A NOVEL EFFECT OF THE NEW ANTILEUKEMIC DRUG, 2-CHLORO-2'-DEOXYADENOSINE, IN HUMAN LYMPHOCYTES

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2-chloro-2'-deoxyadenosine (Cl-dAdo) is a potent drug against hairy cell leukemia and other lymphomas. Its effects on the metabolism of labeled deoxycytidine (5-[³H]dCyd) and deoxythymidine ([³H]dThd) were investigated in short term cultures of human lymphocytes. In the presence of Cl-dAdo, a more pronounced inhibition of DNA synthesis was found, accompanied with a much higher accumulation of radioactivity in dTTP than in dCTP. This difference disappeared in the presence of exogenous dThd. Analysis of intracellular metabolites of 5-[³H]dCyd showed an increase in [³H]dCTP used for liponucleotide biosynthesis and a decrease in [³H]dUMP formation in the presence of Cl-dAdo, suggesting the possible inhibition of dCMP deaminase.

The natural nucleoside, 2-deoxyadenosine (dAdo), is highly toxic to certain lymphoid cells, if for any reason the cell cannot deaminate it by adenosine deaminase (ADA). As it is well known, ADA deficiency causes severe combined immunodeficiency syndrome (SCID). 2-chloro-2'-deoxyadenosine (Cl-dAdo), an ADA resistant analogue, is also highly toxic for nondividing lymphocytes and monocytes, but fibroblasts are resistant for this drug [1-4, 6]. Cl-dAdo is an excellent substrate for mammalian deoxycytidine kinase [5], its phosphorylation is a prerequisite for its toxic effect [1]. Cl-dAdo has a chemotherapeutic activity in hairy cell leukemia, in chronic lymphoid leukemia, and in low grade lymphomas [4,6]. However, the exact mechanism of its effect is still not clear. Recently, relationship of dCyd kinase and the cytoplasmic 5'-nucleotidase activity was published to be responsible for the therapeutic efficacy of Cl-dAdo [7]. DNA polymerase [8] and ribonucleotide reductase[9] were also suggested as target enzymes. DNA strand breaks, and decreased levels of ATP and NAD are also characteristic during Cl-dAdo

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Effect of Cl-dAdo can be antagonized by dCyd, however this effect was markedly different on the various progenitor cells in the bone marrow suggesting a considerable metabolic difference of nucleosides during hematopoetic cell differentiation [11].

In our laboratory, dCyd was shown to be used not only for DNA but also into phospholipid precursor synthesis in tonsillar lymphocytes [14-17]. A significant conversion of dCyd into dTTP was also demonstrated [13,14]. In the present paper, the effect of Cl-dAdo on the metabolism of dCyd and dThd was investigated in short term cultures of human lymphocytes. Evidences are presented for a novel effect of Cl-dAdo: it has a characteristic inhibition on the conversion of dCMP to dTTP. This effect can be antagonized by high concentration of exogenous thymidine.

**METHODS**

*Cell labeling*

Human tonsillar lymphocytes were prepared from surgically removed tonsils of 3-6 year old infants as described earlier [18]. Short-term cell cultures were labeled with either 5-[^3]H]-deoxyribocytidine (dCyd) (1500 GBq/mmol), or with 5-methyl[^3]H]thymidine (dThd) (1480 GBq/mmol) at a concentration of 0.05-0.1 µM for 60 min. at 37°C in Eagles MEM in the presence or absence of inhibitors.

*Separation of labeled metabolites*

After labeling, cells were washed and precipitated with ice-cold 75% ethanol and kept at -20°C overnight. Ethanol soluble fraction was used for separation of nucleotides and liponucleotides on DEAE cellulose sheets as described earlier [12]. Thin layer chromatography (TLC) was used to analyze the nucleotide and liponucleotide fractions, as described earlier [15]. Brie: In case of [^3]H]dThd labeling, nucleoside monophosphates were separated on PEI cellulose using LiCl for development; In case of [^3]H]dCyd labeling, chromatography was performed on Kieselgel 60F254 (1st dimension: butanol-acetate-water 2:1:1; 2nd dimension: chloroform-methanol-ammonia 1:1:1) to separate labeled nucleosides, mono-, di-, and triphosphates from dCDP-choline. Results with this method were similar to the separation technique with HPLC used earlier [14]. The ethanol insoluble material was washed with 0.5N perchloric acid twice, and acid insoluble fraction was hydrolyzed and measured for radioactivity (DNA fraction).

