Studies on Cystic Fibrosis Using Isoelectric Focusing. IV. Distinction between Ciliary Dyskinesia Activity in Cystic Fibrosis and Asthmatic Sera and Association of Cystic Fibrosis Protein with the Activity in Cystic Fibrosis Serum

GREGORY B. WILSON⁽⁴⁰⁾ AND H. HUGH FUDENBERG

Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, South Carolina, USA

Summary

The cystic fibrosis protein (CFP) and ciliary dyskinesia activities (CDA) in sera from CF homozygotes, heterozygote carriers, and individuals with bronchial asthma have been partially purified. Concurrently, the CDA's in sera from patients with cystic fibrosis (CF) or bronchial asthma were shown to be different substances by ion-exchange or gel permeation chromatographic procedures. Sephadex G-200 chromatography indicated that the CF-CDA eluted with a protein fraction of molecular weight (MW) 68,000–150,000 and that the asthma CDA was found in a protein fraction of MW >150,000. The two activities could also be separated by DEAE-cellulose chromatography. Prior acidification of whole normal, CF homozygote, obligate heterozygote, or asthmatic sera to pH 3.7 using EDTA, followed by fractionation on Sephadex G-200 removed all the CDA's from fractions of higher molecular weight and shifted the activities to a protein fraction of MW 1,100–13,700. This procedure afforded a 200-fold purification of the CDA's in sera from patients with asthma or CF. EDTA treatment, however, also generated a CDA in previously nonreactive normal sera. Subsequent fractionation of the various active G-200 fractions on Bio-Gel P10 allowed for the separation of three separate activities (Bio-Gel Fractions I, II-IV, and V). Fraction I was shown to represent the activity in sera from patients with asthma and was determined to be C3a (MW 9,000). Fraction I was also found in normal, CF, and carrier sera and therefore is not a specific CDA. Fraction II-IV is thought to represent a CF-specific CDA (MW 5,000) since it could not be demonstrated in either normal or asthmatic sera but was found in sera of obligate heterozygotes. Fraction III-IV also did not react with antisera to human C3a. Fraction V was

generated from all serum types upon acidification of the serum with EDTA and is thought to be a nonspecific CDA. Bio-Gel P10 filtration of Sephadex G-200 fractions provided 823-fold and 650-fold purification of the asthmatic and CF CDA's, respectively. Concurrent analysis of column fractions for CDA's by bioassay and for CFP by electrofocusing showed CFP only in fractions that contained the CF-CDA. Combined analyses employing acid disc gel electrophoresis, isoelectric focusing, and EDTA treatment of active CF-immunoglobulin (Ig) G and Sephadex G-200 column fractions, followed by Bio-Gel P10 chromatography, provided evidence that CFP was associated with the CF-CDA. It is unknown as yet whether CFP itself is responsible for the CF-CDA activity.

Speculation

We have shown that the ability of CF α_2 -macroglobulin to modulate proteolytic enzyme activity is abnormal (22), and others have shown that polyamine metabolism is also abnormal in CF. We have recently shown that polyamines can inhibit α_2 -macroglobulin and proteinase enzyme functions (24, 25). We speculate that CFP and CF-CDA are both fragments of normal proteinases (proteinase analogs) bound to polyamines or polyamine derivatives and that the CFP-polyamine (or "CF factor"-polyamine) complex is the effector substance responsible for ciliary dyskinesia in the rabbit system and partly responsible for the clinical manifestations of CF. Blockage of α_2 -macroglobulin and proteinase enzyme function by CFP-polyamine complexes or polyamines may give rise to the rest of the clinical manifestations in CF.

INTRODUCTION

In a previous report (33) we described a modified version of the rabbit tracheal bioassay for the detection of cystic fibrosis (CF) ciliary dyskinesia factor (CDF). The assay was perfected to explore the possible biological activity of cystic fibrosis protein (CFP), previously found by us using electrofocusing (27,30), and the relationship of CFP to the pathophysiology of CF and to CDF. Our studies using whole sera indicated complete concordance between CFP positivity and ciliary dyskinesia activity (CDA) in all sera tested, except for sera from patients with bronchial asthma who were CFP-negative and CDA-positive. Our results confirmed the earlier observation of Conover et al. (5) that when whole sera are bioassayed, sera from patients with a variety of respiratory and autoimmune diseases harbor a CDA indistinguishable from the activity found in CF sera.

Our findings using combined electrofocusing and rabbit tracheal bioassay analyses, however, suggested that the CDA found in patients with bronchial asthma may differ from a CF-specific CDF (31,33). To test our hypotheses we initiated purification of CFP and the CDAs in CF and asthmatic sera.

In the present study, CFP and the CDA in CF homozygote, CF heterozygote, and asthmatic sera have been purified over 650-fold. The CDAs in CF and asthmatic sera were shown to be distinct by several chromatographic procedures. With each fractionation procedure employed, CFP was found only in the fractions containing a CF-specific-CDA. Our results support our suggestion (31,33) that CFP is related to a CF-specific CDF. In addition we have found evidence that the asthmatic CDA is C3a whereas the CF-CDA is not.

MATERIALS AND METHODS

Collection and Processing of Samples

Serum samples obtained from clotted venous blood (33) were collected and processed for analysis by electrofocusing, bioassay, and fractionation procedures exactly as previously described (32,33). The concentration of IgG in each sample and in individual column fractions (where noted in the Results) was measured by single radial immunodiffusion as previously described (27). The sera used in this study, with the exception of one from a presumed heterozygote carrier for CF with bronchial asthma (see Results), were from the same individuals analyzed previously by bioassay and electrofocusing (28,32). The diagnoses of CF and bronchial asthma in all patients studied were based on the basis of the criteria previously noted (33). Informed consent was obtained from each subject (or in the case of minors, from their parents) before blood was drawn.

Antisera

Antisera to human C3, IgG, and whole normal human serum were obtained from Meloy Laboratories, Springfield, VA. Antiserum to human C3a (anaphylatoxin) was obtained from Behring Diagnostics, Woodbury, NY, and from M. Michael Glosky, M.D., Chief, Department of Allergy and Clinical Immunology, Southern California Permanente Medical Group, Los Angeles, CA.

