Evidence that the Ciliary Inhibitor of Cystic Fibrosis is not an Antibody

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ABSTRACT An inhibitor of ciliary activity is present in the serum of cystic fibrosis (CF) patients and heterozygotes. Fractionation of CF serum has indicated that the inhibitor is associated with the serum IgG fraction. This study compared the activity of the CF inhibitor to that of rabbit antibody preparations directed against oyster cilia. The aim of this investigation was to determine whether the ciliary inhibitory mechanism in cystic fibrosis is related to a typical immunological reaction. Results from experiments utilizing fluorescent antibody techniques demonstrated that an antiserum directed against oyster ciliated epithelium binds immunologically with both human bronchial and oyster epithelial tissue. Results from experiments utilizing immunofluorescent tracing, passive hemagglutination, double immunodiffusion, and papain digestion, however, indicated that the interaction between the oyster cilia and the CF serum inhibitor was not a typical antigen-antibody reaction.

INTRODUCTION

Cystic fibrosis (CF)[1] is a severe chronic disease that occurs in children due to the homozygous state of an autosomal recessive gene. The incidence in Caucasian children is approximately 1 in 2,500. Symptoms often include recurrent pulmonary infections, pancreatic insufficiency, and other abnormalities secondary to defects in exocrine function. The search for a basic metabolic error has been hindered by the presence of a myriad of clinical abnormalities in the disease (1). Mangos and McSherry (2, 3) described an inhibitor to sodium transport in CF sweat and saliva. Spock, Heick, Cress, and Logan (4) observed a serum factor in CF patients and their parents that caused cilia in rabbit trachea to beat asynchronously. Hanging-drop preparations of oyster gills were observed after exposure to serum from CF patients, obligate heterozygotes, and normal individuals. The typical reaction produced by serum from CF patients and heterozygotes has been described (5). Ciliary action is inhibited by serum from CF patients and heterozygotes, in most cases before 40 min of exposure. Normal serum does not inhibit ciliary activity and the cilia continue to beat for 60 min or longer. Similar results have been obtained in mollusks with cilia of fresh-water mussels (6). Recent observations indicate that a ciliary inhibitor produced by fibroblasts derived from CF homozygotes and heterozygotes has similar if not identical properties with the serum inhibitor (7).

The abnormal serum factor that inhibited ciliary action in the oyster, *Crassostrea virginica,* has been identified as a cationic protein with a molecular weight of 125,000–200,000 (8). The factor is isolated with the IgG fraction of serum from CF patients and heterozygotes (8, 9). Therefore, this investigation was designed to determine whether the serum factor acts as an antibody that interferes with ciliary activity. The formation of immunological complexes between cilia and specific antibodies to cilia has been shown to inhibit ciliary activity in paramecia (10) and mollusks (11). This type of interaction could conceivably produce bronchial obstruction and mucous viscosity typical of CF.

This communication reports the result of the reaction of CF serum with oyster cilia and the comparison of this to the antigen-antibody reaction between oyster cilia and antisera to that tissue. The techniques utilized were immunofluorescence, passive hemagglutination, and immunodiffusion. The specific cilia antiserum reacted im-

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[1] *Abbreviations used in this paper:* CF, cystic fibrosis; FITC, fluorescein isothiocyanate; F/P, fluorescein/protein; PBS, phosphate buffered saline; SRBC, sheep red blood cells.

munologically with ciliated tissue from oyster gills and human bronchial tissue. The CF ciliary inhibitor, in contrast, failed to react similarly to either type of ciliated epithelium.

METHODS

Serum and serum fractions. Whole human serum was obtained from seven normal individuals whose serums did not inhibit oyster ciliary motion, 10 CF patients and 1 heterozygote whose serums inhibited ciliary activity. The IgG fractions were isolated by DEAE chromatography (12), starch block electrophoresis (13), and ammonium sulfate precipitation (14). The serum proteins present after fractionation were identified by immunoelectrophoresis (15). Protein concentrations were determined by the method of Lowry, Rosenbrough, Farr, and Randall (16). Serum that contained no detectable IgG or IgM and only a trace of IgA was obtained from an adult with acquired idiopathic agammaglobulinemia.

