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Characterization of Antibodies to Dihydrothymine, a Radiolysis Product of DNA†

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Received November 17, 1988; Revised Manuscript Received January 27, 1989

ABSTRACT: Antibodies to dihydrothymine were elicited by immunizing rabbits with dihydrothymidine monophosphate conjugated by carbodiimide to BSA. By use of an ELISA assay, the antibodies produced were found to be specific for dihydrothymine. Hapten inhibition studies showed that dihydrothymidine monophosphate was 3 orders of magnitude more effective as an inhibitor than thymidine monophosphate and 4 orders of magnitude more effective than thymidine glycol monophosphate. With DNA containing dihydrothymine, antibody reactivity was observed at 20 fmol of dihydrothymine, which is approximately 0.1 dihydrothymine per 10000 bases. Thus, the assay is very sensitive. The antibody reacted with denatured DNA containing dihydrothymine but not with native DNA containing this lesion. The antibody was used for measurement of in vivo incorporation of dihydrothymidine in wild-type *Escherichia coli* or mutants defective in their ability to remove dihydrothymine from DNA or in the de novo synthesis of thymidylate. Lastly, antibodies to dihydrothymine were used to quantitate the formation of dihydrothymine in DNA X-irradiated under N_2. Production of dihydrothymine in irradiated DNA correlated with the level of reducing species produced by X-rays, and dihydrothymine was produced preferentially in irradiated single-stranded or denatured DNA as compared to irradiated duplex DNA.

Ionizing radiation produces a broad spectrum of DNA base modifications. Many of these have been identified in X-irradiated solutions of purines, pyrimidines, and nucleotides, as well as in DNA [for review, see Teoule (1987), von Sonntag and Schuchmann (1986), and Hutchinson (1985)]. Although damage to DNA bases constitutes the largest class of lesions produced by ionizing radiation, individual damages are produced in low yields, making their detection and quantitation difficult.

The spectrum of base damage produced in DNA by ionizing radiation can differ depending on the irradiation conditions. In irradiated aerated solutions of DNA, products are formed not only from attack by radicals produced from ionized water

† This research was supported by Contract DE-ACOZ-80EV10417 from the U.S. Department of Energy and NIH Fellowship 5 F32GM10567 awarded to K.H.
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0006-2960/89/0428-4382$01.50/0

but also from the subsequent peroxidation events (Scholes et al., 1956). In deaerated solutions, products arise primarily from the interaction of DNA with free radicals produced from ionized water (Teoule et al., 1978). Thymine damages produced in DNA during aerobic irradiation have been extensively studied and characterized. Thymine ring saturation products in general, and thymine glycols in particular, have been measured with enzymatic (Frenkel et al., 1981; Breimer & Lindahl, 1985), chemical (Hariharan & Cerutti, 1972; Hariharan et al., 1975), and immunochemical (West et al., 1982a; Leadon, 1987; Rajagopalan et al., 1984; Hubbard et al., 1988) assays.

The use of immunochemical assays has provided a sensitive means for the detection and quantitation of low levels of base damage in irradiated DNA. Antibodies have been used to detect a number of oxidative radiolysis products such as thymine glycols (West et al., 1982a; Rajagopalan et al., 1984; Leadon, 1987; Hubbard et al., 1988), 8-hydroxyadenine (West et al., 1982b), 5-(hydroxymethyl)deoxyuridine (Lewis et al., 1978), and 8,5′-cycloadenosine (Fuciarelli et al., 1985).

Dihydrothymine is a major anaerobic radiolysis product of thymine and has been detected in DNA irradiated in vitro (Teoule et al., 1978; Dizdaroglu, 1985; Furlong et al., 1986) as well as in DNA extracted from irradiated cells (Furlong et al., 1986). Although dihydrothymidine triphosphate can act as a substrate for *Escherichia coli* DNA polymerase I (Ide et al., 1987b) and does not appear to be a strong replicative block to DNA polymerases (Ide et al., unpublished observations), it is nevertheless removed from DNA by cellular excision repair enzymes. *E. coli* endonuclease III (Demple & Linn, 1980; Melamede et al., 1987; Ide et al., 1988) and *Micrococcus luteus* γ-endonuclease (Furlong et al., 1986; Jorgensen et al., 1988) remove dihydrothymine from DNA containing this lesion in an N-glycosylic followed by an endonucleolytic reaction. These observations suggest that dihydrothymine may have biological consequences.

