The Discocyte–Echinocyte Transformation: Comparison of Normal and ATP-enriched Human Erythrocytes

By Claude J. Féo and Pierre F. Leblond

This work studies the relationship existing between intracellular ATP levels and the discocyte–echinocyte shape equilibrium in normal as well as ATP-enriched human red blood cells. Fresh erythrocytes metabolically depleted by incubation in a glucose-free buffer undergo echinocytic transformation (crenation) as intracellular ATP levels decline below 50% of their original value. When ATP is regenerated by further incubation of the same cells in the presence of glucose, inosine, and adenine (GIA), concentrations required for the complete recovery of the discocytic shape are lower than those which were necessary to maintain this shape in the first place. Addition of inorganic phosphate (Pi) to the GIA medium results in elevation of fresh cell ATP to supranormal levels. When such ATP-enriched cells are then depleted in the presence of the same concentration of inorganic phosphate, echinocytic transformation occurs much more rapidly than in normal fresh cells not previously incubated with Pi despite a similar rate of ATP depletion in both cases. It is suggested that the intrinsic mechanism responsible for shifting the discocyte–echinocyte equilibrium in the human erythrocyte is dependent on one or more intracellular or intramembrane factors occurring in conjunction with ATP depletion or repletion rather than to the absolute concentration of this nucleotide within the cell. This additional factor(s) appears to be temperature dependent and is influenced by the concentration of Pi in the medium.

The relationship between the discocyte-echinocyte transformation and the intracellular adenosine triphosphate (ATP) concentration of the human red blood cell was first established by Nakao and co-workers. Weed et al., a few years later, emphasized the importance of intracellular calcium as well as ATP in determining the shape and deformability of intact erythrocytes and ghosts.

In order to study this relationship in greater detail, we have examined the morphologic events which occur during the depletion of red cell ATP following incubation in a glucose-free medium and during repletion of this nucleotide by subsequent incubation of the cells in a medium supplemented with glucose, inosine, and adenine. Erythrocytes enriched with ATP by preincubation in a medium containing inorganic phosphate (Pi) and then depleted by glucose deprivation were also examined.

It appears from these experiments that the discocyte-echinocyte equilibrium of the human red cell is more critically influenced by the presence of a change in intracellular ATP per se, or that of intracellular factors occurring in conjunc-

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Supported by I.N.S.E.R.M. (France), by the Medical Research Council of Canada, and by U.S. National Heart and Lung Institute grant No. HL-06241.

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Blood, Vol. 44, No. 5 (November), 1974 639
tion with ATP depletion or repletion, rather than by absolute concentrations of this nucleotide itself. The exact nature of these factors remains to be determined.

MATERIALS AND METHODS

Venous blood was collected under heparin (15 IU/ml) from healthy adult donors. The plasma was immediately separated from the cells by centrifugation at 500 g for 10 min at 4°C. The buffy coat was removed as completely as possible and the plasma recentrifuged at 2500 g for 10 min. The supernatant was stored at 4°C and later used for suspending the red cells for microscopic examination. Erythrocytes were then washed three times in 4 volumes of cold, Tris-buffered saline (Tris-(hydroxymethyl)-amino methane, 15 mM) at pH 7.4 by centrifugation at 300 g for 5 min. For incubation, the cells were suspended at a final hematocrit ranging between 15% and 25% as determined by the micromethod. The pH was initially adjusted to 7.6 with a small volume of 15 mM Tris so as to obtain a value close to 7.3 at the end of the incubation period, as determined with a micro-electrode pH meter (Radiometer, Copenhagen) with a precision of 0.01 U.

Incubation Media

ATP depletion. Depletion of ATP was obtained by simply incubating the cells in a glucose-free, Tris-buffered saline solution (pH 7.4, 300 milliosmols), a procedure considered more adequate than the sodium fluoride enzymatic inhibition method utilized by various other authors. After incubation in this medium for periods of 10–15 hr, resynthesis of ATP was induced by further incubation in a new medium containing 5 mM glucose, 10 mM inosine, and 2 mM adenine sulfate (GIA) completed to isotonicity with Tris-buffered sodium chloride at pH 7.4.