Antiserum. Isolated ciliated epithelium was obtained from oysters by separating the membrane to which the cilia were attached from the remainder of the gill tissue with a sharp needle. The membranes, with the attached cilia, were collected and sonicated in physiological saline at 20,000 cycle/s. Each rabbit was injected in the footpads with 5 mg of sonicated oyster gill ciliated epithelium in complete Freund's adjuvant. This was followed by two weekly injections of 2.5 mg of sonicated tissue in physiological saline in the lateral ear vein. Antibody production was verified by double immunodiffusion (17) utilizing sonicated cilia, diluted to a protein concentration of 3 mg/ml, as antigen. A modification of double immunodiffusion described by Kronvall and Williams (18) was utilized to detect immunological reactions between oyster gill tissue and antiserums.

The anti-rabbit IgG preparation used in the indirect staining experiments was obtained by injecting goats with isolated rabbit IgG. The goat IgG was then purified and subsequently labeled with fluorescein isothiocyanate (FITC) (19).

Immunohistological studies. Serum and serum fractions were conjugated with FITC (19). The FITC-labeled protein was separated from unconjugated FITC on a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column. The final protein concentrations ranged from 1 to 5 mg/ml. The molecular fluorescein/protein (F/P) ratio was determined by the method of The and Feltkamp (20). Only those fluorescein-labeled antiserums with an optimum F/P ratio of 1–4 were used in this study.

For direct staining, the tissue sections were treated with FITC-conjugated protein for 15 min, agitated in pH 7.1 phosphate-buffered saline (PBS) for 20 min, and mounted in a 1:1 mixture of glycerol and PBS. Tissue sections stained by indirect immunofluorescence were treated with serum or a serum fraction for 15 min, agitated in PBS for 5 min, and reacted with FITC-conjugated antiserum for 15 min. Slides were washed in PBS for 20 min and mounted in the glycerol-PBS mixture. Slides were examined with a Leitz microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with a vertical illuminator and ultraviolet light (mercury arc HBO 200). Oyster tissue was examined with a 3-mm BG 12 primary filter and a K510 suppression filter, whereas human tissue was observed with a 2-mm UG 1 primary filter and K430 suppression filter. A built-in combination beam-splitting mirror and suppression filter was used to obtain optimum contrast.

Passive hemagglutination. Because of the heterogeneous composition of the antigen (sonicated oyster gill epithelium), sheep red blood cells (SRBC) were instead sensitized with the *antibody* in order to assure complete and invariable attachment. SRBC were sensitized with IgG from antiserum to ciliated tissue with glutaraldehyde as a coupling agent (21). The same technique was utilized for sensitizing SRBC with IgG fractions from either CF, heterozygote, or normal serums. Serial dilutions of antigen (sonicated oyster gill epithelium) were prepared in disposible microtiter plates from an original solution of 16 mg/ml in PBS. 20 μl of a 2% solution of sensitized SRBC were added to each dilution of antigen and to a PBS control. The degree of hemagglutination was judged by grading the sedimentation pattern from 1+ to 3+.

Papain digestion. Papain (Worthington Biochemical Corp., Freehold, N. J.) was utilized for digestion of two preparations of IgG obtained from DEAE chromatography (12) of serum from a CF patient, a CF heterozygote, and a normal individual. Approximately 60 mg of IgG was digested with papain, in the procedure developed by Press, Piggot, and Porter (22). The digested solution was then dialyzed against barbital buffer for starch block electrophoresis (13).

RESULTS

Double immunodiffusion. Each antiserum produced in three different rabbits against ciliated tissue produced two precipitin lines against sonicated oyster gill tissue in immunodiffusion studies. The CF serum and serum fractions (1.5–40 mg/ml) failed to produce precipitin lines with the same sonicate. As demonstrated in Fig. 1, the CF IgG fractions did not deflect the precipitin lines formed between the oyster gill sonicate and the specific anticilia serum. This indicated that the CF

