0031-3998/03/5405-0627
PEDIATRIC RESEARCH
Vol. 54, No. 5, 2003
Printed in U.S.A.

ARTICLES

Evaluation of MRP1-5 Gene Expression in Cystic Fibrosis Patients Homozygous for the ΔF508 Mutation

ILSE HURBAIN, ISABELLE SERMET-GAUDELUS, BENOIT VALLÉE, MARIE-NOËLLE FEUILLET, GÉRARD LENOIR, JEAN-FRANÇOIS BERNAUDIN, ALEKSANDER EDELMAN, AND ANNE FAJAC

Service d'Histologie-Biologie Tumorale, UPRES EA3499, Hôpital Tenon, 75020 Paris [I.H., J.-F.B., A.F.]; Inserm U467, Faculté Necker, 75015 Paris [I.S.-G., B.V., A.E.]; Service de Pédiatrie, Hôpital Necker, 75015 Paris [I.S.-G., G.L.]; Service de Biochimie, Hôpital Necker, 75015 Paris, France [M.-N.F.]

ABSTRACT

Cystic fibrosis (CF), due to mutations of the cystic fibrosis transmembrane conductance regulator (CFTR), exhibits a wide range of disease severity, even among ΔF508 homozygous patients, and the mechanisms of this variability have yet to be elucidated. In view of the close structural homology and possible functional overlap between CFTR and Multidrug Resistance-associated Proteins (MRPs), MRPs were investigated as potentially relevant factors in CF pathophysiology. *MRP1-5* gene expression was analyzed in nasal respiratory epithelial cells from ΔF508 homozygous patients ($n = 19$) and control subjects ($n = 20$) using semiquantitative RT-PCR. Significantly lower *MRP1* and *MRP5* transcript levels were found in CF patients than in control subjects. *MRP1* and *MRP5* transcript levels were strongly correlated ($r = 0.71$). In CF patients, low *MRP1* transcript levels were associated with more severe disease as assessed by the Shwachman score. A relation was also observed between *MRP1* levels and presence of a cAMP-independent chloride conductive pathway, as determined by a halide-sensitive fluorescent assay. These results suggest that MRPs, especially MRP1, might play a role in CF phenotype and might therefore constitute a target for a novel pharmacotherapy of CF. **(*Pediatr Res* 54: 627–634, 2003)**

Abbreviations

CF, cystic fibrosis
CFTR, cystic fibrosis transmembrane conductance regulator
dNTP, deoxynucleoside triphosphate
FEV1, forced expiratory volume
FVC, forced vital capacity
MDR1, multidrug transporter 1
Pgp, P-glycoprotein
MRPs, multidrug resistance-associated proteins
RT-PCR, reverse transcription-PCR
SPQ, 6-methoxy-N-3-sulfopropylquinolinium

Cystic fibrosis (CF) is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a member of the ATP Binding Cassette (ABC) transporter superfamily. The well-established function of CFTR is to act as a cAMP-activated chloride channel. However, CFTR has many other roles, such as regulating epithelial transport proteins (1–3), vesicle trafficking (4) and acidification of intracellular organelles (5). CF, characterized by bronchopulmonary disease and pancreatic insufficiency, presents a wide range of disease severity, especially in terms of lung disease, even among patients harboring the same mutation.

We have previously shown that the cAMP-dependent chloride channel function of CFTR might be relatively conserved in some patients with a ΔF508 homozygous deletion, which is the most frequent mutation among Caucasians (6). We have shown that this heterogeneity of CFTR function is linked to the clinical course of the disease. Therefore, certain factors may modulate or complement CFTR function in some CF patients. The factors responsible for this residual CFTR function have not yet been elucidated, but might be exogenous factors such as environmental factors, or endogenous factors such as so-called "modifier" genes. Whether this residual chloride channel function *per se* causes less severe disease or whether another

Received January 14, 2003; accepted July 16, 2003.
Correspondence: Anne Fajac, M.D., Service d'Histologie-Biologie Tumorale, Hôpital Tenon, 4 rue de la Chine 75020 Paris, France; e-mail: anne.fajac@tnn.ap-hop-paris.fr
Supported by grants from "ABCF Protéines" Association, "Vaincre la Mucoviscidose" Association and by Université Paris VI.

DOI: 10.1203/01.PDR.0000090926.16166.3F

residual nonchannel CFTR function may be more relevant in CF pathophysiology remains to be determined.

Multidrug Resistance-associated Proteins (MRPs), which form a family of at least nine recently identified membrane proteins, share the closest structural homology with CFTR, belonging to the same subfamily C of the ABC transporter superfamily (7–8). Some MRPs, especially MRP1 and MRP5, are expressed in most human tissues, while others, such as MRP2 and MRP3, have a more limited expression pattern mainly in liver and gut (9–10). MRPs are characterized by their ability to export organic anions, either glutathione, glucuronate and sulfate conjugated molecules or unmodified substances, out of cells. Although the physiologic functions of these proteins are still under investigation, there is evidence to suggest that these proteins might be involved in drug resistance, detoxification, inflammation and oxidative stress (11–12).

In addition to the structural homology between CFTR and MRPs, there might be an overlap in functions between these proteins. The hypothesis that CFTR could be complemented by MRPs was driven from the case of a CF patient whose lung function improved after receiving anticancer drugs known to induce MRPs (13). In *in vitro* models, CFTR has been recently shown to efflux organic anions out of cells (14) and transport glutathione (15), which are known to be two main functional characteristics of MRPs.

In the present study, we therefore investigated *MRPs* gene expression and chloride conductive pathways in nasal respiratory epithelial cells from CF patients. We examined *MRP1*,-*2*,-*3*,-*4* and -*5* transcript levels by semiquantitative RT-PCR in ΔF508 homozygous patients and control subjects. In CF patients, we also examined the relationship between *MRPs* gene expression and clinical status on one hand and the existence of a chloride conductive pathway, on the other, by using a halide-sensitive SPQ (6-methoxy-N-3-sulfopropylquinolinium) fluorescent dye assay (6).

