Binding of 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea to L1210 Cell Nuclear Proteins

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INTRODUCTION

The mechanism of action of CCNU has been postulated to relate to its ability to modify macromolecules. It was shown by Montgomery et al. (4) that the spontaneous decomposition of CCNU in aqueous solution probably proceeded by way of an intermediate organic isocyanate, cyclohexylisocyanate, which is a reactive species capable of forming carbamoylated derivatives with primary amino groups. These authors suggested that such modification of primary amino groups in proteins could be relevant to the biological effects of the drug. Subsequently, it was shown by Cheng et al. (1) that the cyclohexyl and chloroethyl groups of CCNU possessed different reactivity and specificity toward different macromolecules. The cyclohexyl group was capable of modifying proteins such as albumin and histone, and also polysynine, but had virtually no reactivity with either DNA or RNA. The chloroethyl moiety showed some capacity to react with protein, albeit only 5 to 10% that of the cyclohexyl group, but had definite reactivity with DNA and rRNA. On this basis it was suggested that CCNU might act in a dual capacity to modify macromolecules by (a) carbamoylation via cyclohexylisocyanate; and (b) alkylation via a carbonium ion generated from the chloroethyl portion of the molecule.

SUMMARY

The binding of 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea (CCNU) to the proteins of the L1210 cell nucleus has been studied using both [cyclohexyl-14C]CCNU and [chloroethyl-14C]CCNU. Most of the bound [cyclohexyl-14C] moiety of CCNU was found to exist in a form that was stable in acid solution but labile and dialyzable in alkaline solution. A small amount of the cyclohexyl moiety was bound to histones in a stable, nondialyzable form. The drug/protein ratio for the H1 histone was about 0.01 to 0.02 mole/mole. No binding of the cyclohexyl group to acidic proteins or of the chloroethyl group to either histones or acidic proteins was observed. Thus, the interaction of CCNU with the proteins of the cell nucleus can be defined in terms of the modification of histones by the cyclohexyl moiety.

MATERIALS AND METHODS

Cell Culture. Murine L1210 leukemia lymphoblasts were maintained in stock culture in Roswell Park Memorial Institute Medium 1630 supplemented with 20% fetal calf serum. The stock culture was established to be free of Mycoplasma. Working cultures were supplemented with penicillin and streptomycin. Cell counts were performed using a Model B Coulter counter, and cell viabilities were estimated by trypan blue exclusion test. Viable cell numbers represent the product of cell number and estimated fraction of viable cells in a given culture.

Radioisotope Incorporation. The incorporation of [3H]thymidine and [14C]valine into perchloric acid-precipitable material by CCNU-treated cells was examined. [methyl-3H]Thymidine and [14C]valine were products of New England Nuclear, Boston, Mass. CCNU (NSC-79037) was obtained from Drug Research and Development Program, National Cancer Institute, Bethesda, Md. The drug was made up in a stock ethanol solution and added in small aliquots to cultures containing 3.0 x 10^6 cells. After the desired period of drug exposure, the culture was pulse-labeled for 10 min with [3H]thymidine, 0.5 μCi/ml, and [14C]valine, 0.2 μCi/ml, then cooled in iced 0.9% NaCl solution, and washed three times. The washed cell pellet was digested in 0.5 N NaOH, then cooled in iced 0.9% NaCl solution, and washed three times. The washed cell pellet was digested in 0.5 N NaOH.
neutralized, and counted in 70% toluene/30% Triton X-100 solvent containing Liquifluor (New England Nuclear), using a Packard Tri-Carb scintillation counter.

Preparation and Chromatography of Nuclear Proteins. Two separate preparations of 14C-labeled CCNU were obtained from Monsanto Research Corporation, Dayton, Ohio, through Dr. H. Wood, Drug Development Branch, Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute. The preparation labeled in the cyclohexyl ring had a specific activity of 12.6 mCi/mmmole, while the preparation labeled in the 2 carbons of the chloroethyl group had a specific activity of 9.9 mCi/mmmole.

