β -Adrenergic Sweat Responses in Cystic Fibrosis Heterozygotes with and without the Δ F508 Allele

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ABSTRACT. Cystic fibrosis (CF) causes early death for most homozygotes, yet has a carrier frequency among Caucasians of about 4–5%, suggesting a heterozygote advantage. The major defect in the CF gene is a three-base deletion leading to loss of a phenylalanine residue at position 508 (Δ F508) that accounts for about 68% of CF alleles in the North American population; the remaining 32% appears to consist of a large assortment of mutations. Sweat secretion in response to β -adrenergic stimulation is completely lacking in CF homozygotes and is reduced to $\frac{1}{2}$ normal in heterozygotes. To determine if this secretory process is affected by different CF alleles, we used the polymerase chain reaction technique with DNA obtained from peripheral leukocytes to determine retrospectively the presence or absence of the Δ F508 allele in 20 CF heterozygotes for whom sweat responses to β -adrenergic stimulation had previously been determined. Twelve of 20 subjects (60%) were positive for the Δ F508 mutation. The variance in sweat responses was not reduced in the Δ F508 group relative to the non- Δ F508 group, but a gender/allele interaction was noted. (*Pediatr Res* 29: 525–528, 1991)

Abbreviations

CF, cystic fibrosis
PCR, polymerase chain reaction
CFTR, cystic fibrosis transmembrane conductance regulator
 Δ F508, phenylalanine deletion at position 508 in CFTR coding sequence

CF is a recessive genetic disease that until recently caused early death for the majority of homozygotes. Despite that severe outcome, the carrier frequency among Caucasians is estimated to be about 4–5%. The CFTR gene, located on chromosome 7, codes for a 1480 amino acid protein that appears to be a transmembrane, ATP-binding protein (1, 2). A three-base deletion leading to loss of a phenylalanine residue at amino acid position 508 (Δ F508) accounts for about 68% of CF chromosomes (3); the remaining 32% appears to consist of a large assortment of mutations, each accounting for no more than a few percent of the remaining mutations (3). The high carrier frequency of such a seemingly improbable mutation is consistent with a heterozygote advantage (4, 5).

Received September 4, 1990; accepted January 30, 1991.

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Supported by Cystic Fibrosis Research, Inc., the Cystic Fibrosis Foundation, NIH Grants R01 DK 39659 and HL42368, and the Ross Mosier Fund.

A heterozygote advantage implies that heterozygotes manifest a desirable phenotype. Although the clinical syndrome of CF is recessive, certain phenotypic measures show a codominant pattern of expression (that is, heterozygotes show responses intermediate between normals and homozygotes). Codominant expression has been demonstrated most convincingly for sweat secretion in response to β -adrenergic stimulation, which is completely lacking in CF homozygotes (6), and is reduced to about $\frac{1}{2}$ normal in heterozygotes (7). This form of sweat secretion has no known function (normal thermal sweating is mediated cholinergically in humans). However, β -adrenergic sweating is of theoretical significance because it appears to depend upon a Cl^- -secretory mechanism for fluid production similar to that used by many other tissues, including intestinal enterocytes (8). In agreement with that possibility, CF homozygotes show an almost complete loss of intestinal Cl^- secretory responses to cAMP and cGMP-elevating agents (9–11). If CF heterozygotes have a proportional reduction in intestinal fluid loss after exposure to such agents, which include toxins that induce secretory diarrhea, they would have an appreciable advantage (5, 12).

Are secretory processes differentially affected by different CF alleles? The CF phenotype is notoriously variable, and some of the variability has recently been related to haplotype differences (3). Behm *et al.* (7) found that the coefficient of variation of the β -adrenergic/cholinergic sweat response ratio was nearly identical for the control and CF carrier populations, which is the predicted result if the magnitude of β -adrenergic sweating were determined completely by the normal allele, with CF alleles contributing nothing, regardless of their type. However, in a subsequent study, Sato and Sato (13), while confirming that heterozygote responsiveness is, on average, about $\frac{1}{2}$ that of normals, emphasized that the “vast individual variation of β -adrenergic responsiveness in CF heterozygotes may suggest that the basic defect of CF is more complex than the simple scenario of a one gene-one protein defect.”

Now that the CFTR gene has been isolated and one major mutation has been identified, it is possible to determine how much of the variation in heterozygote sweat responsiveness can be attributed to variation among CF alleles. In this study, we have retrospectively determined the presence or absence of the Δ F508 mutation in 20 heterozygotes who had previously been tested for sweat secretion (7), and have compared the responses of the Δ F508 group and the group having an unknown and presumably highly heterogeneous set of CF alleles.

