Identification and characterization of a newly recognized population of high-Na^+, low-K^+, low-density sickle and normal red cells

Robert M. Bookchin*†, Zipora Etzion*, Martin Sorette‡, Narla Mohandas§, Jeremy N. Skepper¶, and Virgilio L. Lew‖

*Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461; ‡Bayer Diagnostics, Tarrytown, NY 10591; §Lawrence Berkeley National Laboratory, Berkeley, CA 94720; ¶MultiImaging Centre, School of Biological Sciences, University of Cambridge, Cambridge CB2 3DY, United Kingdom; and ‖Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, United Kingdom

Communicated by Helen M. Ranney, Alliance Pharmaceutical Corp., San Diego, CA, May 2, 2000 (received for review February 23, 2000)

We describe a population of sickle cell anemia red cells (SS RBCs) (≈4%) and a smaller fraction of normal RBCs (<0.03%) that fail to dehydrate when permeabilized to K^+ with either valinomycin or elevated internal Ca^{2+}. The nonshrinking, valinomycin-resistant (*val-res*) fractions, first detected by flow cytometry of density-fractionated SS RBCs, constituted up to 60% of the lightest, reticulocyte-rich (R1) cell fraction, and progressively smaller portions of the slightly denser R2 cells and discocytes. R1 *val-res* RBCs had a mean cell hemoglobin concentration of ≈21 g of Hb per dl, and many had an elongated shape like ''irreversibly sickled cells,'' suggesting a dense SS cell origin. Of three possible explanations for *val-res* cells, failure of valinomycin to K^+-permeabilize the cells, low co-ion permeability, or reduced driving K^+ gradient, the latter proved responsible: Both SS and normal *val-res* RBCs were consistently high-Na^+ and low-K^+, even when processed entirely in Na-free media. Ca^{2+} + A23187-induced K^+-permeabilization of SS R1 fractions revealed a similar fraction of *cal-res* cells, whose ^{86}Rb uptake showed both high Na/K pump and leak fluxes. *val-res*/*cal-res* RBCs might represent either a distinct erythroid genealogy, or an ''end-stage'' of normal and SS RBCs. This paper focuses on the discovery, basic characterization, and exclusion of artifactual origin of this RBC fraction. Many future studies will be needed to clarify their mechanism of generation and full pathophysiological significance.

Normal human RBCs have high K^+ and low Na^+ contents, maintained by the Na^+/K^+ pump balancing modest passive fluxes. Because of their large outward K^+ gradients in physiological conditions, selective K^+-permeabilization of the cell membranes [e.g., via the Ca^{2+}-activated K^+ (K_{Ca}) ''Gardos'' channel, or by exposure to the ionophore valinomycin] results in loss of KCl and water, and cell dehydration.

We recently showed that valinomycin-treated, K^+-permeabilized normal RBCs in low-K^+ media dehydrate at fairly uniform rates (1). Because at high levels of K^+-permeabilization the rate of cell dehydration is limited by the diffusional anion permeability, P_{Cl} (2), this uniformity reflected the narrow distribution of P_{Cl} values among the cells. Here, we investigated the distribution of RBC dehydration velocities by using flow cytometry to follow the changes in hemoglobin concentration (HC), and volume (V) of valinomycin-treated RBCs. When we applied this experimental strategy to density-fractionated sickle cell anemia (SS) RBCs, a substantial portion of the low-density cells failed to shrink. This revealed the existence of a ''valinomycin-resistant'' (*val-res*) cell fraction and raised questions about the mechanism of resistance, origin, and significance of this distinctive population of SS cells. Careful analyses also revealed minute fractions of *val-res* RBCs in normal (AA) blood. We report here the characterization of *val-res* cells, show that they represent a genuine subpopulation of circulating cells (not the result of *in vitro* processing artifacts), explain their failure to shrink, and discuss alternative hypotheses on their possible origins.

Methods

Composition of Solutions. Solution A contained (in mM) 4 KCl, 140 NaCl, 0.2 $MgCl_2$, 20 NaHepes (pH 7.5 at 37°C), and 0.05 NaOH-neutralized EGTA. Solution B differed from A by having 80 mM KCl and 65 mM NaCl. Solution C was solution A plus 1 mM adenine and 10 mM inosine. Solution D differed from A by having 15 mM KCl (to limit the dehydration of K-permeabilized cells and stay within the measurable HC limits of the flow cytometer) and 130 mM NaCl, and solution E was solution D without EGTA. Solution F differed from A by having 100 mM KCl and 45 mM NaCl. For Na-free solutions, NaCl was replaced with choline chloride and pH was adjusted with Tris. For each experiment new stock solutions of ouabain or bumetanide at 100× the final concentration were prepared in DMSO. Control suspensions received the same volumes of DMSO.

Preparation and Density Fractionation of SS RBCs. Venous blood in NaEDTA was obtained after informed consent from normal and SS donors, and RBC density fractions were immediately isolated as described (3). Briefly, the plasma was removed and the RBCs were washed thrice with solution B (filtering the packed RBCs through nylon mesh after each spin to remove white blood cells (WBCs) with minimal loss of reticulocytes), and 50% hematocrit suspensions were layered onto discontinuous arabinogalactan gradients containing high K (as in solution B) (4). The reticulocyte-rich fractions with density (δ) $\leq$ 1.087 g/ml (R1) and $1.087 < \delta \leq 1.091$ (R2), and a discocyte fraction ($1.091 < \delta \leq 1.100$) were washed twice in solution B and twice in the solution for each experiment.

