Disturbed Myeloperoxidase-Dependent Activity of Neutrophils in Cystic Fibrosis Homozygotes and Heterozygotes, and Its Correction by Amiloride[1]

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The present study addresses the question of a possible linkage between the cystic fibrosis (CF) genetic autosomal recessive disorder and disturbance in neutrophil function. Neutrophil-dominated chronic airway inflammation is present at an early age in children with CF, even in the absence of detectable infection. As evidenced by extracellular superoxide anion release (measured by lucigenin luminescence) or intracellular hydrogen peroxide production (measured by 2',7'-dichlorofluorescein (DCF) fluorescence), no significant difference in the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity of isolated neutrophils was observed in noninfected CF children (homozygotes), their mothers or fathers (CF heterozygotes), and controls. In contrast, both myeloperoxidase (MPO)-dependent oxygenation activity (measured by luminol luminescence) and chloramine release were increased significantly in both CF homozygotes and heterozygotes as compared with controls. In the presence of either amiloride (a sodium channel inhibitor and sodium/proton antiport blocker) or EIPA (5-ethyl-*N*-isopropyl-amiloride, a specific inhibitor of the antiport), or choline buffer, intracellular MPO activity was decreased significantly in controls and in the CF homozygotes and heterozygotes, thus bringing intracellular MPO-dependent activity in CF subjects back to the level of controls. Extracellular release of MPO, measured by an ELISA to provide an activity-independent assessment of the enzyme, was increased only in CF homozygotes, and was decreased by amiloride and choline buffer, but not by EIPA. We conclude that a modification of intracellular pH and/or ionic concentrations may be related to the altered MPO enzymatic activity observed in CF neutrophils. *The Journal of Immunology,* **1996, 157: 2728–2735.**

Cystic fibrosis (CF)[4] is an autosomal recessive disorder, common in Caucasians, which is characterized by increased sodium and chloride concentrations in sweat, pancreatic insufficiency, and chronic lung colonization by bacteria, especially *Pseudomonas aeruginosa* (1). CF is caused by mutation of the cystic fibrosis *trans*-membrane regulator (CFTR) gene (2). The product of the CFTR gene is thought to act as a chloride channel on the apical surface of epithelial cells (3).

The chronic airway inflammation of CF occurs at a very early age. This neutrophil-dominated inflammatory process precedes any microbiologic evidence of bacterial infection (4, 5), and raises the critical question of whether such an active inflammatory response may be initiated or at least amplified by the CF genetic effect (6). Polymorphonuclear neutrophil leukocytes (PMN) are essential for host defense, but may produce airway damage through the release of proteases, such as neutrophil elastase (7, 8) and oxidants. Neutrophil oxidative metabolism is activated at the level of NADPH oxidase. This enzymatic complex consists of cytosolic and membrane proteins that assemble on the plasma membrane and ultimately the phagolysosomal membrane. The oxidase generates superoxide anion (O_2^-) (9, 10), and the dismutation of O_2^- produces hydrogen peroxide (H_2O_2) (11). Myeloperoxidase (MPO), an enzyme contained in azurophil granules, catalyzes the H_2O_2-dependent oxidation of chloride (Cl^-) to hypochlorous acid (HOCl), and the reaction of HOCl with H_2O_2 yields singlet oxygen ($^1O_2$) (12–14). HOCl can react with amines to yield chloramines, the so-called long-lived oxidants (15, 16).

An imbalance between proteases and antiproteases (17, 18) and profound disturbances in plasma antioxidant activities (19) have been reported in CF children. However, most of these studies were conducted in children chronically infected with *P. aeruginosa*. Because neutrophils have both an anti-infectious and proinflammatory role in CF, investigation of neutrophil activity is of crucial importance. The aim of the present study was to investigate the possibility of a constitutive abnormality in CF neutrophil oxidant formation. Therefore, we investigated both neutrophil NADPH oxidase- and MPO-dependent oxidant activities in noninfected CF children as compared with their respective parents. The parents are asymptomatic heterozygote CF carriers who are free from the clinical problems associated with the disorder, but who nevertheless express both mutated and normal CFTR in their cells.

Clinical research in CF indicates that amiloride treatment increases the airway mucus hydration and improves CF clinical status (20, 21). Amiloride is both an Na^+ channel inhibitor and a sodium/proton antiport blocker, depending on the cell type and the

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[4] Abbreviations used in this paper: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; CL, chemiluminescence; DA, diacetate; DCF, 2',7'-dichlorofluorescein; DCFH, nonfluorescent 2',7'-dichlorofluorescein; EIPA, 5-ethyl-*N*-isopropyl amiloride; KI, potassium iodide; MPO, myeloperoxidase; NADPH, nicotinamide-adenine dinucleotide phosphate; OZ, opsonized zymosan; PMN, polymorphonuclear neutrophil leukocyte.

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concentration used. The rationale for administering amiloride aerosols to CF patients is based on its Na^+ channel-blocking properties at the level of the airway epithelia. In the second part of our study, we investigate the effect of amiloride on CF neutrophil oxidative activity.

The data show that both CF homozygotes and heterozygotes produce larger amounts of intracellular MPO-dependent oxidants than controls, and that this disturbance is corrected by the inhibition of the sodium/proton antiport activity.

