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Cytotoxicity, DNA cross-linking, and DNA single-strand breaks induced by cyclophosphamide in a rat leukemia in vivo*

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Summary. A study of cyclophosphamide (CP)-induced DNA damage and repair occurring in vivo was conducted in the brown Norway rat myelocytic leukemia (BNML) model. DNA single-strand breaks (SSB), DNA-DNA interstrand cross-links (DIC), DNA-protein cross-links (DPC), and DNA double-strand breaks (DSB) were measured by alkaline and neutral elution. After i.p. injection of 50 mg/kg CP, DIC were detectable at 1 h and peaked at 8 h. DPC were detectable at 2 h and peaked at 6 h. Both DIC and DPC persisted at a relatively high level until 28 h. Dose-response curves for both DIC and DPC were determined at 4 h after CP injection over the dose range of 25–150 mg/kg. These doses ranged from the minimally effective dose to doses curative for rats bearing this leukemia (1- to 9-log kill of leukemia cells). No SSB or DSB was observed at 4 h after CP injection over the dose range of 15–250 mg/kg, but a low level of SSB was observed at 18–28 h after CP treatment. These data suggest that the cytotoxic effect of CP in vivo is mediated mostly by DIC and DPC. SSB appearing late after CP injection in vivo may be a reflection of repair of DIC and DPC and an indication of the optimal timing for administration of DNA-repair inhibitors. This observation is of interest since our earlier work demonstrated that hydroxyurea can potentiate the therapeutic benefit of CP in this model when it is given over the 4-day period immediately after CP treatment.

Introduction

Cyclophosphamide (CP) is a commonly used antineoplastic agent that shows activity against a variety of neoplastic diseases [17]. CP is inactive in vitro but is activated in vivo to 4-hydroxycyclophosphamide, which spontaneously breaks down into the reactive metabolites phosphoramide mustard and acrolein, both of which cause DNA damage [5]. Phosphoramide mustard is an active bifunctional alkylating agent that has been shown to bind to the N^7 position of guanine [2, 4, 15, 16, 18, 30, 42, 48], to bind to the phosphate backbone of DNA [24, 25, 27], and, possibly, to interact with the O^6 position of guanine [29]. The cytotoxic action of CP is thought to result mainly from phosphoramide mustard-induced DNA cross-linking [5]. Acrolein, on the other hand, is reported to bind to proteins [28], to form DNA adducts [26, 41], to create abasic sites [40], and to induce DNA single-strand breaks (SSB) [6, 8]. A contribution of acrolein-induced DNA and protein damage as a mechanism of some of the toxic effects of CP has been suggested [26, 28, 40, 41].

The alkaline-elution techniques originally described by Erickson and Kohn have been used to detect DNA strand breaks and cross-links in vitro [21]. Several investigators have used modifications of these techniques to try to measure DNA damage occurring in vivo [31–34]. Specifically for the study of DNA damage done by CP in vivo, fluorometric assay/alkaline-elution methods and DNA-unwinding techniques have been used [1, 7, 9, 10, 35, 36, 39]. A variety of tissues from several species, including bone marrow, leukemia, lymphoma, brain, testis, and ovary, have been examined in these in vivo studies in the mouse, rat, and human. The patterns and kinetic characterization of CP-induced DNA damage in these studies are somewhat different.

The brown Norway rat myelocytic leukemia (BNML) model, originally developed by van Bekkum and colleagues in the BN rat [11, 13, 14, 43] and later successfully introduced into the LBN rat [46], is an authentic animal model for human acute myeloid leukemia [11, 13, 14, 43,

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44, 46]. This model has successfully been used to study the chemotherapeutic efficacy of CP [37, 45], the synergy of CP with other drugs [45, 47] or ionizing radiation [12], bone marrow purging by 4-hydroperoxycyclophosphamide for bone marrow transplantation [38], and CP resistance [19, 20].

