Modification of L1210 Cell Nuclear Proteins by 1-Methyl-1-nitrosourea and 1-Methyl-3-nitro-1-nitrosoguanidine

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ABSTRACT

1-Methyl-1-nitrosourea (MNU) and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) are potent carcinogens which alkylate nucleic acids. Little is known about their reactions with nuclear proteins. Protein binding occurs via two mechanisms for each compound: alkylation (both), carbamoylation (MNU), or guanidination (MNNG). MNU and MNNG were obtained with 14C labels to investigate these reactions. Nuclear proteins were isolated from L1210 cells following one hr of incubation at 37° with the labeled carcinogens in concentrations from 0.038 to 3.8 mM. Separation of the proteins by sodium dodecyl sulfate gel electrophoresis and Triton-acetic acid-urea gel electrophoresis yielded comparable results. All histones and many nonhistones were modified. At 0.38 mM, binding ranged from the lowest detectable level of 0.1 pmol of [carbonyl-14C]MNU per nmol of H4 (carbamoylation) to 13.1 pmol [methyl-14C]MNNG per nmol of H2A. MNNG is approximately 12 times as potent as is MNU at that concentration and shows more binding than does MNU at all doses for both types of reactions. H1 is more strongly guanidinated than are the other histones (11.0 pmol/nmol at 0.38 mM) whereas H2B and H3 are the major targets of carbamoylation (0.9 and 1.8 pmol/nmol, respectively, at 0.38 mM). H1 and H2A are most strikingly methylated by both drugs. Generally, there is twice as much binding to acid-soluble nonhistones. This study shows that nuclear protein modification occurs with exposure to MNU and MNNG.

INTRODUCTION

MNU and MNNG are potent carcinogens which react covalently with DNA (14, 24), RNA (13), and proteins (25). These reactions are the consequence of spontaneous solvolysis of each compound at physiological pH into 2 chemically reactive entities. Both drugs yield methyl carbononium ions which act as alkylating agents (27). In addition, MNU produces an isocyanate ion that carbamylates ε-amino groups of lysine (27) while the decomposition of MNNG generates a guanidinating group which reacts similarly with lysine to form nitrohomoarginine (16). Previous work has emphasized possible relationships between the carcinogenicity of these compounds and their ability to alkylate DNA (9, 10, 13-15). In view of evidence that methylation at the N7 position of guanine bears little relation to neoplastic transformation, recent attention has focused upon production of O6-methylguanine and upon its rate of excision repair as critical to that process (10, 15). However, in addition to DNA alkylation, it is possible that modification of other nuclear constituents such as histones or nonhistone proteins could alter the fidelity of genetic replication so as to produce a stable clone of transformed cells. Such concepts are not new. In 1963, Pint and Heidelberger (18) suggested that modification of proteins, RNA, or other cellular constituents could contribute to the transition to neoplasia. Direct modification of DNA is not necessarily essential to neoplastic transformation since ethionine produces hepatomas in rats but presumably does not interact directly with DNA (2).

Nitrosoureas and nitrosoguanidines are known to interact with nuclear proteins. Cheng et al. (4) and Schmall et al. (21) demonstrated that 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, which in analogy to MNU produces a carbamoylating isocyanate during decomposition, modifies lysine residues of histones and other proteins in vitro. Alkylation of these proteins was far less extensive than was carbamoylation under the same circumstances. Subsequently, Woolley et al. (30) examined the interaction of CCNU with the nuclear proteins of L1210 cells in culture and observed the carbamoylation of histones, particularly H1. Similar results have been reported for MNNG. Nagao et al. (17) reported guanidination and methylation of whole histone fractions from ascites hepatoma cells treated with MNNG. This was also observed in vitro (20) in histones isolated from the glandular stomach epithelium of rats given intragastric [guanidino-14C]MNNG, although the modification of individual histone species was not examined.

Recent advances in the elucidation of chromatin structure and function (for review, see Ref. 11) make it desirable to extend these data. Individual histone species are located at different sites in the chromatin complex, and since histone modification alters the properties of chromatin (for review, see Ref. 27), identification of carcinogen binding to individual proteins is important.

The present study has examined the modification of nuclear proteins of L1210 cells in culture by MNU and MNNG. This model system was chosen to compare data with other results in the same cells and to develop methodology prior to beginning experiments in animals. Preparations of radiolabeled drug were available that permitted examination of carbamoylation and alkylation by MNU and of guanidination and alkylation by MNNG. The results indicate that both drugs produce modification of histones
and nonhistones, with the guanidination reaction of MNNG being of particular importance.

