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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC FRACTIONATION AND PARTIAL CHARACTERIZATION OF CYSTIC FIBROSIS SERUM ULTRAFILTRATES

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SUMMARY

Analytical separation of serum ultrafiltrates by high-performance liquid chromatography produces a distinctive peak with a retention time of 18.5—21 min (subfraction 18.5) from cystic fibrosis serum ultrafiltrates and obligate heterozygote serum ultrafiltrates, but not in significant concentrations from control or asthmatic serum ultrafiltrates. Semipreparative separation of control serum ultrafiltrates produced a small peak with similar retention time that was approximately 1% of the arbitrary absorbance units found in this cystic fibrosis subfraction. Subfraction 18.5 had biological activity only when separated from cystic fibrosis serum ultrafiltrate, but did not contain measurable amounts of C3a des-arginine and C4a des-arginine. Subfraction 18.5 is a low-molecular-weight material (1000—1400 daltons) that contains 14.9 μg orcinol positive material per 50 μg protein. The spectrum of subfraction 18.5 indicates that it has to be purified to homogeneity.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease that is expressed by dysfunction of the exocrine glands resulting in pulmonary disease, pancreatic insufficiency, intestinal malabsorption and changes in the electrolyte levels of

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various secretions [1]. While elevated levels of ions (sodium and chloride) in the sweat of CF patients have proven to be a reliable diagnostic marker for this disease, no molecular abnormality has been identified to date. CF patients and their parents have a factor or factors in their body fluids (serum, sweat, saliva, and urine) that may be related to the molecular abnormality or to a secondary phenomenon associated with this disease [1—8]. Description of these factors have been based on their biological activities in different experimental systems, including mucociliary dysfunction [2—4, 8], inhibition of glycogen debranching enzyme [5, 6], lectin-like binding [7] and increased K^+ release from submandibular fragments [9]. However, the exact role of this factor or factors in CF still remains unresolved.

These factors have consistently been described as very labile substances that have a molecular weight below 10 000 daltons, with reported p*I* values of either 8.4—9.0 or 4.8—6.0 and may be peptides or glycoproteins [3—10]. This paper describes our attempts to fractionate biologically active CF serum ultrafiltrates by means of high-performance liquid chromatography (HPLC) and to characterize the subfractions that appear to be specific for CF sera.

EXPERIMENTAL

Serum samples

After informed consent and in accordance with the guidelines proposed in the Declaration of Helsinki, venous blood (5—10 ml) was drawn from CF patients (n = 89), asthmatics (n = 25), obligate heterozygotes (n = 11), and control (non-CF) individuals (n = 14) at the CF Clinic, St. Vincent's Hospital and Medical Center over a three-year period. Medical histories excluded autoimmune and other pulmonary diseases. The blood was allowed to clot at 4°C for a minimum of 3 to 6 h and centrifuged at 850 *g* for 15 min [8, 11]. The serum was harvested and either stored in plastic tubes at —70°C until used or immediately subjected to ultrafiltration at 4°C. The latter regimen became routine procedure.

Ultrafiltration was accomplished in an Amicon cell using PM10 Diaflo ultrafiltration membranes with a theoretical molecular weight cut off of 10 000 daltons at 4°C and 580 bar nitrogen gas. Serum (3—5 ml) was ultrafiltered with aliquots of the effluent either used immediately or stored at —70°C. Some ultrafiltrates (effluents) were concentrated three-fold before injection by a second ultrafiltration step using UM05 membranes that have a theoretical molecular weight cut off of 500 daltons with both the concentrated retained material and effluents collected for analysis.

Apparatus

The HPLC apparatus consisted of a Beckman 100A pump (Fullerton, CA, U.S.A.), a Beckman 421 controller, an Altex 210 injector valve fitted with either a 50 or 250 µl loop, a Beckman 100-40 variable-wavelength monitor used at 280 or 254 nm and a Beckman CR-1A integrator. Isocratic separations were carried out at ambient temperatures using a Protein PAK 60 column (Waters Assoc., Milford, MA, U.S.A.) and a mobile phase of 0.1 *M* sodium phosphate, pH 6.8—0.2 *M* sodium chloride with a flow-rate of 0.75 ml/min. The use of elevated levels of buffer and salt allowed for reproducible separation

by reducing hydrophobic and ionic interactions between the stationary phase and the ultrafiltrate sample. Effluents were monitored at 280 nm and subsequently at 254 nm. The Beckman CR-1A integrator was used to determine peak areas and to represent the subfraction (peak) as either a percentage of the total area (concentration) or as the total area under the peak in arbitrary (absorbance) units. Buffer was injected into the column between sample injection to assure a zero baseline and no cross contamination of the column from one sample injection to the next. Subfractions were collected that corresponded to the major absorbance peaks.

