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CORRELATION OF NITROSOUREA MURINE BONE MARROW TOXICITY WITH DEOXYRIBONUCLEIC ACID ALKYLATION AND CHROMATIN BINDING SITES*

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Abstract—All of the clinically available nitrosourea antitumor agents produce serious treatment-limiting bone marrow toxicity. A reduction in this toxicity can be achieved by attaching the chloroethylnitrosourea cytotoxic group to C2 (chlorozotocin) or C1 (1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea, GANU) of glucose. Both glucose analogs are less myelotoxic in mice than 1-(2-chloroethyl)-3-cyclohepyl-1-nitrosourea (CCNU) or 1-(4-amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU), while retaining comparable antitumor activity against the murine L1210 leukemia. To define the nuclear mechanisms for this reduced myelotoxicity, alkylation of L1210 and murine bone marrow DNA was quantitated. With the use of the endonucleases micrococcal nuclease and DNase I, the sites of alkylation within the chromatin substructure were determined. Experiments were performed on L1210 leukemia or bone marrow cells that had been incubated *in vitro* for 2 hr with 0.1 mM [^{14}C]chloroethyl drug. The quantitative alkylation of DNA by GANU was 1.3-fold greater in L1210, as compared to bone marrow, cells. This ratio of DNA alkylation is comparable to the 1.3 ratio we previously reported for chlorozotocin [L. C. Panasci, D. Green and P. S. Schein, *J. clin. Invest.* **64**, 1103 (1979)]. In contrast, the ratio of alkylation (L1210:bone marrow DNA) for the myelotoxic ACNU was 0.66, similar to 0.59 for CCNU. Nuclease digestion experiments demonstrated that chlorozotocin and GANU preferentially alkylated internucleosomal linker regions of bone marrow chromatin, while nucleosome core particles were the preferred targets of CCNU and ACNU. The reduced myelotoxicity of chlorozotocin and GANU may be correlated with the advantageous ratio of L1210:bone marrow DNA alkylation and preferential alkylation of internucleosomal regions of bone marrow chromatin.

The chloroethylnitrosourea class of anticancer agents is active against a broad spectrum of human malignancies, including lymphomas, melanomas, gliomas and cancers of the gastrointestinal tract [1]. The chloroethylnitrosoureas in clinical use produce a unique form of delayed and cumulative myelotoxicity that, after repeated courses of therapy, can result in chronic bone marrow hypoplasia and possible acute leukemia [2, 3]. Structure–activity analyses from our laboratory have demonstrated that this myelotoxicity is reduced by the placement of a glucose carrier on the nitrosourea cytotoxic group [4, 5]. Chlorozotocin§ and GANU are two glucose nitrosoureas that have curative activity for the murine L1210 leukemia at doses that are not myelosuppressive in that species [6, 7]. These data have clinical relevance. In our recent trials of chlorozotocin in patients with advanced melanoma, antitumor activity equivalent to that achieved with the myelotoxic nitrosoureas was obtained, but without evidence of serious or cumulative bone marrow suppression [8].

Alkylation is currently accepted as the mechanism responsible for the antitumor activity of this class of compounds. For chlorozotocin, we have correlated this phenomenon of murine L1210 antitumor activity and reduced bone marrow toxicity with an advantageous ratio of *in vitro* L1210:murine bone marrow DNA alkylation, 1.3 [9]. Comparative studies with equimolar CCNU, a nitrosourea that produces optimal L1210 antitumor activity only at doses that are bone marrow toxic, demonstrated this quantitative DNA alkylation ratio (L1210:bone marrow) to be reversed, 0.6 [9]. To expand our understanding of structure–activity relationships for this class of drugs, we have now extended these DNA alkylation studies to include GANU, the second bone marrow-sparing glucose nitrosourea [7], and ACNU, a myelotoxic pyrimidine analog [10].

To define further the importance of these quantitative differences in alkylation of target and normal tissues, we have determined the sites of alkylation within the chromatin substructure. At the molecular

