Dipyridamole Enhancement of Toxicity to L1210 Cells by Deoxyadenosine and Deoxycoformycin Combinations in Vitro 1

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ABSTRACT

The combination of 2'-deoxyadenosine and deoxycoformycin is known to be markedly toxic to T-lymphocyte cell lines relative to B-cell lines, and this difference appears to be related to the capacity of the cells to accumulate deoxyadenosine triphosphate (dATP). In the presence of dipyridamole and 2'-deoxyadenosine and when adenosine deaminase was inhibited with deoxycoformycin, the L1210 leukemia cell which is a non-T-, non-B-cell type accumulated dATP like a T-cell type. The intracellular L1210 concentration of dATP using the triple combination (1.1 µM deoxycoformycin-40 µM deoxyadenosine-10 µM dipyridamole) reached 400 µM at which concentration ribonucleotide reductase specific activity was reduced by 80%. While this degree of enzyme may be significant, complete inhibition might have been expected, since 400 µM dATP is approximately 40 times the concentration to give 50% inhibition in some purified systems.

INTRODUCTION

Two major mechanisms of dAdo2 toxicity have been proposed, the accumulation of dATP (3, 4, 11, 18, 27), which is known to be a feed-back inhibitor of ribonucleotide reductase (18), and the inactivation of S-adenosylhomocysteine hydrolase with subsequent inhibition of S-adenosylmethionine-dependent methylation reactions (7, 13). Combinations of dAdo with ADA inhibitors have been reported to be more toxic to T-cell lines than to B-cell lines (3, 11, 19, 27). Biochemical studies have shown that T-cells relative to B-cells have an increased capacity to accumulate dAdo nucleotides and that this increased capacity was associated with one form of the severe combined immunodeficiency disease syndrome, where ADA activity was deficient (10, 12, 25). dCF, a potent inhibitor of ADA, which also causes increases in intracellular dATP levels, was found recently to be active in the therapy of ALL in Phase I clinical studies (1, 28). A commonly used laboratory model of clinical ALL is the L1210 murine tumor. The L1210 tumor has surface antigens, which indicates that it is a non-T, non-B type, a characteristic shared with the most prevalent cell type found in ALL of childhood (14).

When dAdo, dCF, and dipyridamole were used in vitro in triple combination, intracellular L1210 dATP levels as high as 400 µM could be obtained, which was correlated with an 80% decrease in ribonucleotide reductase activity. This use of dipyridamole in combination with some nucleoside analogues may possibly have utility in the clinical treatment of certain forms of childhood ALL.

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3 The abbreviations used are: dAdo, 2'-deoxyadenosine; ADA, adenosine deaminase; dCF, deoxycoformycin; ALL, acute lymphocytic leukemia; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid.

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MATERIALS AND METHODS

L1210 Cells in Suspension Culture. The maintenance of the L1210 cell in suspension culture has been described (6). Growth studies were initiated at 104 L1210 cells/ml in Fischer’s medium (K. C. Biological, Inc., Lenexa, Kans.) containing 10% horse serum. In inhibition studies, the medium also contained penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml). The L1210 cells were grown in duplicate in 70-ml Corning flasks at 37° in 5% CO2-95% air in a Forma Scientific Model 329S incubator (Forma Scientific, Marietta, Ohio). Cell counts were performed in a Model ZB1 Coulter Counter (Coulter Electronics, Hialeah, Fla.) Cell viabilities were determined by trypan blue dye exclusion. dCF, dAdo, and dipyridamole used at various concentrations were sterilized by filtration through Millipore filters before addition to the growth flasks.

