Effects of Ion Composition and Tonicity on Human Neutrophil Antibacterial Activity

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Infants with cystic fibrosis (CF) often are infected with *Staphylococcus aureus* (*S. aur.*), which is followed by colonization with *Pseudomonas aeruginosa* (*P. aerug.*). In spite of an excessive, neutrophil-dominated inflammatory response in the respiratory tract, patients with CF often succumb to pulmonary infections with *P. aerug.* Because peripheral blood neutrophils of these patients have normal functions, we examined whether hypothesized alterations of the airway surface liquids (ASL) in these patients significantly impair neutrophil bactericidal activity in the microenvironment of the CF lung. The ionic composition of CF ASL is still not entirely defined and has been speculated to be abnormally high or abnormally low in Na^+ and Cl^- concentrations; estimates of osmolarities have ranged from 200 (hypo-osmolar) to 285 (iso-osmolar) to $>$ 300 meq/L (hyper-osmolar). Our data indicate that bacterial killing activity of human peripheral blood neutrophils against *P. aerug.* or *S. aur.* is not decreased in buffers in which NaCl was replaced with equimolar concentrations of choline Cl, KCl, or *N*-methyl-D-glucamine chloride to maintain isotonicity. Amiloride or benzamil, known modulators of Na^+ transport in neutrophils, did not interfere with this neutrophil function. Deviations from isotonicity of $\pm$ 50% also failed to diminish bactericidal activity of neutrophils significantly. In contrast, superoxide production and enzyme secretion in response to the chemotactic peptide *N*-formylmethionylleucylphenylalanine appeared to be sensitive to the ionic milieu of the assay buffers. Our results suggest that the postulated alterations in the ionic composition of ASL in CF lungs are insufficient to explain why neutrophils fail to clear infections with *P. aerug.* in these patients. **Verghese, M. W., and R. C. Boucher. 1998. Effects of ion composition and tonicity on human neutrophil antibacterial activity. Am. J. Respir. Cell Mol. Biol. 19:920–928.**

Cystic fibrosis (CF) is one of the most frequently inherited disorders in Caucasians and results from genetic mutations in the CF transmembrane regulator (CFTR) gene. The product of this gene regulates the transport of ions across airway epithelia and helps maintain the composition of the surface liquid optimal for host survival and host defense (1). In the absence of normal CFTR function, the ionic composition of the microenvironment may be altered sufficiently to impair host defenses. Indeed, chronic infection of the lung with *Pseudomonas aeruginosa* (*P. aerug.*) is a leading cause of morbidity and mortality in patients with CF (2). These infections are associated with an excessive, neutrophil-dominated inflammatory response in the respiratory tracts of CF patients (3).

Infections in CF patients rarely spread from the respiratory tract to other sites, and no defects in systemic host defenses have been documented. Because the large influx of neutrophils is apparently insufficient to clear these infections, it is likely that the microenvironment of the CF respiratory tract does not support the normal function of neutrophils and/or it interferes with the bactericidal activity of their secreted factors, such as defensins. The exact ionic composition of airway surface liquid (ASL) in CF lungs is not known, but a recent study postulates that defective CFTR function leads to reduced absorption of NaCl and thus generates a hypertonic environment (170 mM NaCl [4, 5]). Because many naturally occurring small cationic antibacterial peptides lose activity at salt concentrations $>$ 50 mM, their activity in such a hypertonic environment would be grossly impaired (6). This scenario is supported by earlier studies suggesting that the ASL in healthy individuals is hypotonic and is closer to isotonicity in the CF respiratory tract (approximately 80 mM or 120 mM, respectively [7]).

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Abbreviations: airway surface liquid, ASL; cystic fibrosis, CF; cystic fibrosis transmembrane regulator, CFTR; colony-forming units, cfu; choline Cl^-, CholCl; 3,3′-dipentyloxacarbocyanine iodide, $Di(O)C_5$; *N*-formylmethionylleucylphenylalanine, FMLP; myeloperoxidase, MPO; *N*-methyl-D-glucamine chloride, NMGCl; *Pseudomonas aeruginosa, P. aerug.*; phorbol myristate acetate, PMA; polymorphonuclear leukocytes, PMN(s); *Staphylococcus aureus, S. aur.*

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Alternative hypotheses focus on data suggesting that CFTR malfunction increases absorption of Na^+ across CF airway epithelia. The concept of raised Na^+ absorption is based on *in vitro* comparisons of rates of Na^+ absorption in CF versus normal airway epithelial cells and on the *in vivo* effects of the Na^+ transport inhibitor amiloride (1). A recent study of the putative consequences of abnormal Na^+ transport focused on the hypothesis that this dysfunction would generate reduced Na^+ concentration in CF ASL and explored the possible importance of decreased Na^+ ion concentrations for host defense mechanisms. These authors found that killing activity of *P. aerug.* by white blood cells was reduced by nearly 60% when the concentration of NaCl was lowered to 62 mM in buffers in which normal osmolality was maintained by the addition of 62 mM choline Cl (CholCl [8]).

In view of these conflicting hypotheses, we systematically varied both the Na^+ concentration and osmolality to test whether postulated alterations in ASL of CF patients would be sufficient to abrogate bactericidal activity of human peripheral blood neutrophils. Specifically, we investigated whether the Na^+ concentration *per se* or other consequences of altered ion composition, such as changes in osmolality, are important for normal neutrophil activity. We assayed bacterial killing activity of neutrophils under varying ionic conditions or in the presence of pharmacologic modulators of Na^+ transport. Because *P. aerug.* infections in children with CF are often preceded by infections with *Staphylococcus aureus* (*S. aur.*), we also used these microorganisms for these studies (2).