Assays

Ultrafiltrates and subfractions corresponding to peaks were assayed for K^+ release from submandibular gland fragments as previously described [11, 12]. K^+ release was presented as stimulated percent K^+ efflux over basal percent K^+ efflux times 100. Percent K^+ efflux was calculated as the K^+ released into the incubation media as a fraction of the total tissue $K^+ \times 100$ [11, 12]. C3a des-arginine (des-Arg) and C4a des-Arg were assayed in both ultrafiltrates and subfractions by means of the radioimmunoassay (RIA) devised by Hugli and Chenoweth [13] for C3a des-Arg and the method described by Gorski [14] for C4a des-Arg. Antibodies and labeled materials for RIA were purchased from Upjohn Diagnostics (Kalamazoo, MI, U.S.A.). The antibody for C3a des-Arg cross-reacts 100% with C3a as well as C3, but not with any of the components of the complement cascade derived from C4 or C5. C3 can be separated from plasma or serum by an acid precipitation step leaving C3a and C3a des-Arg in solution to be determined by RIA [13]. C3 is a large molecule (180 000 daltons) that would easily be excluded by the PM10 ultrafiltration step. Preliminary experiments indicated that the precipitation step in the RIA for C3a des-Arg could be eliminated after ultrafiltration with a PM10 Diaflo membrane that excludes molecules greater than 10 000 daltons. This is also true of the RIA for C4a des-Arg. Protein concentrations were determined by Bradford's method [15] using albumin as a standard, while orcinol positive materials were measured by the method described by Schneider [16] using purified yeast RNA as a standard.

RESULTS

Ultrafiltrates of CF serum have been reported to have biological activities by several investigators including this laboratory [11, 17]. Serum ultrafiltrates are an excellent starting material for chromatography because higher-molecular-weight proteolytic enzymes and other extraneous materials have been removed, leaving a low-molecular-weight fraction with biological activity for analysis.

High-performance liquid chromatography

Analytical isocratic analysis of control serum ultrafiltrates (20 μl) resulted in a very reproducible fractionation of the ultrafiltrates into 6—7 subfractions with characteristic retention times (Fig. 1). Separation of CF serum ultrafiltrates (20 μl) resulted in some changes in the UV profiles of several of the subfractions. Subfractions with retention times of 7—10 min as well as 12—15 min varied in absorbance not only between control and CF subfractions, but

