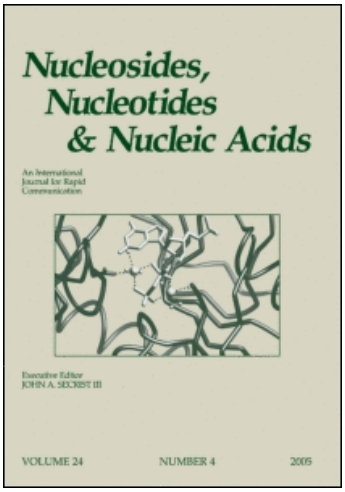

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DETECTION OF 5-HYDROXYMETHYL-2'-DEOXYURIDINE IN DNA FROM CALF THYMUS, HELA CELLS, AND RAT LIVER NUCLEI

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Abstract: HMdU in DNA was quantitated by radioimmunoassay following separation of enzymatic digests. In DNA prepared from calf thymus, HeLa cells, and rat liver nuclei, there was approximately 1 HMdU for every 10^5-10^6 deoxythymidines. Irradiation of calf thymus DNA and HeLa cells increased the level of HMdU.

INTRODUCTION

HMdU (5-hydroxymethyl-2'-deoxyuridine) is a modified thymidine which may occur in DNA as a result of oxygen radicals via the action of ionizing radiation (1) or chemicals (2). HMdU also may be incorporated into DNA following exogenous administration, which results in cytotoxicity (3, 4). The proposed use of HMdU in cancer chemotherapy was based on this cytotoxicity to leukemia cells (5, 6). Furthermore, the mutagenic effect of HMdU in DNA (7) suggests that HMdU in DNA may initiate development of cancer.

Quantitation of HMdU in DNA represents a potentially useful approach to monitoring DNA modification by oxygen radicals and cancer chemotherapy (8). It was possible to measure HMdU in DNA of cultured cells irradiated *in vitro* because thymidine can be specifically prelabeled to high specific activity in DNA of cultured cells prior to

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treatment (9). Unfortunately, prelabeling is not possible in vivo (10). Therefore, only indirect evidence of HMdU formation in vivo has been presented, such as demonstration of repair enzyme activity (11) and urinary excretion of excised bases (12).

The present study describes an approach to quantitate HMdU in unlabeled DNA based on radioimmunoassay (RIA) and high performance liquid chromatography (HPLC). The results demonstrate that it is possible to quantitate DNA base oxidation from tissues without prelabeling, and that spontaneous base oxidation may occur in vivo.

MATERIALS AND METHODS

Materials

Non-isotopic nucleoside standards, calf thymus DNA, enzymes, normal rabbit serum and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. $CsCl_2$ and sucrose were from BRL (Gaithersburg, MD). Venom phosphodiesterase I was from Worthington-Cappel (Malvern, PA). Triton X-100, methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA). Aqueous scintillant was obtained from Amersham Corp. (Arlington Heights, IL). Sodium periodate was from Aldrich Chemical Co. (Milwaukee, WI).

Radioimmunoassay

5-Hydroxymethyluridine (riboside) was conjugated to keyhole limpet hemocyanin by the periodate method of Beiser et al. (13). The conjugate was purified by gel chromatography and had a hapten:protein ratio of ~150:1. To prepare antisera, New Zealand White rabbits were immunized according to a described schedule (14).

Tritiated HMdU was synthesized from [6-^{3}H]-deoxyuridine, 21.4 Ci/mmol (NEN, Boston, MA), by formaldehyde addition (15) and purified by reverse-phase HPLC. The purity of the tritiated HMdU was confirmed by coinjection with authentic non-isotopic HMdU, as all of the radioactivity coeluted with the UV peak. Antisera titers were determined by serial 2-fold dilutions of antisera as previously described (14). The RIA utilized 40 μl of standard or unknown and 40 μl of antisera sufficient for 50% tracer binding, both diluted in 15%