**MATERIALS AND METHODS**

MNU was obtained from the Experimental Drug Branch, National Cancer Institute, and MNNG was purchased from Aldrich Chemical Co., (Milwaukee, Wis.). \([\text{carbonyl-}^{14}\text{C}]\text{MNU (13.3 mCi/mmol)}\) and \([\text{methyl-}^{14}\text{C}]\text{MNNG (19.9 mCi/mmol)}\) were obtained as stock items from New England Nuclear, Boston, Mass. \([\text{carbonyl-}^{14}\text{C}]\text{MNU (7.9 mCi/mmol)}\) and \([\text{guanidino-}^{14}\text{C}]\text{MNNG (14.3 mCi/mmol)}\) were custom synthesized by New England Nuclear at a radiochemical purity of >97%. This purity was confirmed by paper chromatography prior to use. Other chemicals were reagent grade.

L1210 cells in suspension culture were maintained at 37° in Roswell Park Memorial Institute Medium 1630 with 20% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.).

**Assay of in Vitro Carbamoylating or Guanidinating Activity.** As a check that the modification of lysine by these drugs would be observed, MNU and MNNG were incubated with radiolabeled lysine following the method of Wheeler et al. (29). Two μCi of \(L-[^{3}\text{H}]\text{lysine}\) were diluted with 0.42 μmol of the unlabeled compound in sodium phosphate buffer (pH 7.3), and unlabeled MNU or MNNG (0.42 μmol) was dissolved in the same buffer. A control was the amino acid in the absence of MNU and MNNG. Incubation time was 6 hr at 37°. Ten μl of each sample were then spotted on Whatman No. 3 chromatography paper, and modified lysine was separated from the unreacted drugs and amino acid at 3000 V for 75 min in 0.1 M sodium phosphate buffer (pH 6.0). The paper was dried, cut into one-inch squares, and counted. Distinct peaks of radioactivity not coincident with those produced by labeled lysine alone were assumed to represent carbamoylated or guanidinated products. This assumption was shown to be valid for CCNU and lysine in a similar experiment by Wheeler et al. (29). For MNNG, Nagao et al. (17) hydrolyzed histones from MNNG-treated ascites hepatoma cells and found that the major reaction product cochromatographed with authentic nitromonoarginine, the guanidination product of lysine. Carbamoylating or guanidinating activity was expressed as the percentage of total dpm on the strip that was associated with peaks of the reacted products.

**Treatment of Cells with Labeled Drugs.** Two types of experiments were done. In the first, unlabeled L1210 cells were treated with the \(^{14}\text{C}-\text{labeled drugs}, and nuclear proteins were isolated for electrophoresis. In the second, \(L-[^{3}\text{H}]\text{valine}\) (Amersham/Searle Corp., Arlington Heights, Ill.) was added to the cell culture one doubling time prior to dividing the culture into aliquots for drug treatment. Thus, equivalent amounts of radiolabeled valine were incorporated by all cell samples. This procedure was performed for later quantitation of extracted nuclear proteins. In both sets of experiments, cells were harvested by centrifugation immediately preceding carcinogen treatment and resuspended in fresh, serum-free medium to increase their concentration.

All incubations of cells with radioactive MNU and MNNG were for 1 hr at 37°. Thirty μCi of each labeled compound were added to sufficient unlabeled drug to achieve a particular total drug concentration. In the initial experiments where \(^{3}\text{H}-\text{labeled valine was not used}, all cells were suspended at 10⁶/ml, and final drug concentrations were: \([\text{carbonyl-}^{14}\text{C}]\text{MNU, 3.8 mM;}\) \([\text{guanidino-}^{14}\text{C}]\text{MNNG, 2.2 mM;}\) \([\text{methyl-}^{14}\text{C}]\text{MNU, 2.1 mM;}\) \([\text{methyl-}^{14}\text{C}]\text{MNNG, 1.5 mM.}\) In experiments with \(^{3}\text{H}-\text{labeled valine}, all 4 drugs were tested at each of 3 doses: 0.038, 0.38, and 3.8 mM. This was achieved by varying the concentration of cells in suspension: \(10^{8}/\text{ml in 100 ml at 0.038 mM, 10^{7}/\text{ml in 10 ml at 0.38 mM, and 10^{6}/\text{ml in 1 ml at 3.8 mM while maintaining constant amount of carcinogen. After drug treatment, cells were washed in ice-cold spinner salts and frozen at ~20°. Subsequent steps were performed at 2−4° unless otherwise indicated.}

