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STUDIES OF CHLORAMBUCIL–DNA ADDUCTS

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Abstract—Chlorambucil (CLB) is a bifunctional nitrogen mustard whose therapeutic and major side-effects are thought to be caused by binding to DNA. HPLC analysis of hydrolyzed DNA from L1210 cells incubated with [^{14}C]CLB generated two peaks of radioactivity, indicating the formation of two or more major adducts. Since DNA incubated with [^{14}C]CLB in a cell-free system gave rise to the same profile, experiments were conducted with DNA from cells exposed to radiolabeled DNA precursors, which was then reacted with CLB. DNA containing [8-^{14}C]guanine gave rise to one peak of radioactivity, while DNA containing [2,8-^{3}H]adenine gave rise to two peaks. These peaks corresponded to the peaks seen in the experiment with intact L1210 cells treated with [^{14}C]CLB. Experiments with DNA containing [5-^{3}H]cytosine indicated that no cytosyl adducts were formed. No adducts were seen in hydrolysates prepared from labeled DNA incubated with drug solvent alone. These data indicate that the majority of adducts induced by CLB are guanyl adducts, but a substantial quantity of adenyl adducts has also been identified.

Chlorambucil (CLB†) is a bifunctional nitrogen mustard commonly used in the treatment of chronic lymphocytic leukemia and other malignancies [1–5]. Like other alkylating agents, CLF is thought to kill malignant cells by binding covalently to DNA and forming cross-links [6, 7].

Emergence of drug-resistant disease and development of acute non-lymphoid leukemia are major complications of long-term CLB therapy. Work with tissue culture models has indicated that resistance to CLB is related to a reduction in the amount of DNA cross-links formed upon exposure to the drug [7–9]. There is also increasing evidence that alkylating agents induce specific mutations in *ras* oncogenes, and that this property may be responsible for some of their leukemogenic potential [10–12]. Drug–DNA adduct formation, therefore, appears to be of significance in both processes. Consequently, an understanding of the chemical structure of CLB–DNA adducts might aid in the development of strategies to deal with drug resistance and leukemogenesis.

Studies with cell-free systems indicate that CLB has a propensity for forming N-7 guanyl adducts [6, 11, 13–16]. More recently, the induction of N-3 adenyl adducts has been described [17]. However, it remains unclear which DNA adducts are caused by CLB in intact cells. Experiments using neutral thermal hydrolysis of DNA from explanted chronic lymphocytic leukemia (CLL) lymphocytes exposed to ^{14}C-labeled CLB indicated that all measurable radioactivity was found in two HPLC peaks corresponding to purine adducts [18].

In this study, L1210 cells were incubated with labeled DNA bases or CLB. DNA from these cells was hydrolyzed and analyzed by HPLC to investigate the structure of CLB–DNA adducts formed.

MATERIALS AND METHODS

Cell culture. L1210 cells were maintained in continuous culture in RPMI 1640 containing 10% fetal bovine serum (FBS). The cells were grown at 37°, in a humidified atmosphere containing 5% CO_2.

For studies of drug–DNA binding in cells, the cells were washed and resuspended in medium HB 101/1% FBS; cell counts were done in a Coulter counter and were 5–8 × 10^6 cells/mL. An aliquot of freshly prepared 330 mM CLB stock solution containing [^{14}C]CLB in dimethyl sulfoxide (DMSO) was added to the cells for a final drug concentration of 330 μM. [U-*phenyl*-^{14}C]CLB (11.7 mCi/mmol) provided by the National Cancer Institute was used. Incubation was in a water bath at 37°. In some experiments, 2-deoxyglucose (2-DG) was added to a concentration of 5 mM, 10 min before addition of drug. At desired times, aliquots of cells were removed, the cells were washed twice in cold phosphate-buffered saline (PBS), and DNA was extracted by standard methods [19]. Aliquots of initial supernatant medium were mixed with Ecoscint (National Diagnostics, Manville, NJ) and radioactivity was measured to determine drug specific activity at the start of incubation (6–12 dpm/pmol). Cell viability was monitored by Trypan blue exclusion and was at least 89%.

To prepare labeled DNA for CLB binding in cell-free buffer, L1210 cells were washed and resuspended in RPMI 1640/1% FBS and labeled DNA precursor was added to obtain 0.4 to 1.0 μCi/mL. The following DNA precursors were used: [5-^{3}H]deoxycytidine (24.2 Ci/mmol), [2,8-^{3}H]adenine (19.2 Ci/mmol) (NEN, Dupont, Boston, MA) and [8-^{14}C]guanine HCl (55 mCi/mmol) (ICN Radiochemicals, Costa Mesa, CA). After a 2-hr incubation at 37°, the cells

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† Abbreviations: CLB, chlorambucil; FBS, fetal bovine serum; 2-DG, 2-deoxyglucose; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; and CLL, chronic lymphocytic leukemia.

were washed twice in cold PBS and the DNA was isolated by phenol-chloroform extraction [19].