Electrophoresis

Disc gel electrophoresis was performed in 15% polyacrylamide gel with acetic acid - β -alanine buffer, pH 4.5, according to the procedure of Reisfield et al. (17), with the following modifications: (a) A large-pore anti-convection gel was not used. Instead, samples were applied in 20% (w/v) sucrose. (b) The separation gel was increased to a length of 6 cm, and DATD (N,N-dialyltartardiamide; Polysciences, Warrington, PA) was used as a cross-linker. The gel was stained and destained as described for isoelectric focusing (IEF) (30). Samples were electrophoresed for approximately 1 hr at 6 mA per gel using a Buchler electrophoresis apparatus (Buchler Instruments Inc., Fort Lee, NJ). All disc gel electrophoresis and IEF experiments were run at 4°C using a Lauda K-2K controlled-temperature circulating water unit (Brinkman Instruments).

Immunoelectrophoresis at pH 8.6 was performed by standard procedures using Veronal buffer ($\text{pH} = 8.6$) (15). Immunoelectrophoresis at pH 4.5 was performed using acetic acid - β -alanine buffer (17) in 1% agarose. Samples run at pH 4.5 were electrophoresed at a voltage setting of 100 V for 60 min.

Isoelectric Focusing

IEF in disc gels (pH 3.5-10 gradient) was performed by the method of Eder (8), as described previously (23), with the following modifications: (a) Gels of 10% acrylamide were used, and N,N-methylene-bisacrylamide was replaced by DATD as cross-linker (acrylamide:DATD ratio 1:10). (b) The anode and cathode solutions were 1% phosphoric acid and 1% TEMED to conform to the solutions used in thin-layer IEF (20,21,27,30).

IEF in thin-layer polyacrylamide gel was performed using an LKB Multiphor electrofocusing apparatus (Model 2117-411 LKB, Brooma, Sweden) by modification of the procedures described previously (21,23,27). The complete methodology is described elsewhere (20,32).

Reading of the pH gradient and fixing, staining, and destaining of the gel were performed as described previously (27) with minor modification (20). An example of the pH 2.5-10 gradient as measured at 4°C on the surface of the gel is shown in Figure 1. All whole serum samples were analyzed using a volume of serum standardized to contain 300 μ g of IgG (27,32). Serum fractions obtained by chromatography were analyzed using the amounts indicated (see Results).

Ion-Exchange Chromatography, Gel Filtration, and Ultrafiltration

Fractionation of sera on DEAE-cellulose (DE-52, Whatman) was performed using either 0.01 M phosphate (pH 8.0) or 0.005 M Tris-HCl (pH 8.6) buffer and a discontinuous step gradient of NaCl (0.00 M; 0.05 M, 0.1 M, 0.2 M) as described previously (23,26) without EDTA. Gel filtration on Sephadex G200 (Pharmacia) was conducted as previously described (26) using 0.01 M phosphate buffer, pH 8.0 with 0.15 M NaCl (PBS, pH 8.0) for elution. Gel filtration on Bio-Gel P10 (Bio-Rad Laboratories, Richmond, CA) was carried out on a 1X110-cm column at a flow rate of 5 ml/hr. Fractions of 2.0 ml were collected and PBS was used for elution. For EDTA treatment, whole sera or fractions were mixed with solid EDTA (Fisher) to saturation (pH 3.7) and left at room temperature for 15-20 min. Sephadex G200 and Bio-Gel P10 columns were calibrated with markers of known molecular weight (see Results).

All column fractionations were performed at 4°C using siliconized glass columns and plastic tubes. Fractions were pooled, dialyzed using Spectrapor 3 dialysis membrane tubing (MW retention 3500; Spectrum Medical Industries, Los Angeles, CA) to equilibrium against 0.0025 M phosphate, pH 7.2, and

lyophilized. Fractions were reconstituted in distilled water and redialyzed if found to contain excessive electrolytes. Sephadex G200 Fraction I (MW >200,000, V_0 as shown in Results) from CF serum was also concentrated by Amicon ultrafiltration over a UM-2 membrane (MW retention 1000; Amicon Corp., Lexington, MA) to exclude the possibility that lyophilization could have destroyed any activity. Spock et al. (19) have previously reported that this fraction contains ciliary dyskinesia activity.

The protein concentration in different fractions was estimated by reading the absorbance at 280 nm and/or 220 nm. Exact amounts of protein were determined using the procedure of Lowry et al. (13).

Isolation of Human C3a (Anaphylatoxin)

Anaphylatoxin was isolated from human plasma according to the methods of Hugli et al. (10). The final preparation reacted with antisera to human C3a but not to C3 nor with antisera to a variety of other human serum proteins after electrophoresis at pH 4.5, and was of MW ~9000 as determined by chromatography on a calibrated Bio-Gel P10 column.

Bioassay

Determinations of CDA in whole sera and column fractions were performed as described previously for whole sera (33). All fractions (in 0.01 M phosphate, pH 7.2) were mixed 1:1 (v/v) with incomplete medium and warmed to 37°C just prior to bioassay (33). The protein concentration of the fractions tested by bioassay or analyzed by other methods is given as the concentration relative to the original starting material (whole serum equals 1X concentration), unless otherwise indicated. Samples were considered positive if they produced ciliary dyskinesia prior to 45 min. However, it should be noted that some samples were also ciliotoxic (not due to excessive electrolytes), and the majority of the samples tested reacted by 35 min at a 1X concentration.

RESULTS

Distinction between CFP, CF-CDA, and Asthmatic CDA

Whole human serum. Previous comparative analyses of whole sera for CFP and CDA positivity indicated complete agreement between the presence or absence of these two markers for CF in all sera tested, except for patient controls with bronchial asthma (who were CDA+,CFP-). Of particular importance to our studies was the finding of a patient with bronchial asthma who was CFP+,CDA+ (Table 1). This individual provided us with a unique opportunity to study a presumed heterozygote for CF with confirmed bronchial asthma.

DEAE-cellulose chromatography. Analysis of fractions from DEAE-cellulose chromatography demonstrated that CFP was present only in the IgG-containing fractions (Table 2, Figs. 2 and 3). When phosphate buffer was used, the CDAs in CF and asthmatic sera were both found in Fraction I with CFP. When Tris-HCl buffer was used, CFP and the CF-CDA were separated from the asthmatic CDA, the latter being found in Fraction II. The asthmatic presumed heterozygote carrier presented two activities, one with CFP in Fraction I and the other in Fraction II.

Use of Tris-HCl buffer thus afforded one method for distinguishing between CF-CDA and asthmatic CDA, and suggested a close association of CFP with CF-CDA. Of particular interest with regard to the use of Tris-HCl and a stepwise discontinuous NaCl gradient for purification procedures is that Fraction I contains only those serum proteins with the most basic pI values (last cm of stained banding pattern) (Figs. 2 and 3). Immunoelectrophoresis of serum Fractions I and II eluted with either phosphate or Tris-HCl buffer showed that phosphate Fraction I contained only IgG and that Tris-HCl Fraction I contained only the most cathodal IgG proteins (Fig. 3a,d-h). However, we cannot exclude the possibility that other serum proteins which may not react with antisera to normal human serum could be present in Fraction I. Fractions obtained from CF homozygote, CF heterozygote, asthmatic, and normal sera showed similar patterns after immunoelectrophoresis, regardless of whether they were positive or negative for CFP or CDA.

