
Electrode solutions	5% TEMED (N,N,N',N'-tetramethylethylenediamine) and 2.83% H ₃ PO ₄	0.3 M Ethylenediamine and 0.2 M H ₂ SO ₄	A solution of an alkenyldiamine at the cathode is critical since CFP may contain a polyamine or a derivative thereof as part of its structure (18). Composition of anode solution is less critical, but H ₃ PO ₄ is preferred
Polymerization catalyzers	Ammonium persulfate and TEMED	Identical	Ammonium persulfate preferred over riboflavin
Electrofocusing conditions			
Gel prefocusing	Constant current or constant voltage, depending on system used	Constant current	Conditions not critical, either method can be used (10, 19)
Running conditions	Constant power or constant voltage, depending on apparatus and gradient used. Time dependent on gradient and whether constant power (10) or voltage (19) used	Constant voltage for 6 hr	Conditions must be varied according to the apparatus and gradient used (2, 11)
Sample pads	Remain on during entire run, located near anode <i>not</i> cathode	Not stated	Pads should remain on and should be placed at the anode (11, 19)
Fixing and staining of gel			
Fixing	TCA and SSA used (10, 19)	Identical	Fixation with concentrated TCA and SSA is critical to prevent loss of CFP band. Evaporation of methanol at 80°C creates a level of SSA and TCA which is optimal (11, 19)

Table 1, Continued

	Wilson <i>et al.</i>	Scholey <i>et al.</i>	Comments
Staining	Coomassie brilliant blue R-250 (20)	Coomassie brilliant blue G	Apparently, either stain is adequate, but R-250 may be better
Destaining	Ethanol/water/acetic acid or methanol/water/acetic acid (10, 19)	Identical	CFP is a faint blue band which can be overly destained and lost (1, 11). This is especially problematic if TCA and SSA are not used to fix the proteins first
Recording pH gradient and viewing stained gel pH gradient	Recorded at 4° by reading of gel surface with a dual membrane microelectrode	Small sections of the gel eluted and read with a dual membrane microelectrode	Exact method of measuring pH not critical, since distance of CFP from anode (cm) is more consistent
Viewing and photography	Gel placed on x-ray viewing box and viewed through orange filter (19) ¹ or yellow filter (10). ² Gels photographed with Kodak Panchromatic Plus-X film using the same filters	Gels photographed with Kodak Panatomic X film. CFP identified best when examined on x-ray viewing box with Wratten 25 A red filter.	The use of a colored filter and x-ray viewing box is critical for detection of CFP (19). Background staining often obscures CFP (1, 4, 8, 19) when viewed without filter

¹ Tiffen Photar orange filter, series 7.

² Kodak number 8.

with our previous values of 9% (19) and 7% (10). The frequency of CFP-positive CF sera found by them is a bit too low, as we would expect 9 out of 10 CF patients to be CFP-positive. There are several possible reasons for this latter discrepancy.

1. *Not analyzing enough sera.* It is possible that the concentration of IgG determined by single radial immunodiffusion could be erroneously high (relative to values determined in our laboratory), resulting in the analysis of smaller volumes of serum. We suggest that a volume of serum containing 400 µg IgG should be analyzed to check this point. The volume of the samples studied should then range between 15 µl and 45 µl. (A sample volume up to 50 µl can be electrofocused with the LKB Multiphor electrofocusing system (21), and we assume that the Brinkmann-Desaga apparatus can also accommodate samples containing up to 50 µl serum.)

2. *Problems in distinguishing samples that are borderline.* Even when each sample analyzed contains 300 µg IgG, some samples are still only faintly positive (+/-) for CFP. Increasing the volume of serum analyzed, running known standard CF homozygote, obligate heterozygote, and normal control samples for comparison with each sample and increased experience with screening for CFP should lead to successful evaluation of borderline samples.