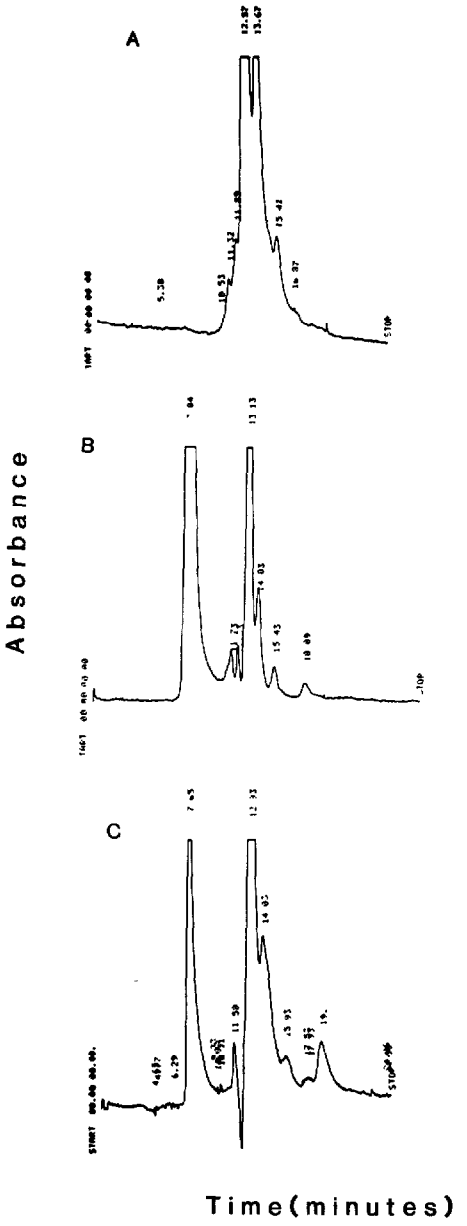

Fig. 1. HPLC separation of serum ultrafiltrates using a Protein PAK 60 column (Waters Assoc.); a mobile phase of 0.2 *M* sodium chloride—0.1 *M* sodium phosphate buffer, pH 6.8; and a flow-rate of 0.75 ml/min at ambient temperatures. Each elution profile was generated by the fractionation of 20 μl of serum ultrafiltrate treated identically. Full scale absorbance corresponds to 0.006 absorbance units at 280 nm. (A) Typical elution profile for control serum ultrafiltrates. (B) Typical profile for asthmatic serum ultrafiltrates. It demonstrates an absorbance peak at 18 min retention time, but no peak was observed at 18.5 min retention time. (C) Typical profile for CF serum ultrafiltrates. It has similar peaks as the separation profiles for control and asthmatic ultrafiltrates plus the peak with a retention time of 19 min.

also between individual CF subfractions as well. However, a distinct new subfraction with a retention time of 18.5—21 min (subfraction 18.5) was present upon analytical isocratic analysis of CF ultrafiltrates (Figs. 1 and 2). The retention time of this subfraction was verified on four different PI-60 columns used over the period of this investigation. The relative amounts of this subfraction varied from 0.7 to 15% of the absorbance units of the CF ultrafiltrate injected onto the column (Fig. 2). In addition, rechromatography after collection of the subfraction with a retention time of 18.5—21 min resulted in a recovery rate of 83.4% as indicated in Fig. 3.

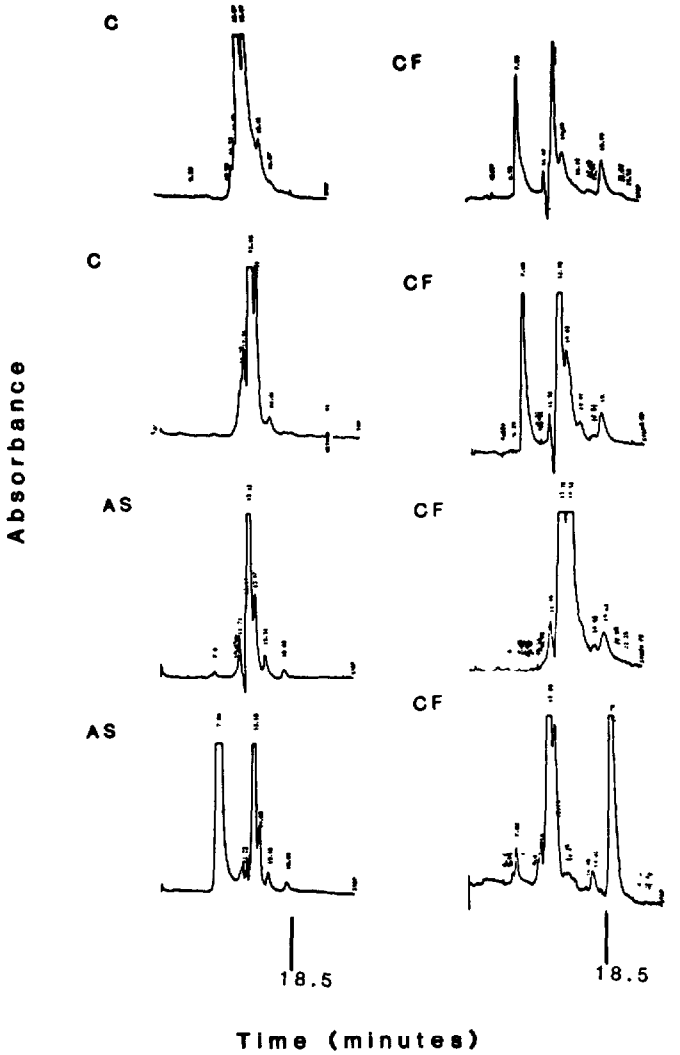

Fig. 2. Typical HPLC separation of control (C), asthmatic (AS), and CF (CF) ultrafiltrates. Each elution profile represents an analysis of the fractionation of 20 μl of serum ultrafiltrate treated identically. The vertical indicator lines represent 18.5 min from the start of the initial injection. Control and asthmatic ultrafiltrates do not produce a peak with a retention time of 18 5—21 min. Separation of CF serum ultrafiltrates consistently produces a peak with a retention time between 18.5—21 min. This peak has a concentration range from 1 to 5% in most cases, but can range up to 15% as in the bottom chromatogram.