HPLC of Intracellular Nucleotide Metabolites. L1210 cells in 500 ml of medium (0.7 to 1 x 106 cells/ml) were incubated with various concentrations of drugs. Cells were preincubated with 1.1 µM dCF for 1 hr before adding dAdo to inhibit ADA. Nucleotides and other acid-soluble metabolites were extracted from the cells with 0.3 ml of perchloric acid at 0°. After 30 min, the extracts were centrifuged at 10,000 x g for 3 min, and the acid soluble extracts were neutralized with KOH solution, and the KClO4 precipitates were removed by centrifugation. These extracts were stored in liquid nitrogen until analyzed. HPLC analyses of acid-soluble extracts of cells were performed on a Beckman Model 334 gradient liquid chromatography system with a system controller (Beckman Instruments, Inc., Berkeley, Calif.) using Ultrasil-NH2 columns. Samples (20 µl) were eluted at room temperature, starting with 100% 5 mM NH4H2PO4 (pH 2.8) and 0% 750 mM NH4H2PO4 (pH 2.6) for 1 min and increasing to 40% 750 mM NH4H2PO4 at 81 min. The flow rate was 2 ml/min, and the effluent was detected at 254 nm with a Hitachi Model 100-10 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Nucleotides were identified by their respective retention times and coelution with known standards. Quantitation was done with a Hewlett Packard Model 3390A integrator (Hewlett Packard, Avondale, Pa.) or by graphical method for small peaks that were not measurable with the integrator. Measurement of [3H]dAdo incorporation was carried out by treating cells with [3H]dAdo and drugs as above. [3H]dAdo metabolites were collected in fractions of 1 ml using an LKB 2111 fraction collector (LKB Produkter AB, Bromma, Sweden), and the sample radioactivity was determined in a Beckman LS-9000 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Incorporation of dAdo into Whole Cells. Suspensions of cells were treated with labeled dAdo as indicated in the appropriate experiments. Duplicate samples (1 ml) of cell suspensions at various times were analyzed for radioactivity in total-cell and acid-insoluble materials. For the determination of total radioactivity, the cells were collected by centrifugation at 1800 x g for 3 min at 4°, and the cells were washed rapidly twice with 5 ml of ice-cold 0.9% NaCl solution and then suspended in 0.5 ml of 0.5 N TCA. This mixture was then heated at 70° for 30 min and counted for radioactivity. For the determination of radioactive acid-insoluble material only, other replicate samples were mixed with 1 ml of n perchloric acid at 0°, and the cellular precipitates were collected by centrifugation at 1800 x g for 3 min at 4°. The precipitates were washed twice with 5 ml of cold 0.5 N perchloric acid and once with 5 ml of cold 0.5 N TCA. The precipitates were suspended in 0.1 ml of 0.5 N TCA, heated at 70° for 30 min, and counted for radioactivity (23).

Ribonucleotide Reductase Assay. L1210 cells from 100 ml of me-
medium (0.6 to 0.8 x 10^6 cell/ml) were homogenized at 4° in 0.3 ml of buffer (0.05 M Tris buffer, pH 7.5, containing 0.025 M dithioerythritol) using a Potter-Elvehjem homogenizer fitted with a power-driven Teflon pestle. These homogenates were centrifuged at 100,000 x g for 1 hr in a Beckman ultracentrifuge (Model L5-65) with a 50 Ti rotor at 4°. The extracts were used for the ribonucleotide reductase assays immediately after preparation. Protein concentrations were measured by the method of Bradford (2). CDP reduction was assayed by the method of Chang and Cheng (9), which is based on the method of Steeper and Steuart (26). Dowex 1-borate ion-exchange chromatography was used to separate CDP from dCDP. The assay mixture contained [14C]CDP (0.2 nCi, 0.15 µM), dithioerythritol (3 mM), MgCl₂ (6 mM), ATP (5 mM), and 30 µl of crude enzyme extract in a final volume of 0.2 ml. Incubations were carried out for 1 hr at 37°.

Chemicals. dCF was obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md. [8-3H]dAdo was purchased from ICN Chemical Radiosotopes Division, Irvine, Calif. and [14C]CDP (trisodium salt) was purchased from New England Nuclear, Boston, Mass. dAdo, dipyridamole, and the nucleotide standards for HPLC analyses were purchased from Sigma Chemical Co., St. Louis, Mo. Dowex 1-borate resin was prepared from Dowex 1-CI (X8, 200 to 400 mesh) resin also obtained from the Sigma Chemical Co.

RESULTS

dATP Accumulation Dependency on dAdo Concentration and Dipyridamole. HPLC analyses were conducted to see to what extent L1210 cells could phosphorylate dAdo to dATP. Chart 1 shows that L1210 cells treated with dCF (1.1 µM) at various concentrations of dAdo and incubated in vitro for 3 hr converted dAdo to dATP. The dATP accumulation was dependent on the concentrations of dAdo and 1 µM dipyridamole. The level of dATP in cells incubated with dCF (1.1 µM) only for 3 hr was less than 11 µM, and the level of dATP resulting from incubation with dAdo (40 µM) only was less than 17 µM.

dATP Accumulation with 10 µM Dipyridamole. Chart 2 shows the result of a typical experiment in which cells were incubated with dCF (1.1 µM) and dAdo (40 µM) with and without dipyridamole (10 µM) for various times. Without dipyridamole, the dATP level reached 28 µM; however, in the presence of dipyridamole, the intracellular concentration of dATP increased markedly to 565 µM.