Residual excess WBCs (>2%) were removed from the R1 fraction, where indicated, as described (5). Briefly, to a 50% RBC suspension in solution A, 1/20 vol of CD45 monoclonal anti-HLe-1 (50 μg/2 ml, Becton Dickinson) was added. After a 20-min incubation while shaking at room temperature (RT), the aggregated WBCs were aspirated.

Preparation and Density Fractionation of Normal RBCs. WBCs and platelets were removed by passing whole blood through a 2:1 mixture of α-cellulose and microcrystalline cellulose (Sigma) by the method of Beutler *et al*. (6) (not used for SS blood, which plugged the column). One milliliter of 30% (hematocrit) RBC

Abbreviations: AA, normal (red blood cells); SS, sickle cell anemia; RBCs, red blood cells; WBCs, white blood cells; *val-res*, valinomycin-resistant; *cal-res*, calcium-resistant; HC, hemoglobin concentration; V, volume; ISC, irreversibly sickled cell(s); RT, room temperature; MCHC, mean cell HC; CV, coefficient of variation.

†To whom reprint requests should be addressed at: Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Rm. 913U, Bronx, NY 10461. E-mail: bookchin@aecom.yu.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.130198797. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.130198797

suspension was layered onto a discontinuous gradient of arabinogalactan and spun for 30 min at 74,000 × *g* at 20°C (Beckman L3–50 centrifuge, SW 28.0 rotor). A light, reticulocyte-rich fraction (R1), from either the $\delta = 1.0829$ or 1.0874 g/ml interface, was washed thrice with solution D.

Valinomycin Treatment of RBCs. To a 5% suspension of density-fractionated RBCs in solution D, valinomycin was added from a 12 mM stock solution in DMSO for a final concentration of 10 μM. After 1 h of gentle shaking at RT, the RBCs were sedimented and washed twice in solution D, and a 50% suspension was layered onto arabinogalactan (at the same density as the RBC fraction) in solution D, and spun for 40 min at 4°C, 17,000 rpm (SS34 rotor, Sorvall RC2B). The *val-res* fractions ($\delta \leq 1.087$ g/ml for R1 or $\delta \leq 1.091$ g/ml for R2) and the dehydrated fractions (with $\delta > 1.087$ g/ml or $\delta > 1.091$ g/ml) were washed twice in the appropriate solution.

Ca^{2+} and Ionophore A23187 Treatment of RBCs. To a 10% suspension of R1 RBCs, 10 μM each (final concentrations) of $CaCl_2$ and the ionophore A23187 (from a 2 mM solution in ethanol/DMSO, 4:1, vol/vol) were added to permeabilize the RBCs to K^+ by activating the K_{Ca} channels. After 40 min of gentle shaking at RT, the RBCs were sedimented, resuspended in solution E, layered on arabinogalactan, and processed as above. The "calcium-resistant" (*cal-res*) RBCs that failed to dehydrate ($\delta \leq 1.087$ g/ml) were washed once in 30 vol of solution D with 1% albumin (BSA) to extract the A23187, and twice with solution A. The dehydrated RBCs ($\delta > 1.087$ g/ml) were washed once with solution F with 1% BSA, twice with solution F without EGTA. To restore cell K content, a 10% suspension of these RBCs was incubated with $CaCl_2$ + A23187 for 30 min at RT and was washed once with solution F with 1% BSA and twice with solution A.

Flow Cytometry. Cells from whole blood or density fractions were analyzed with a flow cytometry-based hematology analyzer, the Bayer-Technicon H*3 RTX (Bayer Diagnostics, Tarrytown, NY). The RBCs are isovolumetrically sphered (7), and Mie scatter theory is used to determine the volume (V) and hemoglobin concentration (HC) of each cell by analysis of low- and high-angle laser light scattering, as described (8, 9). The H*3 RTX instruments used in the present experiments differed from the commercial model of the H*3 in having only a manual, rather than automatic sampling mode.

Using the manual mode, we could select the RBC conditions before H*3 sampling. In preliminary experiments, after valinomycin treatment, the standard H*3 procedure of incubating for 15 min in the dye (oxazine)/buffer solution, with or without the sphering detergent (myristyl sulfobetaine), resulted in RBC swelling, with broadening of HC distributions toward lower values. This swelling was avoided if the RBCs were read by the H*3 immediately after sampling and mixing with the sphering agent, without incubation with the dye. Therefore all RBC samples were sampled two ways in the H*3: immediately after sphering, without RNA staining, and then, to detect reticulocytes, after 15 min of incubation with the dye in the appropriate buffer for the experiment, immediately after adding the sphering agent.

