Use of an indicator sequence of human DNA to study DNA damage by methylbis(2-chloroethyl)amine

(nitrogen mustard/chemotherapy/DNA damage)

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ABSTRACT A highly reiterated sequence of human DNA was used to determine the sites of modification of DNA caused by the anti-tumor drug methylbis(2-chloroethyl)amine (HN2, mechlorethamine, nitrogen mustard) upon treatment of cells in culture and of purified DNA. The lengths of the breakage products of the DNA treated with HN2 were compared to the lengths of DNA scission products produced by chemical reactions used for DNA sequence determination. HN2 was found to create alkali-labile lesions at positions of guanine. The distribution of the guanine modifications was the same for DNA extracted from cells treated with HN2 and for purified DNA treated with HN2. However, the extent of damage was at least 2-fold greater when purified DNA was used as the substrate. Several nitrogen mustard analogues also produced alkali-labile lesions at positions of guanine.

Alkylating agents find wide use as chemotherapeutic agents. Typically, these drugs react with nucleophilic centers within cells. It is likely that the cytotoxic and mutagenic effects occur primarily as a result of DNA modification. Therefore it is of interest not only to determine the sites of DNA modification caused by these agents but also to understand the mechanism of repair of such lesions. Techniques of classical biochemistry have been applied to define the chemistry of DNA modification. For the most part such studies have used purified bases, nucleosides, nucleotides, and DNA as substrates (1, 2). Here we present a different approach to such studies, using the highly reiterated alpha DNA sequence of human cells as a substrate for DNA modification. This approach should permit direct comparison of the sites of DNA modification within a defined DNA sequence when either purified DNA or intact human cells are exposed to agents that damage DNA.

For this study we have used the alkylating agent methylbis(2-chloroethyl)amine (HN2, mechlorethamine, nitrogen mustard). Reactions of the bifunctional agent HN2 or its monofunctional analogue diethyl(2-chloroethyl)amine with purified DNA in aqueous solution have demonstrated that the principal adduct is formed at the N7 position of guanine (1). Modifications at other bases have been detected, notably at the amine at position 6 of adenine, N3 of cytosine, and N3 of thymine, but both the extent and rate of reaction of these sites are considerably lower than those of guanine residues (1). HN2 has also been shown to cause crosslinks between the two strands of a DNA double helix and between guanine residues in DNA (3). Use of the highly reiterated alpha sequence of human DNA as an indicator allows analysis of the sites of DNA modification by HN2 at the level of individual nucleotides within a defined sequence in viable human cells.

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MATERIALS AND METHODS

Chemicals. The HN2 was a commercial preparation of mechlorethamine-HCl (Mustargen hydrochloride; Merck, Sharp & Dohme). Four analogues of cyclophosphamide were kindly provided by Robert Struck. These were phosphoramidate mustard (NCS 69945), isophosphoramidate mustard (NCS 297900), peroxyphosphoramidate (NCS 176986), and 4-hydroperoxyphosphoramidate (NSC 181815) (4).

Cell Lines. The CEM human lymphoblastic leukemia line (5) and HeLa cells (from the laboratory of D. Baltimore) were used. The HeLa cells were grown as spinner cultures in Dulbecco's modified Eagle's medium (6) supplemented with 5% fetal calf serum.

Preparation of Terminally Labeled Alpha DNA. Cells (6×10^7) were harvested by centrifugation and washed twice with phosphate-buffered saline. The pellet was resuspended in 9 ml of 0.15 M NaCl/0.015 M sodium citrate. One milliliter of 10% sodium dodecyl sulfate was added to disrupt the cells. Forty microliters of proteinase K (2.5 mg/ml, predigested for 30 min at 37°C) was added and the mixture was incubated for 30 min at 37°C. A second portion of proteinase K was added and the incubation was repeated. The mixture was extracted twice with chloroform/isoamyl alcohol (19:1, vol/vol). Sodium acetate was added to the remaining aqueous phase to increase the salt concentration to 0.3 M. Nucleic acids were precipitated by addition of 2 vol of cold ethanol and the precipitate was lyophilized.

