Biochemical Pharmacology, Vol. 44, No. 11, pp. 2229–2233, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

INHIBITION OF REPAIR OF BLEOMYCIN-INDUCED DNA STRAND BREAKS BY 2′-DEOXYCOFORMYCIN AND ITS EFFECT ON ANTITUMOR ACTIVITY IN L5178Y LYMPHOBLASTS

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(*Received* 13 *May* 1992; *accepted* 6 *August* 1992)

Abstract—We have observed previously that treatment of plateau-phase L5178Y murine lymphoblasts *in vitro* with 2′-deoxycoformycin plus deoxyadenosine (dCF/dAdo) can inhibit the repair of X-irradiation-induced DNA single-strand breaks (SSB) in these cells and that this effect is associated with synergistic cell kill. In this study we examined the effect of a combination treatment of plateau-phase L5178Y cells with bleomycin (BLM) plus dCF/dAdo. Incubation of BLM-treated cells with dCF/dAdo resulted in significant inhibition of the repair of BLM-induced DNA SSB. However, an additive, but not a synergistic, increase in cell kill was observed when cells were treated with a combination of BLM plus dCF/dAdo.

Bleomycin (BLM)¶ consists of a mixture of glycopeptide antitumor antibiotics that has been used effectively for the treatment of testicular carcinoma, squamous cell carcinoma and lymphomas [1, 2]. The biological activity of this agent has been associated with its ability to interact with DNA and its ability to bind metal ions [3–5]. Antitumor activity has been linked most often to the formation of DNA single- and double-strand breaks [3, 4, 6, 7] which may result from the production of highly reactive hydroxyl radicals [8, 9] or from the action of an oxygen–iron complex of BLM [10–12]. This DNA damage resembles that observed with ionizing radiation. However, other drug targets including RNA [13] and chromatin [14] have also been suggested.

The adenosine deaminase inhibitor, 2′-deoxycoformycin (dCF), is an antitumor agent that is effective against a variety of lymphoid malignancies [15]. The most impressive responses have been in hairy-cell leukemia, where the majority of patients achieve complete remissions [16–18], and in B-cell chronic lymphocytic leukemia [19]. Although the mechanism of the antitumor action of dCF is unknown, the lymphocytolysis following dCF therapy has been attributed to the accumulation of deoxyadenosine (dAdo) and adenosine as a result of adenosine deaminase inhibition. Incubation of human peripheral mononuclear cells [20, 21] or chronic lymphocytic leukemia cells [22] with dCF plus dAdo (dCF/dAdo) *in vitro* resulted in the accumulation of DNA single-strand breaks (SSB), and it was suggested that this effect was due to the inhibition of repair of spontaneously formed SSB by dCF/dAdo.

We recently demonstrated a synergistic increase in cell kill in X-ray irradiated plateau-phase L5178Y lymphoblasts treated with dCF/dAdo *in vitro*. This effect was associated with the cellular accumulation of dATP and inhibition of repair of the irradiation-induced SSB [23]. Additional studies using permeabilized cells indicated that the inhibition of DNA repair by dCF/dAdo requires the phosphorylation of dAdo and involves alteration of the levels of deoxynucleotide triphosphates (dNTP) [24].

Since BLM induces SSB that appear similar to those produced by ionizing radiation, in the current study we examined the effect of combining this agent with dCF. We investigated the effect of dCF/dAdo treatment on the repair of BLM-induced SSB and on the toxicity of this agent in plateau-phase L5178Y lymphoblasts *in vitro*.

MATERIALS AND METHODS

BLM was supplied by Bristol Laboratories of Canada, Montreal, Canada, and dCF was obtained from the National Cancer Institute, Bethesda, MD. Fischer's medium and horse serum were obtained from Gibco Laboratories, Grand Island, NY. Tetrapropylammonium hydroxide was from the Sigma Chemical Co., St. Louis, MO, and polycarbonate filters were obtained from the Nucleopore Corp., Pleasanton, CA.