Materials and Methods

Materials

The standard medium for these experiments was a balanced salt solution containing 124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (Hepes; pH adjusted with NaOH) at pH 7.4 (8). NaCl was replaced by equimolar substitution with either CholCl, KCl, or *N*-methyl-D-glucamine chloride (NMGCl). Hypotonic or hypertonic solutions were prepared by altering the added NaCl concentrations to 62 or 186 mM, respectively, and D-mannitol (100 mM) was added to control osmolality as indicated. All of these reagents and chemicals, including *N*-formylmethionylleucylphenylalanine (FMLP), phorbol myristate acetate (PMA), cytochalasin B, cytochrome C, *Micrococcus lysodeikticus*, *o*-dianisidine, and amiloride, were obtained from Sigma (St. Louis, MO). Benzamil and 3,3′-dipentyloxacarbocyanine iodide [Di(O)C_5] (3) were purchased from Molecular Probes (Eugene, OR). Supplies for bacterial cultures, including Trypticase Soy Broth (TSB) and sheep blood agar plates, were obtained from the Clinical Microbiology services of UNC Hospitals (Chapel Hill, NC), which also supplied *P. aerug.* (PAO1) and a clinical isolate of *S. aur.*

Bacterial Cultures

PAO1 and *S. aur.* were grown overnight in TSB. Bacteria from these stationary cultures were diluted 1:100 in fresh TSB and incubated at 37°C until OD_{630} reached 0.4 or 0.8, respectively, for a density of approximately 10^9/ml. Aliquots from these log growth cultures were centrifuged (10,000 rpm, 5 min) and resuspended in the standard medium, with the addition of 15% heat-inactivated fetal calf serum to provide sufficient protein to avoid nonspecific bacterial adherence. Bacteria were then diluted 1:40 in 25% pooled human serum (Sigma) and opsonized for 45 min at room temperature before use in the bacterial killing assay.

PMN Isolation

Blood obtained by venipuncture from normal healthy adult volunteers (protocol approved by UNC Institutional Review Board) was first sedimented over 3% dextran/saline and then centrifuged through lymphocyte separating medium (Organon Technika, Durham, NC) to isolate polymorphonuclear leukocytes (PMNs) as described (9). Residual red cells were removed by hypotonic lysis and PMNs (> 95% purity) were resuspended in standard medium at 2×10^7/ml. Before use, PMNs were centrifuged and resuspended at the same density in appropriate salt solutions as indicated and incubated for 15 min at 37°C before functional assays were initiated.

Bacterial Killing

Assays were performed in 96-well microtiter plates (Falcon 3072) by sequentially adding 130 μl of appropriate salt solutions, 50 μl of PMNs in the same salt solutions, and 20 μl of bacteria to each well. The ratio of bacteria to PMNs was approximately 2.5:1 (range of 0.8 to 8×10^6 bacteria, 1×10^6 PMN/well). This ratio of bacteria to PMNs consistently resulted in 60 to 80% killing, whereas increasing the ratio to > 100 decreased killing to less than 50%. The same plate also contained bacteria in identical salt solutions in the absence of PMNs. Plates were incubated in a shaker (245 rpm) at 37°C for 90 min. At the end of the incubation, 0.2% Triton X-100 was added to all wells to lyse PMNs where present, and the contents of the wells were diluted serially in 1:2 strength TSB. This concentration of Triton X-100 did not interfere with bacterial survival. Ten-microliter aliquots from the 1:100 and 1:1,000 dilutions were transferred with a multitip pipettor to sheep blood agar plates that were slanted to allow the samples to form tracks as they ran down the plates. Colony-forming units (cfu) were enumerated after overnight incubation at 37°C. All conditions were tested in triplicate wells. The geometric means of the cfu data were used to calculate the percent killing observed in the presence compared with the absence of PMNs.

Superoxide Production and Enzyme Secretion

After PMNs were equilibrated in appropriate salt solutions or pharmacologic modifiers for 15 min at 37°C, they were assayed for superoxide production or enzyme secretion in response to 100 nM FMLP or 10 ng/ml PMA in the presence of 1 μM cytochalasin B in 96-well plates exactly as described (9). The reduction of cytochrome C (OD_{550}) was used to measure superoxide production and was read in a 96-well plate reader. Myeloperoxidase (MPO) was used as an indicator of azurophilic granule secretion and was assayed with *o*-dianisidine (OD_{450}). Lysozyme was quantitated by decreases in turbidity of solutions of *Micro-*

coccus lysodeikticus (OD_{450}). All conditions were assayed in triplicate wells.

Membrane Potential

PMNs were equilibrated in appropriate buffers at 37°C as previously described before adding the membrane potential-sensitive dye 3,3′-dipentyloxacarbocyanine iodide at 0.1 μM for an additional 15 to 30 min (10). The cells were then transferred to a cuvette at 37°C in a model CMIT111 fluorimeter (SPEX Industries, Edison, NJ) equipped with a stirring mechanism. The membrane potential was measured at excitation and emission wavelengths of 475 and 515 nm, respectively, with slit widths set at 10 nm. The initial photon counts for each sample from an experiment were expressed relative to the counts for the cells from the same donor in 124 mM NaCl buffer so that values > 1 or < 1 represent depolarized or hyperpolarized cells, respectively.

Data Analysis

All experiments were performed with PMNs obtained from at least three or more donors, except for the mannitol experiments as indicated. To facilitate comparisons among the different assays, all data were expressed relative to the values obtained under standard buffer conditions, that is, 124 mM NaCl added to the Hepes buffered solutions described above. The averages of these relative values, together with their standard errors from PMNs of all donors, are presented, and the statistical significance was calculated by Student's *t* test and corrected for multiple comparisons (11).

