Anion Transport and 2,3-Diphosphoglycerate in Cystic Fibrosis Red Blood Cells

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Summary

The monovalent and divalent anion transport in red blood cells from patients with cystic fibrosis and controls is not significantly different. The protein pattern of the erythrocyte membrane, as seen by sodium dodecyl sulfate-polyacrylamide gel electropherograms, is similar in cystic fibrosis and control red blood cells. The 2,3-diphosphoglycerate content of the investigated cystic fibrosis red blood cells is normal.

Abbreviations

DIDS, 4,4'-diisothiocyanate-1,2-diphenylethane-2,2'-disulfonic acid

CF, cystic fibrosis

2,3-DPG, 2,3-diphosphoglycerate

SDS, sodium dodecyl sulfate

The molecular defect in cystic fibrosis is still unknown. It is an autosomal, recessive genetic disease characterized by an impairment of the exocrine gland function that leads to serious complications in the pulmonary and digestive tract (32). One of the diagnostic features is an elevation of the sodium and chloride concentration in sweat, collected after stimulation with pilocarpin and a weak galvanic current.

Many experimental approaches have been used to elucidate the primary defect in this disease (6, 22, 32). The easy availability of erythrocytes and their well characterized properties have stimulated many studies in cation transport across the cystic fibrosis red blood cell membrane which will be summarized in the discussion (1, 4, 7, 8, 12-14, 20, 23, 24). Critical review of the conflicting data leads to the conclusion that monovalent and divalent cation transport across the erythrocyte membrane of cystic fibrosis patients and healthy control subjects is not different.

Recently, it has been suggested that a generalized defect in anion or chloride permeability is involved in cystic fibrosis. Quinton's work on isolated perfused sweat gland ducts of patients with cystic fibrosis points to an inhibition of chloride transport across the ductal epithelium (27-29). He postulates that in the patients, in contrast to the controls, chloride cannot be reabsorbed from the ductal sweat in exchange for another anion, such as bicarbonate. The sodium reabsorption is also inhibited, supposedly because of the absence of an electrogenic chloride transport. This leads to an elevation of both sodium and chloride concentration in secreted sweat. Bicarbonate secretion in the

pancreas of patients with cystic fibrosis is also inhibited according to Gaskin *et al.* (10).

Anion transport across the membrane of the human red blood cell is well documented (3, 5, 9, 17, 25, 31, 35). It is mediated by the so-called band 3 protein and can be inhibited by stilbene disulfonates very specifically. In various other types of cells, a saturable anion transport can be found that can also be inhibited by stilbene disulfonates. For example, in Ehrlich ascites tumor cells (21) and rabbit ileum (19), sulfate transport can be strongly inhibited by these compounds, whereas chloride transport is almost unaffected. Active chloride transport in the amphibian cornea is inhibited on both the aqueous and tear side of the cells by DIDS (2). However, these transport systems have not yet been characterized in so much detail that any conclusions can be drawn about the analogy to the anion transporter of the human red blood cell. It has been shown by immunological methods that in various nucleated somatic cells, including human fibroblasts, lung cells, and squamous epithelial mouth cells, there are polypeptides present that have antigenic determinants in common with erythrocyte band 3 (16). Therefore, although it is not yet clear if there exists a ubiquitous protein for the transport of anions across the cell membrane, like the Na/K-ATPase for the transport of cations, there are indications that band 3-like proteins are also present in cell types other than the erythrocyte. So far as the sweat gland is concerned, it should be noted, however, that no effect on sweat secretion and sodium reabsorption is seen when furosemide, also an inhibitor of anion transport in erythrocytes, is applied on both the luminal or contraluminal side of isolated, perfused single human sweat glands (26).

This is the first report on anion transport of the red blood cell in cystic fibrosis. We realize that the study of anion transport in red blood cells in a genetic disease which manifests itself primarily in secretory cells is of disputable significance. However, such investigation has become desirable in view of the hypothesis mentioned above.

MATERIALS AND METHODS

The patients with cystic fibrosis were attending the outpatient clinic and were not selected. Most patients received doxycycline, acetylcysteine, pancreatic extracts, and vitamins. Care was taken that no known inhibitors of anion transport (*i.e.* salicylates, furosemide) were used. Freshly drawn heparinized venous blood from controls (K) and ambulatory CF patients was washed three times in 130 mM NaCl, 1 mM Na₂SO₄, 20 mM EDTA (standard medium), pH 7.4, 0°C. The red cells were then equilibrated in this medium with ³⁶Cl for at least 30 min at 0°C. and samples were taken for the determination of hematocrit, wet and dry weight, and the ratio of intracellular to extracellular chloride concentration (Donnan ratio). The ³⁶Cl efflux was initiated by resuspension of the sedimented cells at a hematocrit of about 0.5% in standard medium, pH 7.4, 0°C. Supernatant was taken

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by the filter technique of Dalmark (5) at suitable time intervals and the radioactivity was determined in aliquots thereof.