In this paper, we describe the specificity and sensitivity of a polyclonal antibody elicited to dihydrothymidine monophosphate coupled to BSA. In addition, we have quantitated the production of dihydrothymine in X-irradiated DNA.

Materials and Methods

Bacteria and Bacteriophages. PM2 bacteriophage and host *Alteromonas espejiana* were obtained from H. Gray and were grown as previously described (Wallace et al., 1981). f1-K12 bacteriophage and host *E. coli* strain SMH77 were kindly supplied by J. Eugene LeClerc (Hayes et al., 1988). *E. coli* strains LAP200 and LAP201 were constructed by Lynn Petrullo in our laboratory and will be described elsewhere (Ide et al., unpublished results). LAP200 was derived from BW372 (Cunningham & Weiss, 1985) and lacks endonuclease III (*nth*$^-$). LAP201 is a *thy*$^-$ auxotroph of LAP200.

DNA, Polynucleotides, and Nucleotides. DNA from bacteriophage PM2 was isolated as previously described (Wallace et al., 1981). DNA was isolated from bacteriophage f1-K12 as described by Messing (1983). Calf thymus DNA, poly(dC), poly(dT), and poly(dG) were purchased from Pharmacia; poly(dA) was purchased from Sigma. 5,6-Dihydrothymidine 5′-monophosphate and thymidine glycol 5′-monophosphate were synthesized as described by Ide et al. (1987). Thymidine 5′-monophosphate was purchased from Sigma.

Preparation of Dihydrothymine-Containing DNA. For the preparation of PM2 DNA containing [*methyl*-^{3}H]dihydrothymine, a nick-translation reaction was employed. A 300-μL reaction mix contained 50 mM Tris-HCl, pH 7.2, 10 mM Mg_2SO_4, 0.1 mM dithiothreitol, PM2 DNA (10 μg), *E. coli* DNA polymerase I (50 units, Pharmacia), bovine pancreas DNase I (0.029 unit, Pharmacia), dATP, dGTP, and dCTP (20 μM each, Pharmacia), dTTP (1 μM, Pharmacia), and [*methyl*-^{3}H]dihydrothymidine 5′-triphosphate (20 μM, 604 cpm/pmol). Reactions were incubated at 20 °C for 1 h and terminated by heating the solution at 100 °C for 5 min. The preparation and HPLC purification of dihydrothymidine 5′-triphosphate have been previously described (Ide et al., 1987). Nicked-translated PM2 DNA was further purified on a Sephadex G-100 (Pharmacia) column (0.7 × 21 cm). The column was eluted with 10 mM Tris-HCl, pH 7.5–1 mM EDTA, and the radioactive fractions in the void volume were pooled. On the basis of the specific radioactivity of purified PM2 DNA, the estimated number of dihydrothymines per PM2 DNA molecule was 209.

E. coli chromosomal DNA containing dihydrothymine was prepared as follows. *E. coli* SMH77, LAP200, or LAP201 was grown in glucose–M9 minimal media (Maniatis et al., 1982) supplemented with amino acids [arginine, histidine, and threonine (20 μg/mL each)], 10 μM $FeCl_2$, and 1 pg/mL thiamin. For LAP201, a *thy*$^-$ auxotroph, thymidine (50 μg/mL) was also included in the medium. An overnight culture of the desired strain was diluted 20-fold in medium (above) and incubated at 37 °C until a cell density of 1×10^8 cells/mL was reached. 5,6-Dihydrothymidine (Sigma) was added to the growing cultures at varying concentrations (final concentrations 0–10 μg/mL), and incubation was continued for 4 h at 37 °C. The cells were harvested, and the DNA was extracted (Schlief & Wensink, 1983). Denatured DNA was prepared by heating at 100 °C for 5 min followed by quick cooling on ice.