Cells. The L5178Y lymphoma used in this study

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¶ Abbreviations: BLM, bleomycin; dCF, 2′-deoxycoformycin; dAdo, deoxyadenosine; dCF/dAdo, 2′-deoxycoformycin plus deoxyadenosine; SSB, single-strand breaks; and dNTP, deoxynucleotide triphosphates.

arose as a spontaneous neoplasm in a DBA/2 mouse [25]. Initial cell inocula were 1×10^5 cells/mL in Fischer's medium containing 12% horse serum, and experiments were carried out following incubation of cells at 37° under 95% air:5% CO_2 for 72 hr at which time the cell concentration had remained unchanged for at least 24 hr at 8×10^5 cells/mL.

Determination of DNA SSB. For DNA SSB studies, plateau-phase L5178Y cells were labeled for 48 hr with [^{14}C]thymidine, as previously described [26, 27]. Cells (1×10^6/mL) were incubated in Fischer's medium containing 12% horse serum at 37° with 200 μM BLM. After 2 hr of incubation the drug was removed and cells were incubated with or without 5 μM dCF plus 50 μM dAdo. At various time points SSB were determined using the alkaline elution assay, as previously described [26, 27]. DNA SSB were expressed as rad equivalents as determined from calibration curves of elution versus X-ray dose. The level of SSB induced by treatment of cells at 37° for 2 hr with 5 μM dCF plus 50 μM dAdo alone was also determined. In one experiment, dCF/dAdo were removed from the incubation medium 4 hr after removal of BLM and the level of SSB in the cells at various times thereafter was determined as above.

Cytotoxicity assays. Plateau-phase L5178Y cells were incubated in Fischer's medium containing 12% horse serum at 37° for 2 hr with various concentrations of BLM. Following removal of drug, cells were incubated at 37° for 6 hr in the presence or absence of 5 μM dCF plus 50 μM dAdo. dCF/dAdo were removed by washing with Fischer's medium and the surviving cell fraction was determined using a soft agar clonogenic assay, as previously described [28, 29]. The expected surviving cell fraction was calculated from the product of the surviving cell fractions of cells treated with BLM alone and dCF/dAdo alone [30].

Effect of time of incubation with dCF/dAdo on cytotoxic activity. Plateau-phase L5178Y cells were incubated in Fischer's medium containing 12% horse serum at 37° for 2 hr with 50 μM BLM alone, with 5 μM dCF plus 50 μM dAdo, or with 50 μM BLM plus 5 μM dCF plus 50 μM Ado. The surviving cell fraction of cells treated with BLM alone was determined using a soft agar clonogenic assay, as described previously [28, 29]. Cells treated with dCF/dAdo alone or with BLM and dCF/dAdo were washed to remove drug and incubated with 5 μM dCF and 50 μM dAdo at 37° for various times. The surviving cell fraction was determined by soft agar clonogenic assay. The expected surviving cell fraction was calculated from the product of the surviving cell fractions of cells treated with dCF/dAdo alone and the surviving cell fraction of cells treated with BLM alone [30].

RESULTS

Effect of dCF/dAdo on the repair of BLM-induced DNA SSB in plateau-phase L5178Y cells. Plateau-phase L5178Y cells were incubated with 200 μM BLM at 37° for 2 hr. Drug was removed and cells were incubated at 37° for an additional 12 hr with or without dCF/dAdo. The levels of DNA SSB in the cells were determined at various times using the alkaline elution assay. BLM treatment induced 156 ± 16 (mean ± SEM) rad equivalents of SSB and in the absence of dCF/dAdo this DNA damage was >90% repaired in 12 hr (Fig. 1). In contrast, BLM-treated cells incubated with dCF/dAdo repaired the DNA SSB more slowly with only 55% of the damage being repaired after 12 hr. When dCF/dAdo were removed from the incubation medium 4 hr after the removal of BLM, the rate of DNA repair increased and nearly 70% of the SSB were repaired after 12 hr. Incubation of cells with dCF/dAdo without prior BLM treatment did not produce significant levels of DNA SSB.

