

CHARACTERIZATION OF CYSTIC FIBROSIS FACTOR AND ITS INTERACTION WITH HUMAN IMMUNOGLOBULIN*

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(Received for publication 2 April 1973)

The recognition of a factor in the serum and media from skin fibroblast cultures established from the majority of patients with cystic fibrosis that affects the movement of rabbit tracheal cilia (1) and oyster cilia (2) has provided additional evidence that this disease can no longer be regarded as a primary disorder of the exocrine glands (3). The relationship of the cystic fibrosis factor activity (CFFA) to the primary defect has not been established. The purpose of this study is to characterize CFFA from skin fibroblast cultures grown in the absence of serum and to examine its association with IgG.

Materials and Methods

Skin fibroblast lines were established from 18 patients having serum CFFA by the oyster cilia test (2) and 12 normal subjects in whom no activity could be detected (4). After 1 mo in culture (two to four subcultures by trypsinization) cells were stained with toluidine blue O and Alcian blue (4).

Cell lines were trypsinized into suspension and approximately 10^6 cells were inoculated into 75-cm² Falcon flasks (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing Waymouth's "special" medium (5) with 10% by volume of fetal calf serum. After the cells had formed an adherent nonconfluent monolayer (usually 12 h), the cultures were washed three times with warmed balanced salt solution, and Waymouth's "special" medium without serum was added (pH 7.5–7.8). Cell growth was monitored by total cell counts and DNA synthesis using tritiated thymidine (6).

An aliquot of medium was removed from cultures 72 h after a medium change and assayed for CFFA. This medium will be referred to as "used medium." The medium was allowed to equilibrate in plastic Petri dishes at 25°C to a pH of 8.0 and tested for CFFA within 1 h. Ciliary action was observed under an inversion microscope by the method of Bowman et al. (2) using gills from oysters *Crassostrea virginica* under the following conditions: (a) Oysters were kept in an aquarium for 1 mo to ensure "clean gills." (b) The gill segments were kept in unused medium at 4°C for 72 h before use to allow the crypts to release debris. (c) Care was taken to eliminate microscopic air bubbles as undissolved air appeared to prevent the inhibition of cilia. (d) Media were not dialyzed against sea water.

Columns (0.9 × 44 cm) were packed with Dowex 1-2X (200–400 mesh) resin in the chloride

* This research was made possible by a grant from The National Foundation-March of Dimes and supported in part by U.S. Public Health Service grants AM 13429, AI 09239, AM 20122, AM 11796, and AM 15103.

form and equilibrated with 0.15 M NaCl (7). 25 ml of used culture medium was precipitated by 1% cetylpyridinium chloride (CPC) and the supernatant containing the small molecular weight, negatively charged substances applied to the column. The eluate of the supernatant will be referred to as the initial volume. After washing with 50 ml of 0.15 M NaCl, elution was performed with increasing concentrations of NaCl (0.4, 0.8, 1.3, 1.5, 1.8, and 2.0 M). Each fraction was precipitated with ethanol at 4°C, redissolved in 1 ml of unused medium, and assayed for protein (8), uronic acid (9), and CFFA. To determine the influence of human IgG1 on the elution pattern of the CFFA, 3.0 mg of human IgG1 was added to 25 ml of used CFFA-positive medium and to normal medium after CPC precipitation and immediately before chromatography.

The interaction between CFFA and different immunoglobulins was studied in the following way. 50 μ g of isolated monoclonal human immunoglobulins (IgG, IgA, IgM, IgD) followed by 100 μ l of rabbit antihuman immunoglobulin of corresponding specificity were added to 1.0-ml samples of used Waymouth's "special" medium from normal or cystic fibrosis metachromatic cultures. Titration experiments indicated that the rabbit antiglobulin was in antibody excess with respect to the 50 μ g of immunoglobulin. Controls included used and unused media, isolated human immunoglobulin, and rabbit antiglobulin in unused media. After incubation at 4°C for 24 h, the samples were centrifuged for 30 min at 1,600 *g* and the supernatant was tested for CFFA. To study the site of interaction of CFFA with the IgG molecule, light and heavy chains of IgG1, papain Fab and Fc fragments, and a pepsin F(ab')₂ fragment of an IgG1 monoclonal protein were prepared.

