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Research Article

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Therapy with Oral Clotrimazole Induces Inhibition of the Gardos Channel and Reduction of Erythrocyte Dehydration in Patients with Sickle Cell Disease

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Abstract

Pathologic water loss from sickle erythrocytes concentrates the abnormal hemoglobin and promotes sickling. The Ca^{2+}-activated K^+ channel (Gardos channel) contributes to this deleterious dehydration in vitro, and blockade of K^+ and water loss via this channel could be a potential therapy in vivo. We treated five subjects who have sickle cell anemia with oral clotrimazole, a specific Gardos channel inhibitor. Patients were started on a dose of 10 mg clotrimazole/kg/d for one week. Protocol design allowed the daily dose to be escalated by 10 mg/kg each week until significant changes in erythrocyte density and K^+ transport were achieved. Blood was sampled three times a week for hematological and chemical assays, erythrocyte density, cation content, and K^+ transport. At dosages of 20 mg clotrimazole/kg/d, all subjects showed Gardos channel inhibition, reduced erythrocyte dehydration, increased cell K^+ content, and somewhat increased hemoglobin levels. Adverse effects were limited to mild/moderate dysuria in all subjects, and a reversible increase in plasma alanine transaminase and aspartic transaminase levels in two subjects treated with 30 mg clotrimazole/kg/d. This is the first in vivo evidence that the Gardos channel causes dehydration of sickle erythrocytes, and that its pharmacologic inhibition provides a realistic antisickling strategy. (*J. Clin. Invest.* 1996. 97:1227–1234.) Key words: K channel • imidazoles • erythrocyte • antisickling agents • clotrimazole

Introduction

The central objective in designing a therapy for sickle cell anemia (SS)[1] is to interfere with sickle hemoglobin (Hb) polymerization—the reaction that causes the hallmark hemolysis and vascular obstruction (1). The reaction rate of this fundamental process is highly dependent on the cellular concentration of Hb S (2). Small decreases in Hb S concentration can slow polymerization to the point where the transit time for erythrocytes to pass through capillaries becomes shorter than the delay time for Hb S polymerization (2). One such successful therapeutic approach decreases the effective Hb S concentration by diluting it with Hb F (an Hb that does not participate in polymerization) using hydroxyurea to turn on silenced gamma globin genes (3–6).

Decreasing cellular Hb S concentration by hypotonic swelling of erythrocytes has also been attempted, using water loading, vasopressin, and a low Na diet. This regimen indeed resulted in cell swelling, decreased cellular Hb S concentration, and decreased sickling (7). Unfortunately the practical difficulty of chronically maintaining significant hyponatremia made these initial promising results unable to be reproduced in a subsequent study (8).

A more selective approach is to specifically inhibit the ion transport pathways that cause sickle erythrocytes to lose K^+ and water. In vitro, K^+ and water loss from sickle cells can be attributed almost entirely to two specific transport pathways: the K-Cl cotransport pathway, and the Ca^{2+}-activated K^+ channel (Gardos pathway). The K-Cl cotransport is activated every time sickle cells are exposed to pH below 7.40, leading to cumulative net loss of K^+, Cl, and water (9–11). The Ca^{2+}-activated K^+ channel, first described by Gardos (12), is activated by the increase in intracellular free Ca^{2+} concentration induced by sickling (13, 14). Approximately 100–150 Gardos channels are present in a normal erythrocyte (15). By using specific inhibitors, it should be possible to determine whether these pathways play a significant role in SS erythrocyte dehydration in vivo and thereby produce a significant reduction in cell Hb S concentration. There are no specific inhibitors of the K-Cl cotransport system which can be used clinically. Cetiedil, a very low affinity inhibitor of the Gardos channel, which also increases the Na^+ permeability (16), has shown some beneficial effects during acute sickle crises (17), but no further studies on this agent have been published in the last nine years.

In vitro work in normal (18) and sickle (19) erythrocytes has shown that clotrimazole (CLT) and other imidazole antimycotics are potent blockers of the erythrocyte Gardos channel, probably by blocking its external pore (19, 20). Studies in a transgenic mouse model (SAD mouse) for SS showed that oral CLT produced inhibition of the erythrocyte Gardos channel, increased erythrocyte K^+ content and decreased mean corpuscular hemoglobin concentration (MCHC) (21). Thus, in this mouse model for SS, the effects observed with oral CLT provide evidence for an in vivo role of the Gardos channel in cell dehydration.

Recently, we have described inhibition of the erythrocyte Gardos channel following oral CLT administration to normal human subjects (22). In these subjects and in control mice (21), CLT-induced inhibition of the Gardos channel was not associ-

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1. *Abbreviations used in this paper:* CHCM, cell hemoglobin concentration mean; CLT, clotrimazole; D_{50}, average erythrocyte density; Hb, hemoglobin; LDH, lactic dehydrogenase; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; R_{60}, median 60% density range; SS, homozygous sickle cell anemia.

