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Factors influencing the inhibition of repair of irradiation-induced DNA damage by 2′-deoxycoformycin and deoxyadenosine*

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Summary. Permeabilized L5178Y cells were used to investigate the mechanism underlying inhibition of the repair of irradiation-induced DNA strand breaks by 2′-deoxycoformycin combined with deoxyadenosine. Permeabilized cells repaired DNA strand breaks as effectively as did intact cells, and at deoxyadenosine concentrations that produced similar levels of deoxyadenosine triphosphate (dATP), repair of DNA strand breaks was inhibited by 2′-deoxycoformycin plus deoxyadenosine to a comparable extent in both types of cells. Accompanying the increase in intracellular dATP produced by 2′-deoxycoformycin combined with deoxyadenosine was a fall in levels of deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP). The addition of dTTP, dGTP, and dCTP reversed the inhibition of DNA repair by 2′-deoxycoformycin plus deoxyadenosine, although the level of dATP was not affected. Reducing the phosphorylation of deoxy-adenosine to dATP by the addition of adenosine prevented the decrease in levels of dTTP, dGTP, and dCTP and the inhibition of DNA repair by 2′-deoxycoformycin and deoxyadenosine. In contrast, increasing the intracellular levels of dATP by the addition of 2′-deoxycoformycin together with dATP, deoxyadenosine diphosphate (dADP), or deoxyadenosine monophosphate (dAMP) had no effect on the levels of the other deoxynucleotide triphosphates and did not inhibit DNA repair. Moreover, DNA repair was not inhibited by the breakdown products of deoxyadenosine, adenine, or deoxyribose. These results suggest that inhibition of the repair of irradiation-induced DNA strand breaks by 2′-deoxycoformycin combined with deoxyadenosine requires the phosphorylation of deoxyadenosine and involves alterations in the levels of deoxynucleotide triphosphates.

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Introduction

The adenosine deaminase (ADA) inhibitor 2′-deoxycoformycin (dCF) is an effective antitumor agent against a variety of lymphoid malignancies [16]. The most impressive responses have been obtained in hairy-cell leukemia, with the majority of patients achieving complete remission [12, 21], and in B-cell chronic lymphocytic leukemia [9]. The lymphocytolysis following dCF therapy has been attributed to the accumulation of deoxyadenosine (dAdo) and adenosine (Ado) as a result of ADA inhibition. It has been suggested that cell death may be due to the conversion of dAdo to deoxyadenosine triphosphate (dATP) [8, 11, 13], which may lead to the allosteric inhibition of ribonucleotide reductase [22], the depletion of ATP [1, 20] or the development of DNA strand breaks (SB) [5, 18]. The development of DNA SB in lymphoid cells following incubation with dCF combined with dAdo (dCF/dAdo) in vitro may be due to inhibition of the repair of naturally occurring SB [5, 6, 18]. We have demonstrated a synergistic antitumor effect in X-irradiated L5178Y murine lymphoblasts treated with dCF/dAdo in vitro that was associated with the cellular accumulation of dATP and the inhibition of repair of the irradiation-induced DNA SB [3].

Inhibition of DNA repair by dCF/dAdo may be related to the increase in dATP, as the accumulation of DNA SB did not occur when lymphocytes were co-incubated with deoxycytidine, which prevented the phosphorylation of dAdo to dATP [6, 19], or in mutant CCRF-CEM cells that were deficient in deoxycytidine kinase and adenosine kinase [14]. Additionally, Cohen and Thompson [6] have suggested that the inhibition of repair is related to an imbalance in deoxynucleotide triphosphate (dNTP) pools, as inhibition could be prevented in intact cells by the addition of thymidine, deoxyguanosine, and deoxycytidine.

In the present study, we used a permeabilized cell system to examine the effects of the decomposition and phosphorylation products of dAdo on DNA repair and related the changes observed in dNTP pools following treatment with dCF/dAdo to the inhibition of DNA repair.