The preparation of single-stranded DNA from hybrid f1-K12 bacteriophage (Hayes et al., 1988) containing 2.6 dihydrothymines per f1 DNA molecule (8.1 kb) will be reported elsewhere (Ide et al., unpublished results).

Preparation of the Antigen. The hapten–protein conjugate was prepared by the carbodiimide conjugation procedure of Halloran and Parker (1966). BSA or RSA (188 mg) and dihydrothymidine 5′-monophosphate (495 mg) were dissolved in 5 mL of distilled water. The pH was adjusted to 7.5 with 0.5 M NaOH, and ethyl [(dimethylamino)propyl]carbodiimide (200 mg, Sigma) was added. The reaction mixture was incubated in the dark at room temperature for 24 h. The solution was then dialyzed extensively against 10 mM Tris-HCl buffer, pH 7.6, followed by extensive dialysis against distilled water.

Immunization Procedure. Immunization of two rabbits (5455 and 5456) was by multiple intradermal injections of a total of 1 mL of the antigen (2 mg/mL) mixed with an equal volume of complete Freund's adjuvant. The first bleeding was taken 5 days after the first booster injection. Bleedings were then taken at 7-day intervals for 2 months.

Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA assay used is a modification of the procedure of Hsu et al. (1980). For the direct assay, either of two methods was used for binding of DNA to microtiter plates. In initial ELISA assays, 0.1 mL of 1 μg/mL DNA in 0.14 M NaCl, 1 mM KH_2PO_4, 20 mM Na_2HPO_4, and 3 mM KCl, pH 7.4 (PBS) was added per well in Linbro "processed" microtiter plates (Flow Laboratories) and evaporated for two nights at 37 °C. In later assays, 0.1 mL of 5 μg/mL DNA in PBS was added per well in UV-irradiated (Zouali & Stollar, 1986) polystyrene plates (Immunolon type I, Dynatech Labs) and incubated for 2 h at 37 °C or overnight at 4 °C. The latter method of binding DNA to microtiter plates is currently in use and is

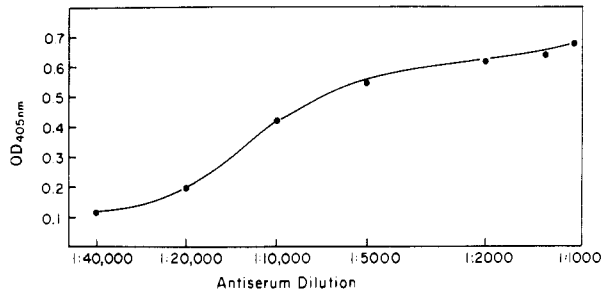

FIGURE 1: Reaction of anti-dihydrothymine antibody (rabbit 5455, 1:1000 dilution) with dihydrothymidine monophosphate–RSA conjugate. Microtitration plates were coated with 1 μg/mL RSA conjugate (100 μL/well), and the ELISA assay was performed as described under Materials and Methods.

preferred because more DNA binds to the plates (180 ng vs 20 ng of DNA/per well). In addition, background levels were extremely low.

The plates with bound DNA were then washed as previously described (Hubbard et al., 1989), and the primary antibody was added (0.1 mL of a 1:1000 dilution in 1% fetal calf serum TPBS) to each well. The ELISA procedure was then followed as previously described (Hubbard et al., 1989) with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). The reactions were developed at room temperature, and the optical density at 405 nm was measured on a Biotech plate reader. The results were corrected for nonspecific reaction of the antibody with untreated DNA. Each assay was done in quadruplicate, and the standard error was less than 10%.