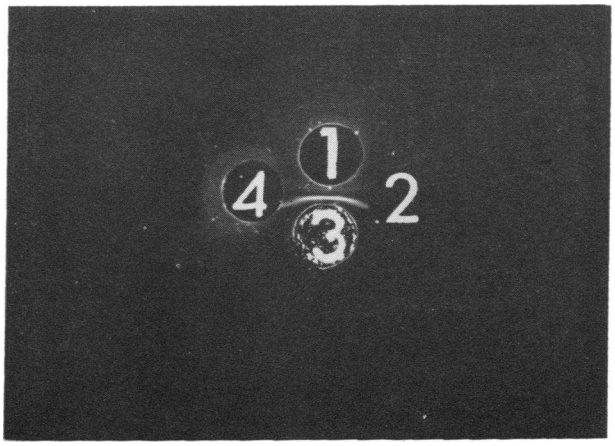

FIGURE 1 Double immunodiffusion plate. Well 1 contains anti-cilia serum, well 2 contains 0.9% saline, well 3 contains sonicated oyster ciliated epithelium, and well 4 contains CF IgG (3 mg/ml), which inhibited ciliary action. Note that two precipitin lines are visible. Neither line is deflected by the presence of CF IgG in well 4 as compared to the control well (2). This indicates that the IgG does not react with either antigen so as to inhibit the formation of the precipitin line.

ciliary inhibitor did not cross-react with the immunological reaction between oyster tissue and its specific antiserum.

Passive hemagglutination. SRBC sensitized with IgG from either CF, heterozygote, or normal individuals failed to produce hemagglutination even when the concentration of antigen (sonicated oyster epithelium) was as high as 1 mg/ml.

A hemagglutination titer of 2.5×10^{-4} mg/ml was established for the antiserum produced against ciliated tissue. The titer reflects the greatest dilution of antigen that resulted in a 1+ agglutination pattern of SRBC sensitized with the anti-cilia antiserum. No significant lowering of the titer was observed by adding from 1.5 to 6 mg/ml of IgG fractions from either CF, normal, or heterozygous individuals as inhibitors to the agglutinator system. These data indicated that the CF ciliary inhibitor, which is fractionated with serum IgG, did not interact with oyster tissue so as to mask the antigenic determinants recognized by the specific anti-cilia antiserum.

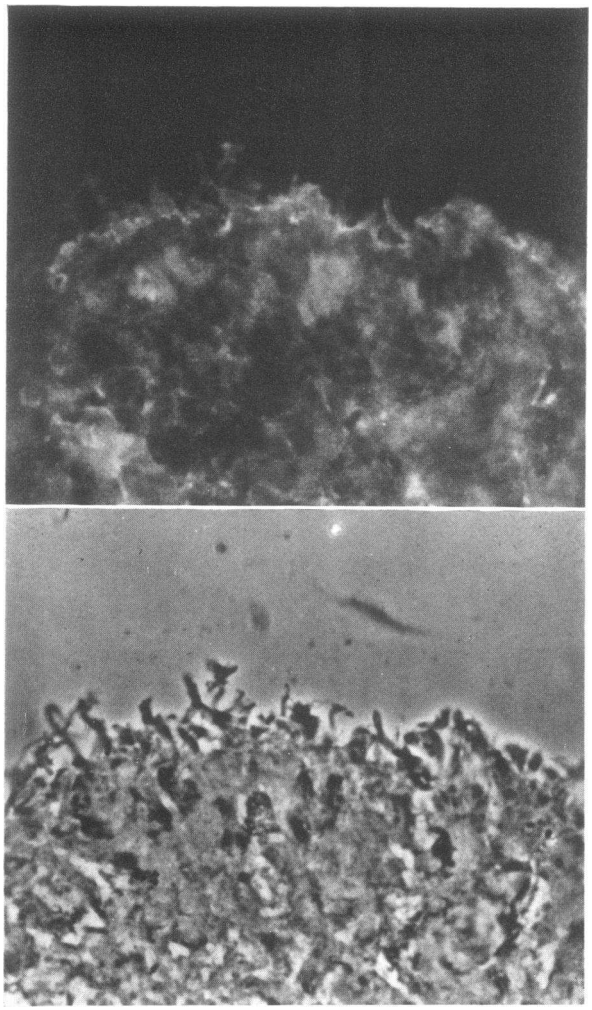

FIGURE 2 Fluorescent (above) and phase (below) images of oyster gill tissue treated with anti-cilia rabbit serum followed by FITC-labeled anti-rabbit IgG. Note the fluorescence along the ciliated epithelium and membranes located deep in the tissue. 400 ×.