The precipitate was resuspended in *Eco*RI buffer (50 mM Tris-HCl, pH 7.5/25 mM NaCl/2.5 mM MgCl₂/50 µg of gelatin per ml/2 mM 2-mercaptoethanol) and *Eco*RI restriction endonuclease was added at a ratio of approximately 3 units of *Eco*RI to 1 µg of nucleic acid. The mixture was incubated at 37°C for 2-3 hr and Na₂EDTA was added to a final concentration of 20 mM.

The mixture (in a volume of approximately 600 µl) was centrifuged through a 4-ml 15-30% sucrose gradient at 50,000 rpm in a Beckman VTi65 rotor for 80 min at 25°C [³²P-labeled 342-base-pair (bp) marker DNA was added before centrifugation]. The gradient was fractionated and the marker was located. DNA from this fraction was precipitated.

The 3' termini of the DNA were labeled in reactions that contained the Klenow fragment of DNA polymerase, [α -³²P] dATP, and [α -³²P] dTTP as described (7). A 92-bp DNA fragment labeled at one terminus was obtained by digestion of the 342-bp fragment with 100 units of *Eco*RI in *Eco*RI* reaction buffer [25 mM Tris-HCl, pH 8.6/2mM MgCl₂/20% (vol/vol) glycerol] for 2-3 hr at 37°C (8). The digest was layered on a

Abbreviations: HN2, methylbis(2-chloroethyl)amine; bp, base pair(s).

nondenaturing 4% polyacrylamide gel and the 92-bp fragment was recovered from the gel as described (9). This protocol was followed for preparation of DNA from cells treated with HN2 as well as for the untreated control cells. The specific activity of the DNA was approximately 5×10^4 cpm/ μ g. *In vitro* reactions contained about 1×10^4 cpm of the DNA.

RESULTS

Treatment of Purified Alpha DNA with HN2. The DNA substrate used for these experiments was a 3'-end-labeled 92-bp fragment of the human alpha sequence. The alpha sequence makes up approximately 1% of human DNA, exists in tandem arrays of a 342-bp sequence, and contains both *EcoRI* and *EcoRI** restriction sites (10, 11). Thus, treatment of total human DNA with the restriction enzyme *EcoRI* yields a well-resolved DNA 342 bp long. For these experiments the 342-bp alpha fragment was labeled at the 3' termini with 32 P. A 92-bp DNA fragment labeled at only one terminus was obtained by treatment of this fragment with the *EcoRI* restriction enzyme in a buffer that relaxes the stringency of the *EcoRI* enzyme and permits cleavage at *EcoRI** sites (8). The terminally labeled 92-bp fragment was isolated from a nondenaturing polyacrylamide gel.

The sequence of the 92-bp fragment is sufficiently homogeneous to permit assignment of all residues (for example, see Fig. 1A, lanes 1-3). Our sequence data confirm the observation of Manuelidis and Wu (12) that an unambiguous sequence can be obtained from alpha DNA. The sequence of the 92-bp fragment is given in the legend to Fig. 1.

To determine the site of modification of purified DNA by HN2, the 3'-end-labeled 92-bp alpha fragment was incubated with HN2 at 1 or 4 mM for 20 min at 37°C. The reaction was terminated by addition of an excess of 2-mercaptoethanol, and then the DNA was denatured before it was layered onto a high-resolution urea/polyacrylamide gel of the type used for DNA sequence analysis (13, 14). Fig. 1B (lanes 3 and 5) shows that the electrophoretic mobility of most of the HN2-treated DNA was the same as that of the untreated control DNA. Thus, HN2 treatment itself does not result in strand scission under these conditions.

Treatment of HN2-modified DNA at alkaline pH with 0.5 M piperidine for 10 min at 80°C resulted in cleavage of the DNA as reflected by the appearance of DNA fragments of increased electrophoretic mobility (Fig. 1A, lane 4; Fig. 1B, lanes 4 and 6). Thus, exposure of DNA to HN2 results in alkali-labile lesions. Comparison of the length of these DNA products with the lengths of those produced by the reaction of the same DNA sequence with dimethyl sulfate in the guanine-specific reaction of the Maxam-Gilbert sequencing protocol (13, 14) demonstrates that the alkali-labile sites occur at all positions of guanine (compare lanes 3 and 4 in Fig. 1A). Strand scission at guanine residues is also evident if the HN2 DNA is first heated to 90°C at neutral pH and then exposed to 0.1 M NaOH for 30 min at 90°C (not shown).

