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Cystic fibrosis : its genetics, the heterozygous state and its detection

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CYSTIC FIBROSIS-ITS GENETICS, THE HETEROZYGOUS STATE AND ITS DETECTION

By

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CYSTIC FIBROSIS-ITS GENETICS, THE HETEROZYGOUS STATE AND ITS DETECTION

In 1912, Garrod and Hurlley described two children born to first cousins parents, who, in retrospect, probably suffered from cystic fibrosis. This description also suggested recessive inheritance of an 'inborn error of metabolism' in the pancreas. Cystic fibrosis is described as a generalized disease of exocrine glands, having its onset in the newborn period, infancy or early childhood. It manifests itself clinically by the following: 1. Loss or diminution of pancreatic exocrine secretions. 2. By increased susceptibility to upper respiratory infections, and pneumonia resulting in the pathologic changes of bronchiectasis, atelectasis and emphysema. 3. Elevation of sodium and chloride concentration in sweat gland secretions.¹

Meconium ileus, a type of intestinal obstruction, was formerly attributed to a complete absence of pancreatic enzymes, however, more recently it has been demonstrated that the inspissation of meconium is primarily due to abnormal intestinal mucoid secretions rather than pancreatic achilia. Meconium ileus is present in 15% of all newborns who will be affected with cystic fibrosis.² It is the earliest and most severe manifestation of this disease in the newborn infant. The affected infant without meconium ileus who appears normal at birth, fails to thrive satisfactorily even though he may have an increased appetite. This deficiency of pancreatic digestion results in bulky, unformed, foul smelling stools in both infants with or without meconium ileus.

The respiratory manifestations present initially as a severe non-productive cough, which later becomes spasmodic and productive. The dysfunction of the mucus-producing glands of the respiratory tree results in the formation of thick tenacious mucus within the trachea and bronchi.

This abnormal secretion hinders normal ciliary movement in the bronchi, plugs the smaller bronchioles and encourages bacterial invasion of the lungs.³

The analysis of sweat for its sodium or chloride content has become the most reliable laboratory method for confirming the diagnosis of cystic fibrosis. The level of sodium chloride in the sweat of a cystic fibrotic patient is consistently four to five times higher than in normal controls in the pediatric age group. No other disease, except adrenal insufficiency, can cause such a marked elevation of salt in the sweat. Use of this sweat assay for studying various groups of adults has revealed that a moderate percentage of heterozygote carriers of the cystic fibrosis gene (parents of cystic fibrotic patients) also manifest a slight moderate elevation of sweat electrolyte. Such studies have also suggested that certain adults with chronic bronchial disease, peptic ulcer or even diabetes mellitus, may have mild forms of cystic fibrosis. These cases of cystic fibrosis could possibly be detected by finding an elevation in sweat electrolyte, but this concept has not been universally accepted. Interpretation of electrolytes in the carrier, have been clouded by an apparent increase in sweat electrolyte level with age.⁴

So, the final diagnosis of cystic fibrosis is usually based on the clinical signs noted in previous paragraphs and laboratory findings of the sweat sodium and chloride excretion, absent trypsin activity in pancreatic secretions from duodenal fluid obtained by intubation or typical pancreatic changes at autopsy.

Although cystic fibrosis is almost undoubtedly a disorder of exocrine gland function, the molecular basis for the disease is not known. But in the great majority of those recessive conditions in which the

biochemical defect has been elucidated this has proven to be a reduction in the activity of a specific enzyme. In most instances the effect of the mutation is an amino acid substitution in the enzyme. Such changes can result in a complete loss of enzyme activity, as well as reduced or altered activity. In other recessive disorders, it appears that there is a complete loss of any protein production as a result of the gene mutation. The possibilities for the enzymatic defect in cystic fibrosis are: a base change which results in a nonsense code; a mutation in an operator or regulator gene, or even a small chromosome deletion. Mutations which are thought to produce a change in a structural protein, such as collagen, are usually dominant. It is therefore, perhaps more likely the defect in cystic fibrosis will be found to be an enzymatic defect, rather than a primary defect in the peptide part of a glyco-protein.²

The familial nature of cystic fibrosis was first described in 1946.⁵ A few years later it was generally concluded that the inheritance pattern could be best explained on the basis of an autosomal recessive gene. Although some groups have attempted to establish a possible linkage between cystic fibrosis, with such remote associations as blood groups and many other linkages, there is now almost total agreement with the single autosomal gene theory.

