Cystic Fibrosis Protein, a Confirmed Diagnostic Marker for Detecting Heterozygote Carriers: Significance in Relation to Future Screening and to a Proposed Primary Defect in Alpha₂-Macroglobulin

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The report by Tully et al. (17) unequivocally demonstrates that when our procedures for sample collection, processing, and storage (26,27) are followed and when our methods for the analysis of sera by isoelectric focusing (IEF) are adhered to (19,24,26) without significant variation (24), then the cystic fibrosis protein (CFP) is readily detected in all cystic fibrosis (CF) genotypes at a frequency close to that reported by our group previously (26). In addition, the results of Tully et al. (17) also confirm our finding that there exists a small (less than 10%) subpopulation of individuals diagnosed as having CF who are CFP negative. This population of "CF patients" may represent either (a) individuals who have mild symptoms relative to all major problems associated with the disease; (b) individuals who do not have CF and who were misdiagnosed primarily due to heavy reliance by some physicians on "sweat test" results, which may be called positive when only borderline positive or which may not be accurate at all [depending on the method employed to measure the electrolytes in sweat, false positive results may be as high as 5% and false negative results as high as 15% (5)]; or (c) individuals who have CF but who present a different propensity from CFP-positive patients for the development of a certain aspect of the disease. For example, hypothetically it may be found that CFP-negative patients do not present extensive pancreatic insufficiency or may have more extensive pulmonary involvement. If either (a) or (c) is correct, it certainly would appear that CF is a syndrome which involves at least two potentially definable disease entities. If CFP-positive and -negative CF patients are carefully compared using multiple criteria involving both the primary and secondary clinical complications normally observed in patients with CF (8), it might be possible to clearly delineate both types, and this could add to our understanding of the role of CFP in the disease. Such documentation might also be helpful to the physician in determining the best therapeutic regimen to follow, and may ensure more effective treatment of the patients.

The present confirmation by Tully et al. (17) of our findings regarding CFP positivity in both carriers and patients, and the previous confirmation by Scholey et al. (11) concerning the detection of CFP in CF patients using our electrofocusing methods (19,26), provide strong evidence that our results are reproducible and accurate and are not "suspect" as stated by Thomas, Merritt and Hodes (16). Indeed, the fact that CFP has now been confirmed as a valid marker for the CF gene suggests that the inability of some investigators (for example, references 14 and 16) to confirm our findings must be related to their failure to adhere to the guidelines proposed previously (19,24,26,27). Alternatively, we may have failed to emphasize some part of the technique that is crucial to reproducing our results, and this may have led some (but not other) investigators to believe that this part could be changed without jeopardizing the successful use of the method. Before discussing the potential of CFP as a screening test for detecting carriers of CF, it may be instructive to other investigators who elect to use our method for screening to examine the methodology set forth in the report by Thomas et al. (16), to determine what "pitfalls" led to their failure to reproduce our work.

Several changes in the methods for sample processing prior to isoelectric focusing (IEF) were made by Thomas et al. (16): (a) the blood was allowed to clot for 2-4 hr, not 4-6 hr; (b) the samples were stored at -20°C or -70°C, not just -70°C (26,27); and (c) the concentration of IgG was not determined in all samples analyzed, so that the standard assay conditions were not employed for all samples tested. Two obvious omissions in the execution of the technique were also evident (16): (a) standards known to be CFP-positive were not employed [at least CFP was never found in any samples reported in reference 7, although, as stated in an earlier abstract (15), some "apparently CFP-positive" specimens were at hand]; and (b) a colored filter was not used to examine the gels. Instead, changes were instituted to "improve" our method for CFP detection. While these changes may have led to better resolution, clearly they did not allow Thomas et al. (16) to detect CFP, for two basic reasons: (a) their use of nonsieving gels, which failed to retard IgG-CFP complexes and probably inhibited the release of the CFP from IgG (24), and (b) their use of 1.0 to 1.2% Ampholine(R) carrier ampholytes in their modified method (a concentration of 2.0 to 3.0% is necessary for proper focusing of the CFP).