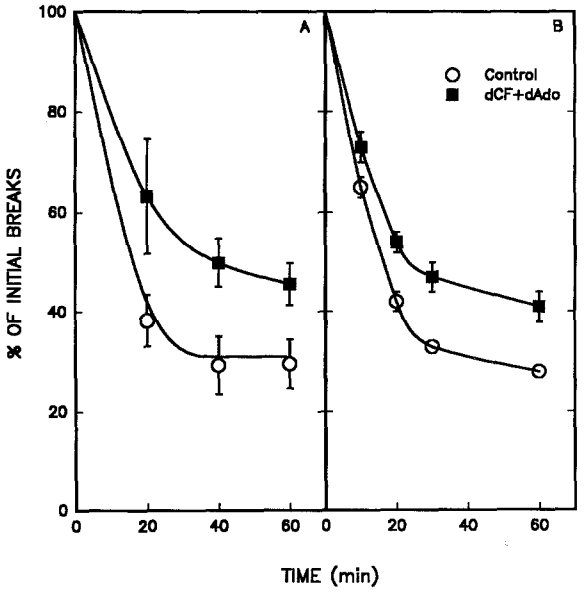

Fig. 1 A, B. Inhibition of repair of irradiation-induced DNA SB by dCF/dAdo. **A** Intact or **B** permeabilized plateau-phase L5178Y cells were incubated at 37° C for 1 h in the absence (O) or presence (■) of dCF and dAdo, then irradiated with 600 rad X-rays and further incubated for the times shown to allow for DNA repair. DNA SB were determined by alkaline elution assay and are presented as a percentage of the level of SB in cells immediately after irradiation. Each point represents the mean value ± SE for 5–44 determinations. On occasion, the confidence intervals were too small to be shown

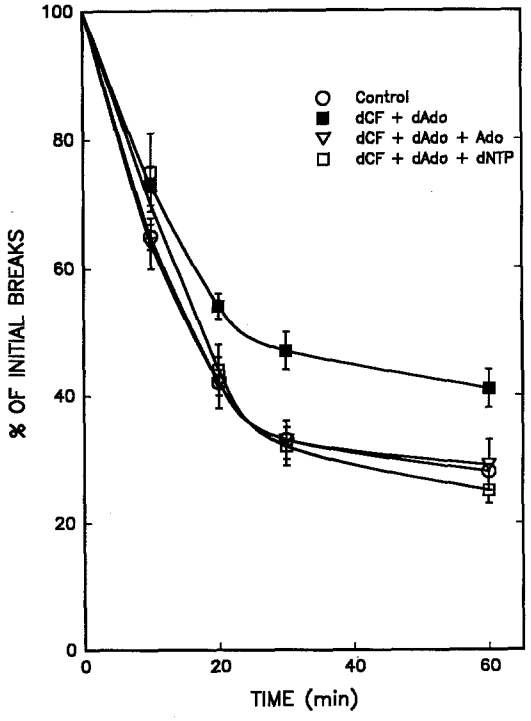

Fig. 2. Effect of Ado and dNTP on inhibition of repair of DNA SB by dCF/dAdo. Permeabilized plateau-phase L5178Y cells were incubated at 37° C for 1 h in the absence of treatment (0) or in the presence of dCF and dAdo (■), dCF, dAdo, and Ado (▽), or dCF, dAdo, dTTP, dGTP, and dCTP (□), then irradiated with 600 rad X-rays and further incubated for the times shown to allow for DNA repair. DNA SB were determined by alkaline elution assay and are presented as a percentage of the level of SB in cells immediately after irradiation. Each point represents the mean value ± SE for 3–44 determinations. On occasion, the confidence intervals were too small to be shown

Materials and methods

Materials. Fischer's medium was obtained from Gibco Laboratories (Grand Island, N.Y.). Horse serum, Poly[d(AT)], Poly[d(IC)], and dCF were purchased from Sigma Chemical Co. (St. Louis, Mo.). [^{14}C] Thymidine, [^{3}H]-dATP, and [^{3}H]-deoxythymidine triphosphate ([^{3}H]-dTTP) were obtained from New England Nuclear (Missassauga, Ontario), and [^{3}H]-deoxycytidine triphosphate [^{3}H]-dCTP), and [^{3}H]-deoxyguanosine triphosphate ([^{3}H]-dGTP) were purchased from ICN Biomedicals (Costa Mesa, Calif.). DNA polymerase I was purchased from Boehringer-Mannheim (Dorval, Quebec), and polycarbonate filters were obtained from Nucleopore Corp. (Pleasanton, Calif.).