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§ Abbreviations: chlorozotocin, 2-[3-(2-chloroethyl)-3-nitrosoureidol]-D-glucopyranose; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; GANU, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea; ACNU, 1-(4-amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea; LD_{10}, dose resulting in death of 10% of normal mice; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

level, chromatin is composed of double tetrameric nucleosome core particles, containing two molecules each of histones H2A, H2B, H3 and H4 that are associated with a helical turn of approximately 140 base pairs of DNA [11, 12]. The core regions are joined by an internucleosomal liner region composed of up to 60 base pairs of DNA complexed with histone H1 and other non-histone proteins. There is evidence that this repetitive nucleosomal arrangement is found in more than 95% of nuclear DNA [13, 14], including both transcriptionally active (euchromatin) and inactive (heterochromatin) regions of the genome [14, 15]. In previously reported studies from our laboratory, endonucleases with defined cleavage sites within chromatin were used to determine the dominant sites of nitrosourea alkylation within the substructure of the HeLa cell chromatin [16]. Micrococcal nuclease initially cleaves DNA in the internucleosomal linker regions of chromatin [14, 17]. Pancreatic DNase I cleaves the DNA of both nucleosome core particles and internucleosomal regions at 10·n base pair intervals, where n is an integer [18, 19]. Under the conditions employed, DNase I preferentially digests chromatin sequences that are involved in the transcription process [19, 20]. Recent studies demonstrate that actively transcribing genes may also have increased susceptibility to micrococcal nuclease [21], but this susceptibility appears to depend on the digestion conditions used [18]. In comparative experiments, we describe the chromatin alkylation sites in murine bone marrow and L1210 leukemia cells for the four chloroethylnitrosoureas with varying bone marrow toxicities, to determine whether the reduced myelotoxicity demonstrated for the glucose analogs can be correlated with tissue-specific differences in chromatin alkylation.

We have previously reported [5, 7] chemical and biological activities of the four nitrosoureas that are relevant to the present studies, and these data have recently been repeated and confirmed by us. Both chlorozotocin and GANU, the glucose analogs, have minimal myelotoxicity at fully active doses against the L1210 system. In contrast to chlorozotocin, GANU has significant chemical carbamoylating activity.

MATERIALS AND METHODS

The chloroethylnitrosoureas investigated in this study were chlorozotocin (NSC 178248), CCNU (NSC 79037), GANU (NSC D 254157), and ACNU (NSC D 245382). [^{14}C]Chloroethyl chlorozotocin (sp. act. 15.8 mCi/mmole) and [^{14}C]chloroethyl CCNU (sp. act. 15.7 mCi/mmole) were provided by Dr. Robert Engle of the Drug Development Branch, National Cancer Institute, Bethesda, MD. [^{14}C]Chloroethyl GANU (sp. act. 9.17 mCi/mmole) was synthesized and provided by Dr. Hisamatsu and his colleagues at the Meiji Seika Co., Tokyo, Japan. [^{14}C]Chloroethyl ACNU (sp. act. 4.03 mCi/mmole) was the gift of the Sankyo Co., Tokyo, Japan. The purity of all radiolabeled drugs was >98.5% as determined by thin-layer chromatography. Chlorozotocin, GANU and ACNU are water soluble compounds and were dissolved in 0.01 M sodium citrate buffer (pH 4); CCNU was dissolved in ethanol. Male BALB/c × DBA/2F (CDF_1) mice, weighing 18–25 g and maintained on Lab-Blox laboratory chow pellets and water, *ad lib.*, were used throughout.

In vitro *alkylation of L1210 leukemia and murine bone marrow cell DNA.* Femurs and tibiae were removed from normal mice, and the bone marrow cells were expressed using McCoy's 5A modified medium (Grand Island Biological Co., Grand Island, NY). L1210 leukemia cells were harvested from the peritoneal cavities of CDF_1 mice on day 7 following implantation with 1×10^5 cells. Erythrocytes were removed by osmotic lysis. The nucleated cells were suspended at a concentration of 4×10^7 cells/ml in McCoy's medium, and HEPES buffer (Sigma Chemical Co., St. Louis, MO) was added at a concentration of 10 mM. Both L1210 leukemia and murine bone marrow cells were then incubated with 0.1 mM [^{14}C]chloroethyl nitrosourea at 37° for 2 hr. This 0.1 mM drug concentration was the peak plasma concentration of chlorozotocin achieved in mice following administration of the optimal L1210 antitumor dose, 64 μmoles/kg [9], and is achieved in humans at the clinical dose of 120 mg/m^2 [22]. Equal amounts of the citrate buffer and ethanol diluents were added to all *in vitro* incubations. Cell viability was confirmed at the end of each incubation by trypan blue exclusion. At 2 hr, the cells were centrifuged for 10 min at 3000 *g*, washed twice with McCoy's medium, and stored at −70° prior to phenol extraction of DNA or differential nuclease digestion of chromatin. All experiments were performed in triplicate. Our previous results with chlorozotocin in combination with hydroxyurea demonstrated that the non-specific reutilization of [^{14}C]chloroethylnitrosourea decomposition products in replicative DNA synthesis during the 2-hr incubation was negligible [9].