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ated with changes in erythrocyte K^+ content, erythrocyte or reticulocyte MCHC, and mean corpuscular volume (MCV), suggesting that under normal circumstances the Gardos channel does not play a role in determining the K^+ or water content of normal human or mouse erythrocytes. These findings were consistent with the predictions of a mathematical model on the regulation of water and ion content in normal human reticulocytes (23).

The use of oral CLT in individuals with SS should clarify the role of the Gardos channel in determining sickle cell dehydration in vivo. In this paper we demonstrate that oral administration of CLT to subjects with SS leads to inhibition of the erythrocyte Gardos channel, and to increased cell K^+ and water content, making it a potentially useful antisickling agent.

Methods

Patients. Individuals over 13 years of age with sickle cell anemia were eligible. Inclusion criteria included normal renal and liver function, no progressive or recent neurologic disease, and performance status of 70% or greater. Individuals who had been transfused within the preceding 90 d, those maintained on chronic medications primarily metabolized by the cytochrome P-450 system, or those who were pregnant were excluded.

Approval for the study was obtained from the Institutional Review Board of Children's Hospital, Boston and by the Food and Drug Administration (Investigational New Drug Application no. 44,004). Written informed consent was obtained from each participant (and/or guardian, if the subject was a minor) after the study had been completely explained by one of the investigators.

Study protocol. The diagnosis of sickle cell anemia was confirmed by cellulose acetate hemoglobin electrophoresis. The following studies were performed at the time of entry, three times weekly during the duration of the treatment, and weekly for one to two weeks thereafter: complete and differential blood counts, erythrocyte and reticulocyte indices, electrolytes, blood urea nitrogen (BUN), creatinine, ALT, AST, total and direct bilirubin, alkaline phosphatase, LDH, urinalyses, erythrocyte phthalate density profiles, and membrane transport studies (described in greater detail below). Female subjects had negative pregnancy tests before beginning the study. All patients were treated as outpatients. No patient was hospitalized during the study.

Subjects were given a starting dose of CLT 10 mg/kg/d divided into two oral daily doses. Vaginal tablets containing 100 mg of CLT (Gyne-Lotrimin®, Schering-Plough Health Care Products, Liberty Corner, NJ) were used for this study. After each week of therapy, subjects were evaluated as to whether they had attained a threshold cellular response to CLT, defined as greater than a 10% reduction in dense cells ($d > 1.120$ measured with the phthalate density profile) or greater than 50% inhibition of the erythrocyte Gardos channel. In the absence of an adequate response, the CLT dose was increased by 10 mg/kg/d for the following week. The approved protocol allowed for weekly dose escalations of 10 mg/kg/d, up to a maximum of 60 mg/kg/d. If the threshold cellular response had been achieved, the subjects continued CLT at the same dose for an additional week and the medication was then stopped. CLT was to be discontinued if the subjects experienced significant clinical or laboratory side effects.

Measurement of ^{86}Rb influx in whole blood. Three 1-ml aliquots of blood collected in Na-heparin were incubated for 60 min at room temperature in 1.5-ml Eppendorf tubes in the presence of A23187 (80 μmol/l blood) and Tris-3-[*N*-morpholino] propanesulfonic acid (Mops; 20 mM in plasma). Tubes were wrapped in aluminum foil, to prevent inactivation of A23187 by light and gently rotated (15 rotations/min). At the end of the incubation, 100 μl of blood was spun through 0.5 ml *n*-butylphthalate and an aliquot of the resulting plasma was taken for measurement of K^+ concentration by atomic absorption. The remaining 0.9 ml of blood was centrifuged at 6,000 rpm for 10 s in a microfuge. ^{86}Rb (2 μCi/ml blood) was added to the supernatant, and the cell pellets were resuspended to initiate the influx assay. At specified time intervals (1 and 3 min), triplicate 0.1-ml aliquots were taken and spun through 0.8 ml of saline medium containing 15 mM EGTA, pH 7.4, layered over a 0.4-ml cushion of *n*-butylphthalate. After aspiration of the supernatant and upper layer of oil, the remaining tube contents were frozen in acetone/dry ice, the tube tip containing the cell pellet was cut off, and the erythrocyte-associated radioactivity was counted in a Micromedics 2000 HE gamma counter. At the 3-min time point, the supernatant was removed for determination of ^{86}Rb specific activity. Preliminary experiments indicated no significant changes in the external K concentration between 0 and 3 min. ^{86}Rb influx was calculated as detailed in references 15 and 22.