For experiments testing the reactivity of the anti-dihydrothymine antibody with the dihydrothymidine 5′-monophosphate–RSA conjugate, 100 μL/well of the RSA conjugate (1 μg/mL) was added to microtitration plates and incubated overnight at 4 °C. Plates containing bound RSA conjugate were then processed as described for the direct ELISA.

Hapten Inhibition. The primary antibody was reacted for 1 h at 37 °C with varying concentrations of hapten (1:1000 dilution of antibody) in PBS in a final volume of 500 μL. Aliquots (0.1 mL) of each mixture were added in quadruplicate, to wells in which dihydrothymine-containing PM2 DNA had been bound. The plates were incubated for 1.5 h at 37 °C and the subsequently incubated with secondary antibody and processed as described for the direct ELISA.

X-Irradiation. X-ray-induced damage was produced by irradiating DNA in a Phillips generator with a beryllium window Machlett tube operated at 50 kVp and 20 mA. The dose rate, determined by Fricke dosimetry, was 1000 Gy/min. Calf thymus and f1-K12 DNA (200 μg/mL) were irradiated in N_2 in either 10 mM NaH_2PO_4, pH 7.0, or 10 mM NaH_2PO_4, pH 7.0, plus 100 mM sodium formate. Poly(dT) was irradiated at a concentration of 110 μg/mL in either of the two buffers and in the presence or absence of oxygen. The remaining homopolymers were irradiated at a concentration of 110 μg/mL in 10 mM NaH_2PO_4, pH 7.0 under N_2 flow.

RESULTS

Antibody Specificity. All of the antisera collected reacted with the RSA–dihydrothymidine monophosphate conjugate. Figure 1 shows that the second bleed of rabbit 5455 reacted with the RSA conjugate at a high antiserum dilution (1:40 000). The other rabbit (5456) exhibited the same or better response, indicating that a high antibody titer was raised in both rabbits.

Figure 2 shows the reactivity of the anti-dihydrothymine antibody with dihydrothymine-containing native or denatured *E. coli* chromosomal DNA. *E. coli* strains SMH77 and

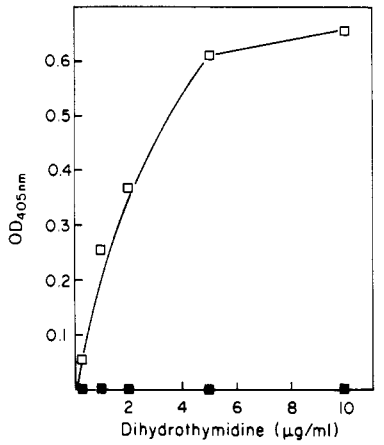

FIGURE 2: Reaction of anti-dihydrothymine antibody (1:1000 dilution) with native (■) and denatured (□) DNA from *E. coli* strain LAP201 (*nth*⁻, *thy*⁻). LAP201 was grown in media containing various amounts of dihydrothymidine (0–10 μg/mL), and chromosomal DNA was isolated from each preparation and examined for immunoreactivity with the anti-dihydrothymine antibody by the ELISA assay.

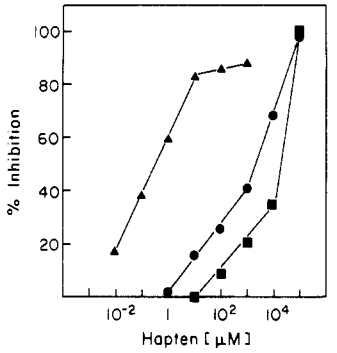

FIGURE 3: Hapten inhibition of the reaction of the anti-dihydrothymine antibody (1:1000 dilution) with denatured dihydrothymine containing PM2 DNA according to the ELISA assay. Competitor haptens: dihydrothymidine 5′-monophosphate (▲); thymidine 5′-monophosphate (●); thymidine glycol 5′-monophosphate (■).