METHODS

Subjects

This study was carried out on healthy control subjects ($n = 20$) and ΔF508 homozygous patients ($n = 19$). All CF patients (9 girls and 10 boys; mean age: 12 y, range: 5–30) presented pancreatic insufficiency. Control subjects had a normal sweat test and had been negatively screened for the 30 most frequent CFTR mutations. Exclusion criteria were recent upper respiratory tract infection, smoking and nasal polyps.

Clinical status was assessed by measuring the forced expiratory volume (FEV1) and the forced vital capacity (FVC) to assess pulmonary function and by the Shwachman score (16) to assess overall disease severity. FEV1 and FVC were expressed as percentages of predicted values for age and sex. Shwachman score is based on four criteria, which are given equal weight, namely 25 points: the general activity, the findings on physical examination, the nutritional status and the findings on chest roentgenograms (maximum score is therefore 100).

This study was approved by our institutional review board. Before the study, written informed consent was obtained from the older children and from the guardian of each child.

Cells

Nasal respiratory epithelial cells (approximately 1×10^6 cells per subject) were obtained by gentle brushing of the middle turbinate. Brushes were immediately placed in cold Dulbecco's Modified Eagle's Medium (DMEM). An aliquot of scraped cells was cytospun on slides (60 g, 7 min, Cytospin Shandon, France) to check the cell type of the samples under light microscopy. The remaining cells were centrifuged (600 g, 2 min, 4°C) and the pellet was placed in Trizol™ reagent (GIBCO-BRL, Les Ulis, France) for further RNA extraction.

Each sample was assessed for cell type by taking into account the presence of necrotic cells, squamous, inflammatory (polymorphonuclear cells) and respiratory epithelial cells (Table 1). Samples containing necrotic cells, squamous cells or no respiratory epithelial cells were eliminated (*i.e.* classes 1, 2, or 3).

RT-PCR Analysis

RNA isolation and cDNA synthesis. Total RNA was isolated using Trizol™ reagent (GIBCO-BRL) by acid guanidine thiocyanate-phenol-chloroform and precipitated by isopropanol at −20°C. RNA (2 μg) was placed in a final volume of 20 μL containing 100 units of MMLV Superscript II reverse transcriptase (GIBCO-BRL), 500 μM dNTP, 10 mM DTT, and 200 ng of random hexanucleotide primers (Pharmacia Biotech, Saint Quentin en Yvelines, France). cDNA was then diluted 5-fold in sterile water.

PCR amplification. The specificity of the primers used (Table 2) has been previously reported by our laboratory (17). Amplification of *β2microglobulin* gene was used as an internal control.

cDNA (5 μL) was added to a 25 μL final volume containing 2 U of Taq DNA polymerase (Boehringer Mannheim, Meylan, France), 500 μM dNTP, variable $MgCl_2$ concentration (Table 2), 20 pmoles of each forward and reverse primer, 10 pmoles of *β2microglobulin* forward and reverse primers and 1 μCi of [αP^{32}]dCTP (Amersham, Les Ulis, France). The samples were amplified for the adequate number of cycles (30 for all genes except *MRP5* and 32 for *MRP5*) corresponding to the expo-

Table 1. *Criteria for the cell type class of nasal samples*

Cell type	Class
Necrotic cells or absence of respiratory cells	
Necrotic cells	1
Absence of cells	2
Squamous cells or rare bare nuclei	3
Presence of inflammatory (polymorphonuclear) cells	
Epithelial cells associated with > 40% polymorphonuclear cells	4
Epithelial cells associated with 20–40% polymorphonuclear cells	5
Epithelial cells associated with < 20% polymorphonuclear cells	6
Absence of inflammatory cells	
Ciliated cells <50%	7
Ciliated cells >50%	8

Table 2. *Sequences of the forward and reverse primers used for PCR amplifications*

Genes	Primer sequences (5′-3′)	$MgCl_2$ (mM)	Fragment length (bp)
MRP1	GGACCTGGACTTCGTTCTCA CGTCCAGACTTCATCCG	1.5	292
MRP2	CTGCCTCTTCAGAATCTTAG ATAACCCAAGTTGCAGGCT	1.8	241
MRP3	CTCAATGTGGCAGACATCGG GGGAGCTCACAAACGTGTGC	1.8	178
MRP4	CCATTGAAGATCTTCCTGG GGTGTTCAATCTGTGTGCA	2.5	240
MRP5	GGGATAACTTCTCTCAGTGGG GGAATGGCAATGCTCTAAAG	2.5	383
MDR1	GGTGCTGGTTGCTGCTTACA TGGCCAAATCACAAGGGT	1.5	291
β2micro-globulin	ACCCCCACTGAAAAAGATGA ATCTTCAAACCTCCATGATG	1.8 (MRP1–4) 2.5 (MRP5)	114

nential range of amplification reaction and consisting of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 1 min 30 s of elongation at 72°C. The positive control used for *MRP1* and *MDR1* genes was the KB8.5 cell line. The positive controls for *MRP2, -3, -4*, and *-5* genes were human liver for *MRP2* and *-3* genes, human lung for *MRP4* gene and human skeletal muscle for *MRP5* gene. Negative controls were included for each amplification (water instead of RNA for reverse transcription and water instead of cDNA for PCR).

Semiquantitative analysis of PCR products. Amplification of the *β2microglobulin* gene was performed for each sample to account for variability in mRNA extraction, mRNA quantitation and cDNA synthesis. Amplification products were submitted to electrophoresis on 8% polyacrylamide gels at 750 V for 3.5 h. After exposure of gels to X OMAT films (Eastman Kodak), autoradiographs were scanned using a Bio-Rad densitometer (Bio-Rad, Ivry sur Seine, France). Each gene mRNA content was expressed as the ratio of the densitometric value of each gene amplification product to that of *β2microglobulin* amplification product.

