PATHOPHYSIOLOGICAL ASPECTS OF CYSTIC FIBROSIS

Genotypes, Phenotypes and Intestinal Current Measurements

Cover: making a CF diagnosis (red area) by Intestinal Current Measurements (ICM) is more accurate compared to the sweat test. Additionally, an alternative chloride secretory pathway (ALT) can be detected.

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PATHOPHYSIOLOGICAL ASPECTS OF CYSTIC FIBROSIS
Genotypes, Phenotypes and Intestinal Current Measurements

ASPECTEN VAN DE PATHOFYSIOLOGIE VAN CYSTIC FIBROSIS
Genotypen, Fenotypen en Intestinale Secretie

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Voor Bernadette,
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mijn ouders

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List of Abbreviations

ATP	Adenosine 5'-triposphate
Ba^{2+}	Barium
BMI	Body Mass Index
Ca^{2+}	Calcium
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
Cl^-	Chloride
DIOS	Distal Intestinal Obstruction Syndrome
FVC	Forced Vital Capacity
FEV1	Forced Expiratory Volume in 1 second
ICM	Intestinal Current Measurements
ICSI	Intracytoplasmic Sperm Injection
I_{SC}	Short Circuit current
K^+	Potassium
M	Mucosal bath
MESA	Microsurgical Epididymal Sperm Aspiration
mRNA	Messenger Ribonucleic Acid
MSD	Membrane Spanning Domain
NBF	Nucleotide Binding Fold
RNA	Ribonucleic Acid
Na^+	Sodium
PI	Pancreatic Insufficiency
PS	Pancreatic Sufficiency
S	Serosal bath
Se	Selenium
tRNA	Transfer Ribonucleic Acid

Preface

Approximately one in twenty individuals of the Caucasian population is a carrier of cystic fibrosis (CF). CF originates from the inheritance of the mutated genes from both parents. CF gene mutations on both alleles lead to an incompetent gene product (CFTR) impairing normal chloride transport in epithelial cells. This causes the characteristic abnormally viscous secretion in epithelial lined organs leading to progressive damage of those organs and ultimately to a reduced life expectancy. The aim of this study was to explore the pathophysiological basis of the variable phenotypic expression of the disease, possibly to be explained by a variable impairment of chloride transport. An Ussing chamber was adapted to perform Intestinal Current Measurements (ICM) in intestinal biopsies. Typically, chloride currents cannot be detected in most patients with CF but residual chloride secretory activity is found in some cases. From this study it appears that the magnitude of the residual currents correlates with the CF phenotype. Residual activity predominantly occurred in patients with uncommon CFTR mutations. However, some patients with a more 'severe' CFTR genotype, including some carrying a null-mutation, displayed residual chloride transport activity as well. The findings of this study suggest the existence of a compensatory chloride secretory pathway.

ICM is a highly sensitive and specific test for CF, and offers several advantages as compared to the 'classical' sweat test. ICM is indicated in selected cases in which the sweat test is inconclusive or cannot be performed. Using this technique we identified mildly affected CF phenotypes with a 'normal' sweat test, which prompts us to reconsider the definition of cystic fibrosis.

The study was funded by the Netherlands Digestive Diseases Fund and carried out in the Laboratory of Paediatrics of the Sophia Children's Hospital, in collaboration with the departments of Cell Biology, Biochemistry, and Clinical Genetics of the Erasmus University.

Chapter 1

Introduction

1.1 Pathological Aspects of Cystic Fibrosis

Cystic fibrosis (CF), is one of the commonest life-threatening autosomal recessive hereditary disease, predominantly affecting Caucasian populations (1). It is listed under number 219700 in McKusick's Mendelian Inheritance in Man (2) and on Internet's Online Mendelian Inheritance in Man (3). In the Netherlands one in every 3600 newborns is affected by the disease (4). The disease is characterized by production of abnormally viscid secretions in epithelial tissues. The different organs involved such as lungs, pancreas, and liver are progressively damaged owing to obstructing mucoid plugs. The clinical manifestations of the disease and consequently the life expectancy range widely. The average life span has increased in the last decade to an age of almost 30 years. It is to be expected that newly diagnosed CF patients will have a better prognosis as a result of more aggressive therapy and preventive measures. Especially, improvement of treatment of meconium ileus, more effective antibiotic regimes, care for optimal nutritional status, and the formation of multidisciplinary teams of CF specialists have contributed in this respect (1).

Abnormal sweat

In 1705 Schmidt described in a book of folk philosophy that a salt taste means a bewitched child and Rochholz (Almanac of Children's Songs and Games from Switzerland, 1857) described: "The child will soon die whose brows tastes salty when kissed". It seems very likely that these children suffered from CF. In 1953 the sweat electrolyte abnormalities of CF patients were first reported (5). In 1959 an improved sweat test using pilocarpine iontophoresis was introduced by Gibson and Cooke (6). The diagnostic criteria for CF were based on the results of tests performed on patients with a typical clinical presentation. In 1983 Quinton and Bijman

(7) observed that defective reabsorption of chloride through a chloride channel in the sweat duct causes the high salt content observed in sweat of CF patients. The excessive losses of sodium and chloride in the sweat account for the abnormal craving for salt, and heat prostration in vigorous activity or febrile episodes.

Intestinal disease

The name cystic fibrosis originates from the cystic malformations observed in the fibrotic and degenerated pancreas (8). In advanced disease, the insulin producing β-cells of the pancreas may become affected, resulting in diabetes mellitus (9).

Typically,, the obstruction makes it impossible for the pancreas to secrete pancreatic enzymes needed for digestion, which leads to large amounts of unabsorbed fat, protein and fat-soluble vitamins. If this condition is left untreated, weight loss and growth retardation may develop.

The malabsorption results from two processes: maldigestion in the absence of pancreatic enzymes and abnormalities of the mucus covering the villus and crypt cells of the intestine (10). Meconium ileus in the neonatal period (11) is seen in 10-20% of the diagnosed CF patients. It is not specific for CF and may be present in other pancreatic diseases. It may also be a functional disorder in preterm babies (12). The equivalent of meconium ileus at later age i.e. Distal Intestinal Obstruction Syndrome (DIOS) is also a typical expression of CF (13). DIOS may be mimicked by other conditions (14) including the recently described colonic strictures related to excessive intake of pancreatic enzymes (15). Both meconium ileus and DIOS are possibly related to liver involvement (16).

Liver disease in CF is caused by fatty degeneration and progressive fibrosis as a result of mucoid material in intra- and extrahepatic bile ducts. Progressive cirrhosis is responsible for the development of portal hypertension with oesophageal varices and hypersplenism (17).

Prolonged neonatal jaundice is also a presenting symptom of CF.

Airway disease

Pulmonary disease is the major cause of morbidity and mortality in CF (1). It results from defective chloride secretion by airway epithelium. Additionally, the airway epithelial cells absorb a higher amount of sodium (18). Not only the defective chloride secretion, but also the increased sodium reabsorption contributes to dehydration of the mucus and formation of mucus plugs. Superimposed airway infections, in most patients initially caused by Staphylococcus aureus or Haemophilus influenzae and later by Pseudomonas aeruginosa (19), may arise distal to the obstruction. Chronic airway inflammation leads to excessive secretion of purulent mucus and to airway obstruction, which in turn causes bronchiectasis, pulmonary hypertension with cor pulmonale and other complications as haemoptysis, pneumothorax, and respiratory failure. In the upper airways chronic sinusitis is associated with the formation of nasal polyps.

Genital disease

Infertility is more common in CF males than in CF females. In males, it originates from the absence of the vas deferens, most likely owing to obstruction and resulting atrophy (20). The diminished fertility in females results from abnormalities in cervical mucus (21).

1.2 Chloride transport

In contrast to the sweat ducts in which mainly chloride reabsorption is impaired (7), other epithelial tissues show defective chloride secretion (22-24). Also, the water flow as a sequel to active ion excretion is diminished, and secretions therefore remain viscous. To facilitate chloride

excretion, chloride is first pumped into the cell through the basolateral membrane by a Na^+-K^+-$2Cl^-$ cotransporter (25) (Figure 1). Sodium recirculates via the Na^+/K^+ pump. This Na^+/K^+ pump together with the Na^+-K^+-$2Cl^-$ cotransporter increases the intracellular potassium concentration. The processes involved in raising the intracellular chloride concentration can only be maintained by opening a basolateral K^+-channel for the required efflux of potassium. This K^+-channel can be activated by cholinergic agents like carbachol (26,27). Through specific channels located in the apical membrane (CFTR channels), chloride will move passively downhill its electrochemical gradient to the mucosal surface. It is at this point, that the CF defect can manifest itself. Normally, the CFTR channel can be opened in the presence of ATP and through phosphorylation of the channel i.e. the cAMP secretory pathway. Other, principally Ca^{2+}-activated chloride channels may also contribute to apical chloride transport in epithelial tissues (Figure 1).

1.3 CFTR protein

The specific chloride channel protein in the apical membrane (28), the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), is a polypeptide of 1480 amino acids with a molecular mass of 168138 Dalton and is encoded by the the CFTR gene (29,30). It contains several domains. In the literature, the CFTR gene is often referred to as the CF gene. Membrane spanning domains (MSDs) contribute to the formation of the Cl^- channel pore that spans the cell membrane. Binding of ATP to the two nucleotide binding folds (NBF-1,2), together with phosphorylation of the regulatory (R) domain, controls the channel behaviour (31). Phosphorylation of CFTR by cAMP dependent protein kinase-A (PKA) or by protein kinase-C (PKC) in the presence of ATP causes the channel to open (Figure 1).

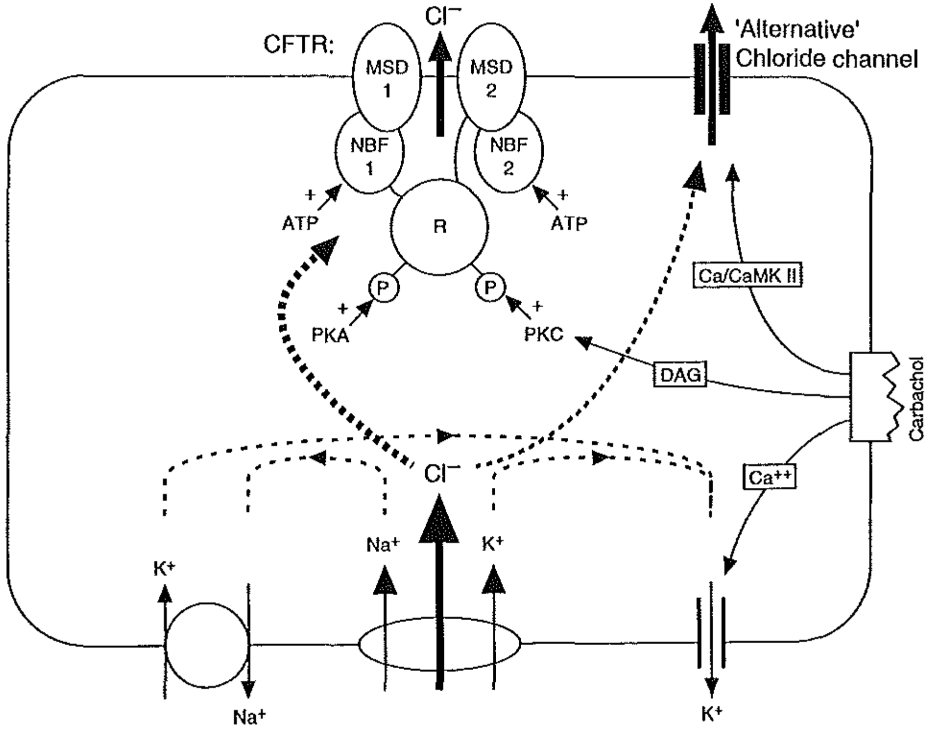

Figure 1. To facilitate chloride excretion in epethelial tissues, chloride is first pumped into the cell through the basolateral membrane by a Na^+-K^+-$2Cl^-$ cotransporter. Sodium recirculates via the Na^+/K^+ pump. This process can only be maintained by opening a basolateral K^+-channel which can be activated by carbachol. In CF, the mutated CFTR channels interfere with the chloride excretion to the mucosal surface. Ca^{2+}-activated chloride channels may also contribute to apical chloride transport.

1.4 CFTR gene

In the case of CF carrier status or CF disease one or two CFTR alleles respectively are defective owing to a mutation. In 1989 the CFTR gene was identified by positional cloning. Within this gene one major mutation,

resulting in a deletion of phenylalanine (ΔF508) represents approximately 70% of the gene mutations in CF in Northern American populations (29,30). In Europe the percentage of CFTR mutations which is ΔF508 ranges from more than 90% in Scandinavia to approximately 50% in the Southern European countries (32). In the Netherlands approximately 60% of the known CF patients are homozygous for the ΔF508 mutation. In the remaining patients, 35% are compound heterozygotes for ΔF508, which means that besides the ΔF508 they carry another CFTR mutation, and 5% carry two non-ΔF508 CFTR mutations (33). In approximately 50% of the non-ΔF508 mutations the disease causing mutation can be identified. More than 400 CFTR mutations have now been recognised, and this number is still increasing (34). For diagnostic purposes, however, only the mutations reported for the specific population from which the patient originates are tested for routine procedures. A negative outcome of mutation analysis therefore does not rule out the diagnosis CF in suspected individuals.

1.5 Classification of disease-causing CFTR mutations

In CF the defect in transepithelial chloride transport results from a mutation in the CFTR gene. There are four main mechanisms by which CF-associated mutations interfere with the normal passage of chloride (35).

Class I mutations result in a largely truncated protein or no detectable protein at all (Figure 2). These mutations, consisting of frame-shifts, splice site abnormalities or nonsense (stopcodon) mutations, generally lead to more severe disease symptoms. Most CF patients carry a *Class II* mutation. Here, the mutated protein is misfolded. The best known mutation leading to this condition is the ΔF508 mutation, a deletion (d) of a triplet coding for phenylalanine (F). Most of the defective protein

molecules are probably recognized by the 'quality control' mechanisms in the cell and will be degraded (Figure 2). Only a small proportion of the protein is able to reach the cell membrane (36,37). Interestingly, at low temperature (approximately 25° C), more mutated CFTR protein is correctly incorporated into the plasma membrane. Importantly, under these conditions, the chloride channel function is partially preserved (38). Also the presence of a third mutation, such as R553Q, may facilitate the escape from the quality control mechanism by a compensatory alteration in the defective CFTR structure (39,40).

In *Class III* mutations the protein is able to reach the cell membrane but the improper binding of ATP at one of the NBFs affects the process of normal CFTR mediated chloride secretion (Figure 2).

Class IV mutations result in alterations in the MSDs and this results in decreased magnitude of the chloride current through the channel (Figure 2). Class III and IV mutations are associated with a milder phenotypic expression of the disease (41), or may not even display CF associated symptoms at all except for male infertility (42).

1.6 Defective intestinal chloride transport in CF

As shown by electrophysiological studies in colonic epithelial cell lines (26,43), carbachol stimulates the basolateral potassium conductance, and creates an increased driving force for chloride excretion through CFTR-chloride channels in the apical membrane (26,27). Furthermore, the protein kinase-C component of the carbachol response may also activate the CFTR-chloride channel by direct phosphorylation (44-46) (Figure 1). In the intestine, however, calcium-activated chloride channels (other than CFTR) contribute little to the apical chloride secretion (43,47,48) in contrast to their prominent role in other epithelial tissues. Chloride secretion in intestine is thus not only defective in response to

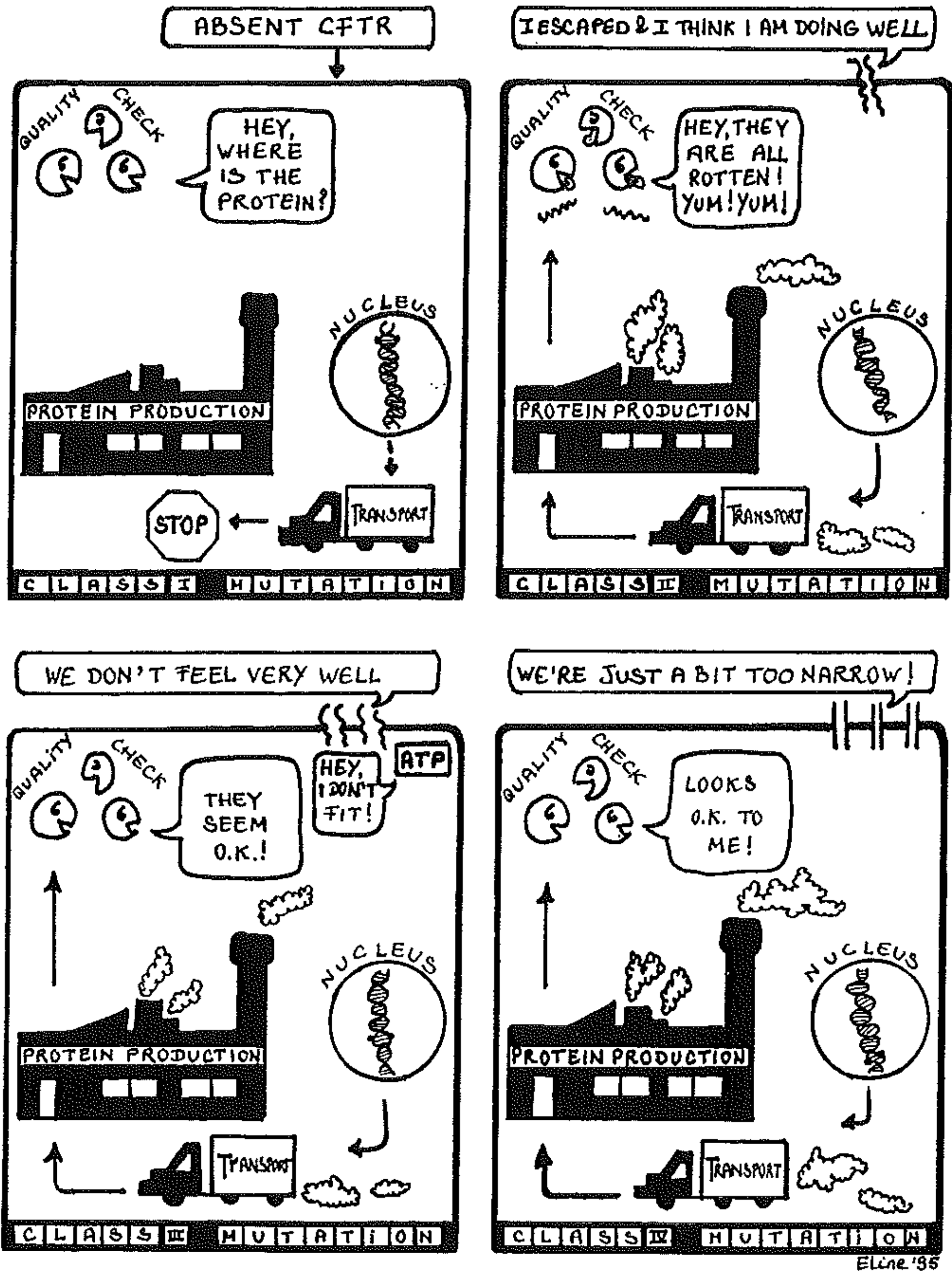

Figure 2. What goes wrong in various classes of CF mutations.

cAMP-linked secretagogues, but also in response to Ca^{2+}-linked secretagogues, including cholinergic stimulation (49-51). In this tissue therefore, CFTR plays a key role in transepithelial chloride transport. However, this thesis proves that Ca^{2+}-activated chloride channels in the intestine may serve to compensate, at least partially, for defective CFTR-mediated chloride secretion in a subgroup of CF patients.

Also, the CF carrier may well have a diminished chloride secretory capacity of the intestine (52,53). This may enhance chances of survival in the case of severe secretory diarrhoea (e.g. cholera), and may thus account for the high frequency of CF carriers (1:30 in the Netherlands, deduced from the incidence of known CF patients).

1.7 Measurement of chloride transport: The Ussing chamber technique

The Ussing chamber was originally developed by Ussing and Zerahn (54). Basically, the Ussing chamber consists of a bath with a physiological salt solution at 37° C and gassed with 95% CO_2 and 5% O_2, divided into two compartments by a piece of epithelial tissue. The potential difference across the tissue is measured by two salt-bridge connected calomel electrodes which give input to a voltage clamp amplifier. The output of the voltage clamp amplifier continuously clamps the potential difference across the tissue at 0 mV by applying a current through two platinum electrodes placed at both sides of the tissue (Chapter 3.1, Figure 3). Under the condition that specific secretagogues or blockers of transport processes are added to the bath solution, the current required to maintain the potential difference at 0 mV, (Short Circuit Current = I_{SC}), is a direct measure for the net movement of ions actively transported across the epithelial cells. With an Ussing chamber, different combinations of stimulating and blocking agents on active transport processes can thus be studied. In the past years this technique was used to measure

intestinal chloride transport, mainly in resection preparations (24,49-51,55). In the present study, an Ussing chamber was adapted to measure Intestinal Chloride Currents (ICM) in intestinal biopsies (Figure 3).

1.8 Study Design

Chapter 2 delineates relationships between CFTR genotypes and clinical symptoms.
Chapter 3 describes the electrophysiological abnormalities of intestinal chloride transport in CF.
Chapter 4 combines the outcomes of the studies of Chapter 2 and 3 in order to further clarify the pathophysiology of CF.

In *Chapter 2*, several clinical phenotypes of CF in relation to different genotypes are described. The relationship between the CF genotype and the age at diagnosis (Chapter 2.1), was a first indication of a possible association between genotypes and phenotypes in CF. Chapter 2.2 describes the clinical manifestations in CF patients in whom two new CFTR mutations were identified. In our hospital, we developed a CF database to establish genotype/phenotype relationships. We selected all our CF patients with only a single identified CFTR mutation. Those patients with a mild clinical phenotype and residual intestinal chloride secretion were identified and an additional mutation analysis was carried out with a panel of 'mild' mutations. The A455E mutation was far more frequently observed than expected. Further investigation of all CF patients with unidentified CFTR mutations in Rotterdam and Groningen revealed

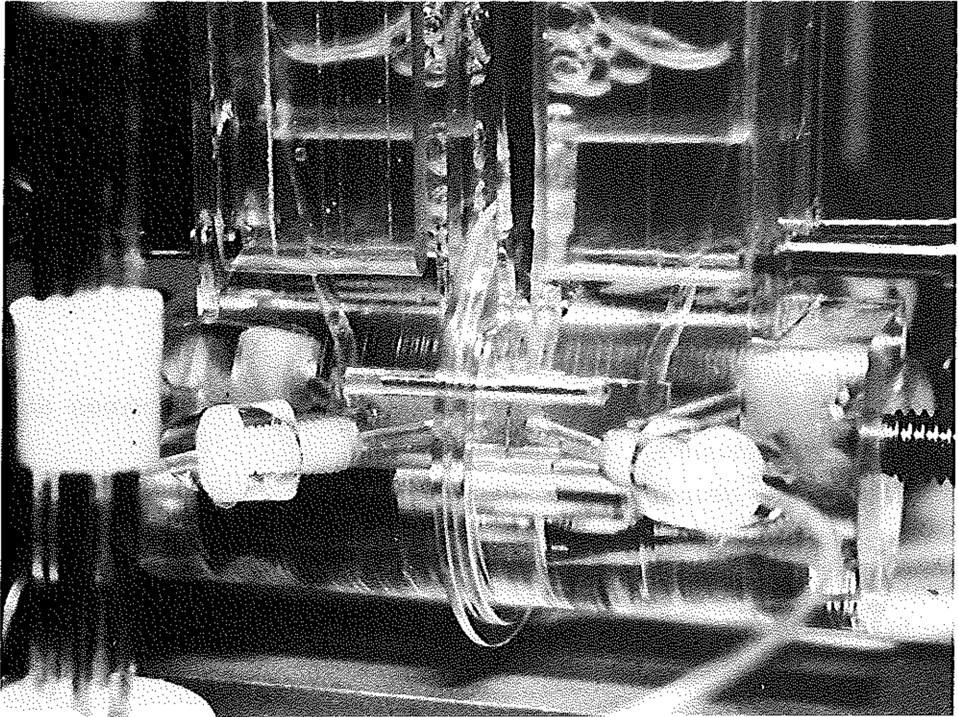

Figure 3. Between the two thin disks, separating both halfs of the micro-Ussing chamber, a small suction biopsy can be placed (exposed area 1.13 mm^2).

that the A455E mutation was the second most frequent mutation in the Netherlands.

The A455E is associated with mild pulmonary disease (Chapter 2.3). As is shown in Chapter 2.4, patients with the A455E mutation often have normal or borderline sweat test results.

In *Chapter 3* studies on electrolyte transport in intestinal tissues are described. The inability to culture non-transformed intestinal cells and the

sparse availability of freshly resected intestinal tissues from CF patients prompted us to develop an assay to measure intestinal chloride currents in biopsies from the rectal mucosa or small intestine with use of an adapted Ussing chamber. Rectal biopsies can easily be obtained without causing pain to the patient. In the past, in fresh tissues derived from meconium ileus CF patients, chloride secretion was undetectable. In contrast, in the present study, in which non-meconium ileus patients were included, residual intestinal chloride secretion could be observed in a minority of the cases. Moreover, our results suggested that these latter CF patients were less severely affected by the disease. The results of sodium and chloride transport measurements in controls and in CF patients are reported in Chapter 3.1 and 3.2.

In *Chapter 4* the relationship between chloride secretion patterns, the clinical presentation, and the CFTR-genotype is described. Following the identification of the CFTR gene in 1989 (29,30), we found that CF patients with residual intestinal chloride secretion often had rare or unidentified CFTR mutations (Chapter 4.1). Furthermore, we could establish a relationship between the clinical phenotype and the intestinal chloride secretion pattern. In Chapter 4.2, the existence of alternative, non-CFTR chloride channels in the intestine is described. These alternative channels, functioning as a compensatory pathway to secrete chloride, may in part compensate for the CFTR defect, and thus influence the severity of clinical manifestations.

Using a large data set of sweat test results, the criteria for a normal sweat test were re-evaluated (Chapter 4.3). It was concluded that CF may exist in case of a negative or borderline sweat test.

Clinically well identified CF patients always showed abnormal ICM whereas in controls the response was always normal. We applied ICM in subjects suspected of CF in whom a sweat test could not be performed (e.g. neonates). When results of CFTR mutation analysis and/or later

performed sweat tests became available, ICM results appeared to be highly reliable (Chapter 4.4). We therefore applied ICM on a larger scale for diagnostic purposes. ICM is especially indicated in individuals with borderline or high-normal sweat test results and an inconclusive CFTR mutation analysis, who can otherwise not be distinguished from CF carriers (Chapter 4.4).

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Chapter 2

Genotype and Clinical Symptoms

Several clinical phenotypes of CF in relation to different genotypes are described.

Chapter 2.1

The Mutation ΔF508 on Dutch Cystic Fibrosis Chromosomes: Frequency and Relation to Patients age at Diagnosis

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Summary

We tested 190 chromosomes from Dutch cystic fibrosis (CF) patients and carriers for the presence or absence of the major CF mutation ΔF508. This mutation was found on 77% of the Dutch CF chromosomes. We observed a significant difference in the distribution of the ages at diagnosis between homozygotes for ΔF508 and the other patients. ΔF508 homozygotes tend to be identified as patients at neonatal or infantile age. The age at diagnosis of patients with at least one unknown allele, on the other hand, ranged between neonatal and young adult age.

Introduction

The recent identification of the CF gene and its most common mutation ΔF508 (1-3) is of great importance both for clinical genetics and for basic CF research. In the absence of a functional test, genetic evidence established the identity of the gene. The three base pair deletion ΔF508 was found on 68% of CF chromosomes, whereas it was never seen on normal chromosomes. Chromosomes with the deletion nearly always carried a specific haplotype for closely linked polymorphic markers (3) that had previously been designated as a "high risk" haplotype for CF (4). The frequency of the "high risk" haplotype on CF chromosomes shows considerable population-dependent variation (4-7). Therefore, population-dependent variation may also be expected for the frequency of ΔF508. The observed frequency of this mutation determines the applicability of mutation analysis for carrier detection and prenatal diagnosis in the population.