Cells were incubated with 10^-5 M CCNU in cultures containing approximately 15 x 10^6 cells/ml. Following incubation for 1 hr, cells were washed in PBS and whole nuclei were isolated by shearing the cells in a Dounce homogenizer (15 strokes with a loose-fitting pestle) in a medium of PBS containing 1.5 mm spermidine and 0.2% Triton X-100 (K. W. Kohn, unpublished observation). Examination of these preparations by electron microscopy showed the nuclei to be free of cytoplasmic tags and to lack an external nuclear membrane. Nuclei were separated from the cell homogenate by centrifugation through 50% sucrose.

Extraction and chromatography of nuclear proteins from preparations of whole nuclei were carried out by 2 methods. In the 1st method, the pelleted nuclei were washed with PBS containing 1.5 mm spermidine and then suspended in a solution of 2 M NaCl/5 M urea to dissociate nuclear proteins. This solution was centrifuged for 18 hr in a Beckman L2-65B ultracentrifuge at 176,000 x g to sediment DNA. The supernatant containing the whole nuclear protein was first dialyzed against a starting buffer of 0.3 M guanidium-HCl/5 M urea. The initial dialysis against a weakly alkaline buffer was performed to remove noncovalently bound products of CCNU which, it was found, were more effectively removed under alkaline conditions than under neutral or acidic conditions (see below). The chromatography of the whole nuclear protein was first carried out on an Amberlite CG-50 column (6 mm x 60 cm) and eluted at 3 ml/hr. After elution of the acidic proteins in the runoff at 0.3 M guanidinium, the histone proteins were eluted in 4 peaks with a gradient of 0.3 to 0.7 M guanidinium, followed by a step to 4 M guanidinium, all in 5 M urea. The order of elution was H3 and H4, H2A, H2B, and H1, as determined by electrophoresis of individual protein peaks on 15% acid-urea polyacrylamide gels (5). The H3 and H4 histones are not resolved but elute together in the 1st peak, which is broad and extends into and overlaps the H2A peak.

Acidic proteins collected in the runoff were extensively dia lyzed against 0.1 M acetic acid, lyophilized, and redissolved in 0.1 M NH4HCO3/5 M urea. Chromatography was performed on a Sephadex A-25 column (6 mm x 55 cm), using a gradient of 0.1 M to 0.8 M NH4HCO3 in 5 M urea, followed by a step to 1.5 M NH4HCO3/5 M urea.

In a 2nd method, H1 histone was dissociated from the remainder of the nuclear proteins by extraction of whole nuclei with H2SO4, pH 1.75/0.05 M NaCl. The residue from this extraction was retreated with 0.15 M H2SO4 to dissolve the remaining histones. Gel electrophoresis of the proteins from these 2 extractions showed that all of the H1 was present in the 1st extract, along with some carryover of other histones. However, the bulk of the histones other than H1 were present in the 2nd extract. The 2 acid extracts were dialyzed extensively against 0.1 M NH4HCO3 to remove noncovalently bound material and lyophilized. Gel filtration of these fractions was carried out on a Sephadex G-100 column (1 x 14 cm), using 0.1 M NH4HCO3, pH 8.1, as eluent. Although these mild conditions produced some aggregation, the H1 fraction eluted well. Order of elution of individual proteins was identified by lyophilizing the individual column fractions and analyzing them by polyacrylamide gel electrophoresis.

Gel Electrophoresis. Histones were analyzed on 15% acid-urea gels using the methods of Panyim and Chalkley (5). In general, gels were pretreated by electrophoresis for 3 hr, and running conditions were 6.5 hr at 125 V using 0.9 M acetic acid in upper and lower wells. Gels were stained overnight in 0.2% Amido black 10B in 7% acetic acid/20% ethanol and destained by soaking in the same solution.

RESULTS

Effects of CCNU on Cell Viability. Chart 1 shows the effects of various concentrations of CCNU upon cell viability as a function of time. The effect of 10^-8 M CCNU is barely detectable, while 2 x 10^-5 M CCNU produces 50% cell death in 24 hr and 10^-4 M CCNU is rapidly cytocidal to all cells in the culture.

Effects on DNA and Protein Synthesis. Chart 2 shows the effects of 10^-4 M and 10^-3 M CCNU upon the incorporation of [3H]thymidine and [14C]valine into perchloric acid-precipitable macromolecules. During the 1st 2 hr of exposure 10^-3 M CCNU is effective in substantially reducing incorporation of both exogeneous labels into perchloric acid-precipitable macromolecules. During the 3rd hr, incorporation of [14C]valine shows recovery. This was a reproducible finding. On the basis of this information, drug incubations were carried out for 1 hr at 10^-3 M CCNU.