Materials and Methods

Study population

Forty-two children with CF (20 males and 22 females) were recruited to the study from patients at the Clinique des Maladies des Enfants (Enfants Malades Hospital, Paris, France). Their mean age was 8 ± 4.5 yr; the range was from 4 to 15 yr. CF was diagnosed according to standard criteria, including a sweat chloride test. Genotyping (22) showed that the allelic frequency of the ΔF508 mutation was 90% among the 42 children; 37% were ΔF508 homozygote; 53% were ΔF508 compound heterozygote with an unknown mutation; and 10% had mutations other than ΔF508 on both alleles. The CF children were not infected chronically with *P. aeruginosa* and had been free from infection during the month before experiments.

Some of their parents, including 31 mothers and 12 fathers, were recruited to the study to form the CF heterozygote group. The control group was made up of 45 healthy blood donors attending the Blood Bank at the Necker Hospital. Parental informed consent (all children) and the child's assent (children older than 7 yr of age) were obtained before entry into the study. This study was approved by the Ethics Committee of the Enfants Malades Hospital.

Neutrophil isolation

Neutrophils were isolated from heparinized (Liquemine Roche, Paris, France; 10 U/ml) venous blood, as previously described (23). Briefly, neutrophils were separated from erythrocytes by sedimentation in plasmagel (Roger Bellon, Paris, France), followed by density gradient centrifugation in Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). Residual erythrocytes were lysed by treating the cell pellet with lysis buffer containing ammonium chloride. Purified neutrophils were resuspended at the desired cell concentration in phenol red-free HBSS (Eurobio, Paris, France), pH 7.4.

Differential measurement of NADPH oxidase and MPO activities by luminescence

Activity-specific chemiluminogenic substrates luminesce by different dioxygenation mechanisms and depend on distinct products of neutrophil oxidative metabolism. For example, the reductive dioxygenation of lucigenin is NADPH oxidase dependent and measures extracellular superoxide anion formation, whereas dioxygenation of the cyclic hydrazide luminol is mostly dependent on MPO activity (measurement of intracellular H_2O_2 and HOCl formation) (24). Basal and chemically stimulated neutrophil oxygenation activities were measured as either the cyclic hydrazide luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) or lucigenin (10,10-dimethyl-9,9-biacridium dinitrate) dioxygenation activities (ExOxEmis, Little Rock, AR). A single photon luminometer (AutoLumat LB953; Berthold Co., Wildbad, Germany) was used for injection and luminescence measurement. Reaction was initiated by injecting 0.1 ml of a neutrophil suspension in HBSS (10^5 cells/0.1 ml) into polystyrene tubes containing 500 μl of a luminogenic substrate (200 μmol/L lucigenin or 150 μmol/L luminol), and 20 μl of either HBSS (resting CL) or stimulating agents such as PMA (1.6 nmol/ml, final concentration), human serum (blood group AB) opsonized zymosan (OZ; zymosan A derived from *Saccharomyces cerevisiae*) (2 mg/ml), or FMLP (1 nmol/ml, final concentration). PMA, OZ, and FMLP were obtained from Sigma Chemical Co. (St. Louis, MO). Luminescence was measured in triplicate over 20-min interval, and results were expressed as specific activity per neutrophil (PMN), i.e., counts/PMN/20 min.

Measurement of intracellular hydrogen peroxide by DCF fluorescence

The hydrogen peroxide-dependent oxidation of nonfluorescent 2′,7′-dichlorofluorescein (DCFH) into a highly fluorescent compound (DCF) was measured by a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA) (25). Briefly, neutrophils were incubated with 2 mM of DCF diacetate (DCF-DA; Eastman Kodak Co., Rochester, NY) at 37°C for 15 min. The DA form of the compound easily penetrates the cell membrane, and after deacylation of the molecule by cellular esterases, it is trapped intracellularly. Neutrophils were washed and resuspended in PBS (10^6 PMN/ml), allowed to equilibrate for 5 min at 37°C, then activated with PMA (1.6 nmol/ml) for 15 min, fixed with 1% formaldehyde, and analyzed. The intracellular H_2O_2 production following activation by OZ could not be measured by FACS, as zymosan particles produced artifacts because of its granulometry.

Neutrophil extracellular chloramine release

Neutrophil release of taurine-chloramine was determined as previously described (26). Briefly, neutrophils (10^6 PMN/ml) were incubated for 1 h at 37°C in the absence (controls) or presence of either PMA (1.6 nmol/ml) or OZ (2 mg/ml). Taurine (15 mmol/L; Sigma Chemical Co.) was added to the incubation medium to promote production of taurine-chloramine. The reaction mixtures were then centrifuged (1200 × *g*, 10 min, 4°C), and the supernatants were stored at −20°C for analysis the next day. Chloramine concentration was estimated by colorimetric measurement of the triiodide ion formed by oxidation of potassium iodide (KI). Two hundred microliters of either neutrophil supernatant or a chloramine-T standard solution (*N*-chloro-*p*-toluene-sulfonamide sodium salt; Sigma Chemical Co.) were first loaded into 96-well microtiter plates (Falcon Labware; Becton Dickinson, Oxnard, CA). The reaction was initiated by addition of 10 μl of 1.16 M of KI. Two minutes later, 20 μl of acetic acid was added, and absorbance at 340 nm was read immediately using a microplate reader (model MR 5000; Dynatech Labs, Chantilly, VA) fitted with a blank containing 200 μl HBSS, 10 μl KI, and 20 μl acetic acid. Absorbance at this wavelength follows Beer's law within the range of 0 to 100 μmol/L.

