In Vivo DNA Cross-Linking by Cyclophosphamide: Comparison of Human Chronic Lymphatic Leukemia Cells with Mouse L1210 Leukemia and Normal Bone Marrow Cells

Wilfried DeNeve, Frederick Valeriote, Mark Edelstein, Carleen Everett, and Michael Bischoff

Wayne State University School of Medicine, Department of Internal Medicine, Division of Hematology and Oncology, Detroit, Michigan 48201 [W. D., F. V., C. E., M. B.]; and the Veterans Administration Medical Center, Allen Park, Michigan 48101 [M. E.]

ABSTRACT

Alkaline elution was done on a variety of cells following cyclophosphamide (CY) treatment in vivo. Cells used were L1210 leukemia, normal mouse bone marrow, and peripheral blood cells obtained from a patient with chronic lymphatic leukemia (CLL). Endpoints used were determination of single strand breaks, DNA-DNA interstrand and DNA-protein cross-links.

After treatment of mice with CY (4 mg/mouse), low levels of single strand breaks were observed in both L1210 leukemia and CDF, normal bone marrow. When a patient with CLL was treated with CY (750 mg/m²), no evidence of single strand breaks could be demonstrated.

Maximum levels of DNA-DNA interstrand cross-links were observed in mice 2 h after injection of CY for the L1210 leukemia cells [175 ± 25 rad-equivalents (req)] and at 4 h for the CY-CAF, normal bone marrow cells (47 ± 9 req). In human CLL, maximum levels were observed 12 h after injection of CY. Peak levels of DNA-DNA interstrand cross-links were approximately 4-fold lower in CY-CAF, normal bone marrow cells than in L1210 leukemia. The frequencies measured in human CLL cells were relatively low at any time point (mean at 12 h = 36 req).

Maximum levels of DNA-protein cross-links were observed 4 h after injection of CY (4 mg/mouse) for both L1210 leukemia [123 req (mean)] and normal bone marrow cells [50 req (mean)]. DNA-protein cross-links were measurable in CLL at timepoints later than 4 h after the start of injection of CY. In order to obtain equitoxicity between L1210 leukemia and CDF, normal bone marrow cells, about 18-fold higher doses of CY had to be given than in the case of the normal bone marrow cells. In contrast, only 4-fold higher doses had to be given to the normal bone marrow to obtain equivalent peak levels of DNA-DNA interstrand cross-links.

INTRODUCTION

CY\(^4\) is a standard anticancer agent which shows activity against a wide variety of neoplasms. CY itself is without cytotoxicity and the cytotoxic activity is due to one or more of its metabolites. As for other bifunctional alkylating agents of the bis-chloroethyl-amino (nitrogen mustard) type, CY is thought to affect its cytotoxic action primarily by cross-linking DNA, although ring cleavage and depurination may also be important (1). The cytotoxic action is thought to result from reactions by phosphoramide mustard or, more precisely, the aziridinium ion thereof. This metabolite has been shown to be the major cross-linking agent (2). Acrolein, another metabolite of cyclophosphamide, is capable of inducing SSB which has been reported after treatment with cyclophosphamide (2-4). Although in vivo models to investigate cross-linking exist, the data are difficult to extrapolate to the human situation because of substantial differences in pharmacokinetics between human and animal models. Data concerning the kinetics of cross-linking by cyclophosphamide in humans are nonexistent.

It was the aim of this study to investigate the kinetics of cross-linking in human CLL after i.v. injection of CY and to compare these data with the kinetics of cross-linking in mice. In the CDF, mouse, both L1210 leukemia and normal bone marrow cells were investigated after i.v. bolus injection of CY.

MATERIALS AND METHODS

Cyclophosphamide. CY (Cytoxan, Mead Johnson) was obtained from PRN Services (Royal Oak, MI). It was dissolved in 0.9 M NaCl immediately before use and administered i.v. via the lateral tail vein in a volume of 0.5 ml. Drug dose is expressed as mg/mouse and can be readily converted to mg/kg since the average mouse weight was 23 g. Thus 1 mg/mouse CY is equivalent to 43.5 mg/kg.

Mice. Twelve- to 24-week-old, 22-24 g CDF, mice were obtained from Frederick, MD. Females as well as males were used. In the assay on leukemia cells, mice received 10⁶ L1210 leukemia cells i.v. on Day 0 and were treated on Day 4. Animals were killed by cervical dislocation at different time intervals after treatment (as specified in "Results"). The spleen (in leukaemic mice) or bone marrow (in nonleukemic mice) was prepared for alkaline elution.