Treatment of Intact Cells with HN2. Intact HeLa cells in logarithmic-phase growth were exposed to the same concentrations of HN2 as were used to modify the purified DNA. After treatment for 20 min at 37°C the cells were washed, the DNA was extracted, and the 3'-end-labeled 92-bp alpha fragment was prepared as above. Total DNA yields were decreased to approximately one half in HN2-treated as compared to untreated control cells extracted at the same time. Lanes 7 and 9 of Fig. 1B show that, when the DNA is not subjected to alkaline hydrolysis, most of the HN2-modified DNA has the same electrophoretic mobility as the untreated control samples do. However, some of the unhydrolyzed DNA migrated more

rapidly than the untreated control DNA. Thus, some of the DNA exposed to HN2 sustained breaks during the treatment, the extraction procedure, or both. This is especially evident for the DNA extracted from cells treated with 4 mM HN2.

Alkaline hydrolysis of DNA samples extracted from cells treated with HN2 results in extensive strand scission. The sites of cleavage are the same as those observed when purified DNA was treated with HN2 (compare lanes 4 and 6, and 8 and 10, in Fig. 1B). However, for a given concentration of HN2, the extent of breakage was noticeably less for the DNA samples from intact cells treated with HN2.

The lowered extent of reaction with intact cells creates the impression that the relative frequency of strand breakage may differ for purified DNA and the DNA of intact cells. However, quantitative measurements of the relative frequency of strand scission at individual guanine residues demonstrates that this is not the case. At very high HN2 concentrations some cleavage is observed at positions of adenine (Fig. 1B, lane 6). Cleavage at these sites probably results from adduct formation at the N3 position of adenine, a modification that should also render the DNA labile to treatment with piperidine (14). The number of breaks at positions of adenine is at most 1/25th of that observed at positions of guanine.

Quantitation of Strand Scission. To obtain a quantitative measure of the difference in the extent of modification of the DNA in the two experiments, the fraction of the 92-bp alpha fragment that was broken after alkaline hydrolysis was determined by measurement of the amount of radioactivity in the more rapidly migrating DNA fragments as compared to the amount that had the mobility of unmodified DNA. The measurements were done by Cherenkov counting of gel fragments. Table 1 demonstrates that treatment of purified DNA resulted in approximately twice as many alkali-labile lesions as were

Table 1. Quantitative determination of strand scission

Sites	Scission by HN2, %			
	Purified DNA		Intact cells	
	1 mM	4 mM	1 mM	4 mM
G ₁ + G ₃	3.2	6.5	0.3	0.3
G ₁₃	1.5	3.6	0.5	0.8
G ₁₆	1.6	3.3	0.5	1.2
G ₁₇	0.7	2.8	0.2	0.6
G ₃₄	1.7	4.0	0.5	1.5
G ₃₇	1.3	2.9	0.6	1.0
G ₃₉	1.0	3.2	0.6	1.2
G ₄₁	0.8	2.3	0.5	1.4
G ₅₁₋₇₂	9.1	17.7	6.6	17.2
Total	20.9	46.3	10.3	25.2