Dependence of Stability of Drug Binding upon pH. Dur-
ing the early stage of this work it was found that variable degrees of apparent drug binding were being obtained. A study was done in which a preparation of nuclear protein treated with [cyclohexyl-\(^{14}\)C]CCNU was adjusted to pH values between 4 and 9 and then subjected to ultrafiltration through an Amicon PM-10 membrane by repeated washes with buffer at the given pH. The removal of [cyclohexyl-\(^{14}\)C] label was very effective (98%) at pH 8 or pH 9 but dropped markedly under mildly acid conditions to about 15% at pH 4 (Chart 3). Thus, the bulk of [cyclohexyl-\(^{14}\)C] label isolated with nuclear protein was in a labile form. This reaction was freely reversible by adjusting the pH of the system back and forth between 4 and 9, and thus appeared to represent the association with nuclear protein of a stable, water-soluble and ultrafilterable metabolite of the cyclohexyl moiety. This reaction is distinct from the covalent interaction between cyclohexylisocyanate and a primary amino group, which forms a substituted urea stable at pH 8. Recognition of the pH lability of a major part of the nuclear-bound drug required close attention to adequate alkaline dialysis of protein preparations.

**Isolation and Chromatography of Whole Nuclear Proteins from CCNU-treated Cells.** Cells were treated for 1 hr with 10^{-3} M [cyclohexyl-\(^{14}\)C]CCNU prior to extraction of the nuclear proteins. The elution of whole nuclear protein from the Amberlite CG-50 column is shown in Chart 4. The order of elution of histone proteins following the runoff was determined by polyacrylamide gel electrophoresis (see “Materials and Methods”). The 1st histone peak contains one-half of the H3 and H4 histones. The remaining half elutes with the H2A histone in the 2nd peak. A small part of the H4 histone elutes with the H2B histone in the 3rd peak. The 4th peak, the H1 histone, contains traces of H4 histone. The elution of CCNU labeled in the cyclohexyl ring is shown in Chart 4. The drug elutes as a broad and poorly defined band which is followed by a definite peak associated with the H1 histone. No peaks are seen in the association with the H2A and H2B histones. Since the H3 and H4 histones were incompletely separated from the other proteins on the Amberlite CG-50 column and overlapped into the H2A and H2B bands, the broad band of drug radioactivity which precedes the H1 peak probably represents, at least in part, binding to H3 and H4.

Chromatography of the nuclear acidic protein fraction on DEAE-Sephadex readily fractionated it into several peaks. No [cyclohexyl-\(^{14}\)C]CCNU was found to be associated with any of the acidic proteins.

When proteins extracted from cells similarly treated with [chloroethyl-\(^{14}\)C]CCNU were analyzed in the same way, no detectable isotope was associated with either the histone proteins or with the nuclear acidic proteins.

An independent method was used to establish that the drug migrating with the H1 histone represented protein-bound material rather than, for example, a free drug product which also eluted from the column with the step to 4 M guanidine. The H1 histone fraction was extracted from whole nuclei using H\(_2\)SO\(_4\), pH 1.75/0.05 M NaCl and, following dialysis against 0.1 M NH\(_4\)HCO\(_3\), pH 8.1, was lyophilized.
and redissolved in a small amount of 0.1 M NH$_4$HCO$_3$. Approximately 500 µg of this material were subjected to gel filtration on a Sephadex G-100 column in 0.1 M NH$_4$HCO$_3$. Although this alkaline pH does not represent ideal conditions for gel filtration of histones (3), it does represent maximum dissociating conditions of noncovalently bound drug from protein; thus, only covalently bound drug would be associated with the proteins. A clear separation of H1 from the other histone fractions was achieved, as confirmed by polyacrylamide gel electrophoresis of the individual column fractions. H1 eluted alone in the 1st peak (Chart 5) along with a peak of protein-bound [cyclohexyl-$^{14}$C] label. A smaller amount of [cyclohexyl-$^{14}$C] label was also eluted with the H2A-H2B-H3 group. The ratio of drug to H1 histone was calculated from the elution in Chart 5 to be 0.01 to 0.02 mole CCNU per mole protein. When this experiment was performed using [chloroethyl-$^{14}$C]CCNU, no $^{14}$C radioactivity was found in the histone fractions.