Cystic fibrosis results from the presence of two pathologic autosomal recessive genes in an individual. In order to produce a recessive homozygote each normal parent must carry a pathologic cystic fibrotic gene. Each parent must also have a normal gene at the cystic fibrosis locus. Other individuals who do not have the pathologic gene would possess two dominant normal alleles at the cystic fibrosis locus.

Three types of individuals with respect to the cystic fibrosis gene

are thus present in the population, normal with both dominant alleles; carrier with one normal allele and one cystic fibrosis gene; and those affected with two pathologic cystic fibrosis genes. Presently carriers can be detected only through the production of affected children. Almost all affected children are produced by carrier parents, since affected persons (homozygotes) with cystic fibrosis rarely live to reproduce.

Carrier parents can produce three types of children: normal who have received only normal cystic fibrosis alleles, carrier or heterozygote who have received a normal allele from one parent and a pathologic cystic fibrosis from the other parent; and affected individuals who have received a pathologic cystic fibrosis gene from each parent. Carrier parents are capable of producing these three types of offspring in the ratio of 1:2:1. Thus, each child produced by such parents has one chance in four of being either completely normal or of being affected, and one chance in two of being a carrier. These chance phenomena do not change from one pregnancy to the next, no matter what genotype the previous offspring have been, the next child has the same genetic potentialities as all of the siblings.

Because of the rarity of carriers in the general population, it is unusual that two carriers will marry and produce affected children. Carriers are much more likely to occur in kindreds of affected cases than in the general population. Because of the familial aggregation of the carrier state, there is a high incidence of consanguinity in the recessive disease, that is, a carrier is much more likely to marry another carrier if the person were related, than if he were not.¹

Overall prevalence of the condition is not informative because of great mortality in infancy and early childhood. This mortality in part, is based on the assumption that of those presenting with meconium ileus

only about one case in three has survived until age five. Ranges of hospital reports, often with referrals, is about 1 in 600, this is probably an unduly high estimate. The best range worldwide seems to be between 1 in 1,000 and 1 in 10,000, as determined by various researches. Confirmed by most studies the incidence in the United States is closer to 1 in 2,500. The incidence of 1 in 2,500 implies a gene frequency of about 1 in 50 in these populations, since cousin marriage rate is low, and a heterozygote frequency of about 1 in 25. This disease is rare both in Negro and Mongolian populations.

If one accepts the figure of 1 in 2,500 and a mortality of two out of three by the age of five, this means that this single condition is responsible for between one and two percent of all deaths under age of five. The incidence of the condition in children of surviving patients will give a direct estimate of gene frequency.^{7, 11, 12}

If the incidence of cystic fibrosis is essentially determined by the mutation rate and hitherto there was a high mortality in childhood, the mutation rate must be of the order of 1 in 2,500. This is quite exceptionally high for a gene mutation rate and so heterozygote advantage is suggested. The selective advantage in the case of cystic fibrosis needs to be relatively small, an increase in fitness of only about 2% of heterozygotes over individuals homozygous for the normal gene. Such a heterozygote advantage will only operate in high incidence areas, and so a clue may well come, as it did with sickle cell anemia and malaria from geographical surveys of the incidence of the disease.

It is, of course, possible that the heterozygote advantage no longer exists. Where malaria has been eliminated there is probably no longer any extra fitness of heterozygotes for the sickle cell gene

but the high frequency will remain in West Africa for centuries to come.

Many attempts to demonstrate a heterozygote advantage in cystic fibrosis have been made, but no definite conclusions concerning the results have been reached. Using the premise that there is an advantage to the carrier which would result in increased fertility, has been examined, in light of knowledge accumulating in the literature regarding changes in mucus and electrolyte secretions in the reproductive tract during the normal ovulatory cycle. Attention has been given to detecting any differences that may exist between carrier cases and normal individuals regarding these secretions, in the hope that this might contribute knowledge to the underlying defect in cystic fibrosis.