Measurement of SS RBC Na and K Contents by Atomic Absorption Spectroscopy. RBCs were washed thrice in ice-cold Na- and K-free solution containing 100 mM $MgCl_2$ and 10 mM Tris–Hepes, pH 7.4, and lysed in 0.2% CsCl, and Na and K were measured in triplicate by atomic absorption spectroscopy (Perkin-Elmer, model 2280). HC was measured as cyanmetHb after addition of Drabkin's reagent. In samples with small amounts of RBCs and many contaminant WBCs (as with isolation of *cal-res* cells in Na-free conditions), the RBCs were selectively lysed in a solution containing 131 mM NH_4Cl and 0.9 mM $(NH_4)_2CO_3$. After pelleting the WBCs, Na and K were measured as above (calibration standards contained the same concentrations of NH_4^+ as the samples).

Measurement of AA RBC Na/K Concentration Ratios by X-Ray Microanalysis. Because normal RBCs yielded only a minute amount of *val-res* cells, preliminary measurements of their Na and K contents were made by x-ray microanalysis, on one whole blood sample and on valinomycin-sensitive and *val-res* samples from two donors (all read anonymously). All RBC samples were washed thrice in isotonic $MgCl_2$, and twice more in isotonic choline chloride. Sedimented RBC aliquots (< 1 μl) on microtome pins were plunged into melting propane cooled in liquid nitrogen, and sectioned at −110°C with a Reichert Ultracut S. Sections (0.25 μm) were pressed onto 100 mesh Formvar/nickel film grids and placed in a covered brass pot containing a reservoir of molecular sieve. The pot was transferred under cold nitrogen gas to an Edwards 360 carbon evaporator, where the sections were freeze-dried, raised to RT over 24 h, and coated with 5 nm of carbon. The evaporator was then vented to atmospheric pressure with dry argon gas.

Using a Philips XL30 scanning electron microscope operated in the STEM mode, we analyzed the sections on a perforated carbon holder overlaying an Oxford Instruments Tetra solid-state detector. Using an Isis microanalysis system running a germanium thin window detector, with a 5-nm-diameter electron probe and a current of 1.5 nA, we collected x-ray spectra from sections of 50 different RBCs, for 50 sec each, for each sample. The net counts under the Na and K peaks were obtained by using the filtered least-squares routine in the Oxford Instruments PB QUANT program, which strips any contribution from the nickel L line to the sodium Kα peak. The means ± SEM of the Na/K ratios were compared by using the Student *t* test.

Measurement of ^{86}Rb Influx. The ^{86}Rb influx was measured as previously described (3). Briefly. ^{86}RbCl (DuPont-NEN) was added to a 10% RBC suspension in solution C at 37°C, to provide 20 μCi/ml (1 μCi = 37 kBq). At times shown, 100-μl samples were delivered into 13 ml of ice-cold solution A and spun, and the pellets were washed with 13 ml of solution A. After the pellets had been lysed in Drabkin's solution, the radioactivity was measured (LKB-Wallace model 1282 γ counter), the HC was measured as cyanmetHb, and the ^{86}Rb uptake was expressed as mmol/200 g of Hb. The ouabain- and high-bumetanide-sensitive components of the K(^{86}Rb) influx were calculated at each time point by subtracting the ^{86}Rb uptake in the presence of 0.1 mM ouabain, or of 0.1 mM ouabain + 1 mM bumetanide, from the uptake in their absence.

Results

Response of AA RBCs to Valinomycin. When unfractionated AA RBCs suspended in low-K^+ media were permeabilized to K with valinomycin (Fig. 1), progressive dehydration, as reflected in increasing MCHC and falling mean cell volume, occurred with minimal changes in the coefficients of variation (CV) of the distributions. This uniform pattern of dehydration indicated similar rates of KCl and water loss for at least most of the cells, consistent with our previous finding of uniform distribution of P_{Cl} among normal RBCs (2).

Detection of *val-res* SS RBCs. Fig. 2 shows the HC histograms of three SS RBC density fractions before and after treatment with valinomycin. After K^+-permeabilization, the lightest fraction (R1) with an initial MCHC of about 20 g/dl, showed a clearly bimodal HC histogram: one large fraction of RBCs failed to shrink, and another showed variable degrees of shrinkage and an