Hematology and chemistry assays. Erythrocyte cation content and phthalate density profiles were determined as described previously (4). Three parameters were evaluated with the phthalate density profile: (*a*) D_{50}, or median density, is the density value at which 50% of the cells are lighter and 50% of the cells are denser. This value is often increased in sickle cell disease. The error of the assay, determined with repeated measurements in a normal subject, is ±0.001 density units. (*b*) R_{60}, or median 60% density range, obtained after subtracting the lightest 20% and the densest 20% cell fractions. This value is increased in sickle cells, reflecting the heterogeneity in cell densities

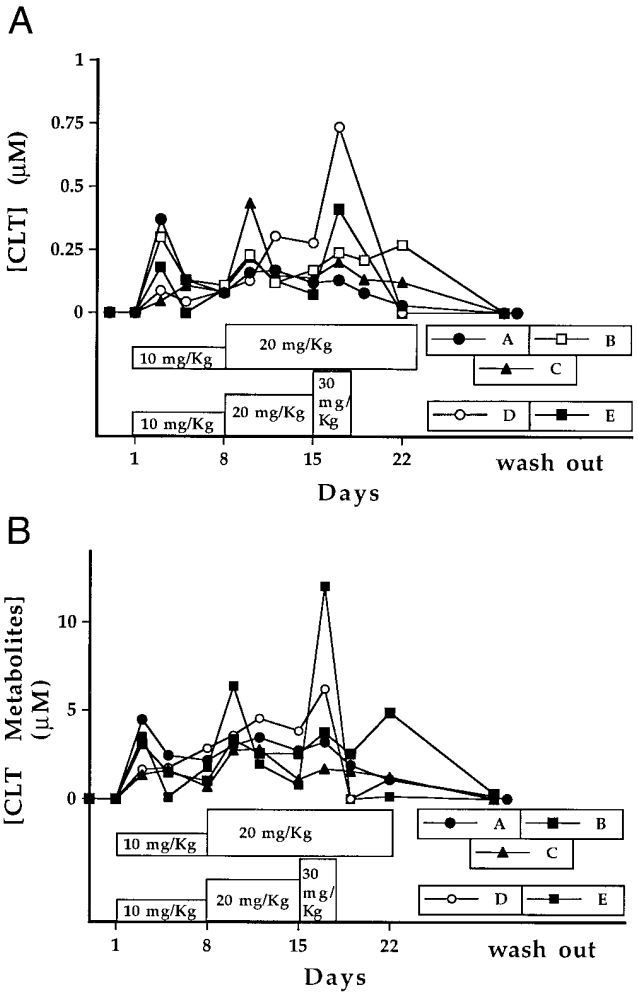

Figure 1. (*A*) Plasma levels of CLT and (*B*) summed plasma levels of two major CLT metabolites (Met A and B) (22, 24) following oral administration of CLT in five subjects with sickle cell disease.

Table I. Laboratory Values at Baseline During and after Treatment with Oral Clotrimazole

	Hematological data					Plasma chemistries					Urine
Patient Time/CLT	Hb	MCV	MCHC	Retics.	WBC	PLT	ALT	LDH	Bili T	Bili Ind	U WBC
days (mg/Kg)	*g/l*	*fl*	*g/dl*	*10^6/mm^3*	*10^3/mm^3*	*10^3/mm^3*	*U/l*	*U/l*	*mg/dl*	*mg/dl*	
A Baseline	6.7	86	34.2	0.26	11.3	310	8	563	5.5	5.2	—
5 (10)	6.8	86	33.7	0.21	10.8	307	6	531	3.3	3	4
12 (20)	7.3	85	33.2	0.26	10.3	363	8	439	1.9	1.5	1
19 (20)	7.2	85	32.7	0.26	10.9	354	9	425	1.7	1.3	2–4
Wash out, 10 d	6.7	82	34.3	0.23	11.2	281	11	521	3.1	2.7	—
B Baseline	7	88	34.5	0.29	9.3	279	13	631	6.8	6.3	—
5 (10)	6.7	87	33.3	0.22	9.4	264	14	560	3.4	3	10
12 (20)	7.4	85	33.6	0.25	8.7	366	12	507	2.1	1.7	1
19 (20)	7.4	85	33.1	0.27	10	333	10	435	2.2	1.7	1
Wash out, 10 d	6.6	85	33.3	0.24	10.9	279	10	582	3.1	2.7	0–1
C Baseline	7.8	89	32.1	0.29	12.3	371	12	617	7.8	7.5	—
5 (10)	8.1	92	32.3	0.42	11.2	401	28	645	4	3.8	2–4
12 (20)	8.5	92	32.6	0.27	8.7	376	15	497	2.2	1.8	—
19 (20)	8.5	90	33.1	0.21	9.3	364	12	499	1.7	1.4	—
Wash out, 16 d	8.5	96	32.9	0.30	11.9	354	34	434	2.5	2.2	—
D Baseline	6.9	96	31.6	0.17	8.0	246	18	476	3.5	3.1	—
5 (10)	6.7	95	31.9	0.35	7.1	362	15	451	2.2	1.7	—
12 (20)	7.1	96	31.4	0.30	6.9	360	15	410	1.7	1.3	—
17 (30)	7.8	93	33.6	0.22	10.7	357	55	505	2.2	1.5	—
Wash out, 16 d	5.8	87	35.1	—	10.1	246	29	540	3.1	2.5	—
E Baseline	9.5	68	31.3	0.31	13.5	360	34	421	2.1	1.7	—
5 (10)	9	67	32.3	0.23	11.5	340	29	397	1.9	1.7	—
12 (20)	9.6	68	31.7	0.29	8.2	368	24	422	1	0.8	—
17 (30)	9.4	65	33	0.11	7.2	267	91	416	1.2	1.0	—
Wash out, 12 d	8.3	68	31	0.17	10.1	499	32	305	1.0	0.8	—