Functional Assay for Chloride Conductive Pathways

Transmembrane chloride conductive pathways were measured in nasal ciliated cells either under basal conditions or after addition of cAMP agonists using a halide-sensitive SPQ fluorescent dye assay, as previously reported (6). Briefly, this method measures the rate of chloride transport as the change in fluorescence of intracellular SPQ in response to exchange of extracellular chloride with nitrate (NO_3^-), an anion that passes through CFTR, but does not quench indicator fluorescence unlike chloride. cAMP agonists consisting of 25 μM forskolin, 100 μM isobutylmethylxanthine and 500 μM 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate were added to activate CFTR resulting in chloride efflux, NO_3^- influx and increased fluorescence. To enhance the sensitivity of the assay, chloride was replaced by iodide (I^-), as CFTR is permeable to I^- and SPQ fluorescence is more strongly quenched by I^- than by chloride.

Basal anion conductance was defined as the maximum fluorescence change ($\Delta F_{basal}/\Delta t$) after replacing I^- with NO_3^-. The cell was considered to have a basal chloride conductive pathway when the maximum fluorescence change $\Delta F_{basal}/\Delta t$ was >0.1. cAMP-dependent anion conductance was defined as the greatest fluorescence change ($\Delta F_{cAMP}/\Delta t$) after adding cAMP agonists. The cell was considered to have a cAMP-stimulated chloride conductive pathway when the maximum fluorescence change after addition of the cAMP agonists $\Delta F_{cAMP}/\Delta t$ was >0.1. Results were expressed for each subject studied as the percentage of ciliated cells with basal chloride conductive pathway and the percentage of ciliated cells with cAMP-stimulated chloride conductive pathway. The experimental protocol used is shown in Fig. 1.

Statistics

Statistical calculations and tests were performed using Stat View 5.0 software (Abacus, CA, U.S.A.). The limit of statistical significance was defined as $p \leq 0.05$.

Means were compared using t tests. Results are expressed as mean ± SEM. Among CF patients, a subgroup with lower *MRP1* levels and another subgroup with lower *MRP5* levels were found (Fig. 2) and relationship between *MRP1* or *MRP5* transcript levels and clinical status (age, FEV1, FVC and Shwachman score) was therefore investigated. The highest *MRP1* and *MRP5* values in the subgroups with lower levels were 0.145 and 0.077, respectively. The values 0.15 and 0.08 were therefore chosen as cut-off values to analyze differences in clinical status according to *MRP1* or *MRP5* levels, respectively.

To investigate whether there was a difference in *MRP1* or *MRP5* transcript levels (≤ 0.15 or >0.15 and ≤ 0.08 or >0.08 for *MRP1* and *MRP5*, respectively) and cell type (inflammatory or non inflammatory), the Fisher exact test was used.

Correlation coefficients were calculated for each combination of mRNA levels.

RESULTS

Cell type of the analyzed nasal samples. Nasal samples containing necrotic cells, squamous cells or no respiratory

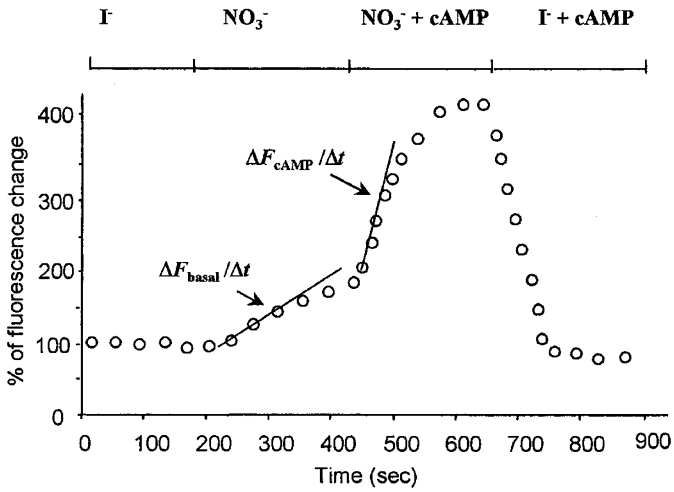

Figure 1. Experimental protocol for SPQ fluorescence assay to measure chloride conductive pathways in nasal ciliated cells. The cells were superfused with I^-, NO_3^- and then with cAMP agonists.

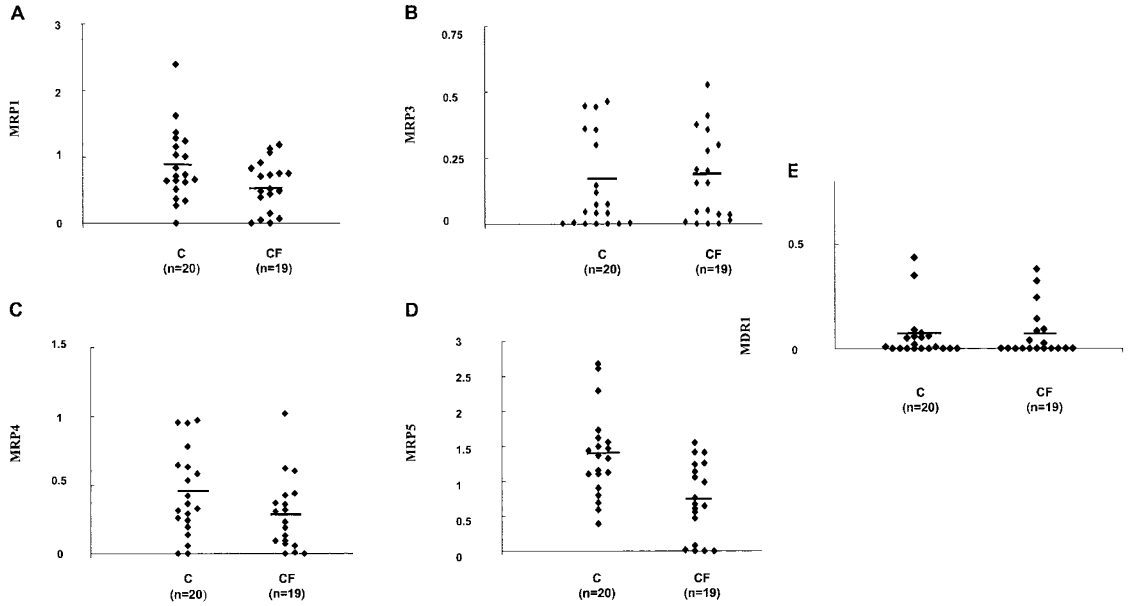

Figure 2. Semiquantitative analysis of *MRP1* (*A*), *MRP3* (*B*), *MRP4* (*C*), *MRP5* (*D*), and *MDR1* (*E*) mRNA levels in controls (C) and ΔF508 homozygous patients (CF) by densitometric evaluation of each gene PCR product compared with *β2microglobulin* PCR product. Horizontal line: mean level of expression for each data set.