The 32 P-3'-end-labeled 92-bp fragment of the human alpha DNA sequence was prepared from HeLa cells with or without pretreatment of the cells with HN2. Purified DNA from the untreated cells was then treated with HN2 as described in the legend of Fig. 1. Concentrations of 1 and 4 mM HN2 were used for both pretreatment of cells and treatment of purified DNA. All samples were treated with 0.5 M piperidine for 10 min at 80°C and layered on a urea-containing 12% polyacrylamide gel. The time of electrophoresis was adjusted such that all radioactivity remained within the gel. The radioactivity was located on the gels by autoradiography and quantitated by Cherenkov counting of gel fragments. Percent scission by HN2 at site X was calculated as $\{[(\text{cpm in band X})/(\text{total cpm in the lane})] \div [1 - (\text{cpm faster than band X})/(\text{total cpm in the lane})]\} \times 100$. Sites of scission were identified by comparison with a lane containing purified 3'- 32 P-labeled 92-bp DNA fragment treated with dimethyl sulfate (13, 14) and by comparison with the DNA sequence for this fragment as defined by Manuelidis and Wu (12). (For numbering see the legend of Fig. 1.) Sites of scission are identified by the guanine residue at that site.

FIG. 1. Effect of HN2 on human alpha DNA. The ^{32}P 3'-end-labeled 92-bp fragment of the human alpha DNA sequence was prepared from cells before or after treatment with HN2. The sequence of the substrate is given below. The italicized bases are ^{32}P labeled. Our sequence confirms that of Manuelidis and Wu (12) for this portion of the alpha sequence.

1 10 20 30 40 50 60 70 80 90
 5' AATTCTCAGTAACTTCTGTGTGTATTCAACTCACAGAGTTGAACGATCCTTTACACAGAGCAGACTTGAAACACTCTTTTGTGG
 3' TTAAGAGTCATTGAAGGAACACACACAATAAGTTGAGTGTCTCAACTTGCTAGGAAATGTGTCTCGTCTGAAC TTTGTGAGAAAAACACCTTAA

(A) The purified ^{32}P 3'-end-labeled 92-bp fragment of the alpha sequence was treated with hydrazine in the cytosine and cytosine-thymine reactions (lanes 1 and 2, respectively) and with dimethyl sulfate in the guanine reaction (lane 3) according to the DNA sequence determination protocol of Maxam and Gilbert (13, 14). The same DNA was treated in 200 μl with 1 mM HN2 in a buffer that contained 50 mM sodium cacodylate at pH 8.0, 10 mM MgCl_2 , and 1 mM sodium EDTA (cacodylate buffer). The reaction was stopped by addition of 0.25 vol of 1.5 M sodium acetate, pH 7.0/1 M 2-mercaptoethanol/100 μg of tRNA per ml. The DNA was precipitated with ethanol, then treated with 0.5 M piperidine for 10 min at 80°C. Reaction mixtures were layered on a urea-containing 8% polyacrylamide gel (13, 14). The HN2 reaction mixture is in lane 4.

(B) The purified 3'-end-labeled 92-bp alpha sequence was untreated (lanes 1 and 2) or treated with 1 mM HN2 (lanes 3 and 4) or 4 mM HN2 (lanes 5 and 6) for 20 min at 37°C in cacodylate buffer (see above). Reactions were terminated as above and the DNAs of lanes 2, 4, and 6 were treated with 0.5 M piperidine for 10 min at 80°C prior to loading the gel. HeLa cells in 100 ml of Dulbecco's modified Eagle's medium at a concentration of 6×10^5 cells per ml were treated with either 1 mM HN2 (lanes 7 and 8) or 4 mM HN2 (lanes 9 and 10) for 20 min at 37°C. DNA was purified from the cells and cleaved with the restriction enzyme *EcoRI*. A 342-bp fragment was isolated from a sucrose gradient, labeled at the 3' terminus with ^{32}P , and cleaved with *EcoRI**, and a 92-bp fragment was purified. The DNA was layered on the gels before (lanes 7 and 9) or after (lanes 8 and 10) treatment with 0.5 M piperidine for 10 min at 80°C. The samples were analyzed on a urea-containing 12% polyacrylamide gel. The sequence of the DNA is indicated next to the panel.

observed in DNA extracted from intact cells when both were treated with HN2 over a concentration range of 1–4 mM. This observation was repeated in three independent experiments.

