Mechanism of Adenosine Triphosphate Catabolism Induced by Deoxyadenosine and by Nucleoside Analogues in Adenosine Deaminase-inhibited Human Erythrocytes

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ABSTRACT

The mechanism of the depletion of ATP, recorded in the erythrocytes of adenosine deaminase-deficient children and of leukemia patients treated with deoxycoformycin, was investigated in normal human erythrocytes treated with this inhibitor of adenosine deaminase. Deoxyadenosine, which accumulates in both clinical conditions, provoked a dose-dependent accumulation of dATP, depletion of ATP, and increases in the production of inosine plus hypoxanthine. Concomitantly, there was an increase of AMP and IMP, but not of adenine, indicating that catabolism proceeded by way of AMP deaminase. A series of nucleoside analogues (9-β-D-arabinofuranosyladenine, N'-methyladenosine, 6-methylmercaptopurine ribonucleoside, tubercidin, ribavirin, and N-1-ribosyl-5-aminomidazole-4-carboxamide riboside) also stimulated adenine nucleotide catabolism and increased AMP and IMP to various extents. The effects of deoxyadenosine and of the nucleoside analogues were prevented by 5'-iodotubercidin, an inhibitor of adenosine kinase. Interestingly, they were reversed if the inhibitor was added after the accumulation of nucleotide analogues and initiation of adenine nucleotide catabolism. Further analyses revealed linear relationships between the rate of phosphorylation of deoxyadenosine and nucleoside analogues and the increase in AMP and between the elevation of the latter above a threshold concentration of 10 μM and the rate of adenine nucleotide catabolism. Kinetic studies with purified erythrocytic AMP deaminase, at physiological concentrations of its effectors, showed that the enzyme is nearly inactive up to 10 μM AMP and increases in activity above this threshold. We conclude that the main mechanism whereby deoxyadenosine and nucleoside analogues stimulate catabolism of adenine nucleotides by way of AMP deaminase in erythrocytes is elevation of AMP, secondary to the phosphorylation of the nucleosides.

INTRODUCTION

Accumulation of dATP has been documented in erythrocytes (1–6), in lymphocytes and bone marrow cells (7), and in platelets (8) of children with ADA deficiency. It is also found in RBC (9–11) and lymphoblasts (12, 13) of leukemia patients treated with the ADA inhibitor, dCF. In addition, dramatic depletions of ATP were recorded in the erythrocytes (9, 10) and lymphoblasts (13) of patients treated with dCF, whereas smaller decreases were observed in the erythrocytes of some children with ADA deficiency (3–6). Bagnara and Hershfield (14), investigating ADA-inhibited human lymphoblastoid cells, have explained this ATP depletion as follows: (a) dAdo, which accumulates when ADA is deficient or inhibited, is phosphorylated to dAMP and subsequently to dATP, a process which utilizes ATP and generates ADP and AMP; (b) the accumulation of dATP, which is greatly facilitated by the fact that dAMP is a poor substrate for AMP-DA (15, 16), stimulates both the deamination of AMP to IMP and the dephosphorylation of IMP to inosine (14, 17), thereby provoking adenine ribonucleotide catabolism. This mechanism is, however, difficult to reconcile with two observations: (a) dATP and ATP are equipotent as stimulators of erythrocyte (15) and lymphoblast (14) AMP-DA; (b) the sum of dATP and ATP in ADA-deficient (4, 5) or in ADA-inhibited cells (9, 10, 13) is not or only slightly higher than that of ATP in control cells. These data and uncertainties about the concentration of AMP in ADA-deficient or -inhibited cells prompted a reinvestigation of the mechanism of the depletion of ATP induced by dAdo. This study was performed with normal human erythrocytes in which ADA was inhibited by dCF. The effect of dAdo has been compared to that of other purine nucleoside analogues which are also substrates of adenosine deaminase (18). Our results provide evidence that all these nucleoside analogues induce catabolism of the adenine nucleotides by a common mechanism, namely elevation of AMP. Part of this work has been presented at a symposium (18).

MATERIALS AND METHODS

Chemicals. [U-14C]Adenine (270 Ci/mol) and [8-14C]AMP (55 Ci/mol) were purchased from the Radiochemical Centre (Amersham, Buckinghamshire, England). ITu was from RBI (Natick, MA) and dCF was from Warner Lambert (Detroit, MI). Adenine nucleotides and adenosine were from Boehringer (Mannheim, Germany). Other nucleotides, nucleosides, and GTP-agarse were from Sigma (St. Louis, MO). The sources of all other chemicals have been given (19, 20).