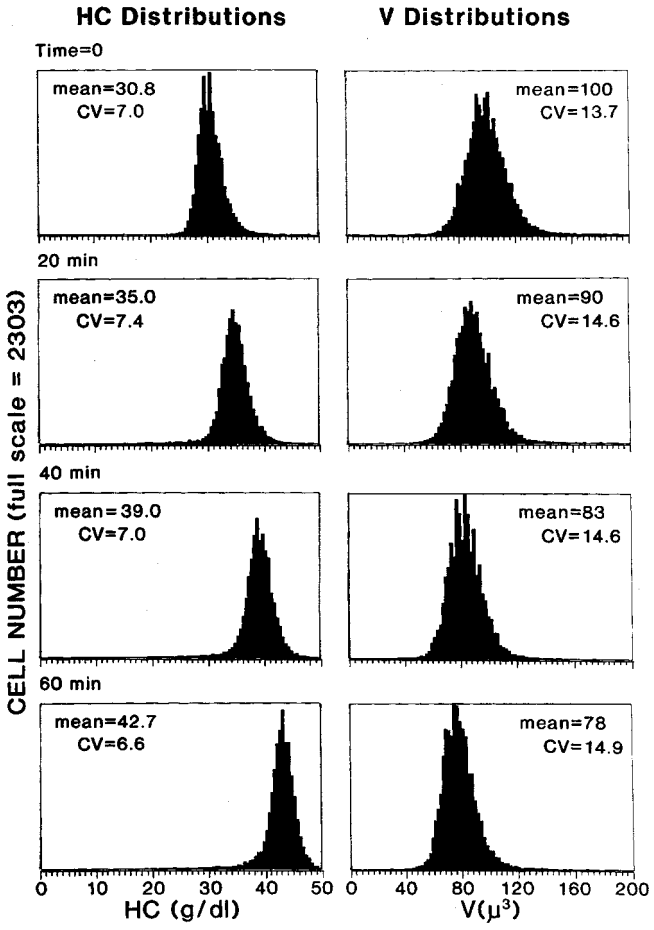

Fig. 1. Progressive changes with time in the HC and V distributions of normal RBCs after K^+-permeabilization with valinomycin. Histograms were obtained with the Bayer H*3 analyzer during a 1-h incubation at 37°C of RBCs suspended in buffer D (15 mM K) and treated with valinomycin. Note the progressive increase in HC and decrease in V, with no evident broadening of cell distribution. CV, coefficient of variation. Samples taken at the times indicated were diluted in the same buffer containing sphering agent and were read immediately without RNA staining.

MCHC of about 34 g/dl. The next denser fractions, R2 (initial MCHC $\approx$ 23 g/dl) and the light discocytes (initial MCHC $\approx$ 26 g/dl), each showed progressively smaller but distinct *val-res* fractions.

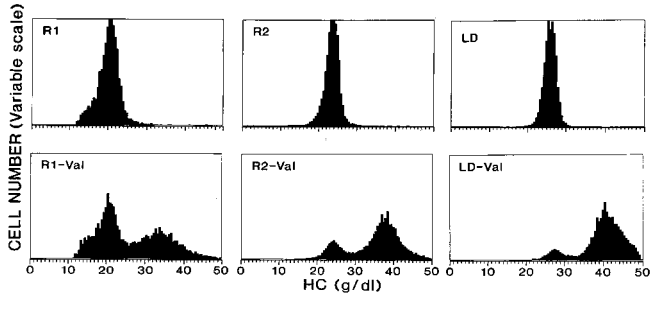

Fig. 2. Effects of K^+-permeabilization with valinomycin on the HC distributions of the light fractions of SS RBCs. After density fractionation, the three lightest fractions of SS RBCs, R1, R2, and LD (light discocyte) (see *Methods* for density boundaries) were treated as in Fig. 1. *Upper* and *Lower* histograms show the HC distributions before and after a 30-min incubation with valinomycin, respectively.

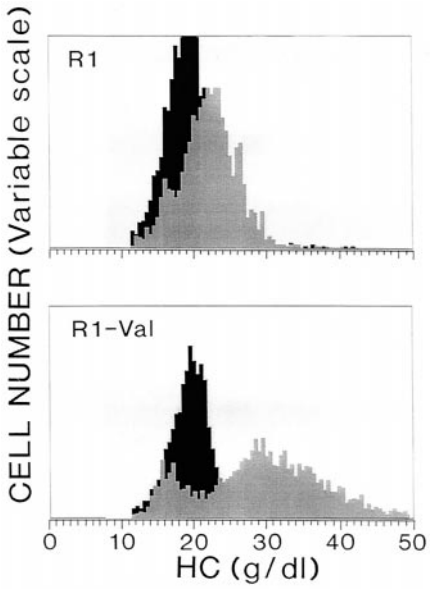

Fig. 3. Effects of K^+-permeabilization with valinomycin on the HC distributions of the reticulocyte and nonreticulocyte components of the lightest, R1, fraction of SS RBCs. The treatment was the same as in Fig. 2, except that the samples of RBC suspension were diluted in buffer D containing RNA stain for the reticulocytes and incubated 15 min before adding the sphering agent. In the histograms, the reticulocytes are shown in gray and nonreticulocyte RBCs in black.

RNA staining of the same cell fractions before flow cytometric analysis, to distinguish the behavior of reticulocytes and mature RBCs (Fig. 3), revealed that most of the *val-res* RBCs were not reticulocytes, and that most of the reticulocytes (constituting 49% of the R1 fraction in the experiment shown) underwent substantial dehydration after valinomycin.

When these procedures were repeated with density-fractionated SS RBCs from a second donor, the overall RBC and reticulocyte distributions were the same as those shown in Figs. 2 and 3.

Fraction of *val-res* RBCs in SS Samples. For a preliminary estimate of the overall proportion of SS *val-res* RBCs, we screened several unfractionated SS blood samples from different donors by flow cytometric analysis after treatment with valinomycin in the same A/15K buffer. On the basis of the observed HC values of *val-res* RBCs in SS R1 fractions, we selected a cut-off HC of <25 g of Hb per dl as indicating resistance to shrinkage after valinomycin. From the HC histograms, the RBCs whose HC remained less than 25 g/dl after valinomycin ranged from 3.3% to 4.5% of the total, with a mean $\pm$ SEM of 4.08% $\pm$ 0.13% ($n = 5$).