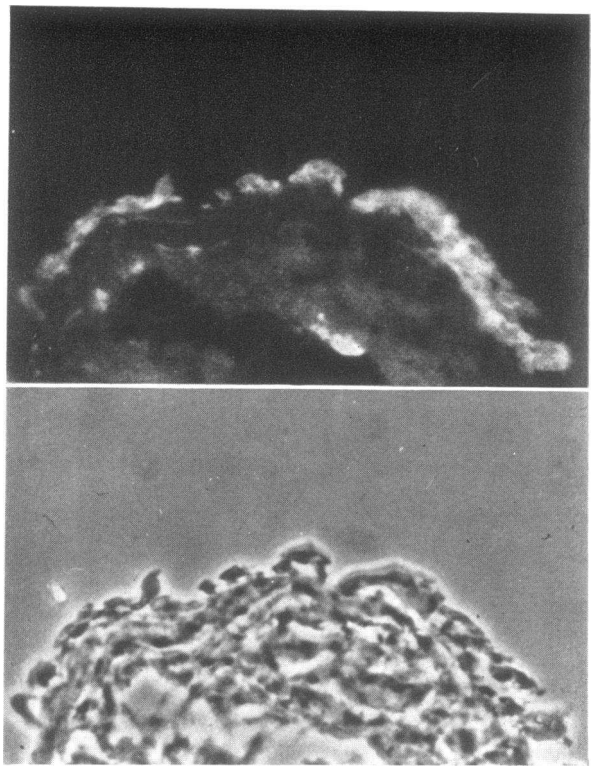

FIGURE 3 Fluorescent (above) and phase (below) images of human bronchial tissue, directly labeled with the same anti-cilia serum described in Fig. 2. The antiserum binds cuboidal epithelium lining the mucus-secreting surface of the bronchial tissue. 300 ×.

Immunofluorescent studies. Frozen sections of oyster gills, treated with FITC-conjugated IgG from anti-cilia antiserum, demonstrated a specific reaction along the ciliated surface including some membrane surfaces indicating sites of specific antigen-antibody reactions. Pretreatment of sections of oyster gills with unlabeled antiserum to ciliated tissue followed by treatment with FITC-conjugated antiserum resulted in a marked decrease in fluorescence. Indirect immunofluorescent tracing of oyster gills utilizing unconjugated cilia antiserum, followed by FITC-labeled anti-IgG, also revealed fluorescence along the ciliated border of gill tissue, indicating the site of antigen-antibody reaction. A fluorescence study of oyster gills treated with anti-cilia antibodies followed by FITC-labeled anti-rabbit IgG is shown in Fig. 2. The same antiserum also caused fluorescence along the ciliated epithelium of human bronchial tissue,

indicating an unexpected immunological cross-reactivity between oyster and human ciliated epithelium. A fluorescence study of the reaction between human bronchial tissue and the cilia antiserum produced in rabbits is shown in Fig. 3.

Sections of epithelial lining from human bronchus and gastric mucosa were examined after treatment with FITC-serums and IgG fractions from CF patients, heterozygotes, and normal individuals. Only autofluorescence was observed on eight slides of bronchus and six slides of gastric mucosa, indicating that no immunological reaction took place between these tissues and IgG fractions from CF patients, heterozygotes, or normals.

Direct and indirect immunofluorescent tracing of normal, heterozygote, and CF serum as well as IgG fractions from these serums consistently revealed fluorescent labeling of the goblet cells. The goblet cells are numerous in oyster gill tissue and are responsible for the production and release of mucus (11). Pretreatment of the oyster tissue with the same unlabeled serum or serum fractions did not diminish the fluorescence even when the FITC-conjugated whole serums were diluted to 0.1 mg/ml.

Ciliary action. The CF ciliary inhibitor causes cessation of oyster gill cilia within 40 min of exposure (5, 8), which is also often accompanied by expulsion of mucus from the oyster tissue. In contrast, the antiserum directed against ciliated tissue caused the cilia to stop beating gradually without causing expulsion of mucus. The antiserum appeared to inhibit ciliary action only slightly; the cilia stopped beating after 50 min of exposure compared to a period of 60 min of exposure to non-CF serum.