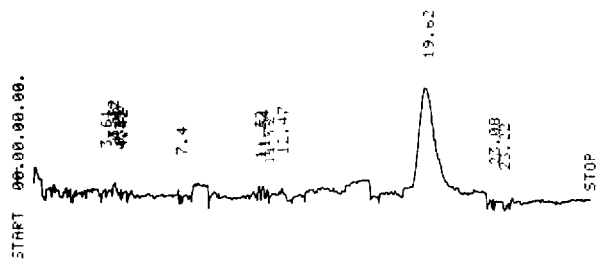

Fig. 3. A 100-μl aliquot of subfraction 18.5 was rechromatographed under identical conditions as the original separation. Of this material 92.4% had the same retention time as the original subfraction with a recovery of 83% in arbitrary absorbance units (area units × volume).

In order to determine the apparent molecular weight of subfraction 18.5, molecular weight markers were dissolved in the mobile phase buffer, injected onto the column and their retention times were determined. Retention times for aprotinin (6512 daltons), substance P (1348 daltons), and serotonin (212 daltons) were 13.5 min, 18.2 min and 26.9 min, respectively. Using this calibra-

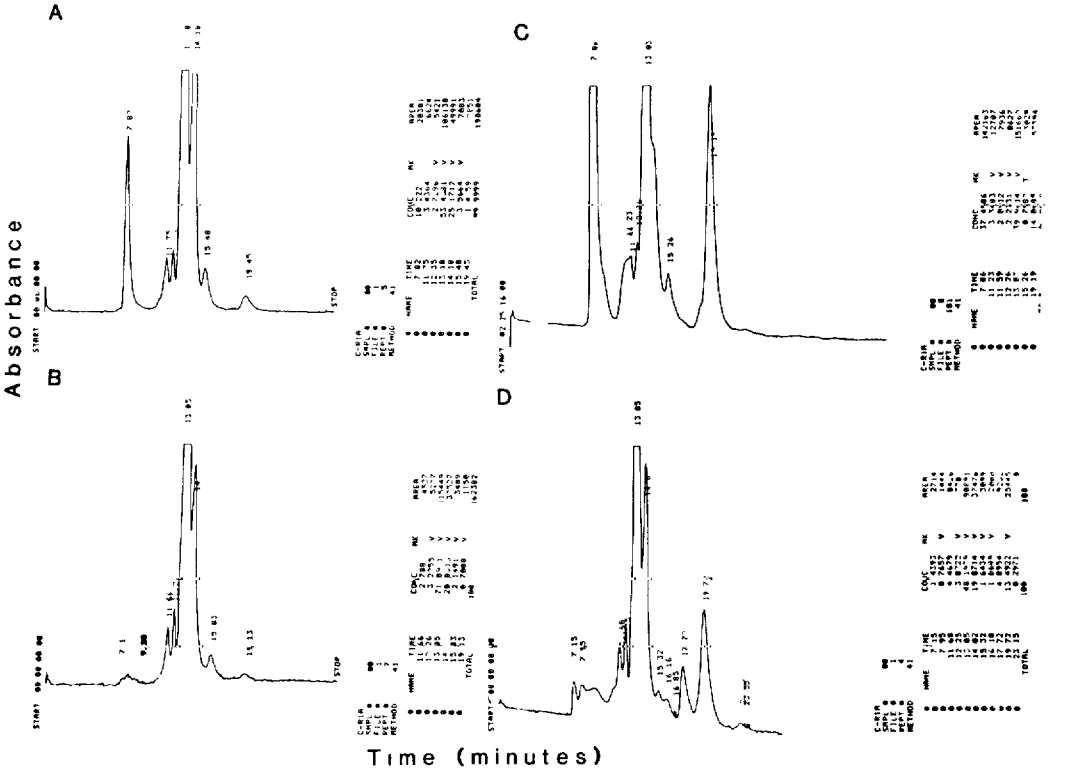

Fig. 4. Chromatograms of the fractionation of serum ultrafiltrates from parent (obligate heterozygote) and affected child (CF) Note that in each case the obligate heterozygote has less than 50% of the arbitrary absorbance units or concentration of subfraction 18.5 of the CF. This is the case when the CF child has a low (A) or high (C) concentration of subfraction 18.5 (A) Fractionation of serum ultrafiltrate from CF child 9. (B) Fractionation of serum ultrafiltrate from parent of CF child 9. (C) Fractionation of serum ultrafiltrate from CF child 6. (D) Fractionation of serum ultrafiltrate from parent of CF child 6. Areas of subfractions 18.5: (A) 2851; (B) 1150; (C) 53 394; (D) 25 445.

tion curve, the apparent molecular weight of subfraction 18.5 was 1000—1400 daltons.