Na^+ and K^+ Contents of SS *val-res* RBCs. We considered the possibility that the *val-res* cells were resistant to K^+-permeabilization or that their chloride permeability was abnormally low, thus limiting the net loss of KCl and water from K^+-permeabilized cells. But preliminary experiments (not shown), indicating that these cells could be swollen in high-K media, ruled out failure of K-permeabilization by valinomycin or low chloride permeability, and suggested that the most likely explanation for the cells' resistance to shrinkage was an altered monovalent cation composition. Indeed, measurements on the *val-res* RBCs from several SS donors (Table 1) showed a distinctly decreased K and increased Na content. Therefore, the RBCs' markedly reduced outward K^+ gradient in low-K^+ media explained their failure to shrink after K^+-permeabilization.

Table 1. Na and K content of SS *val-res* RBC

Experiment/donor	Content, mmol/liter of cell water*	
	Na^+	K^+
J.M.	122.8	56
L.M.	104.1	29.5
K.C.	107.2	62.4
J.M.	99.4	34.5
R.D.	90.5	70.9
J.M.	78.6	30.5
J.M.	89.8	31.8
J.N.	104.2	24.9
J.M.†	79.6	22.7
Mean ± SD	97.4 ± 13.4	40.4 ± 16.8

The light-density fractions (R1, or R1 and R2 combined) of SS RBC were K^+-permeabilized by exposure to valinomycin, and the *val-res* fractions were isolated.

*Cell water: for 20 g of Hb/dl of RBC, with specific volume of Hb = 0.75 ml/g, Hb = 15 ml/dl and water = 85 ml/dl of RBC. Thus [mmol (Na or K)/20 g of Hb]/0.85 = (Na or K)/liter of cell water.

†$Choline^+$ replaced Na^+ in buffers, from the time of the first RBC washes, after sampling.

To test whether the observed high Na of the *val-res* RBCs was largely an artifact, due to *in vitro* manipulations of Na^+-leaky RBCs in high-Na^+ media, we performed the entire isolation procedure, immediately after venipuncture, in media in which Na^+ was replaced by $choline^+$. As shown in Table 1, the *val-res* cells isolated under these conditions also showed high Na contents, which must therefore be a feature of circulating *val-res* RBCs.

Effects of K^+-Permeabilization via the K_{Ca} Channel: Generation of "Ca^{2+}-Resistant" (*cal-res*) RBCs. To confirm that K^+-permeabilization *per se* generated the *val-res* RBC population, and not some other unrecognized effect of valinomycin, the light fractions of SS cells (R1 and R2) were K^+-permeabilized by treatment with the Ca^{2+} ionophore A23187 in the presence of external Ca^{2+} to activate their K_{Ca} channels. In the presence of external Na^+ or in its absence (replaced by $choline^+$, from the time of sampling, or by *N*-methyl-D-glucamine$^+$ from after the density separations), these maneuvers revealed a fraction of *cal-res* RBCs (Table 2) with Na and K contents similar to those of the *val-res* fractions (Table 1). Under the most stringent Na-free conditions (with external $choline^+$), and with selective RBC lysis for Na and K measurements so as to exclude WBCs, the *cal-res* cells had Na contents of more than 100 mmol/liter of cell water and K contents of less than 50 mmol/liter of cell water (Table 2).

Table 2. Na and K content of SS *cal-res* RBC

Experiment/donor	Content, mmol/liter of cell water*	
	Na^+	K^+
J.M.	85.8	11.1
J.M.†	50.9	59.6
F.A.	113.8	35.6
B.B.‡	118.6	46.4
Mean ± SD	92.3 ± 27.0	38.2 ± 17.8

The light-density fractions (R1, or R1 and R2 combined) of SS RBC were K^+-permeabilized by exposure to Ca^{2+} and A23187 and the *cal-res* fractions.

*See Table 1 legend for calculation of Na or K per liter of cell water.

†*N*-Methyl-D-glucamine$^+$ replaced Na^+ in buffers after density fractionation.

‡$Choline^+$ replaced Na^+ in buffers from the time of the first RBC washes, after sampling.