Resealed red cell ghosts were prepared as described by Schwach and Passow (30), 24 h after blood sampling and storage at 4°C in standard medium. A hemolysis ratio of 1 volume of a 50% cell suspension in isotonic NaCl plus 25 volumes of hemolyzing medium (4 mM MgSO₄, 0.5 mM acetic acid, 0°C.) was used. Ghost volumina were determined by means of a Coulter Counter and by determination of the radiosulfate, entrapped by the ghosts.

Rate constants for anion efflux were calculated by fitting a single exponential to the data points by means of a nonlinear curve-fitting procedure. The anion efflux in red blood cells was calculated from the equation: $J = k \times r \times C \times f$ (mM·min⁻¹·kg cell solids⁻¹), where k = the rate constant in min⁻¹, r = the ratio of the chloride concentration inside/outside (Donnan ratio), C = the chloride concentration in mM/liter, and f = the ratio kg cell water/kg cell solids. The flux for red cell ghosts was calculated from the equation: $J = k \times MCV/A \times C_i$ (M·min⁻¹·cm⁻²), where k = the rate constant in min⁻¹, MCV = the average ghost volume in femtoliters, A = the surface area of a human red blood cell [1.42×10^{-6} cm²; (18, 34)] and C_i = the chloride concentration in M·cm⁻³. The flux can also be expressed as M·min⁻¹·kg⁻¹ cell solids by multiplying the flux (in M·min⁻¹·cm⁻²) by the factor 4.4×10^7 cm², which is the surface area of 3.1×10^{13} cells (= 1 kg cell solids).

Spectrophotometric determinations of 2,3-DPG were carried out immediately after blood sampling by means of a test kit purchased from Boehringer.

5% Polyacrylamide gel electrophoresis with SDS was performed after preparing white ghosts by washing the red cells at 0°C with buffer, pH 7.4, containing 0.1% saponin and solubilizing the pellet in 5% SDS and dithiothreitol for 3 min at 100°C.

RESULTS

Figure 1 shows the semilogarithmic plot of ³⁶Cl efflux at 0°C and pH 7.4 in a patient with cystic fibrosis and a control subject. With the filter technique we used, one can only measure the chloride exchange at low temperatures. The isotope efflux is so fast that it reaches equilibrium in about 1 min at 0°C. The rate constant can be calculated from the slope of this plot.

Table 1 shows the chloride equilibrium efflux in red blood cells obtained from four healthy donors and 11 patients with

Fig. 1. One example of chloride equilibrium exchange in erythrocytes of a healthy control subject (●) and a patient with cystic fibrosis (x) at 0°C, pH 7.4. Ordinate, Y , Y_0 , and Y_∞ represent, respectively, the radioactivity in the supernatant at times t , $t = 0$ and $t = \infty$. Abscissa, time in minutes. The rate constant can be calculated from the slope of the straight line through the data points, according to the equation: $\ln(Y - Y_\infty)/(Y_0 - Y_\infty) = -k \times t$, where k represents the rate constant for chloride efflux in min⁻¹ and t represents the time in minutes.

Table 1. Chloride equilibrium efflux in red blood cells*

Subject	k_{Cl}	n	r	f	Flux (mM·min ⁻¹ ·kg ⁻¹ cell solids)
Controls					
K1	3.423 ± 0.507	4	0.63	1.42	398
K2	4.207 ± 0.003	2	0.78	1.41	602
K3	3.287 ± 0.221	2	0.77	1.39	457
K4	3.633 ± 0.128	2	0.47	1.44	320
Mean flux					444 ± 60
Cystic fibrosis					
CF1	2.432 ± 0.338	3	0.86	1.92	522
CF2	2.566 ± 0.084	3	0.87	2.04	592
CF3	2.574 ± 0.158	3	0.85	2.26	643
CF4	2.717 ± 0.251	5	0.48	1.88	319
CF5	3.302 ± 0.012	3	0.61	2.34	613
CF6	3.494 ± 0.244	3	0.72	2.26	739
CF7	3.950 ± 0.043	3	0.89	2.07	946
CF8	4.026 ± 0.351	5	0.37	1.69	327
CF9	3.893 ± 0.075	3	0.79	1.93	772
CF10	3.369 ± 0.158	5	0.55	1.59	383
CF11	3.081 ± 0.198	5	0.40	1.81	290
Mean flux					559 ± 64

* The chloride equilibrium efflux was measured in standard medium at 0°C. k_{Cl} = rate constant (min⁻¹), mean ± SEM. n , Number of experiments; r , Donnan ratio; f , kg cell water/kg cell solids.