Subfraction 18.5 was found in 82% of the CF ultrafiltrates analyzed (n = 89). CF patients with and without pancreatic insufficiency appeared in both

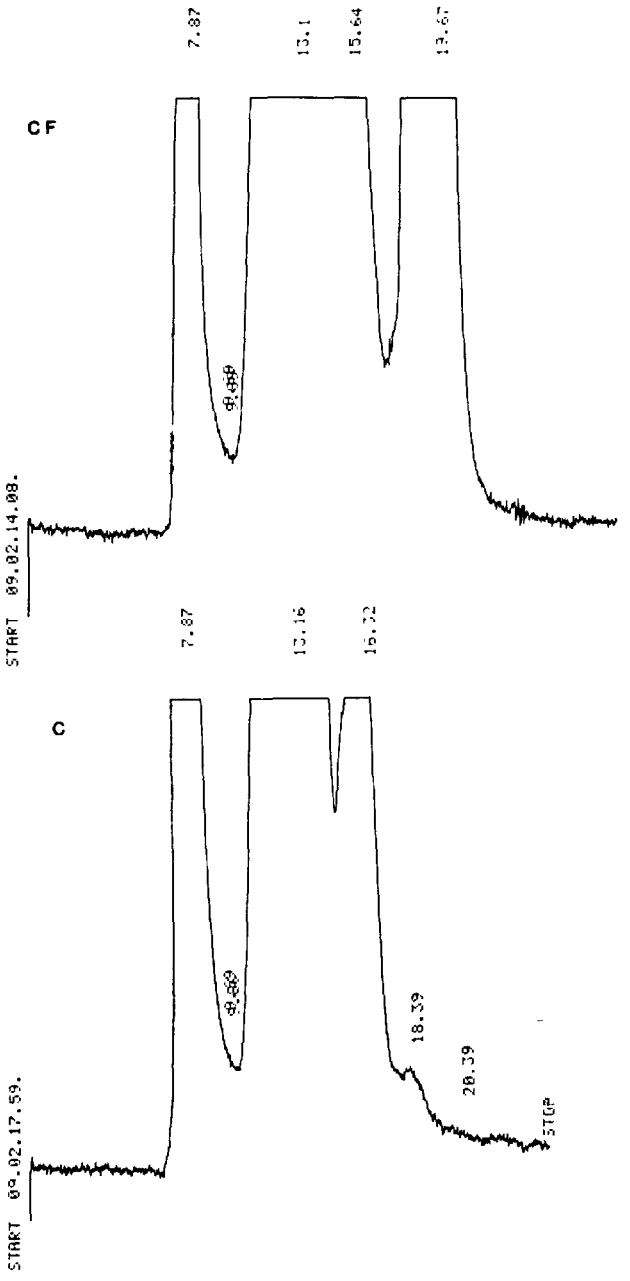

Fig. 5. Separation of concentrated control serum (C) ultrafiltrates now produces a small peak with a retention time of 20.39 min and 4300 arbitrary units. Separation of an equal amount of concentrated CF serum ultrafiltrate produces a subfraction with retention time of 19.67 min that is approximately 99 times greater with 436 000 arbitrary units.

categories. Subfraction 18.5 was not observed upon fractionation of control serum ultrafiltrates (20 μl) from non-CF individuals (n = 14) or from asthmatics (n = 25). The only other population that did have this subfraction (18.5—21 min) present in their serum ultrafiltrates in measurable levels was obligate heterozygotes where 49% of the ultrafiltrates from obligate heterozygotes (n = 11) demonstrated the presence of subfraction 18.5. In addition, subfraction 18.5 from the serum ultrafiltrates of obligate heterozygotes was always significantly less than that observed in CF ultrafiltrates. In these experiments, serum samples were drawn from CF patients (n = 4) and one parent of each patient (obligate heterozygotes, n = 4) on the same day and subsequently processed and analyzed together. Obligate heterozygotes have 50% or less of the arbitrary absorbance units observed in subfraction 18.5 when compared to the same subfraction derived from the heterozygote's affected child. This was true whether the affected individual had a larger or smaller concentration of subfraction 18.5 (Fig. 4).