Sephadex gel filtration. Fractionation of all serum types by gel filtration on Sephadex G200 provided an additional method to partially purify and distinguish the asthmatic and CF-CDAs. A typical chromatogram obtained with 2 ml of serum is shown in Figure 4. Bioassay of all fractions (reconstituted to 1X protein concentration) showed that the CDA in CF sera was contained in Fraction III and in Fraction IV (weak CDA) and was distinct from asthmatic CDA, which was found only in Fraction II (Table 3). Sephadex G200 Fraction I from CF sera, whether lyophilized or concentrated by ultrafiltration, failed to elicit a CDA response. The activities in Sephadex G200 Fractions II, III, and IV and in DEAE-cellulose Fractions I and II were all stable to lyophilization. The asthmatic/heterozygote serum showed activity in Fractions II-IV, and normal control sera did not show activity in any fraction.

Analysis of Sephadex G200 Fractions I-V by electrofocusing indicated that CFP was present only in Fraction III. Representative results from electrofocusing of G200 Fractions II and III from CF homozygote, heterozygote, asthmatic, and normal control sera are shown in Figure 5. A faint CFP doublet can be seen only in G200 Fraction III from the CF homozygote and heterozygote samples.

Analysis of Fractions II and III from CF serum by immunoelectrophoresis is shown in Figure 3b and c, to illustrate the number and relative amount of serum proteins found in each fraction. IgG (large cathodal band) and C3 were found in both fractions by separate analyses using specific antisera (not shown).

Treatment with EDTA. Previous studies have shown that most of the CFP is separated from the bulk of the serum proteins by pretreatment of whole CF serum with 10 mM EDTA, followed by Sephadex G200 gel filtration (23). Subsequent experiments indicated that increasing the EDTA concentration to produce an acid pH more effectively disassociated CFP and afforded a better separation. Therefore, the effect of EDTA on the CDAs in asthmatic and CF sera was tested. Treatment with EDTA prior to gel filtration routinely eliminated all CDA activities in Fractions II-IV and generated a CDA (in all sera) found exclusively in Fraction VI (MW 1,100-13,700; Fig. 4) (Table 3). Analysis of Fraction VI (0.7 mg/ml) (obtained with or without prior EDTA treatment) by immunoelectrophoresis at pH 8.6 against antiserum to whole human serum usually failed to show any reaction. The only reaction ever noted after immunoelectrophoresis was a single anodal arc, which was identified as albumin, in one G200 Fraction VI from normal serum.

Analysis of Fraction VI (25 μ g) by acid disc gel electrophoresis, however, showed the presence of several cathodal bands and indicated obvious differences between the serum types (Figs. 6 and 7). In particular, an extra cathodal band was noted in Sephadex G200 Fraction VI from CF and carrier

samples (Fig. 6), and two additional cathodally migrating bands were noted in EDTA-treated Fraction VI from samples of the same type, as compared with normal or asthmatic samples (Fig. 7).

Electrofocusing indicated that Fractions I-V from EDTA-treated sera of all types were negative for CFP, and that CFP was not generated in normal control or asthmatic sera by EDTA treatment. Figure 8 illustrates the results of analysis of CF G200 Fraction III by electrofocusing before and after EDTA treatment of an otherwise identical serum sample. Note that no bands are found opposite the marker after EDTA treatment of CF serum followed by fractionation on Sephadex G200. The normal control sample was totally devoid of CFP before and after EDTA treatment (Fig. 8). EDTA treatment did not destroy CFP, which was still present when EDTA-treated whole sera from CF homozygotes or heterozygotes were electrofocusing (not shown). This finding, and the occurrence of an additional band in G200 Fraction VI from homozygote and heterozygote sera after EDTA treatment (Fig. 7), suggest that dissolution of a CFP-IgG (or other precursor) complex occurs upon acid treatment, and that subsequent chromatography separates the CFP and CDA from larger macromolecules in CF and asthmatic sera.

Partial direct confirmation of the movement of CFP to Fraction VI was obtained by disc gel IEF. As shown in Figure 9, banding differences were evident when equal amounts (25 µg) of Sephadex G200 Fraction VI from EDTA-treated normal and CF sera were analyzed. Bands near pH 8.5 were evident in the CF sample. Disc gel IEF and acid electrophoresis added further evidence for the association of CFP and several other cationic proteins with the activity in Sephadex G200 Fraction VI from CF homozygote or heterozygote EDTA-treated sera. However, these data do not explain the generation of CDA in normal sera or its movement from Fraction II to VI in asthmatic sera after EDTA treatment.

Bio-Gel P10 Filtration

Analysis of G200 Fraction VI from EDTA-treated samples (from 2 ml of serum) by gel filtration on Bio-Gel P10 is shown in Figure 10, which is a composite for all fractionations on Bio-Gel P10. Subfractionation of Sephadex G200 Fractions II, III, and IV or of IgG yielded peak A and the other peaks shown. (Bio-Gel P10 excludes molecules of MW >20,000.) Peak A corresponds to molecules of MW >20,000 eluted in the void volume (V_0 or Peak A). Bioassay of the Bio-Gel P10 subfractions indicated the existence of three separate activities, in Peaks I, III-IV, and V (Table 4) in CF homozygote and heterozygote G200 Fraction VI, but only two in normal and asthmatic samples. The activity in Fraction III-IV is felt to represent a unique CF-CDA (MW ~5000), since it could not be demonstrated in analogous fractions from normal or asthmatic sera.

Chromatographic analysis of CF-IgG (DEAE-cellulose Tris-HCl Fraction I) and CF G200 Fractions III and IV with and without EDTA treatment indicated that the activities in Bio-Gel P10 Fractions I (MW ~9000) and III-IV were derived from IgG or another macroglobulin-CDA complex. Without treatment, all the CDA found in Sephadex G200 Fractions III and IV or with IgG eluted in Peak A, whereas prior treatment with EDTA effectively disassociated the activity. Figure 8 shows the results of electrofocusing CF DEAE-cellulose Fraction I (Tris-HCl), which was eluted from Bio-Gel P10 before (C) or after (C-EDTA) pretreatment of the sample with EDTA. When equal amounts of the same fraction were analyzed, DEAE-cellulose Fraction I obtained from Bio-Gel P10 Fraction A pretreated with EDTA (as compared with the untreated fraction) was devoid of the CFP doublet. This finding indicates that CFP is disassociated from the IgG fraction and elutes with molecules of lower MW after EDTA treatment. It is unknown at present in which fraction CFP is found.

