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Shape changes of the human red cell studied by aqueous two-phase partition

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Aqueous two-phase systems have been used to study the human red cell during metabolically induced shape changes. When the discoid character of the cells was lost in favour of echinocytic forms, the partition increased both in charge-sensitive and in charge-insensitive two-phase systems. Reversal of the shape transformation by ATP repletion not only led to shape recovery but also restored the initial partition. Therefore it is apparent that red cells exhibit a shape-dependent partition behaviour. As the partition is dependent on surface properties (such as charge and hydrophobicity) of the partitioned material, the results show that the shape changes caused rearrangement of the membrane and thereby exposure of or greater accessibility of binding groups on the cell surface. The similar partition behaviour in the charge-sensitive and charge-insensitive phase systems show that the increased partition was caused mainly by increased hydrophobic interactions between the cells and the upper phase. The observed partition behaviour therefore suggests that the echinocytic cells acquire a higher affinity for the upper phase by repacking the lipid bilayer or at least the outer leaflet into a less efficient packed and thus more fluid structure.

Erythrocyte shape; Membrane; Lipid packing; Two-phase partition; (Human)

1. INTRODUCTION

The characteristic discoid shape of the human red cell is under metabolic control. When cytosolic ATP is consumed a series of shape transformations occurs, including successively the formation of spicules on the surface of the membrane, loss of the discoid character in favour of a spherically symmetric shape, the elongation and in due course loss of the spicules as microvesicles and the formation of a smooth spherocyte [1–3]. The sequence of shape transformations is completely reversible up to the point preceding major membrane loss, and the discoid shape can be recovered if the cell is allowed to resynthesize ATP.

It is obvious that the appearance of protrusions on the membrane surface, due to metabolic depletion, requires repacking of the lipids, at least of those in the outer leaflet. Based on work with the fluorescent probe merocyanine 540, it has been claimed that prolonged depletion of red cells (beyond the point of membrane loss) causes the outer leaflet of the membrane to become more disordered [4]. However, the ability of merocyanine 540 to report changes in membrane fluidity has been questioned [5,6]. Even if merocyanine 540 and other fluorescent probes report changes in lipid packing, such probes are difficult to use in the case of intact red cells due to the strong filter effect.

A simple but yet sensitive method to measure differences in membranes or rather in their surface properties is by partition in aqueous two-phase systems [7,8]. By using phase systems of different composition it is also possible to differentiate between charge and non-charge (hydrophobic) properties. The partition technique has, in fact, been used to distinguish red cells of different species as well as to separate human red cells according to age [9]. As partition in aqueous two-phase systems should have great potential for studying cell surfaces the technique has been explored in monitoring changes in surface properties of the red cell membrane during shape changes. The results show that there is a strong correlation between shape and partition behaviour and that the increased partition of echinocytes depends most probably on increased membrane fluidity.

2. MATERIALS AND METHODS

Fresh human blood was provided by the blood bank at the University Hospital, Umeå. Red cells were washed four times at 4°C in buffer H (130 mM NaCl, 3.7 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 25 mM Hepes, pH 7.5) and twice in complete buffer H (buffer H containing 100 units·ml^{-1} penicillin G, 100 units·ml^{-1} streptomycin (Gibco) and 1 mg·ml^{-1} essentially fatty acid-free bovine serum albumin (Sigma)). Plasma and buffy layers were carefully removed by aspiration after each centrifugation.

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Abbreviations: ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β′-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazinc-*N′*-2-ethanesulfonic acid; PEG, poly(ethylene glycol)

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Metabolic depletion was initiated by resuspending washed cells in complete buffer H at a hematocrit of 10% containing the indicated amount of sodium metavanadate, followed by incubation at 37°C. To initiate ATP repletion, 0.1 vol. of 100 mM glucose and 10 mM adenosine in buffer H was added.

The red cells were fixed by mixing 25 μl of the cell suspension with 0.2 ml 1% glutaraldehyde (Sigma) in 0.1 M potassium phosphate buffer, pH 7.5. After 60 min at room temperature, the extent of shape change was determined by counting at least 400 cells of each sample, using phase-contrast microscopy, and classifying the cells as either crenated or uncrenated.

The partition experiments were done in two different aqueous two-phase systems; a charge-sensitive system and a charge-insensitive (non-charge sensitive) [9]. The charge-sensitive system was made up from stock solutions in a separatory funnel and was composed of 5% (w/w) dextran T500 (Pharmacia), 4.4% (w/w) PEG 8000 (Union Carbide), 0.10 M sodium phosphate buffer, pH 7.3. After thoroughly mixing, the mixture was allowed to settle overnight (20°C), and then the upper and lower phases were separated.

