Cystic fibrosis of the pancreas, the most frequently inherited disease affecting the Caucasian population, is an autosomal recessive disorder primarily affecting exocrine glands. The basic defect is unknown.

Since the demonstration that cultured fibroblasts from both homozygotes and heterozygotes for cystic fibrosis show in vitro abnormalities (metachromasia) (1, 2), variable mucopolysaccharide content (2, 3), increased intracellular glycogen (4), decreased collagen synthesis, and collagenolysis (5), it has become apparent that cystic fibrosis is a disorder affecting, not only the exocrine glands, but all somatic cells.

Sera from both homozygotes and heterozygotes for cystic fibrosis inhibit ciliary motion of rabbit tracheal explants (6), oyster gills (7), and fresh water mussels (8). Saliva and sweat from affected individuals inhibit reabsorption of sodium (9, 10). Whether these two biological activities are caused by the same or different factors is not known and their relationship to the basic defect has not been clarified.

If the skin fibroblast is capable of expressing its abnormal genotype in culture, the possibility is raised that the factor and/or factors found in the saliva, sweat, and serum of cystic fibrosis patients may also be found in the fluid in which the cells are grown.

**Materials and Methods**

A serum sample and skin biopsy was obtained from 36 homozygous children with cystic fibrosis, 36 obligatory heterozygotes, their parents, and 36 normal healthy unrelated adults and children. 10 ml of venous blood was removed, allowed to clot at room temperature, centrifuged, and the serum stored at −4°C. The establishment of the cell lines from the skin biopsies by standard culture methods (1) required about 2 months (six subcultures). The cultures were grown in reinforced Eagle's medium containing 10% by volume newborn calf serum. A cover slip was introduced into each culture at the fourth trypsinization to obtain a cytological preparation for staining. Each cover slip preparation was fixed in methanol and stained with toluidine blue (11) to determine the presence or absence of metachromasia. Patients with cystic fibrosis have been previously shown to consist of at least three primary classes (class I, vesicular metachromasia; class II, generalized metachromasia; and class III, ametachromatic) (12).
For studies employing oyster cilia, approximately 200,000 cells were inoculated into each 2 oz glass flask containing 5 ml of culture medium. After 14 days' incubation, the culture medium was removed and tested. If it was not possible to test the medium immediately, it was frozen and stored at --4°C.

The oyster ciliary test was performed according to the method of Bowman, Lockhart, and McCombs (7). Gill tissues from fresh oysters (Crassostrea virginica) were removed and vertical sections (3 X 3 mm) were suspended in saline in a hanging drop preparation. The authors would like to emphasize the difficulties in developing the oyster cilia test. It was found, as have others (Bowman, personal communication), that only certain oysters are suitable and variability in response is an intrinsic difficulty. After the cilia were observed to be active, the section was removed from the saline with forceps, touched to filter paper to remove excess fluid, and placed in a hanging drop containing either a sample of nutrient medium or serum. A cover slip was placed over the drop and sealed with wax. Each preparation was examined under an inverted microscope every 5 min for 120 min for: (a) expulsion of debris from tubules between mounds, (b) dyskinesis of cilia on mounds, and (c) stoppage of all cilia on individual mounds. The ciliary test was performed on saline, unused medium, and used medium from the cultures from 36 homozygotes, 36 heterozygotes, and 36 normal healthy individuals. Duplicate samples from the same culture were run on gills from different oysters; used media taken at the fourth and sixth subcultures from the same cell lines were tested.

**RESULTS**

The mound cilia in serum from normal healthy individuals remained active with no change in activity for over 30 min (Table I). Although dyskinesis was

| Subjects | Elapsed time necessary to observe changes in activity of oyster cilia |
|----------|---------------------------------------------------------------|
|          | Sera                  | Cell culture media*                  |
|          | Mean Range | Mean Range | Mean Range | Mean Range |
| Normals  |            |            |            |            |
| Children | 18         | 38 30-45   | 71 60-120  | 40 25-60   | 90 60-1201 |
| Adults   | 18         | 40 35-65   | 80 60-110  | 65 30-60   | 110 60-1201 |
| Cystic fibrosis |          |            |            |            |
| Homozygotes |          |            |            |            |
| Metachromatic culture class |          |            |            |            |
| Class I (vesicular) | 12 | 10 5-15 | 20 15-25 | 10 5-15 | 29 15-25 |
| Class II (generalized) | 12 | 5 0§-10 | 15 10-20 | 5 0§-5 | 20 5-25 |
| Class III (ametachromatic) | 12 | 30 25-35 | 90 60-120 | 40 25-60 | 89 60-1201 |
| Heterozygotes |          |            |            |            |
| Metachromatic culture class |          |            |            |            |
| Class I (vesicular) | 12 | 10 5-20 | 25 20-40 | 10 5-15 | 25 15-25 |
| Class II (generalized) | 12 | 5 0§-15 | 20 15-30 | 10 5-20 | 10 5-20 |
| Class III (ametachromatic) | 12 | 35 30-40 | 70 60-120 | 50 40-60 | 70 60-1201 |

* Oyster gill segments in saline or unused culture media showed dyskinesis after 40 min (mean) (range 25-60), and cilia stopped at 95 min (mean) (range 60-1201).
† Cilia still beating when observations terminated at 120 min.
‡ Cilia beating in saline and stopped beating before first observation at 5 min.
seen between 30 and 60 min, the cilia continued to beat during the first 60 observation min. The sera from the heterozygotes and homozygotes for cystic fibrosis could be divided into two groups. The cultures classified as class I and II by metachromatic staining (12) caused dyskinesis (slowing of ciliary movement and irregularity of the beat of individual cilium) in 10 min and cessation of ciliary motility by 30 min. The cultures classified as class III, which show no metachromasia, could not be readily distinguished from sera from normal individuals showing dyskinesis after 30 min.