Neutrophil extracellular MPO release

One hundred microliters of purified neutrophil suspension (5×10^6 PMN/ml) were incubated in a water bath (1 h, 37°C) along with either OZ (2 mg/ml), PMA (1.6 nmol/ml), or FMLP (1 nmol/ml). Neutrophils were pelleted by centrifugation (1200 × *g*, 10 min, 4°C), and the supernatant was collected for measurement of MPO extracellular release by ELISA. The detecting Ab used was a biotinylated form of the capture Ab, a rabbit polyclonal anti-human MPO Ab (Calbiochem, La Jolla, CA) diluted 1/1000. Alkaline phosphatase-conjugated streptavidin (Dako Corp., Glostrub, Denmark) was diluted 1/2000, and *p*-nitrophenyl phosphate (Sigma Chemical Co.) was used as a substrate. The absorbance of the reaction mixture after 1 h was read at 405 nm in a microplate reader.

At the end of the incubation, neutrophil pellets (5×10^5 cells) were solubilized in 50 μl of PBS containing 1% SDS, and the remaining insoluble material was removed by centrifugation. Protein concentration was measured by the bicinchoninic acid (BCA) micro method (Pierce, Rockford, IL) using BSA as standard. MPO release in the supernatant is expressed as nanograms per milliliters, adjusted for 100 μg of protein in the neutrophil pellet.

Modulation of the sodium/proton antiport activity

The activity of the sodium/proton antiport was blocked using the following pharmacologic inhibitors: amiloride (Sigma Chemical Co.) and EIPA (5-ethyl-*N*-isopropyl-amiloride; Molecular Probe, Eugene, OR), which is a highly specific inhibitor (27), or by replacing sodium with choline (Sigma Chemical Co.), an organic osmolyte that is not transported by the antiport (28–30). Where indicated, amiloride or EIPA was added at concentrations of 10 to 100 μM, and luminol chemiluminescence (CL) and extracellular release were measured. The viability of neutrophils (10^6 PMN/ml) following 1-h incubation in amiloride (100 μM) or EIPA (100 μM) was estimated as greater than 96% by trypan blue exclusion. No differences in cell viability were found between control and amiloride- or EIPA-treated neutrophils (data not shown). Experiments in sodium-free buffer were performed using choline buffer, in which NaCl component was substituted with equimolar concentrations of choline, as compared with PBS.

Statistical analysis

Data were analyzed with the Statistica software package (Statsoft, Tulsa, OK). Results expressed as mean ± SEM were compared using ANOVA, Student's two-tailed *t* test (paired or unpaired, as appropriate), or nonparametric tests such as Wilcoxon or Mann-Whitney *U*, as indicated. Differences were considered significant when the *p* value was 0.05 or less.

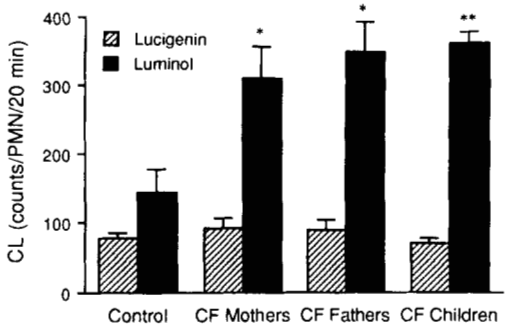

FIGURE 1. Basal neutrophil luminescence activity of control subjects, CF children, and their respective parents. Oxygenation activities were measured as unstimulated lucigenin and luminol CL. One hundred microliters of PMN suspension (10^5 cells/0.1 ml) were automatically injected into a polystyrene tube containing 500 μl of luminigenic substrate (200 μmol/L lucigenin or 150 μmol/L luminol). The resulting luminescence was recorded for 20 min at 37°C and was expressed as counts/PMN/20 min. Results are expressed as mean ± SEM from 20 independent experiments, each including one control (n = 20), one CF child (n = 20), and its mother (n = 20) or father (n = 6). Differences between controls and CF subjects were analyzed by ANOVA (*$p < 0.05$; **$p < 0.01$).