Incubation of Erythrocytes. Fresh blood taken from a cubital vein of healthy human volunteers was collected on heparin. Isolation and washing of erythrocytes were performed in KRB, pH 7.4, containing 5 mM glucose and gassed with 95% O2-5% CO2 as described in Ref. 19. The RBC were resuspended in the same medium as a 20% hematoctrit and their adenine nucleotides were labeled by a 60-90-min preincubation at 37°C with 2–3 μM [U-14C]adenine. This was followed by two washes and resuspension as a 20% hematoctrit in KRB with 5 mM glucose. Unless given otherwise, the concentration of P2 in the KRB buffer was 1.2 mM. In the experiments in which this concentration was increased to 10 mM, that of Ca2+ was reduced from 2.5 to 1.25 mM. Incubations were performed in carefully regassed and stopped vials. In all experiments, suspensions were preincubated for 20 min with 1 μM dCF before addition of nucleosides, in order to allow tight binding of the inhibitor with ADA. We have shown previously that dCF has no effect by itself on erythrocytic adenine nucleotide catabolism (19). In some experiments, 10 μM ITu was used to inhibit adenosine kinase (21).

Assessment of Inhibitor Requirements in Intact Erythrocytes. Preliminary studies were performed in which the nucleotides and the cataabolites produced from the added nucleosides were measured by HPLC, as described below. These showed that the addition of 1 μM dCF and 10 μM ITu inhibited by more than 90% the deamination of up to 0.5 mM...
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RESULTS

Effects of Deoxyadenosine on the Concentrations of dATP and ATP on the Production of Purine Catabolites. Incubation of ADA-inhibited erythrocytes with dAdo resulted in a dose-dependent accumulation of dATP (Fig. 1, left); dAdo, even at the lowest concentration, was not completely utilized over 3 h; dATP represented 80 to 90% of the total amount of deoxyribonucleotides synthesized (not illustrated). Concomitantly, there was a decrease of ATP. At all concentrations of dAdo, the sum of both triphosphates remained approximately equal or became slightly higher than the initial concentration of ATP. The addition of dAdo also induced an accumulation of the terminal purine catabolites, hypoxanthine and inosine (Fig. 1, right), the latter increasing only at high concentrations of dAdo (see Fig. 2). The very small accumulation of purine catabolites, recorded in the control condition, was about doubled upon addition of 20 μM dAdo, the lowest concentration used, and 30- to 50-fold increased in the presence of 0.5 mM dAdo. The purine catabolites nearly completely accounted for the loss of ATP. All these results are qualitatively similar to those recorded by Bagnara and Hershfield (14) in ADA-inhibited lymphoblastoid cells.

Fig. 2 depicts the time course of the effect of various concentrations of dAdo. Concurrently with the depletion of ATP, dose-dependent increases of ADP, AMP, and IMP were recorded. Despite inhibition of ADA, no adenosine could be detected. Together with the increase in IMP, this indicates that the accumulation of inosine and/or hypoxanthine results from deamination rather than from dephosphorylation of AMP.

Influence of Inhibition of the Phosphorylation of dAdo. Similarly to that of adenosine, the phosphorylation of dAdo can be inhibited by the addition of ITu, a potent inhibitor of adenosine kinase (21). When 10 μM ITu was added at the beginning of the incubation, together with dAdo (Fig. 3, left), the accumulation of dATP as well as the depletion of ATP and the elevations of AMP and IMP were prevented, while the production of inosine plus hypoxanthine was inhibited by more than 90%. These results accord with the observation that dAdo did not stimulate adenine nucleotide catabolism in an adenosine kinase- and deoxycytidine kinase-deficient cell line (14). Likewise, addition of ITu 90 min after dAdo, halfway through the incubation (Fig. 3, right), completely arrested the build-up of dATP. Strikingly, however, the addition of ITu also brought about a complete arrest of the degradation of ATP. This was accompanied by a return of the concentration of AMP to nearly its basal value, by a decrease in IMP and, after a 30-min latency,
can be phosphorylated intracellularly. As shown in Fig. 4, adenosine and a series of nucleoside analogues, added at 0.5 mM concentration, all stimulated, although to various extents, the catabolism of the adenine ribonucleotides into inosine and hypoxanthine. Maximal stimulation was obtained by Tu, followed by, in order, N6-MeAdo, dAdo, MMPR, AICA riboside (not illustrated), Rbv, adenosine, and ara-A. Owing to the presence of dCF, it could be concluded that, as with dAdo, the inosine and hypoxanthine produced from the prelabeled adenine nucleotides resulted from the deamination of AMP into IMP rather than from AMP dephosphorylation. In the presence of Tu and of adenosine, some dephosphorylation of AMP occurred, as evidenced by the appearance of labeled adenosine.