Serum ultrafiltrates were concentrated by a second ultrafiltration step using an UM05 Diaflo membrane that retains molecules with a molecular weight of 500 daltons. The amount of serum ultrafiltrate with a molecular weight of 500—10 000 daltons loaded on to the column was increased approximately 30 fold by injecting 200 μl of the retained material from the second ultrafiltration step. Separation of concentrated control serum ultrafiltrates using this semi-analytical technique now produce a peak with a retention time of 20.39 min. This retention time is within the range of 18.5—21 min that defines subfraction 18.5. However, this control subfraction (20.39 min) has a different retention time than the CF subfraction (19.67 min) that was produced under these semianalytical conditions. In addition, the control subfraction contains only 1% of the arbitrary absorbance units of subfraction 18.5 produced by an equal volume of concentrated CF serum ultrafiltrate (4300 vs. 436 000; see Fig. 5). For reference, subfractions from control serum ultrafiltrates with retention times 7.9 and 13.3 min contained 53 and 65% of the arbitrary absorbance units found in the same subfractions separated from CF serum ultrafiltrates. Equal amounts of concentrated serum ultrafiltrates were fractionated for assay of biological activity and characterization studies.

Biological activity

Ultrafiltrates of serum as well as the effluents (subfractions) collected after fractionation of the ultrafiltrates by means of HPLC were tested for their ability to produce K^+ release from rat submandibular gland fragments. As previously reported [11], CF ultrafiltrates were found to produce a significant release of K^+ from the submandibular gland fragments when compared to control serum ultrafiltrates (Table I). Several subfractions of CF ultrafiltrates produced a significant K^+ release from rat submandibular gland fragments. These included subfractions from CF ultrafiltrates with retention times of 10—12 min, 12—14 min and 18.5—21 min. Subfraction 18.5 produced a slightly greater K^+ efflux than the other subfractions. However, the specific activity (percent K^+ efflux divided by absorbance units) of subfraction 18.5 was significantly greater than the other subfractions. The specific activities of CF ultrafiltrate and of subfractions with retention times of 10—12, 12—14, and 18.5—21

TABLE I

BIOLOGICAL ACTIVITY

Percent of K^+ released from rat submandibular fragments above basal after 20 min produced by various subfractions separated from CF serum ultrafiltrates. Values in this table represent the mean ± S.D. of stimulated percent K^+ released above the basal percent K^+ released × 100 after 20 min in four to six experiments. The CF serum ultrafiltrates produced 142 ± 15% K^+ release above basal × 100.

Subfraction of CF serum (retention times, min)	K^+ released above basal × 100 (%)
7 —10	104 ± 9
10 —12	127 ± 11
12 —14	116 ± 9
14 —16	99 ± 8
16 —18.5	107 ± 8
18.5—21	144 ± 12

min were 45.87, 1235, 966 and 5275, respectively. No stimulation of K^+ release was observed upon the addition of subfractions from CF ultrafiltrates with retetion times of 6—8, 8—10, 14—16 and 16—18.5 min or from subfractions collected from control ultrafiltrates.

C3a des-Arg, C4a des-Arg, protein and carbohydrate analysis

The concentration of C3a des-Arg and C4a des-Arg was determined in ultrafiltrates and HPLC effluents (subfractions). C3a des-Arg was found in appreciable amounts in subfractions from CF ultrafiltrates with retention times of 10—12 and 12—14 min and to a lesser extent in subfraction 14—16 min (Table II). C4a des-Arg was found in subfractions with retention times of 10—12 and 12—14 min, but not in any of the other subfractions (Table II). It should be pointed out that these subfractions were from serum samples not optimally processed for detection of C3a des-Arg or C4a des-Arg, but handled as described for HPLC analysis or for biological activity.

Biochemical analysis of subfraction 18.5 indicated that it had 14.9 μg of

TABLE II

RIA FOR C3a DES-ARGININE AND C4a DES-ARGININE

RIA for C3a des-Arg and C4a des-Arg was carried out on subfractions separated from CF serum ultrafiltrates by HPLC. The values in this table represent the mean ± the S.D. of five assays.