Growth Effects of Various Concentrations of dCF, dAdo, and Dipyridamole. Dipyridamole is a known inhibitor of deoxyribonucleoside transport (22). Mitchell et al. (19) obtained decreases in cytotoxicity and dATP amounts in T-lymphoblast cultures in which dipyridamole was added to erythro-9-[3-(2-hydroxyethyl)adenerine and dAdo in combination. We expected similar results with dipyridamole in L1210 cells, since it might also have inhibited dAdo uptake in L1210 cells. However, we found increases in cytotoxicity and dATP amounts when L1210 cells were incubated with dCF, dAdo, and dipyridamole in triple combination. Chart 3 shows the in vitro effects of dCF and dAdo with dipyridamole. Dipyridamole alone had no significant effects on the growth of L1210 cells at concentrations up to 10 µM. dAdo in the presence of dCF (1.1 µM) had no effect on the growth of L1210 cells at concentrations up to 10 µM and showed only 50% inhibition of growth at 72 hr. However, in the presence of 1 or 5 µM dipyridamole, the 50% growth-inhibitory concentration for dAdo in the presence of dCF (1.1 µM) was reduced to 10 and 2.5 µM, respectively. in L1210 cells. Cass et al. (8) have reported a similar increase in toxicity...
dCF, dAdo, and Dipyridamole Cytotoxic Effects

Dependence of dATP Accumulation on dCF Concentrations. dATP accumulation in cells treated with dAdo (40 μM) and dipyridamole (1 μM) with different concentrations of dCF (0.4 to 3.7 μM) did not increase with increasing concentration of dCF up to 3.7 μM. dCF, 1.1 μM, was sufficient to inhibit maximally the ADA activity in L1210 cells (data not shown).

Viability and Recovery of L1210 Cell Growth after Drug Treatments. The viability and recovery of growth of cells treated with the triple combination were studied after resuspending the cells in fresh medium. The dATP amounts in the cells treated with the double combination for 3 hr was 26 to 28 μM, and the surviving cells showed regrowth at 118 hr after resuspension in drug-free medium. The dATP concentrations in the cells treated

of dAdo plus dCF against L1210 cells caused by nitrobenzylthioi- nosine.

dATP Accumulation with Various Dipyridamole Concentrations. dATP accumulation in L1210 cells treated with dCF (1.1 μM) and dAdo (40 μM) increased with increasing concentration of dipyridamole (Chart 4). The highest concentration of dATP was about 400 μM when dipyridamole was used at 10 μM in this study.

Table 1

<table>
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<tr>
<th>Drug combination</th>
<th>dATP (nmol/10⁶ cells)</th>
<th>Ribonucleotide reductase specific activity</th>
<th>% of inhibition</th>
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<tr>
<td>Control</td>
<td>8.43</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dCF (1.1 μM)</td>
<td>6.65</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>dAdo (40 μM)</td>
<td>&lt;15</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Dipyridamole (10 μM)</td>
<td>3.25</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>DCF (1.1 μM)-dAdo (40 μM)</td>
<td>23–25</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>DCF (1.1 μM)-dAdo (40 μM)- dipyridamole (10 μM)</td>
<td>1.59</td>
<td>81</td>
<td></td>
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</tbody>
</table>

* The inhibition given by dipyridamole alone may be artifactual, since the whole-cell homogenates would probably contain dipyridamole eluted from membrane fragments.
with 5 or 10 mM dipyridamole in the presence of dCF (1.1 μM) and dAdo (40 μM) for 3 hr were 240 to 280 μM and 370 to 430 μM, respectively. These dATP concentrations appeared to be too toxic for cell survival. We tentatively conclude from these data that a correlation appears to exist between cytotoxicity and dATP amounts and that there may be a certain limit of intracellular dATP concentrations beyond which few cells survive (Chart 5); however, it is possible that another mechanism is operative (7,13).

Effects of Drugs on [3H]dAdo Uptake into Intracellular Metabolites. Generally, cellular uptake has been an important factor for the biological activity of nucleoside analogues, since natural
dipyridamole, like nitrobenzylthioinosine (21), is a potent inhibitor of nucleoside uptake. Mitchell et al. (19) have shown that dipyridamole was effective in preventing dAdo-induced cytidototoxicity and the elevation of dATP levels in the presence of 5 μM erythrose-9-[3-(2-hydroxynonyl)]adenine in T-lymphoblast cultures. Our data indicate (Chart 6) that total incorporation of [3H]dAdo (40 μM) was not inhibited significantly in whole-cell materials (acid-soluble plus nucleic acids) by either dCF (1.1 μM) alone or a combination of dCF (1.1 μM) and dipyridamole (10 μM) at 2 hr, but incorporation into the acid-insoluble materials (presumably DNA) was inhibited about one half.