We examined whether the higher frequency of the "high risk" haplotype on Dutch CF chromosomes (7) as compared with the data of

Kerem et al. (3) is accompanied by a higher frequency of ΔF508. We analyzed differences in the ages at which the disease is diagnosed between patients who are homozygous for ΔF508 and the other patients.

Methods

We tested 190 CF chromosomes for the presence or absence of the most common mutation ΔF508. We studied the chromosomes from 93 patients with non-consanguineous parents. Hundred and seventy eight chromosomes were derived from 89 unrelated patients and 6 chromosomes were from 2 pairs of affected first cousins. The remaining 6 chromosomes were from unrelated obligate carriers. All individuals were of Dutch origin. The patients' ages at the time of initial DNA analysis ranged between 6 months and 33 years (mean 9, median 12 years). In the families with multiple affected offspring we included the age of the first diagnosed patient in this calculation.

DNA was extracted from leucocytes with the salting out procedure of Miller et al. (8). Mutation analysis by means of PCR and allele specific oligonucleotide hybridization was done essentially as described by Kerem et al. (3) with 5'-GTTTTCCTGGATTATGCCTG-3' and 5'-GTTGGCATGCTT TGATGACG-3' as amplification primers and an annealing temperature of 48° C. The PCR products were blotted onto Genescreen plus membranes using a Schleicher and Schuell Minifold II slot blotting device. We analyzed 2 closely linked D7S23 polymorphisms (XV2c/TaqI and KM19/PstI) (9) on most chromosomes.

Wilcoxon's rank sum test was applied to compare the distributions of the ages at diagnosis between ΔF508 homozygotes and other patients.

Results and Discussion

The overall frequency of ΔF508 in our sample of 190 Dutch CF chromosomes was 77.4% (Table 1). This implies that 60% of Dutch CF patients are homozygous for ΔF508. An equal proportion of prenatal diagnoses in pregnancies at a 1 in 4 risk may now be made by direct mutation analysis (10-12). The obvious advantages of mutation analysis are speed and reliability.

A major restriction of linkage analysis is the requirement of DNA from an index patient. Prenatal diagnosis is now also accessible to 60% of the (Dutch) parents with a deceased affected child in the absence of DNA to establish linkage phase. Mutation analysis would detect 77% of CF carriers among Dutch individuals without a family history of CF. The identification of the remaining CF defects would remove the last barriers to unrestricted possibilities for prenatal diagnosis and carrier detection at

Table 1. Frequency of ΔF508 on Dutch chromosomes. 1. Larger allele; 2. smaller allele; []. phase unknown.

	ΔF508	other mutation	Haplotype [XV2c,KM19]	
	3	8	1	1
	123	14	1	2
	-	6	2	1
	1	2	2	2
	-	1	[]	1
	3	1	[]	2
	4	1	1	[]
	-	3	2	[]
	2	-	[]	[]
	11	7		
Total	147 (77.4%)	43 (22.6%)		

the population level. The principal investigators in molecular genetics of CF have initiated the CF Genetic Analysis Consortium to achieve this in the most efficient way.

The majority of the chromosomes were haplotyped for the XV2c/TaqI and KM19/PstI polymorphisms (Table 1). In agreement with the report by Kerem et al. (3) ΔF508 was found almost exclusively against the background of the [XV2c] 1, [KM19] 2 haplotype. The same haplotype was also present on about half of the phase-known chromosomes carrying other mutations than ΔF508 (14/30, Table 1). The frequency of the 1,2 haplotype on the phase known North American CF chromosomes was 73% (calculated from Kerem et al. (3)). A 1,2 haplotype frequency of 87% was calculated for the present sample of Dutch CF chromosomes (data not shown).

An interesting question would be if the observed clinical heterogeneity in CF patients could be correlated to allelic heterogeneity. Since considerable heterogeneity is expected among the CF defects that remain to be identified, this may be a complicated issue to resolve. However,it has already been possible to indicate a correlation between ΔF508 and pancreatic insufficiency (3).

A parameter that may serve as an indicator for the overall clinical severity is the age at diagnosis. Sofar we have been able to obtain reliable information on the age at diagnosis of 40 patients. All but 3 of these patients were diagnosed in one Clinical Centre, the Sophia Children's Hospital, Rotterdam.

Twenty three patients were homozygous for the ΔF508 mutation. Fourteen of them had been diagnosed as CF patients during the first year of life and 10 of these diagnoses were even made within 2 months after birth (Table 2). No patient from this group had been diagnosed after the age of 4. The other category consisted of 14 compound heterozygotes with one ΔF508 allele and 3 patients with two unknown alleles. More than 50% of the patients from the latter category had been identified after

Table 2. Age at diagnosis for ΔF508 homozygous and other CF patients.

age	ΔF508 homozygotes	Others[1]
<1 month	8	2
1-2 months	2	0
2-12 months	4	4
1-5 years	9	2
6-10 years	0	4
11-15 years	0	2
16-20 years	0	3
Total	23	17

[1]Fourteen patients had one ΔF508 allele, three had two unknown mutations.

6 years of age (Table 2). We examined whether there is a significant difference in the distributions of the ages at diagnosis between the ΔF508 homozygotes and the other patients using Wilcoxon's rank sum test. This did show a significant difference ($T=3.03$, $P<0.001$). We are currently supplementing the data on the age of diagnosis with specific clinical and physiological features.

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Chapter 2.2

Identification of the L927P and ΔL1260 Mutations in the CFTR Gene

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The autosomal recessive disorder cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) (1). Mislocation or dysfunction of CFTR in epithelial cells result in viscous secretion in various organs leading to recurrent pulmonary infections, maldigestion and malabsorption. The phenotypic expression of the disease depends on the level of residual chloride secretion (2). Specific CFTR mutations may lead to residual CFTR activity hereby influencing the clinical outcome (2,3). The CFTR gene has 27 exons scattered over a region of about 230 kb (1,4). More than 400 mutations have been identified, including missense (39%), nonsense (25%), frameshift (20%), splice site (15%) mutations and deletions of amino acids (1%) (CF Genetic Analysis Consortium). The most prominent mutation (67% worldwide) is the deletion of phenylalanine at position 508 (1).

Routine methods detect about 85% of mutations in our Dutch CF patients population (unpublished results). To identify the remaining mutations, genomic DNA was amplified using the primers described by Zielenski et al. (5), followed by SSCP analyses of several exons using 6% polyacrylamide gel electrophoresis, 0.5 x BE buffer with 10% glycerol (overnight at room temperature and 5 Watt). DNA of patient 221 showed an aberrant pattern of exon 20 and this exon was subsequently sequenced. For sequence analysis, PCR products were prepared by using the same primers as for the SSCP analysis except that one of the primers was biotinylated. Single strand DNA was obtained using the Dynabeads method as described previously (6). Direct automatic sequencing primers was performed on an ALF sequencer (Pharmacia-LKB).

Sequence analysis resulted in the identification of a deletion of ACT either at position 3909 - 3911 or at position 3912 - 3914, leading to a deletion of a leucine residue (position 1260 or 1261; ΔL1260). Using SSCP analysis, the ΔL1260 mutation was identified in one out of 148 non ΔF508 chromosomes. The affected sibling of this patient also had the ΔL1260

mutation.

Allele specific oligonucleotide (ASO) hybridisation was done to investigate the presence of the ΔL1260 mutation in non-CF chromosomes. Oligomers used in the ASO analysis are: 5'-GTGTTCAGTAGTCTCA-3' (normal) and 5'-GTGTTCAGTCTCAAAA-3' (mutant). None of 88 non-CF chromosomes tested showed the ΔL1260 mutation and therefore it is very likely that ΔL1260 is not a protein polymorphism.

As the mutation on the other allele of patient 221 was still unknown, the remaining exons, which were not investigated by SSCP analysis, were subjected to direct sequence analysis. PCR of genomic DNA and sequence analysis was performed as described above. Sequence analysis of exon 15 identified a substitution of T at position 2912 to a C resulting

Table 1. Clinical features of CF patients with L927P and ΔL1260 CFTR mutations.

	Family A		Family B	Family C	
	patient 166	patient 290	patient 246	patient 221	patient 209
CFTR mutations	ΔF508/ L927P	ΔF508/ L927P	ΔF508/ L927P	ΔL1260/ L927P	ΔL1260/ L927P
Sex	male	female	male	female	female
Age at diagnosis (yr)	3.7	1.0	0.2	0.6	9.0[1]
Current age (yr)	15.2	13.6	3.3	21.0	18.7
Shwachman clinical score (max. score 100)	80	85	90	80	75
Pancreatic insufficiency	yes	yes	yes	yes	yes
FEV_1 (% predicted)	65	60	-	50	65
Onset of Pseudomonas colonization (yr)	15.0	-	-	6.8	13.0

Patients 290 and 166 as well as patient 221 and 209 are siblings.
[1]Neonatal sweat test was negative, later on the sweat test appeared to be positive.

in a replacement of leucine by proline at position 927 (L927P). ASO hybridisation was performed to determine the frequency of the L927P mutation in our CF patient group using the oligomers: 5'-ACTTTGCTTG-CTATGG-3' (normal) and 5'-ACTTTGCCTGCTATGG-3' (mutant). Two more patients with this mutation were identified, resulting in a total of 3 chromosomes out of 148 non ΔF508 alleles. Two of the patients had affected siblings (see Table 1). The L927P mutation was not present in 60 non-CF chromosomes tested, indicating that L927P is very likely not a polymorphism.

Clinical data of the five CF patients are given in Table 1. Sweat test results in all patients were positive (Na^+ > 70 mmol/L) and none of them had meconium ileus or distal intestinal obstruction syndrome. Patient 290 had liver cirrhosis indicated by nodular findings on ultrasound and elevated activity of gamma-glutamyl transpeptidase, alanine and aspartate aminotransferases complicated by portal hypertension. All five patients showed pancreatic insufficiency, a low Shwachman clinical score and a low FEV_1. From the described clinical features we concluded that most likely both the L927P and ΔL1270 mutations can be classified as severe CFTR mutations comparable with ΔF508.

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Chapter 2.3

Evidence for a CF Mutation Associated with Mild Lung Disease

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Chapter 2.4

High Incidence of the A455E Mutation Identified in Cystic Fibrosis Patients with Negative Sweat Test Results

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Submitted as letter

The diagnosis of cystic fibrosis (CF) is confirmed by the finding of a positive sweat test result (chloride above 60 mmol/L). Clinical suspicion of CF may still be present while sweat tests failed to show a positive result. Usually, these situations remain unresolved although in some cases the diagnosis may be confirmed by the identification of two causative CFTR mutations (1-3). Recently, we detected two CF disease causing CFTR mutations (ΔF508/A455E) in a 9 month old boy with normal sweat test results: in 89 and 59 mg sweat, sodium was 37 and 30 mmol/L while chloride was 33 and 24 mmol/L. He was suspected of CF and referred to our hospital with a lower respiratory infection caused by a Methicillin resistant Staphyloccus aureus.

This has prompted us to reevaluate all sweat tests obtained in our centre of 18 CF genotypes characterized by the A455E mutation (n=17: ΔF508/A455E, and n=1: 1717-1G->A/A455E; median age 5.7 yr). The previously obtained sweat test results relied on sodium values (lower limit for a positive test 70 mmol/L). In 8 out of 18 CF patients, the highest sweat sodium obtained was below 70 mmol/L (Figure 1). To improve the

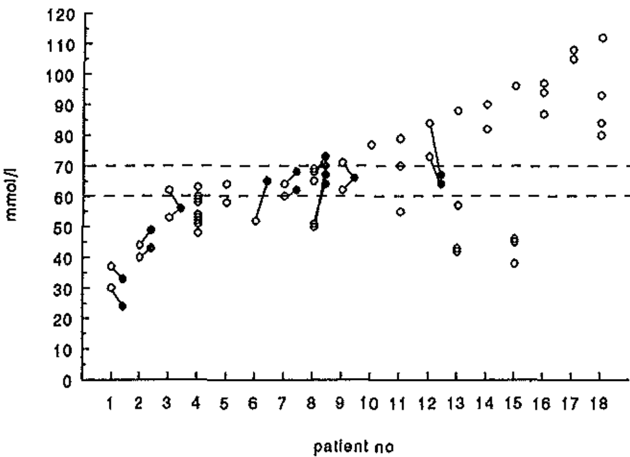

Figure 1: Sweat sodium (O) and if available the sweat chloride values (●) of 18 compound heterozygotes with A455E

predictive value of the sweat test, the determination of sweat chloride was reintroduced in our centre. Sweat chloride (lower limit for a positive test 60 mmol/L), is a more sensitive parameter to detect CF (Figure 1). However, in 3 out of 8 patients both sweat sodium and chloride failed to show a positive result nor could sweat chloride/sodium ratios always be of help to confirm the clinical CF diagnosis (Figure 1). The low sweat test values obtained in these CF genotypes could be explained by a residual chloride transport as has been found in the intestine of such CF patients (4).

In our centre the A455E mutation accounts for 4.8% of all CFTR mutations which is unexpectedly higher than the reported worldwide frequency of 0.1% (5). We suggest to test for the presence of the A455E mutation in clinically suspected cases with borderline or even negative sweat tests. We wonder whether the frequency of the A455E mutation in clinical CF may also be higher elsewhere.

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Chapter 3

Intestinal Secretions

An adapted Ussing chamber was developed to study electrolyte transport in intestinal tissues of CF patients and controls. In most CF patients intestinal chloride secretion in response to specific secretagogues is absent, but residual chloride secretion could be observed in a minorty of cases.

Chapter 3.1

New Insights into the Pathogenesis of Cystic Fibrosis

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Summary

Cystic fibrosis is the most frequent inheritable disease with lethal course during childhood. The characteristic high viscosity of the mucoid secretion products in the lungs, pancreas and gut cause plugging and secondary damage of these organs. In the recent twenty years effective treatment of intestinal obstruction in the neonatal period and the infections of the lungs, has improved the prognosis significantly. Many patients will reach adulthood in the near future.

In the last ten years new insights into the cause of the disease changed diagnostic procedures and, it is to be hoped, soon also treatment. The first development was the estimation of brush-border enzymes in amniotic fluid. With this method prenatal diagnosis is possible in the 17th - 18th week of pregnancy. The recent discovery of the gene on chromosome 7 and its structure is the most important breakthrough. At the same time the process of Cl^- transport across the mucosal membrane of many types of epithelium was subject for investigation by several laboratories.

We have studied the transport of ions in small and large intestines of CF patients. The effect of all three types of intracellular signal transfer is abnormal, although the second messengers themselves (cAMP, cGMP and Ca^{2+}) are present. Evidence is found for K^+ in stead of Cl^- secretion after addition of secretagogues.

Introduction

CF is the most common lethal, autosomal recessive inherited disease in the caucasian population (incidence approximately 1 : 2500). Symptoms are the result of transport abnormalities and tissue destruction in several

organs with secretory epithelium. Destruction is the result from obstruction with mucoid plugs caused by the high viscosity of the secretory product.

Clinical Pathology

The earliest symptom is intrauterine intestinal obstruction, which can lead to perforation, meconium peritonitis, and intestinal atresia (1). Before the early seventies, when surgical procedures were introduced, this symptom caused death in 20% of the patients. In 85% of the newborns the pancreatic secretion is severely disturbed with a loss of more than 90% of the function. Patients with CF suffer from chronic obstructive lung disease, which is at present the most frequent cause of death. Major respiratory complications in CF include haemoptysis, pneumothorax, cor pulmonale, and heart failure. The survival rate has been increasing throughout the past 30 years because of intensive antibiotic- and physiotherapy.

Maldigestion and malabsorption are a common symptom, which results in failure to thrive and makes patients prone to pulmonary complications.

The cornerstone in the diagnosis is an abnormally high content of chloride in sweat. The basis for our present understanding of the pathogenesis of CF are the results of investigations to the chloride transport studied in isolated sweat glands by Quinton and Bijman (2).

Biochemical defect

Movement of water in organs involved in CF is caused by the transport of sodium and chloride ions across the epithelial cell layer lining the lumen

of glands. In most tissues this transport process is characterized by active chloride secretion, whereas sodium follows passively through the intercellular space. Understanding of this process is of major importance for the insight into the pathogenesis of CF (3). The intracellular chloride concentration is build up by a basolateral Na^+-K^+-$2Cl^-$ symporter resulting in an outwardly directed electrochemical gradient of this ion. The required energy for this process is generated by a $3Na^+/2K^+$ ATPase pump. Consequently, the opening of chloride channels in the mucosal membrane results in a rapid chloride secretion, followed by sodium efflux through the paracellular way (Figure 1). The duct of the sweat gland forms the only exception, in that chloride channels are involved in the transport of chloride in the opposite direction, although following the same principle. In this case water movement does not take place because the junctions between the duct cells are very tight, so the sweat production in CF patients is normal.

It is now generally accepted that the high viscosity of the secretion products in organs involved in CF is the result of a diminished water movement, secondary to a defect in the Cl^- secretion. A proper understanding of the pathogenesis was delayed by the high chloride concentration in sweat from CF patients, which is not representative for the composition of other secretion products. This did not seem to offer an explanation for the dehydration of mucoid secretions causing increased viscosity in lung, pancreas, intestine, and liver that is characteristic for the CF condition.

Epithelial chloride and sodium transport through electrogenic transport systems such as ion channels is accompanied by changes in potential difference which can be studied *in vitro* in whole tissue in the Ussing chamber and in isolated cells by patch clamp analysis of single ion channels. *In vivo* studies include electrolyte measurements in combination with potential difference registration across the mucosa.

Application of these techniques in nasal and airway epithelium have

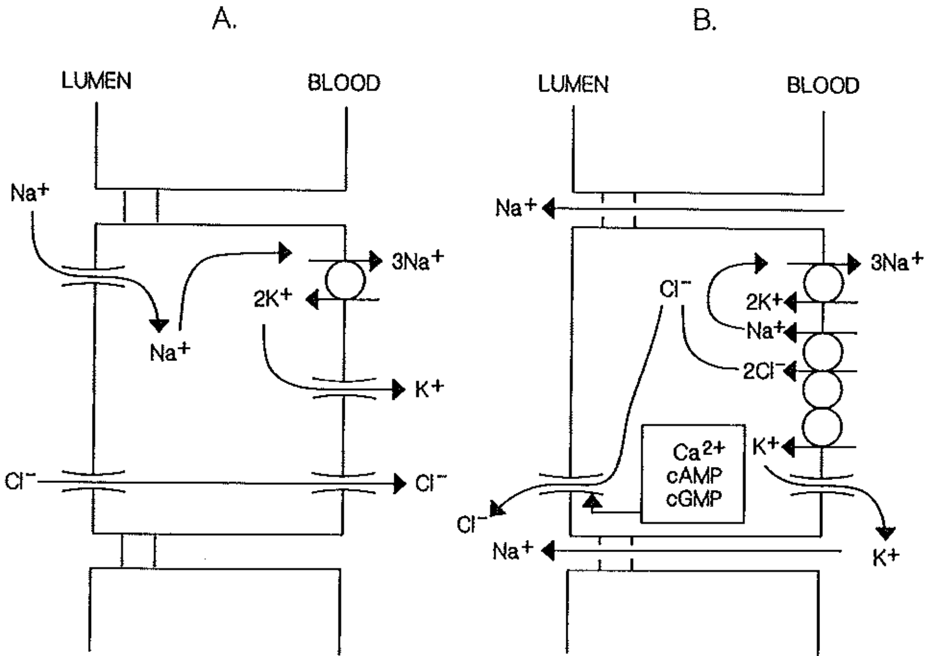

Figure 1. Different models of chloride transport across the mucosal cell membrane. *1A. Sweat gland.* The energy for transport into the cell is provided by the serosal $3Na^+/2K^+$ ATPase pump. This pump constitutes the primary driving force for sodium and indirectly for chloride through a specific channel. *1B. Enterocyte.* The Na^+-K^+-$2Cl^-$ symport accumulates chloride above its electrochemical equilibrium in the cell interior. Opening of a chloride channel allows the passive efflux of chloride into the intestinal lumen. To maintain electroneutrality, sodium ions follow through the paracellular pathway. Na^+ and K^+ ions recirculate across the basolateral membrane through secretogogue-sensitive K^+ channels and the $3Na^+/2K^+$ ATPase pump.

shown both an impermeability for chloride and an increase of sodium conductivity in the mucosal membrane (4). The regulation of chloride channel activity is subject for study in several centres. Values of intracellular second messengers (such as cAMP, Ca, cGMP, and protein kinases) necessary for triggering the opening of the Cl^--channel are normal. Under certain patch clamp conditions *in vitro*, the chloride

channel could still be activated, which indicates that a postulated regulatory protein rather than the channel itself must be defect in CF (5, 6). The identification of candidate proteins for regulation from the chloride channel in the intestine has been facilitated by the fact that in this tissue, besides cAMP and Ca^{2+}, a third second messenger, cGMP is capable of activating the channel. Phosphorylation studies in brush-border membrane preparations of the small intestine have identified a rather unique enzyme species with cGMP-dependent protein kinase activity (PK-G). This enzyme is capable of activating the intestinal Cl^-channel in response to a specific activator of the cGMP generating enzyme guanylate cyclase such as heat-stable Escherichia coli enterotoxin (STA) (7,8) or to cGMP analogues, such as 8-Bromo cGMP.

Genetic defect

Prenatal diagnosis during week 17-18 of pregnancy became possible in 1984 by the estimation in amniotic fluid of the activity of disaccharidases and other brush-border enzymes originating from the fetal intestine. Presumably, the activity is lowered in CF because these enzymes will not pass from the intestine to the amniotic cavity as a result of the sticky meconium (9,10). Since this test is not 100% reliable, the discovery of the location of the CF gene was an important improvement. In 1985 the genetic markers for CF were localized on the long arm of chromosome 7. The identification of the gene by the application of chromosome walking and jumping techniques four years later was an important hallmark in the search for the basic defect (11-13). Since September 1989 it has been known that 68% of the CF genes have a 3-base-pair deletion resulting in the omission of a single amino acid (phenylalanine) at position 508 of a large protein molecule called CFTR (cystic fibrosis transmembrane conductance regulator). It can be anticipated that in the remaining 32% of

the CF genes several other mutations responsible for other defects in the CFTR protein will be identified in the near future. The diagnosis of CF and carriership using DNA technology in an individual in the general population is not possible until the other deletions have been identified. For prenatal diagnosis the application of DNA linkage studies in chorion villus cells is restricted to families with an index patient. If both parents are heterozygotes for the known mutations, one can predict CF without an index patient, but this is until now only possible in about half of the pregnancies at a 1 : 4 risk for CF.

As predicted by the nucleotide sequence of the CF gene transcript, the CFTR protein may belong to a family of transport molecules that includes the multidrug resistance (MDR) proteins. These proteins are also transmembrane molecules and function as excretion pumps for drugs but in all likelihood not for chloride. Whether the CFTR protein functions merely as a regulator of the chloride channel or is identical to the chloride channel itself or a transporter of an inhibitor has still to be elucidated (Figure 2).

Intestinal defect

The intestinal abnormalities in CF are associated with pancreatic insufficiency and subsequently deficiency of digestive enzymes. However, substitution of these enzymes almost never results in normal absorption, relief of abdominal pain, or of intestinal obstruction. The aim of our studies was to investigate to what extent a possible defect in chloride transport in intestinal epithelium is involved in the pathogenesis of CF at the intestinal level (14).

This study was performed *in vitro* by measuring changes in potential difference and short circuit current (I_{SC}) between both sides of the tissue following stimulation of ion transport by specific secretagogues.

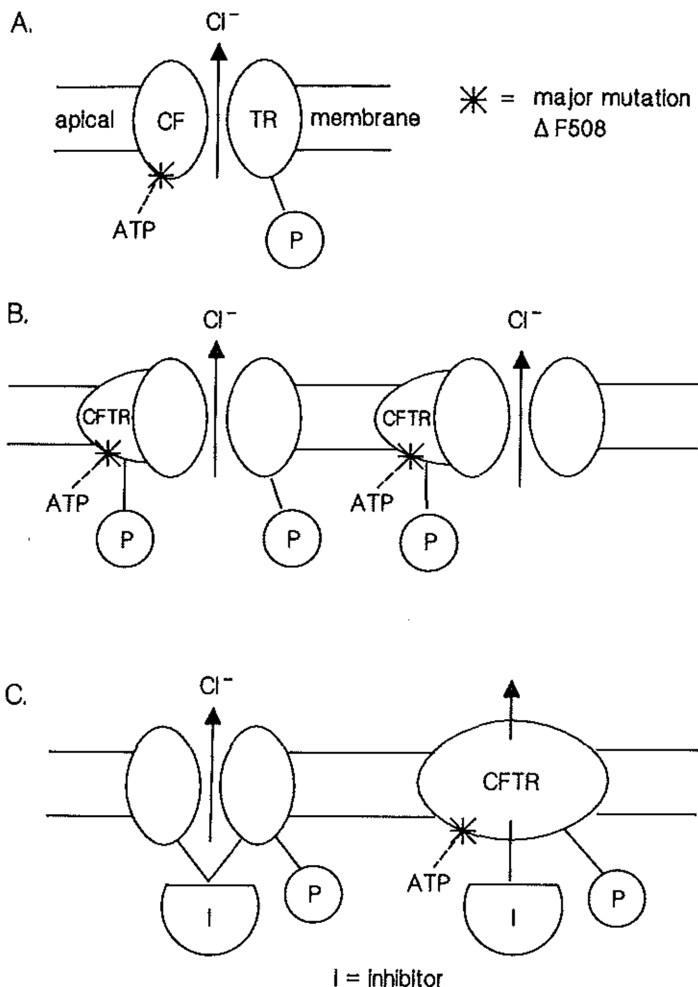

Figure 2. Hypothetical models indicating possible relationships between the CF gene product (CFTR) and epithelial chloride channels. *2A.* CFTR is depicted as a Cl^--conducting transmembrane protein containing additional sites for ATP binding and phosphorylation; phosphorylation of CFTR at P activates the channel. When phenylalanine is deleted from one of the ATP binding domains (ΔF508) its necessary interaction with the regulatory domain is affected. *2B.* CFTR is depicted as a regulatory component of a Cl^- channel complex. When phenylalanine is deleted from the ATP-binding domain, the interaction between CFTR and the Cl^- channel protein is disturbed. *2C.* CFTR is depicted as a pump protein which influences the activity of the Cl^- channel by adjusting the level of aspecific Cl^- channel inhibitor. The deletion of phenylalanine from an ATP-binding domain of CFTR impairs ATP-driven extrusion of the channel inhibitor thereby resulting in a (reversible) blockade of the channel.

After approval was obtained from the Hospital Medical Ethical Committee and from parents by written informed consent, small-intestinal tissue, obtained from CF patients (age 0-12 months) during reconstruction of ileostomies, was stripped and mounted in a conventional Ussing chamber. Rectal suction biopsies derived from patients of the same age range were tested in an adapted micro-Ussing chamber (Figure 3).

Control tissue was obtained from patients studied for Hirschsprung's disease (rectal biopsies) and from patients with non-CF related obstruction of the small intestine.

Tissues were stored directly after collection in ice-cold phosphate buffer and studied in the Ussing chamber within 5 min. During the study the tissue was perfused with Meyler-buffer at 37° C and gassed with 95% O_2 - 5% CO_2.

The I_{SC} was continuously recorded throughout the experiment. The response was defined as the maximal change in I_{SC} provoked by the addition of specific secretagogues or inhibitors.

Positive responses are the result of absorption of positively charged ions (mainly Na^+) or represent excretion of negatively charged ions (mainly Cl^-) on the mucosal side. Negative deflection may plausibly represent excretion of positively charged ions (presumably K^+) to the serosal side.

