BRIEF REPORT


By I. SYLLM-RAPOPORT, G. JACOBASCH, S. PREHN AND S. RAPOPORT

THE CONCENTRATION of adenine nucleotides in red blood cells is maintained at a constant level in the face of a slow renewal of adenine moiety. The mature red cell lacks, however, the ability to synthesize adenine de novo. Therefore an external source in the circulating plasma must be assumed.

We had occasion to observe three cases of arrest of erythrocyte maturation at the level of proerythroblast formation. The bone marrow of the patients was characterized by a great reduction of proerythroblasts, practically complete absence of erythroblasts and absolute lack of reticulocytes. No red cells reached the streaming blood. This condition is known as Erythrogenesis imperfecta (E. i.), Erythroblastopenia or Diamond-Blackfan Disease. The patients depended completely on blood transfusions.

One of the patients (P. C.) exhibited a response to glucocorticoid therapy during the course of her disease and began to produce erythrocytes of her own.

I. ACCUMULATION OF ADENINE NUCLEOTIDES IN ERYTHROCYTES

Venous blood was collected in ice-cold heparinized test tubes. All subsequent procedures were performed at + 4 C. Hematologic and chemical data were determined on the same blood sample. Analyses of donor blood were performed on fresh heparinized samples or taken immediately in ACD-solution; the latter gave lower values for ATP.

Analyses on blood samples of the recipient children were performed after frequent intervals up to 40 days after transfusion. Patient P. G. was followed for 2, R. K. for 5 and Th. M. for 10 transfusion periods. The patients received as a rule 450 ml. of fresh blood from the donors, which was collected either in heparin or in ACD solution.

The determination of acid-soluble phosphates, which gives an approximate indication of the acid-labile phosphate from ATP and ADP was performed essentially as described...
Fig. 1.—Concentration of acid-soluble phosphates (mM/l cells) in erythrocytes of donors (first column) and patient (Th. M.) (second and third column). The data represent 10 transfusion periods.

RESULTS

In a previous communication we reported on the rise of intracellular acid labile phosphates within normal donor erythrocytes during 5 survival periods of the red cells in the organism of the patient (R. K.) with E. i.

In Figure 1 data on the acid-soluble phosphates are shown for patient Th.
Days after transfusion

Fig. 2.—Changes of ATP-concentration with time in transfused erythrocytes in a patient with Erythrogenesis imperfecta. The data represent observations after 6 transfusions over a period of 2½ years (1964–1967). The patient Th. M. was 2 years old when the studies were initiated.

M., which were collected in 10 transfusion periods. The first column represents the values of donor cells, the second and third one data of the patient divided into two groups according to the time interval after transfusion. In the present study the donor erythrocytes transfused to the patients showed the adenine nucleotides to rise to about twice the initial level and to remain high for the rest of the life span of the erythrocytes in the recipient. Figure 2 represents the increase in ATP-concentration of the same patient’s red cells following 6 transfusion periods. The same is shown for patient P. G. in 2 transfusion periods in Figure 3. The main increase in ATP-concentration occurred during a period of about 14 days.

In Table 1 the ATP-concentration of erythrocytes of donors before transfusion and at the end of transfusion periods are shown on patients P. G. and Th. M. The difference in ATP-concentration between patients and donors is statistically highly significant. The relation between ATP, ADP and AMP remained normal, ATP accounting for about 85 per cent. This is shown in Table 2. A factor in the plasma of the recipient causing the accumulation of adenine nucleotides in the erythrocytes was assumed and tested for in the following studies.

2. Crossed incubation experiments

Cross incubation experiments were performed with cells and plasma of normal persons and of a patient at various temperatures. These experiments had variable success. In Figure 4a and b two successful experiments at 4 and 37 C. are shown. No hemolysis occurred in any of the experiments, neither at 4 nor at 37 C. during the observation periods. Details are given in the legend.

As shown in Figure 4a and b higher ATP-levels were found at the end of
Fig. 3.—Changes of ATP-concentration with time in transfused erythrocytes in patient P. G. The data represent 2 transfusion periods. Age of patient at time of observation 2–5 months.