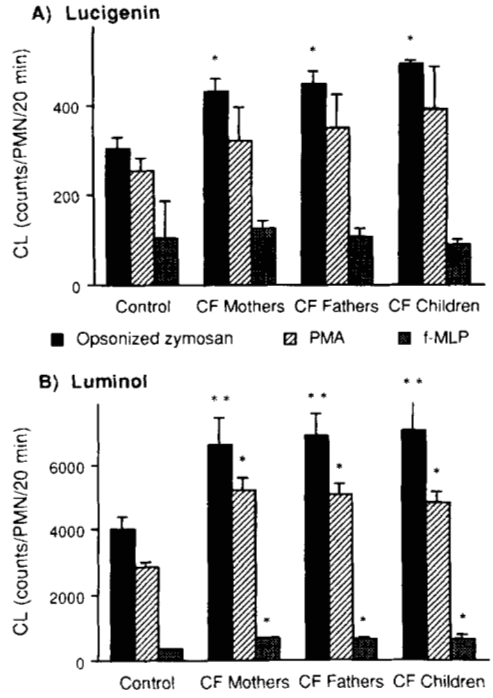

FIGURE 2. OZ, PMA, and FMLP stimulated luminescence activities of neutrophils from control subjects, CF children, and their respective parents. Oxygenation activities were measured as lucigenin CL (*A*) or luminol CL (*B*) following activation with particulate (OZ) or soluble agents (PMA or FMLP). One hundred microliters of PMN suspension (10^5 cells/0.1 ml) were injected automatically into a polystyrene tube containing 500 μl of luminigenic substrate (200 μmol/L lucigenin or 150 μmol/L luminol) and stimulating agents, OZ (2 mg/ml), PMA (1.6 nmol/ml), and FMLP (17 nmol/ml). The resulting luminescence was recorded for 20 min at 37°C and expressed as counts/PMN/20 min. Results are expressed as mean ± SEM from 20 independent experiments, each including one control (n = 20), one CF child (n = 20), and its mother (n = 20) or father (n = 6). Differences between controls and CF subjects were analyzed by ANOVA (*$p < 0.05$; **$p < 0.01$).

Results

NADPH oxidase- and MPO-dependent neutrophil oxygenation activities

The basal or resting state was measured as the CL activity of purified neutrophils from CF children, their respective mothers and/or fathers, and controls in the absence of stimulating agents (Fig. 1). Neutrophils from both CF homozygotes and heterozygotes showed significantly higher luminol CL, corresponding to increased MPO-dependent activity, than controls. The mean activity of controls was 145 ± 34 counts/PMN/20 min; mothers, 312 ± 47 ($p < 0.01$); fathers, 351 ± 45 ($p < 0.01$); and children, 364 ± 17 ($p < 0.01$). However, the differences in lucigenin CL, which is a measure of NADPH oxidase activity (extracellular superoxide anion release), were not significant. The mean lucigenin CL activity of controls was 77 ± 9 counts/PMN/20 min, compared with 93 ± 14 for CF mothers, 90 ± 15 for CF fathers, and 69 ± 8 for CF children.

Stimulated NADPH oxidase- and MPO-dependent activities were measured following activation with either human serum OZ (a particle that can be phagocytosed), PMA (a direct protein kinase C agonist), or FMLP (a soluble chemotactic peptide). OZ caused a significant increase in lucigenin CL (NADPH oxidase activity) in CF children and their parents compared with controls (Fig. 2). No significant difference in lucigenin CL was observed using the soluble stimulants, PMA and FMLP. However, all three agents caused increased luminol CL (MPO activity) in CF children and their parents compared with controls. The largest difference was observed in OZ-stimulated neutrophils. The mean activity of controls was 4041 ± 365 counts/PMN/20 min; CF mothers, 6628 ± 825 ($p < 0.05$); CF fathers, 6907 ± 700 ($p < 0.05$); and CF children, 7081 ± 797 ($p < 0.001$).

To determine whether the increased luminol CL observed in CF homozygotes and heterozygotes was related to MPO activity itself or to an increase in intracellular H_2O_2, we measured the intracellular H_2O_2 generation by DCFH fluorescence (Fig. 3). Neutrophils showed an increase in cellular fluorescence after addition of PMA. The profile of either basal or PMA-stimulated neutrophil fluorescence did not significantly differ among the three groups. Likewise, mean fluorescence was not statistically different between controls, CF homozygotes, and CF heterozygotes.

We next investigated whether increased MPO-dependent activity in CF homozygotes and heterozygotes was associated with increased accumulation of long-lived oxidants in the extracellular medium. PMN from both CF children and, although to a lesser extent, their mothers showed increased chloramine release compared with controls when stimulated with OZ. PMA also stimulated an increase, but this increase was not statistically significant in CF mothers (Fig. 4).

These results support the hypothesis that the hyperactivity observed in neutrophils from CF subjects is related to an MPO-dependent pathway.

Neutrophil extracellular MPO release: an activity-independent assessment of the enzyme

Additional testing was designed to distinguish whether the increased production of chloramines in CF subjects was due to an increased MPO content per neutrophil. Based on ELISA measurements, no difference was found in the total MPO content of neutrophil lysates among CF children (1.27 ± 0.11 μg MPO/100 μg PMN protein, n = 7), CF parents (1.53 ± 0.28, n = 6), and controls (1.24 ± 0.22, n = 6).

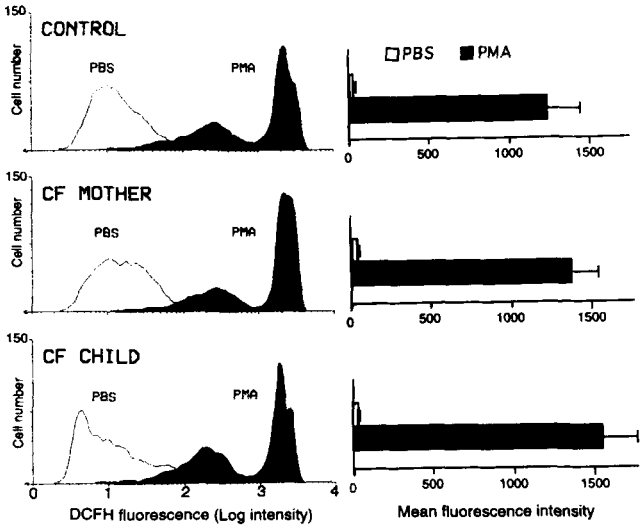

FIGURE 3. Intracellular H_2O_2 production measured by cytofluorometry. After loading with DCFH-DA at 37°C for 15 min, isolated neutrophils from controls (*top*), CF mothers (*middle*), and CF children (*bottom*) were incubated with PBS or with PMA (1 μg/ml). Flow-cytometric analysis of DCF fluorescence was performed as described in *Materials and Methods*. The profile determined from nonstimulated neutrophils is shown in each panel (open curve), along with the fluorescence profiles following stimulation with PMA (closed curve). *Data represent a representative set of experiments including a control*, a CF parent, and a CF child. The mean fluorescence ± SEM from eight experiments is illustrated with histograms on the *right panels*.