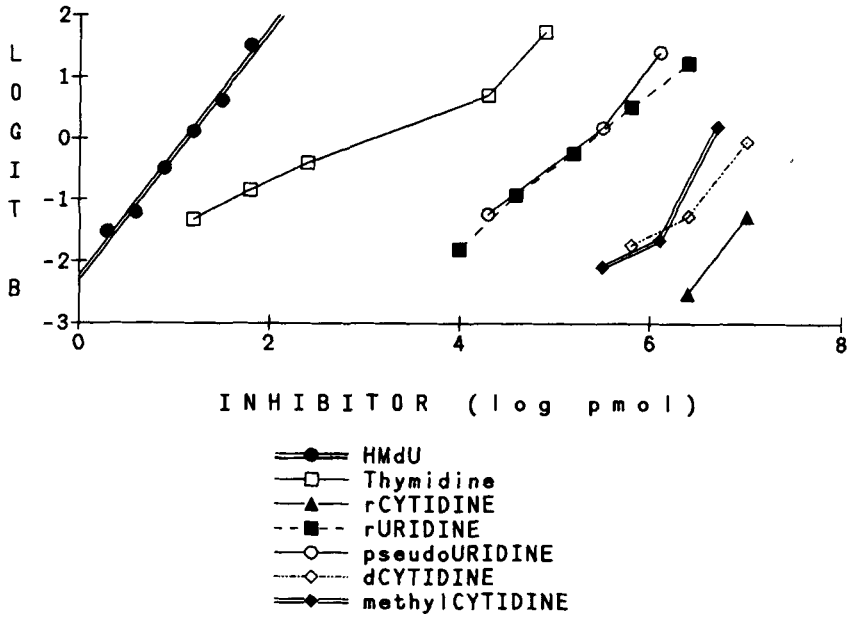

FIGURE 1. Standard curves for HMdU and other pyrimidine nucleosides in RIA. Logit b = ln [% inhibition/(100-% inhibition)]

control rabbit sera prepared with Tris-buffered saline (140 mM NaCl, 20 mM Tris-HCl, pH 7.5; 2 mM NaN_3). Tracer (4000 dpm in 20 μl Tris-buffered saline) was added and the mixture was incubated 2-4 hrs at room temperature. Bound tracer was precipitated by addition of 100 μl saturated $(NH_4)_2SO_4$ for 10 minutes and centrifugation at 10,000 g for 10 minutes. Then 150 μl of supernatant was removed for scintillation counting. Calculations of % inhibition were performed as described by Muller and Rajewsky (16), and a standard curve was established to determine unknowns (Fig. 1).

Sample preparation

Calf thymus DNA (1 mg/ml in H_2O) samples or HeLa cells (10^8/sample) were placed on ice and irradiated (60 krad) using a ^{60}Co source. After irradiation, HeLa cells were lysed (10 mM Tris, pH 8.0; 1% Triton X-100, 0.32 mM sucrose, 5 mM $MgCl_2$) and then digested with Proteinase K, 0.5 mg in 5 mls buffer (10 mM Tris•HCl, pH 8.0; 75 mM NaCl, 24 mM Na•EDTA). Rat liver nuclei were isolated and digested

with 1 mg Proteinase K and 50 mg Sarkosyl in 5 mls buffer (10 mM Tris•HCl, pH = 7.5; 5 mM Na•EDTA) (17). Nuclear digests from HeLa cells and rat livers were sheared and DNA isolated by $CsCl_2$ gradient centrifugation and ethanol precipitation (18). All DNA samples (0.3 - 1.0 mg) were dissolved in 1 ml H_2O and digested enzymatically (16). For quantitation of thymidine (dT) after digestion, 50 μl of digest was analyzed by reverse-phase HPLC using 10% methanol in H_2O, 1 ml/min isocratic conditions. Peak area of dT was compared to external standards for each sample.

