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Letters to the Editor

Electrophoretic Analysis of Serum Proteins in Cystic Fibrosis

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A considerable amount of indirect evidence suggests that fluids from cystic fibrosis (CF) patients and obligate heterozygotes contain a factor or factors which may be relevant to the pathophysiology of CF (2). Much of the evidence suggests that the factor is a low molecular weight, cationic protein which in serum is associated with immunoglobulin G (IgG) (2). Recent reports utilizing electrophoretic techniques claim identification of a "CF serum factor" and development of assays to enable the detection of CF homozygotes and heterozygotes (1, 5, 6). The study by Wilson *et al.* (6) in 1973, announcing the demonstration of serum protein differences in CF by isoelectric focusing (IEF) in thin layer polyacrylamide gels (IEFAG), was followed in 1975 by a report claiming the extension of this technique as a "standardized biophysical assay for the rapid detection of individuals homozygous or heterozygous for CF" (5). In their analyses, identification of the CF genotypes was based on the observation of a protein band with an isoelectric point (pI) of 8.4–8.5 (5). In 1975 Altland *et al.* (1) stated that they could not identify this band with an improved IEFAG technique but were able to demonstrate the presence of a relatively low molecular weight, cationic protein (pI 8–9) in the serum of a few homozygotes and heterozygotes for CF that they tested by two-step IEFAG/thin layer electrophoresis (1). Altland *et al.* (1) implied that their technique may enable the unequivocal detection of patients and carriers by virtue of the fact that their procedure separates the factor from the IgG fraction and thus overcomes any variation in resolution at the IEFAG step.

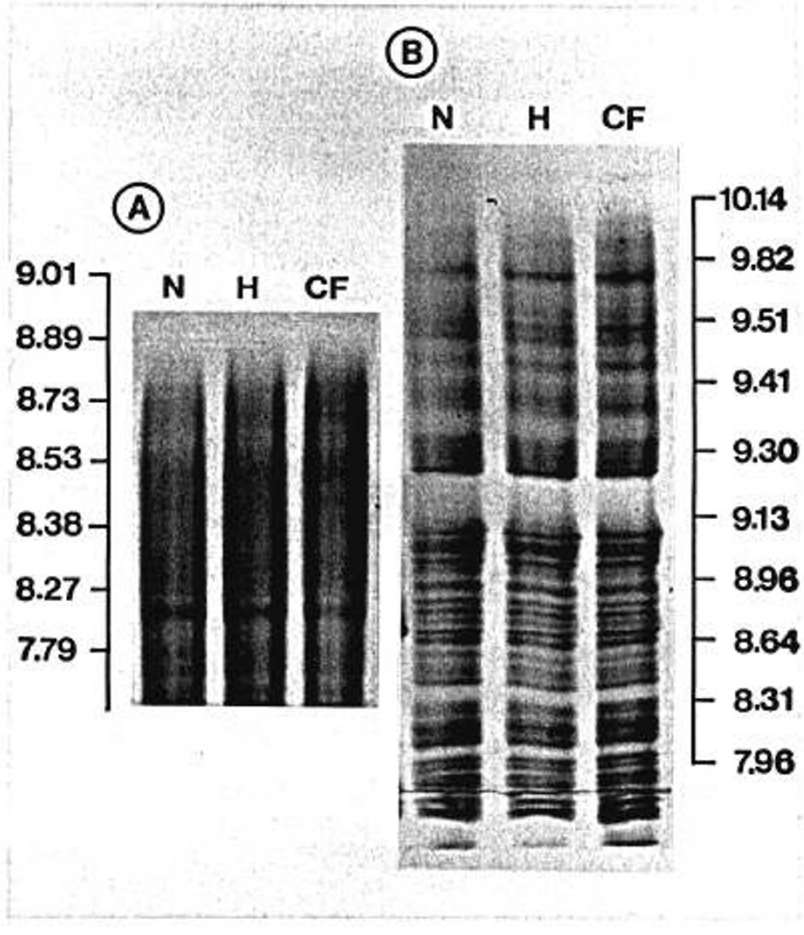

Fig. 1. Comparison of the alkaline isoelectric focusing patterns of serum proteins obtained by two methods: *A:* the technique of Wilson *et al.* (5); *B:* isoelectric focusing in thin layer polyacrylamide gels according to one of our methods (3), which expands the gradient in the pH 8–10 region. Only the relevant portions of the gels are shown. The samples, which were the same for each run, were from one cystic fibrosis (*CF*), one CF heterozygote (*H*), and one normal control (*N*) subject, and each contained 300 μg immunoglobulin G. Urea concentration was 4 M. The measured pH gradients are shown to the side of each gel.