After stabilization of the basal I_{SC} and measurement of tissue resistance, additions to the mucosal (M) and serosal (S) side were made in a standardized order - that is, indomethacin (10^{-5} mol/L, M and S), amiloride (10^{-4} mol/L, M only rectal tissue), glucose (10^{-2} mol/L, M and S) carbachol (10^{-4} mol/L, S), 8-Bromo cAMP (10^{-3} mol/L, M and S) or forskolin (10^{-5} mol/L, S), and, in some experiments, histamine (5×10^{-4} mol/L, S). 8-Bromo cGMP (10^{-3} mol/L, M and S) was used in experiments with small-intestinal tissue only. Addition of Ba^{2+} (5×10^{-3} mol/L) on the mucosal side of the tissue was used to study the contribution of mucosal K^+ excretion to the I_{SC}.

The basal tissue resistance of rectal biopsies (range, 20-22 Ohm/cm^2) and ileal mucosa (range, 34-37 Ohm/cm^2) in CF was not different from controls, demonstrating equal quality of materials.

Sharp responses to the secretagogues demonstrated viability of the tissues and the presence of active transport mechanisms.

In all studies the endogenous prostaglandin synthesis, which causes cAMP-induced Cl^- secretion, was inhibited by the addition of indomethacin on both sides of the tissue. This resulted in a decrease of the I_{SC} in control and CF. After stabilization, this level was considered as the base line.

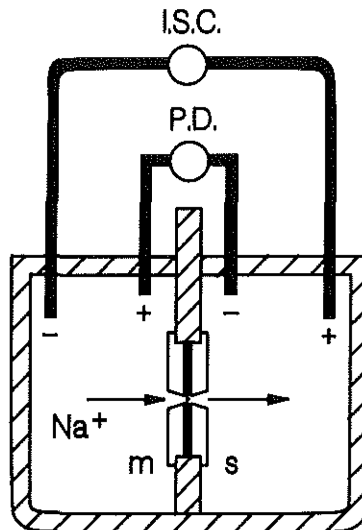

Figure 3. Ussing chamber. The two compartments of the Ussing chamber are divided by a tissue sheet. Biopsies are spread on a plastic disk over a central opening and covered by a second disk. The two halves of the Ussing chamber compress the disks. Potential difference across the tissue is measured through calomel electrodes and KCl agar bridges. The short circuit current (I_{SC}) is applied by platinic electrodes in the mucosal and serosal baths. m=Mucosal side; s=serosal side; P.D.=potential difference.

Addition of glucose to the mucosal side of the ileal tissue resulted in a positive I_{SC}, reflecting coupled Na^+ glucose transport. This response was absent in rectal tissue, as anticipated in view of the lack of expression of Na^+-glucose cotransport protein in this intestinal region (Figure 4).

Because of the absence of Na^+ channels in ileum, amiloride was only applied in rectal tissue experiments. Blocking the Na^+ channels by amiloride led to a negative response in control and CF of a virtually equal magnitude, suggesting a normal level of electrogenic Na^+ absorption in CF rectum.

In ileal and rectal control tissue a positive response was measured following stimulation with the Ca^{2+}-releasing secretogogues carbachol and histamine.

Addition of 8-Bromo cAMP or the adenylate cyclase activator forskolin resulted in a small positive response in normal rectal tissue but in a much stronger response in ileal tissues.

In rectal tissue 8-Bromo cGMP had no effect; in ileal tissue the response was about 40% of that of 8-Bromo cAMP.

In rectum and ileum tissues from CF patients the responses to Ca^{2+}- and cAMP-releasing secretagogues (carbachol, histamine, 8-Bromo cAMP, and forskolin) were usually all negative - that is, in the opposite direction as compared to control tissues. Furthermore, the response to 8-Bromo cGMP was completely absent in CF ileum (Figure 4).

The I_{SC} responses in CF and control ileal tissue were partly inhibited by addition of mucosal Ba^{2+} (results not shown).

Expression of the CF defect in Intestinal Epithelium

Our electrofysiological studies in rectal and ileal tissue in the Ussing chamber demonstrate that at last two absorptive functions of intestinal

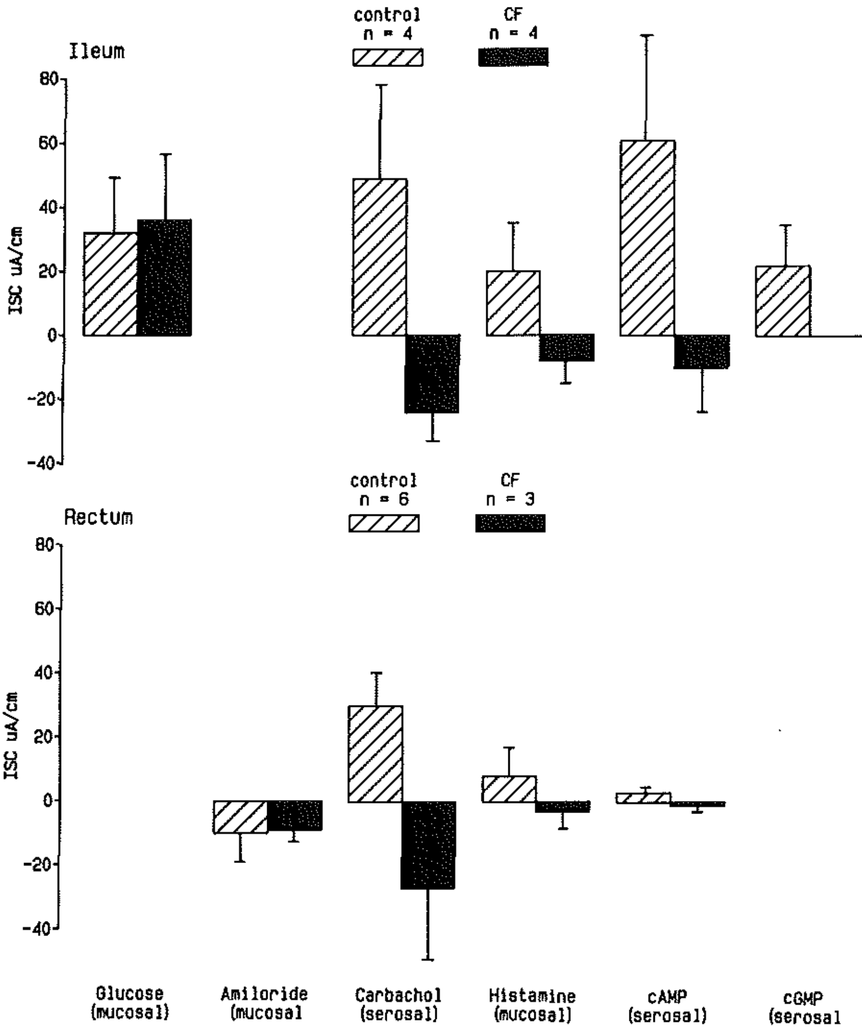

Figure 4. Short circuit current responses to specific secretagogues in ileal and rectal tissue from control and CF patients. A positive I_{SC} represents secretion of a negative charged ion, mainly Cl^-, whereas a negative I_{SC} represents secretion of a positive charged ion (mainly K^+). Rectum is insensitive to cGMP and is unable to absorb glucose, whereas ileum is devoid of amiloride-sensitive Na^+ channels.

epithelium remain unaltered in CF: first, the sodium-coupled absorption of glucose that is expressed in the mature villus cells of the small intestine but not in the colon and rectum and, second, the uptake of sodium through specific amiloride-sensitive sodium channels which is a specific function of surface epithelium in distal colon and rectum but is absent in ileum.

The latter finding is at variance with recent data on CF airway epithelium showing a 2-3 fold increase in Na^+ conductance of the mucosal membrane (4,15).

The molecular basis of the variable response of epithelial Na^+ channels to the CFTR mutation in different tissues remains to be established.

Whereas electrogenic pathways for electrolyte absorption appeared to function normally in CF, conductive pathways for ion secretion was drastically altered. The most dramatic change was the complete loss of a Cl^- secretory response to a variety of intestinal secretagogues including choleratoxin, forskolin, and 8-Bromo cAMP (acting through the intracellular messenger cAMP); carbachol and histamine (acting through Ca^{2+} signals and protein kinase-C); and heat-stable E.coli toxin and 8-Bromo cGMP (acting through cGMP). In analogy to earlier findings in other Cl^- secretory epithelias the failure of CF epithelium to secrete Cl^- is most plausibly owing to the dysfunctioning of the mucosal chloride channels at a point distal to the major activating signals, cAMP, cGMP and Ca^{2+}. It should be noticed, however, that in sweatgland coil and airway epithelium a channel response to Ca^{2+} appeared normal in CF (16). Recent evidence from electrophysiological studies suggest that different types of chloride channels, perhaps with a different sensitivity to the various activating signals and to the CFTR mutation, may co-exist in the same mucosal membrane (17). This finding may candidate the basis for differences in the presentation of the disease among the effected organs.

Another major change in intestinal epithelium of CF patients found in

our laboratory is a hypersecretion of cation, presumably K^+, in response to cAMP- and Ca^{2+}-linked (but not cGMP-linked) secretagogues.

Whether this phenomenon is a direct consequence of the CFTR mutation or reflects intestinal adaptation from a Cl^- to a K^+ secretory state is presently unknown. Nevertheless, the reversed direction of the short circuit current response resulting from electrogenic K^+ secretion that is characteristic for ileum and rectum of CF patients appeared a valuable criterium for the diagnosis of CF.

As indicated in Figure 5, the chloride impermeability in the small intestine and the rectum may explain most of the gastrointestinal symptoms in CF. A plausible consequence of the Cl^- secretory defect is dehydration of the mucus layer, thus creating the risk of luminal obstruction, as in the bronchial tree. The mucus layer is a barrier for macromolecules like micelles. The migration of micelles to the surface of the enterocyte is a crucial step in the absorption of fat and bilesalts. Bile salt absorption from micelles is decreased in CF, resulting in an increased fecal excretion. For the same reason fat absorption from micelles is also diminished.

This explains why, despite adequate substitution with pancreatic enzymes, fat malabsorption persist in most of the patients. In this respect we have to consider the crypt and villus as a gland-like structure that is obstructed by mucoid plugs in CF and is unable to absorb micellar nutrients. Moreover, the viscous mucoid products, when delivered to the intestinal lumen, may increase the risk for obstruction (meconium ileus, DIOS).

The frequency of the CF gene is astonishing high for a disease associated with a considerable mortality before the reproductive age range is reached. This raises the question of the heterozygote advantage.

As discussed above, the Cl^- secretory defect in CF intestine is likely to offer complete protection against secretory diarrhoea, including cholera (cAMP-mediated), and diarrhoea caused by enterotoxin - producing

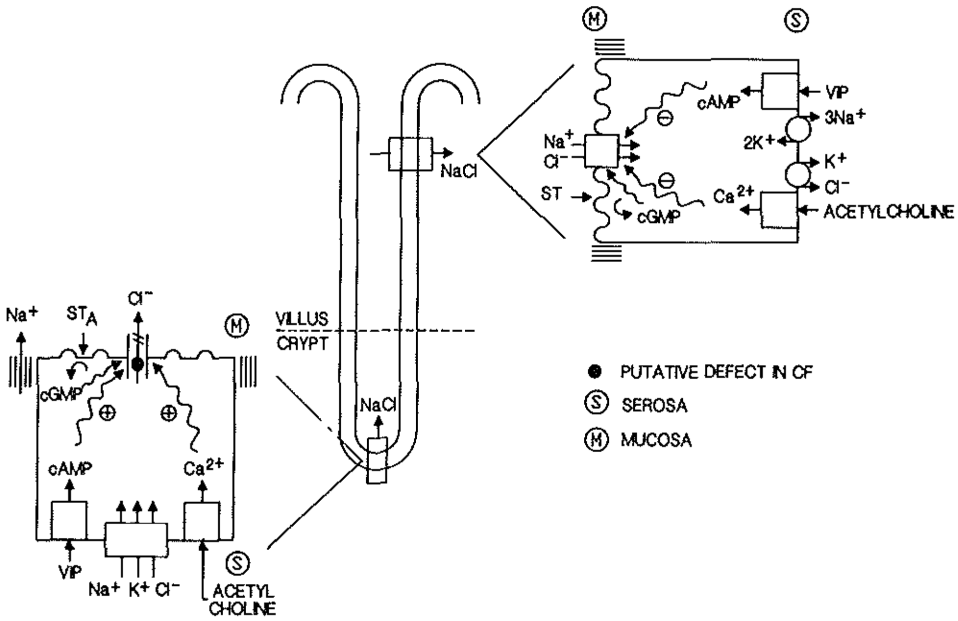

Figure 5. Schematic model of small-intestinal secretion. Cl^- excretion in the crypt cell occurs by chloride channels. In the villus cells Cl^- absorption occurs by sodium-chloride cotransport system. Both mechanisms are regulated by cAMP, cGMP and Ca^{2+}. In CF the crypt cells are unable to secrete Cl^-, whereas Cl^- (and Na^+) absorption in the villus is most likely unaffected. Whereas water transport is diminished in the crypt, its absorption is normal at the level of the villus which enhances the high viscosity of the mucus secreted by the goblet cells in crypt and villus. The consequence is an increased thickness of the mucus layer and an impaired transport of micellar nutrients and bilesalts.

strains of E.coli (cAMP- and cGMP-mediated). Provided that heterozygotes have an intermediate capacity for chloride secretion, the explanation of the high gene frequency could be the protection against life-threatening dehydration caused by microbial enterotoxin (18). However, a further analysis of intestinal Cl^- secretion in CF carriers is needed to support or reject this hypothesis.

The function of the CF-gene-encoded protein, CFTR, and its relation to Cl^- transport is not yet elucidated. As predicted by the aminoacid

sequence of the CFTR, the protein contain consensus sequences for a membrane-spanning domain (possibly acting as a Cl^- channel), a regulatory domain (carrying multiple phosphorylation sites for protein kinases), and two ATP-binding domains, one of which is carrying the major CFTR mutation (ΔF508). In principle, therefore, the CFTR could be the chloride channel itself (Figure 2A), but the other possibilities are that the CFTR is a regulatory protein associated with the chloride channel (Figure 2B) or that CFTR is a pump that extrudes a chloride channel inhibitor (Figure 2C). A defect of the CFTR would than result in an increased intracellular concentration of such a hypothetical inhibitor (Figure 2C). Discrimination between these models is only possible following isolation, expression, localization, and characterization of the CFTR by biochemical and molecular biological techniques.

Additional knowledge of CFTR expression and function, is a prerequisite for the rational design of novel physiologic or pharmacologic therapeutic approaches that are capable of compensating for or bypassing the CF defect.

Acknowledgements:

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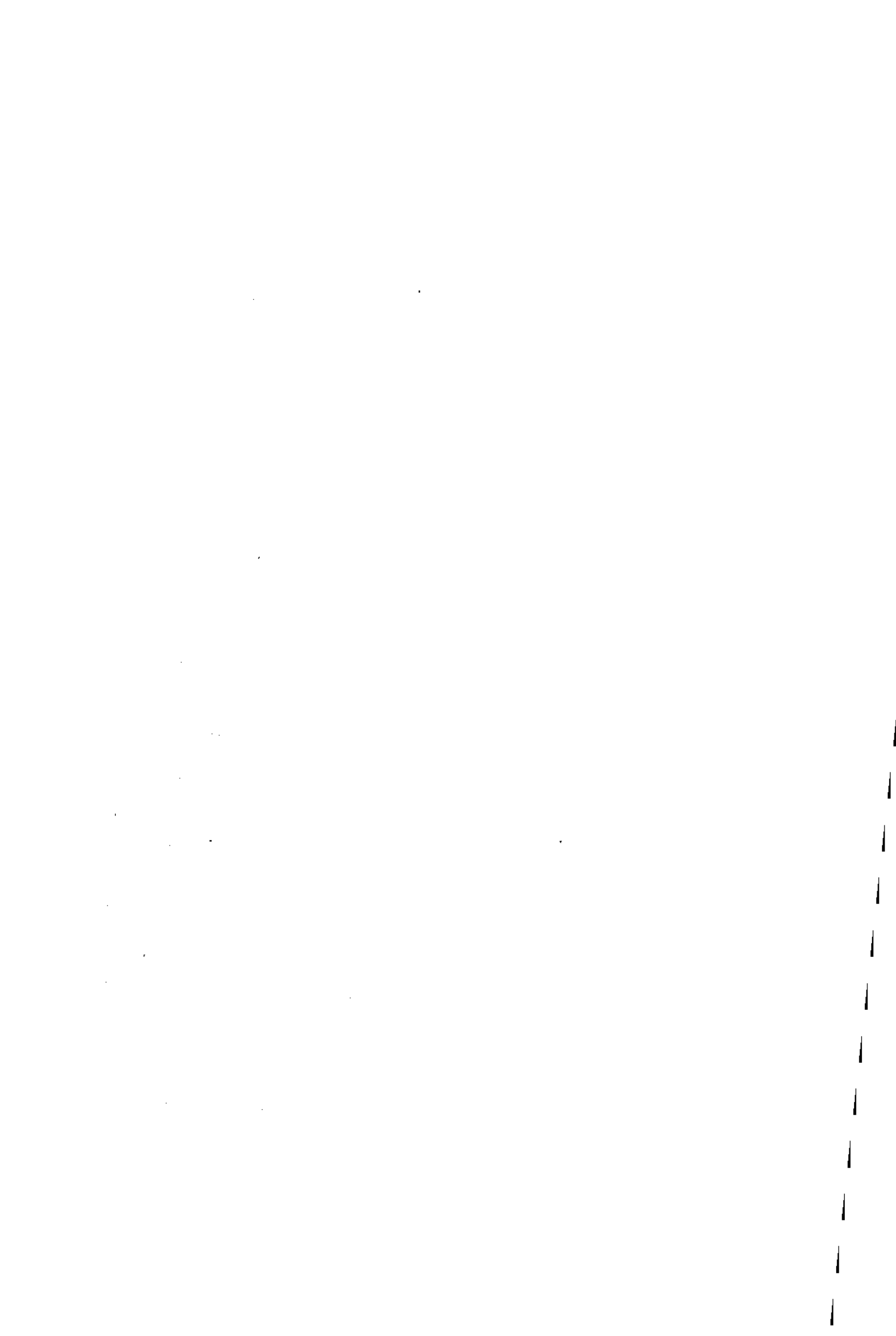

Chapter 3.2

Ion Transport Abnormalities in Rectal Suction Biopsies from Children with Cystic Fibrosis

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Summary

Abnormalities in transepithelial electrolyte transport in cystic fibrosis rectum were analyzed by short-circuit current measurements on 11 control and 11 subjects with cystic fibrosis in a modified Ussing chamber. As judged by the amiloride-sensitive component of the short-circuit current, electrogenic sodium absorption appeared unmodified in cystic fibrosis. In contrast, the short-circuit current response to specific stimuli of both cyclic adenosine monophosphate (cAMP)- and calcium-mediated chloride secretion was drastically altered in all of the cystic fibrosis biopsy specimens examined. Stimulation of the cAMP pathway by 8-Bromo cAMP or forskolin resulted in a sustained increase in short-circuit current in control tissues (+2.51 ± 0.63 $\mu A/cm^2$) but in a slight change in the opposite direction in cystic fibrosis (-0.56 ± 0.49 $\mu A/cm^2$; $P<0.05$). Carbachol, a calcium-linked secretagogue, provoked a transient increase in short-circuit current in all of the control tissues (peak response +26.69 ± 3.63 $\mu A/cm^2$) but in a transient change in the opposite direction in 6 of 11 cystic fibrosis tissues (-12.46 ± 4.64 $\mu A/cm^2$; $P<0.05$). In 2 of 11 patients with cystic fibrosis, however, a significant but subnormal and transient increase in short-circuit current was observed (+2.62 ± 0.04 $\mu A/cm^2$; $P<0.05$), whereas in 3 of 11 patients with cystic fibrosis a transient change in the opposite direction (-9.83 ± 2.20 $\mu A/cm^2$; $P<0.05$) was followed by a small and transient increase (+2.89 ± 0.83 $\mu A/cm^2$; $P<0.05$).

Using the calcium-mediated secretory response therefore, patients with cystic fibrosis could be divided into two categories: a major population showing defective anion secretion but active cation secretion and a subclass (including three siblings) showing residual but subnormal anion secretion. The easy accessibility of rectal samples and the inversed direction of the cAMP- or Ca-provoked short-circuit current is of

considerable advantage in the diagnosis of cystic fibrosis.

Introduction

Cystic fibrosis (CF) is a life-threatening, autosomal recessive disease predominantly occurring in white populations. A major gene defect (ΔF508) responsible for 68% of the gene mutations associated with this illness has recently been identified (1,2). The disease is characterized clinically by recurrent pulmonary infections associated with viscous airway secretions, malabsorption, and intestinal obstruction. Abnormalities in chloride and sodium transport across the apical membrane of epithelial tissues have been identified as the most plausible cellular basis of these symptoms.

In most CF tissues examined, the β-adrenergic (i.e., cyclic adenosine monophosphate (cAMP)-mediated) activation of chloride secretion is completely abolished (3-5). In contrast, the cholinergic (i.e., calcium-linked) activation is either normal (sweat gland coil) (6) or only mildly impaired (airway) (7). However, the intestine was a notable exception. Here, defective Cl^- secretion is defective not only in response to cAMP, but also in response to cyclic guanosine monophosphate (cGMP) and calcium-linked secretagogues (8-12). This conclusion is based on Ussing chamber experiments with resection preparations and a relatively small number of jejunal biopsy specimens (n=6). Therefore the result might be representative only for the category of patients carrying the major CFTR mutation.

However, rectal suction biopsy specimens are readily obtainable on an out-patient basis and allowed us to study Cl^- transport in a larger number of patients with CF, including a subclass in which anion secretion was impaired but not abolished. A further goal of this study was to verify if the inversion rather than the nullification of the cAMP- and Ca-provoked

short circuit current (I_{SC}), observed previously in ileal mucosa from meconium ileus patients with CF (8,12), was manifest also in the distal part of the intestine and in non-meconium ileus patients. This inversion of the I_{SC}, presumably indicating cation rather than anion secretion, has not been reported in similar studies from other laboratories (9-11). However, the direction of the theophylline-induced change in potential difference observed in rectal perfusion studies of patients with CF (13), supported the existence of cAMP-provoked cation secretion in this intestinal segment. Finally, we explored the possibility that the increased activity of amiloride sensitive Na^+ channels in CF airway epithelia (14) is manifest also at the level of the intestine. Because these channels are expressed preferentially in the distal part of the colon (15), rectal biopsies are excellently suited for this purpose. Earlier measurements of rectal potential differences *in vivo* (13,16-18) and a single Ussing chamber experiment on CF colonic tissue (18) led to conflicting results.

Methods

Patients

Eleven children with CF, mean age 11.0 years (range, 0.1-19.8 years), were studied. Two of them had a history of meconium ileus. In each patient the diagnosis was confirmed by at least two abnormal sweat tests. Medication consisted of pancreatic enzyme supplements and 8 patients had antibiotic treatment for pulmonary infections at the time of the experiment. The latter treatment did not show a correlation with the type of Ussing chamber response. The control group consisted of 11 constipated children, mean age 3.6 years (range, 0.6-14.5 years) who underwent a diagnostic rectal biopsy to exclude Hirschsprung's disease. None of these patients showed histological abnormalities or other internal disorders. Approval was obtained from the Hospital Medical Ethical

Committee and from patients or parents by written informed consent.

Experimental procedure

Fresh rectal tissues were obtained with a suction biopsy device (MEDICON, Tuttlingen, Germany). The biopsies were transported in ice-cold phosphate-buffered saline and mounted within 5 minutes in a modified Ussing chamber. The construction of the mounting plates and the mounting procedure are indicated in Figure 1.

The tissue is incubated at 37° C with Meyler-buffer solution (composition in mmol/L: Na^+, 126.2; Cl^-, 114.3; HCO_3^-, 20.2; HPO_4^{2-}, 0.3;

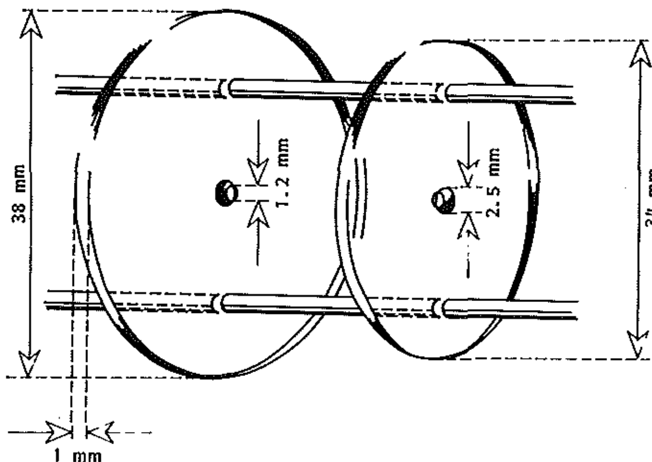

Figure 1. Design of the mounting device for rectal biopsy specimens used in the Ussing chamber studies. The specimen is spread on a plastic disk over the central opening (diameter, 1.2 mm; exposed area, 1.13 mm^2). Another disk is then brought into position by two guiding pins to prevent displacement of the tissue and to locate the central opening just opposite the other. Finally, the two disks are compressed by the two halves of the Ussing chamber. To prevent obstruction by mucus, the opening in the disk is conical, with the larger opening in contact with the Ussing chamber bath. The two disks and the sides of the Ussing chamber (not shown) have different diameters to prevent short circuiting of the two compartments by possible adherent fluid drops.

$H_2PO_4^-$, 0.4; Hepes, 10; pH, 7.4 when gassed with 95% O_2-5% CO_2). Potential difference (PD) across the tissue was measured through KCl-agar bridges which connected the bathing solutions to matched calomel electrodes (K401, RADIOMETER, Copenhagen, Denmark). Calomel electrodes were in turn connected to a voltage-clamp amplifier (QUALITRON, Amsterdam, the Netherlands). The leak noise of the equipment was less than 2 nA, far below the current elicited by the secretagogues. The I_{SC}, was applied to the tissue by platinum electrodes in the mucosal (M) and the serosal baths (S). The resistance was calculated from the change in PD while passing a current of 1 μA through the tissue and was corrected for the fluid resistance between the potential sensing electrodes. The response was defined as the maximal change(s) in I_{SC} provoked by the addition of specific secretagogues or inhibitors observed during continuous registration. Positive values for I_{SC} denote a negative electrical charge flux from serosa to mucosa.

After stabilization of the basal I_{SC}, the tissue resistance was determined. Thereafter glucose (10^{-2} mol/L, M and S), inhibitors and secretagogues were added. Additions were made in a standardized order, i.e. amiloride (10^{-4} mol/L, M), indomethacin (10^{-5} mol/L, M and S), carbachol (10^{-4} mol/L, S), 8-Bromo cAMP (10^{-3} mol/L, M and S). In later experiments forskolin (10^{-5} mol/L, S) was used instead of 8-Bromo cAMP. Thereafter, in two cases the Ca-mediated pathway was reactivated by histamine ($5x10^{-4}$ mol/L, S). All chemicals were obtained from Sigma, St. Louis, USA.

Statistical analysis was performed with the non-parametric Mann-Whitney test.

Results

The basal tissue resistance (Ohm.cm^2 ± SEM (n)) of rectal biopsy

specimens from patients with CF (26.7 ± 3.3 (11)), did not differ from that of controls (30.0 ± 4.9 (11)). A representative tracing of the I_{SC} measurements is shown in Figure 2. The basal I_{SC} (μA/cm² ± SEM (n)) in controls (17.70 ± 4.06 (11)) was different from CF (4.96 ± 2.43 (11), $P<0.05$).

The addition of glucose to the mucosal side of the tissue was unable to change the I_{SC}, indicating the absence of Na^+-coupled glucose transport in this section of the intestine.