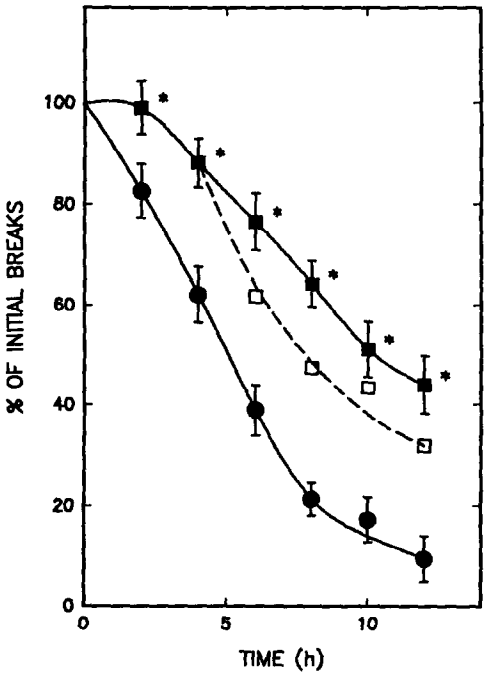

Fig. 1. Effect of dCF/dAdo on the repair of BLM-induced DNA SSB. Plateau-phase L5178Y cells were incubated at 37° with 200 μM BLM for 2 hr, drug was removed and the cells were incubated with (■) or without (●) 5 μM dCF plus 50 μM dAdo. In one experiment (□) dCF/dAdo were removed from the incubation medium 4 hr after the removal of BLM. DNA SSB were measured by alkaline elution assay at the times shown and are expressed as a percent of DNA SSB present immediately following BLM incubation. Treatment of cells with 200 μM BLM for 2 hr induced 156 ± 16 (mean ± SEM) rad equivalents of SSB. The data at each time point represent the mean of 5–14 determinations; bars = SEM. The data were evaluated by a two-tailed *t*-test comparing the significance of the difference of the means of the level of DNA SSB for cells incubated with or without dCF/dAdo at each time point. An asterisk (*) indicates that the difference was statistically significant ($P < 0.05$).

Effect of dCF/dAdo on BLM-induced cytotoxicity in plateau-phase L5178Y cells. Plateau-phase L5178Y cells were treated with various doses of BLM at 37° for 2 hr. Following removal of the drug, cells were incubated at 37° with or without dCF/dAdo for 6 hr.

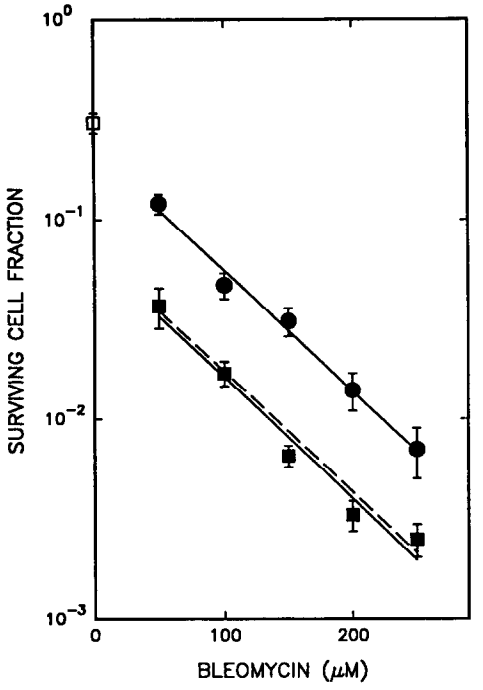

Fig. 2. Effect of dCF/dAdo on the cytotoxic activity of BLM. Plateau-phase L5178Y cells were incubated at 37° for 2 hr with various concentrations of BLM, drug was removed and the cells were incubated with (■) or without (●) 5 μM dCF plus 50 μM dAdo for 6 hr. The surviving cell fraction was determined by a soft agar clonogenic assay, and the expected surviving cell fraction (broken line) for the combination treatment was calculated as described in the text. The surviving cell fraction for cells incubated for 2 hr without BLM and then with 5 μM dCF plus 50 μM for 6 hr is also shown (□). Points, mean of 4–11 quadruplicate determinations; bars, SEM; curves, linear regression of concentration–survival plots.