LAP200 (an *nth*⁻ derivative of SMH77) and LAP201 (a *thy*⁻ auxotroph of LAP200 that also lacks endonuclease III) were grown in medium containing dihydrothymidine. Only denatured DNA from the *nth*⁻ *thy*⁻ strain (LAP201) reacted with the anti-dihydrothymine antibody (data with SMH77 and LAP200 not shown), suggesting that a *thy*⁻ background is required for incorporation of exogenous dihydrothymidine into chromosomal DNA. This figure also shows that the antibody does not react with dihydrothymine in native duplex DNA.

Hapten inhibition experiments (Figure 3) showed that the antibody was highly specific for dihydrothymine. Dihydrothymidine monophosphate was 6100 times more effective as an inhibitor than thymidine monophosphate and 38 000 times more effective than thymidine glycol monophosphate. This specificity is comparable to the anti-thymine glycol antibody generated by the same procedure (Rajagopalan et al., 1984).

Although a number of base damages have been identified in X-irradiated model solutions of purines and pyrimidines (Teoule, 1987; von Sonntag & Schuchman, 1986; Hutchinson, 1985), many are unstable, difficult to synthesize, or difficult to specifically introduce into DNA. To circumvent this problem and to further delineate any potential cross-reactivity of the anti-dihydrothymine antibody, DNA homopolymers were X-irradiated under nitrogen and tested for their reactivity with the antibody by a direct ELISA. The results are shown in Table I. The antibody reacted only with irradiated poly(dT).

Table I: Reactivity of Anti-Dihydrothymine Antibody to X-Irradiated DNA Homopolymers

polymer	dose (kGy)	OD_{405}[a]
poly(dT)	0.84	0.49
	1.20	0.63
poly(dC)	0.60	ND[b]
	1.20	ND
poly(dG)	0.60	ND
	1.20	ND
poly(dA)	0.60	ND
	1.20	ND

[a] Optical densities were corrected by subtracting the background due to nonspecific binding. [b] ND represents no detectable absorbance above background.

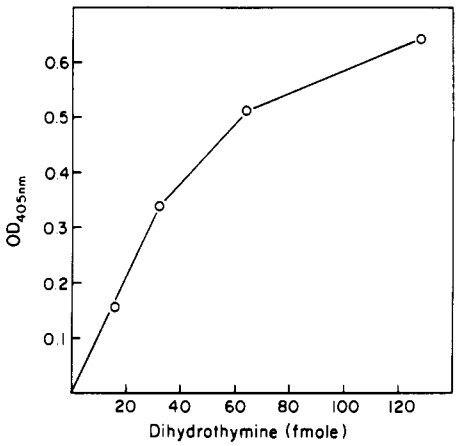

FIGURE 4: Reaction of anti-dihydrothymine antibody (rabbit 5456, 1:1000 dilution) with dihydrothymine-containing single-stranded f1-K12 DNA.

The antibody signal was abolished when PM2 DNA containing dihydrothymine was treated with alkali (pH 12, 25 °C, 16 h; data not shown). This result is consistent with the reported chemistry of dihydrothymine, which undergoes ring opening under alkali conditions to give β-ureidoisobutylic acid (Kondo & Witkop, 1968). Taken together, the data indicate that the antibody is specific for dihydrothymine.

Antibody Sensitivity. When the anti-dihydrothymine antibody was incubated with mixtures of denatured undamaged PM2 DNA and denatured PM2 DNA containing 209 dihydrothymines per molecule, a linear relationship between the direct ELISA signal and the amount of PM2 DNA containing dihydrothymine was observed (data not shown). Since 40 ng of PM2 DNA bound to each microtiter well (Linbro), we were able to dilute the dihydrothymine-containing DNA with untreated PM2 DNA to obtain a concentration range of 0–1.2 pmol of dihydrothymine/well. Although the antibody response was linear, the sensitivity of this particular rabbit (5455 bleed two) was less (26 dihydrothymines per 10000 bp) than was observed for the anti-thymine glycol antibody (Rajagopalan et al., 1984), which could detect about one thymine glycol per 10000 bp.