Papain digestion. Papain cleaved IgG fractions from serum into Fab and Fc components in addition to an undigested protein fraction. These fragments were separated by starch block electrophoresis (13) and their identity established after immunoelectrophoresis of fractions with protein concentrations of 5–10 mg/ml. The fractions were then dialyzed against sea water and examined for ciliary inhibition (5). It was found that neither Fab nor Fc fractions inhibited oyster ciliary activity. In addition, the solution obtained after papain digestion was tested on the oyster gills before dialysis, electrophoresis, or chromatography. Thus, any small fragments produced by proteolysis or chemical disruption would be preserved in the solution. The complete digestion mixture was no longer inhibitory to ciliary action.

DISCUSSION

Both double immunodiffusion and passive hemagglutination experiments showed that the specific antibodies reacted with sonicated oyster ciliated tissue in a typical antigen-antibody reaction. On the other hand, the presence of the CF IgG that contained the ciliary inhibitor did not inhibit the immunological reaction between oyster tissue and its specific antiserum. CF IgG also failed to form an immunoprecipitin line with oyster cilia. These data suggest that the ciliary inhibitor present in serum of CF patients and heterozygotes neither reacts immunologically with oyster tissue nor interferes with the reaction between the oyster tissue and its specific antibodies.

Immunofluorescent tracing by both direct and indirect staining methods demonstrated that there was binding of the specific antibody to the ciliated tissue. The fluorescence could be diminished by pretreating the gill sections with nonlabeled antiserum before treating with FITC-antiserum, indicating that there was an immunological reaction between the ciliated tissue and its specific antibodies. In contrast, fluorescein-labeled fractions containing the ciliary inhibitor from the serum of CF patients and heterozygotes did not produce fluorescent labeling of the oyster gill tissue. The only fluorescence observed was a nonspecific binding of the serum or IgG fractions to the goblet cells, under the ciliated surface of the gill sections. The binding was observed when either serum or the IgG fractions from CF, CF heterozygote, or normal individuals was placed on gill tissue. This reaction was not blocked by pretreating the tissue with unlabeled serum or isolated IgG, indicating that this labeling may be an artifact of fluoresceination.

Another line of evidence that the serum inhibitor of CF patients was not involved in an antigen-antibody response with oyster cilia was furnished by the discovery that the Fab fraction of IgG from CF serum did not inhibit ciliary activity. In a typical immunological reaction the Fab fragment of a specific antibody that contains the antigen binding site would be expected to react immunologically with its specific antigen. Neither the Fab nor the Fc fragments of IgG from a CF patient or a CF heterozygote inhibited oyster cilia. Since the CF factor is no longer active after papain digestion, it may be predicted that the factor is a papain-sensitive molecule that elutes with the IgG fraction of human serum. Evidence supporting this has been obtained from experiments that have revealed that the ciliary inhibitor is destroyed by digestion with papain. When the entire digest was exposed to cilia preparations no inhibition occurred, indicating that the intact protein must be present for ciliary inhibition. Recent investigations on the ciliary inhibitor produced by cultured CF fibroblasts indicated that it has protein properties. Ciliary inhibition was destroyed by proteolysis after treatment with papain and pepsin and by denaturation with heat (7). If the ciliary inhibitor is not an immunoglobulin but is fractionated with the IgG class these results might be expected.

In conclusion, the serum factor that inhibits ciliary

activity in the oyster, *Crassostrea virginica,* does not appear to be a typical antibody, although the serum fraction inhibitory to ciliary activity is purified with the IgG fraction of human serum. Speculation on the occurrence of an inhibitor in CF without strong immunological activity against ciliary tissue has led to the conclusion that a protein in CF serum isolated with the IgG fraction is not an immunoglobulin, but has serum concentrations too low and chemical properties too similar to be differentiated from IgG. An alternate explanation is that the inhibitor of ciliary activity in CF serum is related to an IgG class but has low binding capacity to ciliated tissue and thus was not detectable by the techniques employed. This latter explanation seems unlikely since there were no indications of even slight inhibition of the antigen-antibody reactions between ciliated tissue and its specific antibody by the isolated CF IgG fraction that contained the ciliary inhibitor.

The ciliary-inhibiting fraction recently isolated from culture media of fibroblasts derived from CF patients and heterozygotes shares chemical properties with the serum inhibitor. It is a cationic molecule isolated in the same chromatographic fraction on DEAE at pH 8.5. Further relationships must await additional studies.

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