Quantitation of drug–DNA binding in L1210 cells. DNA extracted from cells that had been exposed to [^{14}C]CLB was redissolved in PBS. Concentration and purity were estimated by measuring the absorbance at 260 and 280 nm (A_{260}, A_{280}). DNA whose A_{260}/A_{280} ratio was 1.8 or greater was used for experiments. Drug–DNA binding was quantified by measuring the radioactivity in a sample of DNA solution.

Drug–DNA binding in cell-free solution. Radioactive DNA extracted from L1210 cells was redissolved in PBS (400–500 mg/mL). A stock solution of CLB in DMSO (660 mM) was added to a final drug concentration of 660 μM. This higher concentration than in experiments with cells was used to maximize adduct formation. The reaction mixture was insulated from light and incubated with gentle shaking at room temperature. After 2 hr, the DNA was subjected to three cycles of ethanol precipitation and redissolution in PBS. The DNA concentration and purity in the final mixture were determined by measuring the absorbance at 260 and 280 nm. Incubation of non-radioactive L1210 DNA with [^{14}C]CLB was carried out in the same way with an approximate drug specific activity of 0.1 mCi/mmol. Drug-free control experiments were performed in the same way, except that the DNA was incubated with DMSO alone.

To make sure that we were not isolating adducts formed between the drug and free guanine in the DNA mixture, the following control experiment was performed. One millimolar guanine, containing [8-^{14}C]guanine at 0.5 μCi/mL, was allowed to react with 660 μM CLB at room temperature, under conditions known to allow the formation of adducts [13]. The reaction mixture was added to unlabeled L1210 DNA, and the above procedure followed. No adduct was isolated, indicating that no adducts were formed between free guanine and CLB under the conditions described above.

To ascertain that free [^{14}C]CLB was not being isolated and mistaken for an adduct, the above procedure was carried out with [^{14}C]CLB without DNA; no radioactivity was found in the final PBS solution. In another control experiment, [^{14}C]CLB was preincubated in aqueous solution for 12 hr to allow hydrolysis of the mustard groups. This mixture was added to DNA and incubated and preincubated as above. Compared to a parallel incubation with fresh [^{14}C]CLB, less than 10% of predicted radioactivity was present in the DNA indicating that covalently bound CLB is found in the final mixture.

HPLC conditions. HPLC was performed on a Waters system equipped with a 250 mm × 10 mm semi-preparative C18 column (Nucleosil, Phenomenex, Torrance, CA) and guard column. A gradient was developed which permitted separation of the four DNA bases from each other and from the adducts. The gradient was as follows: 100% A × 3 min, linear 100% A to 100% B × 60 min, 100% B × 7 min; A = 10% methanol in H_2O, v/v; B = 100% methanol. The flow rate was 2 mL/min, and absorbance at 254 nm (A_{254}) was monitored. Linear standard curves were established for guanine,

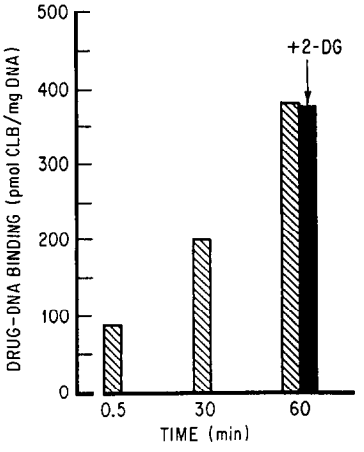

Fig. 1. Time course of [^{14}C]CLB–DNA binding in L1210 cells at 37°. Hatched bars: no metabolic inhibitor; solid bar: cells preincubated with 5 mM 2-deoxyglucose. Each bar represents the average of two experiments.

adenine and cytosine. Cytosine was found to occasionally run as a broad double peak during analysis of the DNA hydrolysates, but there was no overlap with any other peak. Fractions corresponding to peaks of absorption during HPLC of DNA hydrolysates were analyzed by UV absorption spectrophotometry on two separate occasions and compared to standards to confirm that the expected bases corresponded to each peak. Virtually no thymine was found in the hydrolysates.