Table 1.—ATP-Concentration in the Erythrocytes of Donors and 30–40 Days after Transfusion in Two Patients

<table>
<thead>
<tr>
<th>Erythrocyte samples</th>
<th>n</th>
<th>ATP mM/l cells</th>
<th>s</th>
<th>p of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors</td>
<td>9</td>
<td>1.35 ±0.23</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Patients</td>
<td>15</td>
<td>2.67 ±0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.—ATP-, ADP- and AMP-Concentration of Patients' Erythrocytes in mM/l Cells and per cent of Total Adenine Nucleotides

<table>
<thead>
<tr>
<th>Erythrocyte sample</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM/l cells</td>
<td>% of total</td>
<td>mM/l cells</td>
</tr>
<tr>
<td>Patient</td>
<td>2.00</td>
<td>82</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>2.03</td>
<td>83</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>2.10</td>
<td>83</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>2.85</td>
<td>87</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>3.10</td>
<td>89</td>
<td>0.24</td>
</tr>
<tr>
<td>Normal</td>
<td>1.49±0.13</td>
<td>88</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>Average</td>
<td>n=21</td>
<td></td>
<td>n=6</td>
</tr>
</tbody>
</table>

* The AMP-values as performed according to the original method are somewhat high (by about 0.01–0.6 mM/l cells) owing to a variable contamination of the NADH with AMP. This error was avoided in the determination of the normal values, which were obtained later by a separate measurement of the AMP content of the NADH for which a correction was applied.
ADENINE LEVEL

Fig. 4.—Erythrocyte ATP changes during crossed incubation of red cells and plasma of a normal person and of a patient (Th. M.). The red cells were not washed.

(4a) Three combinations of suspensions were set up:
- erythrocytes of the donor suspended in the ACD plasma of the patient
- erythrocytes of the donor resuspended in the ACD plasma of the donor
- erythrocytes of the patient resuspended in the ACD plasma of the patient

The original ATP of the donor’s cells was 1.30 mM/1 cells. The suspensions were kept at 4°C. ATP concentration was determined after 24 hours, 4 and 8 days.

(4b) Heparinized venous blood was treated as above. Two combinations were used:
- erythrocytes of the donor suspended in heparinized plasma of the patient
- erythrocytes of the donor resuspended in the heparinized plasma of the donor

Glucose was added to both suspensions to an end concentration of 30–40 mM/1. The flasks were incubated under gentle shaking for 3.5 hours at 37°C.

the incubation periods in all combinations which contained the plasma of the patient. These results support the assumption of a plasma factor causing the increase of adenine nucleotides in the erythrocytes.

3. PLASMA ADENINE

As to the nature of the plasma factor it appeared most likely that it represented adenine or a compound yielding adenine. Various studies in vitro have shown that adenine or adenosine, particularly in the presence of phosphate may lead to a net increase of adenine nucleotides in the erythrocytes. Decisive proof of the nature of the plasma factor was sought by analysis of the plasma of patients in comparison with normal persons.

The first goal was considered to be the identification of the assumed adenine base, since the isolation of nucleosides presents considerably greater technical difficulties. The presence of nucleotides, which are easier to isolate, appeared
unlikely. Work on the precise identification of the adenine compound in the plasma is in progress.

In Figure 5 a chromatogram is reproduced which shows the larger amount of adenine in the plasma of the patient (Th. M.) as compared with that of a normal person.

The identification was carried out in the following manner: 25 ml. plasma were deproteinized with an equal volume of 1.4 N HC1O4. The precipitated proteins were removed by centrifugation and washed once with 5 ml. 07 N HC1O4. The supernatant fluids were heated for 1h in a boiling water bath to hydrolyze the nucleotides and neutralized to pH 8.5 with KOH. The KC1O4 formed was removed in the cold. The purines were precipitated as Cu (1) salts and were lyophilized after washing and removal of the Cu-ions. The residue was extracted with 2.5 ml.0.1 N KOH in 50 per cent ethanol, which leaves many contaminants undissolved. Amounts of 0.01 and 0.02 ml were chromatographed on MN-cellulose 300, thickness 0.25 cm., with 0.1 N KOH as eluant. The spots were made visible under an UV lamp by spraying with 0.0005 per cent Rhodamin B in ethanol. Under these conditions the RF of adenine was 0.38 and that of uric acid was 0.23. The adenine spots were collected by scraping, eluted repeatedly with 0.01 N HCl and the extinction of the supernatant fluid was measured at 260 μ against the eluate of a blank area of cellulose.