Patients with A, B, and C were treated with CLT for one week at 10mg/kg/d and for two subsequent weeks at 20 mg/kg/d. Patients D and E were treated for one week at 10 mg/kg/d, one subsequent week at 20 mg/kg/d, and 3 final days at 30 mg/kg/d.

typical of this disease. The error of the assay, determined with repeated measurements in a normal subject, is ±0.001 density units. (*c*) Percent dense cells, expressed as the percentage of cells with density higher than 1.120. The presence of dense cells is a defining characteristic of sickle cell disease and no dense cells are present in normal blood.

CLT levels in plasma and whole blood were determined by reverse phase HPLC (24). No attempts were made to standardize the amount of time that transpired between the oral administration of CLT and the subsequent blood sampling for measurements of plasma CLT levels.

Complete blood count (CBC), erythrocyte indices, percent reticulocytes and reticulocyte indices were measured with the H*3 Bayer Hematology analyzer. This instrument measures percent reticulocytes based on the absorbance of the dye Oxazine 750 and quantifies reticulocyte cellular indices such as cell volume (MCVr), cell Hb concentration (CHCMr) and cell Hb content (CHr) (25).

Plasma levels of BUN, creatinine, ALT, AST, ALP, and other blood chemistries were measured using standard assays on a Boehringer/Hitachi 911 chemistry analyzer (Boehringer Mannheim Corp., Indianapolis, IN; Hitachi Sci. Instrs., Mountain View, CA).

Results

Plasma levels of clotrimazole and its metabolites. After the first week of oral CLT treatment at dosages of 10 mg CLT/kg/d, the dosages of all five subjects were escalated to 20 mg CLT/kg/d. Three subjects (A, B, C) were maintained for one additional week at this dosage, while in two subjects (D, E) CLT dosage was escalated to 30 mg/kg. As shown in Fig. 1, oral administration of CLT in subjects A, B, and C was associated with random plasma levels of CLT varying between 0.03 and 0.44 μM. These values are in accordance with published data on blood levels of CLT following oral administration in normal controls or patients with systemic mycoses (26–29). In two subjects (D and E) higher levels of plasma CLT were achieved at dosages of 30 mg CLT/kg/d (0.74 and 0.41 μM, respectively; Fig. 1). These levels were associated with nausea, vomiting, and increased plasma alanine transaminase (ALT) and aspartic transaminase (AST) levels (see below and Table I).

Two additional major peaks were detected in the HPLC extract, consistent with the presence of CLT metabolites (22, 30). Micromolar levels of CLT metabolites were present in plasma following CLT administration in the five subjects (maximum values ranging from 2.8 μM for subject C to 12.5 μM for subject E; Fig. 1 *B*). Given the known sensitivity of the Gardos channel to CLT and its metabolite(s), we predicted that these levels should result in significant inhibition of K^+, Cl^-, and water transport.