The various antisera were then screened with dihydrothymine-containing DNA in order to find individual bleeds that could detect lower levels of dihydrothymine. Figure 4 shows the results obtained with antisera from rabbit 5456 (fifth bleed) with single-stranded f1-K12 DNA containing dihydrothymine that had been incorporated in vivo. The phage DNA contained 2.6 dihydrothymines per molecule as estimated by incorporation of labeled dihydrothymidine (Ide et al., unpublished results). This DNA was diluted with untreated f1-K12 DNA to give a concentration range of 0–120 fmol of dihydrothymine/well. An antibody response was observed at 20 fmol of dihydrothymine/well, which corresponds to approximately 0.1 dihydrothymine per 10000 bases.

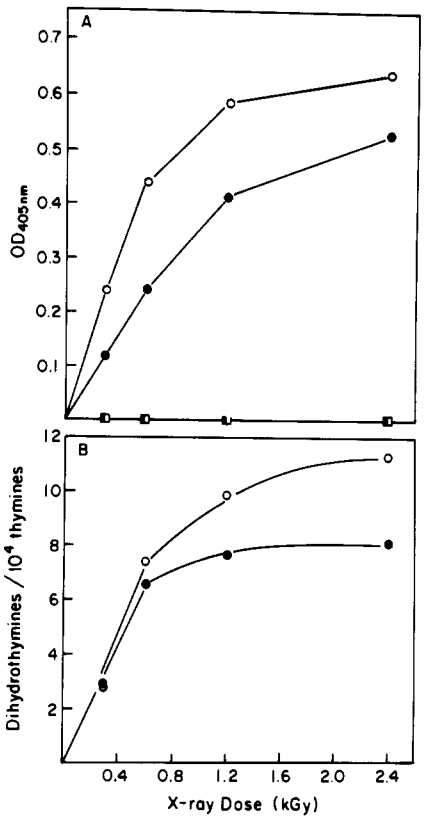

FIGURE 5: Reaction of anti-dihydrothymine antibody with X-irradiated DNA. Panel A shows the reactivity of the antibody with poly(dT) irradiated in a phosphate buffer solution (10 mM NaH_2PO_4, pH 7.0) under nitrogen (●), nitrogen plus 100 mM sodium formate (○), air (■), and oxygen (□). Panel B shows the quantitation of the number of dihydrothymines formed in X-irradiated single-stranded f1-K12 DNA in a phosphate buffer solution (10 mM NaH_2PO_4, pH 7.0) under nitrogen (●) and under nitrogen plus 100mM sodium formate (○).

Quantitation of Dihydrothymine in X-Irradiated DNA. In order to quantitate dihydrothymine in X-irradiated DNA, two irradiation conditions were used, either N_2 or N_2 in the presence of formate. It has been reported that the yield of dihydrothymine is increased by the presence of sodium formate (Wada et al., 1982). Figure 5A shows the anti-dihydrothymine antibody reactivity with X-irradiated poly(dT). When the poly(dT) was irradiated in a solution containing sodium formate, an increase in signal was observed compared to that of irradiation in its absence. In addition, no antibody reactivity was observed with poly(dT) X-irradiated in air or in oxygen, suggesting that the antibody does not cross-react with oxidative thymine damage. These data are in agreement with prior observations that dihydrothymine is produced primarily under deaerated conditions (Furlong et al., 1986).

Panel B of Figure 5 shows the reactivity of the anti-dihydrothymine antibody with single-stranded f1-K12 DNA which was irradiated under N_2 in the presence and absence of sodium formate. As was observed with irradiated poly(dT), the antibody signal increased when the DNA was irradiated in formate. A dihydrothymine standard containing 2.6 dihydrothymines per f1-K12 DNA molecule was used to quantitate the number of dihydrothymines formed under these conditions. The number of dihydrothymines produced at 600 Gy per 10000 thymines was 6.5 for DNA irradiated solely under nitrogen and 7.3 for DNA solutions containing formate.