Evidence of heterozygote advantage, secondary to increased fertility, was sought by studying the size of families of grandparents of patients with cystic fibrosis. Despite the great care taken in matching three different control couples with each grandparental pair, considerable variation existed between the three series of controls. In only one of the control groups does the family size differ significantly from that of the fibrocystic family and even in this instance a possible explanation is suggested. However, in all the comparisons made, the fibrocystic disease grandparents had the larger families. These results were presented with great reservations about their biological significance; and with the realization that the magnitude of the heterozygote advantage apparently shown is very much greater than would be needed to maintain the gene at a steady frequency in the community.

If one uses the data describing larger family size in hetero-

zygotes an explanation of greater fertility is needed. This could operate in two ways, firstly, fertilization could be more efficient; secondly, embedding of ovum and maintenance of pregnancy could be more efficient. Secretions in the female genital tract and their relationship to fertility is not completely known, but it is thought that the increase in sodium chloride in cervical mucus peaks at the time of ovulation. The cervical mucus at this time becomes less viscous and shows spinnbarkeit. This seems to favor sperm passage. This raises the possibility that the heterozygote may possess a better mucus-salt relationship at ovulation for a longer time before and after ovulation. 8,9,10

The difficulties raised by the high incidence of cystic fibrosis would be much less if homozygosity for several different autosomal genes could cause cystic fibrosis. Congenital deafness for example, may probably be caused by up to six different autosomal recessive genes as well as being caused in several other ways, both genetic and environmental. However, in cystic fibrosis the incidence in cousins with affected children and also the low parental consanguinity rate in Caucasian populations indicates that only one gene locus is mainly involved. If the gene frequency is 1 in 50, the proportion of first cousins affected should be about 1 in 200, which it has been found to be. There may well be, however, rare variants of the disease due to mutations similar to but not identical with that causing the common form of the disease.

If the affected sibs of patients, whose disease is mild and of late onset also tend to have a form of the disease which is mild and of late onset, one might have to assume that another mutant gene other than the usual gene is involved, perhaps another mutant at the same locus.

Often two or three mutant genes may cause conditions which are very similar clinically.

Dr. C. O. Carter¹³ summarizes the need for further research into the genetics of cystic fibrosis as follows: 1. Surveys, preferably prospective, of the incidence in the many areas of the world for which figures are not yet available. (A screening test in the newborn would be most valuable here.) 2. Determination of the relative parental consanguinity rate in areas of low incidence of the disease. 3. The detection of heterozygotes which may have to wait, for the determination of the basic biochemical anomaly. 4. The determination of the selective advantage of heterozygotes in the areas of high incidence. 5. The prevention of the disease in the incidence areas will have to come by the routine detection of the 4% of children who are heterozygotes and warning them against marrying each other.

No clinical anomalies have been definitely shown to occur unduly often in those known to be heterozygous for the mutant gene, for example parents of children with cystic fibrosis. Claims to demonstrate a variety of pulmonary and gastro-intestinal disorders in heterozygotes have not been confirmed. Most studies show that such parents had no impairment in respiratory functions. The small increase in sweat sodium and chloride in adults with chronic respiratory disease, if it exists at all, is no reason for suggesting that they are heterozygotes for the gene of cystic fibrosis. The problem is to find what the extra fitness of heterozygotes for cystic fibrosis is due and any substantial proneness to disease in heterozygotes is difficult to reconcile with the incidence of the disease. If such disease proneness exists, it would imply an even greater fitness in other respects. It is conceivable

that a minor degree of unfitness in heterozygotes in adult life might be compensated by increased fitness in childhood, but this is improbable.

The general rule with autosomal recessive conditions, is that it is not possible to pick up those heterozygous for the mutant gene until the direct effect of the gene mutation, at least in terms of the enzymatic activity of the protein product of the gene, has been found. This is in contrast with the X-linked conditions in which, because of the inactivation of one of the X chromosomes, it is often possible to pick up female heterozygous for mutant genes by relatively crude clinical tests.¹⁴

The idea of being able to detect the heterozygous state is the key to the success in the drive to decrease the incidence of cystic fibrosis. Even with the expert medical care given at various University centers for cystic fibrosis the death rate remains high. The survival time is being lengthened gradually and mild cases are living into adulthood, but the basic defect of the disease remains unknown as does the cure. So, at present, the only hope for cure is to find the etiology of the disease and hopefully correct the defect. But the surest and easiest method of controlling the disease, lies in the prevention of the disease. This could be accomplished by being able to detect the carrier state and thus warn carriers of the consequences which could result from their mating with another carrier. Most of the new and exciting research in cystic fibrosis is now being directed toward the goal of being able to detect the heterozygote and thus prevent the disease.