Pretreatment of normal G200 Fractions III and IV with EDTA showed that both the activities found in Sephadex G200 Fraction VI from EDTA-treated normal serum originated from these G200 fractions (Table 4). Pretreatment of normal or asthmatic Sephadex G200 Fraction II with EDTA also resulted in subsequent elution of one of the peaks of activity (Fraction I). These data suggest the possibility that some macroglobulin or a complex found in normal, CF, and asthmatic Sephadex G200 Fractions II, III, and IV liberates the activities in Bio-Gel P10 Fractions I and V after treatment of the Sephadex G200 fractions with EDTA.

Identity of the Bio-Gel P10 Fraction I activity as C3a. Since Conover et al. (6) had previously suggested that C3a might be responsible for the CDA activity in CF and asthmatic sera, we screened representative active samples of Bio-Gel P10 Fractions I-VI for C3a by immunoelectrophoresis at pH 4.5 using a potent rabbit antiserum to human C3a. In previous experiments employing this antiserum and human C3a purified by the method of Hugli et al. (10), a reaction could be observed when as little as 25 µl of a 0.25-mg/ml solution of C3a was analyzed. Analysis of concentrated preparations of the Bio-Gel P10 fractions (0.3-0.6 mg/ml) by immunoelectrophoresis failed to indicate the presence of C3a in any fraction other than Fraction I.

Since only Fraction I from Bio-Gel P10 gel filtration of EDTA-treated asthma G200 Fraction II showed activity (and only G200 Fraction II was active from asthmatic sera), we conclude from these preliminary findings that the CDA in asthmatic sera probably is attributable to C3a. It is not surprising that the activity in Bio-Gel P10 Fraction I could be eluted from either EDTA-treated G200 Fractions III+IV (combined) or Fraction II, since analysis of these G200 fractions by immunodiffusion indicated the presence of C3. It is known that acid conditions favor the dissolution of C3a-C3b complexes (see References in 10).

Regardless of the identity of the activity in Bio-Gel P10 Fraction I, the results in Table 4 clearly indicate the existence of a unique CDA activity derived from CF homozygote and heterozygote sera (Bio-Gel P10 Fraction III-IV) and suggest that Sephadex G200 gel filtration followed by Bio-Gel P10 filtration may afford a useful method to purify both C3a and the CF-CDA. Current research in this laboratory is directed toward the bulk purification and complete characterization of the components responsible for the CDA in Bio-Gel P10 Fractions I, III-IV, and V.

Table 5 summarizes the extent of purification of the CDAs in CF and asthmatic sera obtained by Sephadex G200 and Bio-Gel P10 gel filtration. The data in Table 5 are based on two assumptions: (a) that there is no loss of activity during purification, and (b) that a "unit" of ciliary dyskinesia activity can be defined as the amount of activity per mg protein present in a 1X concentrated preparation. Treatment of CF or asthmatic sera with EDTA followed by Sephadex G200 gel filtration provided a 200-fold purification over the specific activity present in whole serum. When Sephadex G200 Fraction VI was fractionated on Bio-Gel P10, the asthmatic CDA (G200 Fraction II) was purified 823-fold and the CF-CDA 650-fold (Bio-Gel P10 Fraction III-IV).

DISCUSSION

Spock et al. (19) originally described a factor (now termed "ciliary dyskinesia factor" or CDF) that was present in the serum of both homozygotes and heterozygotes for CF and produced dyskinesia of the ciliary beat in explants of rabbit tracheal epithelium. The CDF described by Spock et al. (19) was felt by them to be a specific marker for the CF gene, since the CDF was not found in sera from patients with a variety of respiratory and autoimmune disorders and was not found in normal healthy control sera without a family history for CF.

The CDF was characterized by Spock et al. (19) as a nondialyzable, heat-labile (100°C, 1 min) serum activity associated with the euglobulin fraction. Gel filtration of CF homozygote serum on Sephadex G200 produced two fractions containing CDF activity. One activity (Fraction I, MW >200,000) was found with the proteins excluded from the gel and eluting in the void volume. The other activity (Fraction IV) was associated with serum proteins eluting between IgG (MW 150,000) and albumin (MW 68,000) (19).

Conover et al. (4-6), and more recently Wilson et al. (20,33,34), have confirmed and extended the original observations of Spock et al. concerning the presence of a CDF in sera from homozygotes and heterozygotes for CF (19). Although both groups of investigators could detect a ciliary dyskinesia activity (CDA) in whole sera from both CF genotypes and not in sera from normal healthy controls, Conover et al. (5) found that sera from normal patient controls with a variety of autoimmune and respiratory diseases produced a ciliary dyskinesia reaction indistinguishable from the reaction caused by sera from CF homozygotes. The finding of a CDA in sera from patients with certain respiratory and autoimmune disorders led Conover et al. (5) to suggest that such individuals and CF patients share some functional or structural abnormality in their complement system. The findings of Conover et al. (5,6) seemingly confirmed and extended the original hypothesis of Wilson and Jahn (28,29), who had previously suggested "that CF may have as one of its characteristics a complement related deficiency", manifested as "altered complement components or fragments of normal intact complement components synthesized and excreted into the humoral fluids" (28). Wilson and Jahn felt that the abnormality in complement in CF could be due to an alteration in normal complement function caused by an abnormality either in a complement component inactivator or in an initiator of the alternate complement pathway (9,28).

Subsequently, Conover et al. (6) presented evidence suggesting that the elusive CF-CDF was C3a (anaphylatoxin) and proposed that a defect or deficiency in the anaphylatoxin inactivator (a carboxypeptidase-B-like enzyme) was the primary genetic defect in CF. In addition, Polley and Bearn (16) later found that CF serum showed a decreased ability to convert C3 proactivator to C3 activator and C3 to its hemolytically inactive product (C3i); both results were suggestive of a possible derangement in the alternate pathway of complement activation in CF.