To determine the partition of red cells, an aliquot (25 μl) was withdrawn from the incubation mixture and added to 0.5 ml upper and 0.5 ml lower phase of the charge-sensitive system (in Eppendorf tubes). The phase system was thoroughly mixed (though not shaken) and then left in a vertical position to let the phases separate. After exactly 13 min, 100 μl of the upper phase was added to 1.0 ml water and the absorbance at 420 nm measured. The remaining phase system was remixed, 100 μl of the unseparated system was immediately withdrawn and processed similarly. From these measurements the partition ratio was determined as the ratio of the absorbance at 420 nm of the upper phase to that of the total system. Thus if all cells were to be in the upper phase, the partition ratio would be 2.25.

The charge-insensitive system contained 5% (w/w) dextran T500, 3.6% (w/w) PEG 8000, 0.01 M sodium phosphate buffer, pH 7.3, and 0.14 M NaCl, and was made up similarly. The partition ratio of cells in this system was determined in the same way, except that the separation time was 10 min.

3. RESULTS AND DISCUSSION

During the initial stage of this work, it was observed that the partition of human red cells was highly dependent on the time allowed for phase separation. For instance, increasing the separation time from 5 to 20 min decreased the partition ratio in a charge-sensitive phase system from 1.2 to 0.8. Although longer separation time decreased the partition ratio even further, the decrease was proportionally smaller. Similar observations have been reported previously [10]; using rat red cells (that initially partitioned mainly to the upper PEG-rich phase) it was shown that the partition declined with time and eventually all cells collected at the interface. The partition of red cells of other species have also been shown to be time-dependent and it appears that the partition kinetics differ quantitatively depending on the species [11]. Due to this time-dependency it was necessary to control the separation time accurately to obtain reproducible partition results; in the experiments to be described below exactly 13 and 10 min was allowed for phase separation in the charge-sensitive and charge-insensitive phase systems, respectively.

When human red cells were incubated at 37°C in the absence of extracellular nutrients, i.e. under conditions that are known to lead to a fast consumption of intracellular ATP, the cells lost their discoid form in favour of a spiculated, echinocytic shape (fig.1A). Inclusion of 125 μM vanadate in the incubation medium increased the rate of echinocytosis considerably, as has been reported previously [12]. Addition of glucose and adenine, to replenish the ATP pool, to cells depleted for 18 h and thus largely echinocytic, caused a rapid recovery of the discoid character.

In both the charge-sensitive and the charge-insensitive two-phase systems, the partition of red cells showed a striking dependency on the cell shape, as figs 1B and c show. When discoid cells were slowly transformed into echinocytes by metabolic depletion there was a concomitant increase in the partition ratio. Also during the much faster vanadate-induced echinocytosis the increase in partition correlated with the shape change. Since vanadate, as has been shown before [12], does not influence the level of ATP in the cell but only increases the rate of shape change it is

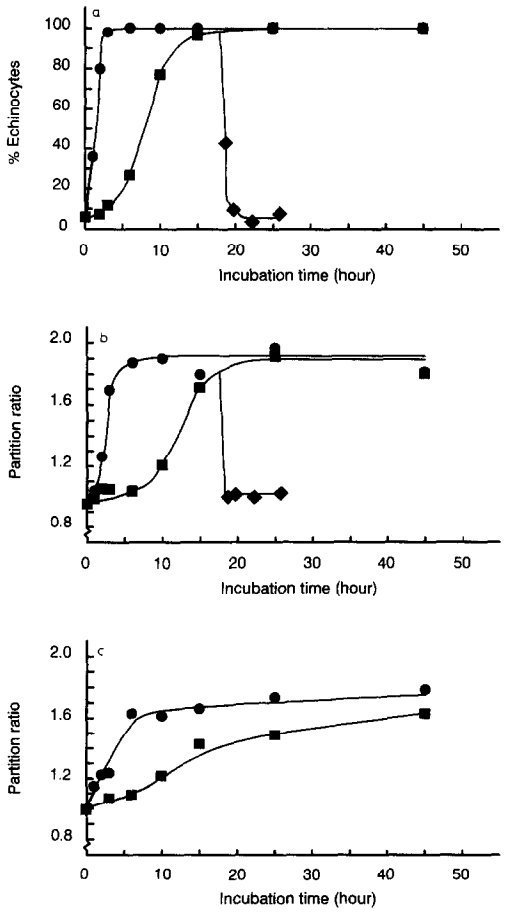

Fig.1. Metabolic depletion of human red cells. Washed red cells were suspended at 10% hematocrit in buffer H and incubated at 37°C in the absence (■) and presence (●) of 125 μM vanadate. At the indicated times, samples were withdrawn and the morphology (A) and the partition ratio in a charge-insensitive (B) and charge-sensitive (C) phase system were determined as described in the text. After 18 h depletion, 0.1 vol. of 100 mM glucose and 10 mM adenosine in buffer H was added to part of the sample incubated in the absence of vanadate (◆) in order to replete the cells.

clear that the change in partition is a consequence of shape and not of the metabolic state of the red cell. In both phase systems, discocytes had a partition ratio of below 1.0 whereas echinocytes (largely type III echinocytes and spheroechinocytes as classified by Bessis [13]) had partition ratios of 1.7 and 1.9 in the charge-sensitive and charge-insensitive systems, respectively. Shape recovery, brought about by ATP-repletion, rapidly restored the partition to its initial level in both phase systems.