**Preparation of Acid-soluble Nuclear Proteins.** Nuclei were prepared by the method of Sporn et al. (22). The pelleted nuclei were extracted twice with 1 ml 0.4 N H₂SO₄ for 2 hr, and these supernatants were pooled. Histones and other acid-soluble nuclear proteins were precipitated in 20% trichloroacetic acid overnight, and the resulting pellets were washed with acidified acetone (10 mM HCl) and pure acetone.

**Triton-Acetic Acid-Urea Gel Electrophoresis.** Acid-extracted proteins from cells not prelabeled with \(L-[^{3}\text{H}]\text{valine}\) were dissolved in a solution containing 8 M urea, 4% 2-mercaptoethanol, 5% acetic acid, and 10% sucrose, and separated on 12% polyacrylamide-Triton-acetic acid-urea gels at pH 2 to 3 (1). The completed gels were stained with 0.2% Coomassie blue in 50% methanol-7% acetic acid, destained in 20% methanol-7% acetic acid, and photographed on Polaroid type 55 film, which renders both negative and positive images. Gels were treated with 20% trichloroacetic acid to fix proteins and then dehydrated with 2 changes of dimethyl sulfoxide. The slab was prepared for fluorography in a third dimethyl sulfoxide solution with 22% PPO and dried on Whatman No. 3 paper after rehydrating with water. Pre-print Kodak SB-5 X-ray films were exposed to the gels at ~70° in a magazine with DuPont intensifying screens. An Ortec 4310 densitometer was used to scan the fluorograms and the corresponding dye patterns from the Polaroid negatives.

**SDS Gel Electrophoresis.** Acid-extracted proteins from cells prelabeled with \(L-[^{3}\text{H}]\text{valine}\) were dissolved in water containing 1% 2-mercaptoethanol. The protein concentration of an aliquot of each sample was assayed using a Bio-Rad determination (3), with a series of solutions of desiccated calf thymus histone (Worthington Biochemical Corp., Freehold, N. J.) as standard. Another aliquot of the acid-extracted protein was counted, and a value was thus derived to relate \(^{3}\text{H} \text{dpm to μg of protein.}

The remainder of each sample was added to buffer containing 0.5 M Tris HCl (pH 6.8), 7% SDS, and 8 M urea and electrophoresed on 15% polyacrylamide slab gels (12). Separating gel and electrode buffer were at pH 8.8. After the gels were stained and photographed (as above), histone bands were cut out with a scalpel. The remainder of each column of the slab contained acid-soluble, nonhistone proteins and was sliced into 7 parts. Gel slices were placed in counting vials with 200 μl perchloric acid and 400 μl 30% H₂O₂ and incubated overnight at 60° with intermittent shak-
RESULTS

In Vitro Carbamoylation and Guanidination. Since lysine is the primary amino acid target of carbamoylation and guanidination by MNU and MNNG (17, 29), the relative efficacy of these compounds in producing modification of lysine was assayed using paper electrophoresis (Table 1). The results show that after 6 hr of incubation, 50% of lysine present in the original reaction mixture with MNU existed in a modified form while 71% of that present with MNNG was modified.

Triton-Acetic Acid-Urea Gels. Quantitative estimates of histone molality were not made in gels run by this method, but qualitative comparisons of the amount of drug bound to different histone fractions were obtained. The densitometric scan of the gel pattern is an index of the quantity of protein separated, while the scan of the fluorogram is a measure of bound carcinogen. These scans are shown (Fig. 1) superimposed for each of the labeled drug preparations, providing estimates of the relative degree of methylation, carbamoylation, and guanidination.

[methyl-1-C]MNU (Scan D). Methylation of proteins by MNU was the least extensive modification and required the longest period of exposure of the fluorogram. Two major peaks of drug binding to proteins near the origin of the gel. These may represent minor histone variants M2 and M3 (7). Corresponding peaks are found for all 4 of the labeled carcinogens. MNU also produced methylation of the HMG proteins. The estimated extent of histone modification was on the order H2A > H1 > H2B > H3 > H4.