To verify the similarity of the mechanism of action of the nucleoside analogues with that of dAdo, further experiments were performed with ITu. Results obtained with two of them, MMPR (Fig. 5, left), which is converted for 90% into a monophosphate, and Tu (Fig. 5, right), which is mainly converted into a triphosphate, are shown. Similarly to that induced by dAdo, the catabolism of the adenine nucleotides provoked by these nucleosides was arrested by the subsequent addition of ITu. This arrest was associated with an interruption of the synthesis of MMPR monophosphate, which reached the concentration of about 1.2 μmol/ml of packed cells after 90 min, and of Tu triphosphate which reached about 0.8 μmol/ml of packed cells after 60 min (not illustrated). As observed with dAdo, addition of ITu also provoked a decrease of AMP and IMP, as well as of the production of inosine and hypoxanthine. Similar results were obtained with adenosine, Rbv, N6-MeAdo, and AICA riboside (not illustrated). These experiments indicate that all the nucleosides provoke an increase in the activity of AMP-DA by a common mechanism, which is independent of the level of their phosphorylation.

Influence of Supraphysiological Concentrations of Pᵢ. Because Parks and Brown (24) had not observed a depletion of ATP after addition of various nucleosides to human erythrocytes incubated in 30 mM Pᵢ, the influence of an elevation of the concentration of Pᵢ was investigated. When Pᵢ in the incubation medium was increased from its physiological level of 1.2, to 10 mM, the effects on adenine nucleotide catabolism of adenosine, dAdo, ara-A, and MMPR, all at 0.5 mM concentration, were completely suppressed (results not shown). The effect of 0.5
of 2 nmol/ml of suspension (corresponding to 10 nmol/ml of
As shown in Fig. 7, below a threshold concentration of AMP
when both processes were switched off by ITu. The correlation
catabolism was induced, and AMP concentration decreased
of Catabolism of the Adenine Ribonucleotides. With all nucleo
since it was also observed, although to a smaller extent, when
The reason for the decrease in P¡ is not clear. It does not seem
by dAdo (Fig. 6, left) or by Tu (Fig. 6, right) was arrested by
dependent on the added nucleoside, decreased by 30 to 60%
control conditions, was not modified by the addition of the
nucleosides. However, the concentration of intracellular P¡,
depending on the added nucleoside, decreased by 30 to 60%
(results not shown). Nevertheless, when in experiments similar
to those depicted in Figs. 3 and 5, the ATP degradation induced
by dAdo (Fig. 6, left) or by Tu (Fig. 6, right) was arrested by
the addition of ITu, intracellular P¡, reincreased only by 20%.
The reason for the decrease in P¡ is not clear. It does not seem
linked exclusively to the phosphorylation of the nucleosides
since it was also observed, although to a smaller extent, when
the synthesis of dATP was prevented by the addition of ITu at
the beginning of the incubation (not illustrated).
Correlation between the Concentration of AMP and the Rate
of Catabolism of the Adenine Ribonucleotides. With all nucleo
studies, the concentration of AMP increased when their
phosphorylation was allowed to proceed and adenine ribonucleotide
catabolism was induced, and AMP concentration decreased
when both processes were switched off by ITu. The correlation
between the concentrations of AMP and the rates of production
of inosine plus hypoxanthine, recorded under control conditions
and with various nucleosides, was therefore investigated.
As shown in Fig. 7, below a threshold concentration of AMP
of 2 nmol/ml of suspension (corresponding to 10 nmol/ml of
packed cells), there was almost no production of inosine and
hypoxanthine. Beyond this value, a steep linear relationship
between the concentration of AMP and the rate of production
of inosine plus hypoxanthine was observed; a 2-fold increase in
AMP enhanced the production of these catabolites by a factor
of 10. The AMP threshold was not due to extracellular AMP,
since its concentration in the medium was below 0.4 nmol/ml,
and the slight hemolysis recorded under control conditions
was not increased by the addition of the various nucleosides.
Fig. 8 shows that the concentrations of AMP obtained with
the various nucleosides were closely related to their rates of
phosphorylation, with the exception of MMPR. This may be
linked to the fact that MMPR is converted to a monophosphate
nucleoside, in contrast with the other nucleosides which are
metabolized to triphosphates.
Kinetic Properties of Erythrocyte AMP Deaminase at
Physiological Concentrations of AMP. The kinetic properties of
purified erythrocytic AMP-DA, including its stimulation by
ATP and K\(^+\), and inhibition by 2,3-BPG and P\(_i\), have been
studied extensively (15, 16, 22, 25). However, to our knowledge,
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Fig. 8. Correlation between the rate of synthesis of nucleotide analogues and the concentration of AMP. AMP concentrations are means of three measurements as in Fig. 7. Rates of synthesis of nucleotides from the added nucleosides were calculated from the concentrations of the nucleotides measured at 30, 60, and 120 min of incubation. r (excluding the MMPR data) = 0.96; P < 0.001. Ado, adenosine.