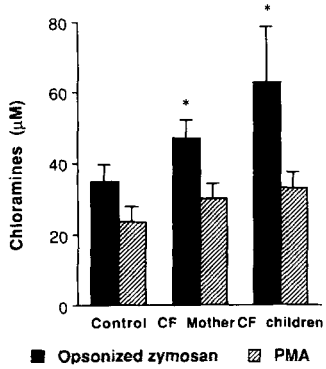

FIGURE 4. Chloramine release from the neutrophils of control subjects, CF children, and their respective mothers. PMN were adjusted to a concentration of 10^6 cells/ml and stimulated with OZ (2 mg/ml) or PMA (1.6 nmol/ml) for 1 h in the presence of taurine (15 mol/L). Chloramine concentrations (μmol/L) were determined by colorimetric measurement of the triiodide ion formed by oxidation of KI (1.16 M). Results are expressed as mean ± SEM from 14 independent experiments, each including one control, one CF child, and its mother. Differences between controls and CF subjects were analyzed by ANOVA (*$p < 0.05$).

We next measured MPO released into the supernatants of neutrophil suspensions (Fig. 5). Purified neutrophils show a basal level of MPO release into the extracellular medium in the absence of a stimulating agent. In contrast to intracellular MPO-dependent oxidant formation, no statistically significant difference in basal MPO release was found among CF children (93 ± 15 ng/ml), their parents (58 ± 9 ng/ml), and controls (64 ± 4 ng/ml). OZ seems to be the most effective agent for triggering MPO release in controls, but PMA (at the concentration used) also induced significant MPO

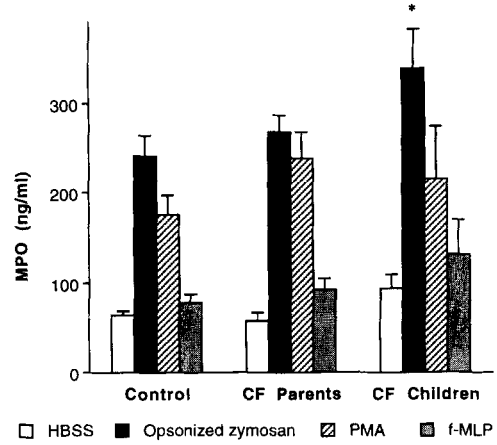

FIGURE 5. Extracellular MPO release in neutrophil supernatants from control subjects, CF children, and their parents. MPO release was measured in PMN supernatants after incubation in the absence of stimulating agents (HBSS) or in the presence of either OZ (2 mg/ml), PMA (1.6 nmol/ml), or FMLP (17 nmol/ml) for 1 h at 37°C. MPO concentrations (ng/ml) were measured by ELISA. Results are expressed as mean ± SEM of 10 independent experiments, each including one control, one CF child, and one of its parents (mother or father). Differences between controls and CF subjects were analyzed by ANOVA (*$p < 0.05$).

release. CF children released more MPO (336 ± 43 ng/ml, $p < 0.05$) than did CF parents (268 ± 18 ng/ml) or controls (241 ± 22 ng/ml) following OZ stimulation. In contrast, PMA- and FMLP-stimulated MPO release was not significantly different for CF children, their parents, and controls: 214 ± 60, 238 ± 29, and 174 ± 23 ng/ml for PMA, and 85 ± 8, 91 ± 14, and 110 ± 40 ng/ml for FMLP, respectively.

Effect of the inhibition of the sodium/proton antiport system on intracellular MPO-dependent oxidant production and extracellular MPO release

We first tested whether amiloride, a blocker of both Na^+ channel and Na^+/H^+ antiport (29), which has been shown to be of potential therapeutic interest in CF, could modulate the disturbance of the MPO-dependent system observed in CF.

In controls, amiloride decreased neutrophil luminol CL both at the resting state and following stimulation with OZ or FMLP. Likewise, neutrophil luminol CL was inhibited in CF heterozygotes and in CF homozygotes. As a result, amiloride (100 μM) corrected the hyperactivity of both resting and stimulated CF PMN and brought MPO-dependent oxidant production back to the level of controls (Fig. 6). The effect of amiloride was dose dependent; inhibition was 35, 52, and 66% in unstimulated controls, and 22, 39, and 59% in OZ-stimulated controls at amiloride concentrations of 10, 50, and 100 μM, respectively. Similar dose-dependence inhibition was observed in neutrophils from CF homozygotes and heterozygotes, irrespective of the stimulating agent used (data not shown). Both addition of EIPA (50 μM), a specific inhibitor of the sodium/proton antiport system, and incubation in choline buffer resulted in significant inhibitory effects on intracellular MPO-dependent oxidant production, as measured by luminol CL, in both resting and stimulated neutrophils (Table I). This effect was observed in neutrophils from controls, as well as from CF heterozygotes or homozygotes.