The production of dihydrothymine in irradiated duplex DNA is shown in Figure 6. Native and denatured calf thymus

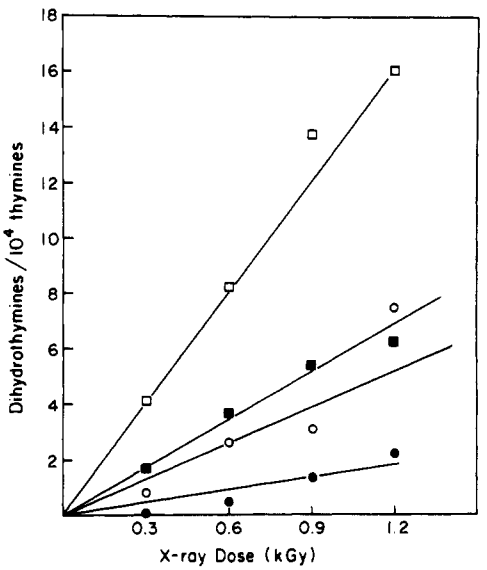

FIGURE 6: Quantitation of dihydrothymine by the anti-dihydrothymine antibody in calf thymus DNA X-irradiated under various conditions: (●) native DNA under nitrogen; (■) denatured DNA under nitrogen; (○) native DNA under nitrogen plus 100 mM sodium formate; (□) denatured DNA under nitrogen plus 100 mM sodium formate. All ELISA data were standardized with f1-K12 DNA containing 2.6 dihydrothymines per DNA molecule.

DNA were irradiated in the presence and absence of sodium formate under N_2. In the absence of formate, the level of dihydrothymine was approximately 8-fold higher in irradiated denatured DNA as compared to that in irradiated native DNA. In the presence of formate, the formation of dihydrothymine was enhanced, and the level of dihydrothymine was 3-fold higher for irradiated denatured DNA as compared to that of irradiated native DNA. Thus, the efficiency of dihydrothymine formation appeared to be reduced in duplex DNA.

Discussion

Immunochemical methods provide a sensitive means to detect and measure specific kinds of DNA damages in a background of other lesions including strand breaks. They are superior to many chemical assays which are less sensitive and can themselves cause chemical changes to DNA.

The anti-dihydrothymine antibody was shown to be both sensitive and specific in its reactivity with dihydrothymine. It can detect as little as 0.1 dihydrothymine per 10 000 bases. It does not cross-react with oxidative radiolysis products of thymine (Figure 5, panel A) or with base damages (Table I) produced in bases other than thymine under anaerobic conditions. The antibody was 6100 times more reactive with dihydrothymidine monophosphate than with thymidine monophosphate (Figure 3). Consequently, this antibody should be able to detect and to measure the production of dihydrothymine in vivo at biologically relevant levels. The antibody's ability to recognize damage only in denatured DNA is similar to that observed with antibodies elicited to unmodified bases (Stollar, 1980). In this regard, the antibody elicited to thymine glycol using a similar method (Rajagopalan et al., 1984) is exceptional since it reacts with thymine glycol in both native and denatured DNA. One possible explanation is that the hindered base stacking interaction due to the two OH groups of thymine glycol may cause enough local disorder (or denaturation) in duplex DNA (Clark et al., 1987) to allow the anti-thymine glycol antibody to access the lesion. In contrast, dihydrothymine in DNA seems to induce only minor perturbations in the local structure compared with thymine glycol (Ide et al., 1987a; Ide & Wallace, 1989) so that the antibody to dihydrothymine may not be able to access the lesion.