Subfraction (retention time, min)	Concentration (ng/ml)	
	C3a des-Arg	C4a des-Arg
7 - 10	0	0
10 - 12	47.5 ± 28.3	57.7 ± 29.7
12 - 14	37.3 ± 33.6	13.1 ± 9.2
14 —16	4.2 ± 5.3	0
16 —18.5	0	0
18.5—21	0	0

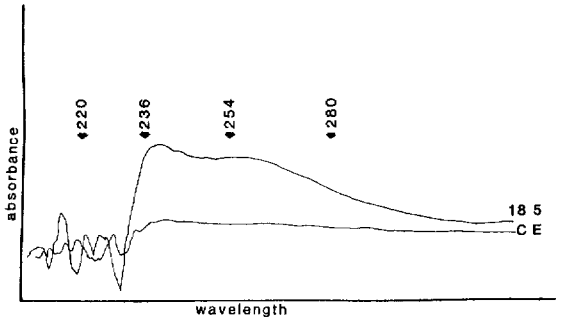

Fig 6 Spectrum of subfraction 18.5 (18.5) which was collected upon fractionation of concentrated CF serum ultrafiltrate and spectrum of control effluent (C E.) collected at 18.5—21 min upon fractionation of control serum ultrafiltrate.

ribose (orcinol positive material) for every 50 μg of protein. The UV spectrum of this material (Fig. 6) is a broad curve with a peak at 236 nm decreasing gradually to 280 nm. Such a spectrum is usually indicative of the presence of a heterogeneous mixture.

DISCUSSION

Fractionation of biologically active serum ultrafiltrates below 10 000 daltons appears to be a viable strategy in the study of CF factors. Ultrafiltrates of serum in this molecular weight range have been documented as having biological activity by several investigators [12, 17]. The ultrafiltrates are free of proteolytic enzymes that could alter the reproducibility of isolation and purification studies by generating fragments from larger proteins. Non-denaturing fractionation of serum ultrafiltrates can be accomplished by means of HPLC employing a Protein PAK 60 column in the gel permeability mode. This stratagem resulted in a separation of ultrafiltrates into several subfractions with reproducible retention times without loss of biological activity. This was accomplished by using a mobile phase consisting of a phosphate-buffered sodium chloride solution instead of organic solvents. Elevated concentrations of sodium chloride have been demonstrated to have a stabilizing effect on low-molecular-weight CF factors [5]. Thus, the effluents contained non-denatured molecules in a physiologically compatible solution that was relatively stable and capatible with several of the bioassays as well as future immunological assessment. The major draw-back to the use of this technology is that significant levels of salts are utilized and must be subsequently removed for complete characterization of the active subfractions.

Our experience with subfractions of CF ultrafiltrates indicates that there are two subfractions with distinct retention times that demonstrate biological activity; one with retention time of 10—14 min, while the other has a retention time of 18.5—21 min. These retention times have apparent molecular weight ranges of 6500—9000 daltons and 1000—1400 daltons, respectively. It appears that there are several biologically active substances in the 6500—9000 daltons molecular weight range including both C3a des-Arg and C4a des-Arg. The presence of these components of the complement cascade were measured in

ultrafiltrate preparations that omitted the addition of the recommended disodium EDTA [13]. This was done to ensure that the measured concentrations were the same as that found in the CF serum ultrafiltrates used for HPLC fractionation or bioassay.

C3a and C4a are biologically active constituents of the complement cascade and have many of the properties that have been assigned to CF factor, i.e. both are cations and have molecular weights of 9038 and 8740, respectively. Indeed, it is possible that some aspects of the ciliary dyskinesia response may be due to cell damage produced by these constituents of the complement cascade. We observed [9] that both CF and control serum produced cell damage subsequent to the time that ciliary dyskinesia was measured and that this damage was far more extensive in the presence of CF sera. Polly and Bearn [18] demonstrated that C3a des-Arg can produce a ciliary dyskinesia, while Kennedy and co-workers [19, 20] demonstrated that CF serum does not produce ciliary dyskinesia in the presence of anti-C3, anti-C4 or anti-C6. There is little doubt that CF serum contains significant levels of complement activation products. CF patients have variable levels of C3 [8, 18, 21]. However, several studies of complement activation products in CF serum revealed normal functional activation of both the classical and alternative pathway as well as normal levels of carboxypeptidase B activity [22, 23]. Thus, the presence of these anaphylatoxins and their des-arginine inactivation products in the subfractions with retention times of 10—14 min is most likely the result of the chronic immunological challenge produced by colonization of the respiratory tract by such infectious agents as *Pseudomonas aeruginosa* and is probably not directly related to the molecular abnormality.