Cells. The L5178Y lymphoma cells used in this study arose as a spontaneous neoplasm in a DBA/2 mouse. Plateau-phase cells were obtained by the inoculation of 1×10^5 cells/ml in Fischer's media containing 12% horse serum and subsequent incubation at 37° C in 95% air/5% CO_2 for 72 h, at which time the cell concentration had remained unchanged for at least 24 h at 8×10^5 cells/ml. Cells were permeabilized using a modification of the method described by Dresler et al. [7]. Plateau-phase cells were washed once with ice-cold 2.7 mM NaH_2PO_4, 13.1 mM Na_2HPO_4, 135 mM NaCl, and 4.9 mM KCl; pelleted and resuspended at a concentration of 1×10^7 cells/ml in a buffer containing 10 mM TRIS (pH 7.6, at 37° C), 4 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, and 3 mM dithiothreitol (DTT); and kept at 4° C for 30 min. The cell suspension was mixed with equal volumes of a buffer containing 70 mM TRIS (pH 7.6, at 37° C), 12 mM $MgCl_2$, 30 mM KCl, and 10 mM ATP, for a final concentration of 40 mM TRIS, 8 mM $MgCl_2$, 15 mM KCl, and 5 mM ATP. Cell permeability was confirmed by demonstrating that [^{3}H]-dATP was incorporated into DNA in permeabilized cells but not in intact cells [4].

Determination of DNA SB. Intact or permeabilized cells were incubated at 37° C for 1 h in the presence or absence of dCF and/or other components and were irradiated with 600 rad X-rays. At various intervals thereafter, SB were determined using the alkaline elution assay as previously described [2]. In these studies, [^{3}H]-thymidine-labeled log-phase L5178Y cells were used as internal controls. The levels of DNA SB were expressed as rad equivalents (rad Equiv.) as determined from a calibration curve obtained by irradiating plateau-phase L5178Y cells with various doses of radiation.

Treatment of nonirradiated cells with dCF/dAdo for 1 h did not induce significant levels of DNA SB. However, to eliminate the possibility that dCF/dAdo-induced DNA SB might result in an underestimation of the amount of DNA repair, elution profiles of nonirradiated cells treated with dCF/dAdo alone were used as controls for calculations of the level of DNA SB at different time points in irradiated cells treated with dCF/dAdo.

Determinations of dNTP levels. Permeabilized L5178Y cells were incubated in the presence or absence of dCF and/or other components at 37° C for 1 h in buffer and the dNTP levels were measured. Nucleotides were extracted using a two-step extraction. First, cells were treated with 60% methanol at a concentration of 1×10^7 cells/ml and were incubated at 30° C for 10 min and then at –20° C overnight. After the cells were pelleted, the supernatant was freeze-dried, extracted with 50 µl 0.4 *N* perchloric acid per 1×10^7 cells, and neutralized with 2 vol. 0.5 M tri-*N*-octylamine in trifluorotrichloroethane. Levels of dNTP were measured using the DNA polymerase assay of Hunting and Henderson [10].

Results

Inhibition of Repair of DNA SB by dCF/dAdo in intact and permeabilized L5178Y cells

Irradiation of intact or permeabilized L5178Y cells resulted in the formation of 466 ± 18 and 364 ± 81 rad Equiv.

Table 1. Intracellular concentrations of dNTPs in permeabilized L5178Y cells

Treatment	Intracellular concentration (pmol/10^6 cells)			
	dATP	dTTP	dGTP	dCTP
None (control)	3.0±0.3	2.3±0.3	0.45±0.05	0.40±0.07
dCF+dAdo	21.7±2.4*	1.5±0.3*	0.27±0.03*	0.19±0.03*
dCF+dAdo+Ado	6.7±1.0*	2.6±0.3	0.34±0.04	0.34±0.05
dCF+dATP	21.2±3.5*	ND	0.51±0.11	0.35±0.10
dCF+dADP	34.0±4.7*	ND	0.55±0.09	0.36±0.10
dCF+dAMP	30.6±3.7*	ND	0.44±0.08	0.37±0.16

Permeabilized L5178Y cells were incubated at 37°C for 1 h with the components shown. Intracellular concentrations of dNTPs were determined as described in Materials and methods. The data represent mean values ± SE for 6–23 determinations and were evaluated statistically using two tailed *t*-tests comparing the significance of the differences between the mean dNTP concentrations measured in control vs treated cells. ND, Not determined

* Significantly different from control values ($P<0.05$) according to a two tailed *t*-test comparing the significance of the difference between the means.