This inhibition of incorporation presumably into DNA is consistent with an increase in dATP levels with indirect inhibition of DNA polymerases.

Effects of dCF, dAdo, and Dipyridamole on Ribonucleotide Reductase Activity in L1210 Cells. Cells grown for 3 hr in the presence of dCF (1.1 μM) alone showed about a 20% inhibition of CDP reduction. The ribonucleotide reductase specific activity was inhibited about 60% with dAdo (40 μM) alone. The cells grown with dAdo (40 μM) in combination with dCF (1.1 μM) did not appear to be inhibited to any further extent. The inhibition of ribonucleotide reductase specific activity increased to 80%, using the triple combination of dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10 μM) (Table 1). Interestingly, cells treated with dipyridamole alone showed a 65% reduction in enzyme activity. This inhibition may possibly be artifactual, since cell-free extracts may have contained dipyridamole eluted from membrane fragments.

HPLC Analysis of Intracellular Acid-soluble Metabolites Using [3H]dAdo. The HPLC profiles of [3H]dAdo metabolites in acid-soluble extracts from L1210 cells that were exposed to dAdo only, a combination of dCF and dAdo, and the triple combination of dCF, dAdo, and dipyridamole are shown in Chart 7. The HPLC pattern shown in Chart 7A was obtained using extracts from cells that were treated with dAdo alone. The dATP peak was too small to be quantitated (less than 17 μM), but it was detectable compared to the control peak. The HPLC pattern in Chart 7B was obtained with cells incubated with dAdo (40 μM) in combination with dCF (1.1 μM). Incubation with this combination resulted in a small increase in dATP pools and showed a dADP shoulder which could not be quantitated. The HPLC pattern in Chart 7C was obtained with cells incubated with the triple combination [dCF (1.1 μM)-dAdo (40 μM)-dipyridamole (10 μM)]. This analysis revealed a large increase in dATP and dADP pools. Despite the marked increase in dATP and dADP pools, the ATP pool was almost constant.

DISCUSSION

The triple combination of dAdo, dCF, and dipyridamole was shown here to give a 40-fold increase in intracellular L1210 dATP levels, and the elevated dATP levels appeared to be correlated with L1210 cytotoxicity. Clinical treatment with dCF alone has been shown to give elevated dAdo levels in serum and urine and increased intracellular dATP levels and, in essence, yields a chemical model of the severe combined immunodeficiency disease syndrome due to deficiency of ADA activity (10, 12, 25). Dipyridamole is known to be an inhibitor of nucleoside transport (22) and blocks the influx of adenosine and deoxycytidine as well as the efflux of uridine and cytidine (16, 17). Dipyridamole (10 μM) has also been demonstrated to inhibit the transport of dAdo into L1210 cells without added dCF by about 90% in short-term kinetic studies carried out for 18 sec at 20°C (15). Our results here are not at variance, since long-term exposure of L1210 cells to dAdo, dCF, and dipyridamole (1 to 10 μM) would allow for a considerable uptake of dAdo even at 90% inhibition. The large increase in dATP levels in the presence of dipyridamole is consistent with a greater inhibition of efflux than influx, although other mechanisms are possible (7, 13). In this connection, when radiolabeled dAdo was used with dCF and dipyridamole, there was a large peak that eluted in the nucleoside region (solvent front) under conditions of HPLC separation of nucleotides from L1210 cell extracts. This compound has been identified as dAdo. Recently, a soluble deoxynucleosidase that preferentially dephosphorylates deoxynucleotides (5) was identified in mammalian cells. It might be interesting to study the effects of dipyridamole on this enzyme if it is found to be a membranal protein.

When dAdo (40 μM) and dCF (1.1 μM) were each used alone or in combination, the L1210 intracellular dATP levels ranged from 11 to 34 μM, and ribonucleotide reductase inhibitions ranged from 20 to 60%. The upper level of inhibition is comparable to the inhibitory effects of 20 to 30 μM dATP on a partially purified ribonucleotide reductase from Ehrlich ascites cells (24). At any rate, these are not potent inhibitions of ribonucleotide reductase and lend support for other models of dAdo toxicity, such as inhibition of S-adenosylmethionine-mediated transmethylation reactions (7, 13). It is possible, however, that both mechanisms are operative. The triple combination of dAdo, dCF, and dipyridamole when used at 40, 1.1, and 10 μM concentrations, respectively, gave a dATP level in L1210 cells of 400 μM, and ribonucleotide reductase activity was inhibited 80%. While this degree of enzyme inhibition may be significant, complete inhibition might have been expected, since 400 μM dATP is approximately 40 times the concentration to give 50% inhibition in some purified systems (20).

REFERENCES


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