RESULTS

A number of variables influenced the ciliary inhibition by used media from metachromatic cystic fibrosis cultures. Ciliary inhibition could be demonstrated only if the media had been in contact with a growing cell population for at least 48 h and only if plastic rather than glass flasks were used to grow the cells in serum-free medium. CFFA was lost when the used medium was stored at room temperature over a 24–48 h period or at 4°C over an 11 day period (however, activity was retained at –20°C for at least 11 days), when the medium was heated to 100°C for 1 min or to 56°C for 30 min, when the medium was exposed to 5% CO₂ with a consequent shift of the pH to the acid range (6.8–7.2) and equilibrated to pH 8.0 for testing. Media with CFFA could be diluted 1:4 before a decrease in activity could be demonstrated. Addition of IgG (50 μ g/ml) had no effect on the stability of CFFA. When used culture media were assayed for ciliary inhibition using the conditions outlined, monitoring of media for CFFA gave reproducible results in 45/46 double blind experiments. After dialysis for 24 h CFFA was present on both sides of dialysis membrane, which retained molecules larger than 5,000 daltons. CFFA was detected in the media of metachromatic cystic fibrosis fibroblast cultures grown in the presence and in the absence of fetal calf serum (Table I).

Anion exchange chromatography provided evidence that the cystic fibrosis factor was bound to IgG. CFFA found in serum-free media eluted only with the 1.5 M NaCl fraction (Fig. 1). This fraction had no significant content of uronic acid or protein and no detectable IgG was present by double immunodiffusion in agar. When IgG was added to the CFFA-positive medium, after

TABLE I
The Influence of Culture Media on Cell Growth (Measured as Uptake of [³H]-Thymidine and Cell Number) and CFFA (Assayed by Oyster Cilia Test) in Skin Fibroblast Cultures from a Cystic Fibrosis Patient and an Age and Sex Matched Normal

| Subjects studied | Media | Culture time in media | Cell growth* | | Cystic fibrosis factor activity [†] (CFFA) |
|------------------|--------------------------------|-----------------------|--|--|---|
| | | | [³ H]Thymidine incorporation cpm × 10 ⁻² /cell [‡] | Cells (× 10 ⁵) per culture after 14 culture days | |
| | | | <i>wk</i> | | |
| Normal | Waymouth with FCS [§] | 2 | 3.90 | 30.0 | - |
| | Waymouth without FCS | 2 | 0.40 | 4.2 | - |
| Cystic fibrosis | Waymouth with FCS | 2 | 3.30 | 27.5 | + |
| | Waymouth without FCS | 2 | 0.60 | 4.4 | + |
| | Waymouth without FCS | 4 [¶] | 0.01 | 1.0 | - |

* Initial cell inoculum per culture flask 1 × 10⁵ cells.
[†] Oyster ciliary inhibition test: positive (+), negative (-).
[‡] [³H]Thymidine added 12 h after subculture. All cultures in fifth subculture.
[§] 10% by volume fetal calf serum (FCS).
[¶] Subcultured after initial 2 wk with 1 × 10⁵ cell inoculum.