Mucosal addition of the Na^+ channel inhibitor amiloride resulted in a drop in the basal I_{SC} that was not significantly different between controls and CF (Table 1).

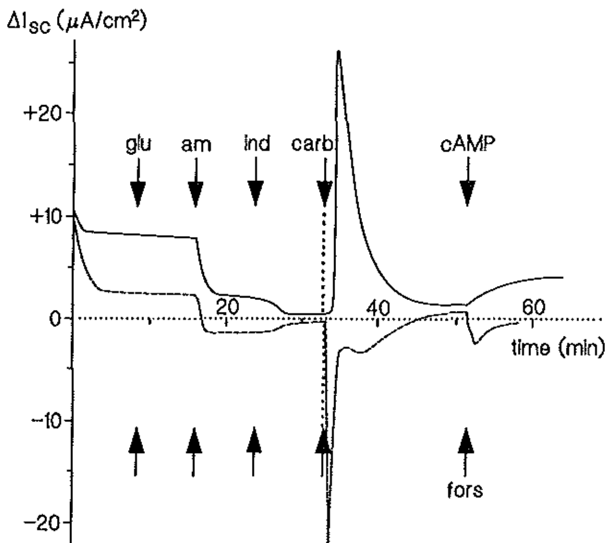

Figure 2. Representative registration of I_{SC} measurements in control (solid line) and CF (broken line) rectal epithelium. Additions, marked by arrows, are the same for both control and CF, unless otherwise indicated. Abbreviation used: glu, glucose (10^{-2} mol/L, M and S); am, amiloride (10^{-4} mol/L, M); ind, indomethacin (10^{-5} mol/L, M and S); carb, carbachol (10^{-4} mol/L, S); cAMP, 8-Bromo cAMP (10^{-3} mol/L, M and S); fors, forskolin (10^{-5} mol/L, S).

Indomethacin, a specific inhibitor of prostaglandin formation, caused a further reduction of the I_{SC} in both controls and patients with CF (Figure 2; Table 1). However, the direction of the response to indomethacin was inversed in CF, suggesting basal cation secretion instead of basal anion secretion.

Subsequent addition of carbachol, a calcium-linked secretagogue, provoked a transient increase in I_{SC} in controls, presumably indicating electrogenic Cl^- secretion (Figure 2 and 3A; Table 2). In CF, however, carbachol-induced secretion showed three patterns (Figure 3B-D): 6 of 11 CF patients displayed a transient change in I_{SC} in the opposite direction, possibly reflecting potassium (K^+) secretion (Figure 3B), 3 of 11 showed a "mixed" response, i.e., a transient increase in I_{SC} was preceded by a change in the opposite direction (Figure 3C), whereas 2 of 11 showed a transient increase in I_{SC} similar to controls but of much smaller magnitude (Figure 3D). A summary of these results is given in Table 2.

In controls, a sustained increase in I_{SC}, presumably indicating anion secretion, was induced by the addition of 8-bromo cAMP or by the adenylate cyclase activator forskolin (Figure 2; Table 1). A persistent change of the I_{SC} in the opposite direction was observed in the CF biopsy specimens. Responses to the cAMP-mediated secretagogues were small in comparison with carbachol responses. This difference in magnitude cannot be readily explained by a desensitization of the Cl^- channel activation mechanism caused by prior addition of carbachol because (a) responses of similar magnitude were observed when 8-bromo cAMP was added prior to carbachol (results not shown) and (b) addition of another Calcium-linked secretagogue, histamine, resulted in a considerable reactivation of the secretory pathway (Table 1).

In four additional experiments (two CF and two controls), stimuli of calcium- and cAMP-pathways failed to induce a change in I_{SC} when the Cl^- in the baths was replaced by gluconate (results not shown).

In a preliminary attempt to correlate the electrophysiological

Table 1. Changes in short-circuit current in response to several secretagogues and inhibitors added serosally and/or mucosally, in control and cystic fibrosis rectal epithelium.

Secretagogue/ inhibitor	Concentration site of addition	Change in I_{SC} ($\mu A/cm^2$)[a]	
		Controls	CF
Amiloride	10^{-4} mol/l;M	-7.14 ± 3.27 (10)	-7.03 ± 1.51 (9)
Indomethacin	10^{-5} mol/l;M,S	-2.34 ± 1.31 (10)	0.33 ± 0.17 (11)[b]
8-Bromo cAMP[c]	10^{-3} mol/l;M,S	2.51 ± 0.63 (10)	-0.56 ± 0.49 (9)[b]
Histamine	$5x10^{-4}$ mol/l;S	12.61 ± 3.71 (5)	-3.19 ± 1.95 (2)

[a]Mean ± SEM(n). [b]Statistically different from control (P<0.05). [c]or forskolin 10^{-5} mol/l;S.

classification of CF patients with clinical parameters, each group was monitored for the age at which CF was diagnosed and for the history of meconium ileus. Within the group of patients with CF displaying no evidence of residual chloride secretion (Figure 3B), the age at diagnosis (age ± SEM (n)) was 1.7 ± 0.8 (6) years. This age was 8.0 ± 2.6 (5) years (P=0.10) for those patients (including three siblings) who showed a transient increase in I_{SC} (Figure 3C-D). One patient in each group had a history of meconium ileus.

Discussion

In comparison with measurements of rectal potential differences and net electrolyte transport *in vivo*, I_{SC} measurements on rectal suction biopsy specimens offer several advantages. First, it permitted us to examine the effects of secretagogues acting through basolateral receptors (carbachol, histamine) while avoiding unwanted side effects on other tissues following mucosal uptake or i.v. application. Second, registrations are unaffected

by motor activity that lead to fluctuations in Cl^- secretion *in vivo* (19). Third, regional differences in ion transport failures can be accurately studied and the inconvenience of this procedure for the control- and CF-groups is minimal.

However, the small size of the biopsy specimens precludes the measurements of unidirectional tracer fluxes of $^{22}Na^+$ and $^{36}Cl^-$. Therefore, electroneutral transport components including coupled Na^+-Cl^- uptake, escape detection. Furthermore, identification of the transport system contributing to the I_{SC} must rely merely on ion substitution experiments and the use of specific transport inhibitors.

In the absence of exogenous secretagogues, the rectal mucosa was found able to maintain an amiloride-sensitive I_{SC} component that was not significantly different between control and CF biopsy specimens. This result is at variance with the 1.6-fold increase in amiloride-inhibitable rectal PD *in vivo* reported for a group of 5 patients with CF by Orlando et al. (18) but is in agreement with the *in vivo* data of Goldstein et al. (13) and Patton et al. (16), who likewise failed to observe a significant increase in amiloride-sensitive PD in CF rectal epithelia. These results taken together, indicate that the strong (three-fold) increase in apical Na^+-channel activity seen in CF airway epithelial cells (14) does not occur in rectal epithelium. The molecular basis of this tissue difference is presently unknown but may become clarified following the elucidation of the biological function of the CF gene-encoded protein, CFTR (1).

In accordance with Ussing chamber studies on human jejunal biopsy specimens (10) and resected intestine (8,9,12), the rectal biopsy specimens of control subjects showed a pronounced but transient increase in I_{SC} in response to cholinergic stimulation. This increase in I_{SC} was abolished in Cl^--free media and could be inhibited, at least in ileum, by serosal bumetanide and Ba^{2+} (specific inhibitors of the basolateral Na^+-K^+-$2Cl^-$ cotransport (20) and K^+ channels (21), respectively) (12). As judged by these criteria, it most probably represents electrogenic Cl^-

secretion provoked by the opening of apical Cl⁻ channels. The intracellular signals enabling communication between the basolateral muscarinic receptors and the apical Cl⁻ channels are still incompletely known. It is likely that both calcium and protein kinase-C play an essential role in this process (22-24).

A less transient and much smaller anion secretory response was observed with 8-Bromo cAMP or forskolin. Both secretagogues are known to trigger the opening of intestinal Cl⁻ channels through the activation of cAMP-dependent protein kinase and the phosphorylation of specific membrane proteins (22,23,25-27). The reason for the much smaller electrical response to cAMP observed in the rectal biopsy specimens compared to jejunal biopsy specimens or human ileal mucosa is presently unknown. Most likely it points to a lower content of cAMP-dependent protein kinase or a lower density of cAMP-sensitive Cl⁻ channels in the apical border of human rectal epithelial cells.

In agreement with earlier studies on jejunal biopsy specimens (10) and ileal mucosa (8,9,12) from CF patients, most CF rectal biopsy specimens examined in the present study showed a failure to secrete anions in response to both calcium- and cAMP-linked agonists. This finding strengthens the concept that the defect in CF is localized at a site shared by both types of agonists. This probably involves the Cl⁻ channel itself or a regulatory protein (CFTR ?) associated with the channel.

However, in two aspects the results of our study were different from earlier observations in CF intestine. First, in 5 of 11 patients with CF (including 3 siblings), a residual carbachol-induced anion secretion was apparent (Figure 3C-D). As suggested by the age at diagnosis, this subclass of patients with CF might also be characterized by a relatively late onset of the disease. Further research in a larger CF population is presently being carried out to establish whether the subclass is also characterized by a mild clinical expression of the disease and by a genotype different from the major CFTR mutation. Second, in 9 of 11

patients with CF, including one with a history of meconium ileus, the initial change in I_{SC} in response to cholinergic stimulation was in the opposite direction in comparison with controls (Figure 3B-C). This inversed response was observed also following exposure to cAMP agonists and has been noted earlier by de Jonge et al. (8,12) in ileal mucosa of CF patients with meconium ileus. As shown by the present study, however,

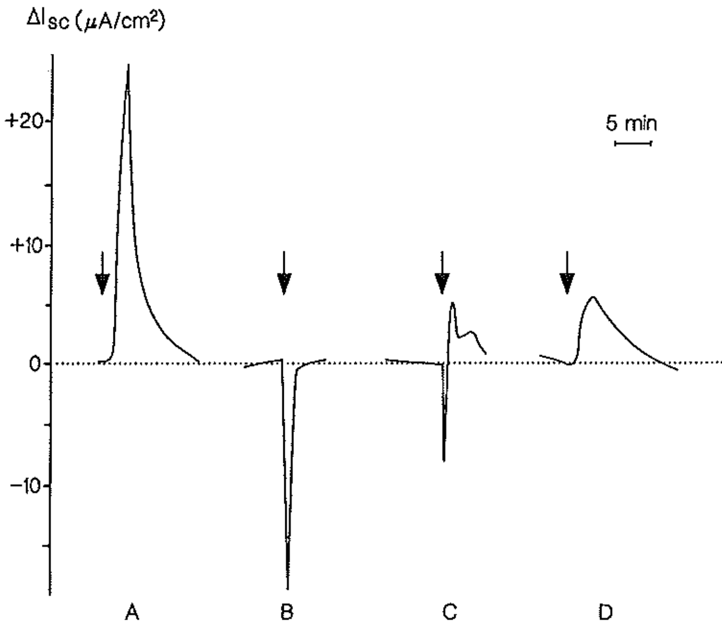

Figure 3. Variability in carbachol (10^{-4} mol/L, S) induced changes in I_{SC} in rectal epithelium in the presence of amiloride (10^{-4} mol/L, M) and indomethacin (10^{-5} mol/L, M and S). Additions are marked by arrows. The transient increase in I_{SC} in (C) and (D) most plausibly indicates residual chloride secretion. A. Controls (11) showing an increase in I_{SC} presumably reflecting chloride secretion. B. Six of 11 CF patients showing an I_{SC} response in the opposite direction, presumably reflecting potassium secretion. D. Two of 11 CF patients showing a greatly reduced increase in I_{SC}. C. Three of 11 CF patients showing a combination of (B) and (D).

Table 2. Changes in the short circuit current in response to serosal addition of carbachol (10^{-4}mol/L) in the presence of amiloride (10^{-4}mol/L) and indomethacin (10^{-5}mol/L), in control and cystic fibrosis rectal epithelium.

Subjects	n	Change in I_{SC} ($\mu A/cm^2$)[a]		Illustrated in Figure:
		Negative	Positive	
Controls	11		+26.69 ± 3.63	3A
CF	6	-12.46 ± 4.64[b]		3B
CF	3	-9.83 ± 2.20[b] →	+2.89 ± 0.83[b]	3C
CF	2		+2.62 ± 0.04[b]	3D

[a]Mean ± SEM. [b]Statistically different from control ($P<0.05$). CF patients with a transient increase in I_{SC} include 3 siblings.

this pattern of responses is not causally related to the history of meconium ileus. Because the response is rapid and transient, it can easily escape detection during discontinuous registration of the PD or I_{SC}. The failure of other groups to register this response (9-11) is unlikely to be explained by minor differences in buffer composition (e.g., bicarbonate content) or pH, because none of these factors appeared critical for observing this phenomenon (de Jonge, unpublished observations, January 1989). As the I_{SC} response in CF ileum was inhibitable for >70% by the mucosal addition of 5 mmol/L bariumchloride, it most likely reflects active secretion of K^+ through carbachol-activatable and cAMP-activatable and Ba^{2+}-inhibitable K^+ channels in the apical membrane (12 and de Jonge et al., in preparation). A cAMP- or Ca-provoked K^+ secretion in CF rectum is supported by the occurrence of a lumen-positive rectal PD response to theophylline described in patients with CF *in vivo* (13). Moreover, physiological evidence exists for a cAMP-stimulated K^+ secretion in rabbit and rat colon, but not in ileum (28). We conclude that the secretagogue induced secretion of cations is either unmasked or induced by the CF condition. Unfortunately, our attempts to verify the possible occurrence of a similar secretory pathway for K^+ in control

subjects were hampered by the well known action of Ba^{2+} as an intracellular activator of Cl^- secretion (29) and as an inhibitor of basolateral K^+ channels (21).

Nevertheless, the abnormal secretion patterns in CF rectal epithelium may be a valuable criterium to diagnose CF for those patients in whom the sweat test and DNA analysis is inconclusive.

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Chapter 4

Genotypes, Phenotypes and Intestinal Secretions

Implications for Diagnostic Approach

We found a correlation between mild clinical symptoms of CF and residual intestinal chloride secretion. Residual secretion can be explained by a mild class of CFTR mutations and the existance of an alternative chloride secretory pathway. Intestinal current measurements appeared to be highly reliable to diagnose CF.

Chapter 4.1

Determinants of Mild Clinical Symptoms in Cystic Fibrosis Patients: Residual Chloride Secretion Measured in Rectal Biopsies in Relation to the Genotype

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Summary

Previous Ussing chamber measurements of secretagogue provoked changes in short circuit current in rectal suction biopsies of cystic fibrosis (CF) patients showed that in a minority of patients chloride secretion in response to cholinergic agonists is reduced but not completely absent. To assess a possible relationship between this phenomenon and both the genotype and the phenotype we performed Ussing chamber experiments on rectal suction biopsies of 51 CF patients. The CFTR mutation was identified in 89 out of 102 CF alleles. No apparent chloride secretion was found in 30 CF patients (Group I). Low residual chloride secretion was found in 11 CF patients (Group II), while a relatively high residual secretion appeared in 10 CF patients (Group III). Pancreatic function was preserved more frequently in CF patients displaying residual secretion: 0% in group I, 27% in group II, and 60% in group III ($P<0.001$). The age at diagnosis (mean ± SEM) in group III (18.4 ± 6.6) was significantly different from group I (1.2 ± 0.4, $P<0.01$) and group II (3.5 ± 1.4, $P=0.05$). Residual chloride secretion was found in some of the 28 ΔF508 homozygous patients (3 in group II, and 1 in group III) disclosing that other factors than the CF gene defect itself, affect the transepithelial chloride transport. The age at diagnosis correlates significantly with the magnitude of the secretory response, even within the ΔF508 homozygous patients ($r=0.4$, $P<0.05$). We conclude that residual chloride secretion in cystic fibrosis is the pathophysiological basis of preserved pancreatic function and delayed presentation of the disease which is not exclusively determined by the CF genotype.

Introduction

In cystic fibrosis (CF) conductive chloride transport is defective in epithelial tissues. This results in viscous secretions associated with pulmonary infections, malabsorption, and intestinal obstruction (1-3). A major gene defect (ΔF508) represents ~ 70% of the gene mutations in CF (4,5). The product of the CF gene, the cystic fibrosis transmembrane conductance regulator (CFTR) functions itself as a chloride channel and may therefore be held responsible for the known defect in transepithelial chloride transport in CF (6,7). In most CF patients, including ΔF508, the mutation in the CF gene results in a mislocalization rather than dysfunctioning of the CFTR-chloride channel (8-13). In a smaller group of CF patients, however, the channel protein is properly inserted into the apical membrane of the epithelial cells but is functionally impaired or inactive (8,14).

The severity of the disease ranges widely. Homozygosity for the ΔF508 mutation was found to correlate with pancreatic insufficiency (15-18), early diagnosis (16-19), poor lung function (16,18), chronic Pseudomonas colonization (18), and high mortality (18). A relationship between clinical manifestations of the disease and the genotype implies that abnormalities in transepithelial electrolyte transport may vary with specific mutations. More than 230 different CFTR mutations have now been described (20).

In contrast to other epithelia of CF patients, intestinal tissues not only show defective chloride secretion in response to cAMP-linked secretagogues, but also in response to Ca-linked secretagogues, including cholinergic stimulation (21-23). As shown by electrophysiological studies in isolated colonic crypts (24) and in colonic epithelial cell lines (25,26), a major stimulating effect of carbachol is on the basolateral potassium conductance creating an increased driving

force for chloride exit through CFTR-chloride channels in the apical membrane (24,25). Furthermore, the protein kinase-C component of the carbachol response may activate the CFTR-chloride channel by direct phosphorylation (27-29). However, in contrast to their prominent role in other epithelial tissues in the intestine, the contribution of calcium activated chloride channels (different from CFTR) to the apical chloride secretion is negligible (26,30,31).

Recently, we demonstrated that in a minority of CF patients chloride secretion provoked by cholinergic agonists was reduced but not completely absent (32). This residual transepithelial chloride transport could help to explain the clinical heterogeneity among CF patients (32, 33). To assess a possible relationship between this phenomenon and both the genotype and the phenotype, we extended our Ussing chamber experiments on rectal suction biopsies to a larger group of CF patients and found a relationship between residual chloride secretion and both a milder phenotype and the CF genotype.

Methods

Patients

This study was approved by the Hospital Medical Ethical Committee and by patients or their parents by written informed consent. During the study period of two yr, 173 paediatric and adult CF patients (78 male, 95 female) with a mean age of 14.8 yr (range, 0.1 - 69.9 yr) who regularly visited our University Hospital were asked to have a rectal suction biopsy taken. Supplemented with 2 patients homozygous for the G542X mutation who came from another clinic, a total of 51 CF patients (25 male, 26 female) with a mean age of 16.5 yr (range, 0.1 - 69.9 yr) were enrolled in the study. Each patient's diagnosis was confirmed by at least two abnormal sweat tests (>70mmol sodium/L or >60 mmol chloride/L).

The medical records were evaluated for a history of meconium ileus, pancreatic insufficiency, Distal Intestinal Obstruction Syndrome (DIOS), chronic Pseudomonas colonization, and nasal polyp extraction. Pancreatic sufficiency was defined as normal fecal chymotrypsin (>6.3 U/g) and fat content values (<5 g/d) without pancreatic enzyme supplements. The date of onset of chronic Pseudomonas colonization was defined as the date of the first isolation of at least two consecutive sputum samples from which Pseudomonas could be cultured. Records of forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) were measured and expressed as a percentage of predicted normal values for age, height, and sex. The results of each patient's most recent pulmonary function tests were used. At the end of the 2 yr study period, each subject was assigned a clinical score with the use of the Shwachman and Kulczycki clinical scoring system as modified by Doershuk et al. (34). This is based on general activity, pulmonary physical findings, growth and nutrition, and X-ray findings resulting in a score from 20 (worst) to 100 (best). Furthermore, each patient's age at diagnosis and maximal derived (current age at the end of the 2-yr study period or age at death) were recorded. Four patients died during the study period.

Electrophysiological study

The procedure of short circuit current (I_{SC}) measurements on rectal tissue has been described earlier (32). Briefly, fresh rectal suction biopsies were mounted in an Ussing chamber with an exposed area of 1.13 mm^2. After stabilization of the basal I_{SC}, various substances were added to the mucosal (M) and/or serosal (S) baths: (*a*) glucose (10^{-2} mol/L) was added to M and S; (*b*) to reduce the contribution of electrogenic sodium absorption to the I_{SC}, specific sodium channels were blocked by amiloride (10^{-4} mol/L) added to M; (*c*) the endogenous prostaglandin synthesis that is possibly linked to cAMP-mediated chloride secretion was inhibited by adding indomethacin (10^{-5} mol/L) to M and S; (*d*) by adding carbachol

(10^{-4} mol/L) to S the cholinergic activation of chloride secretion was initiated. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

To investigate the reproducibility of the experiments without increasing discomfort for the participants, we analyzed two biopsy specimens simultaneously in a subgroup of 18 CF patients.

The mean carbachol-provoked change in I_{SC} values (± SEM) was 35.1 ± 2.6 μA/cm² in 47 healthy volunteers < 18 yr old, and 22.4 ± 4.3 μA/cm² in 8 subjects > 18 yr old. This difference tended to be statistically significant (P=0.07, Mann-Whitney; Pearson's correlation between age and I_{SC}: r=-0.23, P=0.10). The inward current provoked by carbachol in controls (I_{SC} positive; Figure 1) reflects transcellular chloride transport (serosa to mucosa) through the Na^+-K^+-$2Cl^-$ cotransporter in the basolateral membrane and the CFTR-chloride channel in the apical membrane (32). The observed magnitude of this response in controls may well be underestimated by underlying potassium secretion (32).

In the majority of cystic fibrosis patients, hereafter classified as group I, carbachol induces only an outward current response (I_{SC} negative; Figure 1). Most plausibly this reversed current results from apical potassium secretion which is unmasked in the case of absent or largely reduced chloride secretion (32,35,36). The carbachol-provoked I_{SC} changes in CF patients, and possibly also in controls, may therefore be the net result of two opposite currents. In a subclass of CF patients, usually following a negative peak response, a separate and small positive peak response (i.e., inward current) was observed (Figure 1, groups II and III). To facilitate comparison with the data from group I and controls, the net maximal change in I_{SC} was defined as the sum of the negative and positive peak responses (Figure 1, *neg+pos*). This calculated response was either negative (low residual secretion, Group II) or positive (high residual secretion, Group III).

DNA-analysis

We analyzed for the R117H, R347P, A455E, 621+1G→T, ΔI507, ΔF508, 1717-1G→A, V520F, G542X, S549N, S549I, G551D, R553X, R1162X, S1251N, W1282X, and N1303K mutations by PCR amplification of the appropriate exon sequences. Specific mutation detection involved the use of allele-specific oligonucleotides or amplification refractory mutation system (ARMS) (5,37,38). In addition, PCR-amplified exons 10 and 11

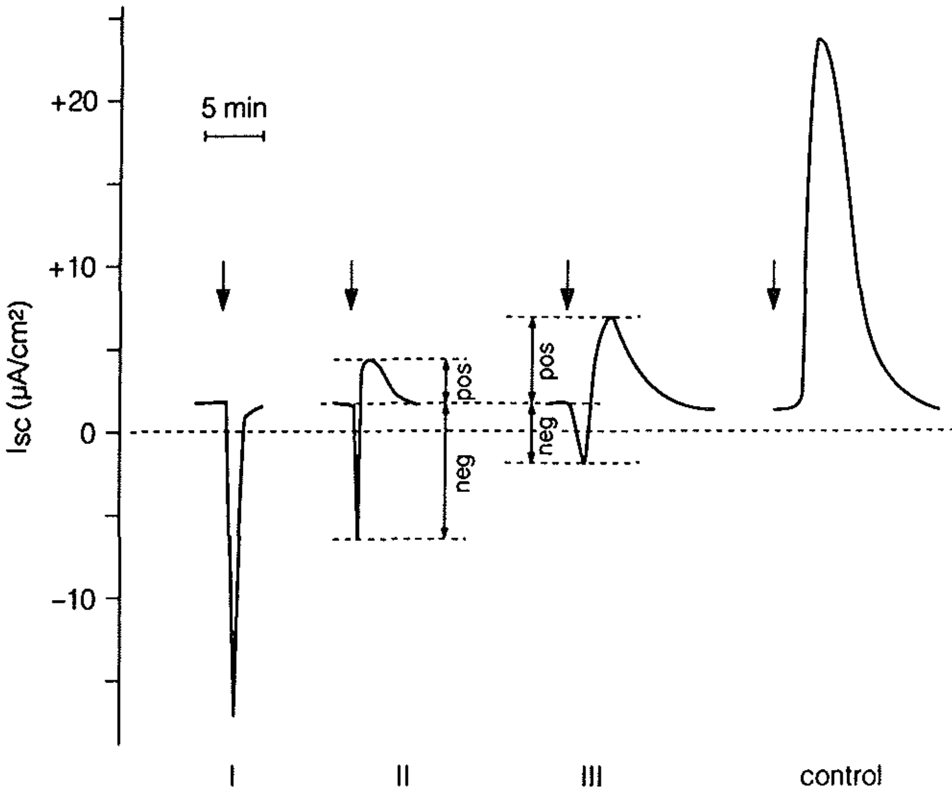

Figure 1. Different patterns of carbachol induced I_{SC} responses in rectal epithelium in the presence of amiloride and indomethacin. Additions are marked by arrows. For concentrations and site of additions, see Methods.

which are known to contain several mutations, were subjected to single strand conformation polymorphism as an aspecific screen for sequence alterations in these exons (39).

Statistics

Differences were considered significant if $P \leq 0.05$. The Pearson's correlation coefficient was calculated between short circuit current and age at diagnosis. Comparing the maximal derived age, a survival analysis was performed using the Log-Rank Test for testing significancy. All statistical tests were two-tailed.

Results

The reduction in basal I_{SC} in response to amiloride (mean ± SEM) in CF patients (-9.2 ± 1.6 $\mu A/cm^2$; n=51) was not different from age-matched control values (-8.7 ± 1.5 $\mu A/cm^2$; n=50). This outcome strengthens our previous conclusion that the strong increase in apical sodium channel activity seen in CF airway does not occur in rectal epithelium (32).

Three groups of CF patients could be distinguished on the basis of the electrophysiological response to carbachol. Group I consisted of 30 patients who failed to demonstrate residual intestinal chloride secretion. In group II, 11 patients showed low residual chloride secretion and group III consisted of 10 patients displaying high residual chloride secretion. From group I to III, the CF patients had a milder clinical presentation of the disease. This is demonstrated by the age at diagnosis which is significantly higher in CF patients with high residual secretion compared to group I and II (Table 1). The difference in the age at diagnosis between group I and II indicates that even CF patients with a low residual secretion are less affected by the disease (P=0.07, Mann-Whitney) than those without. The proportion of pancreatic insufficient CF patients

Table 1. Characteristics of cf patients with a different pattern of carbachol induced changes in short circuit current (I_{SC}) in rectal suction biopsies in the presence of amiloride and indomethacin.