Similar experimental conditions were used to measure neutrophil extracellular MPO release in the presence of either amiloride

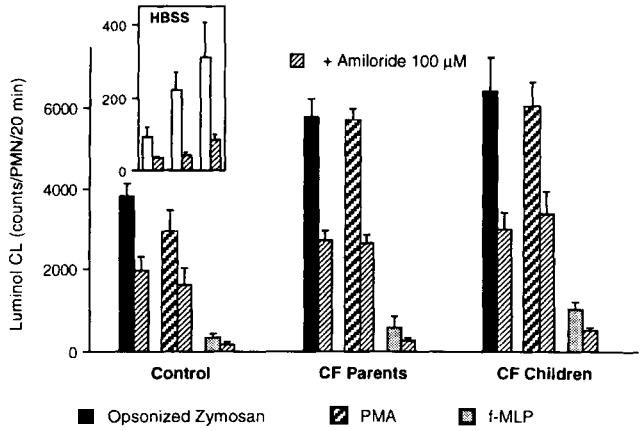

FIGURE 6. Effect of 100 μM of amiloride on luminol CL of neutrophils from control subject, CF children, and their parents. PMN luminol CL was measured following activation with OZ, PMA, or FMLP. One hundred microliters of PMN suspension (10^5 cells/0.1 ml) were injected automatically into a polystyrene tube containing both 500 μl of luminol (150 μmol/L) and stimulating agents, including OZ (2 mg/ml), PMA (1.6 nmol/ml), or FMLP (17 nmol/ml) in the presence or in the absence of amiloride (100 μmol/L). The effect of amiloride on basal luminol CL is shown in the box. The resulting CL was recorded for 20 min at 37°C and was expressed as counts/PMN/20 min. Results are expressed as mean ± SEM from six independent experiments, each including one control, one CF child, and one of its parents (mother or father). Differences between untreated and amiloride-treated PMN were analyzed using the Wilcoxon test (*$p < 0.05$; **$p < 0.01$).

or EIPA, and in a choline buffer. At a 100-μM concentration, amiloride decreased MPO extracellular release in neutrophils stimulated with OZ in controls by 17 ± 6.3%, in CF heterozygotes by 26 ± 7.6%, and in homozygotes by 40 ± 5%. However, no significant inhibition was observed in unstimulated neutrophils or in neutrophils stimulated with PMA or FMLP. A significant inhibitory effect was obtained with choline buffer, whereas specific inhibition of the sodium/proton antiport system by EIPA had either no effect or enhanced extracellular MPO release (Table II).

We conclude, therefore, that the increase in intracellular MPO-dependent oxidant generation observed in CF homozygotes and heterozygotes can be modulated and corrected by inhibiting the activity of the sodium/proton antiport. Possibly an alternative pathway is responsible for the increased extracellular MPO release observed solely in CF homozygotes.

Discussion

The present study investigated both of the main pathways of oxidant formation, NADPH oxidase- and MPO-dependent oxidant production, in resting and stimulated neutrophils. The effects of different stimuli that trigger oxidative burst and degranulation by different mechanisms and to different degrees were analyzed, and the following conclusions were drawn.

The NADPH oxidase activity of OZ-stimulated neutrophils is increased in CF heterozygotes and homozygotes. OZ triggers essentially complete azurophil degranulation and the respiratory burst metabolic activation through an opsonin-opsonin receptor ligation mechanism. No significant increase is observed in either the basal state, or after stimulation with soluble chemical agents such as PMA or FMLP. MPO-dependent oxidant formation is increased in neutrophils of CF homozygotes and heterozygotes, irrespective of the stimulating agent used, but OZ causes a larger increase. As no significant increase in intracellular hydrogen peroxide production was observed in CF homozygotes and heterozygotes, we concluded that the increased luminol CL was due to an MPO-dependent oxygenation pathway. The increase in the release of chloramines in neutrophil supernatants from CF homozygotes and to a lesser extent from CF heterozygotes is proportionally not as great as the increase in luminol CL, thus suggesting that the increase in MPO-dependent activity is mainly intracellular. Chloramine synthesis is relevant as a sort of neutrophil oxidative reserve that may prolong the duration of microbicidal action.

Azurophil degranulation is required for MPO-dependent oxidant formation. CF and control neutrophils show no difference in MPO content. Therefore, a possible explanation for increased MPO-dependent activity is increased mobilization of MPO that is stored in the azurophil granules and released upon stimulation, especially to particulate stimuli such as OZ (30). Although the increase in extracellular MPO release from the neutrophils of CF parents was not significant, the OZ-stimulated neutrophils of CF children released significantly more MPO than controls. Extracellular MPO release following azurophil degranulation results from fusion of azurophil granules with the plasma membrane or, in the case of OZ stimulation, from incomplete or exhausted phagolysosome formation. Most MPO is in the phagolysosome or bound to the plasma membrane because much released MPO rebinds to the neutrophil membranes (31).