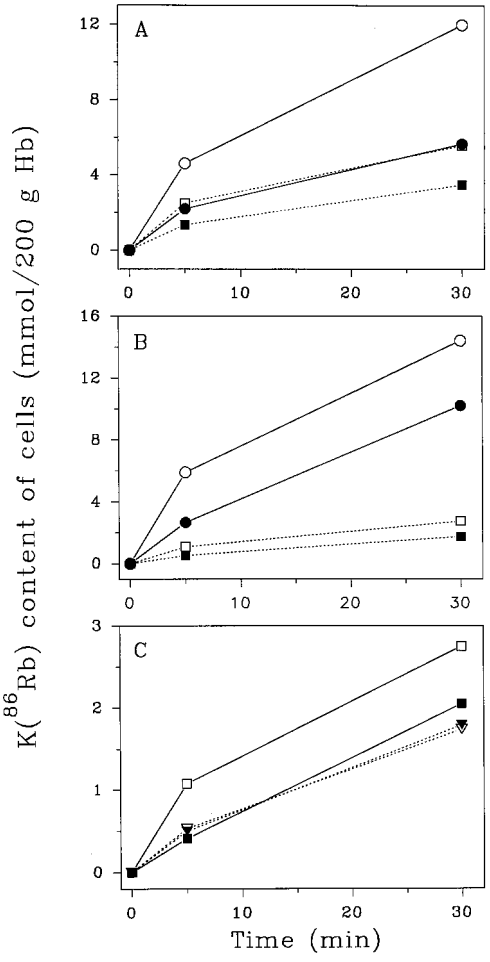

Fig. 4. ^{86}Rb influx measurements in *cal-res* and non-*cal-res* portions of the lightest, R1, fraction of SS RBCs, in the presence and absence of ouabain and high (1 mM) bumetanide. Before measurements, the isolated, dehydrated non-*cal-res* cells were replenished with KCl and rehydrated. *A* and *B* show two patterns of influx seen in four experiments. ○, *cal-res* RBCs; ●, *cal-res* RBCs + ouabain; □, non-*cal-res* RBCs; ■, non-*cal-res* RBCs + ouabain. The experiment in *C* is typical of several showing the effects of added bumetanide on ouabain-treated *cal-res* and non-*cal-res* RBCs. □, *cal-res* RBCs + ouabain; ■, *cal-res* RBCs + ouabain + bumetanide; ▽, non-*cal-res* RBCs + ouabain; ▼, non-*cal-res* RBCs + ouabain + bumetanide.

^{86}Rb Influx Measurements in *cal-res* RBCs: Effects of Ouabain and Bumetanide. The high-Na, low-K contents of the *val-res* and *cal-res* RBCs could result from inhibition of their Na^+/K^+ pumps, from large passive Na^+ and K^+ leak fluxes, or both. Because K^+(^{86}Rb)-permeabilization by valinomycin is irreversible, ^{86}Rb influx was measured in the *cal-res* RBCs and compared with that of non-*cal-res* (Ca^{2+}-sensitive) RBCs from the same fraction, after rehydrating the latter and extracting the ionophore A23187 (see Fig. 4 legend). Fig. 4 *A* and *B* shows the two patterns observed (each pattern was observed twice, in a total of four experiments). Ouabain-sensitive (OS) and ouabain-resistant (OR) ^{86}Rb fluxes were both consistently higher in *cal-res* than in non-*cal-res* RBCs. The high OR flux indicates a high passive K^+ permeability, greater in the *cal-res* RBCs. The higher OS ^{86}Rb influx in the *cal-res* RBCs may be partly due to Na^+/K^+ pump stimulation by the reversed Na^+/K^+ concentration ratio. But such a high absolute value for a Na^+/K^+ pump flux in a low-reticulocyte RBC fraction has never been described. These results suggest that *cal-res* (and, presumably *val-res)* RBCs may have a Na/K pump-leak balance with much larger leaks than

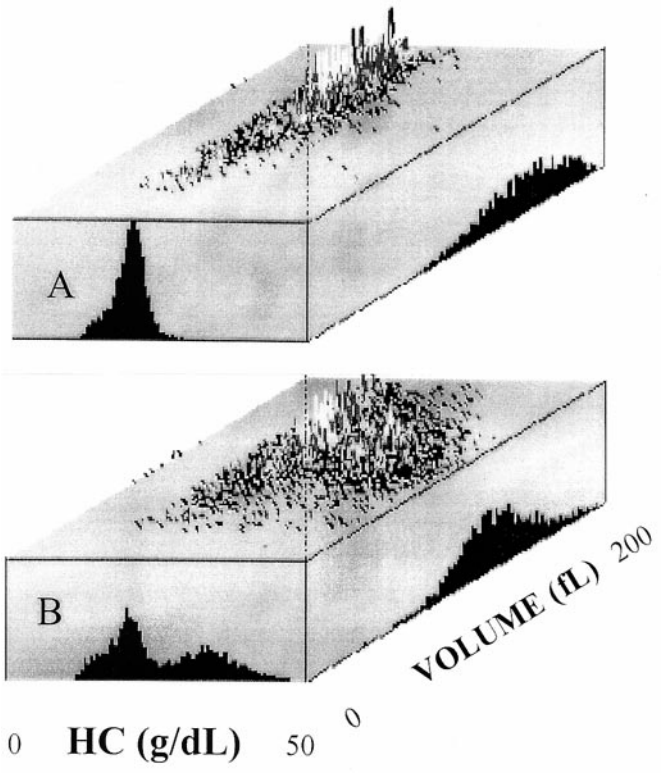

Fig. 5. Cell volume and HC histograms and three-dimensional cytograms of the SS R1 RBCs before (*A*) and after (*B*) K^+-permeabilization with valinomycin. The different RBC volume distributions corresponding to the valinomycin-sensitive and the *val-res* fractions can be distinguished on the cytogram, despite their overlap on the volume histogram: note the broad volume distribution of the lighter (*val-res*) cells, compared with the cells that have dehydrated.

those observed in most reticulocytes and mature RBCs. However, substantial heterogeneity of transport properties within these cell populations (5) prevent precise comparisons between ionic traffic in reticulocytes and *val-res* cells.