In the present report our results (Tables 2 and 3) show that the CDA in asthmatic sera elutes in column fractions different from the CDA found in sera from homozygotes and heterozygotes for CF. Fractionation of all serum types on either DEAE-cellulose (an ion-exchange resin) or Sephadex G200 indicated that the asthma CDA or a CDA complexed with a macroglobulin was more acidic and of higher MW than the CF-CDA or a CF-CDA-macroglobulin complex. Our results from Sephadex G200 gel filtration of CF serum agree in part with those of Spock et al. (19), since both of us found a CF-CDA in a serum protein fraction of MW 68,000-150,000. Unlike Spock et al. (19), however, we could not demonstrate a CDA in Fraction I from Sephadex G200 (Fig. 4, Table 3) when concentrated by either lyophilization or ultrafiltration. We cannot at present explain the discrepancy between the findings of Spock et al. (19) and our own, especially since the CF-CDA in Fractions III and IV is stable to lyophilization and is not lost during concentration of an active fraction by ultrafiltration over a UM-2 membrane (data not shown).

The possibility that the asthmatic CDA and CF-CDA could be the same substance possibly bound to different carriers in asthmatic and CF sera is not excluded by our finding of all CDA-containing fractions in serum from a presumed heterozygote carrier with asthma (Tables 2 and 3). Our data indicate that this serum had all the fractions with activity (DEAE Fractions I and II, G200 Fractions II, III, and IV) but do not permit a conclusion as to whether asthmatics and cystics might produce different CDAs or different macroglobulins with which the same CDAs complex.

Our studies using EDTA treatment prior to Bio-Gel P10 gel filtration, however, strongly indicated that the two CDAs are not structurally the same (Table 4). The asthmatic CDA (Bio-Gel P10 Peak I) was of MW ~9000, reacted with antiserum to human C3a, and could be derived from either normal or asthmatic Sephadex G200 Fractions II or III+IV after acidification with EDTA. On the other hand, a CDA specific to CF sera separated from other nonspecific CDAs on Bio-Gel P10 and was found in Bio-Gel P10 Fraction III-IV (MW ~5000; Fig. 9) (Table 4). Bio-Gel P10 Fraction III-IV did not react with antiserum to human C3a, and the CDA could not be derived from normal or asthmatic G200 Fraction II or III-IV (Table 4).

Our findings suggest, therefore, that a CDF does exist in sera from homozygotes and heterozygotes for CF which may be specific for the CF gene. Unlike the CDA described by Conover et al. (6), our CF-CDA does not seem to be normal intact C3a and does not require IgG for activity (Tables 3 and 4). We are currently exploring the possibility that the CF-CDA may be a fragment of C3a or even possibly C5a or C2-kinin.

Of related interest are the results of Bowman and co-workers (2,3) concerning the specificity and identification of a ciliotoxic factor (CTF) found in sera from homozygotes and heterozygotes for CF. The CTF, which is detected using an oyster gill ciliary bioassay (12), has a MW, cationic charge, and other properties which resemble those of the CDF described by Conover et al. (6). Barnett and Bowman (1) claim, however, that the CTF is specific for CF, is not C3a, and does not require IgG for activity (2,7). These properties distinguish CTF from the CDF of Conover et al. (6) but suggest a close relationship to the CF-CDF described in this report.

Disagreement between our results and those of Conover et al. (17) might best be explained by comparing the techniques used to isolate the CDA activity. Conover et al. (6) employed Amicon ultrafiltration to separate the CDF from other macromolecules. We used acidification of whole serum, Sephadex G200 fractions, or purified IgG-CDA complexes followed by gel filtration on Bio-Gel P10 to separate the CDAs. As our data show (Fig. 9, Table 4), C3a, CF-CDA, and another CDA derived from serum (Bio-Gel P10 Peak V) all are of MW 1,000-10,000 and could never be separated using the ultrafiltration methods of Conover et al.

Since our data (Table 4) indicate that the CF-CDA (Bio-Gel Peaks III-IV) and the C3a activity (Peak I) can both be derived from CF-IgG and CF G200 Fraction III+IV, it is tempting to suggest that Conover et al. (6) may have detected only the C3a activity and either missed the CF-specific CDA or destroyed it during execution of the experiments they described (6).

The CFP detected by isoelectric focusing (28,23,27,32) was previously suggested to be related structurally or metabolically to a CF-specific CDF (33). Our findings in this study indicate that all fractions which contain CF-CDA also contain CFP and support our proposal. We have been unable to determine as yet whether the CFP protein itself is responsible for the activity in Bio-Gel Fraction III-IV; however, we have presented evidence that both the CFP and the CF-CDA are removed from CF-IgG and CF G200 Fraction III after pretreatment of the fraction with EDTA followed by gel filtration on Bio-Gel P10 (Fig. 8). In addition, acid disc gel electrophoresis and isoelectric focusing (Figs. 6, 7, and 9) of active fractions indicate that an additional cationic protein band is present in CF G200 Fraction VI from EDTA-treated serum, and that several proteins with pIs near pH 8.5 are present in this same fraction. Analogous fractions from heterozygote carrier serum, but not from asthmatic sera, also showed all these cationic protein bands.

CONCLUSION AND SUMMARY

The results of this investigation provide evidence that sera from homozygotes and heterozygotes for CF harbor a unique ciliary dyskinesia activity. This CF-CDA is characterized as a small cationic protein (MW ~5,000) which has been shown by two different chromatographic procedures to be distinct from a CDA found in sera from patients with bronchial asthma. Further preliminary evidence suggests that the asthmatic CDA is C3a, whereas the CF-CDA is not, since fractions which contain 800-fold purified asthmatic CDA react with antiserum to C3a, whereas fractions containing 650-fold purified CF-CDA do not (Table 5). Although the results of this study add evidence for our suggestion that CFP may be the CF-specific CDA, further purification of CFP and the CF-CDA will be required to determine whether our specific protein band with a pI near pH 8.5 (CFP) is the CF-CDA.

The identities of our CFP and CF-CDA and the CTF described by Bowman and co-workers (2,7) remain unknown. Although they are not normal C3a, it is still possible that these elusive "CF factors" could be related to some component of the complement or kinin system, since other complement-related defects are evident in CF serum (16,28,29). Of direct relevance to the above are our recent findings that plasma or serum alpha₂-macroglobulin is abnormal in CF (22,24,25). We hypothesized that an abnormality associated with CF alpha₂-macroglobulin can explain the presence of "CF factors" (whatever their exact biochemical nature may be) in various body fluids of CF patients and carriers, as well as much of the pathophysiology of the disease, since a generalized failure of CF homozygote (and to a lesser extent heterozygote) alpha₂-macroglobulin to regulate or modulate serum proteinases such as plasmin or kallikrein could lead to the excessive production of C3a, kinins, and other substances which can alter membrane permeability and electrolyte transport (22,24,25). Our hypothesis of a defect in interactions between alpha₂-macroglobulin and proteolytic enzymes in CF has been confirmed recently by Shapira et al. (18). It is also known that "CF factors" are produced by white cells and fibroblasts in culture (7) and that alpha₂-macroglobulin is a membrane component on B lymphocytes (11) and is synthesized by fibroblasts in culture (14).