The amount of total DNA in the experiments using intact HeLa cells was much greater than that present in experiments with purified DNA. To determine if the amount of DNA in the reactions did affect the extent of modification of the alpha sequence, approximately 300 μg of unlabeled total HeLa cell nucleic acid in 5 ml of double-distilled water was treated with 1 mM HN2 for 20 min at 37°C. This is the same amount of

nucleic acid that is present in 1×10^7 cells. The ^{32}P 3'-end-labeled 92-bp alpha fragment was prepared from the HN2-treated DNA as before. Twenty percent of the DNA was broken upon alkaline hydrolysis of this DNA, the same amount as was broken upon alkaline hydrolysis of the purified alpha DNA treated with 1 mM HN2 in 200 μl in a parallel experiment. Thus, the reduced strand scission observed upon treatment of intact cells with HN2 cannot be attributed to the higher concentration of nucleic acids in the experiment.

The number of alkali-labile lesions per nucleotide can be

calculated for the observed breakage frequency. Treatment of DNA with 3 mM HN2 resulted in about one alkali-labile lesion per 100 nucleotides upon treatment of purified DNA, and about one lesion per 200 nucleotides when intact cells were used.

Quantitative determination of the amount of radioactivity in individual bands identified by autoradiography of the gels permits measurements of the extent of formation of alkali-labile lesions at each guanine residue within the sequence. Inspection of Fig. 1 and the data of Table 1 demonstrates that there are no major differences in the extent of modification of the individual guanine residues, either upon treatment of intact cells with HN2 or upon treatment of purified DNA, except in the case of adjacent guanine residues. For example, the extent of modification of G₁₇ is about half that of G₁₆ and the other guanine residues. The decreased reactivity of G₁₇ was noted for both experimental protocols.

Active Analogues of HN2. The pattern of DNA cleavage within the alpha sequence can also be used to compare the sites of DNA modification by drug analogues. Cyclophosphamide is an alkylating agent that finds wide use as an anti-tumor agent. It is metabolized to a reactive form *in vivo* (15). Several potential reactive metabolic intermediates and analogues of cyclophosphamide have been synthesized (4). To determine if the sites of DNA modification by these compounds were the same as those of HN2, the 3'-end-labeled 92-bp alpha DNA fragment

was incubated with the analogues phosphoramidate mustard (NSC-69945), isophosphoramidate mustard (NSC-297900), peroxyphosphoramidate (NSC-176986), and 4-hydroperoxyphosphoramidate (NSC-181815). The effective concentration of the compounds was 4 mM, and reactions were for 2.5 hr at 37°C. The DNA was layered on a denaturing gel before or after alkaline hydrolysis. Fig. 2 shows that none of the compounds resulted in significant strand scission prior to the alkaline hydrolysis. Significant strand scission occurred upon alkaline hydrolysis of all the samples that were treated with the HN2 analogues. The lengths of the cleavage products corresponded to those observed in the reactions with HN2 and dimethyl sulfate. Thus, these compounds also create alkali-labile lesions at positions of guanine.

Comparison of the extent of strand scission in these reactions shows that the rate of reaction is not the same for all the analogues. The extent of reaction for the analogues varies over a three-fold range. The relative extent of the reaction is: phosphoramidate mustard > 4-hydroperoxycyclophosphamide > isophosphoramidate mustard = peroxyphosphoramidate mustard (Fig. 2).

DISCUSSION

The use of the human alpha sequence permits direct comparison of the sites of DNA modification by the alkylating agent

FIG. 2. Effect of cyclophosphamide analogues on alpha DNA. The purified 3'-end-labeled 92-bp fragment of the alpha sequence was treated for 2.5 hr at 37°C in cacodylate buffer (legend to Fig. 1) with 0.05 mM dimethyl sulfate (lane 1), 4 mM HN2 (lanes 2 and 3), 4 mM phosphoramidate mustard (lanes 6 and 7), 4 mM isophosphoramidate mustard (lanes 8 and 9), 2 mM peroxyphosphoramidate (lanes 10 and 11), 4 mM 4-hydroperoxyphosphoramidate (lanes 12 and 13). [Note that dimethyl sulfate is used at the standard concentration for Maxam-Gilbert sequence determination reactions (13) and that peroxyphosphoramidate contains two bifunctional alkylating groups and is therefore at a concentration equivalent to the other analogues.] Untreated control DNA is shown in lanes 4 and 5. DNA of lanes 1, 3, 5, 7, 9, 11, and 13 was treated with 0.5 M piperidine for 10 min at 80°C prior to loading onto the gel. Analysis of the DNA was done on a urea-containing 12% polyacrylamide gel. The percent scission was calculated as [(amount of radioactivity migrating faster than control untreated DNA)/(total radioactivity in the lane)] × 100. It is 75% for HN2 (lane 3), 59% for phosphoramidate mustard (lane 7), 25% for isophosphoramidate mustard (lane 9), 25% for peroxyphosphoramidate (lane 11), and 42% for 4-hydroperoxyphosphoramidate (lane 13).