Table 2. 2,3-DPG content of red blood cells*

Subject	2,3-DPG (mM/liter red cells)
Controls	
K1	5.29
K2	5.67
K3	6.19
K4	5.16
Cystic fibrosis	
CF4	5.42
CF8	5.72
CF10	5.17
CF11	5.15
	5.24

* Note that the chloride and sulfate fluxes in erythrocyte ghosts from the same subjects are presented in Tables 3 and 4.

cystic fibrosis. The mean flux amounts to 444 and 559 mM·min⁻¹·kg⁻¹ cell solids, respectively. This difference is not statistically significant (Student's t test; $p \leq 0.15$).

Pulmonary complications in cystic fibrosis patients are common. The resulting hypoxemia raises the 2,3-DPG content of the red cell. The oxygen saturation curve of hemoglobin shifts to the left. 2,3-DPG is the most important nonpenetrating anion in the red cell after hemoglobin (11). The other organic phosphates, mainly ATP, are less important nonpenetrating anions. Nonpenetrating anions decrease the Donnan ratio and concomitantly the anion efflux (see "Material and Methods"). This lead us to measure the 2,3-DPG content in the erythrocytes of four patients with cystic fibrosis who had a relatively slow chloride efflux. Table 2 shows that the 2,3-DPG levels in the freshly drawn red blood cells of controls and patients with cystic fibrosis have normal values.

Secondly, we also measured the chloride efflux in "pink" red blood cell ghosts to avoid any influence of impermeant charged solutes on the determination of the anion flux. Red blood cell ghosts lack hemoglobin and other charged constituents, so the Donnan ratio is one (9). Table 3 shows that the mean flux is 9.7 and 7.7 nM·min⁻¹·cm⁻² in erythrocyte ghosts of controls and

Table 3. Chloride equilibrium efflux in ghosts*

Subject	k_{Cl} (min ⁻¹)	MCV (fl)	Flux (M·min ⁻¹ ·cm ⁻²) × 10 ¹⁰
Controls			
K1	2.006	54.2	100
	2.510		125
	2.541	40.8	95
	2.317		87
K2	2.704	36.2	90
	2.969		98
K3	2.267	44.0	91
	1.971		79
K4	2.522	47.2	109
	3.310		104
Mean flux			97 ± 5
Cystic fibrosis			
CF4	1.312	45.6	55
	1.107		46
CF8	1.078	40.4	40
	1.269		47
CF10	1.997	60.5	111
	2.546		141
CF11	1.874	51.5	88
	1.849		87
Mean flux			77 ± 19

* The chloride equilibrium efflux was measured in standard medium at 0°C. k_{Cl} , rate constant (min⁻¹).

patients with cystic fibrosis, respectively. This difference is also not statistically significant (Student's *t* test; $p \leq 0.15$).

As an example for the transport of divalent anions, we examined the sulfate efflux in erythrocyte ghosts that can easily be measured at 37°C since it is much slower than that of chloride. Equilibrium is not reached before 90 min under our conditions. Table 4 shows the sulfate fluxes measured in red blood cell ghosts at 37°C and pH 7.4. No difference is found for the transport of this divalent anion across the membrane of control and cystic fibrosis erythrocytes.

H₂DIDS is a very specific inhibitor of anion transport in the human erythrocyte (17, 25). This inhibitor binds covalently in a 1:1 ratio to the band 3 monomer. The H₂DIDS-binding site and the mechanism by which H₂DIDS binds to this site are well documented (25). We found 98% inhibition of chloride and sulfate efflux in both control and cystic fibrosis erythrocytes by H₂DIDS. The inhibitor concentration in the flux medium was 25 μM. This is also an indication that the function of the anion transport protein in cystic fibrosis is normal.

DISCUSSION

Anion exchange in the red blood cell is mediated by an intrinsic membrane protein, the 'band 3' protein, so-called according to its localization in polyacrylamide gels (31). It accounts for about 25% of the total protein of the membrane. It is a 95-kD polypeptide that mediates the transport of both mono- and divalent inorganic anions as well as organic anions (17). Parts of the amino acid sequence of this protein are now known (3, 15). It is an integral membrane protein and crosses the membrane at least seven times. Each erythrocyte contains about 1 million monomers of the anion transporter. Each monomer forms a functioning unit. Band 3 appears in a rather broad band on polyacrylamide gel electropherograms since the polypeptide is glycosylated with different oligosaccharides. Other minor polypeptides of similar molecular weight migrate in this region as well (31). Figure 2 shows the polyacrylamide gel electropherograms of the red blood cell membrane proteins in a healthy subject and three patients with cystic fibrosis. No abnormal bands can be detected, reconfirming the findings of McEvoy *et al.* (8, 23).