The purpose of the present study was to analyze CP-induced DNA damage in vivo in this rat leukemia model using the most sensitive and precise technique, the radiolabeled alkaline- and neutral-elution methods.

Materials and methods

Rat leukemia model. Female LBN rats (age, 7–8 weeks; weight, 150–170 g) were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). Animals were kept in single hanging cages and were given standard laboratory chow and tap water ad libitum. The BNML cell line was maintained by serial passage in LBN rats and in multiple aliquots of early passages frozen at –90° C. Every eight passages, a new aliquot was thawed to reduce the risk of any spontaneous change in growth pattern or drug sensitivity. Single-cell suspensions of BNML were made from leukemic bone marrow by gentle mincing in RPMI 1640 supplemented with 15% bovine calf serum and serial aspiration through needles decreasing in size to 25 gauge. Each LBN rat was inoculated with 1×10^6 viable BNML cells by tail-vein injection.

Experimental design. CP was obtained from Elkins-Sinn, Inc. (Cherry Hill, N. J.). It was dissolved in sterile water immediately before use and injected i.p. at doses calculated in milligrams per kilogram of body weight. Chemotherapy experiments for advanced disease (analogous to the usual clinical setting) were conducted when the bone marrow of sample rats contained approximately 80% leukemia cells and the spleen was twice its normal weight (around day 14 after i.v. inoculation of rats with 1×10^6 BNML cells). The increased life span (ILS) of drug-treated rats over that of controls was determined. Drug-induced DNA damage in BNML cells as measured by alkaline and neutral elution was conducted when the bone marrow of sample rats contained approximately 100% leukemia cells (days 17–21 after BNML-cell inoculation). In all experiments, control rats survived for 21–24 days after inoculation. Alkaline elution was performed in each experiment on leukemia cells from at least three rats for each experimental point. The experiments themselves were repeated at least once and, sometimes, as many as three times, and overlapping experimental points were included in different experiments. All data obtained for any given set of experimental conditions (dose, time, end point) were combined and expressed as mean values (± SD).

Preparation of BNML cells for alkaline and neutral elution. For in vivo labeling of the DNA of BNML cells, BN rats were injected i.p. with 1.0–1.3 μCi/g [^{3}H]-thymidine (New England Nuclear, Boston, Mass.; sp. act., 50–80 Ci/mmol) q3h for 24 h before drug treatment. All cell-suspension preparation procedures were conducted at ice-bath temperature. Rats were killed by cervical dislocation and kept in the ice bath. The tibias and femurs were removed immediately. BNML cells were obtained by flushing the cells from the tibias and femurs with an ice-cold solution of phosphate-buffered saline (PBS) and ethylenediaminetetraacetic acid (EDTA; PBS solution containing 5 mM EDTA, pH 7.1). A single-cell suspension was prepared by passing the bone marrow through 25-gauge needles. The cell suspension was then passed through a double-layered 250-μm nylon mesh (Tetko. Inc., Briarcliff Manor, N. Y.) and washed twice with ice-cold media, and the cells were counted.

Alkaline-elution assay for SSB, DNA interstrand cross-links, and DNA-protein cross-links. Alkaline elution was performed as described by Kohn and colleagues [21], with minor modifications. L1210 mouse leukemia cells were used as internal standards in all alkaline-elution assays. These were labeled by the overnight addition of 0.005 μCi [^{14}C]-thymidine/ml (New England Nuclear, Boston, Mass.; sp. act., 40–60 mCi/mmol) to cells growing exponentially in RPMI 1640 medium supplemented with 15% heat-inactivated bovine calf serum. Drug-treated BNML cells and L1210 internal-standard cells were washed, suspended in ice-cold PBS-EDTA solution, and irradiated at 0° C with doses of 371 R for the DNA interstrand cross-link (DIC) assay and 2000 R for the DNA-protein cross-link (DPC) assay using a ^{137}Cs irradiator (Gammacell 1000; Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1237 R/min.