In a recent study of ion transport heterogeneity in the lightest SS RBCs, we discovered a rapid turnover (RTO) K^+(^{86}Rb) pool in a small subpopulation, with the distinctive property that its OR ^{86}Rb influx component was inhibited by high concentrations (1 mM) of bumetanide (5). To test whether the high pump-leak *cal-res* RBCs might coincide with that bumetanide-sensitive RTO K^+ pool, we compared the effect of bumetanide on the OR ^{86}Rb influx in *cal-res* and non-*cal-res* RBCs. We found (Fig. 4*C*) that bumetanide inhibited that flux only in the *cal-res* RBCs, and had no effect on the ^{86}Rb influx into the reticulocyte-rich, non-*cal-res* RBCs. Thus, the *cal-res* fraction may be identical to the RTO K^+ pool, and part of the high passive K^+ permeability of *cal-res* RBCs may include a permeation pathway distinct from that in other RBCs, in its sensitivity to high levels of bumetanide.

Cytological Features of *val-res* RBCs. Three-dimensional volume–HC cytograms of the SS R1 RBCs (Fig. 5) show that after valinomycin, the volume distribution of the RBCs that dehydrate is relatively narrow, whereas that of the *val-res* RBCs is rather broad, 80–180 μm^3, with an increased mean cell volume (130–140 μm^3), similar to that before valinomycin treatment.

Morphological Features of *val-res* RBCs. Fig. 6 shows that many of the *val-res* RBCs from the SS R1 fraction have an elongated shape similar to that of ISC. But unlike typical ISC, these cells have low HCs (and low densities) and, despite their increased cell volumes, most have low total Hb content. As discussed below, the ISC-like shape may provide a clue as to the origin of these cells.

Fig. 6. Photomicrograph of the *val-res* component of the R1 fraction of SS RBCs. Note the presence of many RBCs with the typical elongated shapes of ISC.

Identification of a Small *val-res* Fraction Among Normal RBCs. Although the great majority of K^+-permeabilized AA RBCs (with valinomycin) show uniform progressive dehydration (Fig. 1), a small *val-res* fraction could be consistently resolved from the lowest-density fraction (R1) of normal blood samples (Fig. 7). On the basis of their HC histogram patterns, AA RBCs were considered *val-res* if their HC remained less than 32 g/dl after valinomycin. This *val-res* fraction constituted a minute percentage of normal RBCs, ranging from 0.001% to 0.095% of the total, with a mean ± SD of 0.031% ± 0.034% (n = 8).

Na/K Concentration Ratio in Single Normal RBCs Measured by X-Ray Microanalysis. All valinomycin-treated cells showed a large increase in the Na/K concentration ratio relative to untreated

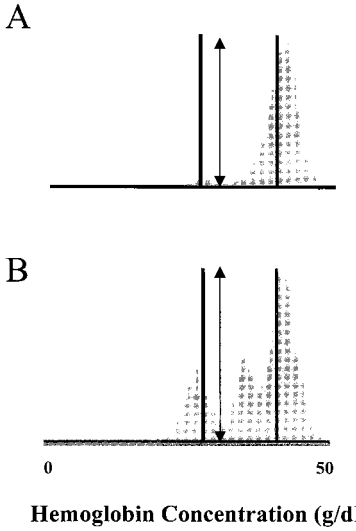

Fig. 7. Effects of K^+-permeabilization with valinomycin on the HC distributions of the lightest fraction of normal RBCs from two individuals. Solid vertical lines show HC gates at 28 g of Hb per dl and 41 g of Hb per dl. The arrows indicate a gate of 32 g of Hb per dl, chosen to distinguish the most valinomycin-resistant fraction from the valinomycin-sensitive majority of RBCs. Histograms *A* and *B* exemplify the heterogeneity of distribution of the normal *val-res* populations and show that in some samples (*B*) a distinct population of intermediately *val-res* cells can be resolved.

Table 3. Na/K concentration ratios in single normal RBC, before and after valinomycin-induced dehydration in low-K media, measured by x-ray microanalysis

Sample	[Na]/[K]
Donor 1	
Whole blood	0.02 ± 0.03
Val-sensitive	1.88 ± 0.47*
val-res	19.8 ± 4.8*†
Donor 2	
Val-sensitive	3.00 ± 0.42*
val-res	53 ± 10*†

Results are mean ± SEM for 50 RBCs. *, $P < 0.001$ compared with whole blood sample; †, $P < 0.001$ compared with valinomycin-sensitive sample from same donor.

controls (Table 3), with highly significant differences between control RBCs and both the valinomycin-sensitive and *val-res* RBCs. But the Na/K ratios of the *val-res* RBCs were an order of magnitude higher than those of the non-*val-res* cells.

Discussion

The present studies describe a previously unrecognized fraction of SS RBCs characterized by many distinctive features (Table 4), some of which appear to provide clues that allow us to speculate about the origin of this cell fraction, and consider their possible pathophysiological significance.

