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ADENOSINE TRIPHOSPHATE RESTORATION AND DISCOCYTIC TRANSFORMATION OF STORED HUMAN ERYTHROCYTES

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Abstract. Erythrocytes in human blood stored for 120 days were collected by centrifugation after dispersion in buffered physiological saline. The aged erythrocytes thus collected were incubated with inosine, adenine, glucose or other media, and their shapes and ATP levels were studied by scanning electron microscopy and a luciferine-luciferase method. The aged erythrocytes incubated in a mixture of adenine and inosine markedly regained their ATP levels, and also showed a marked transformation from spiked spherocytes to normal discocytes. Incubation with inosine alone restored ATP levels of the aged erythrocytes to some extent, but did not result in morphological rejuvenation. Incubation in a mixture of citrate and glucose caused morphological rejuvenation, though it restored ATP levels less effectively than incubation in inosine alone. Incubation with adenine alone neither restored ATP levels nor resulted in morphological rejuvenation of the stored erythrocytes.

Key words : human erythrocytes, rejuvenation, adenine, inosine, ATP, luciferine-luciferase method, scanning electron microscopy, discocyte transformation.

It has been shown by estimating the adenosine concentration with a spectrophotometer that adenosine triphosphate (ATP) levels are decreased in stored or aged human erythrocytes (1). It has also been shown by calculating incorporated radioactive phosphorus using a Geiger-Müller counter or by observing aggregated samples with a light microscope that cellular transformation from discocytes to spherocytes occurs during the storage or aging of the erythrocytes (2, 3). Incubation in a mixture of inosine and adenine markedly restores the decreased ATP levels of aged erythrocytes with reversed transformation from spherocytes to discocytes. The present study reinvestigates these biochemical and morphological changes induced by inosine and adenine in aged human erythrocytes by a recently developed technique for enzymatic (luciferine-luciferase) ATP estimation (4, 5), and by scanning electron microscopy (6, 7).

MATERIALS AND METHODS

Storage and isolation of erythrocytes. Two hundred milliliters of human blood were obtained

intravenously from a healthy Japanese adult, supplemented with 28 ml of ACD mixture containing 0.33 % acid citrate, 2.63 % sodium citrate and 2.32 % glucose and stored in a refrigerator at 4℃ for 120 days. The stored blood was suspended in physiological saline buffered with 10 mM phosphate (pH 7.4) and centrifugated at 1,500 r.p.m. for 5 min. This procedure was repeated 5 times, and purified sediments of erythrocytes were prepared.

Incubation media. The following media were used for incubation of the erythrocytes (physiological saline was buffered with 10 mM phosphate, pH 7.4): 1. Buffered physilogical saline (PBS medium or control), 2. Buffered physiological saline containing 2 mM magnesium adenosine triphosphate (Mg-ATP medium), 3. Buffered physiological saline containing 10 mM adenine hydrochloride (Adenine medium), 4. Buffered physilogical saline containing 0.73 mM glucose, 0.45 mM sodium citrate and 0.23 mM acid citrate (ACD medium), 5. Buffered physiological saline containing 5 mM inosine (Inosine medium), and 6. Buffered physiological saline containing 5 mM inosine and 10 mM adenine hydrochloride (Inosine-adenine medium).

Incubation of stored erythrocytes. One-half milliliter of the purified erythrocyte sediment was mixed with 0.5 ml of each incubation medium and incubated at 37℃ for 3 h. The incubated specimen was then suspended in physiological saline buffered with 10 mM phosphate (pH 7.4) and centrifuged at 1,500 r.p.m. for 5 min. This procedure was repeated 3 times to prepare washed sediments of incubated erythrocytes.

Determination of hemoglobin contents. The washed sediment (0.02 ml) of the incubated erythrocytes was added to 5 ml of cyanmethemoglobin solution (for clinical use, Van Kamper Zijilsra solution) and kept at room temperature for 30 min. The optical density was measured at 540 nm with a spectrophotometer and converted to hemoglobin contents.

Determination of ATP levels. One-half milliliter of the washed sediment of the incubated erythrocytes was added to 4.5 ml of boiling, distilled water and boiled for 10 min to completely inactivate ATP-ase and at the same time to extract ATP from the erythrocytes. The boiled specimen was centrifugated at 3,000 r.p.m. for 15 min and 0.1 ml of the supernatant was diluted with 9.9 ml of distilled water to make a sample solution.

A luciferine-luciferase solution (enzyme solution) was prepared by dissolving FLE-50 (Sigma CO.) in 10 ml of distilled water, followed by centrifugation at 10,000 r.p.m. at ice-cold temperature for one hour (5). The supernatant was used as enzyme solution (5).