Recent research in this laboratory has been directed at extending our findings to determine (a) whether the structure and/or function of cell surface alpha₂-macroglobulin is also abnormal in CF, and (b) whether the CDAs produced by leukocytes from patients with CF or asthma are different substances (34). We have also been exploring our proposal of a possible relationship between an abnormality in polyamine metabolism in CF and defective proteinase regulation by CF alpha₂-macroglobulin (22). We have recently shown (a) that the CDA produced by leukocytes from individuals who harbor the CF gene is distinct from the CDA synthesized by leukocytes from individuals with bronchial asthma (34,35), (b) that polyamines can differentially markedly inhibit the function of alpha₂-macroglobulin and proteinases (24,25), and (c) that the CF-CDA described in this report and CFP may be structurally abnormal proteinase analogs and/or complexed to a polyamine derivative (24,25).

In a future report (24) we clarify the relationship of our findings to the primary genetic defect in CF responsible for the above metabolic abnormalities and other "seemingly unrelated" defects in metabolism observed in CF patients and obligate heterozygote carriers for the CF gene when their cells and humoral fluids are studied in vitro.

REFERENCES AND NOTES

- Barnett, D.R., and Bowman, B.H.: Cystic fibrosis ciliary inhibitor. *Pediat. Res.*, **8**:687 (1974).
- Bowman, B.H.: Factors related to cystic fibrosis. in: J.A. Mangos and R.C. Talamo: Cystic Fibrosis: Projections into the Future. p. 277 (Stratton Intercontinental Medical Book Corp., N.Y., 1976).
- Bowman, B.H., Lockhart, L.H., and McCombs, M.L.: Oyster ciliary inhibition by cystic fibrosis factor. *Science*, **164**:325 (1969).
- Conover, J.H., Bonforte, R.J., Hathaway, P., Pacinc, S., Conod, E.J., Hirschhorn, K., and Kopel, F.B.: Studies on ciliary dyskinesia factor in cystic fibrosis. I. Bioassay and heterozygote detection in serum. *Pediat. Res.*, **7**:220 (1973).
- Conover, J.H., Conod, E.J., and Hirschhorn, K.: Ciliary dyskinesia factor in immunological and pulmonary disease. *Lancet*, **i**:1194 (1973).
- Conover, J.H., Conod, E.J., and Hirschhorn, K.: Studies on ciliary dyskinesia factor in cystic fibrosis. IV. Its possible identification as anaphylatoxin (C3a)-IgG complex. *Life Sci.*, **14**:253 (1974).
- DiSant'Agnes, P.A., and Davis, P.B.: Research in cystic fibrosis. *New Eng. J. Med.*, **295**: 534 (1976).
- Bder, J.: Isoelectric focusing of antibodies in polyacrylamide gels. *J. Immunol. Meth.*, **2**:67 (1972).
- Gewurz, H.: Alternate pathways to activation of the complement system. in: D.G. Ingram: Biological Activities of Complement. p. 56 (S. Karger, N.Y., 1972).
- Hugli, T.E., Vallota, E.H., and Müller-Eberhard, H.J.: Purification and

partial characterization of human and porcine C3a anaphylatoxin. *J. Biol. Chem.*, **250**:1472 (1975).

- James, K., Tunstall, A.M., Parker, A.C., and McCormick, J.N.: The association of alpha₂-macroglobulin with lymphocyte membranes in chronic lymphocytic leukemia and other disorders. *Clin. Exp. Immunol.*, **19**:237 (1975).
- Lockhart, L.H., and Bowman, B.H.: Assay for detection of the cystic fibrosis gene. *Tex. Rep. Biol. Med.*, **31**:631 (1973).
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**:265 (1951).
- Monsher, D.F., and Wing, D.A.: Synthesis and secretion of alpha₂-macroglobulin by cultured human fibroblasts. *J. Exp. Med.*, **143**:462 (1976).
- Ouchterlony, O., and Nilsson, L.A.: Immunodiffusion and immunoelectrophoresis. in: D.M. Weir: Handbook of Experimental Immunology. p. 191 (Blackwell Scientific Publications, London, 1974).
- Polley, M.J., and Bearn, A.G.: Cystic fibrosis, current concepts. *J. Med. Genet.*, **11**:249, 1974.
- Reisfeld, R.A., Lewis, V.J., and Williams, D.E.: Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*, **195**:281 (1962).
- Shapira, E., Rao, G.J.S., Wessel, H.U., and Nadler, H.L.: Absence of an alpha₂-macroglobulin-protease complex in cystic fibrosis. *Pediat. Res.*, **10**:812 (1976).
- Spock, A., Heick, H.M.C., Cress, H., and Logan, W.S.: Abnormal serum factor in patients with cystic fibrosis of the pancreas. *Pediat. Res.*, **1**:173 (1976).
- Wilson, G.B., Arnaud, P., and Fudenberg, H.H.: An improved method for the detection of cystic fibrosis protein in serum using the LKB multiphor electrofocusing apparatus. *Pediat. Res.*, **11**: in press (1977).
- Wilson, G.B., Arnaud, P., Monsher, M.T., and Fudenberg, H.H.: Detection of cystic fibrosis proteins by electrofocusing. *Pediat. Res.*, **10**:1001 (1976).
- Wilson, G.B., and Fudenberg, H.H.: Studies on cystic fibrosis using isoelectric focusing. II. Demonstration of deficient proteolytic cleavage of alpha₂-macroglobulin in cystic fibrosis plasma. *Pediat. Res.*, **10**:87 (1976).
- Wilson, G.B., and Fudenberg, H.H.: Further purification and characterization of serum proteins used to detect cystic fibrosis genotypes by isoelectric focusing. *Tex. Rep. Biol. Med.*, **34**:51 (1976).
- Wilson, G.B., and Fudenberg, H.H.: Role of polyamines in the regulation of proteinase and proteinase-inhibitor function: Emphasis on an alpha₂-macroglobulin-polyamine defect in cystic fibrosis. in: R.A. Campbell and G.D. Daves: Pacific Northwest Polyamine Conference, in press (Raven Press, N.Y., 1977).
- Wilson, G.B., and Fudenberg, H.H.: Polyamines: Inhibition of alpha₂-macroglobulin and proteolytic enzymes. Relationship to the pathophysiology of cystic fibrosis. *Clin. Res.*, **25**: in press (1977).
- Wilson, G.B., and Fudenberg, H.H.: Ciliary dyskinesia factors in cystic fibrosis and asthma. *Nature*, in press (1977).
- Wilson, G.B., Fudenberg, H.H., and Jahn, T.L.: Studies on cystic fibrosis using isoelectric focusing. I. An assay for detection of cystic fibrosis homozygotes and heterozygote carriers from serum. *Pediat. Res.*, **9**:635 (1975).
- Wilson, G.B., and Jahn, T.L.: A comparative immunological study of cystic fibrosis serum. III. A preliminary report on the kinetics and mode of action of cystic fibrosis serum on *Colpidium striatum*. in: Cystic Fibrosis Club Abstracts, p. 59 (Cystic Fibrosis Foundation, Atlanta, 1973).
- Wilson, G.B., and Jahn, T.L.: Decreased rate of cytolysis of *Colpidium striatum* by cystic fibrosis serum. I. Bioassay and evidence for the possible involvement of a C/F factor-IgG complex. *Life Sci.*, **15**:551, 1974.
- Wilson, G.B., Jahn, T.L., and Fonseca, J.R.: Demonstration of serum protein differences in cystic fibrosis by isoelectric focusing in thin-layer polyacrylamide gels. *Clin. Chim. Acta*, **49**:79 (1973).
- Wilson, G.B., Monsher, M.T., and Fudenberg, H.H.: Cystic fibrosis protein factor detected by isoelectric focusing and ciliary dyskinesia. *Clin. Res.*, **24**:295 (1976).
- Wilson, G.B., Monsher, M.T., and Fudenberg, H.H.: Additional notes on the use of analytical isoelectric focusing for the detection of cystic fibrosis protein in serum. *Pediat. Res.*, **11**: in press (1977).
- Wilson, G.B., Monsher, M.T., and Fudenberg, H.H.: Studies on cystic fibrosis using isoelectric focusing. III. Correlation between cystic fibrosis protein and ciliary dyskinesia activity in serum shown by a modified rabbit tracheal bioassay. *Pediat. Res.*, **11**: in press (1977).
- Wilson, G.B., Monsher, M.T., and Fudenberg, H.H.: Distinction between ciliary dyskinesia activity (CDA) in leukocyte culture supernatants from individuals with cystic fibrosis (CF) and asthma. *Clin. Res.*, **25**:34 (1977).
- Wilson, G.B., Stites, D.P., and Fudenberg, H.H.: The ciliary dyskinesia factors produced by leukocytes from individuals with cystic fibrosis and asthma are different substances. *Nature*, submitted (1977).
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40. Requests for reprints should be addressed to: G.B. Wilson, Ph.D., Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, 88 Barre Street, Charleston, South Carolina 29401 (USA).
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TABLE 1
CDA AND CFP IN HUMAN SERA