Isolation of alkylated DNA. Following incubation with 0.1 mM ethyl-labeled nitrosourea, alkylated DNA was extracted from L1210 and murine bone marrow cells using a phenol procedure developed in our laboratory [23]. The purified alkylated DNA had less than 3% uracil compared to thymine and less than 1% protein contamination. DNA concentration was determined by the diphenylamine colorimetric method of Burton [24], and the results obtained were in good agreement with measurements by ultraviolet absorbance at 254 nm. Calf thymus DNA (Sigma) was used as the standard for the Burton method and ultraviolet absorbance after standardization by phosphate determination [25]. Radioactivity in the purified DNA samples was measured by adding 10 ml of Aqueous Counting Scintillant (Amersham Corp., Arlington Heights, IL) and counting in a Searle Mark III scintillation spectrometer (Searle Radiographics, Inc., Des Plaines, IL) with an automatic quench correction and a counting efficiency of 92% for ^{14}C and 35% for ^{3}H.

Nuclease limit digest experiments. Nuclei were isolated from L1210 leukemia and murine bone marrow cells that had been incubated with 0.1 mM ethyl-labeled nitrosourea. Concentrations of 0.3 and 0.5 mM were also compared. Nuclei were extracted using the method of Sporn *et al.* [26], and cytoplasmic

Table 1. *In vitro* alkylation of DNA after a 2-hr incubation with 0.1 mM [^{14}C]chloroethylnitrosourea

Drug	L1210 leukemia cells*	Murine bone marrow cells*	Ratio of alkylation L1210:bone marrow DNA
GANU	44.8 ± 1.8	33.5 ± 2.3	1.3
Chlorozotocin†	56.6 ± 1.7	44.9 ± 4.7	1.3
CCNU†	24.4 ± 5.3	41.5 ± 2.3	0.59
ACNU	36.0 ± 2.8	54.5 ± 2.1	0.66

* Expressed as pmoles of [ethyl-^{14}C]group per mg DNA ± S.D.
† Data were taken from Ref. 9.

fragments were removed by layering the nuclei on 50% sucrose with 2 mM $MgCl_2$ and centrifuging for 25 min at 16,500 *g* according to the method of Snyder *et al.* [27]. Phase contrast microscopy was used to ensure that the isolated nuclei were free of significant cytoplasmic contamination. The nuclei were then washed twice with a 70% ethanol and 0.1 M Na acetate solution.

Differential nuclease digestion of chromatin was performed on the isolated nuclei to determine the preferential alkylation sites of the chloroethylnitrosoureas. Digestion of internucleosomal linker DNA with micrococcal nuclease was carried out using a modification of the method of Cech and Pardue [16, 17]. Each nuclear pellet was suspended in 1.2 ml of a solution containing 50 mM Tris–HCl (pH 7.4), 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose, 15 mM mercaptoethanol and 0.2 mM PMSF. The solution was then prewarmed at 37° for 2 min, and a 200 μl sample was removed and placed on ice as a control. Micrococcal nuclease (Worthington, Freehold, NJ, electrophoretically pure) was added at a concentration of 200 units/ml, and 200-μl samples of the digested product were removed at 1, 2, 5, 10 and 25 min. The digestion reaction was terminated by adding each sample to a tube containing 100 mM EDTA (on ice).

Digestion of the nuclei with DNase I was carried out using the method of Flint and Weintraub [20]. Each nuclear pellet was suspended in a solution containing 10 mM Tris (pH 8) and 3 mM $MgCl_2$. Samples were prewarmed for 2 min and a 200-μl control sample was removed. DNase I was then added at a concentration of 50 units/ml and 200-μl samples were removed at 1, 2, 5, 10 and 25 min of digestion (37°). The reaction was stopped by adding each sample to a tube containing EDTA (on ice). For both nuclease digestions, the percentage of [^{14}C]alkyl-labeled bases in the acid soluble fraction was compared with the percentage of total chromatin digested over the course of the reaction. Previously published studies from our laboratory demonstrated that drug binding did not interfere with the enzymatic digestion of chromatin [16].

Comparable experiments using RNase (Sigma, electrophorectically pure, deoxyribonuclease free) were performed to determine the percentage of chromatin alkylation resulting from nuclear RNA alkylation. Duplicate samples of L1210 leukemia and bone marrow cells were incubated for 2 hr with 0.1 mM [^{14}C]chloroethyl nitrosourea as described previously, except that 5 μCi of [^{3}H]uridine or 10 μCi of [^{3}H]thymidine (Amersham) was added after 75 min. Nuclei were obtained, as described previously, and lysed by agitation in 0.25 mM EDTA (pH 7) with 0.2 mM PMSF. RNase T1 (Sigma) was then added at a concentration of 250 units, and the samples were incubated for 30 min at 37°. Ten units of RNase T2 (Sigma) were then added, and the samples were incubated for an additional 30 min. Each sample was then cooled on ice and precipitated with 10% TCA. The percentage of radioactivity released by T1 and T2 digestion was calculated.