Next, 1×10^6 cells (60% tritium-labeled BNML cells and 40% ^{14}C-labeled L1210 cells) were deposited on a 25-mm diameter, 0.8-μm pore polycarbonate filter (Nucleopore, Pleasanton, Calif.) for SSB and DIC assay or on a 2.0-μm polyvinylchloride filter (Omega Specialty Instrument Co., Chelmsford, Mass.) for DPC assay. In SSB and DIC assays, cells were washed with 20 ml ice-cold PBS, lysed with 5 ml of a sodium dodecyl sulfate (SDS)-EDTA lysis solution (0.1% SDS, 0.1 M glycine, and 0.015 M disodium EDTA, pH 10.0), deproteinized with 2 ml 0.5 mg proteinase K/ml (Merck, Darmstadt, FRG) dissolved in lysis solution for 1 h, and eluted with 50 ml of a Pr_4NOH-EDTA elution solution (a solution of tetrapropylammonium hydroxide containing 0.1% SDS and 0.02 M EDTA, acid form, pH 12.1; RSA Corporation, Ardsley, N. Y.). In the DPC assay, cells were washed with ice-cold PBS, lysed with 5 ml of a Sarkosyl-NaCl-EDTA solution (0.2% sodium lauroyl sarcosinate, 2 M NaCl, and 0.04 M EDTA, pH 10.0), washed with 3 ml 0.02 M EDTA (pH 10.0), and eluted with 50 ml of the Pr_4NOH-EDTA elution solution (pH 12.1). All elutions were carried out at a rate of 0.035 ml/min, and five fractions were collected at 3-h intervals.

The [^{14}C]-DNA and [^{3}H]-DNA radioactivity remaining on the filter and in the eluted fractions was determined according to standard methods [21] using Ready Gel liquid scintillation cocktail (Beckman Instrument Inc.) and a Parkard Tri-Carb 4530 liquid scintillating counter. The retention of label on the filter as a function of the time of elution was calculated.

Neutral-elution assay for double-strand breaks. Neutral elution was performed according to the standard method [3] except that internal-standard L1210 cells were not used. The in vivo and in vitro cell-preparations methods were the same as those described for the alkaline-elution assay except that the radiation dose was 8000 R. Cells (6×10^5) were deposited onto a 25-mm diameter, 2.0-μm pore polycarbonate filter (Nucleopore) washed with ice-cold PBS, lysed and deproteinized with 3 ml of a TRIS-EDTA-SDS lysis solution containing 0.5 mg proteinase K/ml (0.05 M TRIS, 0.05 M glycine, 0.025 M EDTA, and 2% SDS, pH 9.6), and eluted with 50 ml of a TRIS-EDTA-SDS solution containing no proteinase K (pH 9.6) at a rate of 0.035 ml/min. Five fractions were collected at 3-h intervals. The [^{3}H]-DNA radioactivity remaining on the filter and in the eluted fractions was determined by liquid scintillation counting as described above, and the retention of label on the filter as a function of the time of elution was calculated.

Data analysis. DNA SSB factor (SSB-F), DSB factor (DSB-F), DIC factor (DIC-F), and DPC factor (DPC-F) were calculated according to the methods described by Meyn and Jenkins [31], Murray et al. [32–34], Li and Kaminskas [22, 23], and Ataya et al. [1], with some modifications.

SSB-F was calculated using the formula:

$$\text{SSB-F} = \frac{\log (fo/fd)}{\log (fo/fx\text{-}371)},$$

where *fo* indicates the proportion of nonirradiated/non-drug-treated tritium-labeled BNML-cell DNA retained on the filter when 10% of the internal-standard L1210-cell ^{14}C-labeled DNA is retained on the filter and *fd* and *fx-371*, respectively, represent the proportion of drug-treated/nonirradiated BNML-cell DNA and of 371-R-irradiated BNML-cell DNA retained on the filter at the same internal-standard cell-DNA retention.