Hydrolysis of DNA and HPLC analysis. The DNA was treated with 10 N HCl to obtain a 0.1 N HCl concentration and then immersed in a boiling water bath for 45 min; this is known to release purines and purine adducts [20]. After the mixture cooled to room temperature, 12 N NaOH was added until the pH was 6.5 to 7.0. An aliquot of the lysate was removed for determination of DNA base specific activity. The remainder (containing 5–10 × 10^5 cpm) was loaded onto a C18 Sep Pak cartridge (Waters, Milford, MA) which had been washed sequentially with 5 mL of 100% methanol, 5 mL of 10% methanol in H_2O, and 20 mL H_2O. The cartridge was washed with 20 mL H_2O, 10 mL 10% methanol in H_2O, and 10 mL methanol. The methanol fraction was dried in a vacuum centrifuge and then redissolved in 1.2 mL methanol. This sample was centrifuged to remove particulates and analyzed by HPLC as described above. One-minute fractions were collected, and the radioactivity in each fraction was quantitated in a liquid scintillation counter. DNA from cells incubated with [^{14}C]CLB was hydrolyzed as above, filtered and analyzed by the above HPLC method, without the Sep Pak cartridge purification.

Distribution of radioactivity among the DNA bases as well as specific activity of each base in each lysate were determined by filtering the aliquot that was saved before Sep Pak cartridge partitioning and analyzing it using the same HPLC method. The injection volume was 50 μL. The A_{254} peak area of each base in the HPLC profile of the hydrolysate