A second chromatographic separation was performed on MN cellulose 300 (0.25 cm.) with water as eluant after a preliminary lyophilization of the neutralized KOH extract and reextraction with 0.01 N HCl. In this procedure the uric acid is not extracted. The sample was made visible in the same manner as described before. Only a single spot was visible with an RF value 0.78 identical with a reference sample of adenine. Therefore the adenine could be determined directly in the 0.01 N HCl extract at 260 μ.

For further confirmation of the identity and purity of the plasma adenine the following procedures were performed:

1. The absorption curves at pH 2 and pH 9 were compared and found to be identical as shown in Table 3.
Table 3.—Comparison of the UV-Extinction Ratios of Adenine Samples

<table>
<thead>
<tr>
<th>Adenine sample</th>
<th>pH</th>
<th>240 m/μ</th>
<th>270 m/μ</th>
<th>262 m/μ</th>
<th>262 m/μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>2</td>
<td>0.40</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.36</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>2</td>
<td>0.40</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.37</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.—Adenine in Plasma and Urine. Comparison Between Patients with Erythrogenesis imperfecta and Normal Children. The Concentration are Listed with μM/L; the Amounts Exerted in μmoles/24h.

<table>
<thead>
<tr>
<th></th>
<th>Plasma Concentr.</th>
<th>Urine Concentr.</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td>11</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Patients Th. M.  (4 yrs)</td>
<td>29</td>
<td>33</td>
<td>24</td>
</tr>
<tr>
<td>P. G.† (1.5 yrs)</td>
<td>22</td>
<td>37</td>
<td>7</td>
</tr>
</tbody>
</table>

* Mean value of adults.13
† On a noneffective dosage of prednisone with nearly complete block of maturation.

2. Plasma and reference samples were rechromatographed after deamination with HNO₂ on MN cellulose 300 (0.25 cm.) with 0.1 NHCl as eluant. A single spot with an RF value of 0.66 corresponding to a reference sample of hypoxanthine was found. The extinction ratios at 250/260 m/μ were 1.3 and those at 280/260 0.08 in agreement with the values of pure hypoxanthine.

4. Urine Adenine

In view of the higher concentration of adenine in the plasma of the patients as compared with normal persons the adenine excretion in the urines was determined. For this purpose urine was collected from both patients and normal children of comparable age after periods of three days on a meat-free diet. The isolation, identification and determination of the adenine was carried out in essentially the same manner as described for plasma. The procedures for the determination of adenine yielded a recovery of 75 per cent; the values were corrected accordingly. The results of the quantitative determinations of adenine in plasma and urine are shown in Table 4. It may be seen that the adenine concentration in the plasma of the patient (Th. M.) with complete maturation block is about three times as high as in the normal children, while it is somewhat lower in the patient on steroid therapy with incomplete arrest. Concentrations and amounts in the urines of the patients are three to sixfold higher than in control children. The normal data are comparable to those reported,13 considering the differences in age.
DISCUSSION

Plasma Adenine and Erythrocyte ATP

These observations indicate that the level of adenine nucleotides and in particular of ATP in the circulating red cells is determined by the plasma concentration of adenine or an adenine compound.*

There is some evidence that the bone marrow may have insufficient capacity for the de novo synthesis of purine nucleotides and is partly dependent on the supply of preformed purines from the liver.14 Thus it appears possible that the plasma level of adenine normally is governed by the needs of the bone marrow. A possible mechanism for a connection between bone marrow as utilizing tissue of adenine and the liver as a supplier might be a humoral signal, i.e., a hormonal interplay. According to such a hypothesis the hormonal signal originating from the bone marrow would normally affect an inhibition of the adenine supply by the liver to the plasma in a regulated manner. In patients with E. i. the production of the hormone may be supposed to be diminished or lacking, thus causing an uninhibited flow of adenine into the plasma. It may be speculated further, that the production of the postulated hormone is connected with a maturation stage beyond the proerythroblast since this is the level of the maturation arrest. The response of the adenine-producing organs, most likely the liver, to release excessive amounts of adenine in conditions of maturation arrest could perhaps be taken as a frustrate attempt to supply purine for the bone marrow. The scheme in Figure 6 represents the suggested hormonal regulatory system.