Effects on erythrocyte Gardos channel, K^+ content and density. Fig. 2 *A* and Table II present data on the changes in

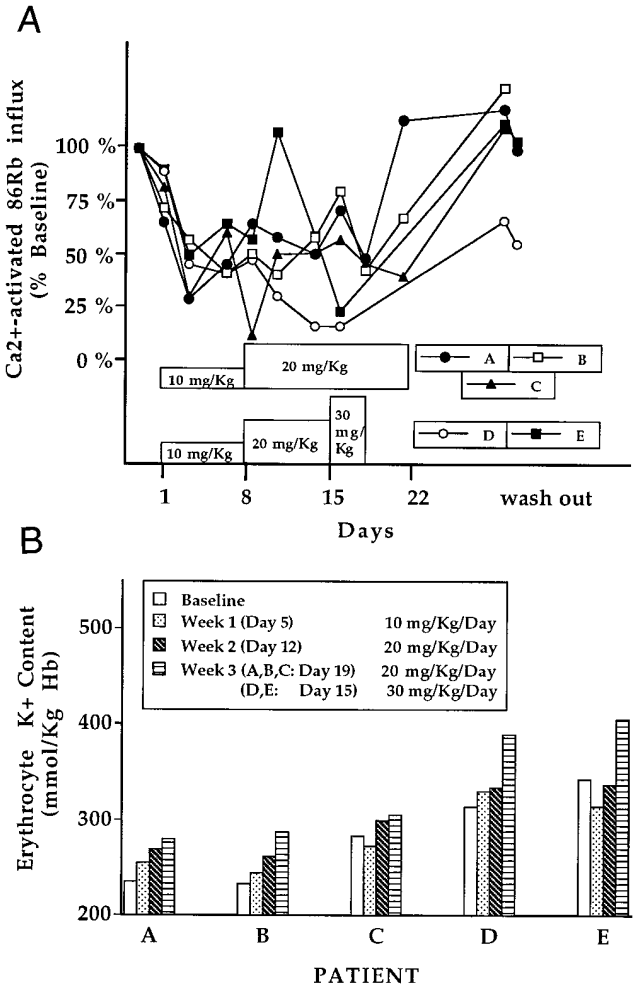

Figure 2. (*A*) Inhibition of Ca^{2+}-activated ^{86}Rb influx by oral administration of CLT as a function of time following oral administration of CLT in five subjects with sickle cell disease. (*B*) Changes in erythrocyte K^+ content during CLT administration in five subjects with sickle cell disease.

erythrocyte Gardos channel activity in the five subjects during CLT administration. This channel is silent under normal conditions, and is studied experimentally by measuring the transport of K^+ in the presence of external Ca^{2+} and a Ca^{2+} ionophore (A23187). The ionophore increases the Ca^{2+} content of the erythrocytes thereby maximally activating the channel (22, 31). Inhibition levels varied from 0 to 89% in the five subjects during the 3 wk of CLT administration (maximum inhibition: 69% in A, 65% in B, 89% in C, 80% in D, and 80% in E). Although there are no data available on the role of the Gardos channel in promoting dehydration in vivo, the levels of inhibition observed in these five patients theoretically should be associated with an increased cell K^+ content and a reduced percentage of dense cells, if the Gardos channel does indeed promote dehydration of sickle erythrocytes.

Fig. 2 *B* and Table II present data on the cell K^+ content and the percentage of dense cells in the five subjects treated with oral CLT. Fig. 3 presents the changes in the phthalate density profile for the five subjects at different CLT dosages. In all subjects, the cell K^+ content increased during CLT administration (Fig. 2 *B*), and returned toward baseline after CLT was discontinued (data not shown). In two subjects (A and B), CLT administration was associated with a significant decrease of the cell Na^+ content, which was markedly elevated at baseline, decreased significantly during CLT therapy (Table II), and returned toward baseline when CLT was discontinued (not shown). This decrease in cell Na^+ partially offset the increase in cell K^+, so that the increase in total cation content was less than the observed increase in cell K^+ (Table II).

The phthalate density method (32) provides valuable information on, and quantification of, the presence of dense cells. As shown in Fig. 3 and Table II, there was a significant reduction in the median erythrocyte density (D_{50}). Four of the five subjects, (A, B, C, and D) had a significant reduction in the percentage of dense cells and the median 60% density range (R_{60}). The percentage of dense cells and R_{60} are typically increased in sickle cell disease, reflecting erythrocyte dehydration and cell heterogeneity, respectively. The subject (E) who showed no changes in D_{50}, R_{60}, or the percentage of cells denser than 1.120 had no dense cells at the beginning of the trial, most likely as a consequence of concomitant α thalassemia (MCV 68 fL, normal iron indices).

In two subjects (A and B) there was a significant increase in K-Cl cotransport activity during CLT therapy. In the five patients combined, no significant changes in K-Cl cotransport were observed (Table II).

Absolute reticulocyte counts and reticulocyte cellular indices [reticulocyte mean corpuscular volume (MCVr), reticulocyte mean cell hemoglobin concentration (CHCMr), and reticulocyte cell hemoglobin content (CHr; reference 25)] were measured in three of the five subjects. No significant changes were observed during CLT administration (data not shown). In the five subjects, red cell MCHC did not change significantly with CLT therapy. This is not surprising, since MCHC is just the mean for a broad distribution of cell hemoglobin concentration values, and is not as sensitive as the phthalate technique to density changes occurring in a subpopulation of red cells. Significant changes were observed in the percent of cells with hemoglobin concentration > 38 g/dl, which is an estimate of dense cells obtained with the H*3 Bayer analyzer (Table II).

