The Deamination of Adenosine and Adenosine Monophosphate in \textit{Plasmodium falciparum}-Infected Human Erythrocytes: In Vitro Use of 2'-Deoxycoformycin and AMP Deaminase-Deficient Red Cells

By Eugene Roth, Jr, Nobuaki Ogasawara, and Seymour Schulman

The role of enzymatic deamination of adenosine monophosphate (AMP) and adenosine in the in vitro growth of the malaria parasite \textit{Plasmodium falciparum} was investigated by means of human red cells deficient in AMP deaminase to which the adenosine deaminase inhibitor 2'-deoxycoformycin was added. Malaria parasites grow normally in red cells lacking one or both of these enzyme activities. As a further probe of adenosine triphosphate (ATP) catabolism, both infected and uninfected RBCs were incubated with NaF (with and without 2'-deoxycoformycin) and the purine nucleotide/nucleoside content was analyzed by high-performance liquid chromatography (HPLC).

THE MALARIA PARASITE \textit{Plasmodium falciparum} is a major cause of morbidity throughout the world and is believed to cause approximately one million deaths per year. The erythrocytic forms of this protozoan pathogen multiply asexually in a 48-hour growth cycle during which they produce 16 to 24 new progeny for each invading merozoite. This requires the rapid synthesis of protein and nucleic acids. Plasmodia have retained one pathway each for the production of purine and pyrimidine nucleotides, respectively. Pyrimidine nucleotides must be synthesized from simple precursors and cannot be salvaged; purine nucleotides, on the other hand, can only be salvaged and not synthesized de novo. Accordingly, purine nucleotides must be synthesized from exogenous hypoxanthine or adenosine, but breakdown of red cell adenosine triphosphate (ATP) and other purine nucleotides may also serve as a source of purine bases. Conversely, preformed pyrimidines such as thymidine cannot be utilized at all.

In order to explore purine nucleotide metabolism in this system, we have tested the ability of malaria parasites to grow in human erythrocytes in which the enzymes adenosine monophosphate deaminase (EC 3.5.4.6 AMP aminohydrolase), adenosine deaminase (EC 3.5.4.4), or both were non-functional. In the case of AMP deaminase, mutant red cells from homozygotes deficient for this enzyme were used. Adenosine deaminase (ADA) deficiency was simulated by means of the inhibitor 2'-deoxycoformycin. AMP deaminase irreversibly converts 5'-AMP to 5'-inosine monophosphate (5'-IMP) by hydrolytic deamination with the release of ammonia; IMP, in turn, can be used to synthesize other purine nucleotides such as guanosine monophosphate (GMP). ADA converts adenosine to inosine and ammonia. Inosine can also be utilized to form IMP and other nucleotide precursors. In addition to parasite growth rates, we have also studied the ability of parasitized and uninfected red cells to catalyze adenosine nucleotides. The catabolism of AMP and adenosine form part of the homeostatic regulation of ATP, 2'-deoxyATP, and other purine nucleotides in red cells.

MATERIALS AND METHODS

The biochemical and genetic characteristics of homozygotes for AMP deaminase deficiency have been previously described. Samples from an adult female homozygote and a normal control (both blood group A) were drawn in standard blood bank citrate-phosphate-dextrose solution (CPD) and sent on ice by air from Japan to New York. Experiments were initiated within 48 hours of phlebotomy. \textit{P.falciparum} (FCR-3 strain, A2 clone) was maintained in continuous culture by the candle jar method of Jensen and Trager. Growth experiments were initiated by inoculation of parasitized normal red cells that had been enriched for trophozoites and schizont forms by gelatin sedimentation. The samples were tested in triplicate, and daily evaluation of the parasitemia was performed by counting 1000 cells on a Giemsa-stained slide using the oil immersion lens of a Nikon microscope. Results were expressed as the mean percentage of parasitized red cells ± SD for each day. Sham cultures were prepared with normal or mutant red cells that were incubated in culture medium in candle jars under the same conditions as infected cells. These were used as controls for enzyme and metabolic studies.

