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Reduced water exchange in sickle cell anemia red cells: a membrane abnormality

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We have measured the diffusional water permeability of sickle cell anemia red blood cells under isotonic conditions using pulsed nuclear magnetic resonance (NMR) techniques. We have found that the equilibrium diffusional permeability for sickle cells is about $1.61 \cdot 10^{-3}$ cm/s, or about 60% of the value measured for normal cells. This abnormality is not related to the heterogeneity generally found in cell populations in sickle red cells with different mean corpuscular hemoglobin concentrations. We speculate that the abnormality of water exchange under isotonic conditions in sickle cells reflects an alteration of membrane proteins responsible for water exchange, possibly caused by oxidation of Band 3 proteins.

Introduction

Various structural abnormalities have been observed in the membranes of sickle cell anemia red blood cells. Loss of lipid bilayer asymmetry has been observed [1,2], and the rate of phosphatidylcholine (PC) flip-flop is altered in sickle cells [3–7]. An abnormal spectrin-actin skeleton has been found in sickle cells [8,9], and a defective association between ankyrin and spectrin in situ on sickle cell inside out vesicles has been detected [10]. Some of these membrane abnormalities have been shown to be related to oxidative damage in sickle cell membranes [1,11–15] and are probably related to the abnormal oxidant-defense systems in sickle cells [12,16, 17]. Direct observations of oxidation of both membrane lipids and membrane proteins have been reported with excess lipid peroxidation [17] and with increased membrane proteins with intramolecular disulfide linkages [14]. There is also evidence for oxidative damage in sickle cell protein 4.1 [18].

Functional abnormalities in sickle cell membranes have also been detected. Altered membrane permeability to cations has been suggested as the cause of sickle cell dehydration [19], while inhibition of the Na^+ pump has been observed in irreversibly sickled cells (ISCs). The kinetics of osmotic water transport by sickle cells is different from that of normal cells [20]. Outward water flux under osmotic stress is about 40% less in sickle cells, as compared to normal red cells.

In this work we have measured diffusional water permeability of sickle cells under isotonic conditions, since this would seem more physiological, using pulsed NMR techniques. We find that the equilibrium diffusional permeability for sickle cells is about $(1.61 \pm 0.20) \cdot 10^{-3}$ cm/s, about 60% of the value measured for normal cells. We speculate that the abnormality of water exchange under isotonic conditions in sickle cells reflects an alteration of membrane proteins responsible for water exchange, possibly caused by oxidation of Band 3 proteins.

Materials and Methods

Blood sample preparations

Normal human adult, packed erythrocytes were obtained from the blood bank of the American Red Cross Society, Chicago Chapter. 11 normal individual blood samples were used in this study. Homozygous sickle blood samples of four randomly selected, asymptomatic sickle patients were obtained. The MCHC of these patients was measured within 24 h by the manual

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Abbreviation: MCHC, mean cell hemoglobin concentration.

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method as described by Mohandas and co-workers [21] Total hemoglobin concentration was assessed with cyanmethemoglobin, and the hematocrit calculated by the method of Strumia [22] Fetal hemoglobin concentration was measured by the alkali denaturation method [23] and A_2 hemoglobin levels by anion-exchange chromatography [24] The average MCHC values and hemoglobin concentrations are listed in Table I

Hemoglobin electrophoresis on cellulose acetate plates was performed on both normal and sickle blood samples just before the NMR experiments to ensure their purity Most of the red blood cell samples were used within one to two days after withdrawal Normal blood samples that were stored up to ten days gave no substantial difference in NMR data

Blood cells were washed at 4°C three to four times with 5 mM sodium phosphate buffer (pH 8) containing 150 mM NaCl (PBS) to remove plasma and buffy coat After the last wash, most of the buffer was removed to obtain samples of packed erythrocytes with hematocrit values that ranged from 92 to 97% Both the normal and sickle cells were processed in pairs

NMR sample preparations

NMR samples consisted of washed packed cells with and without Mn^{2+} ions For samples without Mn^{2+} ions, 0 5 ml of washed packed cells, with hematocrit values of 92–97%, were transferred directly into 5 mm NMR tubes For samples with Mn^{2+} ions, 0 35 ml of the washed packed erythrocytes with the same hematocrit value were mixed thoroughly with 0.1 ml of PBS and 0 05 ml of 20 mM $MnCl_2$ in H_2O, just before NMR experiments, to give a 2 mM Mn^{2+} concentration in the aqueous extracellular environment and a hematocrit value of about 65% It is essential that Mn ions be confined to the extracellular aqueous milieu only Thus, the $MnCl_2$ solution was always prepared at least one week in advance to reduce Mn^{2+} penetration inside the red blood cells [25–27]