We have attempted to reproduce the IEFAG method specified by Wilson *et al.* (5) in our laboratory (7). Since the resolution obtained with their procedure appeared to be inadequate for identifying single protein bands in the relevant region, we have incorporated several methodologic improvements into the isoelectric focusing technique (4). These improvements have significantly enhanced the resolution and have thus enabled us to demonstrate a greater degree of heterogeneity of serum γ-globulins than is visible in the paper of Wilson *et al.* (5). Figure 1 is a comparison of the alkaline IEF patterns of serum proteins obtained in our laboratory by the method of Wilson *et al.* (5) and by one of our methods (3). Despite the significant increase in the number of bands resolved by our method, a difference among CF, heterozygote, and control sera in the pH 8.4–8.5 region could not be detected. In addition, the two-step IEFAG/disc electrophoresis technique of Altland *et al.* (1) has been tested in our laboratory. We have also modified this analytic procedure, enabling the fractionation of several small proteins from a portion of the IgG fraction. Again, despite an improvement in resolution which reveals more heterogeneity, no unique protein has been observed consistently in the CF and heterozygote sera.

The details of our analyses and our conclusions relative to the reports of Wilson *et al.* (5, 6) and Altland *et al.* (1) will be presented at a later time (3).

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7. Confirmation and elaboration of the method were obtained in personal communication with Dr. G. B. Wilson.
8. This is publication no. 76-25 from the Department of Medical Genetics and was supported in part by the Indiana University Human Genetics Center, PHS GM 21054. Dr. J. M. Thomas was supported by National Institutes of Health Training Grant PHS GM 1056.
9. Requests for reprints should be addressed to: M. E. Hodes, M.D., Department of Medical Genetics, Indiana University Medical Center, Indianapolis, Ind. 46202 (USA).

Additional Notes on the Use of Analytic Isoelectric Focusing for the Detection of Cystic Fibrosis Protein in Serum

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In a previous communication (7), written in response to a letter by Smith *et al.* (3), we suggested several possible reasons for the inability of Thomas *et al.* (4) and other investigators (3) to detect cystic fibrosis protein (CFP) using electrofocusing techniques. Those investigators have reported that their techniques were analagous to those used in our laboratory (11) or claimed them to be improvements over our method (3, 4). Our explanation focused on the following three possible major differences between their techniques and ours: (*1*) the apparatus used for electrofocusing, (*2*) slight differences in the collection, processing, and storage of serum samples, and (*3*) the exact methodology used for electrofocusing (7). Also, we provided additional details concerning our methodology and the reagents employed (7).

Before the present exchange of letters, Thomas and coworkers communicated with us for the purpose of determining possible reasons for their inability to detect CFP by electrofocusing. Key points made during these conversations are reiterated below in detail in a firm attempt to alleviate future problems by inexperienced investigators in their attempts to use analytic electrofocusing to detect CFP.

SAMPLE COLLECTION

Extreme care in sample collection, processing, and storage must be exercised (7, 11). CFP and other diagnostic markers used to detect the CF gene and to distinguish homozygotes from heterozygotes for CF (*e.g.*, bands B, C, and D, and α_2Mf (9–12, 14)) are proteins whose biologic activity, structure, function, and isoelectric point (pI) depend on the manner of manipulating the blood before separation of the serum and subsequent serum analysis. In the case of CFP, we recommend the following procedures: (*1*) after removal from the vein, the blood should immediately be placed in glass tubes in an ice bath (if it must be transported from the clinic to the laboratory) or at 4° to clot (11). (*2*) After retraction of the clot has occurred, the blood should be centrifuged at 4°. (*3*) Subsequent removal of the serum should be performed employing plastic pipettes; aliquots should then be transferred to prechilled (in crushed ice) plastic or polypropylene tubes. The serum should then be stored at −70°; the serum should *not* be left at 4° awaiting analysis. When analytic procedures are performed, the serum should be thawed just before its application to the sample pads.

Failure to adhere to the above protocol could result in structural alteration of CFP (and also CF ciliary dyskinesia activity (13)) with a concomitant change in its pI. This structural alteration in CFP may be due to enzymatic degradation either by endogenous enzymes released from cells damaged during the process of clotting or by exoenzymes already present in the serum (especially if CFP coexists in bound and free forms at equilibrium in serum (9)). Degradation of CFP might be a problem, particularly in heterozygote carriers, since any enzyme defect in CF homozygotes appears to be only partly manifested in carriers. An example of a defect is the quantitative abnormalities noted in the ability of heterozygote carrier α_2-macroglobulin (α_2M) to regulate or modulate proteolytic enzymes as determined by the production of detectable α_2-macroglobulin subunits by electrofocusing or other assays (10, 14).

STANDARDIZATION OF ASSAY

As originally reported (11), all serum samples should be analyzed using a volume of serum standardized to contain 300 μg IgG. We have previously shown that CFP is a small molecule (9) associated with the serum IgG fraction (12). Based on the assumption that CFP has a stoichiometric relationship to IgG, we determined a level of IgG (the proposed carrier of CFP in serum) which, if used for analysis, would result in detectable levels of CFP in the majority of homozygotes and heterozygotes for CF (9, 11). One cannot simply run any given level of serum (*e.g.*, 50 μl) and expect to detect CFP reliably in *all* individuals who harbor the CF gene. Analysis of inadequate amounts of serum will result in false negatives. Use of too much serum will result in overloading the region between pH 8 and 9 and else-