[carbonyl-14C]MNU (Scan A). Carbamoylation was quantitatively greater than was methylation by MNU, but the drug concentration was correspondingly greater. H1 was more modified than were core histones which decreased in binding: H2B > H2A > H3 > H4.

[methyl-1-C]MNNG (Scan E). Like methyl-labeled MNU, this compound was most reactive with 2 proteins separating near the origin of the gel. Histone modification followed in the order H2A > H1 > H3 > H2B > H4.

[guanidino-14C]MNNG (Scan B). The most extensive of the reactions was MNNG-induced guanidination. The degree of protein binding was so high that the fluorogram in Fig. 1 is half-scale relative to the others. Once again, 2 peaks of activity near the gel origin were observed. H1 and H2B showed the most guanidination followed by H2A > H3 > H4.

SDS Gels. Data in Table 2 are the ratio of pm quantities of drug to nm quantities of histone associated with individual bands on SDS gels.

At the lowest dose (0.038 mM), modification by the labeled MNU was below limits of detection. At the higher doses, there was more modification by [carbonyl-14C]MNU in the H3 and H2B fractions than in H1 and H2A. Methylation at a 0.38 mM concentration of [methyl-14C]MNU was most pronounced in H1, but at 3.8 mM MNU, the value of H2A (6.0 pmol/nmol) was slightly in excess of that of H1 (4.8 pmol/nmol). This was the same pattern found with the Triton gel separation at the 2.2 mM concentration.

There is little increase in binding at the 3.8 mM concentration of methyl-labeled MNNG compared to the intermediate 0.38 mM dose. H1 and H2A are consistently the prominent

Table 1

<table>
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<th>Compound name</th>
<th>Chemical structure</th>
<th>% Carbamoylated or guanidinated lysine</th>
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<td>1-Methyl-3-nitro-1-nitrosoguanidine</td>
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a △, [methyl-14C]MNU; ▲, [carbonyl-14C]MNU; ▼, [methyl-14C]MNNG; ●, [guanidino-14C]MNNG.

Table 2

<table>
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<tr>
<th>MNU and MNNG binding to L1210 cell histones</th>
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<th>Histones from [14C]MNU- and [14C]MNNG-treated L1210 cells (pretreated with L-[3H]valine) were separated on 15% polyacrylamide-SDS gels.</th>
<th>pmol drug/nmol histone at following drug concentrations</th>
<th>0.038 mM</th>
<th>0.38 mM</th>
<th>3.8 mM</th>
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<td>H1</td>
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* ND, not detectable.
targets of methylation by this compound.

Results for the guanidino label also confirm those obtained with the Triton gel. This reaction is on the order of 100 times as great as methylation of proteins by MNNG. H1 is the predominant modified species, but H2A and H2B are also susceptible.

Acid-soluble nonhistones are considered as a group in Table 3. On the basis of protein weight, there is more binding to the nonhistones than to the histones for all labels at all doses. Binding to nonhistones substantially increased with increasing carcinogen concentration from 0.38 to 3.8 mM for both the carbonyl- and the guanidino-labeled drugs relative to the histones. There is no change in the ratio of nonhistone to histone binding with increasing concentration for the methyl-labeled drugs.

There is preliminary evidence (data not shown) from acid-insoluble nonhistone protein separation (10% polyacrylamide-SDS gels) that the majority of these nuclear proteins are modified by both ends of both MNU and MNNG.

**DISCUSSION**

These data show that nuclear proteins are modified by the carcinogens MNU and MNNG.

In measuring binding, 2 different electrophoretic systems were used for protein separation. Triton-acetic acid-urea gels separate according to hydrophobicity and charge, whereas SDS gels operate on the basis of molecular weight. Furthermore, they are run at different pH values; the Triton gel is acidic (pH 2 to 3), and the SDS gel is alkaline (pH 8.8). The corroborative results diminish a number of objections attendant to the use of a single separatory procedure. Among them is the possibility that radioactivity not covalently bound to a protein could comigrate with the acid extracted histones in an acidic gel. Differential migration of unaltered and drug-bound proteins might have been expected in the Triton gel or proteins of similar molecular weight could comigrate in the SDS gels. To examine the former, Sudhakar et al. ran a similar experiment with [carbonyl-14C]MNU-treated HeLa cells. They separated histones in 2 dimensional gels, Triton in the first dimension and SDS in the second. Drug-labeled proteins migrated to the same position as did marker histones. Some combination of the above factors probably explains the discrepancy found with the carbonyl-labeled MNU where H1 and H2B were the dominant modifications seen in the Triton gel compared to H2B and H3 in the SDS gel. An additional complication is the fact that H3 separates at multiple loci in the Triton gel.