The first area explored in the detection of the carrier state was an evaluation of the idea that the heterozygote might have an increased sodium chloride in sweat as is noted in the patients with cystic fibrosis.

Multiple factors are known to affect the concentration of salt in sweat. The rate of sweat secretion, skin temperature, salt intake, adrenal function, acclimation to heat, and the region of the body from which the sweat is obtained, are all known to affect its chloride concentration. The choice of drug used to stimulate sweating and the analytical method used in the assay can also affect the chloride value obtained. Thus it is difficult to compare different surveys reported in the literature, making it imperative for each laboratory to determine its own normal range of sweat chloride value by their own technique.

It is not surprising to find a binodal population of normal adults since it is now accepted that a percentage of known heterozygotes have a moderate elevation of sweat chloride. There must be an even greater number of unknown heterozygotes in the 'normal' population than there are known heterozygotes who have by chance mated with another heterozygote and given birth to a child with cystic fibrosis. One can only speculate regarding the possibility that normal adults with slightly elevated sweat chloride are heterozygous for cystic fibrosis, since there is not as yet any truly diagnostic tests to perform in an asymptomatic individual. Long term observation of such individuals may be rewarding in the pulmonary problems which may develop in such individuals with increased frequency.

Most data from parents of cystic fibrosis patients indicate there is an increase incidence of elevated chloride levels in the sweat of these individuals. The mean chloride value for this groups was significantly higher than normal controls. The percentage of individuals with sweat chloride values over 40 meq/l. is significantly higher for the parents of cystic fibrosis children compared to the normals.

Normal siblings of cystic fibrosis patients did not show any great deviation from normal children in this regard, but if the trend of the normal data is correct, a greater number of these siblings may develop higher sweat chloride values as they reach adulthood, resulting in a true rise of mean chloride values with age in this group.

It was noted in one study concerning the sweat electrolytes in carriers, that in most instances it is not possible to diagnose heterozygosity for cystic fibrosis, with any degree of certainty, on the basis of a single sweat chloride concentration determination. This is attributed to the fact that the distribution of single values in normal individuals and in known heterozygotes overlap by 70 to 80%. However, the fact still remains that the distribution of the sweat chloride values is higher in known heterozygotes does suggest that the recessive gene is expressed to some extent.

One may postulate that the heterozygotes have two populations of sweat secreting units, normal and affected, each independently distributed on the skin, and each having its own characteristic mean and distribution. A total pooled body sweat test would yield a sweat chloride value which was a mean of both populations of the secreting units and would be a function of the mean of each population and the relative frequency of each, the latter being conditioned by the expressivity of the recessive gene. Those heterozygotes with affected genes of low expressivity might fall within the range of normal sweat chloride values.

The value obtained by weighted patch collection is a sample mean of a number of secreting units. And sample means have a normal distribution around the true mean. The chloride value of sweat collected from a simple patch, then depends on: 1. The means of normal and affected

secreting unit populations. 2. The expressivity of the affected gene. 3. The relative frequency of normal and affected units sampled by the particular patch collection. By performing multiple patch collections on different sites on an individual, the mean and variance of the set of sample means can be determined. In a homozygous normal individual, the mean of the sample set will be low and the variance small, since the secreting units are from only one population. In the heterozygote, the mean of the samples may be normal or elevated, depending on the two populations means and gene expressivity, but the variance will be large, since the population is a double one.

So it was proposed that using both the mean and variance of a set of sweat tests on an individual, it might be possible to distinguish between homozygous normal individuals and carriers of the disease. The problem of assigning an unknown individual, such as a sibling of a child with cystic fibrosis to either normal or carrier population on the basis of these two variables is one of discriminating analysis.¹⁵

The results using the discriminate analysis technique showed 84% of the parents and 83% of the genetically expected number of siblings were identifiable as heterozygotes. Thus, 16% of the parents and 17% of the siblings who fall in the normal zone represent heterozygotes of very low expressivity. These may change zones to a diagnostically significant extent on repeat testing.