DIC-F was calculated using the formula:

$$\text{DIC-F} = \frac{\log (fd/fx\text{-}371)}{\log (fo/fx\text{-}371)},$$

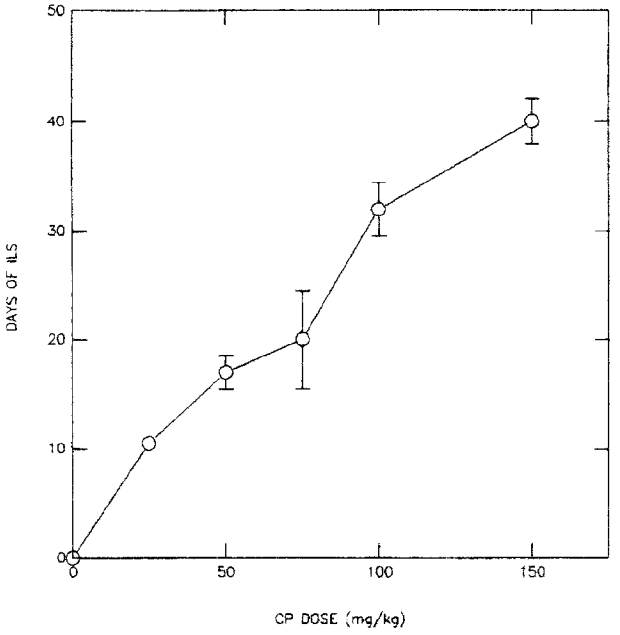

Fig. 1. Effect of CP on the survival of LBN rats bearing BNML

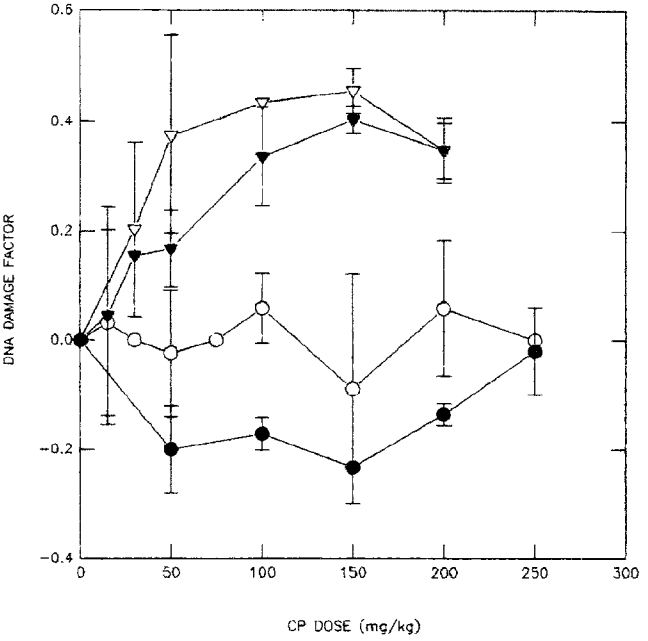

Fig. 2. Dose-response relationship of CP-induced SSB, DSB, DIC, and DPC formation in BNML cells in vivo at 4 h after i.p. CP injection. Data points represent mean values ± SD. ▽, SSB-F; ▼, DSB-F; ▽, DIC-F; ▼, DPC-F

where *fo, fd,* and *fx-371* are defined as described above except that the drug-treated BNML cells also received 371 R irradiation.

DPC-F was calculated using the formula:

$$\text{DPC-F} = \frac{\log(\text{fd/fx-2000})}{\log(\text{fo/fx-2000})},$$

where *fo, fd,* and *fx-2000* are defined as described for DIC-F except that the dose of irradiation is 2000 R.

DSB-F was calculated by the formula:

$$\text{DSB-F} = \frac{\log(\text{fo/fd})}{\log(\text{fo/fx-8000})},$$

where *fo, fd,* and *fx-8000* are defined as described for DIC-F and DPC-F except that the dose of irradiation is 8000 R and internal-standard cells are not used, such that the proportions are calculated at an arbitrary time point of 10.5 h into elution.