Although the time courses of shape changes (under the conditions of incubation employed) were qualitatively very similar to the corresponding partition curves, there were differences. During depletion, when 50% of the cells had turned echinocytic (after about 8 h incubation of untreated cells or after less than 2 h for vanadate-treated cells) the partition ratio had only changed slightly; from just below 1.0 to about 1.1 in both phase systems. Upon repletion and recovery of discoid shape, the major effect on partition was seen when going from 95% to 50% echinocytes. That the partition appeared to be restored before complete shape recovery probably depended on the experimental setup; shape was determined 10 (or 13) min before the partition was measured. As shape recovery continued in the phase system during phase separation, this most likely led to a more extensive recovery of discoid form. Anyhow, it is apparent that the early stage of shape changes (i.e. transforming discoid cells principally into type I echinocytes) did not influence the partition very much. It is equally clear that the major change in partition occurred during the late stages of shape transformation, with the formation of type II and III echinocytes and spheroechinocytes.

The aqueous two-phase system containing low sodium phosphate buffer (0.01 M) and high sodium chloride (0.14 M) concentrations is defined as a charge-insensitive or low-potential phase system [9]. The other system, containing only sodium phosphate buffer (0.1 M) is considered as charge-sensitive or high-potential system. In a charge-insensitive two-phase system the major determinant of partition is noncharge surface properties, mainly hydrophobicity, of the partitioned material. In such a system a change in partition therefore generally signifies altered surface hydrophobicity. As the upper PEG-rich phase is considered to be more hydrophobic than the lower dextran-rich phase, increased surface hydrophobicity of partitioned material leads to higher affinity for the upper phase and thus a higher partition ratio.

In contrast, in a charge-sensitive phase system, there is an electrostatic potential difference between the phases [7,8] that strongly influences the partition. As the upper phase of this type of phase systems is more positive than the lower one, increased net negative surface charge would increase the partition. However, noncharge effects must also be considered in charge-sensitive systems as the contributions to partition behaviour are additive. Thus by comparing the partition behaviour in a charge-sensitive phase system with that of a charge-insensitive one, partition changes due to altered net charge can be distinguished from other (noncharge) effects.

From this it follows that the qualitatively similar partition behaviour in both types of phase systems strongly indicates that the increased partition ratio of echinocytes is due to changed cell surface hydrophobicity. Although altered surface charge effects cannot be excluded completely, it is apparent that the discocyte/echinocyte transformation does not influence the net cell surface charge to any larger extent. This is corroborated by previous findings that the electrophoretic mobility of fresh cells does not differ much from that of cells depleted for 28 h [2].

The remarkable change in red cell morphology undoubtedly requires repacking of the membrane lipids and thus also rearrangement of the glycocalyx. It is therefore easy to envisage how the cell may acquire a higher affinity for the upper phase when the discoid character is lost. Groups or rather binding sites on the surface of the discocyte may become exposed when the cell turns echinocytic. Further, as the change in partition appears to depend on increased surface hydrophobicity, the shape change must lead to increased hydrophobic interactions between the cell and the upper phase. The most obvious way for the cell to increase these interactions would be by making the hydrocarbon core of the lipid bilayer more accessible. This notion is supported by 3 observations. First, the cell surface area increases during shape changes, implying that the components of the cell surface are less efficiently packed in echinocytes [14]. Second, partition of red cells from different species (in a charge-insensitive phase system) shows a strong correlation between partition behaviour and ratio of polyunsaturated to monounsaturated fatty acids [15]. As increasing unsaturation results in a less efficient (i.e. more fluid) packing of membrane lipids, it was suggested that increased unsaturation permits the cell surface to be intercalated to a greater extent by PEG molecules, thereby increasing the affinity of the cell for the upper PEG-rich phase. Third, palmitoylcarnitine not only causes red cells to rapidly turn echinocytic but also alters the molecular dynamics of the membrane [16]. Although the effect of palmitoylcarnitine is complex, it is apparent that the order parameter of the membrane (as measured by a spin-label technique) decreases and consequently the membrane fluidity increases when the cells are transformed into echinocytes. Therefore it seems reasonable to suggest that the observed shape-dependent partition is due to lipid packing efficiency. Hence, altered red cell shape leads to a less ordered membrane and thus a more fluid structure. This would enable the PEG molecules to bind more strongly to the

outer leaflet of the bilayer and thereby increase the affinity of echinocytes for the upper PEG-rich phase, as observed.

It is quite obvious that the largest effects on partition occur during the late stages of shape changes but even so the partition technique is able to discern membrane alterations also during the early phase of depletion. Therefore the partition in aqueous two-phase systems appears to be very sensitive and should be useful to detect changes in membrane fluidity not only in red cells but in all types of cells.

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