Results

Effects of Na^+ Concentration on Killing of PAO1 or *S. aur.* by PMNs

Na^+ substitution under isotonic conditions. To determine whether the removal of Na^+ ions *per se* or changes in membrane potential secondary to Na^+ substitutions affects neutrophil bactericidal activity, we substituted NaCl with equimolar concentrations of either NMGCl, CholCl, or KCl. Isotonicity is thus maintained and only KCl is predicted to depolarize the cells because neither CholCl nor NMGCl have been reported to permeate K^+ channels in PMNs (12, 13). Under our standard assay conditions using 124 mM NaCl, PMNs from 13 different donors killed 60 to 70% of added PAO1 and 50 to 70% of *S. aur.* Substitution of NaCl with 124 mM of NMGCl, CholCl, or KCl did not significantly ($P > 0.05$) decrease PMN killing activity of either *P. aerug.* or *S. aur.* (Figure 1).

Varying Na^+ concentrations under nonisotonic conditions. The results were similar when we altered the Na^+ concentration and tonicity in the assays be decreasing or increasing the concentration of NaCl by 50%: Killing of *S. aur.* was entirely unaffected, and there was a slight but not significant decrease in the killing of *P. aerug.* under hypotonic conditions (Figure 2). In other experiments in which we used 100 mM D-mannitol rather than NaCl to increase osmolality, there were no indications that bacterial killing activity of PMNs was affected. Our *in vitro* studies thus suggest that neutrophil-mediated killing of *P. aerug.* or

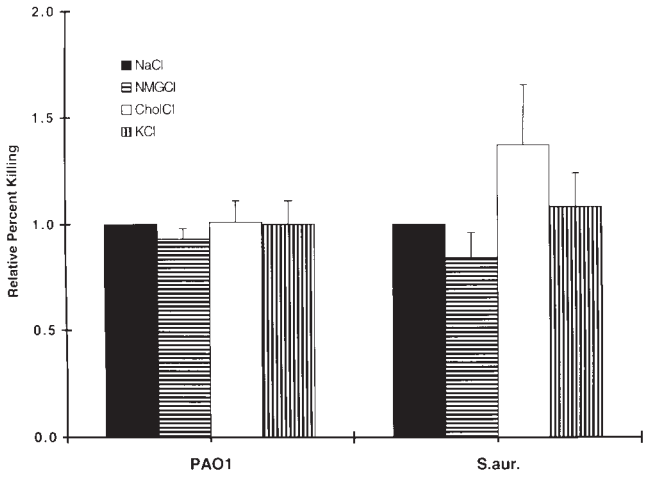

Figure 1. Effects of Na^+ replacement under isotonic conditions on relative percent killing of PAO1 or *S. aur.* Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 124 mM NaCl or equimolar concentrations of NMGCl, CholCl, or KCl before addition of PAO1 or *S. aur.* (*see* MATERIALS AND METHODS). The average percentages of cfu surviving at the end of 90 min in the presence versus the absence of neutrophils in 124 mM NaCl were 76 ± 5% and 68 ± 7% for PAO1 and *S. aur.*, respectively (8 to 12 different donors). All values in an experiment were expressed relative to percent killing observed in 124 mM NaCl before averaging.

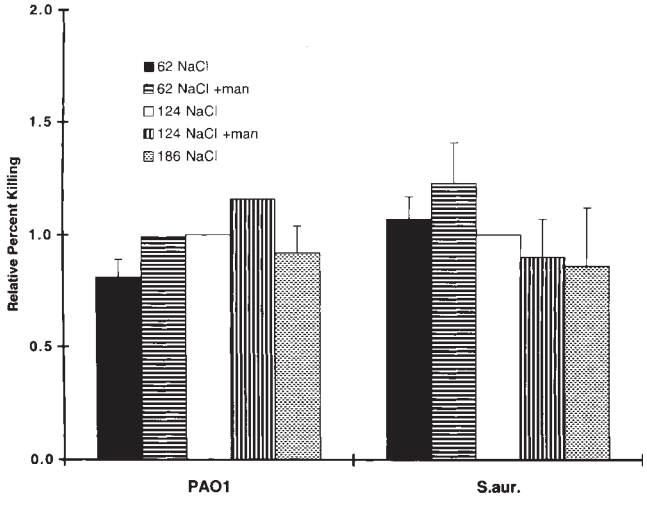

Figure 2. Effects of hypotonicity or hypertonicity on relative percent killing of PAO1 or *S. aur.* Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 62 or 124 mM NaCl without or with addition of 100 mM D-mannitol (+man) or in 186 mM NaCl before addition of PAO1 or *S. aur.* (*see* MATERIALS AND METHODS). The average percentages of cfu surviving at the end of 90 min in the presence versus the absence of neutrophils in 124 mM NaCl were 62 ± 8% and 52 ± 8% for PAO1 and *S. aur.*, respectively (four different donors). All values in an experiment were expressed relative to percent killing observed in 124 mM NaCl before averaging. Only one donor was tested for PAO1 killing in the presence of mannitol.

S. aur. is not dependent on the presence of Na^+ ions and is resistant to ± 50% deviations from isotonic conditions.

Effects of Na^+ Concentration on Superoxide Production

Na^+ substitution under isotonic conditions. Aliquots of neutrophils from the experiments described above were exposed to the chemotactic peptide FMLP or the protein kinase C activator PMA to examine the importance of Na^+ concentrations in isotonic solutions for neutrophil activation through receptor- (FMLP) or nonreceptor- (PMA) mediated mechanisms. The production of superoxide in response to FMLP was reduced moderately (20 to 30%) when NaCl was replaced with equimolar concentrations of NMGCl, CholCl, or KCl, but only the difference between NaCl and NMGCl was statistically significant ($P < 0.05$). There was no decrease in the response to PMA under the same conditions (Figure 3).