Two-tenths milliliter of the enzyme solution was added to 5 ml of ATP standard solution or ATP extract of the erythrocytes, and the intensity of bioluminescence generated from ATP-luciferine-luciferase mixture was measured with a bioluminescence reader (101, Aloca Co.) (5). ATP standard solution (one microgram per ml) was prepared by diluting ATP-2 Na salts with distilled water.

The ATP concentration was computed with a microcomputor (MZ-80, sharp Co.) from the intensity of chemiluminescence according to the correlation equation: $\mathrm{Log}(Y) = K^{*}\mathrm{Log}(X) + C$, where Y denotes the ATP concentration; X, the intensity of bioluminescence; K, the slope, and C, the constant (5). ATP per hemoglobin was also calculated, using the determined value of hemoglobin and the computed value of ATP.

Scanning electron microscopy. Two-tenths milliliter of the washed sediment of the incubated erythrocytes was suspended in 100 ml of 0.1 M phosphate buffer containing 1.0 % glutaraldehyde (pH 7.4) and fixed for 6 h.

The specimen was then filtered through filter paper. The erythrocytes retained on the surface of the filter paper were conductively stained by the tannin-osmium method (2 % tannic acid for one hour, 2 % osmium tetroxide for one hour) (7), dehydrated through a graded series of ethanol, critical-point dried with liquid carbon dioxide; and observed under a scanning

electron microscope (HHS-2R, Hitachi Co.) using an accelerating voltage of 5 kV (6, 7).

RESULTS

Human blood stored for 120 days showed some hemolysis. Repeated washing with buffered saline followed by centrifugation sufficiently eliminated the hemolytic products as well as the serous and plasmatic elements of the stored blood. A similer washing after incubation thoroughly eliminated the incubation medium and allowed a reliable estimation of hemoglobin levels by the cyanmethemoglobin method and of ATP levels of incubated erythrocytes by the luciferine-luciferase method. The erythrocytes were also observed well under the scanning electron microscope with little contamination.

The values of the estimated hemoglobin contents and ATP levels are shown in Table 1 together with the incubation media and calculated values of ATP per hemoglobin. The values of ATP per hemoglobin (ATP/Hb) of the adenine and Mg-ATP incubated samples were almost the same as that of the control. The ATP/Hb value of the ACD incubated sample was slightly increased, about 2 times higher than that of the control.

TABLE 1. ATP LEVELS AND HEMOGLOBIN CONCENTRATIONS OF HUMAN ERYTHROCYTE STORED FOR 120 DAYS AND INCUBATED WITH ADENOSINE, INOSINE OR OTHER MEDIA FOR 3 H.

Incubation media	ATP levels (ng/ml)	Hemoglobin conc. (g/dl)	ATP per Hemoglobin (ng/g)
Control	1.29	3.52	42.21
Mg-ATP	2.14	4.01	55.35
ACD	3.32	3.33	88.86
Inosine	15.31	4.50	340.36
Adenine	2.80	6.98	40.19
Inosine-adenine*	175.86	4.46	3940.91

* See text.

The ATP/Hb value of the inosine-incubated sample was 8 times higher than that of the control. The inosine-adenine incubated sample showed a marked elevated ATP/Hb value, which was 93 times higher than that of the control.

Scanning electron microscopy of the glutaraldehyde-fixed specimens showed that most of the erythrocytes isolated from the control or PBS-incubated samples were spherical or spherocytic and had many thin spicules on their surfaces, resembling the type I spheroechinocytes of Bessis (6) (Fig. 1). Most of the erythrocytes from the Mg-ATP, adenine and inosine incubated samples were also spherocytic and had many thin spicules on their surfaces (Fig. 2).

However, the erythrocytes from ACD and inosine adenine incubated samples had different shapes. Most of them were flattened or discocytic with few spicules on their surfaces, resembling the type I echinocytes or type II spheroechinocytes

