Role of reticulocyte transport heterogeneity in the generation of mature sickle cells with different volumes
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Introduction and background
The question we shall be addressing concerns the origin of dense sickle cell anaemia red cells (SS cells), a fraction of circulating cells rich in irreversibly sickled cells (ISCs), which are thought to play an important role in the pathophysiology of sickle cell disease. The question of their origin was traditionally formulated in terms of their presumed progressive dehydration, and research focused largely on the mechanisms by which sickling might cause the gradual dehydration of SS cells.

Three mechanisms were considered: two triggered by sickling-induced membrane permeabilization and one by acidification of the cells. Those triggered by membrane permeabilization and by the consequent increases in cell [Ca2+]i and in the [Na+]i/[K+]i ratio were the Ca2+-sensitive K+ channels and the Na+ pump respectively. Acid-triggered dehydration was via a K+ :Cl- co-transporter [1, 2]; in all probability the same transporter originally characterized in rabbit and sheep reticulocytes [3–6]. These mechanisms differ fundamentally in their ion transport capacity and consequently in the rate at which they may dehydrate cells. The Ca2+-sensitive K+ channels can dehydrate young and mature cells alike very rapidly if fully activated. There is sound but indirect evidence of activation of this pathway in vivo. Normal and SS red cells have endocytic vesicles capable of Ca2+ accumulation if permeabilized to Ca2+ in vitro [7, 8], but only SS cells accumulate Ca2+ in vivo [9, 10]. Ca2+ accumulation therefore documents episodes of Ca2+ permeabilization with transient [Ca2+]i elevations in vivo [7, [Ca2+], levels high enough to cause endocytic Ca2+ accumulation were also shown to activate K+ channels and dehydrate normal cells [11], but the full extent to which these channels contribute to dehydration of SS cells in vivo remains unknown. The K+ :Cl- co-transporter is highly expressed in young red cells and inactivates on maturation [12, 13]. If it contributes to dehydration in vivo, its contribution would predominate in the immature cell. The Na+ pump would tend to dehydrate young and mature cells continuously at a rate determined largely by the increase in the [Na+]i/[K+]i ratio [14, 15]. In reticulocyte-rich and discocyte-rich cell density fractions the [Na+]i/[K+]i ratio is near normal [16], whereas in dense cells it is highly increased; but in these cells the Na+ pump appears inhibited, partly as a result of altered Mg2+/ATP ratios [17].

The notion of gradual, progressive dehydration of SS cells has prevailed, implicitly or explicitly, in the design and interpretation of all experiments in this area; attention centred on the relative importance of each dehydration mechanism. This ‘gradualist’ view, however, failed to explain the presence of reticulocytes and young red cells within each density fraction of SS cells, as well as the early results of Bertles and Milner [18] which indicated that most of the dense ISCs formed within a few days after cell release from the bone marrow, and were then rapidly cleared from the circulation, whereas the normal density SS discocytes showed the longest survival times. These contradictions were noted but remained unexplained. The work reviewed next describes our attempts to develop alternative hypotheses on the origin of dense SS cells that would integrate all known facts and provide verifiable predictions [19, 20].

Development of a new working hypothesis on the origin of dense sickle cells
The immediate alternative to uniform gradual dehydration is parallel dehydration of distinct cell subpopulations at different rates. If there were young cells on a fast dehydration track, this could account for age heterogeneity within each SS cell density fraction and for the young average age of the densest SS cells [18]. But the crucial question is what could make different cell subpopulations dehydrate at different rates? One well-established possibility is absence of foetal haemoglobin (HbF). HbF interferes with haemoglobin S (Hbs) polymer formation and hence reduces sickling-induced...
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dehydration [21-24]. But for most SS cells, which are not F-reticulocytes or F-erythrocytes, the different dehydration rates would have to arise either from stochastic variations in the intensity or frequency of exposure to sickling episodes in the circulation, or from constitutive differences in the activity of membrane transporters which determine the dehydration response to sickling-induced permeabilization. Stochastic variations could not select young cells for fast track dehydration as required to explain the youth of most dense cells. The pointer was therefore on constitutive differences in transporter expression or activity as the main distinctive characteristic determining dehydration rate. Since such distinction can only arise early in the differentiation of red cells, it was clear that investigation of the parallel dehydration hypothesis had to start with an analysis of the possible effects of sickling-induced permeabilization on reticulocytes, a subject largely ignored, presumably for the following reason. HbS polymerization follows a high power function of haemoglobin (Hb) concentration; the relatively diluted state of Hb in reticulocytes would predictably lead to delayed and reduced overall polymer formation during de-oxygenation cycles in the circulation, and therefore to minor sickling-induced permeabilization. This argument presumed a correlation between extent of sickling and permeabilization which is open to doubt [25]. Before any further analysis, it was therefore necessary to establish whether or not sickling permeabilized light reticulocytes. The results clearly indicated that it did, with unexpected intensity and with characteristics, such as heparin sensitivity, which are absent in mature cells [16, 26].