DNA SB, respectively. Approximately 70% of the SB were repaired in both the whole and the permeabilized cells within 60 min (Fig. 1). Treatment of the cells with dCF/dAdo did not alter the level of DNA SB induced by irradiation but did result in a decrease in the rate and extent of DNA repair in both intact and permeabilized cells, with repair reaching a plateau at 55% and 59% repair of SB, respectively. Intact cells were treated with 5 μM dCF and 50 μM dAdo, whereas permeabilized cells were treated with 5 μM dCF and 200 μM dAdo. These treatments produced intracellular dATP levels of 14.7±2.7 and 21.7±2.4 pmol/10^6 cells in intact and permeabilized cells, respectively.

Effect of decomposition products of dAdo on repair of DNA SB in permeabilized L5178Y cells

Repair of irradiation-induced DNA SB in permeabilized L5178Y cells was compared in untreated cells and cells treated with 5 μM dCF and 200 μM dAdo, 200 μM adenine, 200 μM deoxyribose, or 200 μM adenine and 200 μM deoxyribose. The rate and extent of repair of DNA SB observed in treated cells was the same as that in untreated cells. Treatment of cells with the same concentrations of adenine or deoxyribose in the absence of dCF also failed to affect the repair of SB.

Effect of Ado on inhibition of repair of DNA SB by dCF/dAdo in permeabilized L5178Y cells

Repair of DNA SB induced by irradiation was measured in untreated permeabilized L5178Y cells, in cells treated with 5 μM dCF and 200 μM dAdo, and in cells treated with 5 μM dCF, 200 μM dAdo, and 200 μM Ado (Fig. 2). Although dCF/dAdo significantly inhibited DNA repair, the addition of Ado to inhibit dATP formation reversed this inhibitory effect. dCF and Ado had no effect on the repair of DNA SB.

Effect of phosphorylation products of dAdo on repair of DNA SB in permeabilized L5178Y cells

Repair of irradiation-induced DNA SB in permeabilized L5178Y cells was compared in untreated cells and cells treated with 5 μM dCF/200 μM dAdo, 5 μM dCF/5 μM dATP, 5 μM dCF/20 μM deoxyadenosine diphosphate (dADP), or 5 μM dCF/50 μM deoxyadenosine monophosphate (dAMP). In each case, the concentration of phosphorylated dAdo used was that required to achieve approximately the same intracellular level of dATP that was found in cells treated with 5 μM dCF/200 μM dAdo as determined by measurement of dATP levels. Whereas treatment of cells with dCF/dAdo produced inhibition of DNA SB repair, the repair of SB in cells treated with dCF/dATP, dCF/dADP, or dCF/dAMP was the same as that observed in untreated cells. Incubation of cells with the same concentrations of dATP, dADP, or dAMP in the absence of dCF did not inhibit SB repair, nor did treatment of cells with 100 or 500 μM dATP.

Effect of dNTPs on inhibition of repair of DNA SB by dCF/dAdo in permeabilized L5178Y cells

Repair of irradiation-induced DNA SB in permeabilized L5178Y cells was measured in untreated cells, in cells treated with 5 μM dCF and 200 μM dAdo, and in cells treated with 5 μM dCF, 200 μM dAdo, 5 μM dGTP, 30 μM dCTP, and 20 μM dTTP (Fig. 2). The addition of dNTPs resulted in reversal of the inhibition of DNA SB repair by dCF/dAdo.

Intracellular dNTP concentrations in permeabilized L5178Y cells

The intracellular concentrations of dNTPs in permeabilized L5178Y cells treated with dCF and dAdo, Ado, dATP, dADP, or dAMP are shown in Table 1. The concentrations of nucleosides and nucleotides were the same as those used in the studies of inhibition of DNA SB repair. Treatment with dCF/dAdo produced a 7-fold increase in

the intracellular concentration of dATP, whereas the levels of dGTP, dCTP, and dTTP showed an approximately 2-fold decrease. Following treatment of the permeabilized cells with Ado in addition to dCF/dAdo, the phosphorylation of dAdo to dATP was markedly reduced and the levels of dGTP, dCTP, and dTTP were similar to those observed in untreated cells. Although incubation of permeabilized cells with dCF and dATP, dADP, or dAMP produced intracellular levels of dATP similar to those found in cells treated with dCF/dAdo, the levels of dGTP and dCTP did not fall. We could not measure the levels of dTTP in the experiments in which dATP, dADP, or dAMP was added, as these dNTPs interfered with the assay for dTTP. The addition of dGTP, dCTP, and dTTP did not affect the formation of dATP from dAdo in cells treated with dCF/dAdo.