It is conceivable that under appropriate stress, as applied in determining the carrier of phenylketonuria, the remaining parents and sibling could be found to differ in some respect from the control group. The fact that some heterozygotes are detectable in cystic fibrosis lends weight to the theory that even a so-called recessive gene has

some expression in the heterozygous state. One type of stressful situation tried in order to bring out the hidden heterozygote was a salt deprivation test.¹⁶

It is known that normal individuals depleted of salt will produce sweat of low electrolyte concentration because of the salt conserving influence of aldosterone. Children with cystic fibrosis, however, under the same conditions, continue to produce salt of high electrolyte concentration in spite of normal levels of aldosterone. It was thought then, that perhaps the heterozygote carriers of the cystic fibrosis gene when subjected to similar conditions of salt deprivation, might be unable to conserve salt in the same way as that shown by the affected children. This conjecture became the basis of a study, to subject known carriers to a low sodium chloride diet in hopes it might unmask the recessive disorder. But the results were unproductive as there was a decrease in sweat chloride values of similar magnitude in both parents of cystic fibrosis patients and controls as the diet continued. It was concluded that the study of sweat electrolyte levels in salt deprived subjects is of no value in the diagnosis of cystic fibrosis heterozygotes.

It can be concluded from the studies of sweat chloride levels in adults and children, that a sweat chloride assay is an excellent and simple procedure for confirming a diagnosis of cystic fibrosis in a child or young adult. A normal chloride level should strongly suggest that the patient probably does not have cystic fibrosis and that another diagnosis should be made. In adults, the test has only limited usefulness for detecting heterozygotes or carriers of the cystic fibrosis gene, although known heterozygotes and adults with chronic lung disease were found to have an increased incidence of individuals with slight to

moderate elevation of sweat chloride. In addition most data suggests that the normal adult population is a dual population in regard to the level of chloride in sweat, leaving room for much speculation regarding the significance and meaning of the higher chloride group. Evidence was obtained suggesting that the high chloride normal group appears only in adulthood, and that these individuals may have had lower sweat chloride values during childhood.

The normal range of chlorides values in adults has not conclusively been decided, but any value above 40 meq/l. should probably be viewed with suspicion. This inexact upper limit of normal does not affect the use of the assay as a test of cystic fibrosis, in as much as the characteristic sweat chloride in homozygous is in an extreme range that does not appear to overlap the values found in the remaining population. So at this time the main value of the sweat chloride assay is for the diagnosis of the homozygous cystic fibrosis.^{17, 18}

Another type of study was undertaken in hopes that the minor labial salivary glands would reveal changes that would be used to distinguish not only patients with cystic fibrosis from normal patients, but also patients heterozygous for the gene for cystic fibrosis from normal patients.¹⁹ After intensive studies only one characteristic of cystic fibrosis stood out, plugs of a smooth, eosinophilic material in the lumen of some acini. The presence of these eosinophilic plugs was the criterion used to designate that a minor labial submaxillary or parotid salivary gland was abnormal.

The criterion abnormality, acinar plug of eosinophilic material, was confirmed to be present in over 80% of children with cystic fibrosis. The abnormality was rare in children under 20 years of age who did not have cystic fibrosis. The abnormality became relatively more frequent

in both adult groups, parents of children with cystic fibrosis and normal controls, as the age of the groups increased. This type of testing of the labial minor salivary glands could be obtained with a minimum of trauma, so that studies could be easily done on healthy persons to see if criterion could be found peculiar to the heterozygote carrier of the gene for cystic fibrosis. The impression that parents of children with cystic fibrosis show the criterion abnormality in the mucous salivary glands at an earlier age than do normal adults is strong, but cannot be proved from the presented data. The results may indicate an accelerated rate of change in carriers caused by a basic glandular defect which normally occur slowly over many years. On the other hand, the changes may be entirely nonspecific and could solely be due to morphologic expression of the gland when subject to stress or injury. So in conclusion, these changes appear earlier and more frequently and significantly more severely in adults who are known heterozygous for cystic fibrosis, but this criterion cannot be used as the sole bases to identify the heterozygous. These observations mainly suggest that factors are operating in patients with cystic fibrosis which affect certain tissues or their secretions in such a way that changes occur in childhood which would ordinarily not be seen until later in adult life.