ATP-enriched cells. As shown in Fig. 1, concentrations of 10, 50, 100, and 180 mM inorganic phosphate (Pi) in the form of either NaH₂PO₄ or Na₂HPO₄ added to the GIA-enriched buffer were tried for a period of 15 hr at 37°C, and it was found that the highest possible concentration also gave the highest rise in intracellular ATP. Dipyridamole, an inhibitor of adenosine deaminase found to be useful in preserving erythrocyte ATP levels in stored blood was found not only to have no effect by itself, but to inhibit almost completely the effect of Pi when added to the incubation medium (Fig. 1).

ATP-enriched cells were subsequently depleted by incubation in the same glucosefree buffer as used for normal fresh cells or in a glucose-free medium containing in addition 160 mM inorganic phosphate.

Evaluation of Red Cell Morphology

Red cell morphology was evaluated in wet preparations under a phase-contrast microscope according to a previously described method. The cells were lightly suspended in fresh autologous or ABO-compatible plasma and immediately examined between glass slide and cover slip.
care being taken to avoid compression by allowing for some degree of rouleau formation. The different erythrocyte shapes were identified according to the classification of Bessis, in which echinocyte type 3 is a spherical cell having 30-60 distinct spicules regularly arranged over the surface. Echinocytes types 2 and 3 were distinguished from echinocytes type 1 and discocytes by their absence of membrane flicker.

ATP Measurement

ATP was measured by the firefly bioluminescence method according to Aledort et al. Exactly 0.1 ml of the cell suspension was hemolyzed in 10 ml of deionized distilled water and placed in a boiling water bath for 1-2 min in order to extract protein and abolish ATPase activity. This extract was then stored at −18°C, remaining stable for several months. The assay was performed by placing under a shielded photomultiplier tube a preparation containing suitable proportions of the red blood cell and firefly lantern extracts (Sigma Chemicals Co., St. Louis, Mo.). Light emission was measured as the output current from the photomultiplier tube over an integrated period of 45 sec. For each new bottle of firefly lantern extract a new calibration curve was established with signals obtained from known concentrations of ATP (adenosine-5'-triphosphate solution, disodic salt, extracted from horse muscle, Sigma Chemicals Co.) varying between $5.4 \times 10^{-7}$ and $1.8 \times 10^{-8} M$.

In order to verify the specificity of the luciferin-luciferase-ATP reaction, ADP (adenosine-5'-diphosphate, trisodic salt, Merck Co., Darmstadt, West Germany), and AMP (adenosine-5'-monophosphate, disodic salt, Merck Co.) were tested at the same concentrations used for calibration with ATP.

Intracellular ATP was expressed as μM/ml of red blood cells as derived from the calibration curve and after correction for hematocrit and dilution. It was not considered necessary to express ATP as μM/g of hemoglobin since neither anticoagulation nor incubation induced any significant change in the mean corpuscular volume of the cells.

RESULTS

Erythrocytes With Normal ATP Content

As shown in Fig. 2, a progressive decrease in red cell ATP concentration occurred during glucosefree incubation over a period of 12 hr. For the first 3 hr, no change in morphology was observed when the cells were examined in fresh autologous plasma. Starting at the 4th hr, when intracellular ATP had fallen below 50% of its original concentration, progressive appearance of echinocytes was observed with a marked acceleration in the rate of this shape.

![Fig. 2. Depletion of fresh erythrocytes in glucosefree buffer and resynthesis in GIA supplemented medium: relation to cell echinocytic transformation. Each point represents the mean of two experiments.](image)
Fig. 3. Control experiment to Figs. 2 and 4. Erythrocytes incubated for 15 hr in GIA, but without Pi, do not lose or gain significant amounts of ATP nor undergo disc-echinocyte transformation until placed in a glucose-free medium (depletion). Each point is the mean of four experiments (± 1 SD).