MATERIALS AND METHODS

Twenty CF heterozygotes (parents of clinically defined CF children) were subjects in this study. Eighteen of these had previously participated in a study of pharmacologically stimulated sweat secretion (7); two additional heterozygotes were in-

cluded who had been sweat-tested subsequently, but before determination of their $\Delta F508$ status. For three subjects, allelic status was known as a result of genomic testing done elsewhere at their request. For the remaining 17 subjects, whole blood samples were obtained by venipuncture after informed consent. In addition, blood samples were obtained from two control subjects whose sweat responses overlapped those of heterozygotes.

DNA was isolated from peripheral leukocytes using modifications of the procedure published by Kunkel *et al.* (14). The $\Delta F508$ CF mutation in exon 10 of the CFTR gene was analyzed with the PCR. Using published sequences (3), four primers were synthesized (University of Calgary, Regional DNA Synthesis Laboratory) around the three-base mutation. One set of primers was designed to amplify the normal allele and another set to amplify only the $\Delta F508$ mutation (Fig. 1A). A third set was designed to amplify the entire exon. These three PCR reactions were carried out for DNA from each subject. A primer designated no. 2, consisting of the sequence 5' ACTTCTAATGATGATATGG 3', was common to all reactions. For amplifying the normal and non- $\Delta F508$ alleles, primer no. 2 was paired with a primer designated no. 1: 5' CATCATAGGAAACACCAAAG 3'. For amplifying the $\Delta F508$ allele, primer no. 2 was paired with a primer designated no. 3: 5' CATCATAGGAAACACAATG 3', which differs from primer no. 1 only in that the 3' A was changed to a T. Thus, for CF heterozygotes carrying the $\Delta F508$ allele, both reactions (with primers no. 1 and 2 versus primers no. 2 and 3) will yield products, but heterozygotes carrying a non- $\Delta F508$ mutation and subjects who have two normal alleles will have products only for the reaction using primers no. 1 and 2 (15). Finally, when primers no. 2 and 4 (5'

CTAGTTGGCATGCTTTGATG 3') were used, all of exon 10 was amplified regardless of the allele present. That PCR product was analyzed to detect a heteroduplex migrating aberrantly on a Nusieve (FMC Corp., Rockland, ME) gel (16, 17). The heteroduplex consists of one wild-type and one $\Delta F508$ DNA strand; its presence confirms $\Delta F508$ carrier status (Fig. 1B).

Results were analyzed with statistical programs included as part of the RS/1 data analysis software (BBN Software Products, Cambridge, MA). A *p* value of 0.05 was considered statistically significant. The coefficient of variation was calculated by dividing the SD by the mean.

RESULTS

The results are shown in Tables 1 and 2. Twelve of 20 heterozygotes (60%) tested positive for the $\Delta F508$ allele; eight out of 12 males (67%) and four out of eight females (50%) were positive. This difference is not significant ($\chi^2 = 0.56$), and no gender difference in distribution of the allele has been noted in much larger studies (3). The main result is that the variability of

Table 1. Heterozygote sweat rates and $\Delta F508$ allele

Subject no.*	Sex	Cholinergic response	β -adrenergic response	β -adrenergic/cholinergic ratio	$\Delta F508$
1	M	5.675	0.030	0.005	+
2	F	2.344	0.022	0.009	+
3	M	4.195	0.055	0.013	—
4	F	2.272	0.034	0.015	+
5	M	6.350	0.108	0.017	+
6	M	8.180	0.140	0.017	—
7	M	9.920	0.185	0.019	+
8	M	5.040	0.100	0.019	—
9	F	1.980	0.040	0.020	+
10	M	7.540	0.153	0.020	+
11	M	7.800	0.160	0.021	+
12	M	2.298	0.065	0.028	+
13	M	8.660	0.245	0.028	—
14	M	4.040	0.156	0.039	+
15	M	3.950	0.160	0.041	+
16	F	2.345	0.098	0.042	—
17	F	1.620	0.070	0.043	—
18	F	1.498	0.072	0.048	—
19	F	2.696	0.136	0.050	+
20	F	2.210	0.115	0.052	—

* Ranked in order of increasing β -adrenergic/cholinergic response ratio.

Fig. 1. A, PCR amplification of the $\Delta F508$ region. The first two lanes are reactions in a $\Delta F508$ carrier. The first lane, produced using primers no.1 and 2 (see Materials and Methods), is a 143-bp product from a normal allele (N). The second lane is a 140-bp product using primers no. 2 and 3, and is three base pairs shorter because it derives from a $\Delta F508$ allele (C). A product in both reactions is observed in a carrier of the $\Delta F508$ mutation. A noncarrier or a CF carrier who does not have the $\Delta F508$ mutation has results as shown in the third and fourth lanes: no product using primers no. 2 and 3. B, heteroduplex analysis of exon 10 PCR-amplified products. In this analysis, exon 10 was amplified using primers no. 2 and 4 (see text) nonspecifically, regardless of the $\Delta F508$ mutation. If the mutation is not present, the amplification product is a single band, as shown in N. A $\Delta F508$ carrier has a reaction product as shown in C, in which an extra, slowly migrating band is present. This is a heteroduplex band of $\Delta F508$ and normal DNA strands as indicated. Homozygotes for $\Delta F508$ yield a single band like normals, but a heteroduplex forms when the product is mixed with amplified exon 10 from an individual who totally lacks $\Delta F508$ alleles (not shown).