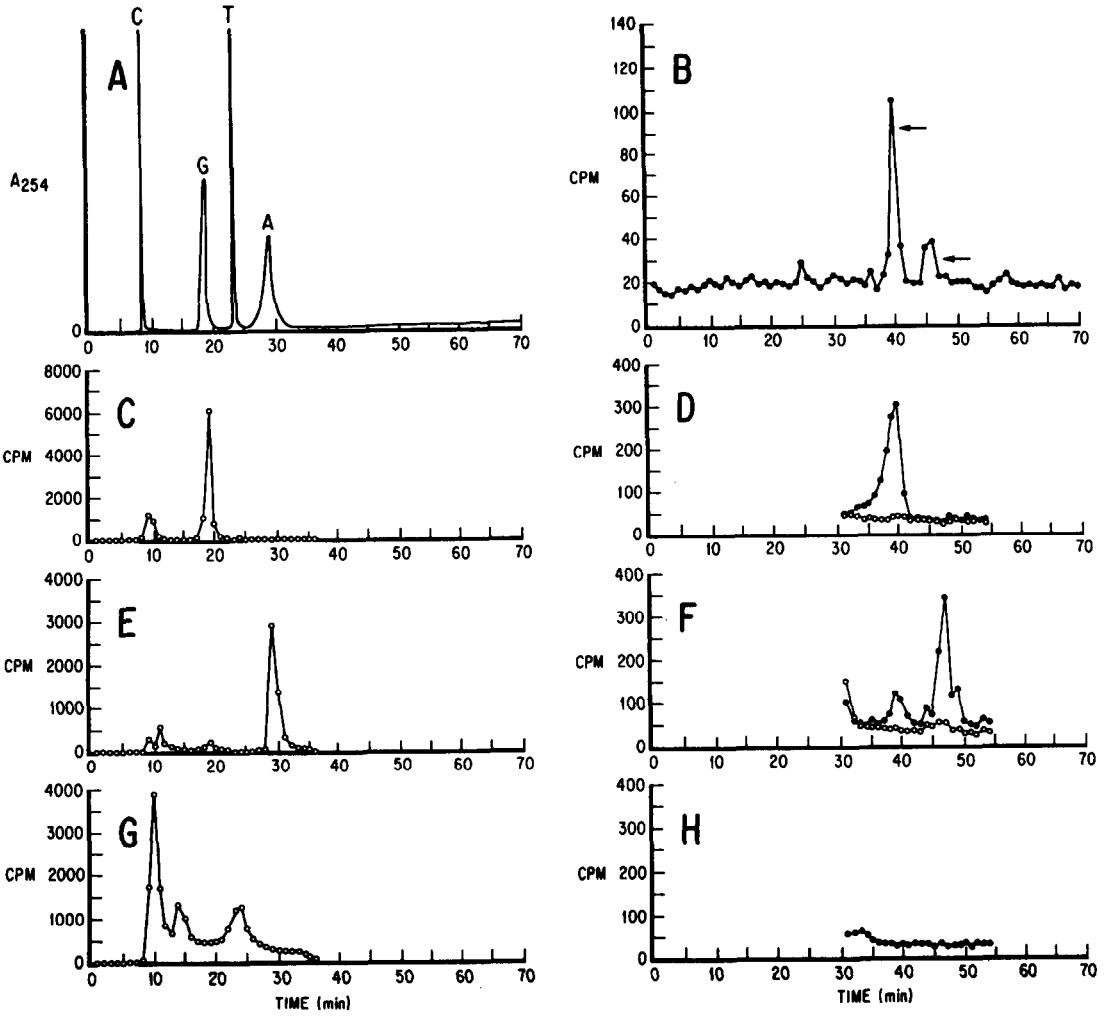

Fig. 2. Panel A: HPLC separation of DNA base standards. Panel B: Elution profile of radioactivity during HPLC of the hydrolysate of DNA from cells incubated with [^{14}C]CLB. The hydrolysate was not pre-purified on a C18 Sep-Pak cartridge. Arrows indicate reproducible peaks of radioactivity. This graph is representative of 4 experiments. Panel C: Elution profile of radioactivity from a hydrolysate of DNA from cells incubated with [8-^{14}C]guanine. Panel D: Elution of radioactivity from a C18 Sep Pak purified lysate of the same DNA as in panel C, but after *in vitro* incubation with CLB (●—●) or solvent alone (○—○). Panel E: Elution profile of radioactivity from a hydrolysate of DNA from cells incubated with [2,8-^{3}H]adenine. Panel F: Elution profile of radioactivity from a C18 Sep Pak purified lysate of the same DNA as in panel E, but after *in vitro* incubation with CLB (●—●) or solvent alone (○—○). Panel G: Elution profile of radioactivity from a hydrolysate of DNA from cells incubated with [5-^{3}H]deoxycytidine. Panel H: Elution profile of radioactivity from a C18 Sep Pak purified lysate of the same DNA as in panel G, but after *in vitro* incubation with CLB (●—●). Each graph in panels C to H is representative of from 2 to 5 separate experiments.

was compared to previously established standard curves. The number of counts in fractions corresponding to each base was measured.

Recovery of counts during filtration and HPLC of the samples was 95–100%. Since all adduct-related radioactivity was found between 35 and 48 min of elution, only 31–54 min samples were routinely counted. Similarly, since control experiments showed no base-related radioactivity after 35 min, only 1–36 min samples were routinely collected for the base analysis.

RESULTS

[^{14}C]CLB binding to DNA in L1210 cells was time-dependent and not affected by the metabolic inhibitor, 2-deoxyglucose, as shown in Fig. 1. This suggests that drug–DNA binding occurs by simple alkylation of the macromolecule by CLB, without any need for energy or drug activation. After a 60-min incubation under the conditions described, an average binding of 390 pmol CLB/mg DNA was seen. This corresponds to about 2.6 adducts/10^4 base pairs [21].

Panel A in Fig. 2 illustrates the ability of this HPLC method to resolve the four DNA bases. This panel can be used for comparison with panels C, E and G. HPLC analysis of hydrolyzed DNA from cells exposed to [^{14}C]CLB indicated the formation of at least two DNA adducts as indicated by one major and one minor peak of radioactivity in Fig. 2, panel B. The minor peak was small, but reproducible in four separate experiments. No C18 Sep Pak pre-purification was used in order to ensure that no identifiable adducts were lost. These results are similar to previously reported results with CLL lymphocytes [18]. Calculations indicated that at least 95% of all adduct-related radioactivity was in these two peaks, showing that virtually all of the CLB–DNA adducts which are formed in intact cells were identified by this method.

Since L1210 DNA alkylated in cell-free buffer by [^{14}C]CLB generated the same HPLC profile after acid hydrolysis and C18 Sep Pak cartridge purification (data not shown), studies with DNA containing labeled bases were undertaken. The C18 Sep Pak step was needed to remove from the lysates the excess labeled, unmodified bases which would make the clear identification of adduct peaks very difficult. Panels C and D of Fig. 2 illustrate the analysis of DNA from cells incubated with [8-^{14}C]guanine, and then alkylated by CLB or reacted with DMSO alone. Panel C indicates that most of the radioactivity is present in guanine whereas panel D shows one peak of radioactivity, with the same retention time as the major adduct peak in panel B. This implies that one major CLB–DNA adduct formed in intact cells is a guanyl adduct. Panels E and F show the results of an analogous experiment with hydrolysate prepared from DNA from cells incubated with [2,8-^{3}H]-adenine. Most of the label in the DNA was seen in adenine, while in panel F two peaks were noted—a larger one with the retention time of the minor peak in panel B, and a lesser one with the retention time of the major peak in panel B. This indicates that two adenyl adducts are formed. In both cases, hydrolysates from labeled DNA that underwent a mock alkylation yielded no peak of radioactivity, as expected. Panels G and H illustrate an experiment with DNA from cells exposed to [5-^{3}H]deoxycytidine. As can be seen in panel H of the figure, no peak was noted indicating that no cytosyl adducts were formed.