Serum type	Number studied	CFP ^a	Onset of Ciliary dyskinesia (min) ^b
CF homozygote	16	+	14 ± 8
Obligate heterozygote	15	+	25 ± 8
Sibling of CF patient	3	-	NR
	4	+	19, 24, 29, 35
Normal individual	13	-	NR
	1	+	20
Asthma	7	-	22 ± 9
	1 ^c	+	15

^a+, present; -, absent.

^bMean ± S.D.; NR, no reaction at 60 min.

^cPresumed heterozygote carrier for CF (see text).

TABLE 3

CDA AND CFP IN FRACTIONS FROM SEPHADEX G200 GEL FILTRATION OF HUMAN SERA

Serum type	CDA and CFP present in fraction ^a					
	I	II	III	IV	V	VI
Normal	-	-	-	-	-	-
Normal-EDTA ^b	-	-	-	-	-	CDA
CF homozygote	-	-	CFP/ CDA	CDA	-	-
CF homozygote-EDTA ^b	-	-	-	-	-	CFP/ CDA
Heterozygote	-	-	CFP/ CDA	CDA	-	-
Heterozygote-EDTA ^b	-	-	-	-	-	CFP/ CDA
Asthmatic	-	CDA	-	-	-	-
Asthmatic-EDTA ^b	-	-	-	-	-	CDA
Asthmatic/heterozygote ^c	-	CDA	CFP/ CDA	CDA	-	-
Asthmatic/heterozygote-EDTA ^b	-	-	-	-	-	CFP/ CDA

^aCFP and CDA positivity as defined in Table 2.

^bSerum pretreated by mixing with solid EDTA to saturation (final pH 3.7).

^cPresumed heterozygote carrier (see text).

TABLE 2

CDA AND CFP IN FRACTIONS FROM DEAE-CELLULOSE CHROMATOGRAPHY OF HUMAN SERA

Serum type ^a	CDA and CFP present in fraction							
	0.01 M phosphate buffer (pH 8.0)				0.005 M Tris-HCl buffer (pH 8.6)			
	I ^b (0.0) ^c	II (0.05) ^c	III (0.1) ^c	IV (0.2) ^c	I ^b (0.0) ^c	II ^b (0.05) ^c	III (0.1) ^c	IV (0.2) ^c
Normal (CFP-, CDA-)	-	-	-	-	-	-	-	-
CF homozygote (CFP+, CDA+)	CFP/ CDA	-	-	-	CFP/ CDA	-	-	-
Obligate heterozygote	CFP/ CDA	-	-	-	CFP/ CDA	-	-	-
Asthmatic (CFP-, CDA+)	CDA	-	-	-	-	CDA	-	-
Asthmatic/heterozygote ^d (CFP+, CDA+)	CFP/ CDA	-	-	-	CFP/ CDA	CDA	-	-

^aSera and fractions are indicated as being cystic fibrosis protein (CFP) positive as determined by electrofocusing, and as having ciliary dyskinesia activity (CDA) as determined by the rabbit tracheal bioassay. Sera and fractions noted as CDA positive produced ciliary dyskinesia by 35 min.

^bFractions containing IgG as determined by immunoelectrophoresis.

^cMolarity of NaCl used for elution.

^dPresumed heterozygote carrier (see text).