Preparation of Spleens for Alkaline Elution. The spleens were removed, cut into small fragments and passed through a 120-mesh sieve (0.0055 wire, metal SS309; Phoenix Wire Cloth, Inc., Troy, MI) while rinsing with an erythrocyte lysing buffer containing 8.29 g ammonium chloride, 1.0 g potassium bicarbonate, 37.1 mg EDTA (disodium salt), and distilled water added up to a total volume of 1000 ml. The suspension was then centrifuged (110 x g) for 20 min at 4° C. After removing the supernatant, the cells were resuspended in 20 ml of PSA-EDTA by gentle pipetting. PSA-EDTA consists of sodium chloride, 0.8% (w/v); potassium chloride, 0.09% (w/v); sodium bicarbonate, 0.035% (w/v) and EDTA disodium salt, 0.186% (w/v), titrated to a pH of 7.4 with 0.1 M NaOH.

Preparation of Bone Marrow Cells for Alkaline Elution. Bone marrow cell suspensions were obtained by flushing the cells from the femurs with PSA-EDTA (3 ml) with a 23-gauge needle. The average yield was 1.5 x 10⁷ cells per femur.

Preparation of Human Peripheral White Blood Cells for Alkaline Elution. As part of his regular treatment, a patient with advanced CLL (Stage III) was treated by i.v. bolus of CY, 750 mg/m², on an outpatient basis or hospitalized depending on his desire. Blood samples were drawn at different times after treatment as specified in "Results." Permission was obtained from the patient for the use of his blood and the purpose of the study was fully explained to him.

Patient blood samples were collected in 10-ml silicone lubricated, stoppered tubes (containing 143 units of USP heparin) and were immediately put in ice water. Within 1 h, the procedure to isolate the lymphocytes was started. The technique was a Ficoll-hypaque centrifugation, described by Sigma (Sigma Diagnostics, St. Louis, MO) as procedure No. 1077 with the following modification: after the second...
centrifugation at 250 × g for 20 min, the supernatant was discarded and the lymphocyte pellet was resuspended in PSA-EpTA solution. Cells were counted and the appropriate dilutions were made so that 5 × 10^6 were loaded on each barrel.

Alkaline Elution. The procedure described by Kohn (5) was used, with a fluorometric determination of the DNA (6). Except where noted, 5 × 10^6 cells were placed onto a 47-mm diameter, 0.8-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA). The cells were lysed with 10 ml of a pH 10.0 solution (2 M NaCl, 0.04 M tetrasodium EDTA, 0.2% Sarkosyl) either with or without PK at a concentration of 0.5 mg/ml. The filters were washed with 10 ml of 0.02 M tetrasodium EDTA, pH 10.3. The DNA was eluted with 0.1 M tetrapropylammonium hydroxide containing 0.2 M EDTA (free acid), pH 12.13 at a constant flow rate of 0.04 ml/min. Fractions were collected every 90 min for 15 h. The entire procedure was conducted with minimal vibration, at room temperature, and under light shields.

Assays for both SSB and DIC were performed under deproteinization conditions; i.e., PK was added to the lysis solution and the lysate on the filter was held 30 min at room temperature.

For both DIC and DPC assays, the cells received 500 rads at 0°C from a Gamma cell 40, 137Cs irradiator (AECL) prior to alkaline elution.

Following elution, filters were removed with forceps, minced into small pieces, and placed into scintillation vials containing 5 ml of washing solution. The DNA retained on the filter fragments was removed by heating to 60°C for 30 min and vortexing for 5 min. DNA remaining in the filter holder or barrel was recovered by flushing with 5 ml EDTA wash solution.

For each experiment, a blank barrel with filter but no cells to correct for background fluorescence and two untreated control samples (one received 500 rads) were run. In the case of patient samples, the untreated control was prepared from a blood sample drawn immediately before treatment of the patient.

DNA Assay. The relative DNA concentration in each fraction was determined using the fluorescent Hoechst dye 33258 (7). A 1-ml aliquot was withdrawn from each fraction, including the filter and wash solutions, and transferred to a 13 × 100-mm borosilicate glass culture tube. Each sample was neutralized with 0.4 ml of 0.2 M KH2PO4 and the volume adjusted to 2.0 ml with 0.6 ml of distilled water. Finally, 1.0 ml of Hoechst dye (1.5 × 10^-6 M in standard saline citrate) was added with a fluorometric determination of the DNA (6). Except where noted, a logarithmic interpolation, where f¡ is the amount of DNA in the ith fraction and n is the number collected was computed by calculating the total DNA per filter before elution (Ao) using the equation:

\[ R_+ = 1 - \frac{\sum f_i}{N_0} \]  

where \( f_i \) is the amount of DNA in the ith fraction and n is the number of fractions. The values of R at 21 ml eluted were used to calculate cross-links. In order to obtain these values, a logarithmic interpolation, between the appropriate samples collected, was performed.