Varying Na^+ concentrations under nonisotonic conditions. In contrast, reducing or increasing tonicity by altering the concentration of NaCl by 50% significantly impaired receptor-mediated stimulation of superoxide production in neutrophils (Figure 4). Changes in tonicity alone could not account for this reduction because the addition of 100 mM D-mannitol to increase the osmolality of the low (62 mM) NaCl solution to the range of the 124 mM NaCl solution did not restore the response to FMLP. Similarly, the presence of 100 mM D-mannitol in addition to 124 mM NaCl was not nearly as detrimental to FMLP-stimulated superoxide production as was raising NaCl concentrations to 186 mM. The results with nonreceptor-mediated activation of the respiratory burst were somewhat different. Changes in tonicity also interfered with neutrophil activation by PMA, but the reduction in superoxide formation was in the 25% range for PMA compared with > 60% for FMLP stimulation, and the PMA response recovered when hypotonicity was corrected by adding mannitol. The trend for PMA-induced superoxide production under hypertonic conditions was similar to that for FMLP in that 186 mM NaCl was more inhibitory than the combination of 124 mM NaCl and 100 mM D-mannitol. These data suggest that receptor-mediated activation of the respiratory burst is dependent on the proper concentration of ions, whereas PMA is effective in low ionic solutions as long as sufficient concentrations of osmolites are present.

Effects of Na^+ Concentration on Enzyme Secretion

Na^+ substitution under isotonic conditions. The results of the ion substitution experiments on enzyme secretion were similar to those observed with superoxide production. The release of lysozyme was monitored as a convenient marker for enzyme release from several compartments, and degranulation of azurophilic granules was followed by measuring the release of MPO. Our data indicate that lysozyme secretion of neutrophils in response to FMLP or PMA was not significantly reduced when NaCl was replaced with equimolar concentrations of NMGCl, CholCl, or KCl (Figure 5a). Similarly, there was no effect

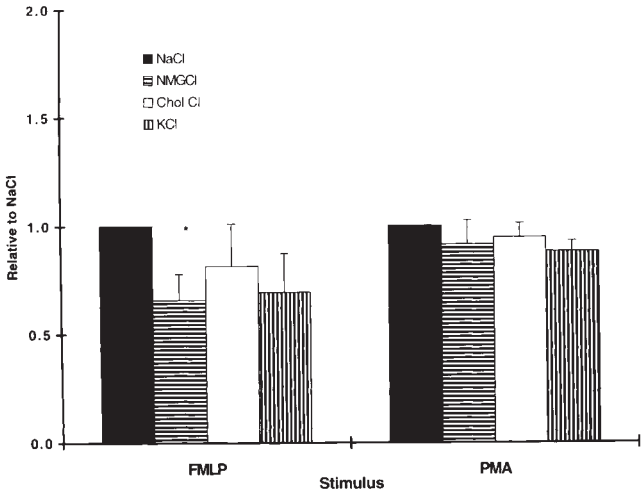

Figure 3. Effects of Na^+ replacement under isotonic conditions on relative superoxide production in response to FMLP or PMA. Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 124 mM NaCl or equimolar concentrations of NMGCl, CholCl, or KCl before stimulation with 100 nM FMLP or 10 ng/ml PMA (*see* MATERIALS AND METHODS). The average change in OD_{550} between stimulated and unstimulated cells at the end of 15 min in 124 mM NaCl was 0.181 ± 0.026 and 0.421 ± 0.024 for FMLP and PMA, respectively (8 to 10 different donors). All values in an experiment were expressed relative to the appropriate change observed for FMLP or PMA in 124 mM NaCl before averaging. *Statistical significance of $P < 0.05$ between treatment and control groups.

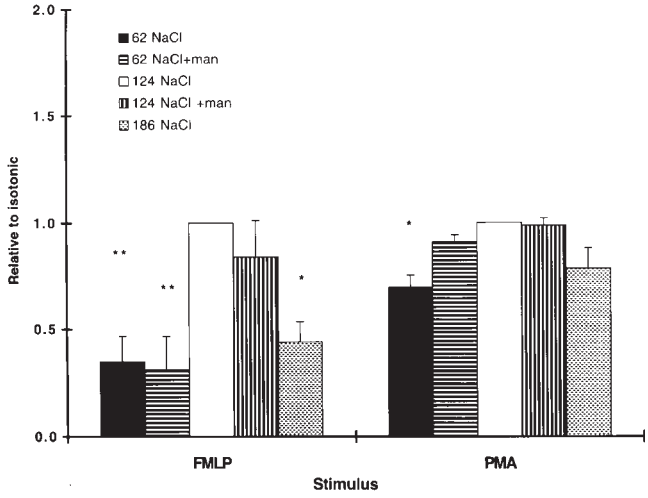

Figure 4. Effects of hypotonicity or hypertonicity on relative superoxide production in response to FMLP or PMA. Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 62 or 124 mM NaCl without or with addition of 100 mM D-mannitol (+man) or in 186 mM NaCl before stimulation with 100 nM FMLP or 10 ng/ml of PMA (*see* MATERIALS AND METHODS). The average change in OD_{550} between stimulated and unstimulated cells at the end of 15 min in 124 mM NaCl was 0.130 ± 0.034 and 0.378 ± 0.043 for FMLP and PMA, respectively (three to six different donors). All values in an experiment were expressed relative to the appropriate change observed for FMLP or PMA in 124 mM NaCl before averaging. * or **Statistical significance of $P < 0.05$ or < 0.01, respectively, between treatment and control groups.

on degranulation of azurophilic granules elicited by FMLP in these experiments (Figure 5b). In contrast, the presence of NMGCl instead of NaCl consistently reduced the secretory effects of PMA on azurophilic granules but sometimes enhanced secretion of lysozyme. Because PMA is generally a weaker secretagogue for azurophilic granules than FMLP (19 ± 3 versus 42 ± 6% degranulation, respectively), PMA-stimulated azurophil degranulation may be more easily modulated by environmental conditions.