*Synthesis of 2-chloro-2-deoxyadenosine*

2-chloro-2-deoxyadenosine was synthesized in the laboratory of Z. Kazimierczuk, as described earlier [20].

**RESULTS**

*Differential effect of 2-chloro-deoxyadenosine on thymidine versus deoxycytidine metabolism in lymphocytes*

Lymphocytes were labeled either from [^3]H]dThd or from 5-[^3]H]dCyd in the presence of increasing concentration of Cl-dAdo (Fig. 1). In accordance with our previous results [12-18], incorporation of [^3]H]dThd into DNA was much higher than the incorporation of [^3]H]dCyd in tonsillar lymphocytes. In addition to this difference, a more pronounced inhibition of DNA synthesis was found in case of [^3]H]dThd labeling than in case of 5-[^3]H]dCyd labeling (Fig. 1, DNA). On the other hand, Cl-dAdo induced a higher accumulation of nucleotides labeled from [^3]H]dThd than from 5-[^3]H]dCyd (Fig. 1, Nucleotides).
As Cl-dAdo is a very good substrate for deoxycytidine kinase [5], one of the possible explanations for the low sensitivity of \([^{3}H]dCyd\) incorporation might be a simple competition between Cl-dAdo and \([^{3}H]dCyd\) during labeling. To exclude this possibility, inhibitory effect of Cl-dAdo on \([^{3}H]dThd\) incorporation was also measured in the presence of trace amount (1µM) of non-labeled dCyd (Fig. 1 DNA: 3HdT + dC). No effect of this low dCyd concentration was found on the inhibitory effect of the drug, suggesting no competitive effect between Cl-dAdo and \([^{3}H]dCyd\) at these concentrations.

**Differential effect of 2-chloro-deoxyadenosine on thymidine versus deoxycytidine metabolism disappears in the presence of exogenous thymidine**

Exogenous dCyd can be used intracellularly for dCTP as well as for dTTP synthesis via deamination of dCMP, methylation of dUMP and its phosphorylation to dTTP. In tonsillar lymphocytes, the conversion of \([^{3}H]dCyd\) to dTTP can exceed 55-70% of the total \([^{3}H]dCyd\) taken up by these cells [13,14], resulting in a low incorporation of 5-[\(^{3}H\)]dCyd into DNA, as the radioactive labeling is lost in the methylation step. As it was shown earlier in our laboratory [14], conversion of \([^{3}H]dCyd\) to dTTP can be inhibited by high concentration of exogenous dThd, leading to an increase of \([^{3}H]dCyd\) incorporation into DNA via the dCTP pathway (see also Fig. 2, inserted table). To investigate whether Cl-dAdo might effect the conversion of dCyd into dTTP, metabolism of \([^{3}H]dCyd\) was investigated in the presence and absence of non-labeled dThd (Fig. 2). Our results clearly show that the differential effect of Cl-dAdo on dThd and dCyd metabolism (Fig. 1 and also Fig. 2, 3HdT vs. 3HdC) disappears in the presence of exogenous dThd (Fig. 2, 3H-dT vs. 3H-dC+dT) suggesting a direct effect of Cl-dAdo on the conversion of dCyd into dTTP.

**2-chloro-deoxyadenosine increased the labeling of dTTP and dCTP from exogenous precursors, while it inhibited the conversion of dCyd into dTTP.**

Cells were labeled either with \([^{3}H]dThd\) (Fig. 3) or with \([^{3}H]dCyd\) (Fig. 4) in the absence or presence of 1 µM Cl-dAdo. Intracellular metabolites of \([^{3}H]dThd\) were analyzed by TLC on PEI cellulose, and an accumulation of label in dTTP fraction was found in the presence of Cl-dAdo [Fig. 3]. Intermediers of \([^{3}H]dCyd\) were separated by 2 dimensional TLC on Silica plates. The results show a significant decrease of \([^{3}H]dUMP\), suggesting a possible inhibition of deoxycytidylate deaminase, while labeling of dCTP, as well as the phospholipid precursors synthesized from it, were elevated. These results, in addition to the results presented above, suggest that Cl-dAdo inhibits the conversion of \([^{3}H]dCyd\) to dTTP in short-termed cultures of lymphocytes without affecting the utilization of exogenous dCyd for the synthesis of phospholipid intermediers.
The effect of Cl-dAdo on 3H-Thd and on 3H-dCyd metabolism

Figure 1. Effect of 2-chlorodeoxyadenosine (Cl-dAdo) on 3H-Thd and on 3H-dCyd metabolism.