RESULTS

The quantitative alkylation of *in vitro* cell cultures of L1210 leukemia and murine bone marrow DNA by the [^{14}C]chloroethylnitrosoureas is presented in Table 1. For L1210 leukemia cells, 0.1 mM GANU resulted in 44.8 pmoles of [ethyl-^{14}C]group bound/mg of phenol-extracted DNA. For bone marrow cells, 33.5 pmoles were bound per mg of DNA. Alkylation of DNA by GANU was 1.3-fold greater in L1210 as compared to bone marrow cells. This ratio of alkylation (L1210:bone marrow DNA) is comparable to the 1.3 ratio we obtained for chlorozotocin [9].

The quantitative alkylation of L1210 leukemia DNA by ACNU was 36.0 pmoles bound per mg of DNA, compared to 54.4 pmoles per mg of bone marrow DNA. In contrast to the results with the glucose nitrosoureas, the myelotoxic ACNU demonstrated preferential alkylation of bone marrow cells, with a ratio of alkylation (L1210:bone marrow DNA) of 0.66. This ratio obtained with ACNU is similar to the 0.59 ratio for CCNU [9], a drug with comparable murine bone marrow toxicity.

Differential nuclease digestion was used to define qualitatively the drug-specific sites of alkylation within the murine leukemia and bone marrow chromatin substructure. The data we have generated using micrococcal nuclease and DNase I, with their selective effects on chromatin, are presented in Figs. 1–6. Figure 1 presents the kinetics of digestion for L1210 cells treated with three concentrations of chlorozotocin labeled in the alkylating moiety. The three graphs in the left column are the results obtained with micrococcal nuclease, and the three on the right present comparative data using DNase I. The percentage of ^{14}C in the acid soluble fraction was compared with the percentage of chromatin digested

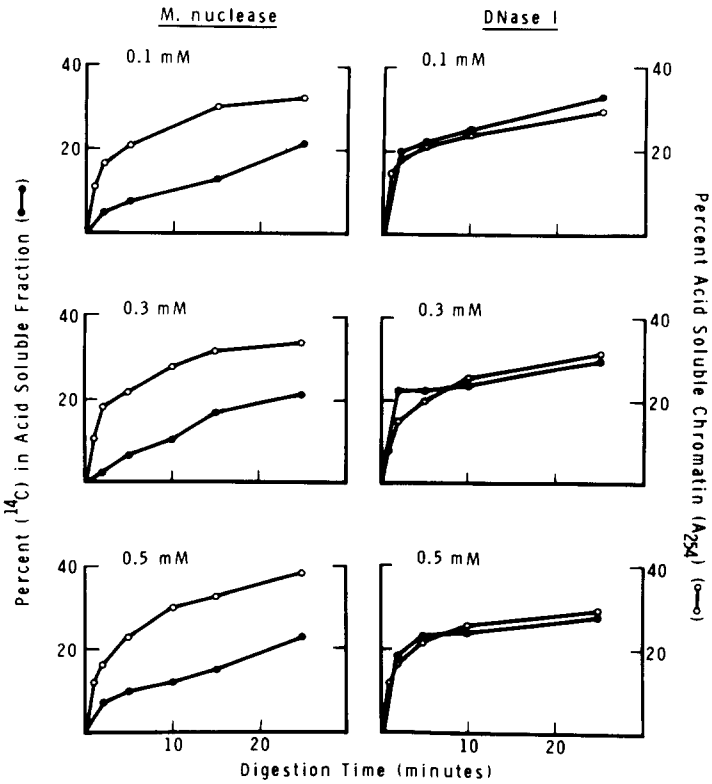

Fig. 1. Limit digest of murine L1210 leukemia cell nuclei from cells treated with 0.1, 0.3 or 0.5 mM [^{14}C]chloroethyl chlorozotocin. Methods for digestion with micrococcal nuclease of DNase I are described in the text. Release of TCA-soluble carbon-14 (●) was compared with the digestion of nuclear chromatin (○).