Fig. 9. Influence of effectors on the substrate saturation curve of AMP deaminase. The activity of the enzyme was measured in the absence of additions () or in the presence of 1 mM ATP () and of 1 mM ATP, 3 mM 2,3-BPG, and 1 mM Pi ( ).

the kinetics of the enzyme had not been investigated at the micromolar concentrations of AMP which prevail in intact RBC. The influence of both the increase in AMP and the decrease in intracellular P, recorded during the metabolism of nucleosides, was therefore assessed. In the presence of 100 mM KCl, but in the absence of other effectors AMP-DA displayed hyperbolic kinetics with a S0.5 for AMP of 0.4 mM (Fig. 9). Addition of 1 mM ATP, the concentration of the stimulator in control erythrocytes, decreased the S0.5 for AMP to 0.15 mM. Further addition of the inhibitors at their physiological concentrations, namely 1 mM for Pi, and 3 mM for unbound 2,3-BPG, rendered the substrate saturation curve sigmoid and the enzyme nearly inactive up to 10 μM AMP. At 50 μM AMP and in the presence of 1 mM ATP and 3 mM 2,3-BPG, P, inhibited the enzyme activity by 40% at 1 mM and by 95% at 10 mM concentration (Fig. 10, left). A decrease in P, from 1 mM to 0.5 mM, as recorded in ADA-inhibited erythrocytes incubated with 0.5 mM dAdo or Tu, would thus increase the activity of AMP-DA by no more than 20%. The influence of ATP and dATP on the activity of the enzyme in the presence of physiological concentrations of its other effectors is shown in Fig. 10 (right).

As described by others (14, 15), dATP and ATP were equally efficient as stimulators, the half-maximal effect being obtained at approximately 1 mM in the presence of 1 mM Pi and 3 mM 2,3-BPG. Under the same conditions, ara-ATP was a 2-fold less potent stimulator than ATP and dATP (not shown). The effect of other triphosphate nucleosides was not tested because they were not available. It was also verified that dAMP, which is not a substrate of AMP-DA (15, 16), was not a positive effector of the enzyme at low concentrations of AMP. Tu monophosphate and ara-AMP were also without effect on the activity of AMP-DA.

DISCUSSION

Several mechanisms have been proposed to explain the loss of ATP accompanying accumulation of dATP in erythrocytes of leukemia patients treated with dCF and of children with ADA deficiency. Simmonds et al. (4) have claimed that erythrocytic ATP is dependent, at least partly, on adenosine produced by the transmethylation pathway and that this production could be impaired in ADA-deficient or -inhibited cells, because of inactivation of S-adenosylhomocysteine hydrolase by dAdo (Ref. 6, and references therein). This mechanism is, however, difficult to reconcile with the finding of increased concentrations of adenosine in the plasma and urine of ADA-deficient patients (1, 7). Recently, Snyder et al. (26) have proposed that adenosine causes a substrate inhibition of adenosine kinase and/or a decreased phosphoribosylation of adenine. On the other hand, the study of Bagnara and Hershfield (14) in ADA-inhibited lymphoblasts and our study in ADA-inhibited erythrocytes show that dAdo increases the rate of degradation of ATP and of total adenine nucleotides. The effect is already seen at 20 μM dAdo, a concentration which is in the range of the concentrations found in leukemia patients treated with dCF (11, 12). Both studies also show that the stimulation of the degradation of adenine nucleotides by dAdo results from an increased activity of AMP-DA. Indeed, catabolism of AMP via dephosphorylation to adenosine can be ruled out because, de-
spite inhibition of ADA, adenosine does not accumulate, and inosine and hypoxanthine are produced. The accumulation of inosine, recorded concomitantly with the high rates of build-up of hypoxanthine, indicates that the activity of purine nucleoside phosphorylase becomes limiting under these conditions, most likely owing to the low intracellular concentration of P_i.