Table 2. Mean β -adrenergic/cholinergic-induced sweat ratios for $\Delta F508$ and non- $\Delta F508$ males and females, and coefficients of variation (CV)

	$\Delta F508$	Non- $\Delta F508$	All alleles
Males			
n	8	4	12
Ratio	0.024 $\pm$ 0.012	0.019 $\pm$ 0.006	0.022 $\pm$ 0.010
CV	0.50	0.32	0.45
Females			
n	4	4	8
Ratio	0.024 $\pm$ 0.018	0.046 $\pm$ 0.005	0.035 $\pm$ 0.017
CV	0.75	0.11	0.49
Males + Females			
n	12	8	20
Ratio	0.024 $\pm$ 0.013	0.033 $\pm$ 0.015	0.027 $\pm$ 0.015
CV	0.54	0.45	0.56

sweat responses in the $\Delta F508$ group was not less than in the non- $\Delta F508$ group. This was true for measures of both β -adrenergic sweat responses and for the ratio of β -adrenergic to cholinergic sweating. The coefficients of variation for the various groups are shown in Table 2.

Table 1 shows subjects' ranks, ordered according to ratio of β -adrenergic to cholinergic sweat responses (as defined in ref. 7). Although this measure is probably the least biased estimate of β -adrenergic sweating (7), it reduced but did not entirely eliminate the large disparity in sweating responses between males and females in our sample. Thus, the mean cholinergic sweat response of males was significantly larger than the corresponding value for females (6.13 ± 2.3 versus 2.12 ± 0.4 , $p = 0.001$) and the mean β -adrenergic sweat response of males was significantly larger than the corresponding value for females (0.13 versus 0.07 , $p < 0.04$). The ratio of the responses just missed significance at the 0.05 level ($p = 0.0512$; all tests two-tailed t tests), and was significant with analysis of variance ($F = 6.16$, $p = 0.025$).

Comparison of mean sweat ratios for all $\Delta F508$ versus non- $\Delta F508$ subjects did not differ significantly (0.024 versus 0.033 , $p = 0.17$), nor did they differ when $\Delta F508$ versus non- $\Delta F508$ males and females were compared separately, although the female difference was close to significance (0.024 versus 0.046 for $\Delta F508$ females versus non- $\Delta F508$ females, $p = 0.054$). A two-way analysis of variance for sweat response ratio versus allele and gender showed a statistically significant interaction ($F = 6.11$, $p = 0.025$).

Two control subjects who had β -adrenergic/cholinergic sweat ratios among the lowest (equivalent to the mean value for the heterozygote population) were both negative for the $\Delta F508$ mutation.

DISCUSSION

In our study, we controlled for one aspect of genotypic variability in CF heterozygotes by determining which subjects had the $\Delta F508$ mutation. The variability of the sweat responses of $\Delta F508$ subjects was not less than that of subjects with unknown CF alleles. Because the number of mutations other than $\Delta F508$ is quite large (18), with none accounting for more than a few percent of CF chromosomes, the major source of the variability in this phenotypic measure does not result from differences among CF alleles. This conclusion also follows from the previous finding (7), that control and CF heterozygote responses had essentially equivalent coefficients of variation, even though the mean β -adrenergic/cholinergic response ratio of heterozygotes was reduced to about $\frac{1}{2}$ of normal. Both results are most consistent with the interpretation that each CF allele is null with regard to the β -adrenergic sweat responses, a conclusion in agreement with the total lack of β -adrenergic sweating in CF homozygotes (6, 7).

The conclusion that all CF alleles are null with regard to β -adrenergic sweating must be tempered by the small size of our sample. The β -adrenergic sweat test is the only known *in vivo* measure that both discriminates absolutely between normal and CF individuals and reveals reliable mean differences between groups of control and CF heterozygote subjects (6, 7). Unfortunately, it is a difficult and time-consuming test that requires repeated measures for adequate reliability. For that reason, the number of subjects in this study was limited, which, together with the significant differences in male and female sweat rates and the unequal gender distribution of the $\Delta F508$ allele in our sample, limited the power of our test to detect phenotypic differences that might have existed among different allelic groups. Given these theoretical and practical reasons for not expecting to find differences, what is the best interpretation of the significant gender/allele interaction? CF is an unusual disease in that it affects females more severely than males (19), so an allele/gender interaction is not implausible. However, we speculate that

this difference arose by chance, and may disappear when larger samples are tested.