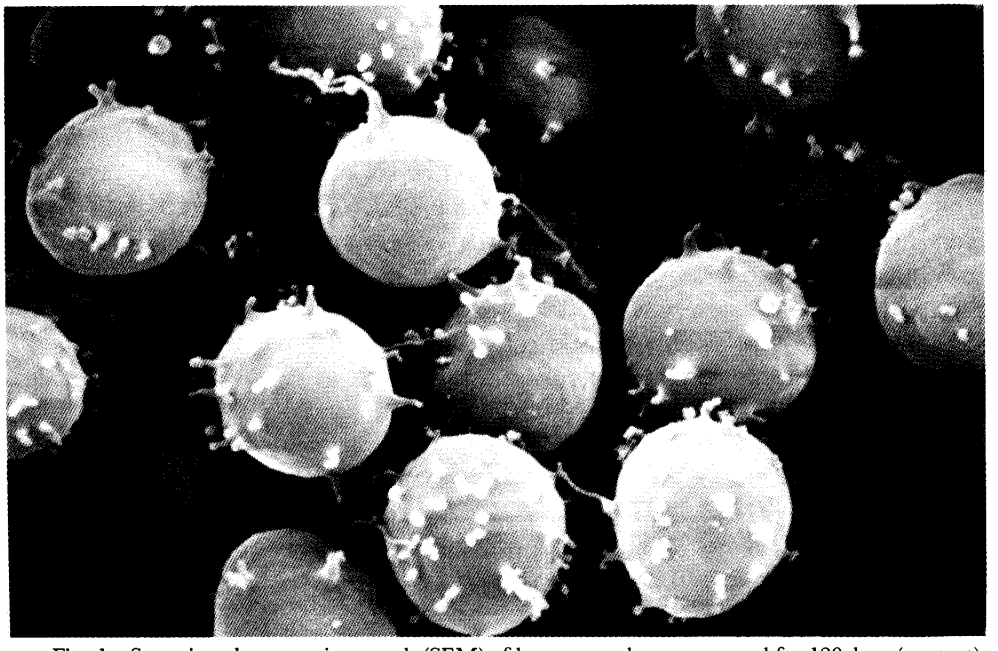

Fig. 1. Scanning electron micrograph (SEM) of human erythrocytes stored for 120 days (see text). × 6,600.

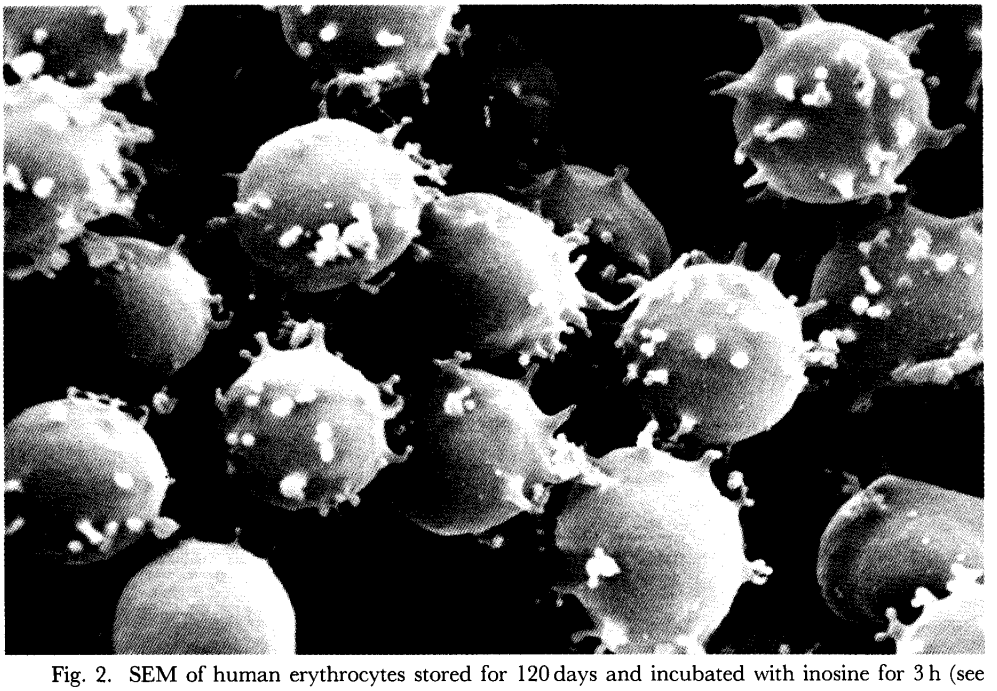

Fig. 2. SEM of human erythrocytes stored for 120 days and incubated with inosine for 3 h (see text). × 6,600.