epithelial cells were eliminated for further analysis (Table 1, classes 1–3). No difference in cell type, as assessed by the cell class, of the remaining samples, was observed between control subjects and ΔF508 homozygous patients (Table 3). In both groups, the majority of the samples (90%) contained epithelial cells (*i.e.* ciliated, goblet and basal cells) with no inflammatory cells and were classified 7 or 8 depending on the proportion of ciliated cells.

MRPs and MDR1 transcript levels. Individual results are presented in Table 3. Variable *MRP1* transcript levels were observed in control subjects (0.87 ± 0.12) and ΔF508 homozygous patients (0.56 ± 0.09) with significantly lower levels in CF patients ($p < 0.05$) (Fig. 2*A*). A subgroup of CF patients (n = 5, about 25%) with undetectable or very low transcript levels (≤0.15) could be distinguished. No difference in cell type was observed between samples from CF patients with *MRP1* mRNA levels lower or higher than 0.15 as percentages of samples without inflammatory cells (*i.e.* classes 7–8) were similar (80% *versus* 92%, respectively; Fisher exact p value = 0.5).

We found no detectable *MRP2* transcripts in any of the nasal samples from all subjects analyzed.

MRP3 transcript levels were similar in controls (0.15 ± 0.04) and CF patients (0.16 ± 0.04) with undetectable or low *MRP3* transcript levels in both groups (Fig. 2*B*).

Variable *MRP4* transcript levels, including low or undetectable mRNA levels, were observed in controls (0.43 ± 0.07) and CF patients (0.28 ± 0.06) (Fig. 2*C*) with no statistical difference between the two groups.

Analysis of *MRP5* mRNA showed variable levels among controls (1.37 ± 0.14) and CF patients (0.73 ± 0.12) (Fig. 2*D*). However, *MRP5* transcripts (>0.08) were observed in all controls, but were undetectable or very low (≤0.08) in about 25% of CF patients (n = 5) and this difference between CF patients and controls was statistically significant ($p < 0.01$). Cell type was similar in samples from CF patients with *MRP5* mRNA levels lower or higher than 0.08 (percentages of samples without inflammatory cells: 100% *versus* 85%, respectively; Fisher exact p value > 0.9). Three patients had low mRNA levels for both *MRP1* and *MRP5* genes (Table 3).

MDR1 gene encodes for Pgp which is an ABC transporter involved in efflux of various compounds and belonging to another subfamily (B) than subfamily C which includes CFTR and MRPs. *CFTR* and *MDR1* genes have been shown to exhibit complementary patterns of epithelial expression, suggesting analogous roles in epithelial cells (18). Therefore, in addition to MRPs, we also analyzed *MDR1* gene expression in CF patients. Similar low *MDR1* levels were detected in controls (0.06 ± 0.03) and CF patients (0.07 ± 0.03) with undetectable transcripts in about one half of subjects in each group (Fig. 2*E*).

Correlation analysis of gene transcript levels. Correlation coefficients (r) for the correlation of *MRPs* and *MDR1* mRNA levels were calculated taking into account all subjects ($n = 39$) (Table 4). Among the various possible combinations of mRNA data sets, *MRP1* and *MRP5* transcript levels demonstrated the highest correlation ($r = 0.71$, $p < 0.0001$) (Fig. 3).

Relation with clinical status. As an overlap in *MRP1* and *MRP5* transcript levels was found between some CF patients and some controls, we investigated whether CF patients exhibiting lower *MRP1* (≤0.15, n = 5) or lower *MRP5* transcript levels (≤0.08, $n = 5$) had a different clinical status on the basis of age, FEV1, FVC and Shwachman score (Table 5). No association was found between *MRP1* or *MRP5* transcript levels and age, FEV1 or FVC (Table 6). However, patients with lower *MRP1* transcript levels had significantly lower Shwachman scores, although in this subgroup one patient had a maximal score of 100. No such relation was observed for *MRP5* gene.

Table 3. *MRPs and MDR1 transcript levels in healthy controls (C, n = 20) and ΔF508 homozygous patients (CF, n = 19)*