Hematological/biochemical changes and side effects. CLT administration was associated with a significant increase in blood Hb levels (1-tail or 2-tail paired *t* test: $P < 0.05$ at day 12, average increase 0.4 g/dl; $P < 0.05$ at day 19, average increase 0.5 g/dl, subjects A, B, and C; $P < 0.05$ at day 17/19, all 5 subjects, average increase 0.5 g/dl; see Table I). Since we did not measure red cell mass, or plasma volume, the increased Hb values have to be interpreted with some caution. Moreover, this increase in blood Hb levels was not associated with a statistically significant reduction in other hemolysis indices, such as plasma lactic dehydrogenase (LDH; Table I) or free hemoglobin (data not shown), even though plasma LDH values decreased during CLT administration in subjects A, B, and C. There were also no significant changes in the absolute number of reticulocytes during CLT treatment.

In all five subjects, CLT treatment was associated with marked reduction in indirect bilirubin levels (Table I). Three subjects (A, B, and C) presented with elevated baseline indirect bilirubin levels (5.2, 6.3, and 7.5 mg/dl, respectively) that decreased during CLT treatment to levels of 1.7, 2.2, and 1.7 mg/dl, respectively.

Side effects of CLT were minimal at doses of 10–20 mg/kg. All subjects experienced mild dysuria, and there was an occa-