	Intestinal chloride secretion		
Intestinal chloride secretion	I: No apparent residual secretion	II: Low residual secretion	III: high residual secretion
n CF patients	30	11	10
Carbachol induced secretion: net change in I_{SC} in $\mu A/cm^2$	-8.8 ± 2.1	-5.8 ± 1.5	5.2 ± 1.4[*]
GENOTYPE:			
1: ΔF508 / N1303K	2	0	0
2: ΔF508 / ΔF508	24	3	1
3: ΔF508 / G542X	1	1	0
4: G542X / G542X	0	2	0
5: ΔF508 / A455E	0	1	3
6: A455E / other	0	0	1
7: R553X / other	0	1	0
8: ΔF508 / other	3	3	5
Age at diagnosis (yr)	1.2 ± 0.4	3.5 ± 1.4	18.4 ± 6.6[‡]
Died in study period	2	2	0
Maximal derived age (yr)	14.3 ± 2.0	13.0 ± 2.4	26.9 ± 6.4[§]
Shwachman score	69.0 ± 4.2	74.1 ± 7.6	74.0 ± 7.0
Pulmonary function			
n CF patients	19	8	7
age (yr)	19.1 ± 1.7	15.4 ± 1.4	33.9 ± 6.8
FVC % predicted	68.1 ± 4.2	75.4 ± 10.3	81.8 ± 7.3
FEV1 % predicted	51.9 ± 5.4	63.9 ± 12.4	58.5 ± 10.7
	n (%)	n (%)	n (%)
Pancreatic insufficiency	30 (100)	8 (73)	4 (40)[ǀ]
Meconium ileus	10 (33)	2 (18)	1 (10)
DIOS	4 (14)	0 (0)	0 (0)
Pseudomonas colonization	21 (72)	7 (64)	4 (40)
Nasal polyp extraction	9 (31)	1 (9)	3 (30)

Values are expressed as mean ± SEM unless otherwise indicated. Differences of group III from group I or II: [*]I, II: $P<0.001$; [‡]I: $P<0.01$; II: $P=0.05$, Mann-Whitney; [§]I: $P<0.05$, II: $P=0.06$, Log-Rank Test; [ǀ]$P<0.001$, Fisher's Exact Test.

decreases significantly from group I to III (Table 1). Additionally, the mean carbachol-provoked I_{SC} response in the 9 pancreatic sufficient patients (3 of group II and 6 of group III) was significantly higher (2.6 ± 2.5 μA/cm²) than in the other 42 pancreatic insufficient patients (-7.1 ± 1.6 μA/cm²; $P<0.01$, Mann-Whitney). The proportion of CF patients with other intestinal symptoms (meconium ileus and DIOS) was also lower in group III, but the differences were not significant (Table 1). The prevalences of Pseudomonas colonization, and nasal polyp extraction are summarized in Table 1. Comparing the lung functions and Shwachman scores (Table 1), as well as the previously mentioned prevalence of pancreatic status, DIOS, Pseudomonas colonization, and nasal polyp extraction, we have to take into account that the time for CF patients of group III to develop more advanced disease was longer. This is demonstrated by their average maximal derived age which was significantly higher in group III than in the other groups (Table 1).

The distribution of genotypes among the three groups defined on the bases of electrophysiological response is indicated in Table 1. Of all genotypes the ΔF508 homozygous patients are most frequent (55%). Most, but not all, of them lack a positive I_{SC} component in their response (Table 1). CF patients carrying one A455E mutation are predominantly found in group III. Considering the variability in I_{SC} responses, even within the ΔF508 homozygous patient group, we also asked ourselves whether or not a relationship existed between the magnitude of the secretory response and the phenotypic expression of the disease. A positive correlation ($r=0.37$, $P<0.01$) was found between age at diagnosis and the net I_{SC} response (Figure 2). This correlation was also significant ($r=0.40$, $P<0.05$) for the ΔF508 homozygous patients.

Two CF patients combined ΔF508 with G542X; one of these showed low residual secretory activity. The two CF patients homozygous for the G542X mutation both displayed low residual secretion (Table 1). All CF patients with one A455E allele showed residual activity (Table 1).

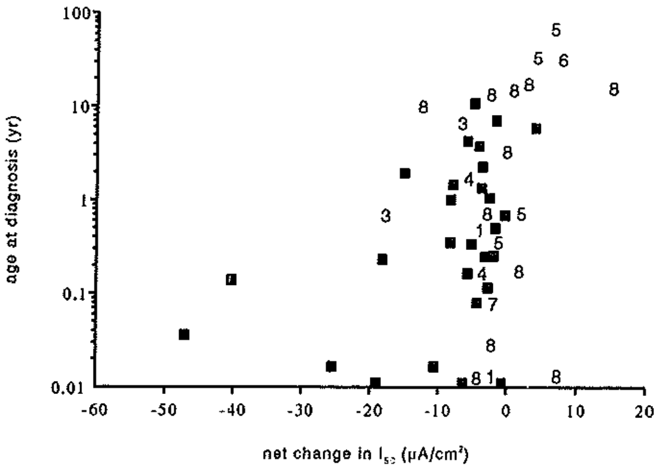

Figure 2. Correlation between age at diagnosis and short circuit current measurements on rectal suction biopsies of CF patients (r=0.37, P<0.01). The correlation was also found in the ΔF508 homozygous patients (■) (r=0.40, P<0.05). The other genotypes, indicated by numbers, are explained in Table 1. Positive net short circuit current changes implicate net residual chloride secretion while a negative response indicates predominant K^+ secretion perhaps blunted by underlying residual Cl^- secretion.

Compared to all other genotypes in this study, these CF patients are diagnosed at a significantly older age, have a higher I_{SC} response, have a higher maximal derived age, and are less frequently pancreatic insufficient (Table 2).

In 18 CF patients duplicate rectal suction biopsies were studied. When every first result (X-axis) equals the results of the duplicate registration (Y-axis) than a line through zero (β=intercept=0) with a tangent=1 can be drawn. In the equation Result1 = α Result2 + β, the β was -1.5 (not different from 0) and the α was 0.7 (not different from 1).

Discussion

The changes in I_{SC} provoked by carbachol represent the net result of all electrical charge fluxes through both apical and basolateral membranes. A positive change in I_{SC} induced by carbachol in a control biopsy specimen is an indicator for net electrogenic chloride secretion. However, in most CF patients, responses to carbachol are characterized by a change in I_{SC} in the opposite direction (32). Apparently, this inversed response reflects potassium secretion through apical potassium channels, most likely unmasked by the chloride channel defect associated with the CF condition (32). In both normal and CF tissue the magnitude of the chloride and potassium current, respectively, may well be underestimated when apical potassium channels are also functioning in normal tissue (36) and if apical chloride channels retain a residual activity in CF cells. However, the relative magnitude of chloride and potassium currents for each biopsy is difficult to estimate. First, the small size of the biopsy as well as the transient character of the changes in I_{SC} provoked by carbachol prevent accurate assessment of unidirectional ^{36}Cl or ^{86}Rb flux rates. Also, specific inhibitors of apical chloride channels (i.e. CFTR) or apical potassium channels are not yet available (31). The positive I_{SC} responses remain representative for residual chloride secretion while negative I_{SC} responses indicate predominant potassium secretion, perhaps blunted by underlying residual chloride secretion.

Considering the recent finding that most mutant CFTR is functionally active but is inappropriately targeted to the plasma membrane (8-13), one possible reason for a variable level of residual chloride secretion, in particular among patients of identical genotype, may be a variable degree of mislocation of the CFTR protein in the enterocytes. Alternatively, the variability may arise from a variable expression level of other chloride channel species in the apical membrane, e.g., calcium activated chloride

Table 2. Characteristics of CF patients with one A455E mutation vs. all other tested mutants.

	Genotype	
	1,2,3,4,7,8 (see Table 1)	5 (ΔF508 / A455E) 6 (A455E / other)
n patients	46	5
Carbachol induced secretion:		
net change in I_{SC} in μA/cm²	-6.4 ± 1.6	3.8 ± 1.7*
Age at diagnosis (yr)	2.7 ± 0.7	26.3 ± 12.4‡
Died in study period	4	0
Maximal derived age (yr)	14.5 ± 1.5	35.0 ± 11.4§
Shwachman score	67.0 ± 12.5	71.5 ± 3.4
Pulmonary function		
n CF patients	30	4
age (yr)	18.4 ± 1.2	42.9 ± 9.8
FVC % predicted	72.7 ± 4.1	72.5 ± 8.0
FEV1 % predicted	57.9 ± 5.1	42.1 ± 7.4
	n %	n %
Pancreatic insufficiency	41 (89)	1 (20)ǁ
Meconium ileus	13 (28)	0 (0)
DIOS	4 (9)	0 (0)
Pseudomonas colonization	29 (64)	3 (60)
Nasal polyp extraction	11 (24)	2 (40)

Values are expressed as mean ± SEM unless otherwise indicated. *$P<0.01$, ‡$P<0.05$, Mann-Whitney; §$P<0.001$, Log-Rank Test; ǁ$P<0.01$, Fisher's Exact Test.

channels, which normally do not contribute much to the carbachol-provoked changes in I_{SC} in intestine (26,30,31). Furthermore, several missense mutations located in the transmembrane domain of CFTR are characterized by an incomplete loss of chloride channel function and may also lead to residual chloride secretion (14).

In Table 1, we demonstrate a relationship between residual chloride secretion and clinical parameters. The positive I_{SC} responses observed in

group III CF patients are not the result of the more advanced mean age in this group, since control values normally decline with increasing age (see Methods). The high residual secretion found in these CF patients might very well be the pathophysiological basis of their milder clinical presentation, as indicated by the age at diagnosis and the preserved pancreatic function. The data from Table 1 additionally indicate that, in comparison with group I and II, and in contrast of what could be expected from their higher current age, CF patients of group III also reach a higher Shwachman score and have a more preserved lung function. The preserved pulmonary function in group III suggests that residual chloride secretion may also occur in airway epithelium. Some of the CF patients (Figure 2) have a calculated response approaching age-matched control values. However, the appearance of their responses was abnormal, due to the (preceding) transient deflection of the I_{SC} that was never observed in controls.

A more continuous relationship between the I_{SC} and the age at diagnosis is illustrated in Figure 2. Even within the group of ΔF508 homozygous patients, a positive correlation between the net I_{SC} response and the age at diagnosis was found. As discussed above, the variability in phenotypic expression of the disease within patients of identical CF genotype can be explained by interindividual differences in the extent of missorting of mutant CFTR or in the expression of other (compensatory) chloride channels. It has been suggested before that genetic factors other than the CF gene may modify the expression of the disease (17). Prediction of the clinical prognosis in individual CF patients may thus not rely on the CF genotype alone.

The previously described genotype-phenotype relationship (16-19) implies that the (residual) function of CFTR may vary with different mutations. In *Xenopus oocytes* it was demonstrated that the chloride conductance depends on the CF genotype (9). In this study we demonstrate that most, but not all, ΔF508 homozygous patients are found

in group I and that CF patients with one A455E mutation can be found predominantly in group III. The latter mutation presumably affects the sorting and/or activity of CFTR to a lesser extent, thus allowing for residual secretion and milder clinical symptoms (Table 2). The A455E mutation was also characterized by others as a mild mutation associated with preserved pancreatic function (40). The two ΔF508/N1303K patients both had pancreatic insufficiency and failed to show apparent residual chloride secretion. This is in agreement with others who characterized the N1303K mutation as a severe one (41).

Interestingly, two patients homozygous for the nonsense mutation G542X had a low residual secretion (in duplicate). Since in other stopcodon mutants no CFTR mRNA was detectable (42), the residual chloride secretion in the two homozygous G542X patients most likely results from compensatory non CF-affected chloride channel species (26) in the apical membrane, rather than from the presence of some functional CFTR.

We conclude that the residual chloride secretion in cystic fibrosis is the pathophysiological basis of preserved pancreatic function and delayed presentation of the disease. Compared to ΔF508, the A455E mutation can be characterized as a mild mutation allowing for residual chloride secretion. The occurrence of residual chloride secretion in the intestine is not exclusively determined by the CF genotype.

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Chapter 4.2

Residual Chloride Transport in Cystic Fibrosis Patients Homozygous for the G542X Mutation is not Related to CFTR Activity

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submitted

Summary

Recently, we have shown that residual carbachol-provoked short circuit current response in rectal epithelium in a minority of CF patients correlates with relatively mild disease and was in part independent of the CF genotype. To investigate this phenomenon, possibly explained by a contribution of non-CFTR chloride channels, mutants without active CFTR, are of particular interest. In this report we show that two patients homozygous for the G542X stopcodon mutation display carbachol-provoked residual chloride secretion in rectal biopsies. We have investigated the possibility that suppression of the G542X stopcodon or activity of the truncated CFTR protein is responsible for the residual chloride transport activity. In cultured cells infected with a Vaccinia virus vector encoding either G542X or R553X CFTR suppression of the UGA stopcodons was not observed. Although the G542X Vaccinia virus infected cells produce large amounts of truncated CFTR, no chloride efflux activity or specific patch clamp signal was observed here. These data, imply that G542X homozygotes can be regarded as true CFTR null mutants. Therefore it is highly unlikely that CFTR activity is responsible for residual intestinal chloride transport observed in G542X homozygotes.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease usually characterized by recurrent pulmonary infections, pancreatic insufficiency, male infertility and elevated sweat chloride levels (1,2). The CF gene product is the cystic fibrosis transmembrane conductance regulator (CFTR) which functions as a chloride channel (3). Mutations within the CF gene prevent a normal transepithelial CFTR-mediated chloride transport.

Different CFTR mutations have been identified in the patient population (1, 2), some of which lead to an incomplete loss of chloride channel function *in vitro* (4,5).

Residual CFTR activity is thought to be an important factor in the large clinical variability of the disease (5). However, if patients of identical CF genotype are compared we still observe large variation in clinical status and development of the disease (6,7,8). Though this could be ascribed to environmental factors, the involvement of genetic determinants, other than CFTR, seems likely. Identification of such determinants might add significantly to the predictive value of diagnosis and to our understanding of the disease.

Recently, we have presented evidence for a correlation between the carbachol-provoked short circuit current response in rectal epithelium and the phenotypic expression of the disease (8). A small carbachol-induced inward current found in a minority of CF patients points to residual electrogenic chloride transport in intestinal tissue and is associated with late diagnosis and pancreatic sufficiency. The residual chloride secretion was not exclusively determined by the CF genotype (8). Residual intestinal chloride secretion may be caused by residual CFTR activity or by a compensatory anion permeability.

CF patients without active CFTR i.e. null mutants, are of particular interest in this context. Patients homozygous for stopcodon mutations have been described, and attracted attention because both mild and severe cases were reported (9-17). First, stopcodons are potentially susceptible to suppression (18,19). Secondly, a truncated CFTR protein can be partially active (20), as has been shown recently in the case of a D836X mutant form (21). In this report we show a low residual chloride transport in colonic epithelium of two patients homozygous for G542X. This prompted us to investigate whether G542X and R553X mutants are true null mutants. We have tested UGA stopcodon suppression and chloride channel activity in cells expressing G542X CFTR after infection

with a Vaccinia virus expression vector. In addition we have studied suppression of the R553X UGA stopcodon mutation which is also observed in CF patients.

Materials and methods

Patients

The study was approved by the Hospital Medical Ethical Committee and by patients or their parents. Patient A was diagnosed at the age of 1.6 yr with recurrent pulmonary infections and pancreatic insufficiency. At the age of 2.6 yr he was in a moderately fair condition, colonized with Pseudomonas and had a Shwachman score of 80/100. Patient B was diagnosed at the age of 1.6 yr with dystrophy and steatorrhoea which improved dramatically by pancreatic supplementary therapy. Intermittent cough and sputum production was present since the age of 5 yr. First clinical admission was at the age of 11.5 yr for a pulmonary infection with Pseudomonas (11). She was studied at the age of 15.6 yr while her pulmonary function tests were excellent (FEV1 and FVC above 100% of predicted) and the Shwachman score was 90/100.

Ussing chamber experiments

Fresh rectal suction biopsies were mounted in an Ussing chamber as described earlier (8,22). After stabilization of the basal short circuit current (I_{SC}), various substances were added to the mucosal (M) and/or serosal (S) baths: glucose (10^{-2} mol/L) was added to M and S; to reduce the contribution of electrogenic sodium absorption to the I_{SC}, specific sodium channels were blocked by amiloride (10^{-4} mol/L) added to M; the endogenous prostaglandin synthesis that is possibly linked to cAMP-mediated chloride secretion was inhibited by adding indomethacin (10^{-5} mol/L) to M and S; by adding carbachol (10^{-4} mol/L) to S the

cholinergic activation of chloride secretion was initiated. A major stimulating effect of carbachol in intestinal epithelium is on the basolateral potassium conductance creating an increased driving force for the chloride current (23). In addition, protein kinase-C may activate the CFTR chloride channel by direct phosphorylation (24). All chemicals were obtained from Sigma, St. Louis, USA.

Cell culture

HepG2, CHANG, CHO, LLC-PK1 (25), JEG-3 (26), and IEC-6 (27) cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with Fetal calfs serum and antibiotics.

Vaccinia virus expression vectors

CFTR expression by a T7 based Vaccinia virus co-infection system has been described (28). VVTG5991 Vaccinia virus contains the CFTR coding sequence with the G542X stop codon mutation, its expression is under control of a T7 promoter. VVTG5996 is a similar construct with the R553X stop mutation, VVTG5959 expresses normal human CFTR. Cells were co-infected with VVTG1193 (expressing the T7 RNA polymerase) and CFTR containing vectors at a multiplicity of 5 pfu/cell for each virus. Infected cells were harvested 16 to 22 hours later.

Gel electrophoretic analysis

Western blotting and immune-precipitation from 35S labeled cells were performed as described (28) with monoclonal antibodies MATG1031 (Transgene, directed against peptide 107-117) or MATG1061 (Transgene, peptide 503-515). Antigen detection on blots was performed by chemiluminescence on X-ray film (Amersham). Samples for Westernblots were an equivalent of 10^5 cells, for immune precipitation 10^6 cells.

Selenium incorporation

For ^{75}Se incorporation, 10^6 PK1 or JEG3 cells were cultured in medium with 10% fetal calf serum. One hour after infection with Vaccinia virus vectors, $Na_2{}^{75}SeO_3$ (800 Ci/g Se) was added to a final concentration of 50 nmol/L. After overnight incubation, 10^6 cells were harvested in 0.75 ml NaCl 100 mmol/L, Tris-Cl 50 mmol/L, Phenyl-methyl-sulfonyl-chloride 0.5 mmol/L, Leupeptin, Triton X114 0.5 % w/w, pH 7.4 at room temperature. After centrifugation to remove nuclei and cell debris the supernatant was collected and incubated on ice for five minutes to precipitate the Triton X114 and extract membrane proteins. Under these conditions about 90% of total full length CFTR could be extracted from expressing cells (data not shown). This method allowed us to load CFTR from an equivalent of 10^6 cells (about 1 microgram) to a single Western blot slot. ^{75}Se-CFTR should contain about 10^4 dpm/microgram. After gelelectrophoresis, proteins were transferred to nylon membrane. ^{75}Se was detected by autoradiography, CFTR antigen was detected using an antibody against peptide 693-716 (CC24, (29)). Experiments with human primary nasal epithelial cells were under identical conditions except for the culture medium (27).

Ion efflux experiments

Chloride efflux rates in HepG2 cells were measured as described in (30). Briefly, cells were loaded with ^{36}Cl and then washed. At one minute intervals the incubation medium was replaced and the amount of ^{36}Cl in the collected samples was measured by scintillation counting. The experiment was terminated by collecting and counting the cells. The efflux rates were calculated as percentage release of chloride content at each point of time.

Patch clamp analysis

Patch clamp experiments were performed essentially as described by

Hamill et al (31). Patch pipettes from borosilicate glass (Clark GC150-TF) were 3-8 MOhm. Data sampling and analysis were as described by Kansen et al (32). Pipette (external) and bath (internal) solutions contained 140.10^{-3} mol/L NMDG (N-Methyl-D-Glucamine), 10^{-3} mol/L EGTA, 3.10^{-3} mol/L $MgCl_2$ and 10^{-2} mol/L Hepes-HCl (pH 7.3, final Cl^- concentration 147.10^{-3} mol/L). Pipet potential refers to the voltage applied to the pipet interior with respect to the (grounded) bath. Positive (upward) current represents negative charge flowing out of the pipet. These conditions are optimal for detection of anion channels as no permeating cations are present in the off cell mode. All HepG2, and about half of PK-1 (35/55 CFTR-G542X infected, 20/45 control infected) Vaccinia virus infected cells were transferred to a 29° C stove 1-6 hours prior to the patch clamp experiments. This was done in order to bypass possible processing defects of the truncated product, as seen with the ΔF508 mutation (33). Patch clamp experiments were performed at 21-24° C. Cells were treated (unless otherwise indicated) with 10^{-5} mol/L forskolin (raising the intracellular cAMP concentration). Patch clamp experiments were performed blind. All GOhm seals were monitored in both cell attached and excised mode, five minutes each. A clamp voltage step protocol (-70/+70 mV) was used routinely to obtain I/V characteristics. Channels active in excised mode were also measured at one third of the bath chloride concentration to check ion selectivity. Linear, 8 pS chloride channels, which were inactivated in excised mode were classified as CFTR.

Results

Carbachol response of colonic biopsies

Two patients homozygous for the G542X stopcodon mutation were studied (see Methods section). Patient B is very mildly affected by lung

disease, however, the clinical symptoms of both patients are within the range observed in ΔF508 homozygous patients. The short circuit current measurements in colonic biopsies of both patients A and B showed that carbachol first induces a negative current response (two biopsies each, A: -4.6 and 0.0 μA/cm²; B: -6.2 and -1.4 μA/cm²). This initial current, reversed compared to healthy individuals, results most likely from apical potassium secretion which is unmasked in the case of absent or largely reduced chloride secretion (8). In both G542X patients, this response is immediately followed by a positive current (A: 0.9 and 2.8 μA/cm²; B: 0.4 and 2.1 μA/cm²) pointing at residual chloride secretion (Figure 1) (8). One possible interpretation of these observations would be residual CFTR activity due to either suppression of the UGA stopcodon or activity of the truncated protein. Alternatively, a chloride permeability unrelated to CFTR

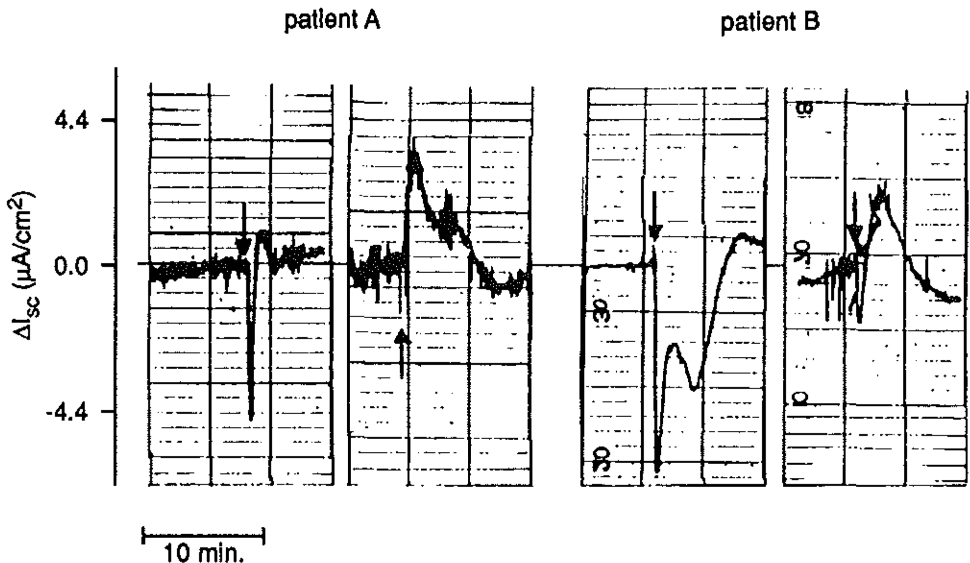


Figure 1. Carbachol induced I_{SC} responses in rectal epithelium. The carbachol addition is marked by the arrow. The duplicate registrations of both G542X homozygous patients are shown.

could be involved.

Suppression of stopcodons

To obtain high and reliable expression of G542X and normal CFTR, we used recombinant Vaccinia virus vectors (28). Cultured cells infected with a vector encoding normal full length CFTR produced large quantities of CFTR antigen as shown by both Western blotting and immune precipitation (Figure 2, lanes labeled CF). The CFTR antigen is partially processed to the mature form (Figure 2, upper arrow) as is seen in Western-blotting of Chang cells (Figure 2a) and pulse chase labelling of HepG2 cells (Figure 2c). The G542X and the R553X CFTR mutant vectors each produced a truncated CFTR antigen of the predicted length (Figure 2, lanes 542 and 553). In none of the experiments full length CFTR could be detected with the mutant vectors, even after prolonged exposure of the autoradiograms. It follows that the level of suppression would be less than 0.1%.

Suppression of UGA stopcodons in epithelial cells occurs during translation of some selenoprotein mRNA's when the UGA termination signal is recognised a seleno-cysteine transfer RNA. In this way selenium is incorporated into the protein (18,19). The efficiency of the process depends on the presence of a stem loop structure in the mRNA (34). It is conceivable that suppression of the UGA stopcodon in CFTR G542X occurs in a similar way. Computer analysis of CFTR mRNA folding indicated that the required AAA()TGAT()AAAA motif did in fact occur downstream of G542X and could form a stem-loop structure (data not shown). Therefore we tested the incorporation of ^{75}Se in CFTR antigen in cells expressing G542X CFTR mRNA. LLC-PKG1' and JEG3 cells, which are cell lines of epithelial origin both used in studies of selenium incorporation via the mechanism described (25,26), were infected with different Vaccinia virus vectors in the presence of ^{75}Se. Membrane proteins were extracted with Triton X114 and transferred to blotting

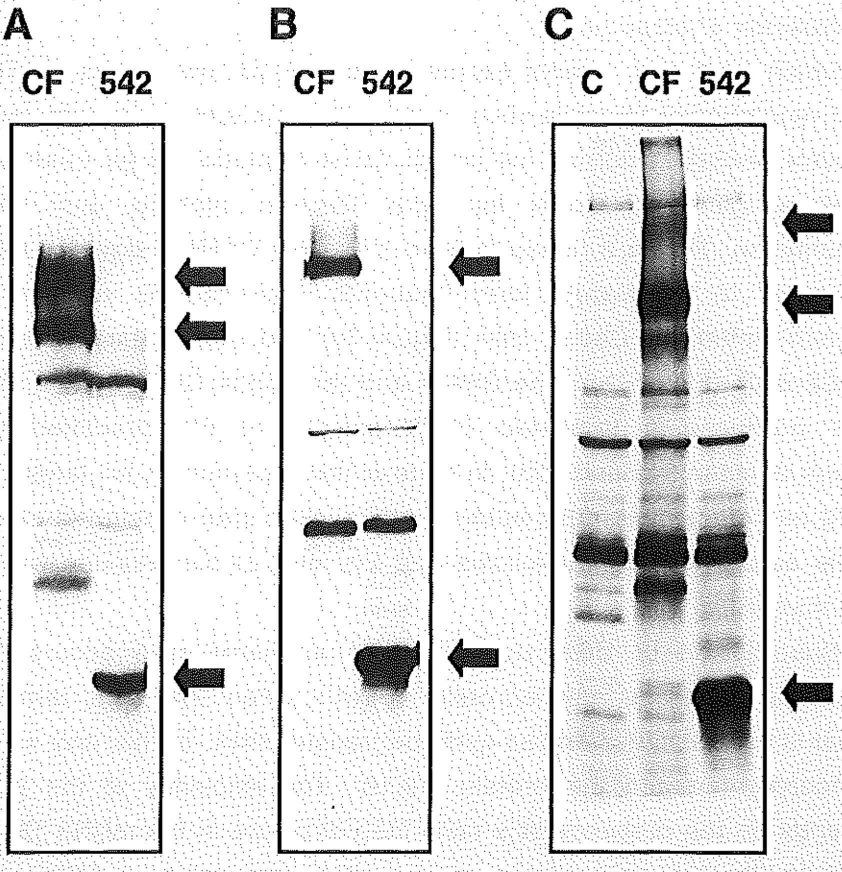


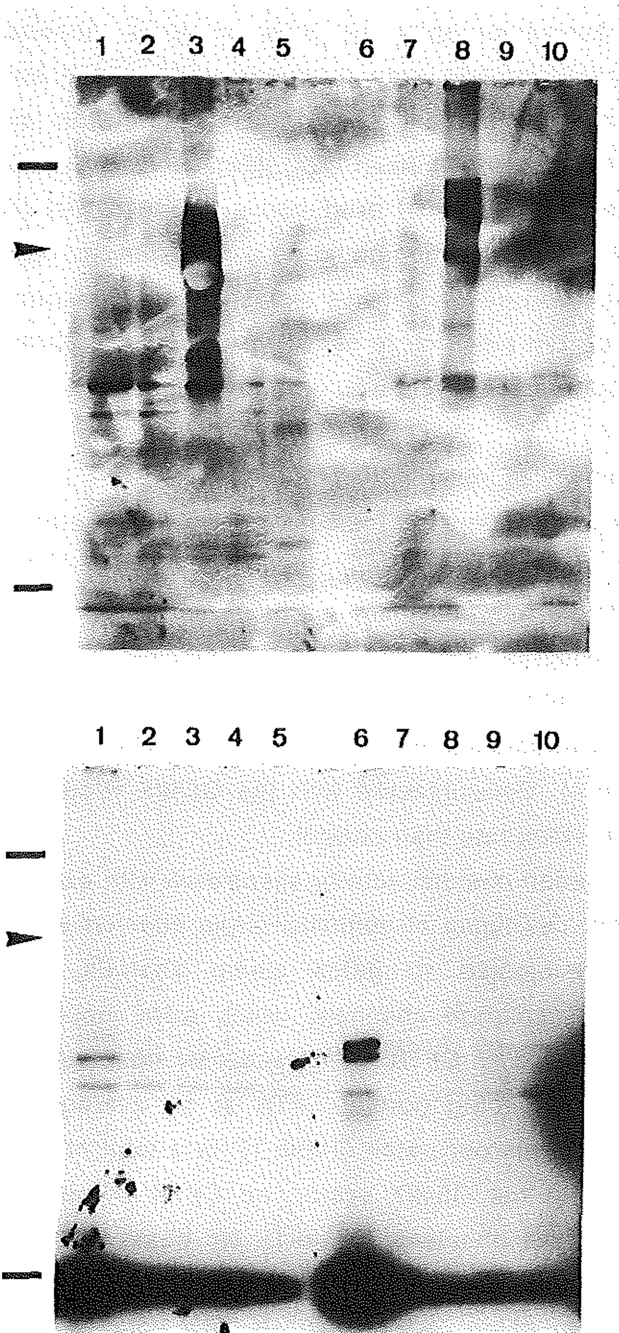
Figure 2. Expression of CFTR antigen in Vaccinia virus infected cells.
Cells were cultured and infected with two Vaccinia virus vectors, one expressing T7 polymerase and the other expressing either normal or a mutant form of CFTR lanes CF: normal CFTR vector (VVTG5959), lanes 542: G542X mutant form (VVTG5991), lanes 553: R553X mutant form (VVTG5996), lanes C: T7 polymerase vector only (VVTG1193) A CHANG cells, western blot with a monoclonal anti-CFTR antibody MATG1031 B CHANG cells Radio-immune precipitation (MATG1031) C HepG2 Cells, Radio-immune precipitation (MATG1031), Pulse chase labeling Upper arrow: glycosylated CFTR (apparent mw: 180 kD), middle arrow: unglycosylated form (150 kD), lower arrow: truncated forms of CFTR (G542X: 55kD, R553X: 57 kD).

membranes. Autoradiography of total samples showed that large quantities of selenoprotein were produced under these conditions (data not shown). When the blots were incubated with an antibody which detects only full length CFTR (CC24, (29)) only cells exposed to the vector encoding normal CFTR produced a strong signal (Figure 3a). No specific selenium incorporation could be detected in the region of 150-200 kD (Figure 3b), even after extensive exposure in a phospho-imager which was found to be ten times more sensitive to ^{75}Se than X-ray film. Again it follows that suppression levels would be less than 0.1 %.