Tully et al. (17) mention two other important points. First, they emphasize the fact that the protein banding pattern in the upper few millimeters of an individual CFP-positive sample may vary somewhat. Second, they strongly feel that known positive and negative controls must be used to assist in the interpretation of specimens being screened. I agree with the first point and offer Figure 1 as further evidence for this point. Figure 1 shows the patterns from two CF patients. Each sample presents a unique pattern in the uppermost centimeter of the profile, yet both are CFP positive and distinctly different from the normal controls (all samples were analyzed using an amount of serum containing 300 µg IgG). I also agree with the second point and would be happy to collaborate in an investigation to determine whether lyophilized sera might be usable as standards. Lyophilized samples may be stable as pertain to prevention of the destruction of CFP, and in theory the sample could be shipped from anywhere in the world to my laboratory (or others) to be screened. Recent research in this laboratory, however, indicates that there is a simpler way to stabilize the sample and ensure that CFP, if present, is not degraded.

Previously, we showed that protein A conjugated to Sepharose CL-4B could be used to remove CF ciliary dyskinesia factors (CDF) from any biological fluid containing IgG (or a structural homolog of IgG). IgG can of course be added to fluids which do not normally contain it. CF-CDF are believed to bind to IgG (25). As part of our continuing efforts to characterize the CFP we have recently determined that incubation of sera with protein A - Sepharose CL-4B removes the CFP along with IgG (18). Examples of the banding profiles obtained for sera before and after incubation with protein A are shown in Figure 1, as are examples of profiles of the material released from the protein A gel itself. The removal of the CFP-IgG complex using protein A stabilizes CFP, since when the Sepharose gel is washed with buffer or saline enzymes are removed which might otherwise inactivate CFP (27). In addition, the amount of IgG can be quantitated, as the binding capacity of protein A for IgG is known (25); this will undoubtedly be of value in the maintenance of standardized conditions for screening.

A Committee of the National Research Council, established for the study of inborn errors of metabolism, published several criteria for screening

tests in a recent monograph on genetic screening (29). Briefly, they felt that screening tests should (a) be reliable, repeatable, and accurate; (b) have their sensitivity and specificity defined; (c) be capable of automation for purposes of efficiency and economy; (d) be subject to a minimum of clerical error; and (e) ensure that processing and delivery of the sample will not compromise the validity of the test through significant alteration of the properties of the sample. It is my feeling that with the recent confirmations of our results, the IEF assay which we have developed now meets criteria (a) through (d). No other assay system currently available can detect carriers of the CF gene as efficiently. In addition, assuming that either lyophilization or adsorption of the sample with protein A - Sepharose CL-4B can be used to preserve the structural integrity of the CFP, then criterion (e) can easily be achieved. The use of protein A offers a simple way to ensure that the validity of the test is not compromised through significant alterations in the biological (or, more important for IEF, the physicochemical) properties of the marker used for detection, while allowing for the shipment of either standards or samples to be screened. Most laboratories have centrifuges, and the amount of protein A needed per sample would not make the cost for its use prohibitive.

When a substance has been shown to be a marker for carriers of a particular disease, certain questions immediately arise. Among the questions which should be asked are: (a) Can the marker be used for antenatal detection of fetuses which harbor the defective gene? (b) Can the marker be used, in combination with others if necessary, to distinguish the homozygous from the heterozygous state? (c) Since the marker is found in carriers and not normal unaffected individuals, and thus probably is related to the basic defect responsible for the disease, what information concerning the basic nature of the gene defect can we gain from knowledge about this marker or from its metabolic relationship to other known abnormalities associated with the disease manifested *in vivo* in the proband or in *in vitro* studies using cells from the proband or carrier? To begin to address these questions properly one must have some conception of the relationship of the marker (in this case CFP) to the known characteristics of the disease.