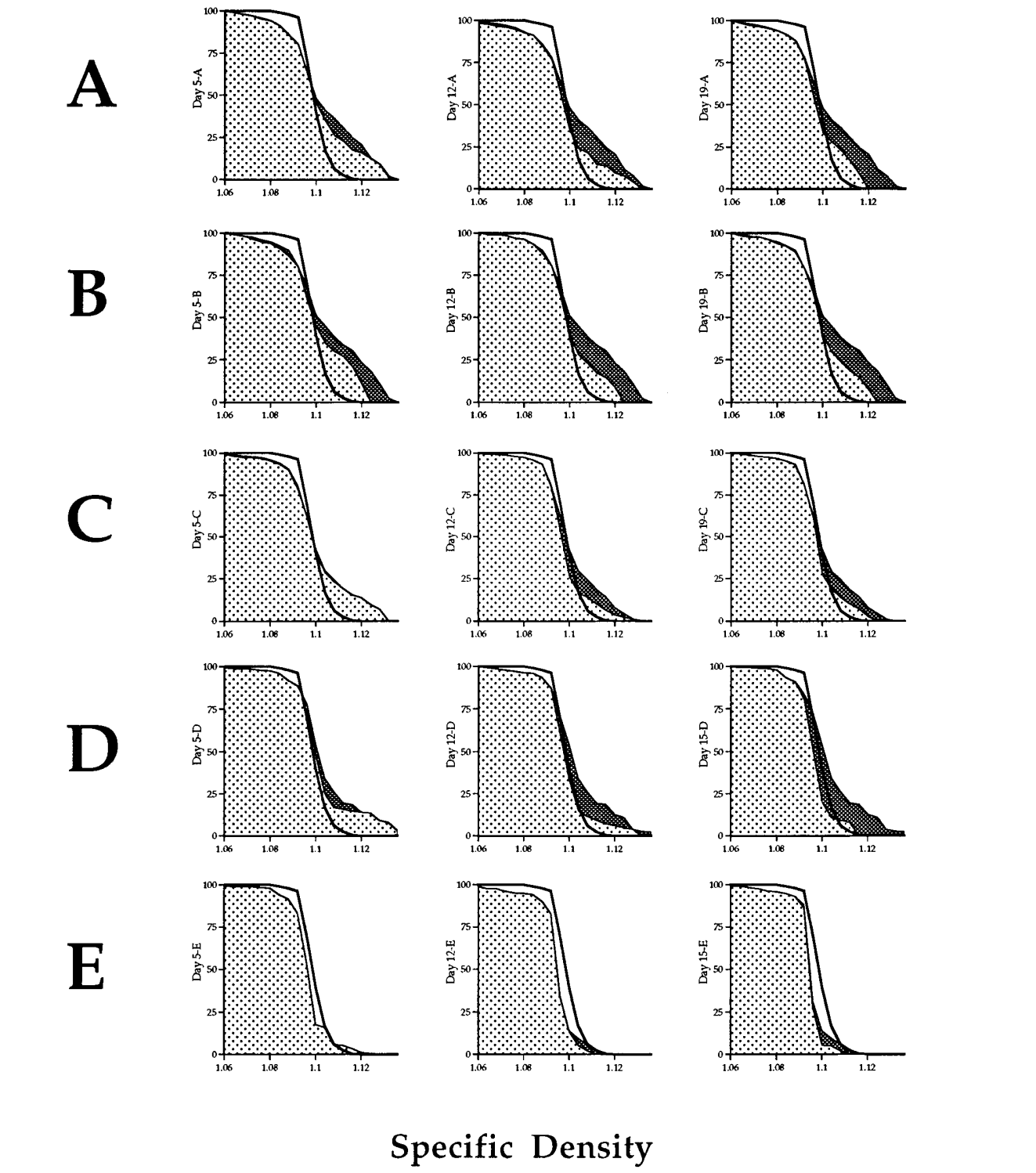

Figure 3. Phthalate density profiles before and during oral CLT administration in five subjects with sickle cell disease. The percentage of denser cells (y axis) is plotted against the density of the phthalate esters used. In each graph, the density profile of normal AA control erythrocytes is also presented, to show the increase in the percentage of dense cells characteristic of sickle cell disease. The shaded grey area corresponds to the decrease in dense cells compared with baseline measurement for each subject.

Table II. Changes in Erythrocyte Cation Content, K^+ Transport, and Cell Density Induced by Treatment with Oral Clotrimazole

Patient Time CLT dose	Cell Na	Cell K	Cell Na + K	D_{50}	R_{60}	Dense cells	CHCM > 38 g/dL	Gardos inhibition	K-Cl COT
days (mg/Kg)	*mmol/kg hemoglobin*					*%*	*%*	*%*	*mmol/l cell × h*
A Baseline	68.3	235.1	303.4	1.099	0.029	31.7	23.3	0	26.0
5 (10)	54.6	254.9	309.5	1.099	0.022	22.7	18.7	73	28.2
12 (20)	47.8	269.3	317.1	1.097	0.018	14.3	14.5	45	31.6
19 (20)	45	280.0	325.0	1.096	0.019	16.7	19.9	55	30.5
B Baseline	72.6	232.9	305.5	1.100	0.031	33.3	24.3	0	25.8
5 (10)	55	244.3	299.3	1.099	0.024	27.1	18.9	51	29.6
12 (20)	47.1	261.4	308.5	1.098	0.017	17.3	14.9	65	32.2
19 (20)	47.7	287.2	334.9	1.098	0.018	18.5	20.4	63	30.5
C Baseline	45.4	283.1	328.5	1.099	0.020	20.5	18.9	0	32.8
5 (10)	53.2	272.6	325.8	1.098	0.020	19.1	16.3	71	29.9
12 (20)	43.8	299.4	343.2	1.097	0.011	10.0	11.9	52	33.4
19 (20)	42.5	305.2	347.7	1.097	0.011	10.0	7.7	56	25.9
D Baseline	36.6	314	350.6	1.101	0.024	16.2	11.4	0	21.1
5 (10)	32.8	330.1	362.9	1.100	0.012	13.9	10.7	44	22.1
12 (20)	28.6	334	362.6	1.098	0.010	6.0	6.4	63	23.4
17 (30)	25.8	388.9	414.7	1.096	0.008	0	6.3	80	24.1
E Baseline	35.7	342.7	378.4	1.095	0.010	0	ND	0	17.5
5 (10)	30.3	314.5	344.8	1.096	0.011	0	ND	53	20.9
12 (20)	33.2	337.3	370.5	1.095	0.007	0	ND	0	21.9
17 (30)	33.2	404.9	438.1	1.095	0.007	0	ND	44	13.8
CLT-induced change (±SD)	−8.35 (10.9)	24.1 (27.6)	13.7 (24.7)	−0.002 (0.002)	−0.008 (0.005)	−8.6 (6.5)	−5.6 (3.0)	58.2 (11.3)	−1.9 (3.7)
$P <$	NS	0.005	0.05	0.005	0.0001	0.0002	0.0001	0.0001	NS

Patients A, B, and C were treated with CLT for one week at 10 mg/kg/d and for two subsequent weeks at 20 mg/kg/d. Patients D and E were treated for one week at 10 mg/kg/d, one subsequent week at 20 mg/kg/d, and 3 final days at 30 mg/kg/d. Baseline values are the mean of two separate pre-study measurements and of two separate post-study measurements. *ND*, not determined; *NS*, not significant. *K-Cl COT*, K-Cl cotransport.

sional episode of nausea or dizziness. Dysuria disappeared with discontinuation of CLT. Urinalysis showed a maximum of 10 white blood cells (WBC)/high power field in one of the five subjects in the absence of urinary tract infection. Urinalysis normalized after cessation of CLT. This side effect had been reported in the past and it is most likely due to the urinary elimination of CLT metabolites (29). Subjects A, B, and C each reported dizziness on a single occasion.