Ion channel activity of mutant CFTR

An alternative explanation for residual activity in CF stopcodon mutants could be that the truncated protein is active as a chloride channel (20, 21). To investigate this possibility we have tested chloride efflux rates in HepG2 cells infected with recombinant Vaccinia virus vectors. Cells infected with a vector producing full length normal CFTR responded to forskolin with a substantial increase in chloride efflux rate, in contrast to control (Figure 4). Cells infected with the G542X vector did not have a higher chloride efflux rate than control.

To identify anion channel activity associated with expression of CFTR-G542X, different cell types of epithelial origin infected with Vaccinia virus vectors were subjected to patch clamp analysis. Linear, forskolin dependent 8 pS chloride channels, which were inactivated in excised mode were classified as CFTR. CFTR was only observed in cells infected with the vector encoding normal CFTR at an average frequency of 8 independent channels per patch (Table 1). In a total of 104 patches from cells infected with the G542X vector, no CFTR or other specific anion channel activity was observed (Table 1). No baseline current shifts were observed after reduction of bath chloride concentration ruling out significant hidden anion channel activity. We conclude that it is highly unlikely that the truncated product of the G542X vector, which is found in


1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

large quantities in infected cells (Figure 2), contributes significantly to chloride conductance.

Discussion

Data presented in a previous paper show that residual colonic carbachol-induced chloride transport observed in some patients correlates with mild disease (8). This effect can be explained by residual CFTR activity (4,5). In fact it could be argued that, since most patients produce a form of CFTR which is potentially active (28,35), all cases of mild disease might be due to residual CFTR activity. Homozygous stopcodon patients are of special interest in this respect. If they really do not produce any functional CFTR than detectable chloride secretion points to an alternative chloride channel. We showed that two patients homozygous for the G542X stopcodon mutation, have a small residual intestinal chloride transport activity. Although both homozygous G542X patients were diagnosed at young age and the residual responses are small, patient B is mildly affected by the disease as is demonstrated by her Shwachman score of 90 and het normal pulmonary function. We tried to establish whether or not the G542X mutated CFTR gene or R553X, another stopcodon mutation, is capable to produce any functional CFTR. To investigate the

Figure 3. Selenium incorporation in Vaccinia virus infected cells.
SDS poly acrylamide gels of Triton X114 extracts from infected JEG (lanes 1-5) and PK1 (lanes 6-10) cells infected with Vaccinia virus vectors in the presence of ^{75}Se lanes 1 and 6: no infection, lanes 2 and 7: VVTG1163 (T7), Lanes 3 and 8: VVTG5959 (CFTR)+VVTG1163, lanes 4 and 9: VVTG5991 (G542X)+ VVTG1163, lanes 5 and 10: VVTG5996 (R553X)+ VVTG1163. *A:* Western blot with anti-CFTR antibody (CC24,(19)) recognizing only full length CFTR.
B: Autoradiogram of the western blot. The arrow indicates the approximate position of CFTR antigen Top bar: top of the gel, lower bar: electrophoresis front.

Table 1. Patch clamp analysis of Vaccinia virus infected cells.

Cell type Vector	Number of channels in (n) experiments				
	Seals (n)	CFTR	3-5pS	8-10pS	no act
HepG2					
T7	19	0	9	10	4
T7+G542X	21	0	12	8	1
T7+CFTR	10	80	-	-	0
PK1					
T7	45	0	17	10	23
T7+G542X	55	0	15	13	27
T7+G542X*	10	0	3	4	3
IEC-6					
T7	33	0	9	9	15
T7+G542X	28	0	4	11	13

Cells were infected with Vaccinia virus vectors encoding T7 RNA polymerase (VVTG1163) plus or minus a vector encoding either CFTR-G542X (VVTG5991) or normal CFTR (VVTG5959) under control of a T7 promoter. After overnight incubation patch clamp analysis was performed as described, in the presence of 10^{-5} M forskolin. Experiments marked * were without forskolin Seals (N): total number of completed experiments in each category. All cell types examined here contained 3-5 pS anion channels and 8 pS non-selective channels in about 25% of the patches which were independent of forskolin, remained active in excised mode, and were not correlated with CFTR G542X expression. No other anion channel activity was observed.

possibility of functional suppression we have constructed and tested Vaccinia virus expression vectors which encode a form of CFTR with either a G542X or a R553X mutation. In all experiments, truncated CFTR proteins were produced in large amounts as shown by western blotting and immune precipitation (Figure 2). No full length CFTR antigen was detected with these methods. Incorporation of ^{75}Se in full length CFTR antigen, as would be predicted in the case of stopcodon suppression by seleno-cysteinyl tRNA (26,34) was not observed (Figure 3). CFTR-G542X expression was not associated with distinct anion channel activity or

chloride permeability (Figure 4, Table 1). In summary, production of full length CFTR from a mRNA carrying either a G542X or a R553X stopcodon was not found. The truncated form of CFTR produced from G542X mRNA does not have detectable anion channel activity. This, combined with the observation that stopcodon mutations tend to severely reduce mRNA levels (15), implies that G542X stopcodon mutants are true CFTR null mutants. Consequently, CFTR activity can not be responsible for residual chloride permeability in G542X homozygotes.

We propose that residual chloride secretion in the homozygous G542X patients described here is due to a parallel anion transporter. A

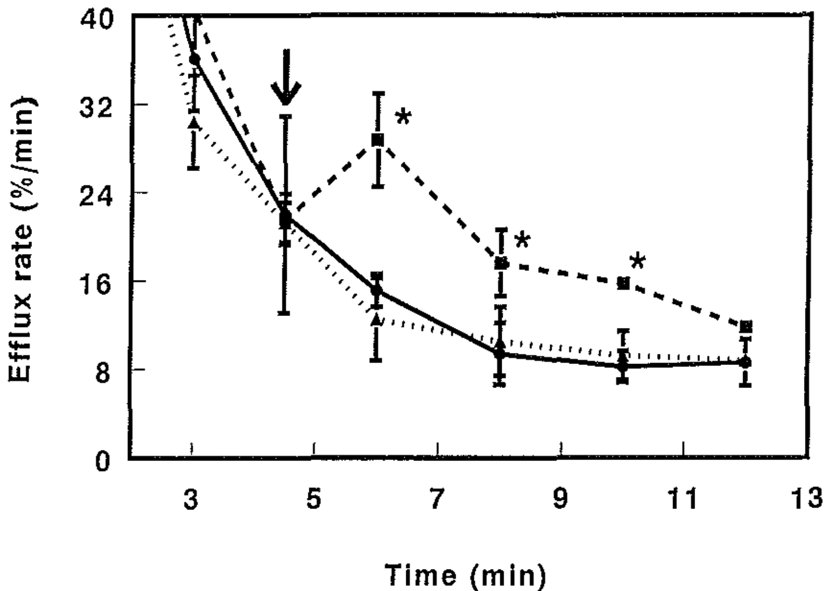


Figure 4. Chloride efflux from HepG2 cells infected with Vaccinia virus. $^{36}Cl^-$ Efflux rates (percent release per minute) from Vaccinia virus infected HepG2 cells were measured as described (31). Cells were infected with VVTG1163 (T7 polymerase) alone: O--O; VVTG1163 plus VVTG5959 (CFTR) ■_■; VVTG1163 plus VVTG5991 (G542X) ▲--▲.

possible candidate would be the calcium dependent chloride conductance found in airway cells (36). Though this conductance seems to be absent in the apical membrane of intestinal epithelium (22), it is conceivable that some individuals carry an allele which increased intestinal expression of the gene. An unusual high expression of calcium sensitive chloride channels in intestine, and possibly the airways, could contribute to milder disease. In support of this hypothesis it has been demonstrated recently, that in CFTR (-/-) knock out mice the severity of organ involvement is determined by the presence of calcium-regulated non-CFTR mediated chloride conductance (37). The fact that only in a minority of ΔF508 homozygotes a positive component in the colonic carbachol response was found (8), is in agreement with the presence of an unlinked genetic factor associated with this phenomenon. After the calcium-regulated chloride channel has been cloned, systematic genetic and electrochemical analysis of patients homozygous for stopcodon mutations may allow to test our hypothesis. The assay for colonic short circuit current that we have developed will be useful in such an analysis.

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Chapter 4.3

Diagnosis of Cystic Fibrosis

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Summary

Applying the sweattest as the first test of choice when suspecting the diagnosis of cystic fibrosis is still common practice. Since the cloning of the CFTR gene more than 400 different cystic fibrosis (CF) mutations have been identified. Using CFTR mutation analysis for diagnostic purposes in CF so far remains therefore be elusive. It is advised to perform sweattests as previously described by Gibson and Cooke.

In this study we re-evaluated the results of sweattests performed in our hospital of 1905 subjects over a period of 9 years (1983-1992). In 1825 subjects where CF was not diagnosed, mean sodium value was 15.5 ± 9.2 mmol/L. The upper limit of the normal range (2SD above the mean) was 34 mmol/L. Re-examination of all 239 sweat sodium values (80.9 ± 19.5 mmol/L) in 80 newly diagnosed CF patients (all: $Na^{+}>70$ mmol/L) revealed that 5% of the values were below 50 mmol/L, the lowest sweat value obtained being 27 mmol/L.

Based on these results, we recommend in case of clinical suspicion of CF and sweat sodium values above 30 mmol/L to repeat the sweattest and also to include chloride measurements for optimal discrimination.

Introduction

Classically, the diagnosis of cystic fibrosis (CF) is based on the high salt content in sweat due to a defect in reabsorbing chloride of the sweat duct (1). The cystic fibrosis transmembrane conductance regulator may be held responsible for the defective chloride transport (2). Since the cloning of the CFTR gene (3), more than 400 different cystic fibrosis (CF) mutations have been identified. Using CFTR mutation analysis for diagnostic purposes in CF so far remains therefore be elusive. In our

Centre (112 paediatric and 57 adult genotyped patients) no CFTR mutation was identified in 2.4% of patients while in 21.9% only one mutation could be found. Of all chromosomes tested, the mutations found are given in Table 1.

Clinical Presentation of Cystic Fibrosis

Applying the sweattest as a diagnostic procedure for CF originates from clinical suspicion of the disease. It is thus crucial to have knowledge of the presenting symptoms of CF. In our hospital (118 paediatric CF patients) 68.6% were diagnosed within the first year of life. Twenty-six (22%) presented with meconium ileus while in all cases of meconium ileus studied in the last years, 42 out of 54 (77.8%) appeared to have CF.

Table 1.

CFTR mutation found in CF Center Rotterdam (118 paediatric and 68 adult patients)	% of 338 chromosomes tested
ΔF508	73.3
A455E	4.1
1717 1G->A	2.7
G542X	2.1
N1303K	0.9
R553X	0.9
L927P	0.9
dL1260	0.6
R1162X	0.3
1078delT	0.3
R75Q	0.3

Although the presence of meconium ileus is very suspicious for CF, it is not specific for this disease (4). In 21.2% of the patients the mode of presentation was related with other intestinal manifestations as steatorrhoea or failure to growth. In 18.6% CF was suspected by respiratory symptoms (chronic sinusitis, nasal polyps, chronic cough, recurrent lower airway infections, suspect sputum cultures with Staphylococcus aureus and Pseudomonas Aeruginosa, or abnormal X-ray findings suspect for bronchiectasis). Furthermore respiratory as well as intestinal symptoms were present in 16.1% of patients. Finally, 8.5% of patients were detected due to positive family history.

The sweattest

For reliable sweattesting it is advised to use the method described by Gibson and Cooke (5). The skin area should be carefully washed with distilled water and Pilocarpine iontophoresis applied for 5 (max. 10) minutes. Use filter paper, cup or capillary tube for collection during 1 hour. Determination should be performed of both chloride and sodium. It is advised that the amount of sweat produced is more than 100 mg. In our hospital (now >400 tests yearly) false results due to a small production were not found when at least 30 mg of sweat was produced. However, we still advise to await a test result with more than 50 mg of sweat. It is important to perform the diagnostic sweattest beyond the age of six weeks, since early tests may give a false positive result. This is due to the physiological low sodium reabsorption at young age. Here, a false positive result may be suggested from the higher sodium content of the sweat compared to chloride.

To be conclusive, a positive test result (chloride above 60 mmol/L or sodium above 70 mmol/L) should be obtained on two separate occasions. Sweat chloride levels provide a better discrimination between normal

persons and CF patients than sodium levels (6-8). Using the above mentioned criteria for the CF diagnosis, sweattest results of some CF patients may be within the 'normal' range (9,10). In the following study attempt was made to unravel the question of what really can be defined as the normal result of the sweattest.

We re-examined all sweattest results in the period from January 1983 until May 1992 (Figure 1). Chloride measurements were not routinely performed. From each of the 1905 subjects the result of the first adequate performed sweattest was used. These data were stratified for the known 80 CF patients diagnosed in this period. In 1825 subjects in whom CF was not diagnosed, the mean value of sodium was 15.5 ± 9.2 mmol/L. The upper limit of the normal range (2SD above the mean) was 34 mmol/L. All 80 CF patients fulfilled our previous criterium of a positive sweattest result (Na^+ >70 mmol/L). To investigate the variability of the sweattest from the 80 CF patients all their 239 sweattest results were examined (Na^+ 80.9 ± 19.5 mmol/L). Five percent of the values were below 50 mmol/L and the lowest sweat value obtained was 27 mmol/L. This demonstrates that the diagnosis CF can not be excluded when sweat sodium is around 30 mmol/L.

Studying Intestinal Current Measurements (ICM) in rectal biopsies of CF patients (11), the magnitude of the chloride current (largely determined by the CF genotype) was found to correlate with the clinical phenotype (12). Using the same technique for diagnostic purposes (13) revealed that it is likely that CF can be diagnosed with ICM in those patients whose sweat sodium values are just above 30 mmol/L. Until now ICM proves to be highly sensitive and specific since chloride channel and sodium channel activity can be studied separately. The sweattest in itself is not specific for CF since low sodium reabsorption (as found in several endocrinopathies) may also cause an abnormal result. Elevated sweat tests (even with chloride in excess of sodium) may also be found in known CF carriers. Diagnostic evaluation of these carriers may be

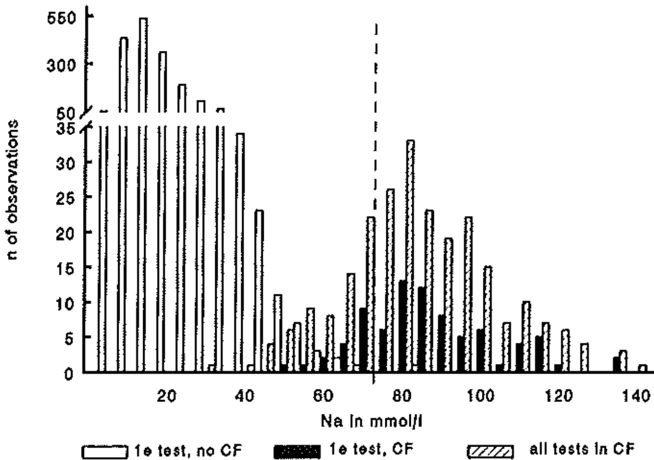

Figure 1. All sweat sodium values of the <u>first</u> (adequate performed) sweattest in a period from January 1983 until May 1992. In 1825 subject (open bars) where the CF diagnosis was not made, the mean sweattest result was 15.5 ± 9.2 mmol/L. From these subjects 144 had a first sweat sodium of more than 30 mmol/L in which the CF diagnosis may well be reconsidered. The mean of the first sweattest result of 80 diagnosed CF patients (filled bars) was 83.7 ± 17.2 mmol/L. All CF patients fulfilled our previously used criterium of a positive sweattest (>70 mmol/L; vertical line). In addition, the variation of all 239 sweat sodium values in the 80 CF patients was considerable (stippled bars). Five percent were below 50 mmol/L, and the lowest sweat value obtained was 27 mmol/L.

troublesome as they may have one detectable CFTR mutation as do most 'borderline' CF patients. Since sweat values tend to rise with age, difficulties in the interpretation of the test can also be found in adults. It is our experience that ICM is especially of value in these situations.

Another fact pointing in the direction that the incidence of CF may be higher than we presently presume, derives from males with Congenital Bilateral Absence of Vas Deference (CBAVD). This is likely to be a genital form of CF although some of these males may also suffer from other CF

manifestations (14,15). In males with CBAVD approximately 12% carry a R117H mutation (15). This is suspected to be a CFTR mutation, occuring very rarely in CF patients. Results: Table 1 suggests that not all CF patients are recognized: The number of found ΔF508 chromosomes in CF patients (73.3%) is less than expected (77.3%) from the number of ΔF508 homozygous patients (59.8%).

In conclusion, the lower limit of a borderline sweattest should be changed from 50 to 30 mmol/L. Repeated, well performed sweattests and in selected cases additional tests (i.e. CFTR mutation analysis, ICM, measurements of nasal potential differences, detection of CFTR anti-bodies) may be of help for the diagnosis of CF.

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Chapter 4.4

The Diagnosis of Cystic Fibrosis: Intestinal Current Measurements, a Highly Accurate Method in Case of a Borderline Phenotype

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Submitted

Summary

In establishing the diagnosis of cystic fibrosis (CF), the sweat test is presently considered the golden standard. Individuals with CF-related symptoms and disease-causing mutations on both alleles of the CFTR gene may, however, have a negative sweat test result. The sweat test measures the results of both sodium and chloride transport processes, and those results are thus not specific for the defective CFTR mediated chloride transport. In control studies, Intestinal Current Measurements (ICM), measuring chloride and sodium channel activity separately, always gave correct results in 55 controls, 22 CF carriers, and 60 known CF patients.

In the present study we applied ICM as a diagnostic tool for CF in 63 cases. In 12 individuals referred to the clinic with CF related symptoms but normal sweat tests, we found abnormal ICM compared with control values of non-CF individuals and CF carriers. CFTR mutation analysis in this group (n=12) revealed: 3 ΔF508/ΔF508, 4 ΔF508/A455E, 4 ΔF508/unknown, and 1 G542X/unknown. In 17 individuals with borderline or positive sweat test results (3 ΔF508/unknown, 13 unknown/unknown, and 1 ΔF508/R117H-7T) the results of ICM appeared to be normal. In conclusion, we found ICM an accurate diagnostic tool for CF, especially in individuals in whom CF is suspected, but sweat test and CF mutation analysis give inconclusive results.

Introduction

Cystic fibrosis (CF) is a recessive inherited disorder, affecting predominantly Caucasian populations, with an estimated incidence of 1 in 2500 births (1). Mutations on both alleles of the CFTR gene (2,3) are

responsible for the defective CFTR mediated chloride transport in epithelial tissues (4). The resulting viscous secretions, which lead to obstruction and progressive fibrosis of various epithelium lined organs, reduce the life expectancy of CF patients. Early diagnostic tests may be prompted by a family history of CF. However, in most cases the diagnosis of CF is considered on the basis of typical clinical manifestations. These include meconium ileus, failure to thrive, fatty stools or other symptoms related to pancreatic insufficiency (PI), prolonged cholestatic jaundice, and recurrent airway infections with suspected micro-organisms such as Staphylococcus aureus or Pseudomonas species (1). Clinical suspicion may also arise from more discrete signs, e.g. like nasal polyps (5) and absence of the vas deferens (6). The sweat test is the standard diagnostic procedure to identify CF patients (7). The sweat salt content remains high on account of defective reabsorption of chloride in the sweat ducts (8). Many clinicians have experienced that in cases in which CF is strongly suspected the sweat test can be negative (9,10), although in some of these cases two CFTR mutations could be identified (11-13). This implies that the sweat test may give false negative results. A more sensitive test in selected cases is therefore highly desirable. Owing to the large number (>500) of mutations found in the CFTR gene (CF Genetic Analysis Consortium) it is still not possible to identify all patients by routine CFTR mutation analysis.

In previous experiments with Intestinal Current Measurements (ICM), we always found differences in the carbachol-provoked chloride currents in colonic biopsies of CF patients with positive sweat test results and those of controls (14,15). In CF patients this inward current is reduced and in most cases preceded by an outward current, most likely representing potassium secretion (14). Because an outward current was never observed in controls, ICM is a valuable diagnostic method for CF. For this reason we applied ICM as a diagnostic tool in subjects suspected of CF in whom the sweat test failed owing to low sweat production or

whose sweat test results were negative despite the clinical manifestations of CF. ICM was also used to test individuals with positive sweat tests whose CF diagnosis was doubted during follow-up. We found that in these cases ICM is a more accurate method to diagnose CF than the sweat test.

Methods

Patients

In a three year period, colonic tissues of 63 individuals suspected of CF were investigated for the absence or presence of a normal chloride current by means of an adapted Ussing chamber (14). Forty of these 63 individuals were referred to our hospital primarily for the application of ICM. The procedure of obtaining rectal suction biopsies or the use of intestinal resection preparations for the purpose of Ussing chamber experiments was approved by the Hospital Medical Ethical Committee. In 26 cases the initial sweat tests failed because of low sweat production. In other cases the clinical suspicion of CF persisted despite a negative sweat test result. Alternatively, earlier CF diagnoses were doubted. Each patient's age was registered on the date of the electrophysiological evaluation. Pancreatic sufficiency (PS) was defined as normal faecal chymotrypsin (>6.3 U/g) and fat content values (<5 gr/d) without pancreatic enzyme supplements.

Sweat tests

Sweat tests were performed by pilocarpine iontophoresis. In our hospital, the criterium for a positive sweat test was based solely on sodium values. For a positive test result sodium value must be in excess of 70 mmol/L on at least two occasions (16). During the study we found that a great number of CF patients detected by the electrophysiological assay had

sweat sodium values below 70 mmol/L; this prompted us to improve the predictive value of the sweat test. In the second half of the study period we therefore also determined chloride values in those sweat samples in which sodium was in excess of 30 mmol/L. Results are given of the highest sweat test obtained unless otherwise indicated.

Intestinal Current Measurements (ICM)

The procedure of short circuit current (I_{SC}) measurements on rectal suction biopsies has been described earlier (14). In 7 cases stripped colonic mucosa of the meconium ileus resection preparations were used. Fresh intestinal tissues were mounted in an Ussing chamber with an exposed area of 1.13 mm^2. After stabilization of the basal I_{SC}, various substances were added in the baths: (1) glucose (10^{-2} mol/L) was added to both the mucosal and serosal side; (2) to reduce the contribution of electrogenic sodium absorption to the I_{SC}, specific sodium channels were blocked by mucosally added amiloride (10^{-4} mol/L); (3) the endogenous prostaglandin synthesis that is possibly linked to cAMP-mediated chloride secretion was inhibited by adding indomethacin (10^{-5} mol/L) to both sides; (4) the chloride current in rectal tissues is best observed by use of serosal addition of carbachol (10^{-4} mol/L) (14). All chemicals were obtained from Sigma, St. Louis, USA. In case of duplicate procedures the technically optimal registration was used for data collection. In case of small inward currents in CF patients (mostly preceded by an outward current), the registration with the highest observed inward current pointing to (residual) chloride secretion was included. Values are expressed as net change in Isc and calculated as the sum of the negative peak response (outward current) and, if present, the positive peak response (inward current) (15). The Reference values in the Results section and Figure 1 are largely obtained from other subjects (49 CF, 21 CF carriers, 32 controls) than described in this paper. They were extended with well defined and undisputable CF patients (n=11, abnormal

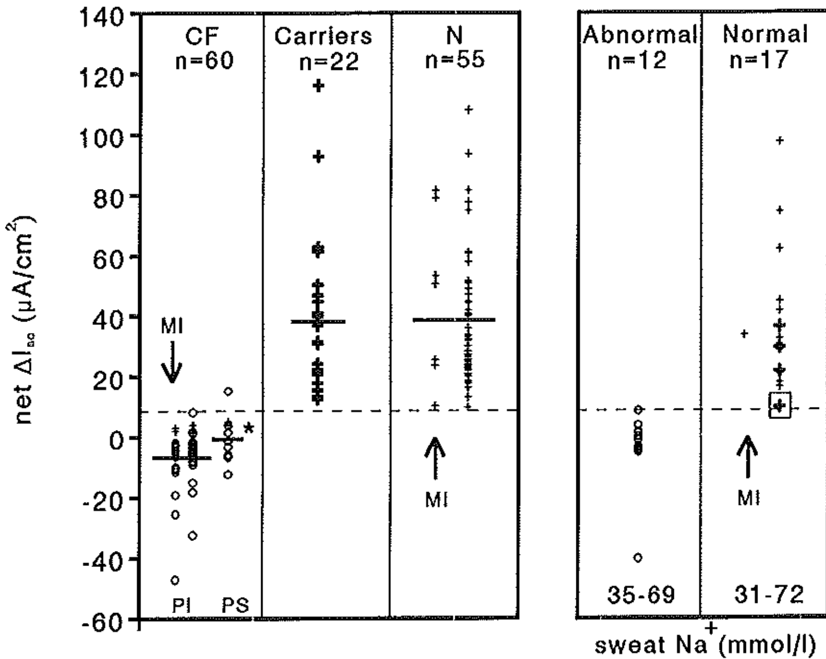

Figure 1. The left panel shows the values of carbachol provoked changes in short circuit current (I_{SC}) of colonic tissues obtained from CF patients, CF carriers and controls. The data indicate the sum of inward and outward current (see Methods). In most CF patients an initial outward current is observed (O). The occurrence of an inward current only is indicated by a (+) sign. *$P<0.05$, Mann-Whitney. On the basis of the lower limit of the control range we have defined a normal ICM as an I_{SC} response higher than 9 μA/cm² without a preceding outward current (dashed line). The right panel shows the values obtained in CF patients without any sweat sodium above 70 mmol/l and those in non-CF patients (+) with elevated sweat tests. Four carried one copy of ΔF508 (+). One of these (horizontal arrow) had an additional CFTR mutation (7T variant of R117H).