change until the 12th hr where 90%-100% of the cells had become echinocytes. This shape transformation was, qualitatively speaking, a gradual phenomenon, and the population examined at any one time was heterogeneous, i.e., composed of various intermediate stages between the discocyte and the echinocyte type 3. Control cells, preincubated in saline supplemented with glucose or in the GIA medium (Fig. 3), maintained their initial ATP concentration and discocytic shape for a period of 15 hr before undergoing depletion and echinocytic change when later transferred into the glucose-free buffer. Subsequent incubation of these ATP-depleted cells in the GIA-supplemented medium resulted in a dramatic reversion of the morphologic picture after only 2 hr (Fig. 2). At this time, virtually all cells had returned to a disc configuration while intracellular ATP had risen from 0.2 to 0.45 μM/ml. It should be noted that during the depletion incubation, when the mean ATP concentration reached this level, it was accompanied by 15%-20% echinocytes in the cell population. In order to explain this difference in morphology for the same intracellular ATP concentration, a duplicate set of similar incubations was carried out with addition of either CaCl₂ (2 mM) or EGTA (10 mM) to the incubation media. The depletion phase was maintained this time for 20 hr and the repletion for 3 hr. In both cases, the rate of echinocytic transformation and reversion was the same and corresponded to similar concentrations of ATP throughout the incubations; thus no conclusive evidence was obtained for an influence of added extracellular calcium, at the concentration utilized, on this phenomenon.

Erythrocytes With Supranormal ATP Content

As seen in Fig. 4, the preincubation of fresh cells for 15 hr in the GIA medium that also contained 160 mM Pi resulted in a threefold increase in the mean ATP concentration of the population over its original level. Control cells incubated for the same period of time in absence of Pi (Fig. 3, preincubation) did not show such a rise in intracellular ATP. Despite this high ATP level, a few cells (5%-10%) always underwent echinocytic change during the preincubation period; their number however could be reduced (1%-3%) if the preparation of the cells immediately after blood collection was carried out more quickly. Once loaded with ATP, the cells would be maintained in the discocytic shape
for up to 40 hr at 37°C by simply replacing the incubation medium with fresh GIA with or without Pi added. Over this period of time the intracellular ATP remained equal to or above 2 μM/ml, i.e., twice the amount present in freshly drawn cells.

ATP-enriched cells were suspended in 24-hr incubated autologous plasma to see if their elevated ATP content would protect them against the immediate extrinsic echinocytic transformation that usually occurs to normal cells in this environment.15 No difference was found between these and normal fresh cells even after serial dilution of the plasma to unmask a difference in sensitivity between the two cell populations.

Finally, ATP-enriched cells were incubated in glucose-free buffer with or without added Pi to examine their morphologic evolution during depletion. Incubation in saline alone (Fig. 4) resulted in slow echinocytic change involving only 20% of the population after 12 hr when ATP had fallen from 4 to 1 μM/ml; the rate of echinocytic change increased rapidly thereafter to reach more than 75% of the cells after 18 hr with a mean intracellular ATP falling below 0.5 μM/ml.

Incubation in saline and Pi (Fig. 5) resulted in a much more rapid echinocytic change than in saline alone, but without significant difference in the rate of
ATP depletion. Thus, for a concentration of 1 \( \mu M/ml \) of ATP, for instance, there were 50\% echinocytes in the presence of \( \Pi \), compared to only 10\% when \( \Pi \) was absent.

**DISCUSSION**

Bessis and Lessin\textsuperscript{16} defined and described in detail the discocyte-echinocyte transformation of the normal and pathologic erythrocyte and called attention to the possible artifacts that can arise from examination of red blood cells under the phase-contrast microscope. The factors which determine this reversible morphologic transformation are generally regarded as being either extrinsic or intrinsic to the cell. Among extrinsic factors are listed the effect of glass surfaces,\textsuperscript{17,18} of elevated pH,\textsuperscript{18,19} of 24-hr incubated plasma,\textsuperscript{15} and that of a number of anionic or noncharged amphiphilic drugs\textsuperscript{18,20} when used in appropriate concentration. The only known intrinsic factor to date is intracellular ATP depletion.\textsuperscript{1-5}

Our experiments generally agree with Nakao and co-workers' observations and contribute additional information on the relationship between red cell ATP and morphology by showing that a given absolute concentration of ATP does not necessarily correspond to a constant morphologic picture within the discocyte-echinocyte equilibrium. As can be seen by comparing Figs. 2, 4, and 5, a concentration of ATP of 1 \( \mu M/ml \), which is within the range obtained by our technique for freshly drawn normal cells, can correspond to anywhere between 0\% and 50\% echinocytes in the population depending on the conditions of the incubation.