One of the most promising studies in the field of cystic fibrosis is being carried on by Dr. A. Spock.²⁰ His group is studying the effect of sera from patients with cystic fibrosis upon human and animal respiratory tract epithelium. First observations indicated that these sera produced an asymmetrical ciliary beat. The study was designed to elucidate the significance of this abnormal beat and to detect factors within the sera of patients with cystic fibrosis responsible for the beat,

and possibly help clarify the pathogenesis and genetics of cystic fibrosis.

It has been shown that ciliary motility is essential for maintenance of proper respiratory function by constantly moving the mucus blanket of the respiratory tract upward toward the oral cavity. Although it has not been proven that ciliary motility is impaired in patients with cystic fibrosis, these individuals do show damage to cilia and changes in the volume and consistency of the mucus which reduce effective transport and result in bronchial obstruction with pulmonary dysfunction.

The nature of the study was in vitro, the serum from a patient with cystic fibrosis was applied to a tissue specimen, rabbit tracheal epithelium. An asymmetrical beat was observed within five to ten minutes and persisted for a period up to one hour. After this time the border became obscured with desquamated cells and was no longer suitable for study. The degree of altered activity was unrelated to the age of the patient, severity of the disease, or sweat chloride level. In contrast, sera from all of the control patients produced no alterations in the ciliary beat in a similar time period.

By bioassay technique the fraction in the sera of these patients which was responsible for the asymmetrical beat was identified. The major component was found to reside in the euglobulin, water-insoluble fraction. To determine whether the abnormal serum factor was also present in heterozygotes, sera from some of the parents of the patients used in the study were tested. The sera from 28% of the parents was capable of alternating the ciliary rhythm. In further testing, the remainder of the parents whose whole sera failed to produce an asymmetrical beat, euglobulin (water insoluble) fraction was prepared and

examined. With a concentrated euglobulin fraction produced, an asymmetrical beat in all these parents was noted. Whereas, the euglobulin fraction prepared in a similar manner from the sera of an equal number of adult volunteers with no family history of cystic fibrosis was positive in only one case. This one subject had a normal sweat chloride test.

The presence of this unique protein in the serum is significant in view of the identification of mucoprotein in body fluids of cystic fibrosis patients by other investigators. Dische, initially observed the presence of abnormal mucoprotein in duodenal fluid. Mucoprotein complexes have subsequently been described in sweat, submaxillary saliva, in urine and most recently in the feces. At present no direct correlation has been made between the protein fraction which has been found in the sera and the mucoprotein noted by these other workers in body secretions of patients with cystic fibrosis.

The findings of a serum factor as well as the abnormal protein found in the urine, suggests that the suspected enzymatic defect in cystic fibrosis may not be limited to the exocrine glands. The ability of cystic fibrosis sera to cause an asymmetrical beat in cilia of respiratory tract epithelium obtained from animals and humans initially led to postulation that the asymmetrical beat could be responsible for the retention of mucus by cystic fibrosis patients. This abnormality of ciliary function could potentiate the effect of the viscid secretions seen in this disease. It should be emphasized that the in vitro effect reported above has not been seen in vivo. Examination of nasal polyps from patients with cystic fibrosis immediately upon removal show normal ciliary motility.

Dr. Spock's group is currently designing experiments to improve purification and identification of the serum protein by various immuno-

chemical techniques. It is hoped that this will enable them to determine the nature of the protein and its constituents. This information will provide a more rapid and simpler assay technique and perhaps a possible clue to the pathogenesis of cystic fibrosis.

Another attempt to detect heterozygotes is being carried on by Danes and co-workers.²¹ Initially this work has looked very promising. Skin fibroblast cultures were used as the means of detecting the cystic fibrosis patient and the heterozygous carrier, as they showed easily recognizable cytoplasmic intravesicular metachromasia. The existence of a cellular abnormality recognizable in the fibroblasts of these individual offers an experimental opportunity to study the primary defect of this common inherited disease at the molecular level.