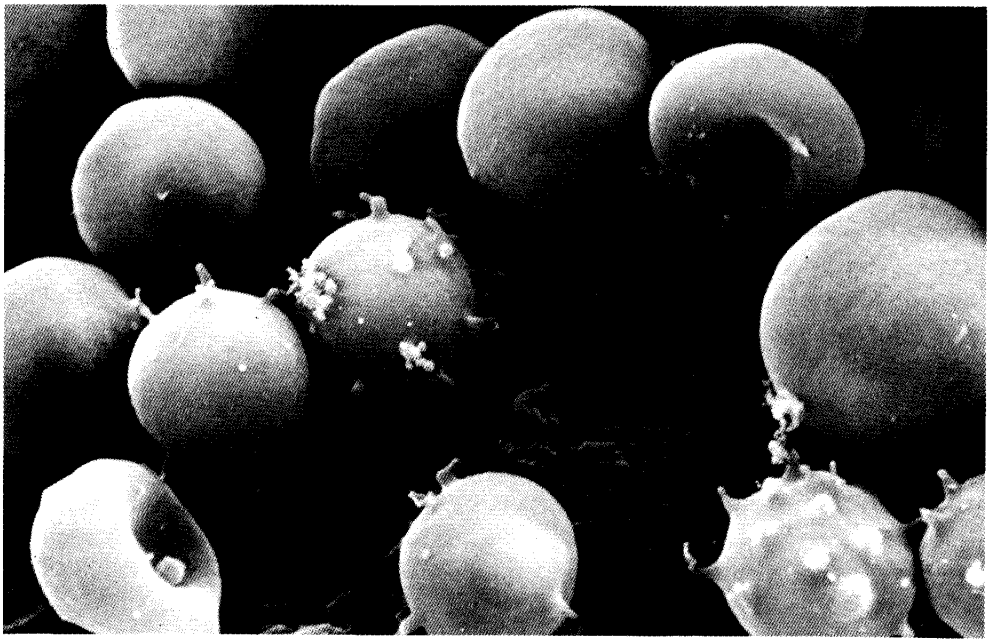

Fig. 3. SEM of human erythrocytes stored for 120 days and incubated in a mixture of glucose, sodium citrate and acid citrate for 3 h (see text). × 6,600.

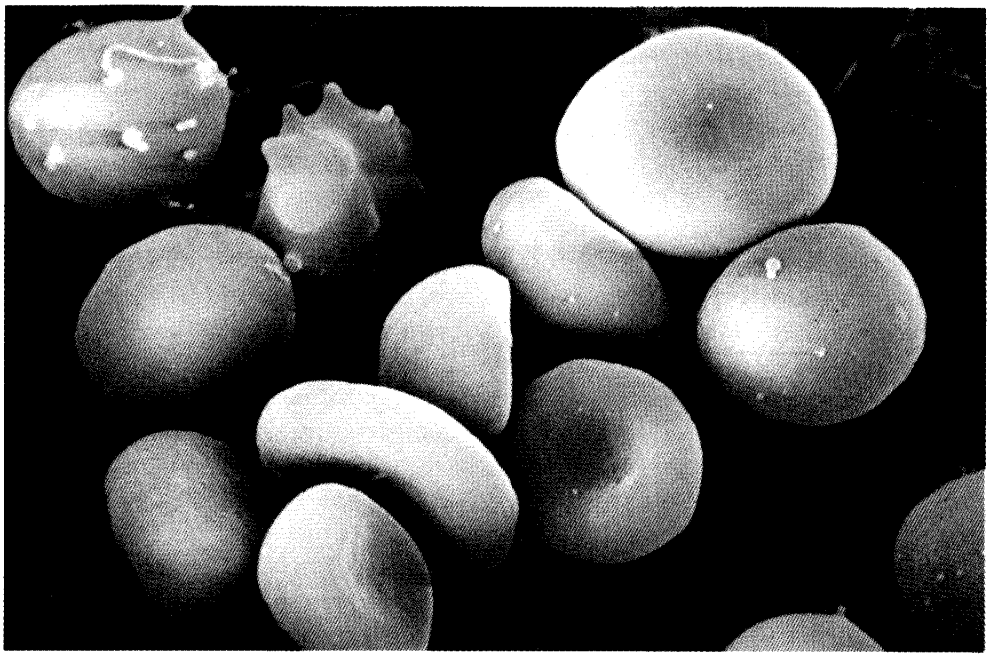

Fig. 4. SEM of human erythrocytes stored for 120 days and incubated in a mixture of inosine and adenine for 3 h (see text). × 6,600.

of Bessis (6) (Figs. 3, 4). Only a few spherical cells with spicules were noted in the ACD and inosine-adenine incubated samples (Figs. 3, 4).