Much information on the function of the anion transport

Table 4. Sulfate equilibrium exchange in ghosts*

Subject	k_{SO_4} (× 10 ⁻²)	MCV	Flux (M·min ⁻¹ ·cm ⁻²) × 10 ¹⁴
Controls			
K1	1.49	47.5	50
K2	1.44	36.2	37
K3	1.21	44.0	38
K4	1.47	47.2	49
Mean flux			44 ± 4
Cystic fibrosis			
CF4	1.25	45.6	40
CF8	1.31	40.4	37
CF10	1.34	60.5	57
CF11	1.21	51.5	44
Mean flux			45 ± 4

The sulfate equilibrium efflux was measured in standard medium at 37°C. k_{SO_4} = rate constant (min⁻¹) and MCV = mean ghost volume (fl).

Fig. 2. SDS-polyacrylamide gel electropherograms of a healthy subject (A) and three patients with cystic fibrosis (B-D). The outer lane on the right contains proteins of known molecular weight as indicated in kilodaltons. The enumeration of the protein bands of the erythrocyte membrane according to Steck (31) is presented on the left. The apparent accessory bands in lane B are caused by mild proteolysis (observed in about 50% of the gels).

protein of the red blood cell membrane has now accumulated (3, 5, 9, 17, 35). This protein exchanges chloride for bicarbonate and vice versa (Hamburger shift). The capacity of the blood in transporting carbon dioxide from the tissues to the lungs is thus

increased because 80% of the carbon dioxide is transported as bicarbonate ions (35). Inhibition of this system (*e.g.* by salicylate poisoning) causes an increase in the PCO_2 of the blood leading to hyperventilation. Hypercapnia is not found in cystic fibrosis unless pulmonary complications are present. From this, one would not expect a serious impairment of the anion transport in red blood cells of cystic fibrosis patients.

Studies on monovalent and divalent cation transport in cystic fibrosis red blood cells have often led to contradictory results. In the following, we summarize briefly these data beginning with the monovalent cations.

Balfe *et al.* (1) found that the ouabain-sensitive and insensitive Na efflux was decreased by 5 and 35%, respectively. With ethacrynic acid, he was able to further inhibit the ouabain-insensitive efflux by two-thirds. This ouabain-insensitive, ethacrynic acid-sensitive Na efflux was diminished to about the same extent for homozygotes and heterozygotes, suggesting it to be useful in heterozygote detection. Lapey and Gardner (20) also observed a decrease in the ethacrynic acid-sensitive Na efflux except for homozygous young females and heterozygotes. Grinwald and Segal (12) found ouabain-insensitive Na efflux diminished in cystic fibrosis erythrocytes for 35%. These findings could not be confirmed by Feig *et al.* (7) and Horton *et al.* (13) who found no abnormalities in the active Na and K transport in cystic fibrosis red blood cells.

The Na/K-ATPase was normal according to Feig *et al.* (7) and Katz (14). However, Balfe *et al.* (1) found in contrast to their transport data that only the ouabain-sensitive Na/K-ATPase in homozygotes was diminished by 32%. Cole and Dirks (4) reported a significant decrease of 10% for the ouabain-insensitive Na/K-ATPase but found no difference for the ouabain-sensitive component.

In spite of these conflicting data, it seems improbable that the active Na/K transport in the cystic fibrosis erythrocyte is primarily impaired. Differences in medication and clinical situation of the patients, as well as slightly different experimental conditions may explain the variation in the results.

Furthermore, where divalent cation transport in the cystic fibrosis red blood cell is concerned, a normal Ca-ATPase activity was observed by Feig *et al.* (7) and McEvoy *et al.* (23). However, the latter found that the apparent affinity for calcium in cystic fibrosis was higher in contrast to Katz's finding of a 50% reduced activity of the Ca-ATPase with a normal affinity (14). A decreased enzyme activity corresponding to the severity of the pulmonary disease was reported by Horton *et al.* (13). According to Miner *et al.* (24), red blood cells from patients with cystic fibrosis have a reduced number of functional calcium pumps with a decreased affinity. The intracellular calcium level, however, is normal because the passive calcium permeability is also reduced. The Mg-ATPase activity was found to be normal by several workers (13, 14, 23).

The findings summarized above do not permit one to draw a definitive conclusion about an alteration in cation transport of the red blood cells of patients with cystic fibrosis. The present study shows that the transport of mono- and divalent inorganic anions across the red cell membrane of patients with cystic fibrosis and controls is not significantly different. Secondly, we reconfirm that the polyacrylamide gel electrophoreses of the erythrocyte membrane proteins from patients with cystic fibrosis and healthy subjects show the same pattern (8, 23). So far, both morphological (33) and functional studies, including the transport studies presented above, suggest that the erythrocyte in cystic fibrosis is normal and therefore not a useful tool for the study of the pathogenesis of this disease.

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