In this connection it is of great interest that in one of the patients (P. C.) maturation could be induced by treatment with glucocorticoids. With the return of the ability of the bone marrow to produce mature red cells the level of the adenine nucleotides in the circulating erythrocytes declined to nearly normal values.

Next to adenine the phosphate may influence the level of the adenine nucleotides as well as of 2,3 diphosphoglycerate.15 Lack of phosphate leads to a lowering of all organic phosphate compounds in the red cells.16,17 The higher levels of adenine nucleotides in the erythrocytes of infants and children as

*The "simple explanation" (suggested by a reviewer), that the elevated adenine levels in plasma and urine stem from the destruction of the immature erythroid cells is contradicted on two accounts: 1) The breakdown of adenine compounds in all cells including those of the bone marrow does not yield free adenine. By deamination and oxidation hypoxanthine and finally uric acid are produced. 2) The amount of adenine conceivably liberated is only a fraction of that accumulated in the body fluids and excreted in the urine, as the following example shows: the patient Th. M. with a body weight of 15 Kg. may be assumed to produce daily an amount of immature erythroid cells corresponding to 5 ml. of erythrocytes; this is based on a potential total red cell mass of 500 ml. and a daily renewal of 1 per cent, i.e., a normal rate of cell production. This corresponds to 8.5 μmoles of adenine, assuming the normal values of adenine nucleotides in erythrocytes and their total conversion to adenine. The actual urinary excretion amounted to 24 μmoles per day. In addition there must be an increased utilization of adenine by various tissues corresponding to the increased plasma level.26,27
ADENINE LEVEL

Liver | Blood | Bone-marrow

\[
\text{ATP} \rightarrow \text{ADENINE} \rightarrow \text{Pro-Ery bl.} \rightarrow \text{Ery bl.} \rightarrow \text{Ret.} \rightarrow \text{Ery.}
\]

Fig. 6.—Scheme of the regulation of the "adenine" level in the blood plasma by the interplay of liver and bone marrow. The interrupted line indicates the postulated hormonal signal from the bone marrow to the liver, the solid line the flow of adenine.

compared with adults may be related to their higher inorganic phosphate in the plasma.\(^{18}\) It may be surmised that in children with E. i. the high inorganic phosphate concentration of the plasma supports the accumulation of adenine nucleotides in the erythrocytes.

**ATP-Level and Life Span of the Erythrocyte.**

It has been suggested frequently that the ATP level is a limiting factor in the life expectancy of red cells in vivo. This assumption is supported by observations of low ATP concentrations in older erythrocyte populations.\(^{19}\) (See however\(^\text{20}\)). An indirect argument is the close connection between ATP levels and the maintenance of the biconcave shape of the erythrocyte in stored blood.\(^\text{21,22}\)

In an earlier publication (3) we studied the life expectancy of the transfused donor cells with acquired high ATP levels in a patient with E. i. These showed evidence of ageing by the criteria of decreasing cell diameter and glucose-6-phosphate dehydrogenase activity, despite a high ATP content. The life expectancy of the erythrocytes was not prolonged. Subsequent studies on the two other patients showed if anything a slight reduction of the life span (unpublished data). It has been reported that stored erythrocytes which had been enriched in ATP-content likewise failed to show a prolonged survival after transfusion.\(^\text{23}\) One must conclude therefore that factors other than ATP can be limiting for erythrocyte survival. Conceivably several factors, among them ATP, either individually or together may be critical depending on the conditions.
SUMMARY
In 3 cases of Erythrogenesis imperfecta (Diamond-Blackfan) the concentration of adenine nucleotides in the red cells from normal donors was found to rise to twice the normal level. A factor in the plasma of the patients was postulated and proved to be an adenine compound. The concentration was 2–3 times as high in the plasma of patients as compared with normals and the excretion in the urine was likewise several fold higher. From these data it is deduced that the plasma adenine level is regulated by a hormonal interplay between the bone marrow and the liver. It is hypothesized that the bone marrow secretes an inhibitor of adenine production in the liver, which is connected with a maturation stage beyond the proerythroblast.

The life expectancy of the donor cells was not prolonged despite their high ATP level. It is concluded that factors other than ATP are limiting for the survival of the red cell.

REFERENCES

I. SYLLM-RAPOPORT, G. JACOBASCH, S. PREHN and S. RAPOPORT

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