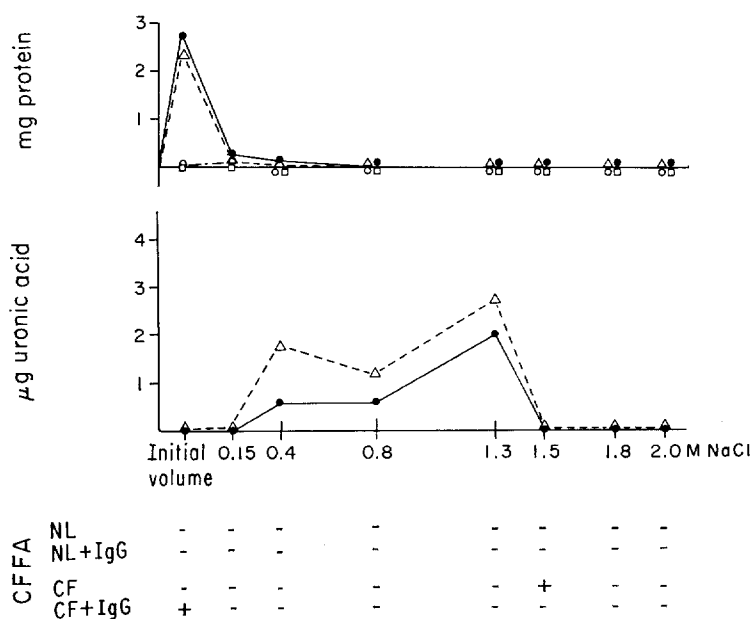


FIG. 1. Elution diagrams from Dowex 1-2X chloride columns (0.9 × 44 cm) of CPC supernatants of 25 ml of used medium from cystic fibrosis (CF) skin fibroblast cultures with cystic fibrosis factor activity (CFFA) and normal (NL) cultures with (3 mg/ml) and without IgG1 added to the CPC supernatant. Elution was performed with increasing NaCl concentrations (7). Ethanol precipitates of each fraction were assayed for protein content (8), uronic acid content (9), and oyster ciliary inhibition (CFFA) (2). Symbols: medium from normal cultures, □--□; medium from normal cultures with IgG1 added, ●--●; medium from cystic fibrosis cultures, ○--○; medium from cystic fibrosis cultures with IgG1 added, △--△.

CPC precipitation, CFFA disappeared from the 1.5 M NaCl eluate and appeared in the initial volume with the IgG1.

CFFA was lost when used media from cystic fibrosis cultures were mixed with IgG subclasses 1 and 2, or their isolated heavy chains and specific antisera (Table II). Human immunoglobulins and precipitating antisera removed CFFA when a minimum of 8–12 μg of immunoglobulin was used. IgG1 and IgG2 subclasses with antisera were equally effective on a quantitative basis in

TABLE II
Effect of Various Immunoglobulins and Their Specific Antiglobulin Antisera on CFFA of Cystic Fibrosis Culture Media

| Immunoglobulin* | No. of samples | Cystic fibrosis factor activity [†] (CFFA) |
|---------------------------|----------------|---|
| IgG1 | 3 | – |
| IgG2 | 4 | – |
| IgG3 | 2 | + |
| IgG4 | 2 | + |
| IgG1 heavy chain | 1 | – |
| IgG1 light chain κ | 1 | + |
| λ | 1 | + |
| IgG1 Fab | 1 | + |
| Fc | 1 | + |
| F(ab') ₂ | 1 | + |
| IgA1 | 2 | + |
| IgA2 | 1 | + |
| IgM | 2 | + |
| IgD | 1 | + |

* Monoclonal proteins separated by starch block electrophoresis were the source of the whole immunoglobulins, heavy and light chains, and papain Fab, Fc, and pepsin F(ab')₂ fragments. The IgG subclasses (IgG1–4) and IgA subclasses are shown. The IgG1 proteins included samples with both kappa and lambda light chains; two were Gm(f+), one was Gm(a+).

[†] The experiments involved addition of 100 μl of the appropriate class-specific antisera and 50 μg of various immunoglobulins to 1 ml of culture medium having CFFA. In control experiments the addition of the immunoglobulin or the antisera separately did not alter CFFA. Results are shown as presence (+) or absence (–) of CFFA.

influencing CFFA. Heat-aggregated IgG1 without antisera had no effect on CFFA. IgG subclasses 3 and 4, immunoglobulin classes IgM, IgA, IgD, kappa and lambda light chains, and fragments Fc, Fab, F(ab')₂, and their specific antisera had no influence on CFFA (Table II). Of the three other human serum proteins tested (Table III), only β_2 -microglobulin in the presence of its antiserum removed CFFA.

DISCUSSION

Earlier observations that CFFA in serum and used culture media containing serum could be found in the same chromatographic fraction as IgG (2, 10) suggested that CFFA might be either an immunoglobulin, a molecule with properties similar to immunoglobulin, or a molecule bound to immunoglobulin.