In this study, skin biopsies were obtained from the cystic fibrosis patients, their parents, healthy controls and persons with miscellaneous illnesses. The cystic fibrosis patients all were diagnosed positive by abnormal sweat tests. Monolayer skin fibroblast cultures were established by tissue culture procedures which were then stained and examined. Final results showed subcultures from healthy unrelated individuals and patients with a variety of other disease contained an occasional cell showing metachromatic inclusions. While cultures derived from the patients with cystic fibrosis contained large numbers of fibroblast showing evident cytoplasmic metachromasia. The metachromatic material appeared to be contained within vacuoles or vesicles and was not distributed evenly throughout the cytoplasm. The size and shape of the inclusions were not uniform and they were often restricted to one cytoplasmic area within the cell. The proportion of cells containing metachromatic inclusions increased for approximately two months after the establishment of the monolayers and then remained constant.

Although the proportion of metachromatic cells did not vary significantly after the fifth subculture, the number of vesicles per cell continued to increase.

Fibroblast cultures derived from clinically unaffected carrier parents contained cells with metachromatic material similar to that seen in skin fibroblast obtained from their affected children. The time sequence for the appearance of metachromasia was similar to that seen in cultures from the affected individuals. Although there was some variation in the percent of metachromatic cells in the heterozygous carriers, as well as the number of inclusions per cell, multiple biopsy specimens from the same area from a single individual showed only slight variation. Cytopathic effects including vacuole formation and cytoplasmic granulation have been noted in human cell cultures as a result of mycoplasma infection. A considerable effort was made to determine whether there was any relationship between the appearance of cytoplasmic vesicles and the infection of cultures with mycoplasma. No correlation between the presence of mycoplasma and cytoplasmic vesicles was evident. Since mycoplasma infection was randomly present in the cultures here, the possibility that it was responsible for the cytoplasmic vesicles in the positive cases is highly unlikely.

Several genetic disorders can be investigated in cultured fibroblasts. The addition of cystic fibrosis to this list is noteworthy, since both clinical and chemical studies have suggested that cystic fibrosis is an exocrine abnormality rather than a multicellular disorder involving connective tissues. The detection of a recognizable cell marker in both the affected and carrier states provides an in vitro model for investigation of the primary molecular abnormality in this disease.

The metachromatic appearance of the cultured cells derived from patients with cystic fibrosis differs from that observed in the mucopolysaccharidoses in which the metachromatic granules are more evenly distributed throughout the cytoplasm. The striking variation in the degree of cellular metachromasia in cultures derived from both affected individuals and known carriers is similar to that seen in cell cultures established from the autosomal recessive mucopolysaccharidoses. As in the mucopolysaccharidoses, there seems to be correlation between the severity of the clinical disease, the number of metachromatic cells or the number of inclusions per cell in the cultured skin fibroblast.

Despite intensive clinical and laboratory investigations extending over many years, the primary inherited defect in cystic fibrosis remains unknown. The glycoprotein-rich secretions which are produced in excessive amounts in cystic fibrosis do not seem to differ qualitatively from those found in healthy individuals. Findings indicate that skin fibroblasts, derived from affected individuals and carriers grown in cell cultures, contain characteristic metachromatic inclusions which may reflect the primary inherited defect in this disease. The chemical nature of the metachromasia material has not yet been elucidated. It is noteworthy that carbohydrate containing proteins have not been found to give strong metachromasia. An increase in sulfate groups attached to the carbohydrate containing protein, may be responsible for the staining reaction.

In this series all the known carriers showed cellular metachromasia, and the results with unknown carriers were compatible with the estimated carrier frequency of approximately 2 to 8 per cent. So, the approach here seems to provide a simple method to identify healthy carriers, and affords a tool for determining directly the carrier frequency of this

highly lethal gene in the healthy population. Additional family studies will be required before the reliability of cell marker for detecting carriers can be established. The possibility that the application of this technique will reveal genetic heterogeneity in cystic fibrosis can also be entertained. The recognition of a cellular abnormality in cultured fibroblast permits a more direct investigation of the molecular basis for this inherited disease..