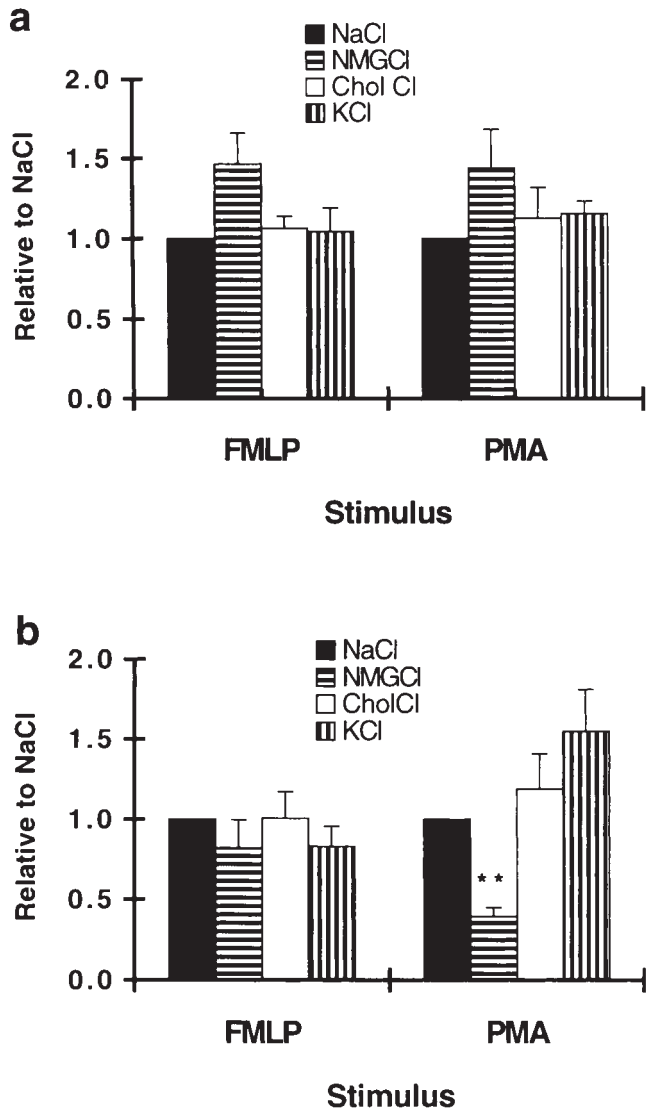

Figure 5. Effects of Na^+ replacement under isotonic conditions on relative lysozyme (*a*) or MPO (*b*) secretion in response to FMLP or PMA. Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 124 mM NaCl or equimolar concentrations of NMGCl, CholCl, or KCl before addition of 100 nM FMLP or 10 ng/ml PMA (*see* MATERIALS AND METHODS). The average net percent of Triton X-100 releasable lysozyme between stimulated and unstimulated cells at the end of 15 min in 124 mM NaCl was 48 ± 9% and 64 ± 9% for FMLP and PMA, respectively (6 to 10 different donors). The average net percent of Triton X-100 releasable MPO between stimulated and unstimulated cells at the end of 15 min in 124 mM NaCl was 42 ± 6% and 19 ± 3% for FMLP and PMA, respectively (6 to 10 different donors). All values in an experiment were expressed relative to the appropriate change observed for FMLP or PMA in 124 mM NaCl before averaging. **Statistical significance of $P < 0.01$ between treatment and control groups.

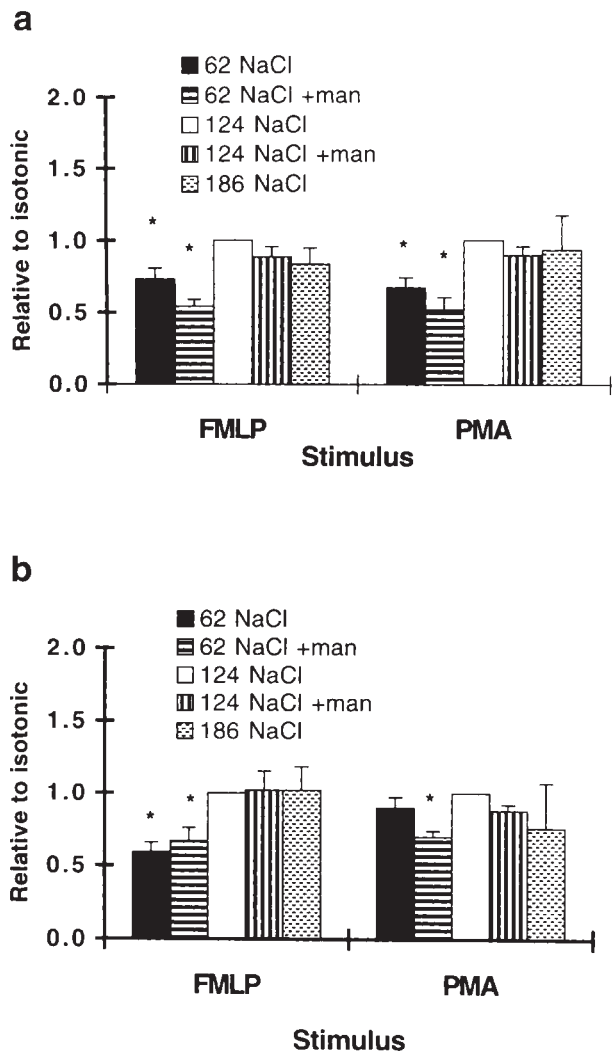

Figure 6. Effects of hypotonicity or hypertonicity on relative lysozyme (*a*) or MPO (*b*) secretion in response to FMLP or PMA. Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 62 or 124 mM NaCl without or with addition of 100 mM D-mannitol (+man) or in 186 mM NaCl before stimulation with 100 nM FMLP or 10 ng/ml PMA (*see* MATERIALS AND METHODS). The average net percent of Triton X100 releasable lysozyme between stimulated and unstimulated cells at the end of 15 min in 124 mM NaCl was 48 ± 9% and 64 ± 9% for FMLP and PMA, respectively (six to seven different donors). The average net percent of Triton X-100 releasable MPO between stimulated and unstimulated cells at the end of 15 min in 124 mM NaCl was 57 ± 5% and 19 ± 3% for FMLP and PMA, respectively (6 to 10 different donors). All values in an experiment were expressed relative to the appropriate change observed for FMLP or PMA in 124 mM NaCl before averaging. *Statistical significance of $P < 0.05$ between treatment and control groups.