DIC were computed according to:

\[ \left(1 - R_+(r)/1 - R_+(rd)\right)/2 - 1\right) \times \text{(dose in rads)} \]  

(B)

DPC were computed according to:

\[ \left[\left(1 - R_+(r)\right)/\left(1 - R_+(rd)\right)\right] \ln \left( R_+(r)/R_+\right) \]  

(C)

A derivation of this formula is given in Ref. 9. \( R_+(r) \) and \( R_+(rd) \) are the fractions retained in the irradiated and lysed samples, respectively, with or without PK. \( R_-(rd) \) and \( R_-(rd) \) are the fractions retained in the irradiated and lysed samples after drug treatment, respectively, with or without PK. \( R_0 \) is the fraction retained in the control sample lysed with PK. No correction for drug-induced SSB [as described by Ewig and Kohn (10)] was applied. Both types of cross-links are expressed as req.

Statistics. Statistics were according to Ferguson (11). The t test was used. All tests were two-tailed. \( P < 0.05 \) was considered significant.

RESULTS

When mice were treated with 4 mg CY per mouse for 1, 2, 4, and 8 h, low levels of SSB could be observed both in normal bone marrow and L1210 leukemia cells (results not shown). The profiles obtained for normal bone marrow, removed 2 h after treatment with different doses of CY, are shown in Fig. 1. The shape of the curves suggest that these SSB represent alkali labile sites because the curves bend down at later times of elution. There was no clear dose-effect relation. In the CLL patient, no evidence of SSB was demonstrable following CY treatment (Fig. 2).

DIC. Figure 3 shows the formation and repair of DIC for mice treated with a CY dose of 4 mg/mouse. For leukemia, the maximum level of DIC was observed 2 h after CY injection and DIC were gradually repaired afterwards. For normal bone marrow cells, the maximum was observed at 2–4 h. Maximum levels of DIC were about 4-fold lower for normal bone marrow than for leukemia cells at this dose (\( P = 0.009 \)).

Figure 4 shows the dose-effect relation for both leukemia and normal bone marrow at 2 h following CY administration. For leukemia cells, a dose-response relation with an increase of about 50 req of DIC per 1 mg/mouse increase in dose of CY was observed. For normal bone marrow, an increase of about 15 req of DIC per mg/mouse of CY was observed.

For human CLL, maximum DIC were observed at 12 h after CY injection given as a 2-h infusion (Fig. 5, solid symbols). The number of DIC was relatively low (mean at 12 h = 36 req) and close to the level that could be detected by alkaline elution. They were significantly different from zero at 12 and 24 h (\( P < 0.05 \)). In the mice significant levels of cross-linking were detectable in leukemia cells as early as 1 h after i.v. bolus injection. For background fluorescence and two untreated control samples (one received 500 rads) were run. In the case of patient samples, the untreated control was prepared from a blood sample drawn immediately before treatment of the patient.

DNA Assay. The relative DNA concentration in each fraction was determined using the fluorescent Hoechst dye 33258 (7). A 1-ml aliquot was withdrawn from each fraction, including the filter and wash solutions, and transferred to a 13 × 100-mm borosilicate glass culture tube. Each sample was neutralized with 0.4 ml of 0.2 M KH2PO4 and the volume adjusted to 2.0 ml with 0.6 ml of distilled water. Finally, 1.0 ml of Hoechst dye (1.5 × 10^-6 M in standard saline citrate) was added with a fluorometric determination of the DNA (6). Except where noted, a logarithmic interpolation, between the appropriate samples collected, was performed.

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IN VIVO DNA CROSS-LINKING BY CYCLOPHOSPHAMIDE

Fig. 2. Alkaline elution profiles of CLL cells after treatment of a patient with 750 mg/kg CY i.v. Blood samples were drawn at 4 h, A; 8 h, B; 12 h, C. Control cells were obtained from blood drawn immediately before injection of CY (O). A control sample was irradiated with 500 rads of γ-rays in vitro on ice (•).

Fig. 3. Formation and repair of DIC on CDF1 normal bone marrow (O and — — —) and L1210 leukemia cells (• and — — —) after treatment i.v. with 4 mg/mouse of CY.

Fig. 4. Dose-effect relation for L1210 leukemia (O and — — —) and CDF1 normal bone marrow (O and — — —) cells 2 h after treatment with CY.