Subject		Cell class	MRP1	MRP3	MRP4	MRP5	MDR1
1	C	8	0.708	0.443	0.535	1.367	0.436
2	C	8	0.730	0.447	0.632	1.441	0.349
3	C	8	0.386	0	0	0.589	0
4	C	6	1.149	0.360	0.242	1.158	0.075
5	C	7	1.366	0	0.644	1.730	0
6	C	8	0.658	0.005	0.363	0.694	0
7	C	8	1.280	0	0.056	2.291	0
8	C	8	0.644	0.001	0.138	1.103	0.007
9	C	8	0.639	0.046	0	0.799	0.009
10	C	8	1.237	0.356	0.581	1.325	0
11	C	8	0.834	0.041	0.781	1.502	0
12	C	8	2.391	0.298	0.971	2.613	0.051
13	C	8	1.617	0.146	0.423	2.679	0
14	C	8	0.002	0.075	0.313	1.103	0
15	C	8	1.001	0	0.329	1.467	0
16	C	8	1.030	0.073	0.258	1.124	0.020
17	C	8	0.266	0.004	0.194	0.392	0.089
18	C	8	0.335	0.119	0.954	1.622	0.058
19	C	8	0.619	0.463	0.950	1.556	0.059
20	C	6	0.509	0.041	0.290	0.901	0.054
21	CF	8	0.145	0	0	0	0
22	CF	7	0.001	0	0.091	0	0.025
23	CF	8	0	0.375	0.622	1.242	0.091
24	CF	8	0.438	0.201	0.426	0.608	0.083
25	CF	8	0.749	0.298	0.319	0.643	0
26	CF	4	0.066	0.154	0.073	0.470	0
27	CF	nd	0.707	0.155	0.057	0.762	0.039
28	CF	7	0.724	0.206	0.188	1.134	0
29	CF	7	0.516	0	0.008	0.077	0
30	CF	8	0.909	0.014	0.231	1.256	0
31	CF	8	0.822	0.045	0.603	1.414	0
32	CF	7	0.391	0.035	0.368	0.670	0.322
33	CF	8	1.119	0.409	0.131	1.411	0.243
34	CF	8	0.481	0.007	0.307	0.558	0
35	CF	8	1.064	0.034	0.437	0.001	0
36	CF	6	0.752	0.051	0	0.989	0
37	CF	8	0.044	0.277	0.094	0.017	0
38	CF	8	0.485	0.526	0.359	1.056	0.142
39	CF	8	1.177	0.356	1.021	1.554	0.380

Table 4. *Correlation coefficients (r) for the correlation of transcript levels in controls and ΔF508 homozygous patients (n = 39)*

	MRP1	MRP4	MRP5	MDR1
MRP3				
r	0.14	0.42	0.28	0.55
p	0.39	0.008	0.09	0.0003
MRP1				
r		0.37	0.71	0.03
p		0.02	<0.0001	0.85
MRP4				
r			0.55	0.34
p			0.0003	0.03
MRP5				
r				0.15
p				0.36

Relation with basal and cAMP-dependent chloride conductive pathways. Given that the cAMP-dependent chloride channel function of CFTR varies between ΔF508 homozygous patients and that this variation is related to the severity of CF (6), we investigated whether *MRP1* or *MRP5* gene expression could be related to CFTR function. We measured cAMP-dependent chloride conductive pathway using a halide-sensitive SPQ fluorescent dye assay in nasal ciliated cells from CF patients. We also measured chloride conductive pathway under basal conditions, *i.e.* before addition of cAMP agonists. As we previously reported (6), the percentage of ciliated cells exhibiting basal chloride conductance varies among healthy controls as it is the case for cAMP-dependent chloride conductance. The lowest percentages were 63% and 50% for basal and cAMP-dependent chloride conductances, respectively (6). These values were therefore chosen as cut-off values to analyze conductances in CF patients (see Table 5 for individual results).

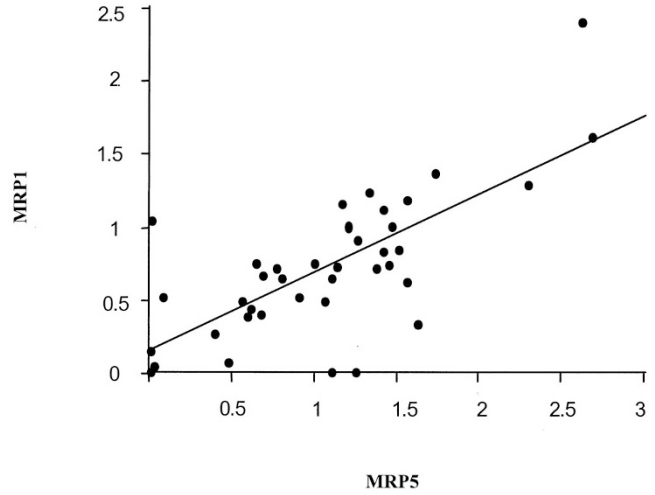

Figure 3. Correlation between *MRP1* and *MRP5* transcript levels in controls and ΔF508 homozygous patients ($n = 39$). $r = 0.71$, $p < 0.0001$.

As expected, there was a significant difference in the percentage of ciliated cells exhibiting a cAMP-dependent chloride conductive pathway between controls and CF patients (83 ± 5% *versus* 45 ± 7%, $p < 0.01$). There was also a significant difference in the percentage of ciliated cells exhibiting a basal chloride conductive pathway between controls and CF patients (98 ± 2%*versus* 68 ± 7%, $p < 0.01$).

When considering cAMP-dependent chloride conductive pathway, no difference in *MRP1* or *MRP5* transcript levels was observed between CF patients with less than or more than 50% cells showing cAMP-dependent chloride conductive pathway. However, when considering basal chloride conductive pathway, significantly lower *MRP1* transcript levels were found for CF patients with less than 63% cells exhibiting basal chloride conductive pathway ($p < 0.01$) (Fig. 4). Although lower *MRP5* transcript levels were also found for CF patients with less than 63% cells with basal chloride conductive pathway (0.49 ± 0.19 *versus* 0.84 ± 0.15%), this difference was not significant.

DISCUSSION

The main findings of the present study are: *i*) the lower *MRP1* and *MRP5* transcript levels in ΔF508 homozygous patients compared with control subjects and *ii*) the associations of *MRP1* transcript levels with both clinical status and presence of a cAMP-independent chloride conductive pathway in CF patients. These results suggest a potential interrelation between

Table 5. *Clinical status (according to age, FEV1, FVC, and Shwachman score) and chloride conductive pathways in CF patients*