The specific activities of radioactive bases in each lysate were determined by measuring counts per minute in fractions corresponding to peaks of absorbance at 254 nm during HPLC of samples not subjected to the C18 Sep Pak step (not shown). Standard curves for cytosine, guanine and adenine at A_{254} were linear over this range. The specific activities of bases in runs of drug-treated and control DNA were comparable, as were the total amounts of radioactivity which were processed to generate the data; therefore, the absence of peaks during elution of the control hydrolysates was not due to inadequate amounts of radioactivity.

The HPLC A_{254} profiles of the hydrolysates not pre-purified on C18 Sep Pak cartridges contained three peaks. On two occasions, fractions corresponding to each peak were collected and their UV absorption spectra were compared to standards of cytosine, guanine and adenine. In this way it was determined that the first peak was cytosine, followed by guanine and adenine, as expected from the elution profile of standards in panel A. Occasionally hydrolysates prepared from DNA containing tritiated cytosine gave rise to a double peak of radioactivity. Thymine was not detected in the hydrolysates. Panels C and E show that the majority of counts in the DNA were associated with the expected bases, although about 15–20% were found in cytosine. This was not of consequence, however, since no cytosyl adducts were detected, as shown in panel H.

DISCUSSION

Two major biological effects of CLB, cytotoxicity and leukemogenesis, are most likely caused by the ability of the drug to modify DNA. Consequently, the structures of DNA adducts induced by CLB in target cells are of interest.

Based on earlier studies with cell-free systems, N-7 guanyl adducts have been assumed to be the major adducts induced by CLB and other nitrogen mustards [6, 11, 13–15]. More recent reports indicate that a larger variety of adducts is formed. Melphalan and CLB have been shown to induce adenyl adducts *in vitro* in plasmid DNA [16, 17]. The mutations caused by melphalan in a shuttle vector were found to be predominantly transversions at adenine sites, suggesting adduct formation at this base [22]. The alkylating agent phosphoramide mustard, an active metabolite of cyclophosphamide, produces several adducts, including a phosphoester DNA complex [23].

The identification of drug–DNA adducts that are formed *in vivo* is difficult. Usually, small quantities are formed; therefore, the lack of sensitivity of the techniques that are used is a potential problem. In addition, certain adducts are unstable and may be lost during DNA extraction or other steps. In this report, more than 95% of the radioactivity present in DNA extracted from L1210 cells incubated with [^{14}C]CLB was eluted in two peaks during HPLC of the DNA hydrolysate (Fig. 2, panel B). This suggests that the major adducts are being identified, although minor or unstable ones might not be. Studies with DNA containing labeled bases and alkylated by CLB indicated that these adduct peaks correspond to at least one guanyl and two adenyl adducts. Since, as indicated in Fig. 1, CLB–DNA binding in living cells appears to be a simple alkylation requiring no energy, it is reasonable to believe that the adducts formed in the cell-free system are indicative of those formed in intact cells.

The exact structure of the adducts cannot be deduced from the above data. Work by others has indicated that the N-7 position of guanine and the N-3 position of adenine are the most susceptible to alkylation by nitrogen mustards [6, 11, 13–17]. It is probable that these are the sites of bond formation in these adducts. An approximate ratio of 3:1 was noted in the amounts of radioactivity in the major and minor peaks in panel B, indicating a preponderance of guanyl adducts.

Cytosyl adducts were not detected (Fig. 2, panel H); the incomplete elution of tritium during HPLC of DNA hydrolysate containing [^{3}H]cytosine (Fig. 2, panel G) was likely due to the relative resistance of cytosine to acid hydrolysis and does not change this conclusion. This is because recovery of counts was complete during the HPLC analysis represented by panel B, indicating that all measurable CLB adducts are susceptible to acid hydrolysis and identifiable by the HPLC method used to generate the profile in panel H. Panel G also indicates that cytosine was substantially converted by the cells, but this also does not alter the conclusions indicated by panel H. As thymine was not released during the acid hydrolysis, experiments with DNA containing labeled thymine were not necessary.

In conclusion, our results suggest that the major DNA adducts induced by CLB in leukemic cells are guanyl and adenyl adducts. The guanyl adducts appear to be predominant, but a substantial number of adenyl adducts are formed. These results are in keeping with reports by others using different systems [11, 14–17, 22] and indicate that both types of adducts need to be considered in studies of clinical drug resistance and mutagenesis.

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