TABLE III
Effect of Addition of Different Serum Proteins and Their Specific Antisera on CFFA of Cystic Fibrosis Culture Media.

| Serum protein | No. of individual samples studied | No. of determinations | Cystic fibrosis factor activity* (CFFA) |
|----------------------------------|-----------------------------------|-----------------------|---|
| Haptoglobin (Hp1-1) | 1 | 4 | + |
| Group-specific component (Gc2-1) | 1 | 4 | + |
| β_2 -microglobulin | 3 | 12 | - |

* Assayed by oyster cilia test after addition of serum protein and specific antiserum. Positive (+), negative (-).

Bowman et al. (10) had previously suggested that the substance with CFFA was not an immunoglobulin, as the ciliary inhibitor associated with cultured fibroblasts grown in serum-free medium for short culture periods did not demonstrate any immunological reaction with antisera specific for IgG. Beratis et al.¹ have reported a ciliary dyskinesia factor (mol wt 1-10,000) in the used culture medium from both homozygotes and heterozygotes, which when incubated with IgG became active in the rabbit tracheal cilia assay. In the present study skin fibroblasts were grown in serum-free medium to avoid contamination with fetal calf immunoglobulins. Immunologic analysis failed to reveal any immunoglobulins in used culture medium with CFFA, indicating that immunoglobulins were not synthesized in detectable amounts by the cultured fibroblast. It was concluded from these experiments that CFFA was not an immunoglobulin, and that binding to IgG was not required for CFFA.

The following properties were noted for CFFA. CFFA was associated with a negatively charged substance that had a molecular weight of less than 5,000 daltons as determined by dialysis. CFFA was bound to IgG1 and IgG2 as shown in immunologic studies (Table II) and by modification of its elution pattern on chromatography with IgG1 (Fig. 1).

The association between human immunoglobulins and cystic fibrosis factor does not represent an antigen-antibody reaction since the latter did not bind to immunoglobulin fragments containing the antibody binding sites. The interaction of immunoglobulin and cystic fibrosis factor occurs in the constant region of IgG1 and IgG2 heavy chains that is lacking in papain or pepsin fragments. The absence of disulfide bonds does not interfere with binding. The binding is IgG class and subclass specific. Since β_2 -microglobulin, a structural homologue of immunoglobulin G, is synthesized by human skin fibroblasts grown in medium containing fetal calf serum,² the possibility that this protein is also synthesized in serum-free cultures cannot be excluded.

¹ Beratis, N., J. Conover, E. Conod, R. Bonforte, and K. Hirschhorn. 1973. Studies on ciliary dyskinesia factor in cystic fibrosis. III. Skin fibroblast cultures and amniotic fluid cells. *Pediatr. Res.* Manuscript submitted for publication.

² Hütteroth, T. H., H. Cleve, S. D. Litwin, and B. S. Danes. 1973. β_2 -microglobulin synthesis in different human cell types in culture. Unpublished results.

SUMMARY

Cystic fibrosis factor activity (CFFA), assayed as the ability to stop oyster ciliary movement, was present in serum-free medium from actively growing cystic fibrosis skin fibroblast cultures. CFFA was associated with a low molecular weight, negatively charged molecule that contained no uronic acid and was heat and pH labile. When CFFA-positive media were mixed with human IgG1, the CFFA was chromatographically displaced and emerged with the IgG1 fraction on column chromatography. Experiments in which various immunoglobulins were added to CFFA-positive culture media and then incubated with specific anti-immunoglobulins suggested that CFFA binding was class specific for human IgG, subclass specific for IgG1 and IgG2, and occurred with intact unaggregated heavy chains but not with κ - and λ -light chains, or Fab, Fc, and F(ab')₂ fragments. The serum protein β_2 -microglobulin, which has structural homology to IgG, also bound CFFA.

The authors wish to thank Dr. Henry G. Kunkel for his interest and advice. We thank Dr. M. D. Poulik for the gift of β_2 -microglobulin antiserum and M. F. D. for her encouragement during this research.

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