To analyse how the volume, pH and ion content regulation systems of a reticulocyte may respond to sickling-induced permeabilization we developed a theoretical model of the reticulocyte [19, 20] using an approach of proven predictive value in epithelial cells [27-29] and red cells [30-33]. Our starting point was consideration of a cell in which the main pathway for passive ion transport was the acid and volume-sensitive K⁺:Cl⁻ co-transport, with the acid and volume sensitivity parameters defined from results obtained in experiments with reticulocyte-rich SS cell fractions by Brugnara et al. [1]. The model produced many novel predictions [20], two of which are particularly relevant in the present context. The first was that isotonic replacement of external Na⁺ by an impermeant monovalent cation would rapidly dehydrate reticulocytes but not mature red cells. Such an experiment would test a basic assumption of the model, that short term volume stability of a cell with high K⁺:Cl⁻ mediated traffic requires Na⁺-dependent Cl⁻ entry pathways to balance Cl⁻ loss via K⁺:Cl⁻ co-transport [20]. The second, and most important one, was that brief K⁺ permeabilization would cause delayed and irreversible dehydration of reticulocytes with high expression of K⁺:Cl⁻ co-transporters as long as they are deficient in acid-stimulated Na⁺:H⁺ antiports. This prediction led to the formulation of the following working hypothesis of a rapid dehydration mechanism for fast track cells. Sickling-induced membrane permeabilization would cause increased Ca²⁺ influx, elevated [Ca²⁺], and transient activation of Ca²⁺-sensitive K⁺ channels. This extra Ca²⁺-induced K⁺ permeabilization would trigger net KCl and water loss and consequent cell acidification by the following process [20, 30, 31]: the isotonic loss of KCl from K⁺-permeabilized cells dilutes internal Cl⁻; this increases the [Cl⁻]i/[Cl⁻]o ratio and causes the powerful Jacobs-Stewart mechanism [34, 35] to rapidly restore the [H⁺]i/[H⁺]o = [Cl⁻]o/[Cl⁻]i equilibrium by increasing [H⁺]i. The resulting cell acidification in turn stimulates K⁺:Cl⁻ co-transport with further KCl and water loss and cell acidification in a positive feedback loop leading to full gradient dissipation and a new dehydrated Donnan equilibrium with a cell relative density of above 1.117. Although a single sickling-induced Ca²⁺ permeabilization episode would suffice to trigger this self-sustaining loop, repeated sickling episodes would speed up dehydration directly, and also indirectly through additional acid-stimulation of the K⁺:Cl⁻ co-transport. This mechanism would not operate in reticulocytes with few K⁺:Cl⁻ co-transporters, or with acid-stimulated Na⁺:H⁺ antiports. In cells with comparable acid-sensitive K⁺:Cl⁻ and Na⁺:H⁺ mediated ion traffic, the reticulocyte model indicated that these transporters, together with the Na⁺ pump and the Jacobs-Stewart mechanism, would combine to prevent sickling-induced acidification and dehydration [20]. Fast-track dehydration would therefore only be expected from a subpopulation of reticulocytes with high K⁺:Cl⁻ mediated ion traffic, deficient in Na⁺:H⁺ antiports, and then only for as long as the high K⁺:Cl⁻ traffic persists during maturation.