The fact that both CF parents, who are clinically asymptomatic and free of infection, show a similar but lesser disturbance of the neutrophil MPO-dependent oxygenation activities further supports the hypothesis that the increased intracellular MPO-dependent activity is linked to the genetic defect. Nevertheless, although the expression of CFTR gene has been reported in neutrophils and in other cell lines of nonepithelial origin (32), to date, expression of the CFTR protein in neutrophils has not been demonstrated. In interpreting our data, the following points need to be taken into consideration. First, neutrophil priming can increase the rate of MPO-dependent oxidant formation, especially following OZ stimulation, since opsonin receptors are rapidly up-regulated by priming agents (33). To clarify this issue, we investigated whether CF neutrophils were primed in vivo by measuring the functional opsonin receptor expression of whole blood phagocytes as an index of inflammation in CF homozygotes and heterozygotes (34). We found that the functional ratio of circulating to maximum opsonin receptor (e.g., CD11b/CD18, CD35) expression of blood phagocytes was increased in CF children as a consequence of increased in vivo inflammation. (Witko-Sarsat et al., manuscript submitted for publication). Second, if the inflammatory state of the homozygous CF child and the heterogous CF parent is dependent on common exposure to environmental factors, we hypothesized that on the same day a CF child and its mother would be primed to the same extent. Therefore, we performed another set of experiments, testing on the same day a noninfected CF child and an unrelated CF heterozygote, as compared with a control. In addition, the CF heterozygote's own child was not included in the study. We obtained exactly the same results in both sets of experiments, thus allowing us to rule out the possibility of a collective environmentally dependent primed state relating a CF child and his own parent.

Neutrophil function may also be affected by the isolation procedure that may amplify the state of activation relative to that of the circulating neutrophil (35). The purification process can trigger a low level respiratory burst activity when the cells are allowed to contact a surface such as polystyrene. Although neutrophil priming by an infectious agent could partly explain the increased MPO-dependent activity in CF children, it is unlikely to be the case for

Table I. *Effect of the inhibition of the sodium/proton antiport activity on intracellular MPO-dependent oxidant production*[a]

A) No stimulation (PBS)	No Additive	Amiloride (100 μM)		EIPA (50 μM)		Choline	
Controls (n = 9)	38 ± 11	25 ± 2*	−34%[b]	33 ± 4*	−13.2%	20 ± 3*	−47.3%
CF parents (n = 10)	42 ± 6	30 ± 5*	−28.1%	37 ± 6*	−11.9%	24 ± 2*	−50%
CF children (n = 8)	51 ± 9	28 ± 3*	−45.1%	32 ± 4*	−37.3%	27 ± 3*	−47.1%
B) Opsonized zymosan (2 mg/ml)							
Controls (n = 9)	1946 ± 156	1330 ± 103**	−31.7%	984 ± 101**	−49.4%	1028 ± 115**	−47.2%
CF parents (n = 10)	2255 ± 165†	1455 ± 116**	−35.5%	1228 ± 62**	−45.6%	940 ± 85**	−58.3%
CF children (n = 8)	3124 ± 271‡	1798 ± 297**	−42.4%	1330 ± 203**	−57.4%	906 ± 114**	−70.9%
C) PMA (1.6 nmol/ml)							
Controls (n = 9)	868 ± 49	580 ± 42*	−40.0%	728 ± 37*	−24.8%	53 ± 3**	−94.6%
CF parents (n = 10)	1178 ± 78†	562 ± 46*	−52.3%	712 ± 68*	−39.6%	74 ± 17**	−93.7%
CF children (n = 8)	1324 ± 125†	564 ± 37*	−57.4%	564 ± 30*	−57.4%	100 ± 31**	−84.9%

[a] Neutrophils were adjusted to a concentration of 10^6 cells/ml and luminol CL was measured in the absence of any stimulating agent (A) or in the presence of opsonized zymosan (B) or PMA (C). Incubations were performed in PBS in the presence or absence of either amiloride (100 μM) or EIPA (50 μM) or in a choline buffer (sodium-free buffer). The resulting CL was recorded for 20 min at 37°C and expressed as counts/PMN/20 min.

[b] Results are given as the mean ± SEM of the indicated number (in parentheses) of independent experiments. Percentage of variation as compared with luminol CL from neutrophil in PBS alone. Statistical significance at $p < 0.05$ (†) or $p < 0.01$ (‡) when comparing with controls by Mann Whitney test. The effect of the modulation of the sodium/proton activity was evaluated for each treatment in a given group (controls, or CF parents or CF children) by comparing the amiloride or the EIPA group to the control group, with the Wilcoxon test (nonparametric paired test, * $p < 0.05$; ** $p < 0.01$).

Table II. *Effect of the inhibition of the sodium/proton antiport activity on MPO extracellular release*[a]

A) No stimulation (PBS)	No Additive	Amiloride (100 μM)		EIPA (50 μM)		Choline	
Controls (n = 8)	57.3 ± 7.3	45.7 ± 6.3	−20%[b]	62.9 ± 9.3	+10%	39.7 ± 6.6	−30.6%*
CF parents (n = 6)	62.6 ± 7.2	56.4 ± 8.2	−10.1%	65.8 ± 8.5	+5%	49.4 ± 6.2	−21.1%*
CF children (n = 5)	81 ± 11.1	89.7 ± 12.9	−10.8%	90 ± 11.5	+11%	64.8 ± 8.7	−20.1%*
B) Opsonized zymosan (2 mg/ml)							
Controls (n = 8)	200 ± 36.1	158 ± 19.1	−21.2%*	255 ± 31.2	+26.8%	121 ± 19.6	−40%**
CF parents (n = 6)	264 ± 39.5	191 ± 25.6	−27.6%*	300 ± 35.1	+13.7%	211 ± 25.2	−20%*
CF children (n = 5)	337 ± 41.8†	229 ± 31.4	−32.4%*	381 ± 52.4	+13%	216 ± 25.3	−36%*
C) PMA (1.6 nmol/ml)							
Controls (n = 8)	128 ± 33.5	133 ± 15.9	+3.6%	139 ± 15.8	+8%	105 ± 22.7	−18.3%
CF parents (n = 6)	158 ± 35.7	162 ± 25.5	+2.3%	188 ± 28.6	+18.6%	139 ± 26.3	−15%
CF children (n = 5)	149 ± 24.8	153 ± 22.2	+2.3%	183 ± 25.7	+22%	112 ± 25.2	−25.5%