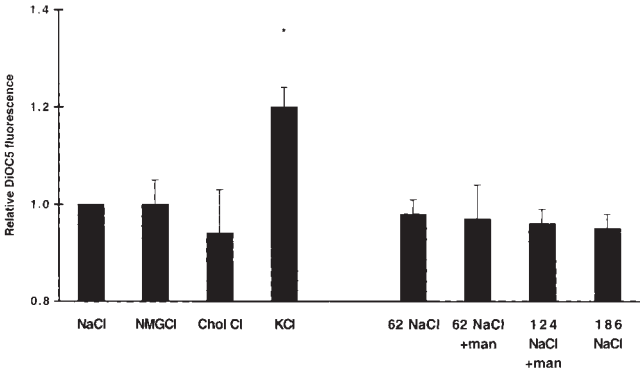

Figure 7. Effects of Na^+ replacement under isotonic conditions or changes in tonicity on membrane potential [$Di(O)-C_5$]. Human neutrophils were equilibrated for 15 min at 37°C in solutions containing 124 mM NaCl or equimolar concentrations of NMGCl, CholCl, or KCl to maintain isotonicity or in solutions containing 62 or 124 mM NaCl without or with addition of 100 mM D-mannitol (+man) or in 186 mM NaCl solutions for hypotonic or hypertonic conditions. $Di(O)C_5$ at 0.1 uM was added for an additional 15 to 30 min before measuring fluorescence (*see* MATERIALS AND METHODS). Arbitrary fluorescence units observed for cells kept in standard 124-mM NaCl solutions were set as 1.00, and all other fluorescence values in an experiment were expressed relative to this value before averaging. Data are averages of these relative values for four or three different donors for ion replacement and tonicity studies, respectively. *Statistical significance of $P < 0.05$ between treatment and control groups.

Varying Na^+ concentrations under nonisotonic conditions. Similar to the results obtained with superoxide production, low (hypotonic) NaCl buffers inhibited the secretory activity of neutrophils in response to FMLP, and adding 100 mM D-mannitol did not reverse this effect (Figure 6). Interestingly, enzyme secretion did not decline significantly in hypertonic buffers, whereas superoxide production was impaired in hypertonic (186 mM) NaCl-containing buffers. The results obtained with PMA indicate that the combination of 100 mM mannitol with low NaCl appeared to interfere with degranulation from both specific and azurophilic granules ($P < 0.05$), but the effects of low NaCl concentrations reached statistical significance ($P < 0.05$) only for lysozyme secretion.

Effects of Na^+ Concentration on Membrane Potential [$Di(O)-C_5$]

Na^+ substitution under isotonic conditions. Because changes in the extracellular concentration of Na^+ could affect neutrophil functions through the effects of Na^+ activity *per se* or through changes in membrane potential, we assayed membrane potential first in buffers where we had substituted 124 mM NaCl with equimolar concentrations of NMGCl, CholCl, or KCl. For these studies, we studied neutrophils from at least three separate donors in the presence of the membrane potential-sensitive dye $Di(O)C_5$ and monitored fluorescence intensity. The data in Figure 7 indicate that buffers containing 124 mM KCl depolarized neutrophils, as indicated by an increase in the fluorescence signal compared with cells in 124 mM NaCl. None of the other ion substitutions, including NMGCl or CholCl, yielded significant alterations in the membrane potential of neutrophils.

Varying Na^+ concentrations under nonisotonic conditions. Placing neutrophils in hypotonic buffers of 62 mM NaCl or hypertonic NaCl buffers did not affect membrane potential. Changing osmolality with noncharged molecules such as D-mannitol also did not alter the membrane potential of resting neutrophils.

Effect of Amiloride, Benzamil, or $ZnSO_4$ on Killing of PAO1 or *S. aur.* by Neutrophils

Because subjects with CF exhibit abnormalities in Na absorption, amiloride has been investigated as a pharmacologic blocker of Na^+ transport in CF patients (1). The effects of amiloride and benzamil on various functions of human neutrophils have been investigated previously to study neutrophil Na/H and Na/Ca exchangers (13–15). Although both of these agents reduced superoxide production, chemotaxis was inhibited only by benzamil, and enzyme secretion was not affected by either amiloride or benzamil. We extended these studies with amiloride and benzamil and included $ZnSO_4$ as an additional blocker of Na/Ca exchange to determine whether these modulators of Na^+ transport affected bactericidal activity of human neu-

TABLE 1
Effects of amiloride, benzamil, or $ZnSO_4$ on neutrophil functions

Treatment	Concentration (*mM*)	Bacterial Killing		Superoxide Production		Lysozyme Release		MPO Release	
		PAO1	*S. aur.*	FMLP	PMA	FMLP	PMA	FMLP	PMA
Buffer	—	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Amiloride	1.00	1.01 ± 0.05	0.98 ± 0.03	0.50 ± 0.20*	1.22 ± 0.05	0.76 ± 0.16	1.35 ± 0.43	1.17 ± 0.15	1.53 ± 0.26
Benzamil	0.1	1.06 ± 0.04	0.91 ± 0.11	0.65 ± 0.17	1.08 ± 0.08	1.16 ± 0.11	1.10 ± 0.18	1.22 ± 0.26	0.84 ± 0.19
$ZnSO_4$	0.03	1.09 ± 0.10	1.13 ± 0.08	1.06 ± 0.25	0.82 ± 0.16	0.98 ± 0.04	0.89 ± 0.11	1.01 ± 0.17	2.11 ± 0.24
	0.1	0.72 ± 0.03**	0.89 ± 0.19	0.74 ± 0.04*	0.82 ± 0.09	0.98 ± 0.10	0.91 ± 0.04	1.00 ± 0.18	2.16 ± 0.79
	0.3	0.42 ± 0.42	0.06 ± 0.06**	0.28 ± 0.07**	0.31 ± 0.14**	1.16 ± 0.26	0.86 ± 0.17	0.55 ± 0.28	1.03 ± 0.36