HN2 upon purified DNA and upon the same sequence of DNA in intact cells. The results demonstrate that the pattern of DNA modification was similar in both experiments. Treatment of either purified DNA or intact cells results in modification of the DNA such that it is labile to alkaline hydrolysis at positions of guanine residues.

We conclude, with the exceptions mentioned below, that the overall structure of the chromatin and the cellular environment does not markedly alter the reactivity of the guanine residues of the alpha sequence. For a given HN2 concentration, the extent of guanine modification was reduced only 50% in experiments with intact cells as compared to naked DNA and the relative reactivities of individual guanine residues within the sequence were unchanged. This is remarkable because the DNA in the two experiments is found in very different environments. Within the cell the alpha is complexed with histones and surrounded by cellular constituents that are absent when naked DNA is used as the substrate.

Two effects of the different DNA environments were noted: the total amount of DNA damage was decreased to approximately half in intact cells relative to purified DNA, and the amount of DNA recovered from HN2-treated cells was approximately half that recovered from untreated control cells. The lack of specific regions of protection or enhanced reactivity within the defined sequence is consistent with the results of McGhee and Felsenfeld (16), who demonstrated no significant difference in the reactivity of N7 of guanine with dimethyl sulfate when treatment was performed on naked DNA as compared to DNA of core nucleosome particles. The reduced extent of damage is probably the result of reaction of HN2 with cell constituents other than DNA (nucleophilic centers on RNA and proteins). The reduced extent of damage may also be due to limited diffusion of HN2 within the cell. The reduced yield of DNA obtained from cells treated with HN2 may be due to crosslinks formed between DNA and proteins (17). Additionally, we note that the 92-bp alpha fragment extracted from cells treated with HN2 contains more internal nicks prior to alkaline hydrolysis than does untreated control DNA or purified DNA treated with the same concentration of HN2. These nicks may have been created in the cells or during the extraction procedure.

Alkali sensitivity of HN2-modified DNA at positions of guanine is consistent with modification at the N7 position, which has been reported previously (1). Addition of the alkyl group to the N7 position weakens the glycosylic bond of the modified nucleotide at neutral pH. Thus, a treatment at high temperature at neutral pH should produce an apurinic site, which is labile to subsequent treatment at high pH. The N7 adduct should also labilize the 8-9 guanine bond to alkaline hydrolysis (18). Thus, piperidine treatment of the DNA modified at N7 should lead to ring opening and elimination of the base, followed by β -elimination reactions and strand scission (14). HN2 is a bifunctional reagent and should form DNA crosslinks between guanine located on opposing strands of the double helix (3). The guanine residues involved in such crosslink formation should also be labile to alkaline hydrolysis and there-

fore would not be apparent in analysis of piperidine-treated DNA. Formation of DNA crosslinks may account for the fraction of DNA that migrates more slowly than control DNA in samples of HN2-treated purified DNA fragments that have not been exposed to alkaline conditions (Fig. 1B, lanes 3 and 5).

The use of the alpha sequence also permits rapid comparison of HN2 analogues. We conclude that the derivatives of cyclophosphamide studied react with DNA in a manner qualitatively similar to HN2, albeit at a slower rate.

These experiments demonstrate that the alpha sequence of human DNA can be used as an indicator to investigate the effect of alkylating agents upon purified DNA in intact cells at the level of individual nucleotides. This indicator sequence can also be used to study the repair of DNA lesions in viable cells.

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