AMP deaminase activity was determined using the kinetic method of Campbell et al., which is based on the evolution of ammonia as detected by the glutamate dehydrogenase-dependent oxidation of NADH monitored at 340 nm on an LKB recording spectrophotometer (Ultraspex, Pharmacia-LKB, Gaithersburg, MD). Purified rabbit muscle AMP deaminase was obtained from the Sigma Chemical Co, St Louis, and used for comparison with red cell AMP deaminase. Adenosine deaminase was measured by the method of Beutler, which determines kinetically the decrease in absorption at 265 nm as adenosine is converted to inosine. Hemoglo-
The extracts were stored frozen at -70°C. Acid was made and immediately neutralized with 3 mol/L K₂C₅O₃.

Enolase, and 2'-deoxycoformycin is an inhibitor of ADA. At the end of each experiment, washed red cells were divided into three portions: (1) 30 mmol/L glucose; (2) 30 mmol/L glucose + 10 mmol/L NaF; (3) 30 mmol/L glucose + 10 mmol/L NaF + 0.01 mmol/L 2'-deoxycoformycin. NaF inhibits glycolysis through inhibition of enolase, and 2'-deoxycoformycin is an inhibitor of ADA. At the end of the incubation period, a deproteinized extract with 6% perchloric acid was made and immediately neutralized with 3 mol/L K₂C₅O₃. The extracts were stored frozen at -70°C. Analysis of nucleotide content was performed in duplicate on a Gilson-Rainin high-performance liquid chromatography (HPLC) apparatus using a Whatman Partisil 10-SAX ion exchange column and a UV detector at 254 nm. A starting buffer of 0.01 mmol/L H₃P₀₄, KOH (unadjusted) at ambient temperature was measured at 540 nm as cyanmethemoglobin. A previous study had confirmed that even though malaria parasites partially catabolize about 25% of the hemoglobin content of each infected red cell, no error in hemoglobinometry occurs because the hemolysate cannot be metabolized. Since the cyanmethemoglobin method measures chiefly heme that remains constant, the hemoglobin concentration as measured by this method remains a suitable denominator for all red cell enzyme and metabolite measurements.\(^\text{13}\)

The catabolism of purine nucleotides was assayed by incubating infected or control red cells in an isotonic medium, pH 7.4, with 8.5 mmol/L Na₃H₂P₀₄, 1.4 mmol/L NaH₂P₀₄, 133.5 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1% bovine albumin with 30 mmol/L glucose for 90 minutes at 37°C in a shaking water bath. For each experiment, washed red cells were divided into three portions: (1) 30 mmol/L glucose; (2) 30 mmol/L glucose + 10 mmol/L NaF; (3) 30 mmol/L glucose + 10 mmol/L NaF + 0.01 mmol/L 2'-deoxycoformycin. NaF inhibits glycolysis through inhibition of enolase, and 2'-deoxycoformycin is an inhibitor of ADA. At the end of the incubation period, a deproteinized extract with 6% perchloric acid was made and immediately neutralized with 3 mol/L K₂C₅O₃. The extracts were stored frozen at -70°C. Analysis of nucleotide content was performed in duplicate on a Gilson-Rainin high-performance liquid chromatography (HPLC) apparatus using a Whatman Partisil 10-SAX ion exchange column and a UV detector at 254 nm. A starting buffer of 0.01 mmol/L H₃P₀₄, adjusted with KOH to pH 2.85, was run isocratically at ambient temperature at the rate of 2 mL/min for five minutes. Then a linear gradient to 100% 0.7 mol/L KH₂P₀₄, pH 4.40, in 37 minutes was introduced at a flow rate of 2 mL/min. Concentrations of nucleotides were calculated by reference to purified standards and their peak areas.\(^\text{14}\) ATP, adenosine diphosphate (ADP), and ATP were also measured enzymatically.\(^\text{15}\) Nucleosides were determined on the same extracts using an IBM C-18 column with an isocratic solution consisting of 86% 10 mmol/L KH₂P₀₄ (pH unadjusted) and 14% methanol at a flow rate of 2 mL/min.\(^\text{16}\) All reagents and standard nucleotides and nucleosides were purchased from Sigma except for HPLC grade potassium phosphate and methanol, which were supplied by the Fisher Scientific Corp (Springfield, NJ).