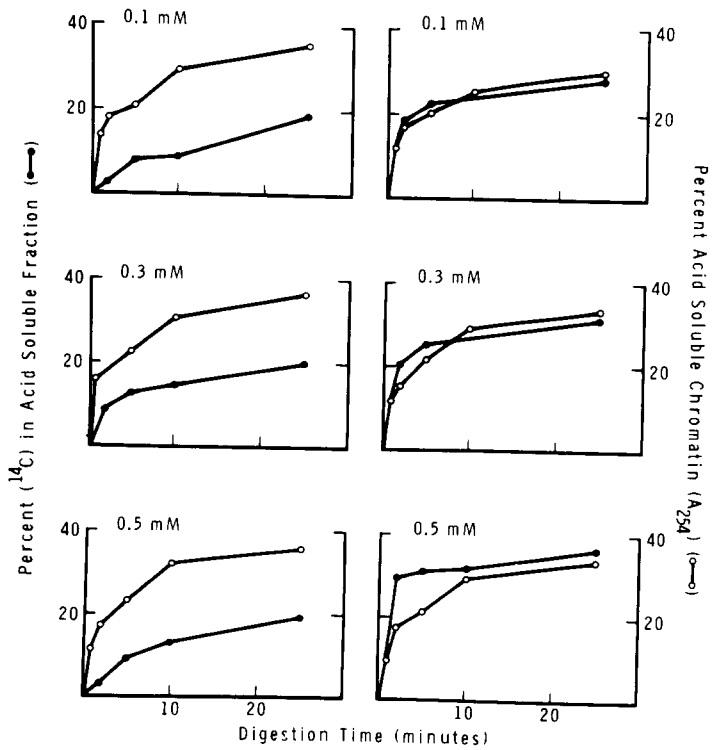

Fig. 2. Limit digest of L1210 leukemia cell nuclei from cells treated with [^{14}C]chloroethyl CCNU.

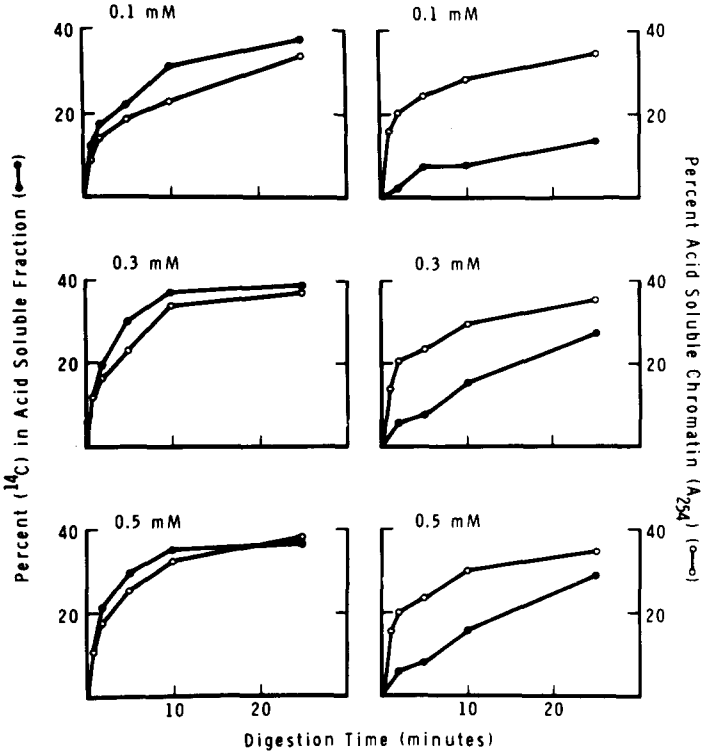

Fig. 3. Limit digest of murine bone marrow nuclei from cells treated with [^{14}C]chloroethyl chlorozotocin.

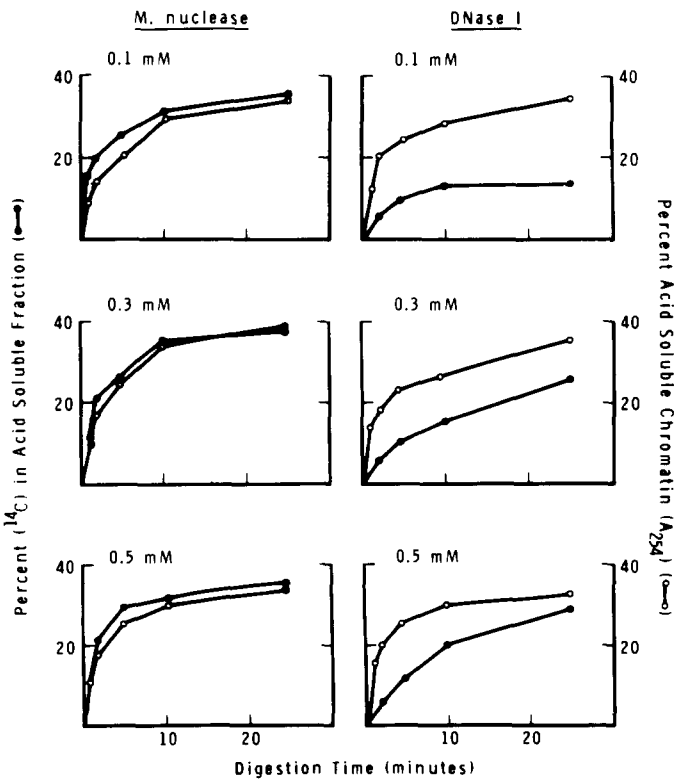

Fig. 4. Limit digest of murine bone marrow nuclei from cells treated with [^{14}C]chloroethyl GANU.