The antibodies could be used to show that exogenous dihydrothymidine was incorporated into the chromosomal DNA of *E. coli* strain LAP201, a strain lacking endonuclease III (*nth*$^-$) and requiring exogenous thymidine (*thy*$^-$) to grow. We also tested the effect of the *nth* mutation alone on the in vivo dihydrothymidine incorporation and found that LAP200 (*nth*$^-$) did not incorporate dihydrothymidine into its DNA. This was surprising since endonuclease III removes dihydrothymine from DNA in vitro (Demple & Linn, 1980; Melamede et al., 1987; Ide et al., 1988). These data strongly suggest that one of the key factors for in vivo dihydrothymidine incorporation is the nucleotide pool balance between dihydrothymidine and thymidine 5′-triphosphates. We are currently assuming that for LAP201 exogenous dihydrothymidine went through the same salvage pathway as thymidine (Kornberg, 1980); that is, it first is phosphorylated and then incorporated into DNA in competition with dTTP. Since SMH77 and LAP200 both have the normal de novo pathway for dTTP biosynthesis, the amount of dTTP may overwhelm the amount of dihydrothymidine triphosphate, thus preventing its incorporation.

The antibody detected the formation of dihydrothymine in X-irradiated single- and double-stranded DNA. The production of dihydrothymine was increased in DNA solutions containing sodium formate. In deaerated solutions containing formate, OH radicals and H atoms are converted to $CO_2^{\bullet-}$ (Wada et al., 1982). As a result, the primary reducing species are e^-_{aq} and $CO_2^{\bullet-}$. The conversion of thymine to dihydrothymine by e^-_{aq} is achieved by an electron adduct formed with thymine (Teoule & Cadet, 1978) followed by a subsequent protonation of the electron adduct. In the case of the $CO_2^{\bullet-}$ a possible mechanism for the production of dihydrothymine is that of an electron transfer to yield a thymine radical anion (Wada et al., 1982), which is then protonated. The increase in reducing species in deaerated formate solutions is 1.8 times (Wada et al., 1982) that normally produced. The increase in antibody signal for poly(dT) and single-stranded f1-K12 DNA irradiated in formate roughly approximates this value. For calf thymus DNA, whether irradiated in the native or denatured state, the increase was at least 2-fold higher in formate-containing solutions. Thus, the formation of dihydrothymine as measured by the antibody correlates well with predicted values that are based on the level of reducing species present.

The formation of dihydrothymine was greater in irradiated denatured than in irradiated native calf thymus DNA. Since the formation of dihydrothymine is partially dependent on the reaction of the e^-_{aq} with DNA (Teoule & Cadet, 1978), one limiting factor should be the rate of reaction with duplex DNA versus single-stranded DNA. Schragge et al. (1971) found that the relative rate constant for reaction of the e^-_{aq} with DNA is dependent on secondary structure. That is, as the DNA molecule becomes more structured (base stacking, hydrogen bonding, and shielded from water), the rate constant decreases. This was inferred from data showing that the difference was 5.77 times less for a duplex structure [poly(A+U)] as compared to that for a single-stranded structure [poly(U)]. We found that the formation of dihydrothymine in irradiated denatured DNA, regardless of the addition of formate, was greatly increased over that found in irradiated native DNA. The actual number of dihydrothymines produced in irradiated denatured DNA was slightly lower than that in single-stranded f1-K12 [1.09 dihydrothymine $(10^6$ thymines$)^{-1}$ Gy^{-1} for f1-K12 and 0.61 dihydrothymines $(10^6$ thymines$)^{-1}$ Gy^{-1} for denatured

calf thymus DNA]. These results are consistent with the decreased reaction rate of the e^-_{aq} (and probably $CO_2^{\bullet-}$) with duplex DNA.

At present, the biological consequences of dihydrothymine are not known. Since we now have an antibody against dihydrothymine that is specific and sensitive at biologically relevant levels of this damage, we should be able to gain some insight as to the significance of dihydrothymine formation during anoxic irradiation conditions.

ACKNOWLEDGMENTS

We are grateful to Lynn Petrullo for the construction of *E. coli* strains LAP200 and LAP201 and to Yoke W. Kow and Kihei Kubo for their helpful discussions.

Registry No. Dihydrothymine, 696-04-8.

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