Subjects D and E experienced nausea and vomiting ~ 24 h after CLT was increased to 30 mg/kg. CLT was discontinued after this occurred and there was no further nausea or vomiting within 24 h of stopping CLT. Both subjects had marked elevation of liver enzymes at the time of the nausea and vomiting (subject D, peak plasma levels: AST 158, ALT 78; subject E, peak plasma levels: AST 122, ALT 91; also see Table I). Bilirubin, LDH, and alkaline phosphatase were not elevated during this period. Liver enzymes returned to baseline following CLT withdrawal (subject D, 25-d follow-up, and subject E, 12-d follow-up).

Discussion

We have shown that oral administration of clotrimazole leads to marked inhibition of the erythrocyte Gardos channel in five subjects with sickle cell disease. In four subjects who had a significant percentage of dense, dehydrated sickle cells before treatment, CLT caused a marked reduction in the number of these pathologic cells, and overall erythrocyte density (Table I and Fig. 3). This reduction in cell density was associated with increased cell K^+ content, indicating for the first time that the erythrocyte Gardos channel plays a significant role in the formation of dense, dehydrated sickle erythrocytes in vivo. One of the subjects (E) lacked dense cells, probably as a result of the concomitant presence of α thalassemia. In this subject, inhibition of the Gardos channel was associated with no significant changes in cell density.

Interestingly, no significant changes were observed in the cell volume or hemoglobin concentration of reticulocytes (measured in three subjects), suggesting that the Gardos channel may play a greater role in the dehydration of mature sickle cells than reticulocytes. It remains to be established in vivo that dehydration of sickle reticulocytes can be attributed to K-Cl cotransport, as suggested by in vitro data (33, 34).

These effects on erythrocyte density and K^+ transport were obtained at dosages of 20 mg CLT/kg/d, which are substantially lower than those previously used to treat systemic mycoses (100–160 mg/kg/d) (29). At the relatively low dosages of 20 mg/kg/d, CLT was well tolerated, with the major side effect

being mild/moderate dysuria, sometimes with pyuria, likely due to urinary excretion of CLT metabolites (29). The two subjects treated with higher dosages (30 mg/kg/d) experienced significant nausea and vomiting, with increased ALT and AST that returned to baseline after CLT was discontinued. Such toxicity was unexpected at 30 mg CLT/kg/d, since it had been described mostly at higher dosages, and may reflect an increased sensitivity of subjects with sickle cell disease.

This short-term study was designed to evaluate the cellular effects and possible toxicity of oral CLT in subjects with sickle cell disease. We were surprised by the increase in Hb levels and decreased indirect plasma bilirubin levels observed after only two weeks of CLT administration (Table I). Although these results indicate that CLT may reduce hemolysis, we did not observe significant change in plasma LDH or free hemoglobin levels, and the dramatic decrease in plasma indirect bilirubin level could be the result of hepatic microsomal induction, which has been described for CLT and the cytochrome P450 system (29).

The inhibition/induction of cytochrome P450-dependent enzymes is not a feature of the CLT metabolites, probably because of the enzymatic removal of the imidazole ring (30). These data, together with our finding that CLT metabolites are active as inhibitors of the Gardos channel (22), open the possibility for rational design of a second generation of Ca^{2+}-activated K^+ channel inhibitors, with a different toxicity profile from that of the "first generation" imidazole derivatives.

Other biological effects of CLT will need to be evaluated in long-term clinical trials. These effect include inhibition of Ca^{2+} entry via the dihydropyridine-sensitive Ca^{2+} channels of the plasma membrane (35, 36), inhibition of cell proliferation (37), and blockade of release of von Willebrand factor from cultured endothelial cells (38, 39). Studies in a mouse model for β thalassemia have indicated that combination of CLT with either recombinant human erythropoietin (r-HuEPO) or hydroxyurea induces a greater increase in Hb levels compared to therapy with r-HuEPO or hydroxyurea alone (40). While the bases for this potentiation induced by CLT are not yet clarified, its applicability to human SS needs to be evaluated.

This study indicates that it is possible to prevent dehydration of sickle erythrocytes in vivo by specifically blocking one of the pathways involved in the loss of cell K^+. The effects of CLT are more evident in patients with a significant number of dense cells as baseline. It remains to be determined whether it will benefit patients with few or no dense cells, such as S/β thal or SS/α thal. Future studies will also address the issues of long-term treatment and combination therapy with other antisickling agents.

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