Multiple NMR samples were prepared from each blood preparation to give a total of 26 NMR samples from 11 normal red blood samples Seven sickle blood preparations from four sickle patients were used to prepare 20 NMR samples

NMR measurements

The methods of Pirkle and co-workers [27] were used with minor modifications All NMR measurements were performed on a Nicolet NT 200 MHz spectrometer (Nicolet Magnetics Corporation, CA) within 30 min of the Mn^{2+} addition to the erythrocytes to minimize Mn ions entering into the erythrocytes [27] The magnet was shimmed with a spinning standard sample (sodium acetate) to give a water line-width of about 0.5 Hz Pulsed NMR measurements of the water proton spin-spin relaxation time, T_2, were made at 37°C without spinning the sample, using the standard T2ECHO pulse sequence (a spin-echo pulse sequence) A 90° pulse was introduced and followed by a series of 180° pulses which were separated by time-delays of 500 ms for packed cell and 100 ms for Mn^{2+}-doped cells This pulse sequence was achieved by using a continuously variable delay trigger after the composite 180° pulse which consisted of 90 – 180 – 90 degree pulses A total of 256 amplitude measurements were taken at the top of each echo A 2 s recycling time and a 512 ms acquisition time were used Signals were averaged over 16 scans, with a total data acquisition time of 0 53 min Spectral widths were set at ±500 Hz for samples of cells without Mn^{2+}, and ±2183 9 Hz for cells with Mn^{2+} The non-spinning line-width of water signal in samples with and without Mn ion were about 60 and 10 Hz, respectively Duplicate NMR measurements were obtained from each NMR sample

NMR measurements were also obtained on normal cells that were first treated with $MnCl_2$ and then washed with PBS

Atomic absorption experiments

Atomic absorption experiments were performed on sickle cells treated with Mn ions with and without subsequent washes to ensure that no substantial amounts of Mn ions entered into the cells, since sickle cells are known to have various cation permeability abnormalities Sickle blood samples containing 2 mM $MnCl_2$, similar to those used in NMR measurements, were incubated for 30 min and washed three times with PBS The supernatant was discarded and the packed cells were used for atomic absorption experiments Control samples were sickle blood samples without $MnCl_2$

Blood samples (50 μl of packed cells) were added to 2 95 ml lysing medium (Triton X-100) for atomic absorption measurements A Perkin-Elmer Model 5000 Atomic Absorption spectrometer, equipped with a Mn lamp, was used The wavelength was set at 279 5 nm No significant amounts of Mn ions were measured in the washed samples that were treated with $MnCl_2$ (less than micromolar concentration), indicating that no significant amounts of Mn ions entered sickle red blood cells under our NMR experimental conditions

NMR data analysis

The data analysis procedure used by Pirkle and co-workers [27] was followed The data from packed cell samples, without Mn ions, were fitted, with SAS (Statistical Analysis System from SAS Institute, Research Triangle Park, NC) nonlinear regression methods, to a single exponential function, $M(t) = A \cdot \exp(-t/T_{2a}) + B$, where $M(t)$ was the decay of magnetization, or spin-echo amplitude, measured at time t T_{2a} was the spin-spin relaxation time of water protons inside the red blood cells, and B was a constant to adjust the baseline

to zero after complete relaxation had occurred For cells suspended in buffer doped with Mn^{2+}, the decay of the echo amplitudes could be resolved into two exponential components, a fast component, and a slow component, using the theory of two-site exchange [27] $M(t) = P'_a \exp(-t/T'_{2a}) + P'_b \exp(-t/T'_{2b}) + B$, where T'_{2a} was the apparent relaxation time of water molecules inside the cell (slow component) and T'_{2b} was the relaxation time for water outside the cell (fast component), P'_a and P'_b were the apparent fractional echo amplitudes from the intra- and extracellular water molecules, respectively, and B was the baseline correction factor With the values of T_{2a} obtained above from samples without Mn ions, and T'_{2a}, T'_{2b}, P'_a and P'_b obtained from samples with Mn ions, the values of T_{ex}, the water diffusion exchange time, can be obtained from the following equations [27]