MNNG was considerably more reactive than was MNU. This could be a function of the kinetics of solvolysis of the 2 compounds. A half-life of 48 min was reported for MNNG in phosphate buffer at pH 7.85 (14), whereas Wheeler et al. (27) reported a 7-min half-life for MNU in phosphate buffer at pH 7.4. Cellular incubations were for 1 hr. Nonetheless, in the 6-hr chemical incubation with lysine, MNNG was more active. The presence of intracellular thiols might have accelerated the decomposition and subsequent activation of MNNG in the L1210 cell incubations (14).

In an earlier study (17), MNNG binding to whole histone fractions of ascites hepatoma cells was found to be of the same order of magnitude as in the present report. These workers identified nitrohomoarginine as the major guanidination product, indicating that lysine was the primary target residue as indicated by McCalla and Reuvers (16). This is consistent with the observations that the very lysine-rich histone H1 is most affected by [guanidino-14C]MNNG. It also agrees with the high reactivity of MNNG with lysine seen in the *in vitro* guanidination experiments.

H4 was shown to be the least modified of all the histones following either MNU or MNNG treatment. Such reduced drug binding may relate to the nucleosome core location of histone H4 (11), this region being sterically less accessible to these drugs. Additionally, the Triton gel pattern showed polyacetylation of H4 and was similar to H4 acetylation patterns seen in cells treated with sodium butyrate and CCNU (8, 26). Since lysine residues are primary sites of acetylation (19), carbamoylation, and guanidination, it is possible that prior histone H4 acetylation interfered with drug binding.

Other workers have compared MNNG binding to proteins and nucleic acids. At a concentration of 0.35 mM in ascites hepatoma cells, there are 1.1 nmol of [methyl-14C]MNNG per mg of DNA. This is greater than the total binding to the histones which was reported as 0.3 nmol/mg for guanidination and 0.17 nmol/mg for alkylation (17). Guanidination of DNA and RNA was not observed. Methylation of RNA was slightly in excess of that seen for DNA (1.4 nmol/mg). In *vivo*, the total binding to protein was approximately equal to DNA binding (20). Such data are consistent with the hypothesis that the modification of all 3 types of macromolecule might contribute to the action of these carcinogens.

The present studies in a transformed cell line do not show directly which of these protein modifications may be relevant to the carcinogenic activity of MNU and MNNG. Since the histones are identical in L1210 and nontransformed cells, they do provide a model that indicates which protein species may be expected to be modified *in vivo* and thus represent a useful point of departure for further studies in animals. Nonhistone proteins are believed to be important in the regulation of gene expression, and most of the nonhistones examined in this study showed evidence of drug binding. It may be that no single site of chromatin modification will adequately correlate with carcinogenicity.
More plausibly, a combination of carcinogen binding to nucleic acids and to nuclear proteins involved in repair of DNA and regulation of gene expression is involved in neoplastic transformation. For example, Fornace et al. (6) demonstrated that the ligase step of excision repair of DNA is inhibited by the carbamoylating moiety of 1,3-bis(2-chloroethyl)-1-nitrosourea, cyclohexyl isocyanate. More insight will be obtained by extending these experiments to animal tissues that exhibit varying susceptibility to tumor induction by these agents.

REFERENCES

Fig. 1. Binding of MNU and MNNG to nuclear proteins. Top, typical stain pattern representing acid-extracted proteins on a Triton-acetic acid-urea gel from carcinogen-treated L1210 cells. All show densitometric scans of the stained gels and their corresponding superimposed fluorograms. The scale for the stain patterns is the same for all scans. The fluorogram scale for Scan B is one-half that of the others. Scan D is taken from a fluorogram exposed approximately 3 times as long as the others. Left, origin. Proteins are labeled following the procedure of Ref. 6: Scan A, [carbonyl-14C]MNU (3.8 mM); Scan B, [guanidino-14C]MNNG (2.1 mM); Scan C, [methyl-14C]MNU (2.2 mM); Scan D, [methyl-14C]MNU long exposure fluorogram (2.2 mM); Scan E, [methyl-14C]MNNG (1.5 mM). The scans are not linear over the range of densities obtained. ——, stain; - - - -, fluorogram.
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