over the 25-min period. Micrococcal nuclease initially cleaves the DNA of the internucleosomal regions of chromatin. Therefore, these kinetic data demonstrate a preferential alkylation of the DNA of L1210 nucleosome core particles: the release of ^{14}C was less than the percentage of total chromatin digested. The initial specificity of micrococcal nuclease for internucleosomal regions has been well documented, and early digestion products that become acid soluble are predominately small base pair fragments from the linker regions. In contrast to micrococcal nuclease, DNase I attacks DNA of both core particle and linker at early time points. Digestion of alkylated products with DNase I is a useful comparison to micrococcal nuclease, since proportionately more of the absorbance at 254 nm represents core particle DNA. The digestion kinetics for DNase I are consistent with the results obtained with micrococcal nuclease, demonstrating preferential alkylation of core particle DNA. The time course of chromatin digestion (as measured by percent acid soluble chromatin) was comparable at the three drug concentrations, so drug binding to chromatin did not alter the kinetics of enzymatic digestion.

Figure 2 presents the kinetics of chromatin digestion for L1210 cells incubated with [^{14}C]chloroethyl CCNU. These results demonstrate that CCNU, like chlorozotocin, preferentially alkylated the DNA of L1210 nucleosome core particles. Similar results were obtained for GANU and ACNU.

Figure 3 presents the kinetics of nuclease digestion for chlorozotocin-treated murine bone marrow cells. These digestion kinetics differ from the results with L1210 cells. Micrococcal nuclease digestion resulted in a slightly greater percentage of ^{14}C counts released than the percentage of chromatin digested, demonstrating a preferential alkylation of internucleosomal linker regions of bone marrow chromatin by chlorozotocin. Drug concentrations up to 0.5 mM gave comparable results. The DNase I digestions were consistent with preferential alkylation of internucleosomal regions. Figure 4 presents the kinetics of digestion for GANU-treated bone marrow cells. GANU, like chlorozotocin, preferentially alkylated the internucleosomal regions of bone marrow chromatin.

In contrast to these results with chlorozotocin and GANU, Figs. 5 and 6 show that the bone marrow toxic CCNU and ACNU preferentially alkylated nucleosome core particles of bone marrow chromatin.

Experiments were performed using RNase to determine the percentage of chromatin alkylation due to alkylation of nuclear RNA. Digestion with RNase T1 and T2 released approximately 85% of the tritium incorporated during the [^{3}H]uridine pulse, confirming that the digestion conditions used released most of the chromatin-associated RNA. Less than 1% of the [^{3}H]thymidine pulse was released. For all four chloroethylnitrosoureas, approximately 15–18% of the total nuclear alkylation

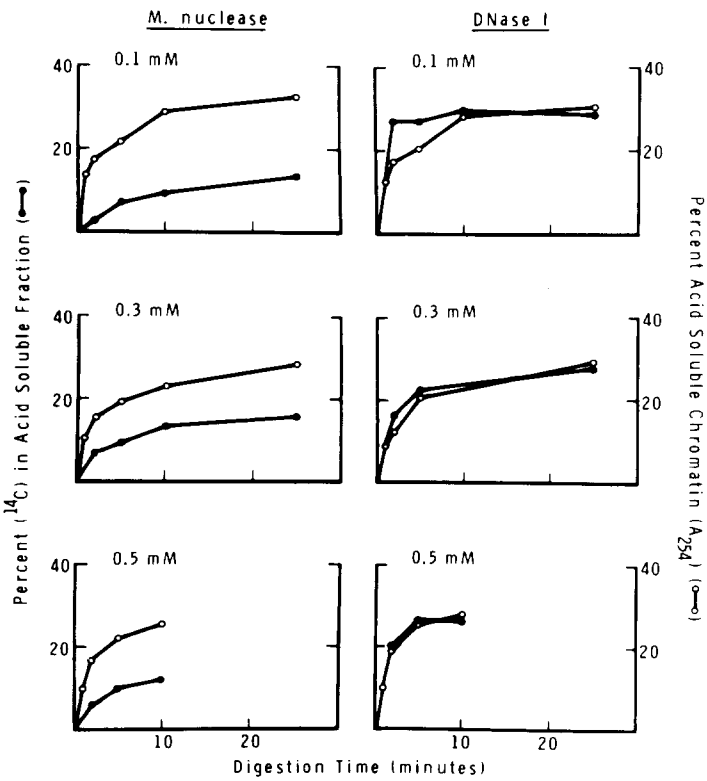

Fig. 5. Limit digest of murine bone marrow nuclei from cells treated with [^{14}C]chloroethyl CCNU.