$$1/T'_{2a} = 1/2\left[1/T_{2a} + 1/T_{2b} + 1/T_{ex} + 1/t_b\right] - 1/2\left\{(1/T_{2b} - 1/T_{2a} + 1/t_b - 1/T_{ex})^2 + 4/T_{ex}t_b\right\}^{1/2}$$

$$1/T'_{2b} = 1/2\left[1/T_{2a} + 1/T_{2b} + 1/T_{ex} + 1/t_b\right] + 1/2\left\{(1/T_{2b} - 1/T_{2a} + 1/t_b - 1/T_{ex})^2 + 4/T_{ex}t_b\right\}^{1/2}$$

$$P'_b = 1/2 - 1/4\left\{(P_b - P_a)(1/T_{2a} - 1/T_{2b}) + 1/T_{ex} + 1/t_b\right\} \Big/ \left\{1/2\left[(1/T_{2b} - 1/T_{2a} + 1/t_b - 1/T_{ex})^2 + 4/T_{ex}t_b\right]^{1/2}\right\}$$

$$P'_a = 1 - P'_b$$

where $P_a = 1 - P_b$ and $P_a/T_{ex} = P_b/t_b$

The diffusional water permeability constant, P_w, is then related to the exchange time by the equation $P_w = (V/A)\ 1/T_{ex}$, where V is the volume of cellular water and A is the surface area of the red blood cell [28] A volume of 0 63 10^{-10} cm^3, assuming a cell volume of 9 0 10^{-11} cm^3 and 70% water content inside the red blood cells, and a surface area of 1 42 10^{-6} cm^2 [29] were used to give a V/A ratio of 4 4 10^{-5} cm

Results

Fig. 1 shows a typical plot of the spin-echo amplitude as a function of data acquisition time for blood samples A total of 256 amplitude values were collected for each sample, with or without Mn ions For samples with Mn ions, the amplitude values approach zero at about 50 ms (curve A) For samples without Mn ions, the amplitude values are still relatively high at 50 ms (curve B), as expected, since the water relaxation time is much longer, but approach zero at about 250 ms (insert) The spin-echo amplitude values were analyzed to give relaxation times The relaxation times of individual patients are shown in Table I Substantial differences in the relaxation values between normal and sickle cell samples were observed As shown in Table II, the averaged T_{2a} of sickle cells from duplicate runs of 20 NMR samples ($n = 40$) was 85 8 ± 14 3 ms and that of normal red cell blood samples was 153.4 ± 12.4 ms ($n = 52$) A paired Student's t-test with the null hypothesis showed that the differences between sickle and normal cells are statistically significant with a t value of 20 52 and a P value of 0 0001.

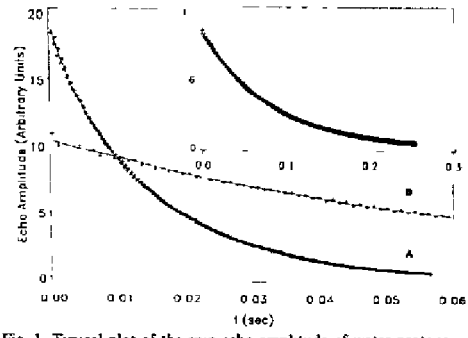

Fig 1 Typical plot of the spin echo amplitude of water protons as a function of data acquisition time for blood samples. See text (NMR measurements) for spin echo pulse sequence 256 amplitude values were collected for each sample Curve A is for a red blood cell sample containing 2 mM Mn ions at a hematocrit value of about 65% Curve B is a partial plot for a packed red blood sample without Mn ions and with a hematocrit value of about 95% A full plot of Curve B is presented in the inset The solid lines are the fitted curves as discussed in the text (Data analysis)

TABLE I

Average hematologic values and water relaxation times of the sickle patients

Patient	MCHC (gm/dl)	HbS (%)	HbF (%)	HbA_2 (%)	T_{2a} (ms)
1	33	90 1	6 4	3 6	83 9 ± 4 5 (n = 14)
2	37	92 0	3 9	4 0	82 6 ± 7 7 (n = 14)
3	34	95 8	0 7	4 2	109 5 ± 0 4 (n = 8)
4	34	91 3	6 7	2 0	56 0 ± 3 3 (n = 4)