3. *Genetic heterogeneity in cystic fibrosis.* Current evidence from several lines of research indicates that the clinical entity known as cystic fibrosis may be a syndrome rather than a single disease entity; *i.e.*, there may be several forms of CF. (This would be analogous to hemophilia, which was originally thought to be a single disease but later shown to include two separate entities, one due to factor VIII deficiency and another to factor IX deficiency, and Wiskott-Aldrich syndrome, in which some patients appear to have monocyte defects but others have normal monocytes.) For example, in our own work on the α_2 -macroglobulin in CF we have clearly delineated two types of CF patients (16, 18); and with regard to the CFP, there is always a small population of "sweat test-positive" homozygotes for CF who, along with their obligate heterozygote parents, are negative for CFP (10, 17, 19). This apparent discordance might be due to problems inherent in the sweat test, which is known not to be 100% accurate (23), but is used in conjunction with other clinical findings in the diagnosis of CF. We suggest, however, that this population of CFP-negative homozygotes may rather represent individuals with a possibly more mild form of the disease. Indeed, impressive evidence for

genetic heterogeneity in CF and for the contention that CF may represent a syndrome rather than a single disease is provided by the studies of Danes and coworkers (3).

Although we can appreciate the fact that our isoelectric focusing assay for the detection of CFP may be complex and require painstaking attention to detail, as noted by Scholey *et al.* (4), we do not feel that this precludes the use of CFP in screening for the defective CF gene. The electrofocusing technique, once mastered, becomes easier to perform the more it is used. Moreover, several samples can be analyzed on each gel (10, 19), which greatly reduces the time required for screening a given population of samples. We do agree with Scholey *et al.* (4) that there is no absolute correlation between the presence of the CFP band and the clinical status of the CF patient as judged by Shwachman scoring *when the samples are analyzed using a standardized amount of serum* (*i.e.*, containing 300 µg IgG) (19). We have, however, found some correlation between the *amount* of CFP in serum and the severity of the disease in CF patients as measured by Schwachman scores when samples containing decreasing volumes of serum were screened (17). A good correlation was found for patients with severe forms (Shwachman score of 40 or below) or very mild forms of the disease (scores of 71–100), but the correlation seemed to dissolve for those with Shwachman scores of 41–70 (mild to moderate) (17). We must agree, therefore, that total concordance between the presence of the CFP marker and the pathogenesis of CF may never be found. Indeed, this is not surprising, since CFP is also found in serum from obligate heterozygotes, who do not present any of the clinical symptoms commonly associated with CF, at least not to a degree that requires treatment (19, 20), except possibly a predisposition to certain allergies (9, 21).

In order to determine the possible relationship of CFP to the disease process in cystic fibrosis, we previously initiated investigations to explore the biological activity of CFP and its possible metabolic relationship to the CF-ciliary dyskinesia factors (17, 18, 21). In addition, we have been studying the possible role of abnormal regulation of serine proteases by the α_2 -macroglobulin found in CF plasma in the generation or abnormal metabolism of C3a, kinins, CFP, and ciliary dyskinesia factors (16). Recently, using short term leukocyte cultures and long term lymphoid lines (12, 13, 15), we have found evidence that α_2 -macroglobulin may be partly responsible for controlling the metabolism of C3a and the ciliary dyskinesia factors, and that it is most likely also

responsible for controlling the metabolism and function of CFP. Interestingly, it has been shown that monocytes secrete α_2 -macroglobulin in culture, and we have suggested previously that an abnormality in protein production by granulocytes or monocytes may be implicated as a possible reason for CF factor production or accumulation in individuals homozygous or heterozygous for the CF gene (19).

Since the obligate heterozygote carrier expresses only a partial defect in α_2 -macroglobulin, as evidenced by electrofocusing (16, 18) and other techniques (5), the carrier may be essentially asymptomatic *in vivo* due to the existence of other as yet unknown mechanisms which compensate in part for the α_2 -macroglobulin defect. *In vitro*, these compensatory mechanisms may not operate, and therefore the gene defect (*i.e.*, the generation of CFP and ciliary dyskinesia factors by cells in culture) may be fully expressed in both the obligate carrier and homozygote for CF (20).

The exact nature of CFP and the other ciliary dyskinesia factors is only partially known, and all aspects of their metabolism are not yet understood. In addition, the role of α_2 -macroglobulin in the maintenance of normal homeostasis is only now beginning to be understood. Therefore, it may be some time before we comprehend the incomplete concordance found between CFP and the pathogenesis of CF.

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