Patient		Sex*	Age	MRP1	MRP3	MRP4	MRP5	MDR1	FEV1	FVC	Shwachman score	Number of cells tested	% cells with $\Delta F_{basal}/\Delta t>0.1$**	% cells with $\Delta F_{cAMP}/\Delta t>0.1$**
21	CF	M	16	0.145	0	0	0	0	74	61	60	5	0	0
22	CF	M	8	0.001	0	0.091	0	0.025	120	123	100	7	0	100
23	CF	M	12	0	0.375	0.622	1.242	0.091	77	92	70	3	33	0
24	CF	M	7	0.438	0.201	0.426	0.608	0.083	98	62	90	13	39	31
25	CF	F	11	0.749	0.298	0.319	0.643	0	97	89	80	28	46	46
26	CF	M	16	0.066	0.154	0.073	0.470	0	85	90	80	15	60	60
27	CF	F	17	0.707	0.155	0.057	0.762	0.039	67	71	70	21	67	19
28	CF	M	11	0.724	0.206	0.188	1.134	0	81	84	80	21	67	19
29	CF	F	8	0.516	0	0.008	0.077	0	90	92	80	13	69	39
30	CF	M	30	0.909	0.014	0.231	1.256	0	75	71	90	17	71	82
31	CF	F	12	0.822	0.045	0.603	1.414	0	99	91	90	8	75	50
32	CF	F	11	0.391	0.035	0.368	0.670	0.322	59	73	70	11	82	18
33	CF	M	9	1.119	0.409	0.131	1.411	0.243	107	110	100	17	88	29
34	CF	M	5	0.481	0.007	0.307	0.558	0	100	100	100	16	94	100
35	CF	F	17	1.064	0.034	0.437	0.001	0	75	75	80	5	100	60
36	CF	F	7	0.752	0.051	0	0.989	0	80	113	100	7	100	100
37	CF	F	19	0.044	0.277	0.094	0.017	0	79	70	60	9	100	22
38	CF	M	6	0.485	0.526	0.359	1.056	0.142	120	120	100	16	100	31
39	CF	F	10	1.177	0.356	1.021	1.554	0.380	100	100	100	18	100	50

* M, male; F, female.

** Results concerning basal and cAMP dependent chloride conductive pathways are expressed as percentages of nasal ciliated cells with maximal fluorescence change $\Delta F_{basal}/\Delta t>0.1$ and $\Delta F_{cAMP}/\Delta t>0.1$, respectively.

Table 6. *Age, FEV1, FVC, and Shwachman score according to* MRP1 *(A) and* MRP5 *transcript levels (B) in CF patients (*n = *19). Results are expressed as mean ± SEM.*

A.

	MRP1 ≤ 0.15 (n = 5)	MRP1 > 0.15 (n = 14)	p
Age	14 ± 2	12 ± 2	0.4
FEV1	87 ± 8	89 ± 4	0.8
FVC	87 ± 11	89 ± 5	0.8
Shwachman score	74 ± 7	88 ± 3	0.05

B.

	MRP5 ≤ 0.08 (n = 5)	MRP5 > 0.08 (n = 14)	p
Age	14 ± 2	12 ± 2	0.6
FEV1	88 ± 8	89 ± 4	0.8
FVC	84 ± 11	90 ± 5	0.5
Shwachman score	76 ± 7	87 ± 3	0.1

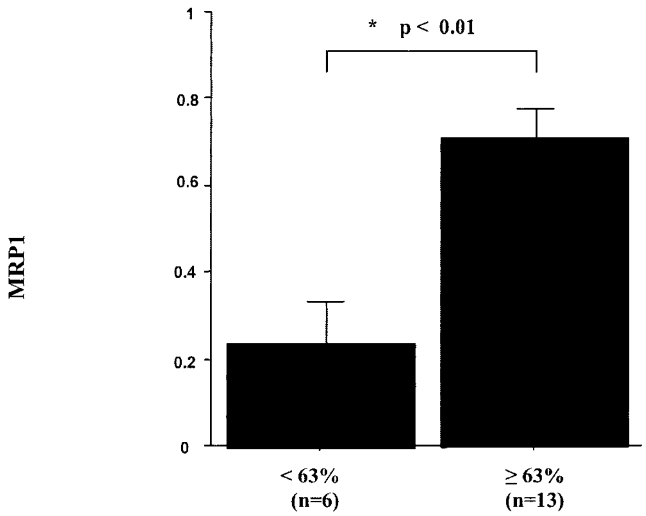

Figure 4. MRP1 transcript levels according to the percentage of nasal ciliated cells exhibiting a $\Delta F_{basal}/\Delta t$ fluorescence change >0.1. The 63% value was the lowest percentage observed in controls as previously reported (6) and was therefore chosen as cut-off value. Significantly lower *MRP1* transcript levels were found for CF patients with less than 63% cells exhibiting basal chloride conductance: 0.23 ± 0.12 *vs* 0.7 ± 0.09%, $p < 0.01$.

MRPs and CFTR and that MRPs, especially MRP1, might play an important role in the pathophysiology of CF.

The lower expression of *MRP1* and *MRP5* genes observed in CF patients compared with controls, which was not due to differences in cell type of the analyzed nasal samples, raises the question of the mechanisms of this down-regulation. Regulation of expression of *MRPs* genes is still poorly known. *MRP1* expression has been shown to be modulated by drugs and toxins (19), oxidative stress (20), Nmyc (21) and p53 (22–23) and down regulated by elevated glutathione levels (20) and wild-type p53 (23). CF lung disease, which is the major cause of death, is characterized by chronic inflammation and severe oxidative stress in the airways. Oxidative stress may therefore be involved in *MRP1* regulation in CF patients. Even fewer data are available concerning the mechanisms of *MRP5* gene regulation. In contrast with *MRP1*, *MRP5* does not appear to be regulated by pro-oxidants (24).

The highest correlation among *MRPs* genes between *MRP1* and *MRP5* transcript levels for control subjects and CF patients (Fig. 3) suggests that these two genes may be co-ordinately regulated under physiologic conditions and in CF. Interestingly, in another non-CF pathologic setting, namely lung cancer, no such strong correlation was found between *MRP1* and *MRP5* mRNA levels, while the highest correlation was observed between *MRP1* and *MRP3* mRNA levels (25). The mechanisms of *MRPs* gene regulation and consequently their co-regulation may therefore be different in different diseases.