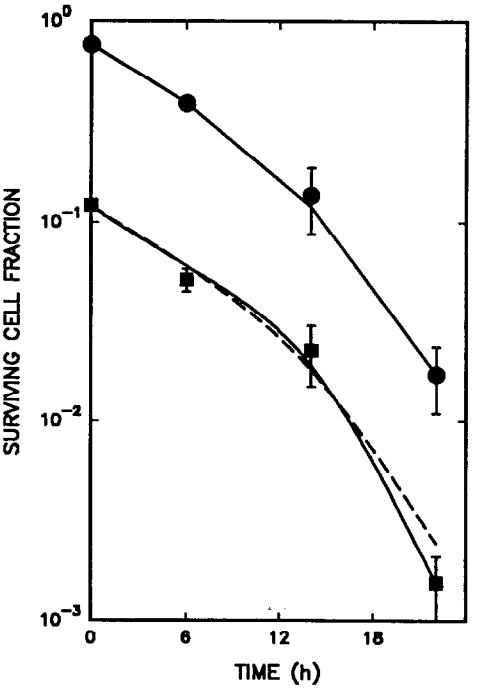

Fig. 3. Effect of time of incubation with dCF/dAdo on cytotoxic activity. Plateau-phase L5178Y cells were incubated at 37° for 2 hr with 50 μM BLM alone, with 5 μM dCF plus 50 μM dAdo (●), or with 50 μM BLM plus 5 μM dCF plus 50 μM dAdo (■). Cells treated with dCF/dAdo alone or with BLM and dCF/dAdo were washed to remove drug and then were incubated with 5 μM dCF and 50 μM dAdo for the times shown. The surviving cell fraction at each time was determined by a soft agar clonogenic assay, and the expected surviving cell fraction (broken line) for the combination treatment was calculated as described in the text. Points, mean of 6–12 determinations; bars, SEM.

Incubation of cells with dCF/dAdo without prior BLM treatment resulted in a surviving cell fraction of 0.34 ± 0.04. Figure 2 shows the concentration–survival curves for BLM-treated cells with or without dCF/dAdo incubation as well as the concentration–survival curve expected for these cells if the cytotoxic effects of BLM and dCF/dAdo were additive. Incubation of BLM-treated cells with dCF/dAdo did produce increased cell kill compared to BLM alone; however, the effect of the combination therapy was additive and not synergistic.

Effect of time of incubation with dCF/dAdo on the antitumor activity of combination therapy. Plateau-phase L5178Y cells were incubated at 37° for 2 hr with 5 μM dCF and 50 μM dAdo in the absence or presence of 50 μM BLM. Following removal of the BLM, cells were incubated at 37° with dCF/dAdo for various times up to 22 hr. Incubation of cells at 37° for 2 hr with BLM without subsequent dCF/dAdo treatment resulted in a surviving cell fraction of 0.14 ± 0.01. Figure 3 shows the survival curves for cells treated with dCF/dAdo with or without BLM as well as the survival curve expected for these cells if the cytotoxic effects of BLM and dCF/dAdo were additive. The cell kill produced by incubation of cells with dCF/dAdo alone increased with increasing treatment time. In addition, treatment of cells with BLM and dCF/dAdo increased cell kill compared to dCF/dAdo alone; however, the effect of the combination therapy was additive and not synergistic.

DISCUSSION

dCF is an effective agent in the treatment of hairy-cell leukemia [16–18] and shows useful activity in other lymphoid malignancies [15, 19]. It is an inhibitor of the enzyme, adenosine deaminase, and treatment with dCF results in a build-up of adenosine and dAdo, which initially arise from normal turnover of marrow cells. In lymphoid tissues dAdo can be phosphorylated to dATP and this may lead to the cytotoxic effects of dCF [16, 31, 32]. Thus, for *in vitro* studies it is necessary to add both dCF and dAdo to simulate the *in vivo* effect. BLM has been used in the treatment of testicular carcinoma, squamous cell carcinoma and lymphomas [1, 2]. Its

antitumor activity has been attributed to the formation of DNA strand breaks resulting from the generation of oxygen free radicals [3, 4, 6, 7].

We have demonstrated previously that dCF/dAdo can produce synergistic cell kill in plateau-phase L5178Y murine lymphoma cells and cells from patients with chronic lymphocytic leukemia when combined with X-irradiation *in vitro* [23]. X-radiation produces its cytotoxic effects by the formation of oxygen free radicals and the induction of DNA strand breaks, and the enhanced toxicity observed when X-rays were combined with dCF/dAdo appeared to be due to the inhibition of repair of DNA SSB by dCF/dAdo [23]. Further studies indicated that the inhibition of repair of irradiation-induced DNA strand breaks by dCF/dAdo requires the phosphorylation of dAdo and involves alterations in the levels of dNTP [24]. Ayusawa *et al.* [33] have previously observed increased sensitivity to BLM in mutant mouse mammary tumor FM3A cell lines having altered dNTP levels and particularly increased dATP levels. In this study we have investigated the effect of combining dCF/dAdo, which also alters dNTP levels [24], with BLM on the formation and repair of DNA SSB and cytotoxicity in plateau-phase L5178Y lymphoblasts. Plateau-phase cells were used in this study as these cells represent a model of slow growing cells found in chronic lymphoid malignancies and because we have previously observed a greater effect of combination therapy in these cells than in log-phase cells.