ICM, positive sweat test, clinical phenotype of CF), a CF carrier (n=1, normal ICM, highest sweat sodium below 30 mmol/L, one CF allele present compared with index CF patient), and controls (n=23, highest sweat sodium below 30 mmol/L, normal ICM) from this study.

DNA-analysis

In most cases DNA was extracted from blood leucocytes (17). Sometimes buccal swabs were used for the isolation of DNA (18). Mutations analyzed in this study were: R117H, R347P, A455E, 621+1G→T, ΔI507, ΔF508, 1717-1G→A, G542X, G551D, R553X, R1162X, S1251N, W1282X, and N1303K. Mutation analysis was performed using either the amplification refractory mutation system (ARMS) (19) or PCR amplification of the appropriate exon in combination with a restriction enzyme digestion, or an allele specific oligonucleotide hybridisation. Haplotype analysis was carried out using the polymorphic dinucleotide repeat markers IVS6A, IVS8CA, IVS17BTA, and IVS17BCA. The conditions have been described by Morral and Estivil (20,21). The length of the T-stretch in intron 8 was determined using the method described by Kiesewetter et al (22) with one modification. The oligonucleotide used to identify 9 T's in intron 8 was modified. The sequence used was: 5'-TGTGTGTTTTTT AACAG-3'.

Statistics

Values are expressed as range (median) or mean ± SD. All tests are two-tailed.

Results

Reference values of ICM

In a total of 60 CF patients (average age 13.2 yr; SD=13.1) with an undisputable CF diagnosis (clinical manifestations and sweat sodium above 70 mmol/L), the mean short circuit current (I_{SC}) response by ICM was -5.3 ± 9.5 μA/cm^2. The response in 11 pancreatic sufficient (PS) CF patients (0.65 ± 7.5 μA/cm^2) was significantly higher than in 49 PI CF patients (-6.7 ± 9.4 μA/cm^2, $p<0.05$, Mann-Whitney, Figure 1), which is suggestive of a higher (underlying) residual chloride secretion in PS

patients (15). In 55 healthy volunteers and patients with non-CF related symptoms and sweat sodium below 30 mmol/L (average age 6.4 yr; SD=10.3), the average (±SD) I_{SC} response was 38.5 ± 22.5 μA/cm². Responses tend to decrease with increasing age (15). Despite their higher age, 22 CF carriers (29.4 yr; SD=14.4) showed no reduction in their responses (38.7 ± 26.1 μA/cm², Figure 1). Outward currents, as seen in most CF patients (Figure 1), were never observed in controls and CF carriers.

On the basis of the lower limit of the control range we have defined a normal ICM as an I_{SC} response higher than 9 μA/cm² without a preceding outward current (Figure 1).

Abnormal ICM; diagnosis CF

Of 63 individuals, 23 showed an abnormal ICM (Figure 2). Eleven of these 23 later on had a positive sweat test, thus confirming the diagnosis of CF. However, a positive sweat test was not found in the remaining 12 individuals with an abnormal ICM (Table 1); the lowest sweat sodium value recorded in this group was 34 mmol/L (Table 1). In 7 of these 12 cases the CF diagnosis was confirmed by the presence of two CFTR mutations (Table 1). In the other 5 cases only one CFTR mutation could be identified. In two families, both the index patients and the asymptomatic siblings carried one ΔF508 mutation ([6,7] in Table 1, Figure 3). Haplotype analysis using intragenic polymorphic CFTR markers was used in these families to identify the genotype of the siblings of the index patients. In these families some siblings appeared to be either carrier of the ΔF508 mutation (Figure 3A II:2 and 3B II:3) or carrier of the allele with the unknown mutation (Figure 3B II:2); others were not carriers at all (Figure 3A II:3 and 3B II:1). Only ICM enabled to identify the index patients as CF patients and to distinguish them from their siblings; sweat tests and CFTR mutation analysis apparently were not conclusive (Figure 3B II:3).

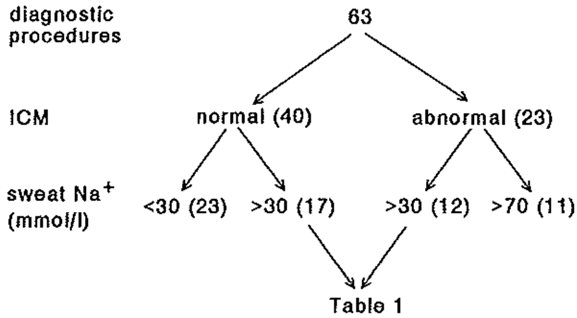

Figure 2. Diagnostic procedures with application of ICM in 63 cases.

Normal ICM; CF diagnosis rejected

Forty of the 63 diagnostic cases showed a normal ICM (Figure 2). On this basis the CF diagnosis was rejected. Twenty-three of these 40 later showed a normal sweat test (<30 mmol/L of sodium), which makes a CF diagnosis unlikely. However, in 17 individuals normal ICM was associated with elevated sweat test results (>30 mmol/L sodium) (Table 1). Four individuals with a history of positive sweat tests had been followed as 'CF patients' for 5 to 11 years. None of them had a detectable CFTR mutation, none were PI, and in combination with a mild clinical course the CF diagnosis was doubted. One (8 yr, present sweat chloride/sodium 40/44 mmol/L) presented with meconium ileus and a neonatal sweat chloride of 80 mmol/L (Table 1). The second (39 yr and sweat sodium 68 mmol/L) had oligospermia, the third one (15 yr, sweat chloride/sodium 47/69 mmol/L) had a history of nasal polyps. The fourth subject (19 yr) still has a sweat sodium of 72 mmol/L with sweat chloride of 44 mmol/L.

Another individual (out of 17) carrying one copy of ΔF508 and showing a suspect sweat test result (chloride/sodium of 55/46 mmol/L), had a I_{SC} of 36.3 μA/cm², which is well within the normal range. This individual must be a CF carrier.

Table 1.

Diagnosis by ICM ΔI_{SC} μA/cm^2 (median)	age range (median)	CFTR mutations found	n	PI	MI[3]	sweat Na^+ or Na^+/Cl^- (mmol/l) highest	(lowest)
Abnormal ICM diagnosis CF and highest sweat $Na^+ < 70$ mmol/l: -40.4 to 8.5 (-0.6)	0 to 17 (1.1)	ΔF508/ΔF508	3	3	2	61	(50)
						62	(56)
						68	(52)
		ΔF508/A455E	4	1	-	44/49	(40/43)[5]
						64/68	(60/62)
						68/73	(50/67)[5]
						64	(58)
		ΔF508/-	4	1	1	35/55	(34/53)[6]
						51/60	(34/53)
						58/65	(37)[7]
						69/-	(35/39)
		G542X/-	1	1	1	52/86	(43/78)
normal ICM CF rejected highest sweat $Na^+ > 30$ mmol/l: 9.6 to 97.4 (32.4)	0 to 47 (7.6)	ΔF508/-	3	-	-	39/31[7]	
						46/55	
						38	
		ΔF508/R117H[1]	1	-	-	52	
		none	13[2]	-	1	31 to 72	

[1]7T variant of R117H which is associated with CBAVD and PS CF patients. [2]Four subjects had a history of positive sweat tests and had been in follow up as 'CF patient' for 5 to 11 years. [3]MI Meconium ileus. [4]presented with meconium ileus and a neonatal sweat chloride of 80 mmol/l, at the age of 8 yr. sweat sodium/chloride 44/40 mmol/l. [5]CF-siblings. [6]see also Figure 3A,[7]see also Figure 3B.

ICM appeared normal (9.7 μA/cm^2; -1.3 SD from the mean) in one individual with two CFTR mutations (ΔF508/R117H). His sweat sodium was 48 and 52 mmol/L. At the age of 14 years he is in good condition, is suffering mildly from asthmatic bronchitis and is pancreatic sufficient. Analysis of intron 8 of the CFTR gene on the allele carrying the R117H mutation revealed 7T repeats that may be associated with CBAVD without

additional symptoms of CF (22). At this moment we are in doubt whether in this case the CF diagnosis is justified.

Discussion

The observed abnormal currents in intestinal biopsies of CF patients demonstrate the known defect of chloride transport through CFTR channels. The outward current, most likely reflecting an abnormal K^+ secretion through the apical membrane, serves as an additional typical characteristic of CF when applying ICM for diagnostic purposes (14,15).

In the CF mouse model, CF carriers have a diminished intestinal response to the cAMP agonists (23). This would support the theory that human CF carriers have a selection advantage because in response to cholera toxin, salt losses are less severe (24). In this study with ICM, CF carriers could not be distinguished from controls. This apparent discrepancy may be explained in several ways: (I) the fact that carbachol was used instead of cAMP as a secretagogue (cAMP-provoked currents in rectal biopsies are too small (14)), (II) the measured carbachol-induced currents were modulated by some unusual expression of Ca^{2+}-activated chloride channels, (III) the control group included CF carriers (with a low response), and (IV) the variation of responses between individuals may be too large to detect the relatively small differences expected between CF carriers and controls.

Nevertheless, ICM measurements in intestinal tissues of identified CF patients were always different from those obtained from healthy, non-CF individuals (14,15). Moreover, differences in the pancreatic status (Figure 1) and other phenotypical characteristics of the disease are related to the magnitude of the carbachol-provoked current (15). One individual presented with meconium ileus, typically in the terminal ileum. In this case ICM showed no outward current and a normal magnitude of

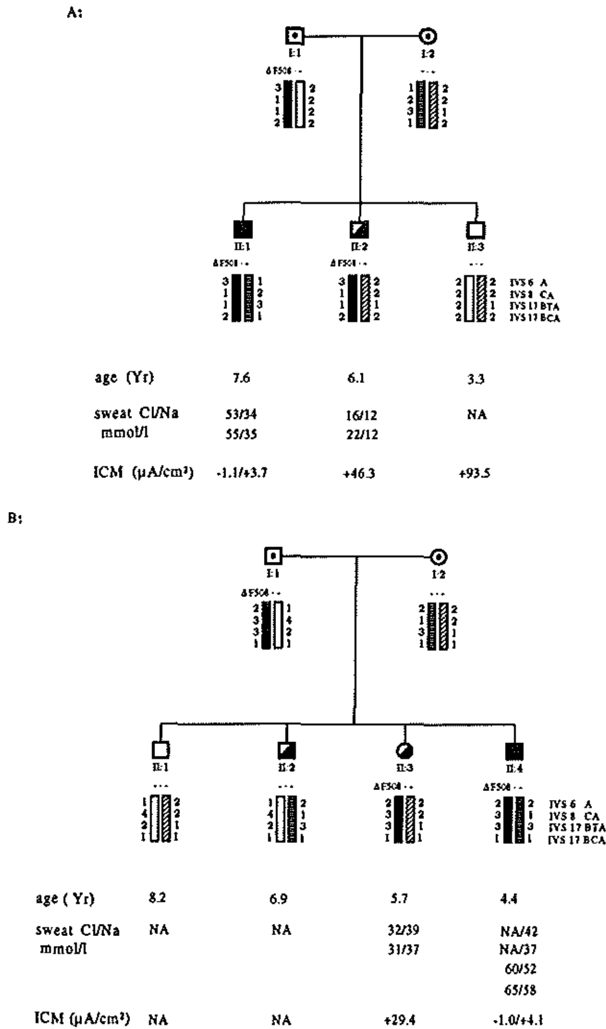

Figure 3. In two families, both the index patients and their asymptomatic siblings carried one ΔF508 mutation. Haplotype analysis using intragenic polymorphic CFTR markers was used to identify the genotype of the siblings of the index CF patients identified by ICM. In these families siblings were identified who were carriers of the ΔF508 mutation (Figure 3A II:2 and 3B II:3) or carrier of the allele with the unknown mutation (Figure 3B II:2), or who were not carriers at all (Figure 3A II:3 and 3B II:1). Only ICM enabled to identify the index patients as CF patients and to distinguish them from their siblings, when sweat tests and CFTR mutation analysis were not conclusive (Figure 3B II:3).

inward response (23.7 μA/cm^2). The absence of CF was later confirmed by a normal sweat sodium value of 15 mmol/L, and CFTR mutation analysis was negative, while two of his siblings were homozygous for the ΔF508 mutation. Interestingly, these CF patients had not presented with meconium ileus. It was also noted that the magnitude of ICM, both in CF and non-CF patients, is not related to the condition of meconium ileus (Figure 1). These observations indicate that the pathological origin of meconium ileus is in part independent of CF.

We have shown that ICM can be used to demonstrate CF in individuals with borderline or normal sweat tests. Occasionally, ICM may also exclude CF in individuals with a suspect clinical history and elevated sweat tests. In this study, the individual with meconium ileus and a neonatal (false) positive sweat test was the most striking example.

Interestingly, a normal ICM was found in one individual carrying two CFTR mutations. It has been demonstrated that CFTR mutated by R117H is partially functional (25). Moreover, the 7T variant of intron 8, as found in this case, is likely to produce a higher level of full length CFTR mRNA than the 5T variant. Clinical evaluation of 14 subjects carrying ΔF508/R117H-7T revealed that 7 were mildly affected PS CF patients, 6 were males with CBAVD and one was a completely asymptomatic women (22). As our patient suffers only mildly from asthmatic bronchitis, it may well be that 'CFTR' disease will be limited to CBAVD (not evaluated in this child) or that perhaps with advancing age other CF related symptoms may arise.

How may we explain that the sweat test, being a useful and widely applied diagnostic method, is not accurate in all cases? It is important to realize that the present criteria for an abnormal sweat test are based on sweat test results of CF patients with a typical clinical phenotype (26). With the identification of CF phenotypes showing milder symptoms and less severe sweat test abnormalities, the validity of the currently used sweat test criteria can be questioned. In 1825 subjects without a CF

diagnosis the mean sodium sweat value plus 2SD is 34 mmol/L (27). Moreover, the sweat test measures the result of both sodium and chloride transport processes and is thus not specific for CF in evaluating the CFTR defect (11-13). In general, chloride values and moreover chloride/sodium ratios, are better discriminators between CF and non-CF individuals than sweat sodium values (Table 1) (28). However, sweat chloride may be lower than sodium in CF patients (29), and in CF carriers chloride may be in excess of sodium (Table 1). Other possible pitfalls of the sweat test are the considerable variation within one and the same CF patient (27,30), and the age dependency in normal sweat tests (31).

In conclusion, we found ICM an accurate and useful complementary diagnostic tool for CF. It is especially indicated in individuals in whom CF is suspected, though sweat test and CF mutation analysis give inconclusive results.

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Chapter 5

General Discussion

5.1 General discussion

This thesis explores the pathophysiological basis for the clinical variability in cystic fibrosis and the consequences of this variability for the interpretation of diagnostic tests.

Different CFTR mutations lead to differences in severity of the disease. 1. The ΔF508 mutation and the newly identified CFTR mutations L927P and dL1260 can be characterized as 'severe' mutations since they are associated with early diagnosis, pancreatic insufficiency and severe lung disease. 2. The A455E mutation is associated with a milder phenotypic expression of CF as appeared from the higher age at diagnosis, the more often observed pancreatic sufficiency, the less severe lung disease with a lower prevalence of Pseudomonas colonization. Worldwide, the A455E mutation is rare, but in the Netherlands it is the second most common mutation (Chapter 4.3) and may therefore be a typically Dutch mutation. The high number of non-positive sweat tests observed in a proportion of these patients, together with the need to perform ICM to establish the CF diagnosis (n=9), suggests a false low detection rate elsewhere. 3. The R117H is a very 'mild' mutation, which leads to male infertility as single manifestation of CF.

Intestinal chloride current measurements (ICM) can be used to quantify the pathophysiological defect in CF. It appears that most CF patients have no measurable intestinal chloride secretion, but that in a small subpopulation a residual (=inward) current is present. In this subgroup symptoms of CF present later in life, suggesting a causal relation between residual chloride secretion and mild clinical phenotype. The abnormalities in intestinal chloride transport and the resulting diminished water flow to the epithelial surface will contribute to the malabsorption observed in CF. Supplemental pancreatic therapy will therefore not lead to complete normalisation of this malabsorption.

Whether the observed outward current represents the upregulation of a previously silent process has not yet been fully clarified. Nevertheless, the carbachol-provoked outward current serves as a typical characteristic of CF when applying ICM for diagnostic purposes. The outward current presumably indicates an abnormal transport process because (I) it occurs quickly after the addition of carbachol, within a 'silent' period after which a normal current can be expected in controls (Chapter 3.2, Figure 2), and (II) because unlike in controls the outward current is followed by an inward current. The outward current probably reflects active secretion of K^+ following the opening of cation channels in the apical membrane in response to carbachol (Chapter 3.2).

The upregulation of sodium channel activity observed in airway cells of CF patients does not occur in colonic epithelium. At a later stage of our investigations this could be confirmed in a larger study group (51 CF patients, 50 controls, Chapter 4.1). We presume that the abnormal K^+ secretion in the CF intestine is the equivalent of the abnormal Na^+ transport in CF airway cells. How these abnormalities are related to the mutated CFTR remains to be elucidated.

Although the pattern of chloride secretion is largely determined by the CFTR genotype, it is in part independent of the specific mutation (Chapter 4.1). Variations in the chromosomal background, such as the CFTR intron 8 variants (see discussion Chapter 4.4), can also affect the function of CFTR and may therefore have impact on the clinical outcome of CF. Furthermore, it can not be ruled out that a third CFTR mutation may have a beneficial effect on the mutated CFTR protein configuration (1,2). Additionally, the existence of an alternative Ca^{2+}-mediated chloride secretory pathway may well explain the residual secretion we observed in two patients homozygous for stopcodon mutations who are not capable of producing any functional CFTR (Chapter 4.2). This assumption is supported by studies in transgenic mice. The pancreas (3,4) and airways (4,5) of these mice display a high level of expression of Ca^{2+}-activated

chloride channels. In contrast, in the CF mouse intestine the expression of these alternative channels is low. Strikingly, in this animal model pancreatic and pulmonary disease is mild, whereas intestinal symptoms are severe (4). This indicates a beneficial role of Ca^{2+}-activated chloride channels in the mildly affected organs. In cultured human T_{84} colon carcinoma cells, Ca^{2+}-activated chloride channels distinct from CFTR also contribute to apical chloride secretions (6-8). Preliminary experiments from our lab, applying ICM with inhibition of the possibly Ca^{2+}-activated chloride channels, also reveal the presence of a compensatory chloride channel in intestinal tissues of some CF patients. Current studies are aimed at demonstrating compensatory non-CFTR chloride channels in very mildly affected patients homozygous for ΔF508, the genotype most frequently associated with the typical, more severely affected CF patients. First results show that a compensatory channel indeed probably contributes to the chloride current in a mildly affected ΔF508 homozygous patient. A high level of expression of Ca^{2+}-mediated chloride channels implies that mild CFTR dysfunction may be fully compensated. We observed several patients with normal chloride secretory responses (one case ΔF508/A455E and 4 males with CBAVD with CFTR genotypes 2 ΔF508/R117H-7T, 1 R553X/R117H, and 1 A455E/R117H-7T) in whom despite two identifiable CFTR mutations, the clinical manifestation of CF is absent or minimal. Since both CFTR- and Ca^{2+}-mediated chloride channel activity influences the clinical phenotype, CF is likely to be a polygenic disease. It is essential to find the gene involved in the Ca^{2+}-mediated secretory pathway. Moreover, stimulating chloride secretion via the alternative chloride secretory pathway may be more easily to achieve than gene-therapy. It is important that subjects with an atypical phenotype and 'mild' mutations are clinically and functionally investigated in a CF centre. Only after long-term follow-up the ultimate outcome will be known.

5.2 Implications for establishing a CF diagnosis

Clinical diagnosis:

The diagnosis of CF is justified only in the presence of clinical symptoms. The initial symptoms may be as mild as only nasal polyps, isolated bronchiectases or obstructive azospermia (CBAVD). More typical presenting symptoms are meconium ileus, recurrent airway infections with Staphylococcus aureus or Pseudomonas species, fatty stools and malnutrition (see Figure The CF diagnosis). Knowledge of the clinical features of CF is thus crucial in establishing the diagnosis.

A number of diseases with characteristics that can also be found in CF, such as Young syndrome, azospermia and chronic obstructive sinobronchitis (McKusick 279000), and CBAVD (McKusick 277180) need to be re-evaluated with new diagnostic tools (CFTR mutation analysis and ICM) to establish whether they are in fact a mild form of CF. It is likely that CFTR dysfunction is the underlying cause of infertility in a large proportion of males with CBAVD (9-12). In CF-related CBAVD the disease is perhaps limited to the vas deferens because at a crucial stage of its embryonal development it failed to express or activate sufficient Ca^{2+}-activatable chloride channels.

Sweat test:

In case of clinical symptoms of CF, the sweat test (pilocarpine iontophoresis with sweat collection onto pads as described by Gibson and Cooke (13) or into capillary tube (Macroduct) (14)) remains the test of choice. Chloride sensing electrodes should <u>not</u> be used since they very often give false results. Collection of at least 100 mg sweat is recommended in the literature, but in our experience as little as 40 mg can be used to obtain reliable results. The sweat test is not specific for chloride transport measurement. In fact the outcome is the result of both

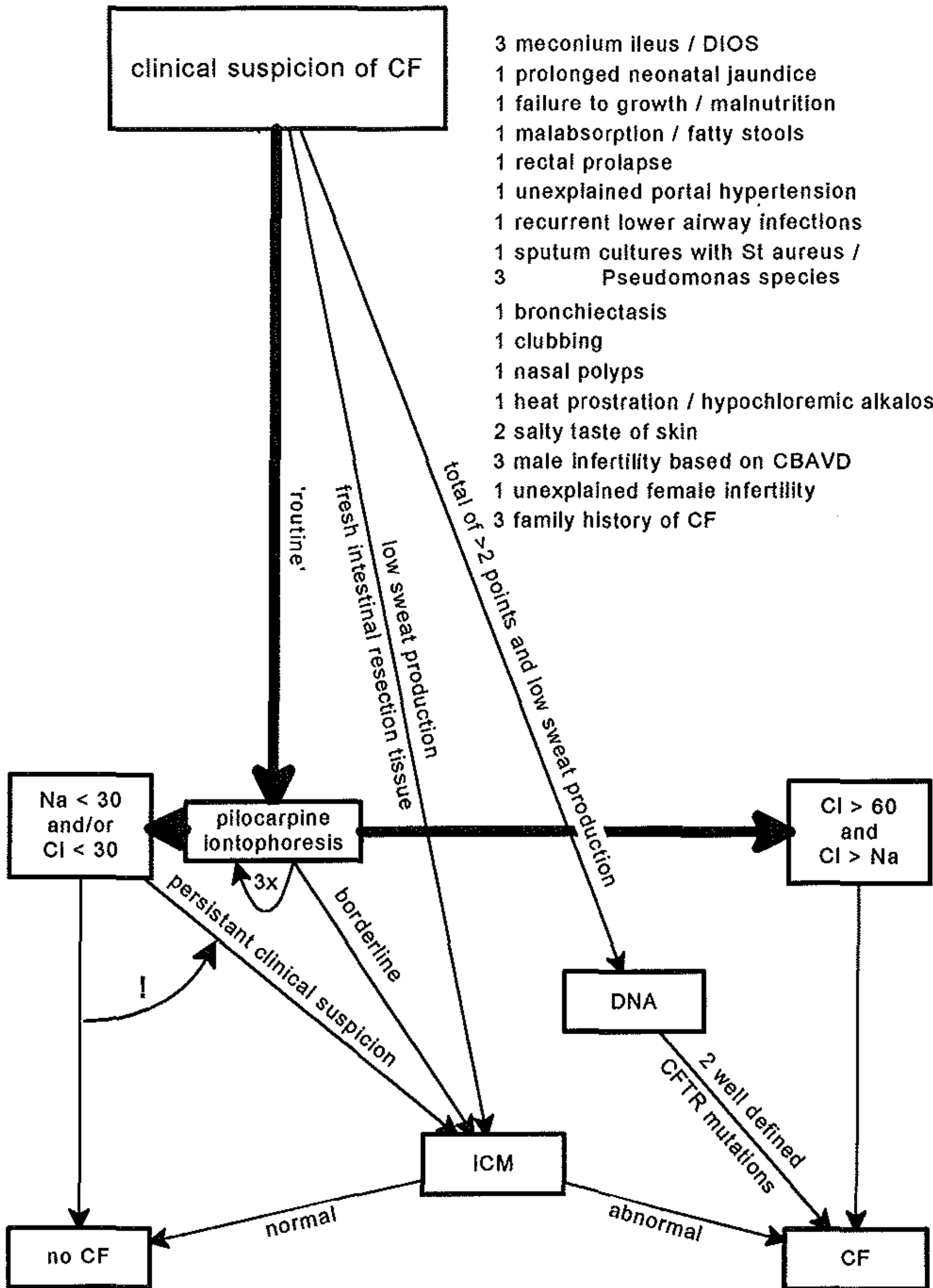

Figure. The CF diagnosis.

chloride and sodium transport processes. If the reabsorption process of one electrolyte is impaired, the ion of opposite charge is also accumulating to adjust for electroneutrality. Therefore, in case of low sodium reabsorption (e.g. neonates, anorexia, adrenal insufficiency) (15-17), chloride levels will also be higher than normal, though lower than those of sodium owing to the presence of other anions like bicarbonate. Therefore, to improve discrimination between CF and non-CF individuals, when initial sweat sodium is in excess of 30 mmol/L, both chloride and sodium concentrations should be measured and their ratio determined (18-20). Typically, in CF a positive sweat test result implies that (I) chloride is in excess of 60 mmol/L and (II) chloride is in excess of sodium. Sweat tests may also be abnormal ($Cl^- > 60$ mmol/L but $Na^+ > Cl^-$) in case of low sodium channel activity as in the case of (pseudo-) hypoaldosteronism and other endocrinopathies. Under these conditions the observed sodium channel activity in ICM may also be low (results not shown here). In adult subjects electrolyte concentrations in sweat after stimulation can be physiologically elevated (21). Based on our experience we advise for routine clinical practice to suspect CF when clinical symptoms are present and sweat sodium or chloride values are in excess of 30 mmol/L (instead of 50 mmol/L which is the current lower limit of a borderline sweat test). This value of 30 mmol/L is almost 2 SD above the mean and can, also on these grounds, be considered as the upper limit of a normal sweat test (Chapter 4.3). Mildly affected CF phenotypes may have borderline sweat tests without the typically high chloride/sodium ratio (ΔF508/A455E: in 59 mg sweat $Na^+ = 30$ mmol/L, $Cl^- = 24$ mmol/L, Chapter 2.4).