**Results**

**Parasite Growth**

The FCR-3 strain of *P. falciparum* proliferated normally in adenine deaminase-deficient (AMPD) red cells (Fig 1A). Normal growth was also seen in AMPD-deficient cells in the presence of 10 μmol/L 2'-deoxycoformycin, an ADA inhibitor (Fig 1B). In previous studies, we have found that 2'-deoxycoformycin does not inhibit parasite growth in normal red cells (data not shown). These data show that the combined loss of AMPD and ADA activities has no effect on parasite growth.

**Enzyme Studies**

ADA and AMPD content of infected red cells diverged markedly. There was a 57% decline in AMPD activity in infected normal red cells, which is suggestive of digestion of red cell cytosolic proteins. This pattern has been seen with other red cell enzymes that are not synthesized by the parasite, such as glucose-6-phosphate dehydrogenase (G6PD) and diphosphoglycerate mutase (DPGM).\(^\text{14}\) Infected AMPD-deficient red cells continued to show no AMPD activity (Table 1). ADA activity was increased markedly in both infected normal and infected AMPD-deficient red cells (Table 1). When extrapolated to 100% parasitemia, the increment in activity was approximately 1800%. All detectable ADA activity was ablated by 10-μmol/L 2'-deoxycoformycin. ATP levels were approximately twice normal in AMPD-deficient red cells maintained in sham culture conditions (Table 1). Ten-μmol/L 2'-deoxycoformycin had a minimal inhibitory effect (less than 10%) on AMPD activity derived from either human red cells or rabbit muscle (Sigma).

| Table 1. Some Biochemical Characteristics of Normal and AMPD-Deficient Red Cells in Sham Culture and After Infection With *P. falciparum* |
|-------------------|------------------|-----------------|
|                   | ATP              | Adenosine Deaminase  |
|                   | μmol/g Hemoglobin| U/g Hemoglobin   | 5'-AMP Deaminase U/g Hemoglobin |
| Normal RBCs in sham culture | 3.85            | 0.96 ± 0.09      | 9.62 ± 0.22                     |
| Infected normal red cells (parasitized cells = 75%) | 4.19            | 13.04 ± 0.48     | 4.27 ± 0.14                     |
| AMPD-deficient RBCs in sham culture | 6.83            | 1.10 ± 0.48      | *                               |
| Infected AMPD-deficient (parasitized cells = 83%) | 5.15            | 12.50 ± 0.50     | *                               |

*Not detectable.*
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Table 2. Nucleotide, Nucleoside, and Hypoxanthine Content of Normal and AMPD-Deficient Red Cells: The Effect of Fluoride and 2'-Deoxycoformycin

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>GTP</th>
<th>HYPO</th>
<th>ADO</th>
<th>GUANO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal RBC + GLC</td>
<td>9</td>
<td>392</td>
<td>3510</td>
<td>207</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPD-deficient RBC + GLC</td>
<td>59</td>
<td>569</td>
<td>7030</td>
<td>25</td>
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<td></td>
<td></td>
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<tr>
<td>Normal RBC + GLC + NaF</td>
<td>370</td>
<td>343</td>
<td>58</td>
<td></td>
<td>840</td>
<td></td>
<td>21</td>
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<td>AMPD-deficient RBCs + GLC + NaF</td>
<td>883</td>
<td>1010</td>
<td>250</td>
<td>859</td>
<td></td>
<td></td>
<td>41</td>
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<tr>
<td>Normal RBCs + GLC + NaF + 2'-deoxycoformycin</td>
<td>183</td>
<td>364</td>
<td>57</td>
<td>25</td>
<td>2678</td>
<td>178</td>
<td></td>
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<tr>
<td>AMPD-deficient RBCs + GLC + NaF + 2'-deoxycoformycin</td>
<td>4970</td>
<td>1107</td>
<td>270</td>
<td></td>
<td>22</td>
<td>532</td>
<td></td>
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</tbody>
</table>

Concentrations given as nanomoles per gram hemoglobin (after 90 minutes at 37°C).
Abbreviations: HYPO, hypoxanthine; ADO, adenosine; GUANO, guanosine; GLC, glucose.