TABLE II

Water relaxation time, exchange time, and diffusional permeability of normal and sickle cells and their t and P values from Student's t-test with the null hypothesis

Sample	T_{2a} (ms)	T_{ex} (ms)	P_w (cm/s) ($\times 10^3$)
Sickle cells (n = 40)	85 8 ± 14 3	29 2 ± 7 3	1 61 ± 0 39
Normal cells (n = 52)	153 4 ± 12 4	16 4 ± 2 6	2 76 ± 0 48
t value (n = 40)	20 52	10 54	8 28
P value	< 0 0001	< 0 0001	< 0 0001

No significant differences were observed between normal cells that were treated with $MnCl_2$ and then washed with PBS, and normal cells that were not treated with $MnCl_2$, in agreement with the published results [48] These NMR data on normal cells and the atomic absorption data on sickle cells indicate that no significant amounts of Mn ions entered red blood cells to affect the relaxation times of intracellular water molecules

The average water exchange time, T_{ex}, for sickle cells was calculated to be 29.2 ± 7.3 ms, while for normal cells it was 16.4 ± 2.6 ms Again, the difference was statistically significant, with a t value of 10 54 and a P value < 0.0001 A longer exchange time for sickle cells implies that the water permeability across sickle cells is lower than that for normal cells The average diffusional water permeability, P_w, at 37°C was calculated to be $(1.61 \pm 0.39) \cdot 10^{-3}$ cm/s for sickle cells, and $(2.76 \pm 0.48) \cdot 10^{-3}$ cm/s for normal cells These values differ significantly, as shown by the Student's t-test with a t value of 8 28 and a P value < 0.0001

Discussion

Water exchange measurements

The main techniques used to measure water exchange across erythrocyte membranes are isotope tracer, either efflux or influx, and NMR methods The diffusional water permeability of normal red cells at room temperature determined by NMR techniques is $(2.4–3.2) \cdot 10^{-3}$ cm/s [27,30–32], which agrees with results obtained by measuring the efflux of tritiated water from labeled normal red cells by the continuous flow method [29] The values obtained by influx measurements, $(4–5) \cdot 10^{-3}$ cm/s, are somewhat higher, however, the reason for the discrepancy is not clear [33] It is not likely that the difference is related to the addition of manganous ions to the extracellular medium in NMR studies, since recent studies have shown that manganous ions at concentrations as high as 19 mM do not change diffusional water permeability [29]

Our NMR results indicate that the exchange time of water across membranes under isotonic conditions increased from about 16 ms in normal cells to about 29 ms in sickle cells, an increase of about 81% The diffusional permeability in sickle cells $(1.61 \cdot 10^{-3}$ cm/s) is decreased by about 42% from that of normal cells $(2.76 \cdot 10^{-3}$ cm/s) It has been shown that NMR techniques are unique and useful in studying the physiology and pathology of water permeability in erythrocytes of patients with disease For example, using NMR techniques, the exchange time of water through erythrocyte membranes increased by 13–55% in patients with Gaucher's disease, essential hyperlipemia, obstructive jaundice, chronic hepatitis and the nephrotic syndrome [34], while the permeability decreased significantly in patients with Duchenne muscular dystrophy [35]

Homogeneity of cell population

In these studies and in previously published NMR studies of normal and abnormal blood samples, the cell populations have been considered to be uniform A single cell population, in terms of hemoglobin and water concentration, was assumed for data analysis and was probably applicable, particularly for normal red cells The assumption, however may not be applicable to sickle cells, since sickle cells are known to be heterogeneous in their hemoglobin concentrations The values for the MCHC of various fractions obtained from density-separated sickle cells range from 34 g/100 ml to 48 g/100 ml [36] Furthermore, the magnetic relaxation time depends on the hemoglobin concentration [26,37,38] Dense cells have a low T_{2a} due to the high intracellular hemoglobin concentration, since different values for MCHC may lead to different relaxation times, this leads to uncertainties in water permeability values Thus, the effects of these dense cells on membrane permeability obtained from NMR measurements should be addressed