One possible explanation for this phenomenon lies in the natural heterogeneity of any red cell population obtained from a normal donor. If, as suggested by LaCelle et al.,\textsuperscript{21} the amount of intracellular ATP varies according to cell age, it is expected that the actual variation around the measured mean concentration of this nucleotide in a population of fresh erythrocytes may indeed be quite large. Hence, because of averaging, echinocytic transformation of 100\% of the cells during substratefree incubation may have occurred at a mean ATP which was lower than the actual threshold at which each individual cell becomes echinocytic. Once profound depletion has occurred, however, it is conceivable that the population had become more homogeneous, in which case the addition of substrate could have reversed the morphology back to 100\% discs at a mean ATP closer, this time, to the individual cell threshold value. This interpretation, although probably valid, would have to be supported by experimental evidence, making use, for example, of cell separation techniques based on density gradient\textsuperscript{22} or angle-head\textsuperscript{23} centrifugation. An even better, but more delicate, technique of the type recently developed by Weed and Bessis\textsuperscript{24} would be one sensitive enough to measure ATP inside individual erythrocytes.

Another possibility would be the role played by intracellular calcium during the course of these incubations, as it is possible that, in one way or the other, \( \Pi \) may influence the amount or distribution of intracellular calcium in erythrocytes. Since calcium was not measured in our experiments, no strong argument can be made for the occurrence, for instance, of a change in the intracellular ATP/calcium ratio which could explain the observed data.\textsuperscript{4,5}

A third possibility, as proposed recently by Shohet and Haley,\textsuperscript{25} would be a
decrease in the ATP-dependent acylation and turnover of erythrocyte membrane phospholipids. This, however, can hardly be invoked since the cells here were incubated in absence of plasma or serum. Nevertheless, it is recognized that even washed red cells can retain small amounts of endogenous membrane lysolecithin, the concentration of which might have risen with the various manipulations and incubations to which they were submitted. The fact that ATP-enriched cells were not protected from immediate echinocytic transformation when suspended in 24-hr incubated plasma is not surprising in view of the observation by Lichtman et al.26 that in order to adapt morphologically to an added load of extrinsic lysolecithin, metabolically intact erythrocytes require a finite period of time, and our cells were examined immediately after suspension in the aged plasma. From this, it would seem that intracellular ATP, even in high concentration, cannot be considered as an intrinsic “anti-echinocytic” agent acting through the same pathway as that of extrinsic echinocytic inducers.

The effect of Pi on red cell energy metabolism has been studied by numerous investigators.27-29 The threefold increase in intracellular ATP obtained in our experiments by preincubating the cells in GIA and Pi is essentially similar to that recently reported by Warrendorf and Rubenstein.30 The only reported effect of altered extracellular Pi on red cell morphology is the echinocytic or spherocytic transformation that occurs in vitro when cells are incubated in the presence of hypophosphatemic plasma,29 or in vivo in profoundly hypophosphatemic patients.31,32 In both cases, however, red cell ATP is also below normal and has been held responsible for the change in erythrocyte shape.

Our results indicate that red cells containing high levels of intracellular ATP rapidly become echinocytic when incubated without substrate, provided Pi is present in the incubation medium (Fig. 4). This phenomenon was not observed in the absence of Pi (Fig. 5), when the incubation temperature was lowered (4°C or 22°C), or even when cell morphology was examined immediately after addition of the phosphate. Cells in which ATP was prevented from becoming elevated by addition of dipyridamole to the Pi-containing medium (see Materials and Methods) did not become echinocytic either, even after a 15-hr incubation at 37°C. A dramatic difference in the permeability of the cell membrane to Pi at the low temperatures mentioned above is unlikely in view of the fact that Pi enters the cell mostly by passive diffusion.33

From this study, it can only be concluded that the intrinsic mechanism responsible for the echinocytic transformation of the human erythrocyte is more closely related to one or more intracellular or intramembrane factors occurring in conjunction with ATP depletion than to the absolute amount of this nucleotide itself. Such a factor(s) does not operate at reduced temperatures and is influenced by the concentration of Pi in the incubation medium. Further studies will be required to elucidate the exact nature of this intrinsic echinocytic inducer.

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