Separation and Quantitation of HMdU

The 1 ml samples of hydrolyzed DNA were clarified by centrifugation (10,000 g for 3 min) and chromatographed on 2 serially connected 4.6 mm x 12.5 cm Partisphere C18 Cartridge columns (Whatman, Inc., Clifton, NJ) using 1 ml/min 25 mM aqueous ammonium acetate (pH 5.0) containing 2% acetonitrile. One minute fractions were collected and the entire fraction (corresponding to the HMdU retention time) or an aliquot thereof was dried under reduced pressure, and RIA was performed. The column was washed with H_2O and methanol between runs.

RESULTS AND DISCUSSION

Antisera was obtained which caused 50% tracer binding at a 1:128 dilution. This antisera was used to establish the RIA standard curve for HMdU in Fig. 1. The limit of detection of HMdU at 20% inhibition was 2 pmol. Twenty percent inhibition was chosen because results consistently demonstrated linearity of a log-logit transformation of the RIA standard curve down to 10% inhibition. Cross-reactivity with other pyrimidine nucleosides is also shown in Fig. 1. The cross-reactivity of the HMdU antisera at 50% inhibition was 0.02 against thymidine. This prevented the use of the RIA to quantitate HMdU in hydrolyzed DNA without separation.

The chromatographic separation of HMdU from enzymatically hydrolyzed DNA is depicted in Fig. 2. The retention time of HMdU (usually 10-11 minutes) was determined before and after each series of

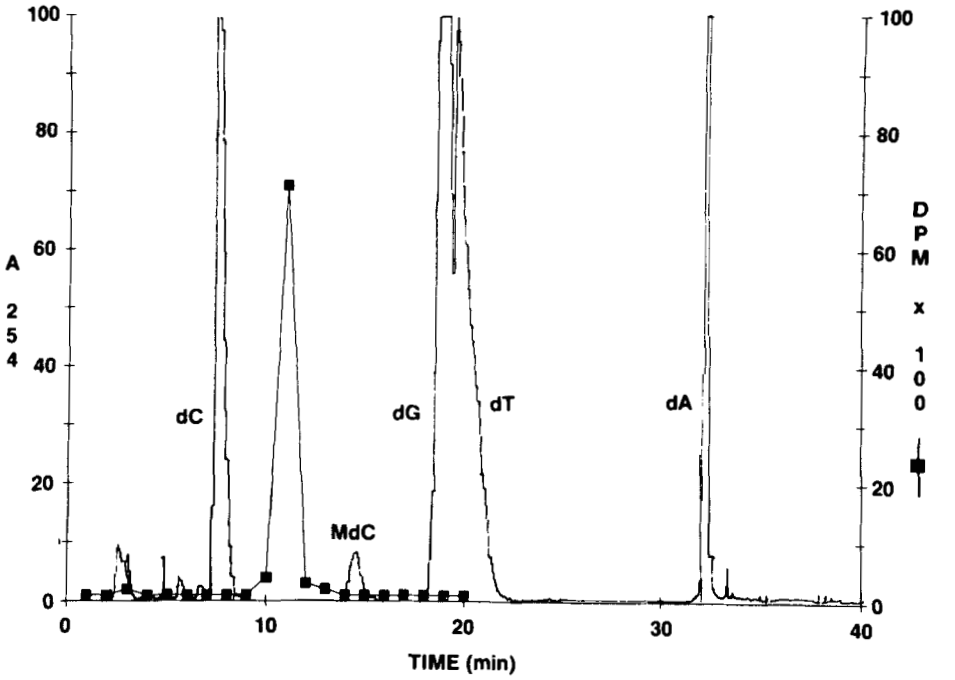

FIGURE 2. Chromatogram of enzymatically hydrolyzed DNA to which 8000 dpm of tritiated HMdU was added. Nucleoside peaks:

dC (deoxycytidine), dG (deoxyguanosine),
dT (thymidine), dA (deoxyadenosine),
MdC (methyldeoxycytidine)

samples by injection of a sample of DNA hydrolysate spiked with tritiated HMdU and monitoring its elution by liquid scintillation counting of fractions.