DISCUSSION

The present study showed that stored or aged erythrocytes markedly regain their ATP levels in a mixture of inosine and adenine. Similar results have been reported by Nakao and his associates who incubated 56-day-stored human erythrocytes in various media, including the inosine and adenine mixture. Nakao *et al.* estimated their ATP levels by calculating P^{32}-labelled phosphorus incorporated into nucleotide by a conventional Geiger-Müller counting method, but they did not estimate the ATP concentration per hemoglobin (ATP/Hb) which may provide more reliable information than simple estimation of ATP levels (2, 3).

However, Nakao and his associates have given reasonable and acceptable explanations that this ATP restoration is mediated by the well-known Warburg-Dickens pathway where ribose phosphate is produced from inosine, and that adenine is utilized as the adenine bases of adenosine phosphates, including ATP (8, 9).

The present ATP/Hb estimation shows that incubation with adenine or Mg-ATP alone is not affective in restoring the ATP levels of aged erythrocytes, that ACD-incubation is moderately effective, and that incubation with inosine restores considerable ATP levels though inosine is much less effective than when mixed with adenine. It is conceivable that glucose in the ACD medium is used as an energy source to convert ADP to ATP in the Embden-Meyerhof pathway.

Adenine may not supply energy for such conversion, and may only be utilized as the adenine base of adenosine phosphate (see above). Ineffectiveness of Mg-ATP may be due to its difficulty in penetrating the erythrocyte membrane.

The present scanning electron microscopy reveals clearly that human erythrocytes, when stored, lose their original discocytic shapes and gain spherocytic ones and that re-transformation or rejuvenation of the aged erythrocytes from spherocytes to discocytes is induced by incubation in the mixture of inosine and adenine. Nakao and his associates have also demonstrated such transformation by a conventional light microscope method, but they failed to observe the fine spicules on the surface of the spherocytes because of the limited resolution of their light microscope (9).

It is generally accepted that ATP is an important intrinsic factor in the regulation of the forms of erythrocytes; erythrocytes with high ATP content maintain their discocytic form and those with low ATP content maintain their spherocytic form (3, 9).

This belief was confirmed by the present data. Ferrel and Huestis also confirmed by scanning elecron microscopy that ATP-depleted human erythrocytes lose their discoid shape and adopt a spiny and crenated form (10). They further showed biochemically that such change in shape coinsides with the conversion of phosphatidylinositol-4, 5-bisphosphate to phosphatidyl-inositol and phosphatidic

acid to diacryglycerol, and that both crenation and lipid dephosphorylation are accelerated by iodoacetamide (10).

Consequently, the work of Ferrel and Huestis suggests strongly that the metabolic crenation of erythrocytes resulted in a loss of the inner monolayer of the cell membrane secondary to the degradation of phosphatidylinositol-4, 5-bisphosphate and phosphatic acid (10).

It is interesting that the inosine-incubated erythrocytes did not show discocytic transformation (Fig. 2) though their ATP levels were considerably restored (15 ng/ml) (Table 1), and that the ACD-incubated erythrocytes showed a marked discocytic transformation (Fig. 3) in spite of their slight ATP restration (Table 1). The former result may suggest that a definite elevation of ATP restration (much higher that 15 ng/ml) is needed for the visible transformation to discocytes, and the latter result that the forms of erythrocytes are determined factors other than ATP. Wall and his associates have described a decreased activity of glucose-6-phosphatase in stored or spherocytic human erythrocytes (11). Estimation of this enzyme was omitted in the present study.

However, it may be that glucose contained in the ACD medium and available as an energy source is also utilized as the glucose base of glucose-6-phosphatase to restore or elevate the activity of this enzyme. In this connection, it may be worthwhile to add that in our preliminary experiments stored or spherocytic human erythrocytes incubated in saline neither regained ATP levels nor showed discocytic transformation.

Great importance of the fluidity of the membrane lipid bilayer has been documented in maintaining cell shape. Fujii and his associates described that amphipathic drugs with an anionic polar group induce externalization or crenation of human erythrocytes (*i.e.*, transformation from discocytes to spheroehinocytes), and those with a cationic polar group induce internalization or invagination (*i.e.*, transformation from discocytes to spherostomacytes) (12).

Utsumi and his associates reported that some agents such as DDT fuidize the lipid bilayer and cause a transformation from discocytes to spherocytes with marked invagination (spherostomatocytes) (13, 14). It is well known that some ionized metals such as Ca^{++} and Mg^{++} are also important factors in maintaining or stabilizing the cell membrane or cell shape. Estimation of these ionized metals was omitted in the present study. It is also well known that morphological changes are induced by some extrinsic factors such as osmolarity. In the present study, such factors were minimized by adjusting the incubation media to physiological conditions with saline.

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