Therefore, a value of 0 or less for SSB-F or DSB-F indicates a lack of SSB or DSB formation. A value of 0 for DIC-F or DPC-F indicates the absence of DIC or DPC formation whereas a value of less than 0 for DIC-F or DPC-F indicates that more SSB formation is produced by drug and ionizing irradiation combined than by ioning irradiation alone (radiosensitizing effect). SSB-F, DSB-F, DIC-F, and DPC-F values exceeding 0 indicate relative amounts of DNA damage. An SSB-F value of 1 is indicative of DNA damage equivalent to that produced by a radiation dose of 371 R.

Results

The maximum tolerated dose (MTD) and dose-response curve of CP have been well established in this animal model [12, 47]. The MTD of CP is 200 mg/kg by i.p. administration. A linear dose-response curve for increased life span (ILS) was also observed over the dose range of 25–150 mg/kg CP following i.p. injection in this multiply repeated experiment (Fig. 1).

In the first experiment, SSB, DIC, DPC, and DSB were determined at 4 h after the administration of a single dose of CP over a span of doses ranging from a minimal effective dose of 15 mg/kg to curative doses ($\geq$200 mg/kg). No SSB was detected at 4 h over the range of CP doses given (Fig. 2). DIC and DPC were seen, showing a positive slope relationship between strand-break frequency and increasing dose up to 50–150 mg/kg CP, at which point the numbers did not further increase with dose, presumably a reflection of the limits of detection of the assay or experimental conditions. No DSB was detected at 4 h after any dose of CP; in fact, DNA of cells from CP-treated animals eluted more slowly than did that of cells from control rats (Fig. 2).

Subsequent experiments examined the time course of SSB, DIC, and DPC occurrence and resolution following CP administration. At doses of 100 mg/kg and higher, the cell yield was insufficient after 4 h. The results obtained at 15 mg/kg were erratic and hard to reproduce, but those obtained at 50 mg/kg were more consistent and were studied at time points ranging up to 28 h (Fig. 3). Both DIC and DPC peaked at 6–8 h after the administration of 50 mg/kg CP but persisted at significant levels for more than 24 h. SSB were not detected through 14 h but were detectable at 18, 20, and 22 h after the injection of 50 mg/kg CP.

Discussion

The results presented herein demonstrate that the alkaline-elution technique developed by Kohn et al. [21] and the neutral-elution technique developed by Bradley and Kohn [3] can be used to investigate chemotherapy-induced DNA damage and repair in vivo. Due to the differences between in vitro and in vivo exposure, some modifications were made from the original methods. The DNA of BNML cells

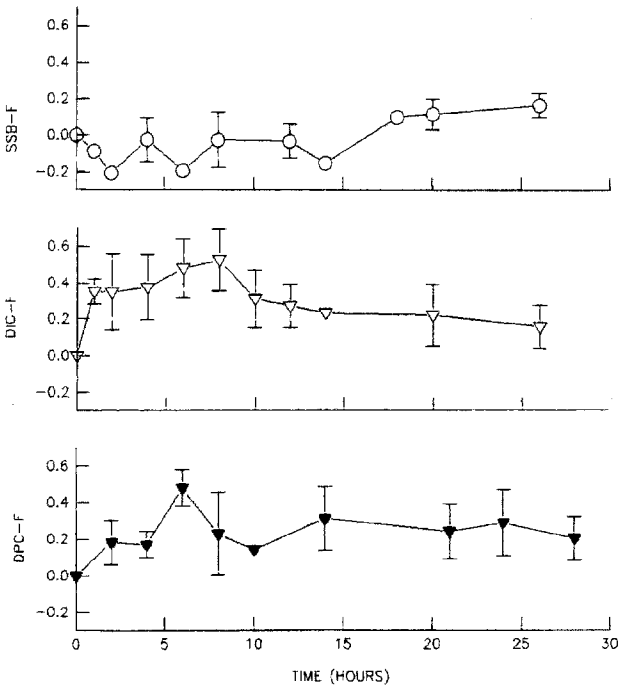

Fig. 3. Time-response relationship of DNA damage and repair induced by 50 mg/kg CP in BNML in vivo. Data points represent mean values ± SD. ▽, SSB-F; ▽, DIC-F; ▽, DPC-F