The latest effort to detect the cystic fibrosis heterozygote has been reported by J.W. Balfe and associates.²² Their motivation for this study stemmed from the work of Mangos and McSherry in which they demonstrated that the sweat from patients with cystic fibrosis inhibits sodium transport in the duct system of the rat parotid gland. This latest study examines the components of active transport in another cell membrane system, the erythrocytes of patients with cystic fibrosis and their parents.

Balfe's study shows that the mean concentration of sodium per liter of red blood cell, for affected and the control group was very similar. The sodium efflux studies were then done with erythrocytes from patients with cystic fibrosis and parents of patients each of these studies were coupled with a control.

Two sodium pumps were described: 1. Pump I is that component of sodium efflux which is inhibited by the addition of ouabain to a medium which contains sodium and potassium. 2. Pump II is another decrement in the efflux of sodium which is due to the addition of ethacrynic acid to a medium which contains maximally inhibitable concentrations of ouabain. The remnant of the efflux, that is, the component of the total efflux unaccounted for by pumps I and II is referred to as the "leak". This presumably correlates with the permeability

of the red blood cell membrane.

After data for normals was compiled, patients with cystic fibrosis were studied under identical circumstances. Results showed the mean decrease in pump I is 15% of control values. Pump II differences were even more strikingly decreased in patients with cystic fibrosis as pump II was only 53% as active as their controls. These differences were statistically significant. The "leak" parameter was statistically significantly less in the patients as well.

So, patients with cystic fibrosis have a normal concentration of sodium in red blood cells, but a diminished active efflux of sodium. This suggests that the passive influx of sodium, the "leak" parameter, might possibly be diminished, and the "leak" parameter of sodium efflux measured with isotopes is diminished.

In erythrocytes from parents of these patients, there was no difference in pump I nor in the associated ouabain sensitive ATP activity, but there was a 56% diminution in pump II. The magnitude of the depression in the activity of pump II is similar to that seen in the patients with cystic fibrosis.

The final conclusion of this investigation demonstrates a diminished active transport of sodium in the erythrocytes of patients with cystic fibrosis. Of special significance is the marked decrease in that which is defined as pump II in the parents. This quantitative marker of the heterozygous carrier of the disease may be useful in genetic studies and in genetic counseling.

In summary the following may be concluded about the carrier of a cystic fibrosis and its detection. No consistent clinical abnormalities have been found in these individuals. Studies of parents of patients with cystic fibrosis have shown no increased prevalence of respiratory

and gastrointestinal disease. The mean sweat sodium concentration and the sodium content of nails are frequently increased in known carriers, unfortunately, the heterozygous values overlap those found in normal individuals, and so these tests are unsuitable for the detection of carriers. The most promising studies in the area of cystic fibrosis detection are: (1) The bioassay technique reported by Spock and co-workers in 1967,²⁰ this test is based on the observation that sera of carriers cause a dyskinesia of ciliary motility of rabbit tracheal cells in vitro. (2) The detection of metachromasia in fibroblast of carriers of cystic fibrosis by Dr. Danes and associates.²¹ (3) The detection of a red blood cell transport defect of patients with cystic fibrosis and in their parents. This investigation of J.W. Bulfe and co-workers,²² gives a quantitative marker of the heterozygous carriers of cystic fibrosis.

With further research and modifications of present data it seems reasonable that soon there will be a practical and simple means to detect the 2-8% of the population that are carriers for the cystic fibrosis gene. This type of testing would be of special value to high risk groups, such as sibs of affected patients and related married couples, as well as in the general population. If the information of being a carrier were made available to newly married couples good genetic counseling could be provided. The risks involved of having affected children could be explained to these couples. Then each of the carrier couples could decide for themselves with the aid of genetic counseling if they would want to have children or not. So, theoretically this might be the solution to end cystic fibrosis simply with its prevention. Of course, the incidence would never be reduced to a zero level but with the knowledge of known carriers it seems likely the incidence rate can

be reduced to a lower level than the present estimated incidence of 1 in 2,500 live births. With the continued ingenious research in cystic fibrosis and the ever present effort in the area of carrier detection, there remains hope that the molecular basis of the disease will be discovered. And with this information available a correction of the defect might be possible.^{23, 24, 25}

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