Results of analysis of DNA samples for HMdU are shown in Table 1. Production of HMdU in DNA irradiated in solution and in DNA from irradiated HeLa cells is consistent with previous reports (9). DNA from livers of rats treated with WY-14,643 (0.1% in diet for 28 days) was analyzed because it was suggested that oxidative damage of DNA may be responsible for the hepatocarcinogenicity of this compound (19, 20). However, production of HMdU by WY-14,643 treatment was not demonstrated by this analysis.

TABLE 1.

Quantitation of HMdU in DNA: Effect of Source and Treatment

DNA Source and Treatment	HMdU* (pmol/μmol dT)
Calf thymus Control	14.5 ± 1.5
Calf thymus 60 Krad (in vitro)	3164 ± 405
HeLa Control	4.0 ± 0.4
HeLa 60 Krad	12.3 ± 2.3
Liver Control	2.7 ± 0.5
Liver WY-14,643	4.9 ± 1.9

*Values depicted as mean (n=2) ± difference/2 (2 separate DNA extractions were performed on HeLa and liver samples)

The presence of HMdU in control samples of DNA from all three sources evaluated (Table 1) has not been reported. Furthermore, it is unlikely that cross-reacting inhibitors were being detected by the RIA. The separation of HMdU by HPLC suggested that thymidine was not the source of inhibition. The separation of deoxycytidine and 5-methyldeoxycytidine by HPLC coupled with their low cross-reactivity in the RIA also precluded their contribution to inhibition. The various pyrimidine ribonucleosides were unlikely sources of inhibition because of their low cross-reactivities and the isolation of DNA from RNA in the samples. Acetone extraction or filtration (MW $>$ 2000) of DNA hydrolysates prior to HPLC did not reduce background inhibition (data not shown), suggesting that protein contamination of the HMdU fraction was not occurring. In addition, the level of HMdU/dT in a synthetically constituted DNA sample (thymine, adenine, guanine, and cytosine deoxyribonucleosides plus enzymes) was 10^{-2} pmol/μmol, considerably lower than levels in control DNA samples. Thus, control samples of DNA contained quantifiable levels of HMdU.

Recently published studies in which different oxidative base modifications were analyzed by different techniques also suggested that endogenous levels of oxidized DNA bases may occur. Kasai *et al.*

(21) demonstrated 8-hydroxydeoxyguanosine (~ 10 pmol/μmol deoxyguanosine) in DNA from HeLa cells and mouse liver in the absence of treatment, using HPLC with electrochemical detection. Dizdaroglu et al. (22) demonstrated 8,5'-cyclo-2-deoxyguanosine (~ 25 pmol/μmol deoxyguanosine) in DNA from lymphoblasts in the absence of treatment, using gas chromatography with trimethylsilyation and mass spectrometry (selected ion monitoring). The use of phenol extraction for isolation of DNA raised the possibility that oxidants in phenol caused DNA base damage during isolation (21). However, in the present study, DNA isolation by an alternative method (density centrifugation) eliminated phenol as a potential source of background oxidation.

The demonstration of thymine glycol and hydroxymethyluracil in urine of untreated animals (12) suggested that oxidative damage to DNA bases occurs in the absence of radiation or chemical injury. Therefore, our results strongly suggest that background levels of HMdU are present in nuclear DNA from livers of untreated rats. Furthermore, the level of HMdU in rat liver nuclei (~ $3/10^6$ dT) was lower than that of 8-hydroxydeoxyguanosine (~ $30/10^6$ deoxyguanosine) recently reported (23).

In summary, quantitation of HMdU in DNA by HPLC and RIA obviates the need to incorporate radioisotopically labeled thymidine in DNA of cells or animals in vivo. The results of this study indicate that HMdU is present in DNA of untreated cells and animals. The investigation of potential sources of HMdU in DNA such as oxidative damage of cells and animals is warranted. Furthermore, spontaneously occurring HMdU in DNA should be anticipated in future studies on the efficacy and toxicity of HMdU in cancer chemotherapy.

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