The very high Na^+/K^+ pump expression and activity of *val-res* RBCs are intriguing, because they are mostly nonreticulocytes, and so presumably older than the non-*val-res* or *cal-res* R1 cells which show less pump activity. Their higher pump-leak turnover of K^+(^{86}Rb) is thus particularly surprising, because normally, postreticulocyte maturation involve shifts toward ionic traffic levels about 1/10 of those in reticulocytes (10, 11). One possibility is that the maturational loss of Na^+/K^+ pump activity is not irreversible, and that the high level of pump activity results from reactivation of dormant pumps from normally matured cells. But this possibility only transfers the puzzle from a transport to a metabolic domain. To maintain such an enhanced Na pump activity *in vivo*, these cells would require a level of glycolytic ATP production far greater than that allowed by the endowment of hexokinase and phosphofructokinase enzymes in mature RBCs. Since a small fraction of reticulocytes are also *val-res*, the *val-res* cell population may be either young cells, or older cells with partially impaired maturation (deficient loss of reticulum, retention of high Na pump and metabolic activity). The presence of a unique bumetanide-sensitive ^{86}Rb transport pathway in these RBCs suggests the possibility that *val-res* cells might descend from a distinct erythroid lineage, with persistence of passive transporters from early erythroid cell precursors which have a high pump-leak turnover. This may represent a minute subpopulation of cells in normal conditions, but in conditions with accelerated hematopoiesis, the proportion of such cells in the systemic circulation may become substantially increased.

Alternatively, the high cation permeability may represent an acquired abnormal response. The ISC-like shape of many of the *val-res* RBCs raises the possibility that these cells may have previously been dense ISC, which have subsequently become cation-leaky and less dense. An *in vitro* model describing such a sequence has been described previously (12). It should be noted, however, that ISC were found to have slightly inhibited, or at most, normal levels of Na^+/K^+ pumping. Furthermore, the low Hb content of a substantial portion of the *val-res* RBCs is not a typical feature of ISC. To lose Hb content, the cells would have to undergo either significant fragmentation or transient lysis and resealing.

Table 4. Features of SS *val-res* and/or *cal-res* RBCs

Reversal of cell Na^+/K^+ content ratio
High mean cell volume with wide range
Low MCHC and cell density
Large increase in passive fluxes of Na^+ and K^+
High-bumetanide (1 mM)-sensitive K^+ flux component
Very high Na^+/K^+ pump expression and activity
Many RBCs with morphological features of ISC
Low proportion of reticulocytes
Average proportion about 4% of total SS RBCs

In preliminary experiments we have demonstrated the presence of high-Na^+, low-K^+ *val-res* RBCs (more than 1% of the total) in β-thalassemia intermedia blood samples (R.M.B., Oded Shalev, and Z.E., unpublished data). Because this condition involves both accelerated hematopoiesis and oxidative membrane damage, this finding supports the possible general pathophysiological significance of *val-res* RBCs, but does not further distinguish the mechanisms of their origin.

val-res cells were also found among AA RBCs, but in a much smaller proportion than in SS cells. It is uncertain whether the properties of AA *val-res* cells are similar to those of SS cells because the yield of the former was too small to measure tracer fluxes and determine Na and K concentrations directly by atomic absorption spectroscopy. Preliminary measurements of Na/K concentration ratios in single cells by x-ray microanalysis of two AA cell samples (Table 3) showed an increase in Na/K ratio in the valinomycin-sensitive RBCs relative to untreated controls, which simply reflects the selective loss of K during cell dehydration. The much larger increase in Na/K ratio in the *val-res* cells, on the other hand, suggests that in AA cells, as in SS cells, resistance to dehydration in the presence of valinomycin results from inversion of the normal Na/K concentration ratio, and hence from a reduced driving force for dehydration.

The population of SS *val-res* cells described here may play an important role in the pathophysiology of sickle cell disease, and further detailed studies of the cells' structural and functional properties, and their possible roles in the mechanisms of hemolysis, vasoocclusion, and thrombotic events, in sickle as well as in other red cell disorders, should be enlightening.

We are grateful to Mark Venczel for technical assistance. We thank the National Institutes of Health (Grants HL28018, HL20985, HL58512, and HL31579), the Wellcome Trust U.K. (V.L.L.), and the MultiImaging Centre, Cambridge, U.K. (J.N.S.) for funds.

1. Raftos, J. E., Bookchin, R. M. & Lew, V. L. (1998) *J. Physiol. (London)* **499,** 17–25.
2. Raftos, J. E., Bookchin, R. M. & Lew, V. L. (1996) *J. Physiol. (London)* **491,** 773–777.
3. Ortiz, O. E., Lew, V. L. & Bookchin, R. M. (1986) *Blood* **67,** 710–715.
4. Sorette, M. P., Shiffer, K. & Clark, M. R. (1992) *Blood* **80,** 249–254.
5. Etzion, Z., Lew, V. L. & Bookchin, R. M. (1996) *Am. J. Physiol.* **271,** C1111–C1121.
6. Beutler, E., West, C. & Blume, K.-G. (1976) *J. Lab. Clin. Med.* **88,** 328–333.
7. Kim, Y. R. & Ornstein, L. (1983) *Cytometry* **3,** 419–427.
8. Tycko, D. H., Metz, M. H., Epstein, E. A. & Grinbaum, A. (1985) *Applied Optics* **24,** 1355–1365.
9. Mohandas, N., Kim, Y. R., Tycko, D. H., Orlik, J., Wyatt, J. & Groner, W. (1986) *Blood* **68,** 506–513.
10. Wiley, J. S. & Shaller, C. C. (1977) *J. Clin. Invest.* **59,** 1113–1119.
11. Rapoport, S. M. (1986) *The Reticulocyte* (CRC Press, Boca Raton, FL).
12. Horiuchi, K. & Asakura, T. (1987) *J. Lab Clin. Med.* **110,** 653–660.