was labeled with [^{3}H]-thymidine and the internal-standard L1210 cells were labeled with [^{14}C]-thymidine in our experiments. This revision enabled us to use the less expensive and lower-energy [^{3}H]-thymidine for in vivo labeling of the large number of experimental animals required. The cell-cycle time (Tc) for BNML cells in vivo at 18 days after i. v. injection is about 14 h [13]. To chase the radioactivity into high-molecular-weight DNA so as to avoid the influence of CP on [^{3}H]-thymidine incorporation into the DNA of BNML cells and to obtain adequate DNA labeling, we labeled the DNA of BNML cells with [^{3}H]-thymidine using a q3h injection schedule for 24 h before drug treatment of the rats.

Despite this schedule, the in vivo labeling efficiency of DNA was relatively low as compared with its in vitro labeling efficiency. Thus, the concentration of [^{14}C]-thymidine for labeling of the DNA of internal-standard L1210 cells was decreased to 0.005 μCi/ml, and $6-7 \times 10^5$ BNML cells but only 4×10^5 internal-standard L1210 cells were employed in each of the in vivo alkaline-elution procedures. Under these conditions, the ratio of [^{3}H]-thymidine to [^{14}C]-thymidine radioactivity was kept at around 1 : 3, thus minimizing cross-counting between the two isotopes.

One of the problems encountered in alkaline and neutral elution involves the analysis of the data from several individual experiments repeated over time. This variation may be especially large when the DNA damage being measured has occurred in vivo. The variation results from a multitude of factors [3, 21, 49] such as the quality of the filter, the pH of the elution solution, the elution speed, the room temperature, the cell-labeling time, the relative purity of the population of leukemia cells separated from the bone marrow, and any individual variation in the pharmacokinetic parameters of rats. Some investigators have used DNA strand-scission factor [31–34] or DNA cross-link factor [1] to calculate radiation- or chemotherapy-induced DNA damage. We modified this method by using two controls in each individual experiment, one negative control and one positive control. For SSB-F and DSB-F, fo was the negative control and fx-371 or fx-8000 was the positive control. For DIC-F and DPC-F, fo was the positive control and fx-371 or fx-2000 was the negative control. The value for the negative control was set at 0, and that for the positive control was set at 100%. The relative value (%) for drug-induced DNA damage was calculated on the basis of the increased percentage of DNA damage over the negative control. By this method, the variation in results obtained in different individual experiments was greatly decreased.

The patterns of DNA cross-link formation observed in the present study after the exposure of BNML cells in the LBN rat to alkylating agent were found to be in general agreement with the findings of other investigators [1, 7, 9, 10, 31–36, 39]. However, significant variation has been observed in the time to maximal DIC and DPC formation when different tissues and species have been studied. The reasons for these variations are not known but do not bear any obvious relationship to known pharmacokinetic differences between species or cell-cycle-parameter differences between cell types.

The dose-response effects of CP-induced DIC and DPC in vivo have been studied in L1210 cells and normal bone marrow [7]. The present studies in BNML cells in LBN rats further confirm a positive slope to the dose-response curve for CP-induced DIC and DPC in vivo over a substantial range of doses. The reasons for the apparent decrease in the response curve for CP-induced DIC and DPC at very high doses is not clear. Crook et al. [6] reported a similar phenomenon in K562 human leukemia cells treated in vitro with hepatocyte-activated CP. In those studied, DIC formation peaked at 50 μM but decreased at concentrations exceeding 50 μM. The authors suggested that this pattern resulted from high levels of drug-induced SSB formation. In our studies in BNML cells, the levels of DIC and DPC peaked at 150 mg/kg but appeared to decrease at 200 mg/kg in the absence of detectable SSB formation. One possible explanation for this observation would be a higher rate of cell loss during drug treatment and cell preparation for the more heavily damaged cells treated at higher doses. Such an effect might manifest as high levels of secondary SSB occurring during autolysis of cells in vitro but not in vivo, as the elimination of lethally injured cells would be expected to be more rapid in vivo. The apparent demonstration of reduced DSB elution of CP-treated cells versus untreated cells may represent an artifact resulting from the stabilization of any DSB that may have occurred during elution by DIC and DPC in the drug-treated cells.