Neutrophils were treated with indicated concentrations of compounds for 15 min at 37°C before addition of bacteria, 100 nM FMLP or 100 ng/ml PMA. Values are expressed relative to buffer-treated neutrophils and represent four to five different donors. The following values were set as 1.00 for buffer-treated neutrophils: bacterial killing was 72 ± 8% and 79 ± 3% for PAO1 and *S. aur.*, respectively; superoxide production was 0.126 ± 0.013 and 0.531 ± 0.019 OD_{550} units for FMLP and PMA, respectively; lysozyme release was 54.5 ± 7% and 56.3 ± 6.5% of Triton X-100 releasable enzyme for FMLP and PMA, respectively; and MPO activity was 0.190 ± 0.069 and 0.079 ± 0.022 OD_{450} units for FMLP and PMA, respectively.

* or ** denotes statistical significance of $P < 0.05$ or < 0.01, respectively, between treatment and control groups.

trophils (15). Our results clearly indicate that at concentrations customarily used to differentiate between their effects on Na/H of Na/Ca transport, neither amiloride (1 mM) nor benzamil (0.1 mM) interfered with the killing of *P. aerug.* or *S. aur.* in our assays (Table 1). Although our data suggest that high concentrations of $ZnSO_4$ affected neutrophil-mediated killing of these bacteria, the antibacterial effects of 0.3 or 0.03 mM $ZnSO_4$ in the absence of neutrophils were 90 or 50%, respectively. Thus it is unlikely that Na/H or Na/Ca exchange activity are necessary for neutrophil-mediated killing of *P. aerug.* or *S. aur.*

Effect of Amiloride, Benzamil, or $ZnSO_4$ on Superoxide Production and Enzyme Secretion

To confirm that our treatment protocol with amiloride, benzamil, or $ZnSO_4$ was effective in modulating neutrophil activation, we compared the effects of these pharmacologic modifiers on receptor-mediated (FMLP) and nonreceptor-mediated (PMA) activation of neutrophils. Our results (Table 1) confirm those of others; that is, amiloride and benzamil inhibited approximately 50 and 30%, respectively, of FMLP- but not PMA-elicited production of superoxide. Amiloride did not interfere with neutrophil degranulation, as reported previously (15, 16), and benzamil also failed to affect enzyme release.

The effects of $ZnSO_4$ on superoxide production appeared to be nonspecific because this response was inhibited to the same extent when neutrophils were activated with FMLP or with PMA. Because 0.3 mM $ZnSO_4$ was incompatible with our bacterial killing assays, we did not extend these dose-response curves beyond this concentration. However, our results for enzyme release were similar to previous reports in which 3 mM $ZnSO_4$ selectively inhibited the degranulation of azurophilic granules without affecting secretion from secondary granules (17). The observation that $ZnSO_4$ enhanced the effects of PMA on the release of MPO from neutrophils supports the concept of independent activation pathways for this stimulus as compared with FMLP.

Discussion

Although CF patients frequently succumb to chronic infections of the respiratory tract with *P. aerug.*, it is still not clear which specific defects in airway defense contribute to the phenotype of chronic infection. Does CFTR malfunction change the ASL composition from a normally hypotonic to a hypertonic solution so that salt-sensitive defensins are ineffective in the CF respiratory tract (4, 5, 7)? Experimental support for this hypothesis comes from two recent publications in which ASL from CF-cultured epithelial cells or CF bronchial xenografts had abnormally high Na^+ and Cl^- concentrations (approximately 180 mM) and failed to kill bacteria unless these fluids were diluted with water (4, 5); however, Smith and colleagues could not reproduce Cl^- concentration measurements (18). When the dysfunctional CFTR was corrected with adenoviral vectors, salt concentrations declined to the 120 mM range and antibacterial activity increased dramatically.

Alternatively, are Na^+ concentrations in CF airways lower than normal, and does this lead to impaired bacterial killing by neutrophils, as recently suggested (8)? Hyperabsorption of Na^+ was observed in freshly excised CF airway epithelial cells and could be corrected with the Na^+ transport blocker amiloride (19). *In vivo* studies supported the concept of hyperabsorption of Na^+ because administration of amiloride reduced the abnormally high electrical potential difference of the nasal epithelium of CF patients (20). In one scenario, the functional consequence of raised Na^+ transport is a reduced ASL Na^+ concentration, likely leading to hypotonicity, consistent with data of sputum Na^+ concentrations recently reported by Wills and associates (21). However, Na^+ concentrations *per se* would not necessarily be decreased if Na^+-dependent liquid absorption is isotonic, as postulated by others (1, 22). Because there are published reports supporting each of these hypotheses, we wanted to address the question whether any of the proposed alterations in ASL tonicity in the CF respiratory tract would be sufficient to explain the lack of bacterial clearance by the infiltrating neutrophils. For this purpose, we examined the role of Na^+ concentrations under varying conditions of tonicity in neutrophil-mediated killing of pathogens frequently observed in early (*S. aur.*) and late (*P. aerug.*) stages of CF lung disease. We also evaluated the effects of altered ionic environments on other neutrophil functions that contribute to antibacterial activity.