Nucleotide Catabolism

Uninfected red cells. Uninfected red cells metabolizing glucose appeared to be in a steady state (Table 2). No hypoxanthine, guanosine, or adenosine were detected. These nucleosides are evidence of ATP breakdown. As noted, ATP was approximately twice normal in AMPD-deficient red cells, and AMP was about six times higher in concentration. On the other hand, the guanosine triphosphate (GTP) concentration was about one-third normal. In the presence of 10-mmol/L fluoride, ATP levels declined in both normal and AMPD-deficient red cells after an incubation period of 90 minutes. GTP disappeared entirely, and there were large increments in AMP that were more marked in normal red cells where the initial level had been lower to start with. Hypoxanthine and guanosine were detectable in both cell types.

The addition of 2'-deoxycoformycin to the fluoride incubation did not affect the degree of ATP degradation, but there was a marked effect on the metabolites that accumulated. Of interest was the massive accumulation of AMP in the AMPD-deficient red cells—a 25-fold increase over the AMP content in normal red cells treated similarly. Purine accumulation patterns were also affected in that hypoxanthine accumulated in large amounts in normal red cells but not in AMPD-deficient cells. Guanosine was not found, but for the first time, adenosine was detected in both normal and AMPD-deficient red cells. The accumulation of adenosine can be attributed to the inhibition of ADA, which otherwise would have converted the adenosine into inosine and thence into other metabolites such as hypoxanthine. It appears that under these conditions, normal red cells catabolized ATP mostly to hypoxanthine, whereas AMPD-deficient cells (lacking the functions of both AMPD and ADA) could not metabolize ATP beyond AMP.

Infected red cells. The nucleotide/nucleoside profiles of infected normal and AMPD-deficient red cells are in general more alike than are the patterns of their uninfected counterparts (Table 3). In the presence of glucose, ATP is maintained, and GTP and ADP are increased in both normal and mutant infected red cells. However, AMP is also elevated in normal cells, but perhaps surprisingly not in AMPD-deficient red cells. The failure to find AMP accumulation in infected AMPD-deficient red cells (in contrast to the considerable accumulation in uninfected red cells) suggests that the AMP was further degraded by the parasite for hypoxanthine salvage. There are small amounts of hypoxanthine present in both types of cells; this is evidence of purine nucleotide catabolism. Upon the addition of NaF, there is a near total loss of ATP and GTP with some increment in AMP, but most of the nucleotides appear to have been degraded into hypoxanthine and other metabolites that were not detected. The concomitant addition of NaF and 2'-deoxycoformycin appeared to accentuate the accumulation of hypoxanthine and adenosine.

The most salient feature of the combined deaminase deficiencies was the difference between uninfected and

Table 3. Nucleotide, Nucleoside, and Hypoxanthine Content of P Falciparum-Infected Normal and AMPD-Deficient Red Cells: The Effect of Fluoride and 2'-Deoxycoformycin

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>GTP</th>
<th>HYPO</th>
<th>ADO</th>
<th>INO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal RBCs* + GLC</td>
<td>1134</td>
<td>449</td>
<td>4190</td>
<td>378</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPD-deficient RBCs† + GLC</td>
<td>35</td>
<td>691</td>
<td>5150</td>
<td>488</td>
<td>11</td>
<td></td>
<td></td>
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<tr>
<td>Normal RBCs + GLC + NaF</td>
<td>967</td>
<td>309</td>
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<td></td>
<td>759</td>
<td></td>
<td>177</td>
</tr>
<tr>
<td>AMPD-deficient RBCs + GLC + NaF</td>
<td>392</td>
<td>407</td>
<td>115</td>
<td></td>
<td>1715</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Normal RBCs + GLC + NaF + 2'-deoxycoformycin</td>
<td>158</td>
<td>650</td>
<td>177</td>
<td></td>
<td>1320</td>
<td>133</td>
<td>21</td>
</tr>
<tr>
<td>AMPD-deficient RBCs + GLC + NaF + 2'-deoxycoformycin</td>
<td>197</td>
<td>28</td>
<td>139</td>
<td></td>
<td>1935</td>
<td>179</td>
<td>23</td>
</tr>
</tbody>
</table>

Abbreviations: HYPO, hypoxanthine; ADO, adenosine; INO, inosine; GLC, glucose; tr, trace.
*Percent parasitized red cells = 88% (trophozoites/schizonts).
†Percent parasitized red cells = 83% (trophozoites/schizonts).
infected red cells. The uninfected doubly deficient red cells were unable to accumulate hypoxanthine in the presence of fluoride. On the other hand, significant amounts of hypoxanthine in the presence of fluoride were found in malaria-infected red cells with activity of both deaminases blocked (Table 3). In addition, infected AMPD-deficient cells did not accumulate AMP, as did their uninfected counterparts under the influence of 2'-deoxycoformycin. In this respect, both AMPD-deficient and normal red cells behaved similarly when infected with \textit{P. falciparum}.