The repair of DIC and DPC in this model can also be estimated from these experiments by the kinetics of DNA cross-link removal. DIC and DPC were removed relatively slowly and persisted at a relatively high level (about 1/3 of the peak level) for 28 h after i.p. CP injection. Thus, the

repair system for CP-induced DNA cross-linking in BNML cells is not very effective. This may explain in part why this leukemia model is particularly sensitive to CP.

CP-induced SSB formation has been reported to occur in vivo [7, 9, 10, 35, 36, 39], and the data we obtained from BNML cells in LBN rats confirm these findings. As for DIC and DPC, however, the kinetics of CP-induced SSB formation and repair in vivo may be different in various models. In BNML cells in LBN rats treated i.p. with 50 mg/kg CP, a low level of SSB was detectable at as late as 18 h and persisted until at least 28 h. No substantial SSB or DSB formation could be detected at 4 h after i.p. injection of CP over the entire dose range of 15–250 mg/kg.

The reasons for this late SSB formation are not known, and hypothesis generation is difficult since the nature and mechanisms of CP-induced SSB formation in vivo are unclear. Acrolein, an active metabolite of CP in vivo, has been reported to induce SSB both in vivo in cultured mouse L1210 cells at 0–6 h following 1 h exposure to 20 μM acrolein [8] and in cultured K562 human chronic lymphatic leukemia immediately following 1 h exposure to 5–20 μM acrolein [6]. Although details on the kinetics of acrolein-induced SSB formation and repair have to our knowledge not been reported, the formation of acrolein-induced SSB seems to occur soon after drug exposure and would not explain SSB formation in BNML cells at 18–28 h after CP injection. Because the predominant repair of DIC and DPC occurred at between 18 and 28 h, our data favor the hypothesis by Ford and Warnick [9] and Franssen et al. [10] that excision repair of DNA cross-links might produce secondary SSB. If this were true, CP-induced SSB formation could be classified into SSB that occur soon after drug treatment, presumably induced by acrolein (early-appearing or primary SSB formation), and those that occur relatively late after drug treatment, consistent with the timing of removal of DNA cross-links (late-appearing or secondary SSB formation). The hypothesis that late-appearing SSB are secondary to repair of DNA cross-links is further supported by our observations on hydroxyurea's potentiation of the therapeutic response to CP in this model. On a dose schedule that over 4 days has no effect on survival, single-agent hydroxyurea increases the life span of CP-treated rats when it is given during the 4-day period immediately after CP injection but does not affect animal survival when it is given during the 4-day period immediately prior to CP treatment [47].

In conclusion, in the present investigation we applied the sensitive and precise radiolabeled alkaline-elution [21] and neutral-elution [3] techniques to the study of CP-induced DNA damage and repair in BNML cells in LBN rats in vivo. The prominent DNA damage induced by CP in BNML cells in vivo included DIC and DPC formation, with dose- and time-response relationships and evidence of delayed repair suggesting that DNA cross-link formation seems to play a major role in the cytotoxic effects of CP on this model in vivo. The variations observed in the kinetics of formation and clearance of various forms of DNA damage between this model and other models do not seem likely to be due to technical differences and suggest that in clinical trials of drug combinations designed to achieve synergy, investigators will need to consider that the sequence and timing of drug administration may be important parameters. The mechanism and significance of CP-induced SSB formation warrant further study.

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