The only remaining subfraction with biological activity separated from CF serum ultrafiltrates is the low-molecular-weight subfraction with a retention time of 18.5–21 min. Subfraction 18.5 has a molecular weight of approximately 1000—1400 daltons. It does not appear in appreciable amounts in the control serum ultrafiltrates. The absorbance peak separated from concentrated control serum ultrafiltrate with a retention time of 20.36 min is approximately 1% of the amount found in CF absorbance peak with a retention time of 19.6 min. If this material is the same as that found in the CF subfraction 18.5, than it might represent a metabolite that is not completely degraded in CF serum, but would be metabolized in control serum. However, these absorbance peaks were separated by a gel permeation HPLC method which produces subfractions that contain different substances of similar molecular weight. Subfraction 18.5 does not contain C3a des-Arg or C4a des-Arg, but did appear in approximately half of the obligate heterozygote ultrafiltrates tested in appreciable amounts. Thus, subfraction 18.5 appears to be directly related to this disease entity in some manner.

Several other investigators have also identified low-molecular-weight factors in CF serum with biological activities including lectin-like binding [10] and inhibition of glycogen debranching enzyme [5, 6]. In addition, CF subfraction 18.5 also inhibits glycogen debranching enzyme [24]. We have identified a low-molecular-weight material that contains both a peptide or peptides with a ribose or other orcinol positive group. Subfraction 18.5 appears to be related in some undetermined manner to CF. However, it is not possible at this time to

assign a specific structure to this material. The spectrum of this subfraction indicated that it is heterogenous. We are now in the process of purifying and characterizing this subfraction in order to determine the chemical nature of this material and its relationship to CF.

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REFERENCES

1 P.A. DiSant'Agnese and P.A. Davis, N. Engl. J. Med., 293 (1977) 481.
2 J.D. Mangos and N.R. McSherry, Science, 158 (1967) 135.
3 M.C. McNeely, Y.C. Awashti, D.R. Barnett, T. Iwasumi, L. Schneider, S.K. Srivastawa and B.H. Bowman, Pediatr. Res., 16 (1982) 21.
4 J.H. Conover, R.J. Bonforte, P. Hathaway, S. Paciuc, E.J. Conod, K. Hirshhorn and F.B. Kopel, Pediatr. Res., 7 (1973) 220.
5 J.E. Impero, G.M. Harrison and T.E. Nelson, Pediatr. Res., 12 (1978) 108.
6 J.E. Impero, G.M. Harrison and T.E. Nelson, Pediatr. Res., 15 (1981) 940.
7 J. Lieberman and W.M. Kaneshiro, Am. J. Med., 77 (1984) 678.
8 G.B. Wilson, in E. Shapira and G.B. Wilson (Editors), Immunological Aspects of Cystic Fibrosis, CRC Press, Boca Raton, FL, 1984, p. 150.
9 B.I. Bogart, E.J. Conod, P.F. Gaerlan and J.H. Conover, Pediatr. Res., 12 (1978) 15.
10 E.J. Carson and B.H. Bowman, Pediatr. Res., 16 (1982) 13.
11 B.I. Bogart, J. Picarelli, P.A. Gaerlan and C.R. Denning, Pediatr. Res., 16 (1982) 223.
12 B.I. Bogart and J. Picarelli, Am. J. Physiol., 235 (1978) C256.
13 T.E. Hugli and D.E. Chenoweth, in R.M. Nakamura, W.R. Dito and E.S. Tucker, III (Editors), Immunoassays Clinical Laboratory Techniques for the 1980s, Alan R Liss, New York, 1978, p. 443.
14 J.P. Gorski, J. Immunol. Methods, 47 (1981) 61.
15 M.M. Bradford, Anal. Biochem., 72 (1976) 248.
16 W.C. Schneider, Methods Enzymol., 23 (1955) 680.
17 E.J. Conod, J.H. Conover and P.A. Garelan, Pediatr. Res , 11 (1977) 45.
18 M.J. Polly and A.G. Bearn, J. Med. Genet., 11 (1974) 249.
19 J.R. Kennedy and P.L. Allen, Pediatr. Res., 14 (1980) 1173.
20 K.D. Lin and J.R. Kennedy, CF Club Abstracts, 24 (1983) 29.
21 R.B. Moss and N.J. Lewiston, Am. Rev. Respir. Dis., 12 (1980) 23.
22 J. Lieberman, Am. Rev. Respir. Dis., 111 (1975) 100.
23 P.O. Schiotz, N. Hoiby, F. Juhl, H. Permin, H. Nielson and S.E. Svehag, Acta Pathol. Microbiol. Scand., C 85 (1977) 57.
24 T. Kelleher, CooperBiomedical Co., personal communication, 1985.