In case of borderline sweat tests (30-60 mmol/L chloride), considering the day to day variability in the reabsorption mechanisms of the sweat duct (Chapter 4.3), it is recommended to repeat the test three to five times. However, even a completely normal sweat test in the presence of suspect clinical symptoms is an indication for ICM.

Note: It is common policy to perform sweat tests in siblings of a CF patient to exclude the diagnosis. One should realize that a sweat test result may be elevated due to the carrier status.

Intestinal Current Measurements (ICM):
ICM is strongly recommended in individuals suspected of CF in whom none of the sweat tests revealed a positive result ($Cl^- > 60$ mmol/L and $Cl^- > Na^+$ mmol/L). An increasing number of ICMs for diagnostic purposes is being performed in our department. Up to January 1995, 124 individuals suspected to have CF, including 17 males with CBAVD, were investigated. In nine subjects the diagnosis CF was withdrawn on the basis of ICM (naturally with as much circumstantial evidence as could be provided by the sweat test, CFTR mutation analysis and haplotype analysis).

Nasal Potential Differences (nasal PD):
Measuring nasal transepithelial electric potential differences *in vivo* is another method to evaluate epithelial transport processes (22). In CF the basal PD is higher and the change in PD induced by superfusion with amiloride is larger than in controls and can thus be used as a diagnostic method. However, this technique mainly relies on the increased sodium channel activity in CF airway epithelium which may not be a valid criterium to detect mild abnormalities especially in subjects with borderline or high normal sweat tests. Moreover, one should be very experienced to obtain reliable results and in active nasal infection the typical abnormalities can not be seen. Measuring the chloride permeability is even more difficult to use for a routine procedure. Nevertheless, it is of interest to seek for different modes of expressions of the CF defect or possible compensatory chloride channels in different epithelia (23) and for this reason we have started nasal PD measurements in our laboratory.

Indications for CFTR mutation analysis:

1. To establish the diagnosis CF in term newborns presenting with meconium ileus because these patients are too young for reliable sweat testing owing to a low sweat production and a low sodium channel activity, which may result in falsely high sweat test results. In case of CF most of them will have two identifiable CFTR mutations (31 of 42 CF patients with meconium ileus had two identifiable CFTR mutations). A blood sample (2-5 ml heparin blood) should be sent to a genetic centre, with information about the ethnic origin of the patient (required for the panel of mutations to be tested). If the patient has received a blood transfusion, a mucosal smear (sterile procedure and initial rinsing of the oral cavity to prevent contamination) should be taken. If two CFTR mutations are found, the parents have to be examined to confirm the segregation of the two mutations. In meconium ileus patients without two identified CFTR mutations, CF has to be excluded by other diagnostic procedures.
   Note: In meconium ileus patients fresh resection preparations can be analyzed in the Ussing chamber. A diagnosis may be established within 1 to 2 hours.
2. To exclude CF in siblings of a CF patient. This may be indicated in case of clinical symptoms to promptly start CF therapy and preventive measures. Also anxiety of the parents may call for CFTR mutation analysis. For further genetic analysis in the family the index patient and both parents must be investigated first. False conclusions could be drawn in case of abnormal segregation of the two CFTR mutations.

An unwanted side effect of CFTR mutation analysis may be the burden of knowing that one is a CF carrier. Generally, when young children are tested, it could be agreed with the parents to give the results as non-CF or CF. The decision to be informed about possible carrier status (with a chance of 2 out of 3) can then be made by the individual at an older age.

3. To perform carrier testing in close relatives of a CF patient, including the partners of males with CBAVD, in whom Microsurgical Epididymal Sperm Aspiration (MESA) and Intracytoplasmic Sperm Injection (ICSI) are going to be performed (24).
4. To confirm the diagnosis of CF, to search for new mutations, to contribute to further research on genotypes/phenotypes or to contribute to epidemiological studies.

Note: It is our experience that a high proportion (32%) of subjects with borderline sweat tests (>30 mmol/L sodium or chloride) show only one identifiable CFTR mutation. This includes the A455E mutation which accounts for 29% of CFTR mutations in CF patients with borderline sweat tests. In these cases, CFTR mutation analysis will not distinguish between CF carriers and CF patients.

5.3 Reflections on the incidence of CF

There are several indications that the true incidence of CF is higher than presently is believed:

1. The intrapatient variability of sweat tests (Chapter 4.3) may lead to underdiagnosis of CF. In individuals in whom the result of the first sweat test is within the classic 'normal' range the diagnosis of CF may unjustly be rejected.

2. Forty-seven tests performed in our hospital in 40 CF patients (in which both sodium and chloride values were determined) yielded a considerable number of normal and borderline results (53% of tests had a sodium value of less than 70 mmol/L; 28% of tests had a chloride value of less than 60 mmol/L).
3. With application of ICM, the annual number of newly diagnosed CF patients in the Sophia Children's Hospital has increased from approximately 9 per year in 1983 - 1992, to 17 in 1993 and 21 in 1994 (Patients living outside the normal referral region of the hospital, and males with CBAVD are excluded).
4. The prevalence of 'mild' CFTR mutations in newly recognized patient groups, like the one with CBAVD, is much higher than was expected on the basis of findings in 'classic' CF population.
5. The percentage of ΔF508 chromosomes found on 338 CF chromosomes (73.3%) is less than expected (77.3%) from the number of ΔF508 homozygous patients (59.8%) (Chapter 4.3).

5.4 Consequences for the detection of CF carriers and CF patients

At present, the carrier detection rate in the Netherlands is approximately 85%. The assumption that improved diagnostic methods will reveal a higher number of patients with a diagnosis of CF than currently is considered, implies that the carrier rate in the general population is also higher. A higher number of diagnosed CF patients will mainly result from detection of patients with 'mild' CFTR genotypes, in whom the detection rate of the CFTR mutations involved is lower. We therefore expect that the detection rate of all CF carriers will drop in future. This may effect the cost/benefit ratio of a population based screening programme and it is thus crucial to establish the true prevalence of CF.

When considering screening for CFTR mutations, practical implications with respect to counselling must be considered. For instance, the question should be raised whether it is wise to screen for very 'mild' CFTR mutations like R117H.

Besides population based screening programmes, another approach might be to initiate neonatal CF screening programmes in the hope that early detection and treatment will improve the prognosis. Until now there is little evidence that CF patients will benefit from early detection before clinical symptoms arise (25); moreover the evidence gained sofar is not convincing because patient groups were small and the results could have been influenced by selection bias.

5.5 Definition of CF

This study prompts us to redefine CF.

CF predisposition:

An impaired CFTR-chloride channel function originating from a mutated CFTR gene seems most appropriate for the definition of 'CF predisposition'.

CF disease:

Whether 'CF disease' will develop depends on the severity of the CFTR defect in combination with the expression of compensatory chloride secretory pathways. Only if CF-related symptoms exist in the presence of the 'CF predisposition' it would be justified to diagnose 'CF disease'.

- A patient with classic pulmonary and intestinal disease, a positive sweat test and two identified ΔF508 mutations clearly suffers from 'CF disease'.

- Individuals with 'mild' CFTR genotypes have a 'CF predisposition'. The largely reduced chloride secretion is responsible for the associated clinical symptoms and these individuals have 'CF disease', even if the sweat test is non-positive, as is often the case in this category.
- Male infertility associated with a mutated CFTR gene is a case of 'CF predisposition'. In the absence of other CF-related symptoms, establishing 'CF-disease' depends on the assumption that infertility is a disease characteristic of CF. If so, CBAVD when associated with a CFTR gene mutation, can be categorized as one of the variants of 'CF disease'.
- When an individual carrying two CFTR gene mutations has no clinical symptoms of CF owing to compensatory chloride channel activity (Chapter 4.1), 'CF predisposition' is obviously present but 'CF disease' does not exist. Nevertheless, genetic counselling aspects should then be considered.

If CF disease is defined as the occurrence of CF-related clinical symptoms combined with an impaired CFTR chloride channel function, it can now be understood that CF comprises a large variety of clinical entities depending on the type of CFTR mutation and the expression of alternative chloride channels. It will be a challenge for the medical profession and also for the community to understand and acknowledge the broadening scope of the diagnosis CF.

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Summary

Chapter 1: Introduction

Cystic fibrosis (CF) is one of the most common life-threatening autosomal recessive hereditary disease, predominantly affecting Caucasian populations. The disease is characterized by the production of abnormally viscid secretions in epithelial tissues. The different organs involved suffer from progressive damage owing to obstructing mucoid plugs. Typically, in CF there is excessive loss of sodium and chloride in the sweat owing to defective reabsorption of chloride in the sweat duct. This phenomenon is the basis for the abnormal sweat test result generally found in these patients. In other epithelium lined organs, it is the process of chloride excretion through the apical membranes that is impaired. Pulmonary disease, a result of mucus plugs and superimposed airway infections causing various serious complications, is the major cause of morbidity and mortality in CF. Also the genital tract in CF patients can be affected, which especially in males leads to infertility. In the gastrointestinal tract, the disease manifests itself in liver, pancreas and small and large bowel. The clinically most relevant result is pancreatic insufficiency which in combination with viscous mucus - a result of defective chloride secretion - covering the villus and crypt cells leads to malabsorption. Intestinal chloride secretion takes place mainly through CFTR chloride channels located in the apical membrane. The chloride secretion processes can be studied *in vitro* by mounting intestinal tissues in an Ussing chamber. Adding specific secretagogues to the bathing solution will achieve changes in the transepithelial transport of chloride. The current required to maintain the potential difference across the tissue at 0 mV is recorded and is a measure for the net amount of transported anion. In this study the Ussing chamber was adapted to perform intestinal current measurements (ICM) in intestinal biopsies. The CFTR chloride channel is encoded by the CFTR gene. More than 400 different mutations affecting

the CFTR function have so far been identified. Depending on the place of the specific mutation within the CFTR gene, there are several mechanisms by which the mutation is able to interfere with the normal passage of chloride.

Chapter 2 delineates the relationship between CFTR genotypes and clinical symptoms.

Chapter 2.1: The frequency of ΔF508 mutations in the Dutch population is described. It was found that 60% of the CF patients were homozygous for this CFTR mutation. Of the remaining patients, 35% were compound heterozygotes for ΔF508, and 5% of the CF patients carried two non-ΔF508 mutations. The age at diagnosis, which is inversely related to the severity of the disease, was lower in the ΔF508 homozygous group. This was one of the first findings suggestive of an association between genotypes and phenotypes in CF.
Chapter 2.2: The clinical manifestations in a small group of patients with two newly identified CFTR mutations - L927P and dL1260 - are described.
Chapter 2.3: To investigate whether A455E, the second most frequent CFTR mutation in the Netherlands, is associated with mild clinical symptoms, including mild pulmonary disease, we studied embargo 33 A455E compound heterozygotes (all with a severe CFTR mutation on the other allele) and compared them with age/sex matched ΔF508 homozygotes. Patients with the A455E mutation were diagnosed at a later age, fewer patients were suffering from pancreatic insufficiency, and none had diabetes mellitus. Pulmonary function was significantly superior, and fewer patients were infected with Pseudomonas aeruginosa.
Chapter 2.4: In our centre the A455E mutation accounts for 4.8% of all CFTR mutations, which is unexpectedly higher than the reported worldwide frequency of 0.1%. All sweat tests obtained in 18 CF patients

with the A455E mutation were re-evaluated. The results were often negative and in one case as low as 24 mmol/L chloride. Even chloride/sodium ratios were not always conclusive.

Chapter 3 describes the electrophysiological abnormalities of intestinal chloride transport in CF.

Chapter 3.1: The transport of ions in the small and large intestine of CF patients was studied using the Ussing chamber. The effects of all three types of intracellular signal transfer were abnormal, although the second messengers themselves (cAMP, cGMP and Ca^{2+}) were present. In CF an indication is found for an abnormal K^+ secretion instead of Cl^- secretion.
Chapter 3.2: Abnormalities in transepithelial electrolyte transport in rectal tissues of 11 control and 11 cystic fibrosis subjects were analyzed by short-circuit current measurements in a modified Ussing chamber. As judged by the amiloride-sensitive component of the I_{SC}, electrogenic sodium absorption appeared unmodified in cystic fibrosis intestine, which is in contrast to what is found in airway epithelium. The I_{SC} responses to specific stimuli of both cyclic adenosine monophosphate (cAMP)- and calcium-mediated chloride secretion were drastically altered in all of the cystic fibrosis biopsy specimens examined.
On the basis of the calcium-mediated secretory response, patients with cystic fibrosis could be divided into two categories: a major population showing defective anion secretion but active cation secretion, and a subclass showing residual but subnormal anion secretion. The easy accessibility of rectal samples and the inverse direction of the cAMP- or Ca-provoked changes in I_{SC} are of considerable advantage in the diagnosis of cystic fibrosis.

Chapter 4 combines the outcomes of the studies in Chapter 2 and 3 in order to further clarify the pathophysiology of CF.

Chapter 4.1: In search of a possible relationship between residual intestinal chloride secretion on the one hand, and both the genotype and the phenotype on the other hand, we performed Ussing chamber experiments on rectal suction biopsies of 51 CF patients. The CFTR mutation was identified in 89 of 102 CF alleles. No apparent chloride secretion was found in 30 CF patients (Group I). Low residual chloride secretion was found in 11 CF patients (Group II), whereas a relatively high residual secretion was observed in 10 CF patients (Group III). Pancreatic function was preserved more frequently in CF patients displaying residual secretion: 0% in group I, 27% in group II, and 60% in group III. The age at diagnosis in group III was significantly higher than in groups I and II. Residual chloride secretion was found in some of the 28 ΔF508 homozygous patients (3 in group II, and 1 in group III), disclosing that other factors than the CFTR gene defect itself, could affect the transepithelial chloride transport. The age at diagnosis correlated significantly with the magnitude of the secretory response, even within the ΔF508 homozygous patients. We conclude that residual chloride secretion in cystic fibrosis is the pathophysiological basis of preserved pancreatic function and delayed presentation of the disease in some CF patients. This residual secretion is not exclusively determined by the CFTR genotype.

Chapter 4.2: Two patients homozygous for the G542X stopcodon mutation displayed carbachol-provoked residual chloride secretion in rectal biopsies. We could not confirm that suppression of the G542X stopcodon or activity of the truncated CFTR protein lead to residual chloride transport. Therefore it is highly unlikely that CFTR activity is

responsible for residual intestinal chloride transport observed in G542X homozygotes. This observation suggests the existence of an alternative chloride transport pathway.

Chapter 4.3: In this study we re-evaluated the sweat test results of 1905 subjects performed in our hospital over a period of 9 years (1983-1992). In 1825 subjects in whom the CF diagnosis was not made, the mean sodium value obtained was 15.5 ± 9.2 mmol/L. The upper limit of the normal range (2SD above the mean) is 34 mmol/L. Re-examination of all 239 sweat sodium values (80.9 ± 19.5 mmol/L) in 80 newly diagnosed CF patients with a positive test on at least one occasion, revealed that 5% of the values were below 50 mmol/L the lowest sweat value obtained being 27 mmol/L.

In view of these results, we recommend to repeat the sweat test in case of clinical suspicion of CF and sweat values above 30 mmol/L, and also to determine both sodium and chloride values for optimal discrimination.

Chapter 4.4: ICM was applied as a diagnostic tool for CF in 63 cases. Compared with control values of non-CF individuals and CF carriers, 12 individuals had abnormal ICM whereas they did not have a positive sweat test. In this group the lowest sweat sodium obtained was 34 mmol/L, and chloride was as low as 39 mmol/L. CFTR mutation analysis revealed: 3 ΔF508/ΔF508, 4 ΔF508/A455E, 4 ΔF508/unknown, and 1 G542X/unknown. In 17 individuals with elevated sweat test results up to 72 mmol/L sodium (3 ΔF508/unknown, 13 unknown/unknown, and 1 ΔF508/R117H-7T), ICM appeared to be normal. In conclusion, we found that ICM is an accurate diagnostic tool for CF. It is especially indicated in individuals in whom CF is suspected, though both sweat test and CFTR mutation analysis give inconclusive results.

Chapter 5: Discussion

In this chapter a relationship was established between different CFTR gene mutations, the magnitude of ICM and the CF phenotype. There is evidence that in some CF patients an alternative, Ca^{2+} regulated chloride channel plays a compensatory role. ICM is a highly sensitive and specific test for CF, more so than a sweat test. The implications for a diagnostic approach are discussed.

Samenvatting

Hoofdstuk 1: Inleiding

Cystische fibrose (CF), ook wel taaislijmziekte genoemd, is een van de meest voorkomende, levensbedreigende, autosomaal recessief overervende ziekte, voornamelijk voorkomend bij Kaukasische bevolkingsgroepen. De ziekte wordt gekenmerkt door produktie van buitengewoon taai slijm door epitheliale weefsels. Diverse betrokken organen ondervinden toenemende schade als gevolg van obstruerende slijmproppen. Kenmerkend in CF is het overmatige verlies van natrium en chloride in zweet als gevolg van gestoorde reabsorptie van chloride in de ductus van de zweetklier. Dit verschijnsel vormt de basis voor afwijkingen in de zweettest die in het algemeen bij patiënten gevonden worden. In andere met epitheel beklede organen is het juist de chloride excretie via membranen aan de apicale zijde van de cel die gestoord is. De ziekteverschijnselen in de longen, een gevolg van obstruerende slijmproppen en luchtweginfecties met daaropvolgende complicaties, is de voornaamste oorzaak van morbiditeit en mortaliteit bij CF. Ook kunnen de genitale organen bij CF patiënten betrokken zijn bij het ziekteproces. Vooral bij mannelijke patiënten leidt dit tot onvruchtbaarheid. In het maagdarmkanaal kan de ziekte tot uiting komen in lever, alvleesklier en dunne- en dikkedarm. Klinisch het meest relevante gevolg hiervan is pancreas insufficiëntie welke tezamen met het abnormaal taaie slijm welke villus en crypten van de darm bedekt - een gevolg van onvoldoende chloride secretie - leidt tot malabsorptie. In de darm vindt chloride secretie plaats door CFTR chloride kanalen, gelegen aan de apicale zijde van de cel. Deze chloride secretie kan *in vitro* worden bestudeerd door darmweefsel te bevestigen in een zogenaamde Ussing kamer. In dit apparaat wordt de chloride stroom gemeten (ICM) als reactie op blootstelling aan diverse secretagogen die de kanalen activeren. In dit onderzoek werd de Ussing kamer aangepast, zodat ook metingen aan

darmbiopten konden worden verricht. Meestal werd gebruik gemaakt van rectum zuigbiopten. De ingreep is pijnloos en vergelijkbaar met rectaal temperaturen.

De codering voor het CFTR chloride kanaal ligt besloten in het CFTR gen. Er zijn al meer dan 400 verschillende mutaties bekend. Afhankelijk van de plaats van een specifieke mutatie binnen het CFTR gen, zijn er verschillende aangrijpingspunten waar het transport van chloride verstoord kan worden.

Hoofdstuk 2 omschrijft relaties tussen CFTR genotypen en klinische verschijnselen.

Hoofdstuk 2.1: Het voorkomen van de ΔF508 mutatie in Nederland wordt omschreven. Vastgesteld werd, dat 60% van de patiënten homozygoot was voor deze mutatie. Van de overige patiënten had 35% een ΔF508 met nog een andere mutatie, terwijl 5% twee andere dan de ΔF508 mutatie bezit. De leeftijd waarop de diagnose wordt gesteld, welke een indicatie vormt voor de ernst van de ziekte, was lager in de groep van de homozygoten voor ΔF508. Dit was één van de eerste aanwijzingen voor het bestaan van een relatie tussen genotype en fenotype in CF.
Hoofdstuk 2.2: De klinische verschijnselen van een kleine groep patiënten met twee kort tevoren geïdentificeerde mutaties - L927P en dL1260 - worden beschreven.
Hoofdstuk 2.3: Om vast te stellen of A455E, de nu op één na meest frequent voorkomende mutatie in Nederland, gepaard gaat met milde klinische verschijnselen, waaronder ook mildere ziekteverschijnselen van de longen, werden 33 patiënten met de A455E mutatie (allen met een 'ernstige' CFTR mutatie op het andere allel) bestudeerd en vergeleken met leeftijds- en geslachtsidentieke ΔF508 homozygoten. Patiënten met de A455E mutatie hadden een hogere leeftijd bij diagnose, minder vaak pancreasinsufficiëntie en hadden geen diabetes mellitus. Tevens was de

longfunctie significant beter en hadden minder patiënten infecties met Pseudomonas aeruginosa.

Hoofdstuk 2.4: In ons centrum komt de A455E mutatie in 4,8% van alle CFTR mutaties voor, hetgeen hoger is dan de gerapporteerde mondiale frequentie van 0,1%. Alle zweettests van 18 patiënten met de A455E mutatie werden opnieuw bestudeerd. De uitslagen waren vaak 'normaal', éénmaal zelfs slechts 24 mmol/L chloride. Ook de chloride/natrium ratio kon niet altijd uitsluitsel geven.

Hoofdstuk 3 omschrijft electrofysiologische afwijkingen van chloride transport in de darm bij CF.

Hoofdstuk 3.1: Het transport van ionen in dunne- en dikkedarm van CF patiënten werd bestudeerd met behulp van een Ussing kamer. Het effect van alle drie typen signaaloverdracht was afwijkend, ofschoon de signaalstoffen zelf (cAMP, cGMP en Ca^{2+}) aanwezig waren. Er waren aanwijzingen dat K^+- in plaats van Cl^- secretie optrad na toevoeging van secretagogen.

Hoofdstuk 3.2: Afwijkingen in elektrolyt transport in het epitheel van rectumweefsel bij 11 CF patiënten en 11 controles werden bestudeerd door middel van ICM met een aangepaste Ussing kamer. Te oordelen naar de respons op de toediening van amiloride, bleek de elektrogene absorptie van natrium niet gestoord in de darm van CF patiënten, hetgeen in tegenstelling is tot wat in luchtweg epitheel wordt gevonden. De veranderingen in de kortsluitstroom in respons op stimuli van zowel door cyclisch adenosine monofosfaat (cAMP) als door Ca^{2+} geïnduceerde chloride secretie was sterk veranderd in alle biopten van de CF patiënten.

Gebruik makend van de Ca^{2+}-afhankelijke secretoire respons, kunnen CF patiënten in twee groepen worden onderverdeeld: een grote groep met afwezige anion (chloride) secretie maar actieve kation secretie en een groep met verminderde (residuele) chloride secretie. Zowel het feit dat

rectumbiopten eenvoudig af te nemen zijn, alsook de omkering van cAMP- of calcium-geïnduceerde kortsluitstroom, maken ICM een aantrekkelijk diagnosticum bij CF.

In Hoofdstuk 4 worden de resultaten van de studies van Hoofdstuk 2 en 3 samengevoegd om de pathofysiologie van CF te verduidelijken.

Hoofdstuk 4.1: Om vast te stellen of er een relatie bestaat tussen residuele intestinale chloride secretie enerzijds en geno- en fenotypen anderzijds, werden Ussing kamer experimenten verricht met rectum zuigbiopten van 51 CF patiënten. De CFTR mutatie was geïdentificeerd bij 89 van de 102 CF allelen. Geen zichtbare chloride secretie werd gevonden bij 30 CF patiënten (groep I). Geringe residuele chloride secretie werd gevonden bij 11 CF patiënten (groep II), terwijl relatief hogere residuele chloride secretie werd vastgesteld bij 10 patiënten (groep III). De pancreasfunctie was vaker behouden bij patiënten met residuele chloride secretie: 0% in groep I, 27 % in groep II en 60% in groep III. De leeftijd waarop de diagnose werd gesteld was in groep III significant hoger dan in groep I en II. Residuele chloride secretie werd vastgesteld bij sommigen van de 28 ΔF508 homozygote patiënten (3 in groep II en 1 in groep III), hetgeen aangeeft dat andere factoren dan het CFTR gen defect zelf het transepitheliale chloride transport beïnvloeden. De leeftijd ten tijde van diagnose bleek sterk gerelateerd aan de grootte van de secretoire respons, zelfs bij homozygote ΔF508 patiënten. We stelden vast dat residuele chloride secretie in CF de basis vormt voor behoud van pancreasfunctie en verlaat optreden van ziekteverschijnselen. Deze residuele secretie wordt niet uitsluitend door het CFTR genotype bepaald.

Hoofdstuk 4.2: Twee patiënten homozygoot voor de (stopcodon) mutatie G542X vertoonden in hun rectumbiopten residuele chloride secretie in reactie op carbachol. We konden niet vaststellen dat suppressie van het

G542X stopcodon of activiteit van het ernstig beschadigde CFTR eiwit leidde tot residueel chloride transport. Om deze reden lijkt het zeer onwaarschijnlijk dat CFTR activiteit verantwoordelijk is voor residueel intestinaal chloride transport welke bij G542X homozygoten werd vastgesteld. Deze bevinding is een sterke aanwijzing voor het bestaan van een alternatieve route voor chloride transport.

Hoofdstuk 4.3: In deze studie werden zweettest resultaten in ons ziekenhuis verricht in 9 jaar tijd (1983-1992) bij 1905 individuen bestudeerd. In 1825 personen bij wie de diagnose CF niet werd gemaakt, bedroeg de gemiddelde natriumconcentratie 15,5 ± 9,2 mmol/L. De bovengrens van het normale gebied (2SD boven het gemiddelde) is 34 mmol/L. Herevaluatie van alle 239 natriumconcentraties in zweet (80,9 ± 19,5 mmol/L) van 80 nieuw gediagnostiseerde patiënten (bij allen was tenminste een positieve zweettest vastgesteld), toonde aan dat 5% van de waarden onder de 50 mmol/L waren. De laagste waarde bedroeg 27 mmol/L. Op grond van deze resultaten bevelen wij aan om in geval van klinische verdenking op CF en zweettest waarden boven 30 mmol/L de zweettest te herhalen en zowel natrium als chloride te meten voor optimale beoordeling.

Hoofdstuk 4.4: Onderzoek met behulp van ICM werd bij 63 individuen toegepast. Vergeleken met controle waarden van gezonde individuen en CF dragers, waren er 12 personen met een afwijkende ICM die geen positieve zweettest hadden. In deze groep was de laagst verkregen natriumwaarde 34 mmol/L, de laagste chloridewaarde bedroeg 39 mmol/L. CFTR mutatie analyse wees uit: 3 ΔF508/ΔF508, 4 ΔF508/A455E, 4 ΔF508/onbekend, 1 G542X/onbekend. Bij 17 individuen met verhoogde zweettest waarden tot 72 mmol/L (3 ΔF508/onbekend, 13 onbekend/ onbekend en 1 ΔF508/R117H-7T) bleek ICM normaal. Concluderend stellen we vast dat ICM een nauwkeurig diagnosticum is voor CF. ICM is vooral geïndiceerd in gevallen waarbij verdenking bestaat op CF, terwijl zweettest en CFTR mutatie analyse geen uitsluitsel kunnen bieden.

Hoofdstuk 5: Discussie

In dit onderzoek werd een verband aangetoond tussen verschillende CFTR mutaties, de grootte van ICM en de klinische presentatie van CF. Er zijn sterke aanwijzingen dat er bij sommige CF patiënten sprake is van een alternatief, door Ca^{2+} gereguleerd chloride transport. ICM is een zeer gevoelige en specifieke test voor CF, meer nog dan de zweettest. De gevolgen hiervan voor de diagnostische benadering bij CF werden besproken.

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Curriculum Vitae

31 maart	1958	: Geboren te Aerdenhout.
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april	1988	: Stage afdeling Kindergeneeskunde Zuiderziekenhuis Rotterdam (Dr. R.N. Sukhai).
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oktober	1990	: Werkzaam als chef de clinique in het Sophia Kinderziekenhuis.
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overige		: Lid van Steering Committee International Registry of Cystic Fibrosis. Voorzitter consensus bijeenkomst "Diagnostiek en Behandeling van Cystic Fibrosis" van het CBO.

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