**DISCUSSION**

Previous studies have suggested that CCNU may act in a dual capacity to modify macromolecules (1, 7). One route is carbamylation of primary amino groups by cyclohexylisocyanate and the other is an alkylation reaction by a carbonium ion generated from the chloroethyl group (1). Both of these processes would be expected to yield stable covalent bonds. An important finding in this work was that the cyclohexyl group binds to nuclear proteins in a form that is labile under mildly alkaline conditions, yet stable under mildly acid conditions, in addition to binding in a form that is stable under both these conditions. The exact nature of the labile form of the drug has not yet been identified, but it appears to represent the binding of a stable decomposition product of CCNU, since the reaction was freely reversible with adjustments in pH. An alternative hypothesis would be that it represents the interaction of cyclohexylisocyanate with some group other than a primary amino group. However, the isocyanates are unstable compounds in solution and this type of reaction could not explain a situation in which the drug-protein interaction remained freely reversible over a prolonged period of time.

This work indicates that interaction of the decomposition products of CCNU with nuclear proteins results primarily in reaction of the cyclohexyl group with histones. The most readily modified histone is the lysine-rich H1, which undergoes reaction to the extent of 0.01 to 0.02 mole drug per mole protein. Quantitation of drug yields within the other histone fractions was difficult because of the low specific activities, but variable binding of the cyclohexyl group to the other 4 histones appears also to occur. By contrast, there was no demonstrable modification of the histones by the chloroethyl group of CCNU and no modification of acidic proteins by either the cyclohexyl or chloroethyl groups.

This pattern of reactivity is consistent with current concepts of chromatin structure, as well as with previous knowledge regarding the interaction of CCNU with macromolecules. For example, the lysine-rich H1 histone is known by a variety of evidence, such as its ease of dissociation (3) and ability to undergo chemical modification (8), to be a physically accessible molecule within the chromatin complex. This accessibility, as well as its high lysine content, makes H1 an ideal candidate for reaction with cyclohexylisocyanate. The H2B histone, however, is also lysine rich, but the present data suggest that it is less reactive than H1. This lack of reactivity may indicate that these lysine groups are sterically unavailable. Thus, stereochemical considerations, as well as availability of lysine groups, may govern the interactions of CCNU with nuclear proteins. The low reactivity of the acidic proteins may reflect both low lysine content and steric restriction. The failure to observe modification of histones by the chloroethyl moiety of CCNU is in accord with the data of Cheng et al. (1), which indicated that the reactivity of the chloroethyl group with protein is lower than that of the cyclohexyl group.

However, the data demonstrating the interaction of the cyclohexyl group of CCNU with amino groups of protein have been obtained in vitro (2, 7, 9). It seems reasonable to extrapolate these findings to the in vivo situation, but it remains to be demonstrated rigorously that the in vivo products of this reaction are indeed those observed in vitro.

Our data regarding the extent of drug binding to nuclear protein appear to agree well with those of other authors. Recently, Connors and Hare (2) have reported that the histones are especially susceptible to attack by the cyclohexyl moiety of CCNU. They were able to recover about 15,000 dpm of $^{14}$C derived from the cyclohexyl group of CCNU per mg dry weight of nuclear protein. This converts to about 0.01 mole drug per mole protein using the stated drug specific activity of 12.62 mCi/mmmole and assuming the molecular weight of histone to be 20,000. Also Cheng et al. (1) reported binding of about 4.0 nmoles [cyclohexyl-$^{14}$C]CCNU per mg histone in in vitro studies with isolated proteins, which corresponds to an approximate drug/protein ratio of 0.1 mole/mole. These same authors reported that about 430 pmoles [cyclohexyl-$^{14}$C]CCNU could be recovered per mg leukemia cell protein when leukemia-bearing mice were.
treated with the drug. Thus, the molar quantities of the cyclohexyl moiety of CCNU that have been observed bound to protein under various conditions are of the order of 0.01 to 0.1 mole/mole protein, and our data are in accord with these observations. Further studies would be useful to determine whether detectable structural or functional alterations of the chromatin complex result from the interaction with nitrosoureas. This is of particular interest in view of the report that alkylating agents such as chlorambucil can produce profound alteration in the protein composition of chromatin (6).

REFERENCES

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