**DISCUSSION**

Purines play very different roles in uninfected and infected red cells. Uninfected red cells are metabolically sluggish elements that require small amounts of ATP to maintain electrolyte-water gradients and cell shape, as well as levels of NAD and NADP. The low metabolic rate of human red cells (1-2 mM glucose metabolized to lactate per liter of red cells per hour) is perhaps advantageous for the organism in view of the large red cell mass which would otherwise consume more calories than might be available.

Once the malaria parasite invades the erythrocyte, glucose consumption may increase as much as 100-fold, and a 48-hour growth cycle begins, which requires DNA, RNA, and protein in an abundance that the red cell alone cannot supply. Other researchers have stressed that the parasite may derive its purines from exogenously supplied hypoxanthine or adenosine, but as the data in this study show, hypoxanthine and adenosine can be supplied as a result of breakdown of endogenous adenine and guanine nucleotides as well. In the uninfected state, red cell hypoxanthine is an end product that plays little if any role in the normal red cell and may diffuse out and be metabolized in the liver or gut by xanthine oxidase (which is absent in red cells) to uric acid.

Fluoride and other glycolytic inhibitors have been used to perturb cellular metabolism in such a way as to highlight ATP breakdown when ATP synthesis is blocked. The increased accumulation of ATP catabolites (even if transient) aids in identifying potential pathways of interest in the breakdown of ATP. Fluoride acts by inhibiting enolase and thereby decreases the production of ATP and the regeneration of NAD+ via lactic dehydrogenase (LDH). Meanwhile, ATP continues to be consumed by the hexokinase and phosphofructokinase reactions (but not without a net gain in ATP) and thus begins the depletion of ATP and the appearance of its catabolites.

In a previous study with uninfected ADA-deficient red cells using both fluoride and iodoacetate as inhibitors of glycolysis, Mills et al.\textsuperscript{22} found that with fluoride, nearly all of the AMP was converted to IMP via AMP deaminase; significant amounts of adenine were also seen but only in the ADA-deficient red cells. A different pattern was seen with iodoacetate inhibition in the same cells. With this agent, 20% to 30% of the degraded AMP was metabolized via 5'-nucleotidase to adenosine. Using similar techniques in normal human red cells, Bontemps et al.\textsuperscript{23} found that the normal pathway in the presence of glucose for the breakdown of AMP proceeds via AMP deaminase to IMP followed by dephosphorylation of IMP via 5'-nucleotidase to inosine and thence via nucleoside phosphorylase to hypoxanthine. In glucose-deprived red cells, the catabolism of ATP proceeds via dephosphorylation of AMP (5'-nucleotidase) to adenosine and then via adenosine deaminase to inosine and thence to hypoxanthine, with some recycling of adenosine detected. The process was very dependent on pH and inorganic phosphate levels.

Clearly, then, the catabolic pathway for purine nucleotides is not a single file of consecutive conversions without branching or other alternative pathways. A concatenation of unidirectional chemical reactions would be highly susceptible to blockade by mutation or environmental inhibition at one step and would very likely be at a selective disadvantage from an evolutionary viewpoint. Thus, it is very likely that malaria parasites possess alternative purine catabolic pathways that readily bypass the two blocked deaminase steps in this study. Further work will be required to elucidate this pathway, which may involve enzymes not found in the host red cell.

Finally, an important difference in plasmodial sensitivity to 2'-deoxycoformycin should be noted. Webster et al.\textsuperscript{24} have previously described successful therapy with this agent against \textit{Plasmodium knowlesi} in rhesus monkeys. The lack of effect against \textit{P. falciparum} reported here in vitro suggests significant differences in susceptibilities between species of malaria. Above all, these findings should serve as a warning against any extrapolations to human malaria from rodent or primate malarials.

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The deamination of adenosine and adenosine monophosphate in Plasmodium falciparum-infected human erythrocytes: in vitro use of 2'deoxycoformycin and AMP deaminase-deficient red cells

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