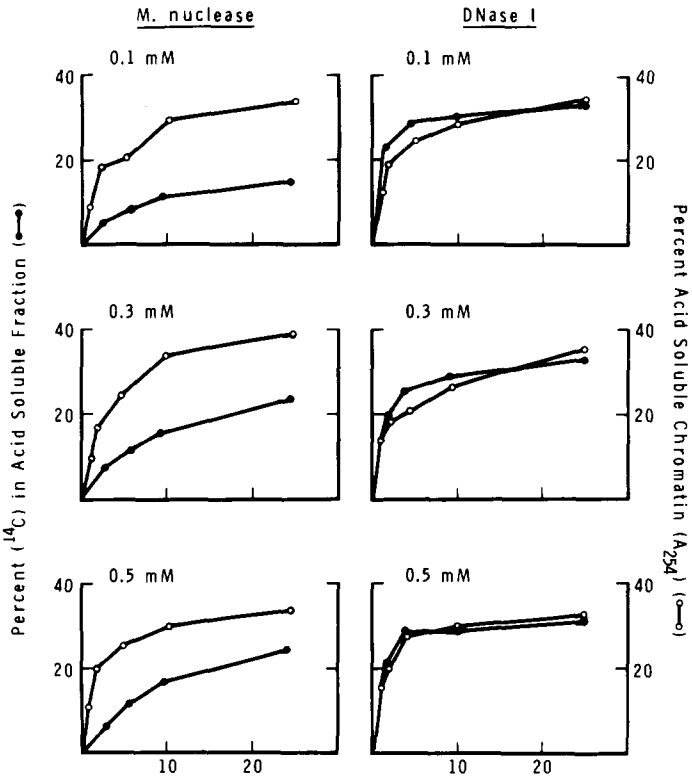

Fig. 6. Limit digest of murine bone marrow nuclei from cells treated with [^{14}C]chloroethyl ACNU.

was in RNA. DNase I digests nRNA, while micrococcal nuclease does not. However, since 18% or less of total nuclear alkylation was in nRNA, this value may not be quantitatively significant.

DISCUSSION

The chloroethylnitrosoureas, like many anticancer agents, are characterized by their inability to discriminate effectively between target and normal tissues. All of the clinically available compounds of this class of alkylating agents produce serious treatment-limiting bone marrow toxicity in humans [2, 3]. Chlorozotocin, a new glucose chloroethylnitrosourea, is therefore unique in possessing curative *in vivo* antitumor activity against the murine L1210 leukemia at doses that are minimally bone marrow toxic [5, 9]. GANU, a second glucose analog, has similarly demonstrated the property of reduced bone marrow toxicity at optimal therapeutic doses [5, 7]. This is in contrast to BCNU, CCNU and ACNU, nitrosoureas that produce optimal L1210 antitumor activity only at doses that are severely myelotoxic [5, 10].

To determine possible mechanisms that contribute to this reduction in bone marrow toxicity, we quantitated the *in vitro* alkylation of DNA produced by equimolar concentrations of chlorozotocin, GANU, CCNU and ACNU, For GANU, alkylation of DNA was 1.3-fold greater in L1210 leukemia as compared to murine bone marrow cells. This ratio of L1210:bone marrow DNA alkylation (1.3) was comparable to the ratio we have previously reported for chlorozotocin [9]. In contrast, the ratio of alkylation for the myelotoxic ACNU was 0.66, similar to our previous finding of 0.59 for CCNU. The consistency of these results suggests that the DNA alkylation ratio may provide a quantitative explanation for the decreased bone marrow toxicity of chlorozotocin and GANU. This differential alkylation of bone marrow and leukemia DNA may be related to the mechanism of drug transport across cell and/or nuclear membranes in the two cell types. Differences in the metabolism of these compounds by bone marrow as compared to L1210 cells may also be a contributing factor. In addition, preliminary data from our laboratory demonstrate that the alkylating moiety of CCNU has a higher covalent binding affinity for macromolecules associated with the nuclear matrix in HeLa cells [28]. In comparison, chlorozotocin demonstrates less specificity for matrix binding and selectively attacks the functional chromatin subfractions, which may contribute to the increased cytotoxic potential of chlorozotocin for tumor cells.