Gill sections placed in unused culture medium showed the same activity as in normal saline. Little change in ciliary motion was seen until 40 min when dyskinesis followed by immobility by 95 min. Gill sections placed in used medium from cultures derived from all 36 normal individuals showed activity similar to that observed in normal saline or unused medium.

A study of the medium in which fibroblasts from homozygotes and heterozygotes had been grown enabled a classification to be made that was similar to that observed when serum was used as the testing medium. Culture classes I and II (metachromatic) (12) caused dyskinesis in 10 min, and stoppage of cilia by 30 min. None of the used media from these cultures permitted ciliary motion as long as that from normal cultures. Although, there was no overlap between the medium from normal individuals and these cystic fibrosis cultures, it was not possible to discriminate between the homozygous and heterozygous individuals, by the length of time to produce ciliary cessation. Cultures classified as class III (ametachromatic) (12) could not be readily distinguished from the gills placed in normal saline, unused medium, or used medium from control cultures. The inhibitory activity of culture medium and serum from the same individual appeared similar.

In all gill preparations studied, the cessation of ciliary movement appeared to be reversible. If, after cessation of ciliary movement, the cover slip was removed or the gill section was placed in fresh normal saline or unused medium, the cilia started to beat again and could not be distinguished from unused gill segments from the same oyster.

DISCUSSION

Although cystic fibrosis has been recognized as a genetic disorder involving the exocrine glands, abnormalities recently observed in the cultured fibroblasts from both homozygotes and heterozygotes for cystic fibrosis suggest that, at least, in culture the abnormal genotype may be expressed (1-5).

The abnormalities noted in culture have all been nonspecific rather than identifying a specific cytoplasmic defect. Such diverse observations as metachromasia (1, 2), variable mucopolysaccharide content (2, 5), increased glycogen storage (4), quantitative increase in lysosomes (13), and decreased collagen synthesis (5) suggest that a number of metabolic pathways are being affected by, as yet, an unknown defect caused by the cystic fibrosis gene.

Sera from both homozygotes and heterozygotes for cystic fibrosis inhibit the
ciliary motion of rabbit tracheal explants (6), oyster gills (7), and fresh water mussels (8). This factor has been characterized as a protein (125,000–200,000 mol wt) associated with immunoglobulin G (14). Saliva and sweat appear to contain a sodium-transport inhibitory factor (9, 10). Whether these two factors are one or two distinct entities is not known.

Of the three observations made on the oyster gill in this study, the expulsion of debris from the tubules between the gill mounds occurred inconsistently in all sera and media tested. The sera and media tested from culture class II often, but not invariably, gave a massive discharge of material which formed a blanket over the mound surface, similar to that described previously (7). The accumulation of debris covering the cilia on the mounds was markedly influenced by ciliary activity. Thus, if the expulsion occurred and ciliary activity was brisk, the debris accumulated at one end of the gill section, whereas if dyskinesia had started, the debris accumulated on the individual mounds. The other two observations (time of dyskinesia and total cessation of ciliary motion on the mounds) could be monitored (Table I).

As sera from cystic fibrosis patients have been found to inhibit cilia motility in three different biological preparations (6–8), it has been proposed (14) that a factor, probably an altered protein or a protein carrying a bound compound, occurs in the sera of both homozygotes and heterozygotes for cystic fibrosis.

Observations in cell culture have suggested genetic heterogeneity within this clinically defined disorder (12). Cellular metachromasia indicated morphological heterogeneity of the cultured fibroblast. The morphological distribution of the metachromasia in the fibroblast cultures derived from each patient had a sufficiently consistent morphological appearance that each could be assigned to one of three morphological classes: class I, vesicular; class II, generalized; and class III, ametachromatic. The reason for this difference in staining characteristics is unknown. The observations reported in this preliminary communication on the inhibition of oyster cilia by both sera and used culture media are supportive of heterogeneity. Both the fluids of classes I and II showed inhibitory activity similar to that reported for sera (6–8). Those in Class III appeared not to inhibit ciliary activity to the same degree.

SUMMARY

Using the oyster ciliary test, sera and fibroblast culture fluid from certain homozygotes and heterozygotes for cystic fibrosis can be shown to contain an inhibitory factor. The tissue culture fluid derived from the metachromatic fibroblast cultures (cystic fibrosis classes I and II) show ciliary inhibition. The fluid derived from the ametachromatic fibroblast cultures (cystic fibrosis class III), thus far studied, cannot be invariably distinguished from the normal noncarriers. Similarly sera from classes I and II showed marked ciliary inhibition, whereas sera from class III individuals do not consistently. These experiments support the notion of genetic heterogeneity in cystic fibrosis. The nature of the
inhibitory factor in tissue culture fluid and its relationship to the serum factor remain to be explored.

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Dr. Barbara H. Bowman, University of Texas Medical Branch, Galveston, has recently examined chromatographic fractions of some of our fibroblast media on oyster cilia and has isolated a fraction from the media of cystic fibrosis fibroblasts from classes I and II patients which inhibits ciliary action (Bowman et al., manuscript in preparation).

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