To examine the effect of dense cells on the T_{2a} value of packed cells, in addition to the single-exponential fit used for obtaining the T_{2a} values as discussed in Materials and Methods, we have also assumed 10% or 20% very dense cells with a short T_{2a} and fitted the data to a two-exponential term equation, $M(t) = A' \cdot \exp(-t/T'_{2a}) + A \exp(-t/T_{2a}) + B$, where $A'/(A' + A)$ is either 10% or 20%, and T'_{2a} is the T_{2a} value for very dense cells with a value of 60 ms (from Fig 2, Ref. 37) From a set of 30 relaxation measurements of sickle red cells, the averaged T_{2a}, assuming 10% very dense cells, increased by about 4% above the value obtained if no very dense cells were assumed to be present (single-exponential fit) An approx. 9% increase occurred if 20% very dense cells were assumed to be in the cell population A 4% T_{2a} measurement error introduces a 2% error into the measured T_{ex} or P_w, and a 9% error introduces a 3% error into the measured T_{ex} or P_w (from Fig 2, Ref 27) Thus, the assumption of a single MCHC value in sickle cells does not appear to introduce significant error into the apparent cell water lifetime or into measured water permeability

In our data analysis, we have also assumed that both normal and sickle cells have a water volume of $0.63 \cdot 10^{-10}$ cm^3, assuming a cell volume of $9.0 \cdot 10^{-11}$ cm^3 and a 70% water content inside the red blood cells If we assume that the water content in some of the sickle cells is smaller than that of normal cells, the calculated P_w values will be smaller than the ones reported in our result section, since P_w is directly proportional to water volume in cells Thus, if the sickle cell samples have

some populations of cells with smaller cell volumes, the P_w values will be even lower than $1.61 \cdot 10^{-3}$ cm/s

Decreased water exchange and sickle cell abnormalities

Our measurements show that the water permeability value in sickle cells under isotonic condition is only about 60% of the value of normal cells If one considers that water equilibration across cell membranes is very rapid, this alteration in water permeability may have little physiological effect in sickle cells However, since membrane permeability to water is governed by membrane proteins and lipids, significant changes in membrane permeability do indirectly indicate changes in the state of membrane proteins and lipids Extensive studies on diffusional water permeability reveal that water normally travels through the human red blood cell membrane via two parallel pathways approx 90% flowing through water channels, and 10% diffusing through the lipid portion of the membrane [29,32,39] The water channels have an equivalent pore size of 4 5 Å [40], and it has been suggested that the channels are lined with integral proteins [41] The anion transporter, Band 3, also appears to be involved in water transport [39]

Excessive lipid peroxidation has been detected in sickle cell membranes [1,11–13], this might affect water transport Since only 10% of the water diffuses through the lipid portion of the membrane in normal cells [32], however, we believe that the 40% decrease in sickle cell diffusional water permeability which we observed can not be explained by altered lipid organization alone

Some sulfhydryl-reactive reagents, such as pCMB or pCMBS, which modify the proteins, reduce the diffusional transport of water to a 'ground' value of about $(1.2–1.6) \cdot 10^{-3}$ cm/s [29] General thiol oxidation of all membrane proteins has been detected in sickle cells [14], probably due to the excessive spontaneous generation of oxygen radicals [42]. It is tempting to speculate that the reduction of diffusional transport of water in sickle cells to the 'ground' value is due to the oxidation of Band 3 sulfhydryl groups in sickle cell membranes

The kinetics of osmotic water transport by sickle cells, in light-scattering studies, has been found to decrease by about 40% from that of normal cells [20] These workers suggested that the decrease was probably due to hemoglobin association with membranes Hemoglobin binds with high affinity to the cytoplasmic portion of Band 3 molecules [43] However, the inhibitory effects of pCMBS on water transport cannot be attributed to cytoplasmic SH groups The sites inside the transmembrane aqueous pore and near the extracellular surface are critical for water transport [44] Band 3 contains six sulfhydryl groups [45,46] Five of these react with both *N*-ethylmaleimide and pCMBS, while the sixth sulfhydryl, which reacts only with pCMBS, appears to be responsible for the inhibition of water transport in intact cells [44] This sulfhydryl group is located on the 15 kDa transmembrane fragment near the anion transport inhibitor site [47] Therefore, we further speculate that this sulfhydryl group in Band 3 of sickle cells may be oxidized However further work is needed to prove these speculations

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