Our ion substitution experiments with NMGCl, KCl, or CholCl demonstrate that under isotonic conditions, Na^+ was not required for neutrophil-mediated killing of *P. aerug.* or *S. aur.* Although we assayed bacterial killing activity in the same NaCl- and CholCl-substituted buffers containing 2.5% human serum as described in the studies cited previously (8), we consistently failed to detect any impairment of neutrophil-mediated killing when CholCl replaced NaCl. It is possible that interactions between lymphocytes and monocytes, or monocyte bactericidal activity, are more sensitive to alterations in ionic composition because Mizgerd and coworkers (8) used unfractionated leukocytes in their studies. Our results also indicate that changes in the tonicity of solutions by approximately $\pm$ 50% from isotonicity had no effect on killing of *P. aerug.* or *S. aur.* by peripheral neutrophils. Apparently, more extreme deviations from isotonicity are required to impede antibacterial activity of neutrophils. Our findings confirm earlier studies in which phagocytosis and killing of *S. aur.* or *Escherichia coli* were barely affected at 400 mOsm/liter but were severely compromised when neutrophils were assayed at $>$ 500 mOsm/liter (23, 24). The only regimen that inhibited bacterial killing activity in our assay system was to hold neutrophils at 4°C during the entire assay period, demonstrating the expected temperature dependency of neutrophil activation (data not shown). It is possible that more prolonged incubation under the different ionic conditions could affect antibacterial activity of neutrophils, but we did not want to confound these studies with possible effects on neutrophil viability or apoptosis.

Like bacterial killing, Na^+ substitution under isotonic conditions did not significantly decrease superoxide production or enzyme release. In contrast, superoxide production and degranulation in parallel experiments of identical neutrophil aliquots were clearly compromised in hypo-

tonic NaCl solutions. The inhibitory effects of low Na^+ concentrations were not due to hypotonicity *per se* because restoring isotonicity with mannitol did not reverse this inhibition. Hypertonic Na^+ concentrations also impaired generation of superoxide but affected enzyme secretion only marginally. Again, these effects on superoxide production could not be explained simply by increased tonicity because they were not mimicked by adding mannitol to create hypertonic conditions. Apparently, the ionic strength of the solutions, or possibly the Cl^- concentration, rather than osmolality, is important in maintaining receptor-mediated activation of neutrophils.

The hyperabsorption of Na^+ by CFTR-deficient respiratory epithelial cells can be blocked with amiloride *in vitro*, and the drug appears to be similarly effective *in vivo* (1, 22). The use of amiloride and other Na^+ transport blockers in CF patients could therefore affect neutrophil functions indirectly by altering the extracellular Na^+ concentration or directly by inhibiting Na^+ transport paths in neutrophils. Neither amiloride nor benzamil interfered with neutrophil-mediated killing of *P. aerug.* or *S. aur.* in our experiments, indicating that Na^+ transport paths mediated via the Na/H or Na/Ca exchange mechanism are not essential for this function. Interestingly, Zn was very effective as an antimicrobial agent by itself and therefore it was difficult to segregate effects of Zn on neutrophil function from those on bacteria. In contrast, all three of these modulators of Na^+ transport inhibited superoxide production, confirming previous reports (15, 16). However, amiloride and benzamil did not reduce enzyme secretion substantially, whereas Zn selectively reduced secretion of MPO, as expected from previous reports (15–17). Our findings indicate the *in vivo* use of amiloride or related Na^+ transport blockers should not interfere with bacterial killing activity of neutrophils. Because these compounds inhibit superoxide production only partially and do not interfere with degranulation, it is unlikely that they act as anti-inflammatory agents *in vivo* or interfere significantly with neutrophil defense mechanisms.

Although the exact ionic composition of ASL is still debated, it is unlikely that tonicity deviates from isotonicity by more than 50% in either direction (7, 21, 25, 26). Two recent studies reported values for Na^+ of 80 to 85 meq/L, K^+ of 15 meq/L, and Cl^- of 75 to 80 meq/L for lower airways ASL from normal human volunteers, with an estimated osmolality of approximately 200 mOsm/liter (22, 25). The authors suggest that osmolality may be closer to isotonic (285 mOsm/liter for plasma) under basal conditions and that the measured hypotonicity reflects stimulation of gland secretion induced by sample collection. Liquids from nasal surfaces were isotonic (approximately 300 mOsm/liter) with Na^+ of 110, K^+ of 30, and Cl^- of 125 meq/L. Importantly, the ionic composition of liquids from lower airway or nasal surfaces did not differ detectably between CF or normal subjects. These data, with the results presented in our study, imply that alterations in ionic composition of CF airways are insufficient to explain why neutrophils cannot successfully deal with infections in the respiratory tract of CF patients. However, our *in vitro* studies were limited to evaluate the direct effects of alterations in ionic composition on neutrophils and therefore we cannot exclude the possibility that minor alterations in ion composition could affect bactericidal activity of neutrophils in the complex environment of the respiratory tract.

It is likely that one of the most important factors that predispose CF infants to infections is a defect in the primary defense mechanism of the respiratory tract, namely, deficient mucociliary clearance. Rather than differences in ionic composition of ASL, it is possible that the key difference between a CF and a normal microenvironment is the concentration of mucus. Mucus that has been concentrated because of excessive isotonic volume absorption may be more difficult to transport and may also sequester invading pathogens from normal host-defense mechanisms. Such an environment may provide sufficient opportunities for microorganisms to evolve mechanisms that enable them to evade the second line of host defense, that is, infiltrating neutrophils. A variety of such mechanisms have been proposed, including sequestration of bacteria in biofilms or release of bacterial enzymes that cleave recognition molecules important for phagocytosis (27). Additionally, many of the known antibacterial factors released from neutrophils or other cells, such as defensins, lysozyme, and lactoferrin, carry strongly positive charges and thus could be bound to mucins or to DNA (6, 28). A combination of factors in the microenvironment of the CF lung thus subverts effective host-defense mechanisms, and factors other than ionic imbalances likely affect neutrophil bacterial killing activity.

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