Defective cAMP-dependent chloride transport in the apical membrane of secretory epithelia has been considered to be the

underlying source of CF pathophysiology. However, there is growing evidence that CF pathogenesis might be not solely related to the defective chloride channel function, but also to other nonion channel functions of CFTR and to "modifier" genes (26–27). An interesting finding of the present study is the association between *MRP1* transcript levels and clinical course of CF disease, as low *MRP1* levels were associated with more severe disease, as assessed by the Shwachman score, than high *MRP1* levels. Although this result may be incidental and needs further investigation on a larger series of patients, it suggests that MRP1 could be involved in CF pathophysiology and could constitute a "modifier" gene in CF. Although MRP1 is localized to the basolateral membrane of epithelial cells, especially respiratory ciliated cells (10), as opposed to CFTR which is on the apical membrane, CFTR and MRP1 may have similar functions, such as efflux of organic anions (14) or glutathione (15). The function of MRP1 possibly involved in the clinical course of CF might be related to either a nonchloride channel function and/or the chloride channel function of CFTR. MRP1 is thought to be involved in various physiologic or pathologic processes, some of which may be potentially relevant in CF pathophysiology, such as inflammation and antioxidant defense.

Among MRPs, MRP1 has the highest affinity for leukotriene C4 (28), a potent mediator of the inflammatory response causing edema and increased vascular permeability. A decreased inflammatory response was observed in a MRP1-deficient mouse model, consistent with a role of MRP1 in mediating leukotriene C4 export (29). As bronchial cells are known to produce various types of leukotrienes, especially leukotriene C4 (30), one of the functions of MRP1 in respiratory cells could be efflux of endogenously synthesized leukotriene C4. The absence of an effective inflammatory response *via* MRP1 could therefore be involved in the more severe clinical course of CF in patients with low *MRP1* transcript levels.

Oxidative stress is increased in CF patients due to chronic inflammation and evidence for inadequate antioxidant defense has been reported (31). In particular, glutathione (GSH), which is a major antioxidant in the lung, is decreased in the apical fluid from CF airway epithelial cells (32). As MRP1 can transport GSH out of cells into the interstitial compartment, MRP1 might play a protective role against toxic compounds generated by oxidative stress (11). Oxidative stress has been shown to increase paracellular permeability (33). GSH might therefore reach the airway lining fluid from the interstitial compartment *via* the paracellular route during oxidative stress. CF patients with lower *MRP1* levels might have lower GSH in airway fluid *via* lower efflux of GSH by MRP1 and subsequently they might have a more severe disease *via* a lack of adequate protective role in the lung against oxidants due to defective MRP1.

The striking relation between *MRP1* transcript levels and the existence of a basal chloride conductive pathway in CF patients (Fig. 4) strongly suggests that MRP1 may have a chloride channel-related function. Therefore, MRP1 could be involved in the better clinical status of CF patients with high *MRP1* levels *via* this chloride channel-related function in addition to the possible functions discussed above.

A chloride channel-related function has been previously reported in human cell lines overexpressing MRP1 (34–35). Whether MRP1 might act as a chloride channel by itself or regulate, directly or indirectly, a chloride channel remains to be determined. Given the basolateral localization of MRP1 and its possible implication in CF pathophysiology, we hypothesize that MRP1 rather indirectly regulates a chloride channel localized to the apical membrane as CFTR. Our results suggest that the channel regulated by MRP1 would be different from CFTR, as no association was demonstrated between *MRP1* mRNA levels and the existence of cAMP-dependent chloride conductance characteristic of CFTR.

Although we found a correlation between *MRP1* and *MRP5* transcript levels, we found no association of *MRP5* with either clinical status or chloride conductive pathway. A possible explanation is that the group of patients with low *MRP1* mRNA levels was not the same as the group with low *MRP5* levels.

As no effective treatment for CF is yet available, many different treatment strategies are being developed in addition to transfection of normal *CFTR* gene, especially activation of defective CFTR or non-CFTR chloride channels by pharmacological agents. Although there is need to further evaluate MRPs in CF at the protein level, our results suggest that MRP1 could constitute a new target for pharmacotherapy for CF. Based on the findings that (i) low *MRP1* gene expression was associated with more severe disease and (ii) levels were positively associated with the existence of a cAMP-independent chloride conductive pathway, a new therapeutic approach might consist of inducing MRP1 in CF patients. Such pharmacological modulation of MRP1 in CF patients, associated with restored chloride conductance, has been very recently reported in an abstract (36).

Acknowledgments. The authors thank C. Durrant and J. Lehmann-Che for skillful assistance and J.H. Calvet for helpful discussion.

REFERENCES

1. Stutts MJ, Rossier BC, Boucher RC 1997 Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. J Biol Chem 272:14307–14040
2. Gabriel SE, Clarke LL, Boucher RC, Stutts MJ 1993 CFTR and outwardly rectifying chloride channels are distinct proteins with a regulatory relationship. Nature 363:263–268
3. Schreiber R, Nitschke R, Greger R, Kunzelmann K 1999 The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. J Biol Chem 274:11811–11816
4. Bradbury NA, Jilling T, Berta G, Sorscher EJ, Bridges RJ, Kirk KL 1992 Regulation of plasma membrane recycling by CFTR. Science 256:530–532
5. Barasch J, Kiss B, Prince A, Saiman L, Gruenert DC, Al-Awqati Q 1991 Defective acidification of intracellular organelles in cystic fibrosis. Nature 352:70–73
6. Sermet-Gaudelus I, Vallee B, Hurbain I, Torossi T, Marianovski R, Fajac A, Feuillet MN, Bresson JL, Lenoir G, Bernaudin JF, Edelman A 2002 Normal function of the cystic fibrosis conductance regulator protein can be associated with homozygous ΔF508 mutation. Pediatr Res 52:628–635
7. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley GR 1992 Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650–1654
8. Allikmets R, Gerrard B, Hutchinson A, Dean M 1996 Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the expressed sequence tags database. Hum Mol Genet 5:1649–1655

9. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJT, Juijn JA, Baas F, Borst P 1997 Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1) in human cancer cell lines. Cancer Res 57:3537–3547
10. Brechot JM, Hurbain I, Fajac A, Daty N, Bernaudin JF 1998 Different pattern of MRP localization in ciliated and basal cells from human bronchial epithelium. J Histochem Cytochem 46:513–517
11. Leslie EM, Deeley RG, Cole SPC 2001 Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology 167:3–23
12. Borst P, Evers R, Kool M, Wijnholds J 2000 A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 92:1295–1302
13. Lallemand JY, Stoven V, Annereau JP, Boucher J, Blanquet S, Barthe J, Lenoir G 1997 Induction by antitumoral drugs of proteins that functionally complement CFTR: a novel therapy for cystic fibrosis? Lancet 350:711–712
14. Linsdell P, Hanrahan JW 1998 Adenosine triphosphate-dependent asymmetry of anion permeation in the cystic fibrosis transmembrane conductance regulator chloride channel. J Gen Physiol 111:601–614
15. Linsdell P, Hanrahan JW Glutathione permeability of CFTR 1998 Am J Physiol 275:323–326
16. Shwachman H, Kulczycki LL 1958 Long term study of one hundred and five patients with cystic fibrosis. Am J Dis Child 96:6–15
17. Decleves X, Fajac A, Lehmann-Che J, Tardy M, Mercier C, Hurbain I, Laplanche JL, Bernaudin JF, Scherrmann JM 2002 Molecular and functional MDR1-Pgp and MRPs expression in human glioblastoma multiforme cell lines. Int J Cancer 98:173–180
18. Trezise AE, Romano PR, Gill DR, Hyde SC, Sepulveda FV, Buchwald M, Higgins CF 1992 The multidrug resistance and cystic fibrosis genes have complementary patterns of epithelial expression. EMBO J 11:4291–4303
19. Schrenk D, Baus PR, Ermel N, Klein C, Vorderstemann B, Kauffmann HM 2001 Up-regulation of transporters of the MRP family by drugs and toxins. Toxicol Lett 120:51–57
20. Yamane Y, Furuichi M, Song R, Van NT, Mulcahy RT, Ishikawa T, Kuo MT 1998 Expression of multidrug resistance protein/GS-X pump and γ-glutamylcysteine synthetase genes is regulated by oxidative stress. J Biol Chem 273:31075–31085
21. Haber M, Bordow SB, Gilbert J, Madafiglio J, Kavallaris M, Marshall GM, Mechetner EB, Fruehauf JP, Tee L, Cohn SL, Salwen H, Schmidt ML, Norris MD 1999 Altered expression of the MYCN oncogene modulates MRP gene expression and response to cytotoxic drugs in neuroblastoma cells. Oncogene 18:2777–2782
22. Sullivan GF, Yang JM, Vassil A, Yang J, Bash-Babula J, Hait WN 2000 Regulation of expression of the multidrug resistance protein MRP1 by p53 in human prostate cancer cells. J Clin Invest 105:1261–1267
23. Wang Q, Beck WT 1998 Transcriptional suppression of multidrug resistance-associated protein (MRP) gene expression by wild-type p53. Cancer Res 58:5762–5769
24. Lin-Lee YC, Tatebe S, Savaraj N, Ishikawa T, Kuo MT 2001 Differential sensitivities of the MRP gene family and γ-glutamylcysteine synthetase to pro-oxidants in human colorectal carcinoma cell lines with different p53 status. Biochem Pharmacol 61:555–563
25. Young LC, Campling BG, Voskoglou-Nomikos T, Cole SPC, Deeley RG, Gerlach JH 1999 Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin Cancer Res 5:673–680
26. Schwiebert EM, Dale JB, Egan ME, Stutts MJ, Guggino WB 1999 CFTR is a conductance regulator as well as a chloride channel. Physiol Rev 79:S145–S166
27. Bronsveld I, Mekus F, Bijman J, Ballmann M, de Jonge HR, Laabs U, Halley DJ, Ellemunter H, Mastella G, Thomas S, Veeze HJ, Tümmler B 2001 Chloride conductance and genetic background modulate the cystic fibrosis phenotype of ΔF508 homozygous twins and siblings. J Clin Invest 108:1705–1715
28. Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG, Keppler D 1994 The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. J Biol Chem 269:27807–27810
29. Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P 1997 Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. Nat Med 3:1275–1279
30. Holtzman MJ 1991 Arachidonic acid metabolism. Implications of biological chemistry for lung function and disease. Am Rev Respir Dis 143:188–203
31. Brown RK, Wyatt H, Price JF, Kelly FJ 1996 Pulmonary dysfunction in cystic fibrosis is associated with oxidative stress. Eur Respir J 9:334–339
32. Gao L, Kim KJ, Yankaskas JR, Forman HJ 1999 Abnormal glutathione transport in cystic fibrosis airway epithelia. Am J Physiol 277 (Lung Cell Mol Physiol 21):L113–L118
33. Rao RK, Baker RD, Baker SS, Gupta A, Holycross M 1997 Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation. Am J Physiol 273 (Gastrointest Liver Physiol 36):G812–G823
34. Jirsch J, Deeley RG, Cole SPC, Stewart AJ, Fedida D 1993 Inwardly rectifying K^+ channels and volume-regulated anion channels in multidrug resistant small cell lung cancer cells. Cancer Res 53:4156–4160
35. Hainsworth AH, Henderson RM, Hickman ME, Hladky SB, Rowlands T, Twentyman PR, Barrand MA 1996 Hypotonicity-induced anion fluxes in cells expressing the multidrug-resistance-associated protein, MRP. Pflugers Arch 432:234–240
36. Pradal U, Delmarco A, Morganti M, Cippoli M, Mini E, Cazzola G 2002 Azithromycin induces over-expression of the multidrug resistance-associated protein (MRP) and restores chloride conductance in cystic fibrosis. J Cystic Fibrosis 1:S52 (Abstr)