**Experimental tests**

We briefly review next the experiments carried out to explore our hypothesis [16]. We fractionated light, reticulocyte-rich, and denser, discocyte-rich, SS red cells on Stratagam gradients, and examined
the effects of deoxygenation-induced sickling, external Ca<sup>2+</sup>, acidification, and replacement of external Na<sup>+</sup> by N-methyl-D-glucamine (NMG<sup>+</sup>). Light SS reticulocytes rapidly shrank when NMG<sup>+</sup> replaced Na<sup>+</sup>, thus supporting predictions of Na<sup>+</sup>-dependent volume control systems (although NMG<sup>+</sup> appeared to be partially permeable in these cells [16]). Sickling permeabilized light reticulocyte-rich cell fractions to Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>; the permeability increase was larger than in discocytes. Without external Ca<sup>2+</sup>, Na<sup>+</sup> influx matched K<sup>+</sup> efflux, and cell volume remained stable. With Ca<sup>2+</sup>, many light, low-HbF reticulocytes dehydrated rapidly. Dehydration was prevented by quinine, a Ca<sup>2+</sup>-dependent K<sup>+</sup>-channel inhibitor [36]. These results demonstrated that sickling did permeabilize reticulocytes to cations, that sickling-induced rapid dehydration of light reticulocytes was strictly Ca<sup>2+</sup>-dependent, and that the dehydrating effects of Ca<sup>2+</sup> required activation of Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels.

Acidification of oxygenated discocytes with high mean HbF contents, and of light reticulocyte-rich cell fractions, yielded denser, reticulocyte-enriched cells with the relatively low HbF content characteristic of the original reticulocyte-rich fractions, and much lower than that of the discocytes. This indicated that low-HbF reticulocytes, and also low-HbF cells which had lost their reticular structures, but were young enough to have retained sufficient K<sup>+</sup>:Cl<sup>-</sup> transport activity to rapidly dehydrate on acidification, were present among discocytes with high mean HbF contents. These are precisely the properties expected from fast track cells on their way to high density by the hypothesized mechanism, with acid stimulation of K<sup>+</sup>:Cl<sup>-</sup> co-transport more powerful than inhibition by reduced volume.

Since the proportion of high-F red cells and reticulocytes was unchanged in the fraction of light cells which became denser on acidification, the presence of HbF did not inhibit acid activation of the K<sup>+</sup>:Cl<sup>-</sup> co-transport. On the other hand, the reticulocytes from the lightest cell fraction that became denser on deoxygenation (and sickling) excluded most high-F cells [16]. These results pointed to the importance of sickling rather than acidification as the major trigger of dehydration, since only the former can account for the great enrichment of HbF in the relatively mature discocyte fractions.

**General conclusions**

The analysis and results briefly reviewed above pose new questions concerning the origins of reticulocyte heterogeneity and provide general insights into the vulnerability of cells in transitional states of differentiation or division. The experiments, which exposed the volume instability of some reticulocytes, also revealed an extraordinary diversity of volume responses to different experimentally induced perturbations among SS reticulocytes [16]. Rapid shrinkage could be elicited by medium acidification, low-Na<sup>+</sup> solutions or sickling, but different groups of cells were responding with different intensities to each of these challenges. SS reticulocytes were therefore clearly heterogeneous in the nature and quantity of the transporters expressed in their membranes. Reticulocyte transport diversity may be unique or part of a more general variability in cell enzymes and membrane components. Nothing is known about its origin, whether it is normal or appears only during stimulated erythropoiesis, and if so whether the diversity is independent of, or linked to, the nature of the erythropoietic stimulus. There is complete ignorance of the transport characteristics of erythroid precursor cells and of how these change in successive generations. It is therefore impossible at present to approach questions such as those concerning the stage at which the established reticulocyte heterogeneity begins to appear in the erythroid precursor lineage.

In stable cells, the makeup of active and passive membrane transporters secures the restoration of steady resting states after transient perturbations such as those that may be elicited by physiological agonists or by toxic agents, bacterial and parasitic infections, hypoxia, physical injury, membrane permeabilization or extracellular pH changes. In cells in transitional states of division or differentiation, the continuously changing makeup of membrane transporters, selectively timed for optimal completion of a physiological transition, but not necessarily adequate to secure volume stability and uniform mature cell products if perturbed during the transitional state, renders the cells and their maturation or division products vulnerable to irreversible abnormalities or early demise. The vulnerability exposed in experiments with light SS reticulocytes [16] could be induced by a brief sickling pulse. Thus, brief membrane permeabilization by HbS polymers, which causes relatively small and transient ion and water shifts in the more mature and stable SS discocytes [16], can irreversibly destabilize a transitional cell such as a reticulocyte and generate a dense young red cell.

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