We show in this work that the main factor responsible for the increased activity of AMP-DA in erythrocytes incubated with dAdo is neither the accumulation of one of its stimulators, dATP, nor a decrease of its inhibitors, P_i and 2,3-BPG, but an increase in AMP brought about by the utilization of ATP in the phosphorylation of dAdo. That the elevation of AMP plays a major role in this catabolism is indicated by the observation that the arrest of the synthesis of dATP by ITu (Fig. 3) reverses the elevation of AMP and stops the catabolism of ATP, despite the presence of 0.5 mM dATP in the cells. ITu, an inhibitor of adenine kinase, has no effect by itself on AMP-DA (19, 27).

The role of the elevation of AMP is validated by our results obtained with other nucleosides, which are also substrates of adenosine kinase (28, 29). Similarly to dAdo, the nucleoside analogues induced an elevation of AMP and an acceleration of the catabolism of the adenine ribonucleotides, which were both counteracted by the subsequent inhibition of adenosine kinase by ITu.

The relationship between the intracellular concentration of AMP and the rate of production of purine catabolites (Fig. 7) shows that erythrocytes are very sensitive to an elevation of AMP above a threshold concentration of 5–10 μM. One hypothesis to explain this threshold, is that part of AMP, similarly to other erythrocytic metabolites (30), is bound to hemoglobin and thus not accessible to AMP-DA. We, however, favor the explanation that AMP-DA is not active at 5–10 μM AMP, owing to the kinetic characteristics of its substrate saturation curve in the presence of physiological concentrations of effectors (Fig. 9). The kinetic data given in Fig. 10 indicate that both the decrease of intracellular P_i from 1 to 0.5 mM and the substitution of ATP by dATP are not the triggering factors of the catabolism of the adenine nucleotides induced by dAdo.

The reason why nucleosides induce a decrease of intracellular P_i, (Fig. 6) is not clear, since it occurs even when the synthesis of nucleotides is prevented by a prior addition of ITu. The fact that AMP-DA is strongly inhibited at supraphysiological concentrations of P_i (Fig. 10) most likely explains why the degradation of the adenine nucleotides induced by nucleosides can be prevented by incubation in a high-P_i medium. Indeed, at 10 mM extracellular P_i, intracellular P_i increases from 1 to 4–5 mM (19). This probably also explains why Parks and Brown (24), who incubated erythrocytes in a high-P_i medium, did not observe a depletion of ATP with various nucleoside analogues. Why Henderson et al. (31) recorded nucleoside-induced adenine nucleotide catabolism in the presence of 25 mM P_i, is, however, not clear.

The increase of the concentration of AMP provoked by nucleosides is clearly related to the rate of their phosphorylation or, more precisely, to the amount of high-energy phosphate required for the synthesis of analogue nucleotides (Fig. 8). This is evidenced by the lower increase of AMP induced by MMPR (which becomes a monophosphate nucleotide) than by Tu (which becomes a triphosphonucleotide), although both nucleosides are phosphorylated at the same rate. These results also indicate that the rate of glycolysis in erythrocytes is not quite sufficient to provide high-energy phosphates upon addition of nucleosides, although we have observed a small (10–20%) stimulatory effect of their addition on the production of lactate. The rates of accumulation of nucleotides, shown in Fig. 8, although potentially also influenced by their concomitant degradation which may vary from one analogue to another, reflect approximately the affinities and substrate efficiencies of the respective nucleosides toward adenosine kinase from rabbit liver (29). Adenosine is, however, an exception since, although it is the best substrate of adenosine kinase, it induced only a limited elevation of adenine nucleotides, including AMP. This is most likely due to the known inhibition of adenosine kinase by excess of substrate (26, 29).

The relationships between the rate of phosphorylation of nucleosides and the elevation of AMP and between the latter elevation and the rate of AMP catabolism most likely explain why the depletion of ATP is usually smaller in erythrocytes of ADA-deficient children (3–6) than in patients treated with dCF (9–11). Indeed, in the latter, higher concentrations of dAdo in the plasma have been recorded (11, 12).

Taken together, our results suggest that catabolism of the adenine nucleotides of human erythrocytes can be induced by all nucleosides, and perhaps by other compounds, that are phosphorylated at a sufficient rate. The degradation is caused by an elevation in erythrocytic AMP and proceeds by way of AMP-DA. This catabolism may be responsible for the anemia recorded not only in ADA deficiency (6) and dCF treatment (9, 32) but also with other nucleoside analogues used in anticancer and in antiviral therapy (33). Whether a similar nucleoside-induced catabolism occurs in other tissues remains to be established. Its existence may depend on various factors, among which are the kinetic characteristics of the AMP-DA isozyme of the tissue. For liver AMP-DA for instance, the concentrations of the effectors of the enzyme seem to play a more important role than that of its substrate (27, 34).

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