Figure 2 presents a scheme which attempts to link together what are, in this author's conception, the more important clinical, biochemical, and pathophysiological findings in cystic fibrosis. In this scheme a defect in the alpha₂-macroglobulin (A₂M) synthesized by individuals who harbor the CF gene is considered to be a primary defect, and abnormal regulation of the activity of certain serine, thiol, or carboxyl proteases by A₂M is felt to be responsible for the presence of increased amounts of CFP and other biologically active substances in the body fluids (such as C₃a or C₅a) (22,23). Alterations in the activity of these enzymes can lead to the other changes noted (see additional explanatory statements in the legend of Figure 2). Certain clinical problems are felt to occur by more than one mechanism. For example, the hallmark of the disease, namely, an abnormal electrolyte composition of the secretory fluids found in patients with CF, is envisioned to result from at least two mechanisms: (a) by the direct action of CF factors on ion transport, and (b) through disruptions in stimulus-coupled secretion or reabsorption of electrolytes by the exocrine glands due to an alteration in membrane receptors (Figure 2). The possibility that CFP or the CF-CDA (25) can have a direct effect on ion transport is derived from the fact that these substances are polycations and from documentation that polycations can mimic the effects produced by hormones, as they can stimulate or inhibit adenylate cyclase depending on their concentration (28). In addition, a direct defect on A₂M-enzyme regulation could lead to a third mechanism for abnormal electrolyte levels, involving the defective metabolism of a hypothetical factor called "kinin-E" (3). Dann and Blau have proposed that kinin-E initiates ion reabsorption in exocrine glands (3), and that it is missing or decreased in the proximal ducts of CF glands, due to a deficiency in an arginine esterase activity believed to activate kinin-E (3). Obviously, in my scheme, A₂M would be responsible for controlling the arginine esterase.

The impact of defective A₂M-enzyme interactions on the disease process is probably exacerbated by the fact that another major protease inhibitor, namely, alpha₁-antitrypsin, may transfer inhibited proteases such as trypsin or elastase to A₂M (9). Thus, in CF certain proteases would be completely immune to regulation, especially during acute or chronic illnesses. Certain secondary defects such as alterations in membrane receptors or in glycosyltransferase activity are envisioned to escalate the disruption in homeostatic mechanisms initially caused by the defective A₂M-enzyme interactions. It is possible, for example, that the alterations in glycosyltransferase activity noted by Rao et al. (10) and Louisot et al. (6) could lead to changes in the carbohydrate portion of certain enzymes or of A₂M itself and thus affect their binding properties (1).

A correlation between defective A₂M-enzyme interactions and the generation of CF-CDA and CFP is especially apparent when one examines cells in culture (20). For example, the *in vitro* generation of CF-CDA by CF or carrier cells has been shown primarily using cell types which in the normal individual are also capable of the synthesis and secretion of A₂M. This is true for both peripheral blood leukocytes (4) (monocytes) and fibroblasts (7). Fibroblast cultures in particular have been used in several studies on the cellular metabolism of CF factors (2), and I have recently found that CF monocytes may secrete at least two CDAs (20), which were shown previously to be present in cell-free supernatants from CF or carrier peripheral blood leukocyte and long-term lymphoid cell cultures (25).

The fact that A₂M and CF-CDA are made by fibroblasts and monocytes points to the fact that these markers and the CFP will be of value for the detection of the CF gene antenatally. Screening for CFP and CF-CDA by IEF or radioimmunoassay in conjunction with quantitation of the functional capacity of A₂M could distinguish a fetus homozygous from one heterozygous for the CF gene, since carriers show only a partial defect in A₂M regardless of whether IEF (22) or other methods (12,13) are used. As part of the ongoing research in my laboratory, I am utilizing purified peripheral blood monocytes or pulmonary macrophages as isolated model systems to study the possible role of a defective A₂M in the generation or accumulation of CDAs and the role of the A₂M-CDA metabolic interrelationship in the disease process (particularly pulmonary disease). I hope that the above-related studies in CF will define more clearly and strengthen the concepts presented in Figure 2.

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Figure 1. Isoelectric focusing of whole serum and of serum samples incubated with protein A - Sepharose CL-4B in a pH 2.5-10 gradient using the LKB Multiphor electrofocusing apparatus (19,27). N, normal control; H, heterozygote for CF; CF, homozygote for CF. The figure shows (from left to right) whole

untreated serum; those proteins not adsorbed to the protein A gel; and the material desorbed from the protein A gel. The CF band is indicated by a marker (lower band when two bands are marked). 3% Ampholytes were used.

Figure 2. Scheme relating what seem to be the most important clinical, biochemical, and pathophysiological findings in CF. Dotted lines denote "feedback loops". Normally these connections serve to escalate disruptions in homeostatic mechanisms initially caused by some other more fundamental abnormality in metabolism. Double-headed arrows show interrelationships where in certain instances it may be difficult to separate cause and effect. One important aspect of the disease not accounted for in the diagram is the

problem of sterility. In males this could involve the pathway shown on the extreme right (alterations in the quantity or quality of membrane receptors), leading ultimately in most cases to a failure of the wolffian duct to develop properly during early fetal life, due to a lack of proper signals for induction of development. In females there are multiple reasons for sterility (30).