[a] Neutrophils were adjusted to a concentration fo 5 × 10^6 cells/ml and incubated in the absence of any stimulating agent (A) or in the presence of opsonized zymosan (B) or PMA (C) for 1 hour at 37°C. Incubations were performed in PBS in the presence or absence of either amiloride (100 μM) or EIPA (50 μM) or in a choline buffer (sodium-free buffer). MPO has been determined by ELISA and is expressed in ng/ml for 100 μg neutrophil protein.

[b] Results are given as the mean ± SEM of the indicated number (in parentheses) of independent experiments. Percentage of variation as compared with MPO release from neutrophil in PBS alone. Statistical significance at $p < 0.05$ (†) when comparing with controls by Mann Whitney test. The effect of the modulation of the sodium/proton activity was evaluated for each treatment in a given group (controls, or CF parents or CF children) by comparing the amiloride or the EIPA group to the control group, with the Wilcoxon test (nonparametric paired test, * $p < 0.05$; ** $p < 0.01$).

CF heterozygotes, as they are clinically asymptomatic, and show no increased susceptibility to infections or inflammatory disorders (36). The question of whether CF heterozygotes are particularly susceptible to pulmonary dysfunctions has also been studied. While one study concluded that they had increased airway reactivity (37), another study has shown that CF heterozygotes are less susceptible to asthma (38). We cannot rule out the possibility that CF could be associated with some additional genetic abnormality. A significant degree of inbreeding in CF family lineages may favor this possibility. However, no such evidence of inbreeding could be found in the CF population we have studied.

Phagocyte disturbance in uninfected CF homozygotes and clinically normal CF heterozygotes has been reported. Adherence-induced monocyte luminol CL was higher than that observed for healthy age-matched controls and for patients with chronic obstructive pulmonary disease unrelated to CF (39). Some authors showed that both CF neutrophils and monocytes exhibited greater oxidative responses than controls, and concluded that phagocytes from CF patients were primed (40, 41). Phagocyte degranulation studies show increased release of elastase, a serine protease contained in azurophil granules, in *P. aeruginosa* mucoid exopolysaccharide-stimulated monocytes from CF patients (42). Moreover, eosinophils and neutrophils from patients with CF are reported to have an increased propensity to release their granular proteins, including eosinophil cationic protein and MPO, as compared with patients with bronchial asthma (43).

We investigated the effects of amiloride because it provides a pharmacologic tool for investigating the mechanism underlying

neutrophil disturbance in CF, and has also been shown to have a therapeutic value in this condition (20). In neutrophils, amiloride mediates its effect through modulation of the sodium/proton exchange system (2) and inhibition of cytosolic alkalinization that follows the respiratory burst (44, 45).

Study of the effect of sodium/proton antiport inhibition, in particular the effect of amiloride, EIPA, or the incubation in choline buffer further supports the hypothesis that the sodium/proton antiport activity is involved in the intracellular MPO-dependent oxidant generation in neutrophils from control and in neutrophils from CF heterozygotes or homozygotes. Moreover, inhibition of the sodium/proton antiport activity abrogates the disturbance in MPO activity observed in CF homozygotes and heterozygotes. A similar experimental approach has been used to demonstrate that the sodium/proton antiport activity is involved in the neutrophil volume increase associated with neutrophil migration (28). Although MPO extracellular release was inhibited both by amiloride and by choline buffer, which is in agreement with the finding that sodium ions are involved in the selective azurophil granule mobilization (30), no inhibition by EIPA, a specific inhibitor of the sodium/proton antiport activity, was observed. Based on the available evidence, we conclude that the mechanisms leading to increased extracellular MPO release observed in CF children may be distinct from those involved in the increased intracellular MPO-dependent oxidant formation.

The possible clinical consequences of increased chlorinated oxidant release might be the inactivation of the antiprotease shield, especially α_1-antitrypsin, and contribute to the imbalance between elastase and its inhibitors (4–6). We have shown previously that sputum from CF patients contain high concentrations of chloramines, along with high levels of taurine (46).

At the molecular level, the activation of the NADPH oxidase and associated pathways triggers an important proton secretion (47). A disturbance in pH compensation to the proton load might modify intracellular pH and/or chloride concentration, and could thus greatly affect MPO activity, as assessed by luminol CL (48, 49). Cytoplasmic and phagolysosomal pH regulation in neutrophils involves a complex of mechanisms that may include sodium/proton antiport activity, H^+ conductance, and an H^+/ATPase (50).

Our observations suggest that the genetic alteration responsible for CF may also affect neutrophil function, thus modifying the classical picture of CF-related inflammation. Instead of being simply an actor in the host defense against infectious agents, the CF neutrophil could be considered as being genetically modified.

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