At the molecular level, our experiments demonstrate that the chloroethylnitrosoureas interact with L1210 leukemia, and murine bone marrow, chromatin in a non-random manner. All four drugs preferentially alkylated DNA within the nucleosome core particles of L1210 chromatin. CCNU and ACNU also preferentially alkylated core particle DNA of bone marrow chromatin. In contrast, the glucose analogs, chlorozotocin and GANU, predominately alkylated the internucleosomal linker regions in this tissue. These results demonstrate that members of the same class of antitumor agent may have

different tissue-specific binding sites within the chromatin substructure. The differential toxicity of chlorozotocin and GANU for bone marrow and tumor cells, as compared to CCNU and ACNU, may be mediated in part by these observed qualitative differences in sub-nucleosomal alkylation sites. The specific site of drug interaction within the cell nucleus may be as important as the type of lesion produced.

The repetitive nucleosomal organization of eucaryotic chromatin is well established. However, the importance of chromatin substructure in DNA replication, transcription and repair is not yet resolved. Compton *et al.* [29] have reported the nucleosomal repeat length of hamster ovary cells to be conserved in mitotic chromosomes, with a repeat length comparable to that found in interphase chromatin. Similarly, no difference in repeat length was found between confluent and exponentially growing C6 rat glial tumor cells [30]. These results suggest that in our studies the proportion of S phase cells should not be a factor in determining the percentage of alkylation sites in core particle compared to internucleosomal regions.

It has been shown that the nucleosomal substructure of chromatin influences the repair of DNA. The kinetics of repair of alkylated DNA lesions within specific regions of chromatin are currently under investigation, and experiments from several laboratories have demonstrated DNA lesions within internucleosomal linker regions to be more rapidly repaired than comparable lesions in the nucleosome core particles. Bodell [31] found DNA repair to be concentrated in the internucleosomal regions of mouse mammary cell chromatin after methyl methanesulfonate methylation. Similarly, studies by Cleaver [32] and Smerdon *et al.* [33] demonstrated that the initial sites of repair in human fibroblast cells following u.v.-induced DNA damage were in the internucleosomal linker regions. An increased efficiency of repair of internucleosomal DNA would be consistent with increased accessibility to repair enzymes and with the decreased myelotoxic properties of chlorozotocin and GANU, which produce proportionately more lesions within linker regions of bone marrow chromatin. In preliminary experiments, we have quantitated alkylation of murine bone marrow, and L1210 leukemia, DNA to determine the role of the glucose moiety and carbamoylating activity on the repair of alkylated lesions [34]. Binding to phenol-extracted DNA was quantitated at 2 hr of drug incubation and after 6 and 12 hr of repair. In both cell types, CCNU and GANU demonstrated only 5–13% repair after 6 hr, in contrast to 40% repair with chlorozotocin. At 12 hr, there was 50% repair with chlorozotocin compared to 20% with GANU and 10% with CCNU. These results suggest that the carbamoylating activity of CCNU inhibits repair of alkylated lesions. GANU (non-myelosuppressive) similarly inhibits repair, consistent with its carbamoylating activity. The reduced myelotoxicity of chlorozotocin and GANU may be more related to the localization of DNA lesions within chromatin than to the rate and extent of repair. This is consistent with our previous results with the methylnitrosoureas, streptozotocin and 3-β-D-glucopyranosyl-1-methyl-1-nitrosourea [35].

This study has expanded our understanding of the structure–activity relationships for this class of alkylating agents, since there are important differences in the chemical activities of the four nitrosoureas. CCNU and GANU have significant chemical carbamoylating activity, in contrast to chlorozotocin and ACNU. Chlorozotocin, GANU and ACNU are water soluble, but CCNU is highly lipid soluble. Our previous studies demonstrated that neither antitumor activity nor myelotoxicity could be correlated with carbomoylating activity or water solubility [4, 5]. These experiments demonstrate no correlation between the relative chemical carbamoylating activities of the four drugs and either the DNA alkylation ratio or the preferred chromatin alkylation sites.

In summary, both chlorozotocin and GANU, the glucose analogs, have significant L1210 antitumor activity with reduced murine bone marrow toxicity. This may be correlated with an advantageous ratio of L1210:bone marrow DNA alkylation and preferential alkylation of the internucleosomal regions of bone marrow chromatin. The results of these structure–activity studies should be important in future rational design of new alkylating agents, particularly the nitrosoureas. It will be important to determine whether addition of a glucose or glucosamine carrier results in a comparable reduction in bone marrow toxicity for other classes of alkylating agents.

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