Plateau-phase L5178Y cells required approximately 12 hr to repair >90% of BLM-induced DNA SSB, while similar repair of irradiation-induced SSB requires only 6 hr [23]. As was observed previously with irradiation-induced SSB, dCF/dAdo signficantly inhibited both the rate and the extent of repair of the DNA damage induced by BLM. However, dCF/dAdo only inhibited approximately 50% of the repair. When dCF/dAdo were removed from the cell incubations 4 hr after the removal of BLM, there appeared to be an increase in DNA repair. We observed a similar result previously for repair of irradiation-induced DNA damage.

Treatment of plateau-phase L5178Y cells with BLM followed by dCF/dAdo resulted in increased cytotoxicity compared to treatment with BLM alone. However, the combination therapy did not produce a synergistic increase in toxicity since the levels of cell kill observed were nearly identical to those predicted for additive cell kill. As our studies of inhibition of DNA repair indicated that there was increased repair following removal of dCF/dAdo, we were concerned that a similar removal of dCF/dAdo after 6 hr in the cytotoxicity studies might have allowed the cells to fully repair the BLM-induced SSB and thus might have accounted for the lack of synergistic activity. As a result, we examined the effect of increasing the time that the cells were exposed to dCF/dAdo. Although the level of cell kill increased with longer dCF/dAdo incubation, this effect could be accounted for by increased cytotoxicity produced by dCF/dAdo alone. Thus, longer treatment with dCF/dAdo did not produce a synergistic increase in cell kill with BLM.

Previous studies have shown that inhibitors of poly(ADP-ribose) synthesis can potentiate cell killing by BLM in Ehrlich ascites tumor cells *in vitro* and *in vivo* [34, 35] and in Chinese hamster ovary cells *in vitro* [36]. Poly(ADP-ribose) has been associated with DNA repair [21] and the inhibitors of poly(ADP-ribose) synthesis appear to inhibit repair of BLM-induced DNA damage [36]. However, it was not clear in these studies whether the inhibitors produced synergistic cell kill. In contrast, the thymidine analog, 5-bromo-2'-deoxyuridine, produces a synergistic increase in cell kill in a human squamous cell carcinoma cell line *in vitro* when combined with BLM [37]. While the mechanism of this effect is unclear, it was not due to a change in the rate of repair of BLM-induced DNA SSB and may result from a direct interaction of BLM with analog-substituted DNA.

The findings of the current study provide further evidence that dCF/dAdo can inhibit repair of DNA damage. However, as was observed with irradiation-induced damage [23] not all the repair could be inhibited by these agents. The lack of complete inhibition of repair may be due to the use of insufficient dCF/dAdo to fully inhibit the repair mechanism or may result from the involvement of multiple repair processes not all of which are inhibitable by dCF/dAdo. Unlike our previous study with irradiation-induced DNA damage, the inhibition of repair of the BLM-induced SSB did not result in a synergistic increase in cell kill. This may be due to the level of inhibition of repair being too small to significantly alter cytotoxicity. Alternatively, it is possible that formation of DNA SSB may not be the most important cytotoxic mechanism for BLM. Recent studies have suggested other critical targets for BLM [13, 14].

In summary, we have demonstrated that incubation of plateau-phase L5178Y lymphoma cells with dCF/dAdo following treatment with BLM resulted in inhibition of repair of BLM-induced DNA SSB. Combination therapy with these agents *in vitro* produced additive, but not synergistic, cell kill in the same cells. Because of the different toxicities exhibited by these agents *in vivo*, it may be possible to use these antitumor agents in a combined regimen for the treatment of lymphoid malignancies.

Acknowledgements—The authors thank Linda Verburg for technical assistance. This work was supported by a grant from The National Cancer Institute of Canada.

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