TABLE 4

CDA IN SUBFRACTIONS FROM BIO-GEL P10 FILTRATION OF ACTIVE FRACTIONS

Sample	CDA present in subfraction ^a						
	A	I	II	III	IV	V	VI
CF-EDTA G200 Fr.VI ^b	NP	CDA	-	CDA	CDA	CDA	-
Normal-EDTA G200 Fr.VI ^b	NP	CDA(+)	-	-	-	CDA	-
Heterozygote-EDTA G200 Fr.VI ^b	NP	CDA	-	CDA	CDA	CDA	-
Asthma-EDTA G200 Fr.VI ^b	NP	CDA	-	-	-	CDA	-
Asthma G200 Fr.II	CDA	-	-	-	-	-	-
Asthma G200 Fr.II-EDTA ^c	-	CDA	-	-	-	-	-
Normal G200 Fr.II-EDTA ^c	-	CDA	-	-	-	-	-
CF G200 Fr.III, IV	CDA	-	-	-	-	-	-
CF G200 Fr.III, IV-EDTA ^c	-	CDA	-	CDA	CDA	-	-
Normal G200 Fr.III, IV-EDTA ^c	-	CDA	-	-	-	CDA(+)	-
CF IgG	CDA	-	-	CDA(+)	-	-	-
CF IgG-EDTA ^c	-	CDA	-	CDA	CDA	-	-

^aNP, no peak; CDA as defined in Table 2; CDA(+), weak activity. Subfractions were 2X concentrated before testing.

^bFractions from EDTA-treated samples as in Table 3 but not treated with EDTA again before Bio-Gel P10 filtration.

^cSample pretreated by mixing with solid EDTA to saturation (final pH 3.7) before Bio-Gel P10 filtration.

TABLE 5

RELATIVE PURIFICATION OF CILIARY DYSKINESIA ACTIVITIES IN CF OR ASTHMATIC SERA

Test material	Average protein content (mg/ml) ^a	Specific activity (units/mg) ^b	Purification ^c
Whole serum	65.0	0.015	-
Sephadex G200 fraction			
II	11.00	0.091	5.90
III	9.50	0.105	6.84
IV	19.0	0.053	3.42
VI ^d	0.330	3.03	197.0
Bio-Gel P10 EDTA subfraction ^e			
I	0.079	12.66	823.0
III	0.046	21.74	1413.0
IV	0.055	18.18	1181.8
V	0.069	14.49	942.0
I,V	0.148	6.76	439.19
III,IV	0.100	10.00	650.00

^aAverage of 3-5 determinations made on different samples with ciliary dyskinesia activity, except for whole serum, which is the average for 6 active samples (3 asthma, 3 CF homozygote).

^bOne unit is defined as the reciprocal of the protein content in a 1X concentrated fraction that shows activity no later than 35 min (assuming that the entire protein content is required for activity).

^cPurification of activity relative to whole serum (assuming that there is no loss of activity).

^dFraction obtained from EDTA-treated serum.

^eSubfractions obtained from Sephadex G200 Fr.VI of active preparations of EDTA-treated asthma or CF homozygote sera.

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

Fig. 2. Results of electrofocusing on the LKB Multiphor (pH 2.5-10) of serum fractions obtained from DEAE-cellulose chromatography using phosphate buffer, pH 8.0 (phosphate), or Tris-HCl buffer, pH 8.6 (Tris-HCl). N, C, H, A, normal control, CF homozygote, CF heterozygote, and asthmatic sera respectively. Left to right, Fraction I obtained after DEAE-cellulose chromatography of N, A, H, or C sera using phosphate buffer; Fraction I and II from DEAE-cellulose chromatography of C and H serum respectively using Tris-HCl buffer; whole CF and normal sera. All serum samples and DEAE-cellulose samples contained 100 µg of IgG. See Table 2 for definition of fractions. Line indicates location of cystic fibrosis protein (CFP). A scale (right) shows the distance in centimeters from the bottom of the sample application pad (0.5 cm from the anode).

Fig. 3. Analysis of whole serum and fractions obtained from Sephadex G200 gel filtration and DEAE-cellulose chromatography by immunoelectrophoresis at pH 8.6. Patterns developed with goat antiserum to whole normal human serum. (a) Whole serum. (b) Sephadex G200 Fraction II. (c) Sephadex G200 Fraction III. (d) DEAE-cellulose Fraction I, phosphate buffer. (e) DEAE-cellulose Fraction I, Tris-HCl buffer. (f) DEAE-cellulose Fraction II, phosphate buffer. (g) DEAE-cellulose Fraction II, Tris-HCl buffer. (h) Whole serum. See Tables 2 and 3 for definition of fractions from DEAE-cellulose chromatography and Sephadex G200 gel filtration.

Fig. 4. Typical chromatogram obtained by fractionation of 2.0 ml of serum on Sephadex G200. HSA, human serum albumin; V_0 , V_T , void volume and total column volume respectively. Similar patterns were obtained with regular or EDTA-treated sera.

Fig. 5. Results of electrofocusing on the LKB Multiphor of Sephadex G200 Fractions II and III from untreated sera. Left to right, whole N, A, H, and C sera; G200 Fraction III from N, H, C, or A sera; G200 Fraction II from A, C, or N sera. A, C, N, H, and scale as defined in Figure 2.

Fig. 6. Disc gel electrophoresis of Fraction VI from Sephadex G200 gel filtration of untreated sera. Dots depict cathodally migrating bands of interest. H, N, C, and A as in Figure 2. Note presence of extra cathodal migrating bands in H and C G200 Fraction VI. Each sample contained 25 μ g protein.

Fig. 7. Disc gel electrophoresis of Fraction VI from Sephadex G200 gel filtration of EDTA-treated sera. Dots depict six bands of interest. Untreated normal and CF G200 Fractions VI are shown (c,d) next to G200 Fractions VI from H, N, and C sera (a,b,e) to facilitate direct comparison of the results. Note the occurrence of additional cathodal bands (bands 4, 5, and 6) in samples a and e as compared with b, c, and d. Each sample contained 25 μ g of protein, except for e, which contained 35 μ g.

Fig. 8. Effect of EDTA treatment on serum positivity for CPP as shown by electrofocusing on the LKB Multiphor (pH 2.5-10). Left to right, whole N, H, and C sera; Fraction A from Bio-Gel P10 filtration of CF DEAE-cellulose Fraction I (Tris-HCl) without and with prior treatment with EDTA (C and C-EDTA); Sephadex G200 Fraction III from N or C sera without or with prior treatment with EDTA. Scale as in Figure 2.

Fig. 9. Results of disc gel electrophoresis of G200 Fraction VI from EDTA-treated CF or normal serum (25 μ g). Arrows mark cathodal bands with pIs near pH 8.5. Note that the normal sample fails to show these bands, even though the intensity of banding elsewhere in the gel is similar.

Fig. 10. Composite chromatogram for elution of Sephadex G200 or DEAE-cellulose (Tris-HCl) fractions on Bio-Gel P10. Fractions of 2.0 ml were collected. Absorbance was recorded at 220 nm or 280 nm as indicated. V_0 as in Figure 4. See Table 4 and text for details.