Discussion

We have previously demonstrated that a synergistic antitumor effect can be obtained in plateau-phase L5178Y lymphoblasts by combining irradiation with dCF/dAdo and that this effect was associated with the inhibition of repair of DNA SB [3]. Earlier studies have suggested that the accumulation of dATP [3, 6, 13, 14, 19] and alterations in dNTP pools [6] may be responsible for the inhibition of DNA repair produced by dCF/dAdo. In the present study, we used permeabilized plateau-phase L5178Y cells to examine directly the effect of changes in intracellular dNTP levels on DNA repair.

Permeabilized cells could repair irradiation-induced DNA SB as effectively as intact cells, and this activity was inhibited by dCF/dAdo. Higher concentrations of dAdo were required to achieve dATP levels in permeabilized cells that were similar to those obtained in intact cells, probably because of the increased ability of phosphorylated dAdo metabolites to efflux from the former. As Zunica et al. [23] had observed that D-ribose could inhibit DNA repair synthesis in irradiated human lymphocytes, we examined the effect of the breakdown products of dAdo, deoxyribose and adenine, on DNA repair. However, we observed no inhibition of DNA repair when either the sugar or the base or a combination of the two was added to irradiated cells.

Measurement of the intracellular dNTP concentrations following treatment with dCF/dAdo showed that the level of dATP increased 7-fold whereas the dTTP, and dCTP levels were significantly reduced. The addition of Ado along with dCF/dAdo prevented both the accumulation of dATP and the decrease in levels of the other dNTPs (Table 1) and reversed the inhibition of DNA repair (Fig. 2). As Ado can compete with dAdo for kinase activity, these findings suggest that the phosphorylation of dAdo to dATP is necessary for inhibition of DNA repair. These results are similar to those obtained by Seto et al. [19] and Cohen and Thompson [6] using deoxycytidine, which in their cell systems also prevented the phosphorylation of dAdo to dATP. However, when we added the phosphorylation products of dAdo (dAMP, dADP, or dATP) along with dCF to permeabilized cells to produce an intracellular level of dATP similar to that obtained following treatment with dCF/dAdo, we observed no effect on the levels of dGTP or dCTP or on DNA repair. Matsumoto et al. [14] have reported that very high concentrations of dATP are required to inhibit semiconservative DNA synthesis in isolated nuclei; however, in the present study, the addition of up to 500 μM dATP failed to inhibit DNA repair. These findings suggest that the initial phosphorylation of dAdo may be required both to produce changes in dGTP and dCTP levels and for the inhibition of DNA repair. The ability of added dNTPs to reverse the inhibition of DNA repair produced by dCF/dAdo (Fig. 2) provides further evidence that perturbation of dNTP levels is involved in the mechanism underlying inhibition of DNA repair.

The present study indicates that phosphorylation of dAdo and altered dNTP levels may play a role in the inhibition of DNA repair by dCF/dAdo; however, the mechanisms involved are not clear. The effects on the dNTP pools may have been mediated through the allosteric inhibition of ribonucleotide reductase by dATP formed from dAdo. Although the finding that exogenously added dATP did not affect dNTP levels argues against this mechanism, it cannot be ruled out with certainty, since the lack of an effect under these circumstances might be explained by (a) physical compartmentalization, in that dATP formed from dAdo may have a different cellular distribution than exogenously added dATP, or (b) physiological compartmentalization, in that only dATP formed from dAdo can inhibit ribonucleotide reductase and DNA repair [15, 17]. We could not test this hypothesis directly because the level of ribonucleotide reductase activity in the plateau-phase cells was too low to be measured. Alternatively, the initial phosphorylation of dAdo to dAMP may produce altered dNTP levels via unknown mechanisms. In summary, this study demonstrates that inhibition of the repair of irradiation-induced DNA SB in plateau-phase L5178Y lymphoblasts by dCF/dAdo requires the phosphorylation of dAdo and involves alterations in the levels of dNTPs. Further studies should be carried out to determine whether the inhibition of DNA repair is related to a depletion or an imbalance in dNTP pools.

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