Freshly isolated lymphocytes were labeled either from [3H]dCyd (Δ-Δ) or from [3H]Thd in the absence ( ------ ) or presence ( o - o ) of 1 µM dCyd and increasing concentrations of Cl-dAdo. After one hour of incubation, the radioactivity incorporated into the intracellular metabolites (nucleotides, measured on DEAE cellulose sheets from the ethanol soluble fractions of the cells, see insert) as well as their incorporation into DNA (DNA, measured from the acid insoluble fraction of cells after hydrolysis), was determined.

DISCUSSION

The management of hairy cell leukemia has been changed in the last decade as a result of some new effective agents including alpha-interferon, 2'-deoxycoformicin and 2-chloro-2'-deoxyadenosine (Cl-dAdo). According to a recent review [21], Cl-dAdo has yielded the best overall response rate of 95% and complete remission rate of 82% among these drugs in the treatment of hairy cell leukemia. The high response rate and the easy administration of Cl-dAdo might make it the first-line treatment of choice. Thus it is not surprising that the molecular mechanism of the effect of Cl-dAdo became in focus of investigations.
Figure 3. Separation of the intracellular metabolites labeled from $[^{3}H]$dThd.
Lymphocytes were labeled with $[^{3}H]$dThd in the absence (I--;<--) or presence (o--o) of 1 µM Cl-dAdo for one hour. After labeling, nucleotides were separated on PEI-cellulose TLC eluted with 1 M LiCl and analyzed for radioactivity. Spots were identified by non-radioactive standard in UV light.

Cl-dAdo has a preferential effect on lymphoid cells, as deoxycytidine kinase, the enzyme needed for its intracellular metabolism, is highly expressed in lymphoid cells [5]. Evidently, the biochemical mechanism of the effect of Cl-dAdo should be in connection with the metabolism of deoxycytidine (dCyd). dCyd can be incorporated into DNA either through the production of dCTP, or through its conversion to dTTP. While dTTP production via deoxythymidine kinase is highly cell cycle dependent, production of dTTP from exogenous dCyd seems to be independent from DNA synthesis [24], and has a high rate in tonsillar lymphocytes [13,14]. In this paper, evidences are presented for the inhibition of the metabolism of dCyd by Cl-dAdo through the pathway of dTTP formation, probably by the inhibition of deamination of dCMP.

According to Plunkett's group [22,23], dCMP deaminase, a non cell-cycle dependent enzyme, is one of the main regulator of the proper intracellular ratio of dCTP to dTTP. The ratio of the two deoxyxypyrimidine nucleotide was suggested to regulate the intracellular activity of dCMP deaminase [22,23]. Different inhibitors of DNA synthesis (aphidicoline, hydroxyurea, araC [24] and dThd [25]) have been shown to have contradictory effects on the dTTP/dCTP pools.
Plunkett's group have suggested that the strictly cell cycle dependent dTTP synthesis via thymidine kinase might involve a specific enzyme complex, while the dTTP synthesis via dCMP deaminase and thymidylate synthase is not cell cycle dependent [24]. The compartmentation of dCTP pool was also suggested, one serving for liponucleotide synthesis beside dTTP synthesis [15-17]. In accordance with the results of Plunkett and his colleagues [23], we found that dCTP, produced by the salvage of dCyd is not an efficient substrate for DNA replication, it is rather used for dTTP and liponucleotide synthesis [15-17]. Experimental data presented in this paper suggest that Cl-dAdo inhibits conversion of exogenous dCyd to dTTP.

One of the key enzymes in the interconversion of exogenous dCyd to dTTP is dCMP deaminase. Our experiments, presented in this paper show a lower intracellular labeling of dUMP in the presence of Cl-dAdo, suggesting dCMP-deaminase as a possible target enzyme of Cl-dAdo treatment. As Cl-dAMP is similar in structure to dGMP, and dGMP is a natural inhibitor of CMP-deaminase [26], a direct inhibition of Cl-dAMP is possible. On the other hand, changes in dCTP/dTTP pool ratio has also a regulatory effect on this conversion pathway [22]. Presently, experiments are in progress to distinguish between the direct and the indirect effects of the drug.

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