Fig. 5. Frequencies of DIC in human cells after injection of 750 mg/m² CY by either a 2 h infusion (O) or i.v. bolus injection (C). Time was determined from the start of i.v. administration.

Fig. 6. DNA-DNA interstrand crosslinks (Rad-Equivalents) for human CLL, no DPC could be demonstrated at 4 h or earlier after CY injection (Fig. 7). From 6 through 48 h significant levels of DPC were measured.

DISCUSSION

The aim of this study was to determine the kinetics of cross-linking by CY in mouse L1210 leukemia and normal bone marrow cells, to correlate them with cytotoxicity data, and to compare them with the kinetics obtained in human CLL, with all treatments done in vivo.

CY is selectively cytotoxic to L1210 leukemia as compared to the normal bone marrow stem cells in the syngeneic CDF1 mouse (12). This selective cytotoxicity has been explained by high levels of aldehyde dehydrogenase in normal bone marrow cells, leading to the transformation of aldophosphamide to carboxycyclophosphamide, an inactive compound (13).

The dose-response curves for CY is linear both for L1210 leukemia (14) and on CDF1 normal bone marrow (12) in vivo. Using these published data, a 3-log cell kill was calculated per 1.0 mg CY per mouse for L1210 leukemia. For CDF1 normal bone marrow, we calculated a 1-log cell kill per 6.0 mg CY per mouse. This means that it requires 18-fold more CY in order

(P = 0.04; Fig. 3). In order to investigate whether the delay in the onset of the appearance of DIC for human CLL could be explained by the infusional administration of CY, a subsequent treatment was performed as a i.v. bolus (Fig. 5, open symbols). No DIC could be demonstrated at times shorter than 4 h.

DPC. For L1210 leukemia cells, while DPC was detectable 1 h after CY administration, maximum number of DPC was observed at 24 h (Fig. 6). A significant number of DPC disappeared by 24 h (P = 0.02). For normal bone marrow cells, the maximum number was observed by 1 h and a plateau was observed between 1 and 24 h (Fig. 6). The number of DPC was more than twofold higher in leukemia than in normal bone marrow cells at 2 h. In both cell types, DPC were already detectable 1 h after injection of CY.

For human CLL, no DPC could be demonstrated at 4 h or
measured by the NCFU assay (12), as these account for less than 1% of the cells of mouse bone marrow. It is of interest that Sahovic et al. demonstrated heterogeneity of CY sensitivity between mouse marrow progenitor cells (15).

This study shows that measurement of CY-induced DNA adducts is possible in human CLL, without high dose treatment. The frequencies measured are close to the level that can be reproducibly detected by alkaline elution. CLL provides an easily accessible tissue for studies of cross-linking; however, potential difficulties arise in the interpretation of data due to differences in sensitivity to CY. It has still to be investigated whether differences in DIC correlate with differences in response to CY in these cells. As alkaline elution measures global DIC, resistance in a small subset would be masked by the cross-linking in the majority of cells. In the mouse model, low levels of SSB have been observed. No clear dose-effect relationship of SSB could be derived from the elution rates, although for higher doses of CY, increasing SSB could be masked by increased cross-linking. It is likely that these SSB reflect alkali labile sites by the shape of the elution curves. In the human cells, SSB or alkali labile sites could not be demonstrated. It is of interest that Ford et al. (16) observed the presence of strand breaks and no indication of cross-links by alkaline sucrose sedimentation in patients treated with high dose CY.

The patient response could be characterized as stable; his lymphocyte count, typically 70,000–90,000 with 95% lymphocytes at the time of treatment decreased to 40,000–50,000 at Days 10–12, to increase again at the time of treatment 3 weeks later. Lymph nodes similarly would decrease in size transiently by somewhat less than 50% of their initial size.

Significant differences in DPC were observed between L1210 and normal bone marrow cells and DPC were easily measurable in human CLL. The significance of DPC induced by CY and therefore further discussion would be speculative. Differences in the kinetics of cross-linking between murine and human leukemia cells is most likely due to differences between rates of CY activation in mouse and humans. In vitro studies with active forms of CY have shown little differences in the time course of DNA cross-linking (2, 3, 17).

This paper shows that DNA-cross-linking can be measured in vivo. It describes the establishment of an in vivo model which we will use for further study of modulation of cross-linking by cyclophosphamide both on normal and malignant cells and shows the feasibility of the determination of cross-linking in human CLL at regular doses of cyclophosphamide. Whether this cross-linking can be used as an early indicator of cytotoxicity in patients has to be studied further.

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