The evidence that all CF alleles are null with regard to β -adrenergic sweat secretion contrasts with clear evidence that pancreatic function is strongly affected by different alleles (3, 20). To our knowledge, a specific comparison of β -adrenergic sweat responses in pancreatic-sufficient and pancreatic-insufficient CF homozygotes has not yet been carried out, so it may yet emerge that pancreatic-sufficient subjects also have some sparing of their sweat responses. However, a true difference in allele-related variability among tissues might arise in several ways. The function of the CFTR in different tissues may well differ. Alternatively or in addition, the CFTR protein itself may differ among tissues, inasmuch as alternative splicing, now demonstrated for CFTR message (21), may give rise to isoforms of CFTR that are differentially affected by various mutations. In extreme instances, a mutation that completely disrupts one isoform may have no effect on a splice variant. If CFTR isoforms display tissue specificity, some mutations might spare certain tissues.

In summary, our results suggest that the major determinant of the β -adrenergic/cholinergic sweat secretion ratio in CF heterozygotes is the single, wild-type CFTR allele. However, the possibility that other CF alleles may contribute to β -adrenergic sweat secretion cannot be excluded unequivocally for the reasons given above.

Acknowledgments. The authors thank our subjects for their interest and cooperation, Anne Camber and Margit Nemeti for assistance in DNA analysis, and Grace Hagiwara for assistance in preparing the manuscript. We also thank anonymous reviewers for helpful suggestions.

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Announcements

Charles E. Culpeper Foundation Scholarships in Medical Science

The Charles E. Culpeper Foundation is currently accepting applications for its 1992 Scholarships in Medical Science Program designed to support the career development of academic physicians.

Up to three awards of \$100,000 per year for 3 years will be made to United States medical schools on behalf of candidates who are U.S. citizens, who have received their M.D. degree from a U.S. medical school in 1983 or later, and who are judged worthy of support by virtue of the quality of their research proposals. All scientific research relevant to human health is eligible for consideration. No institution may nominate more than one candidate.

In selecting awardees, emphasis will be on identifying young physicians with clear potential for making substantial contributions to science as academic physicians. Since January 1988, twelve physicians have been selected as Charles E. Culpeper Foundation Medical Scholars, three each in the years 1988, 1989, 1990, and 1991.

Deadline for applications is August 15, 1991. Awards will be announced by January 15, 1992 for activation on or about July 1, 1992. Application forms and instructions may be obtained by contacting the Charles E. Culpeper Foundation at Financial Centre, 695 East Main Street, Suite 404, Stamford, CT 06901.

Peter J. Hotez, M.D., Ph.D. Receives Charles E. Culpeper Foundation Scholarship in Medical Science

Peter J. Hotez, M.D., Ph.D., has been named a recipient of the Charles E. Culpeper Foundation Scholarship in Medical Science for 1991. Through this award, Dr. Hotez will receive \$100,000 a year for 3 years to fund his research at Yale University School of Medicine in New Haven, CT, where he is currently an instructor in both the Departments of Pediatrics and Epidemiology and Public Health.

"The Charles E. Culpeper Foundation Scholarships in Medical Science are designed to support the career development of young academic physicians," said Francis J. McNamara, Jr., President of the Culpeper Foundation. "The Foundation's objective with this scholarship program is to provide significant support to young physicians of high potential achievement, like Dr. Hotez, who are committed to careers in academic medicine," added McNamara.

Dr. Hotez and two additional investigators were selected for the scholarship awards from a competitive field of applicants nominated by their medical schools. The two additional scholarship recipients are Sandra Lee Hoffman, M.D., Ph.D., Assistant Professor of Internal Medicine and Molecular Genetics at the University of Texas Southwestern Medical Center, Dallas, TX and Michael Whitlow, M.D., Ph.D., Assistant Professor of Dermatology at the New York University Medical Center in New York City.

Dr. Hotez's research as a Charles E. Culpeper Medical Scholar will focus on understanding the molecular basis and prevention of hookworm disease, a debilitating iron-deficiency anemia that frequently occurs in children in underdeveloped nations.

The Charles E. Culpeper Foundation Scholarships in Medical Science Program was established in 1987 as an extension of the Foundation's ongoing commitment to medical science, which has totaled more than \$20 million since 1970. The Foundation is a private, nonprofit, charitable foundation established under the will of the late Charles E. Culpeper, one of the early pioneers in the bottling and marketing of Coca-Cola. In recent years, the Foundation has dispensed more than \$6 million a year to activities in health, science, technology, education, youth, the arts, and the administration of justice.