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ANNUAL TECHNICAL PROGRESS REPORT

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AN IMMUNOCHEMICAL APPROACH TO THE STUDY OF DNA DAMAGE AND REPAIR

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DISCLAIMER

Progress Report

1. Antibody Development

A. Antibodies to Thymine Glycol.

The most studied radiation-induced modified DNA base is thymine glycol. Thymine glycols are produced in relatively high yields in irradiated DNA (28, 29) and are also formed as a consequence of oxidative stress (30). Thymine glycol has been shown to be an *in vitro* replicative block to DNA polymerases (43-46) as well as a cytotoxic lesion (49-51). DNA glycosylases that remove thymine glycol from damaged DNA are found in both prokaryotes and eukaryotes (for a review see 48). Thus this lesion provides a good model for studying the potential biological consequences of pyrimidine ring saturation products.

In order to elicit antibodies that would react with unique modified bases on a damaged DNA molecule, we have chosen to chemically synthesize the hapten of interest and conjugate it to a protein carrier. Thymine glycol monophosphate was synthesized, conjugated by the carbodiimide method (23,24) to bovine serum albumin (BSA), and used as an immunogen. Initially, polyclonal antibodies were produced in rabbits. These antibodies had high affinity and specificity as measured by both immunoprecipitation with thymine glycol monophosphate conjugated to rabbit serum albumin (RSA) and by enzyme immunoassay using either the conjugate or DNA oxidized by osmium tetroxide. In the competitive ELISA assay, antibody reactivity was inhibited by thymine glycol, thymidine glycol and thymine glycol monophosphate haptens. The latter inhibited to the greatest extent indicating antigenic determinants for the phosphate group in addition to the thymidine glycol. In both the direct and competitive assays, this antibody reacts with osmium tetroxide-treated DNA containing *cis*-thymine glycols and DNA X-irradiated *in vitro* at a femtomole level of sensitivity (23,24,66).

To produce monoclonal antibodies, BALB/c mice were immunized with the above conjugate in complete Freund's adjuvant. The immunization protocol was that of Holmdahl *et al* (67). A 50 μl volume of antigen (1 mg/ml) in adjuvant was injected subcutaneously in each hind foot pad. Nine days after the immunization, cells were isolated from the draining popliteal lymph nodes and were fused with mouse myeloma P3x63-Ag8-653 (68). Screening by dot-immunobinding and ELISA procedures gave 8 clones that bound OsO_4-treated DNA. One of them, 2.6F.6B.6C, an IgG2a kappa, was characterized further. Hapten inhibition studies with OsO_4-treated DNA showed that the antibody was specific for thymidine glycol monophosphate. Among the various inhibitors tested, thymidine glycol monophosphate > 5,6-dihydrothymidine monophosphate > thymidine glycol > thymine glycol. Inhibition by 5,6-dihydrothymidine, thymidine, thymine, AMP and CMP was negligible. In OsO_4-treated DNA, as few as 0.5 thymine glycols per 10,000 bp was detectable by direct ELISA. Like the polyclonal antibody, the monoclonal antibody was equally reactive with native or denatured DNA containing thymine glycol. Among X-irradiated homopolymers, dC, dA, dG, and dT, only dT reacted with the antibody. Thus, this monoclonal antibody is of potential use in assays for DNA damage caused by X-rays or other agents that damage DNA by free radical interactions. This work has been submitted for publication to *Radiation Research* (Chen *et al*).

B. Antibodies to Dihydrothymine

Dihydrothymine is the major product of radiolysis produced in DNA X-irradiated under anaerobic conditions (69-71). 5,6-Dihydrothymidine monophosphate was synthesized by catalytic hydrogenation of thymidine monosphosphate and extensively purified by anion exchange high performance liquid chromatography as described below. 5,6-Dihydrothymidine monophosphate was conjugated to BSA using the carbodiimide method (7) and then used as an immunogen in rabbits. Using an ELISA assay, the antibodies produced were found to be specific for dihydrothymine. Hapten inhibition studies showed that dihydrothymidine monophosphate was three orders of magnitude more effective as an inhibitor than thymidine monophosphate and four orders of magnitude more effective than thymidine glycol monophosphate. With DNA containing dihydrothymine, antibody reactivity was observed at 20 fmole dihydrothymine, which is approximately 0.1 dihydrothymine/10,000 bases. Thus, the assay is very sensitive. The antibody reacted only with denatured DNA containing dihydrothymine but not with native DNA containing this lesion. This preference is in contrast to the anti-thymine glycol antibody which reacts equally well with the lesion in native or denatured DNA. One possible explanation for this difference in reactivity is that the hindered base stacking interactions due to the two OH groups of thymine glycol may cause enough local disorder (or denaturation) in duplex DNA (72) to allow the anti-thymine glycol antibody to access the lesion. In contrast, dihydrothymine in DNA may induce only minor perturbations in the local structure compared with thymine glycol (40,73) so that the antibody to dihydrothymine may not be able to access the lesion. The results of these studies are have been published in Biochemistry (Hubbard et al, 1989).

C. Quantitation of Abasic Sites in DNA

Abasic (apurinic, apyrimidinic, AP) sites are the most common cellular DNA lesion (35). They are produced by radiation, a variety of chemical agents and by free radicals generated during oxidative metabolism. Abasic sites are also intermediates formed during the removal of modified bases by DNA glycosylases. Because they are labile in alkali, abasic sites are relatively easy to measure when they are selectively produced in vitro. However, they are more difficult to quantitate in cellular DNA, especially when they are generated by environmental agents that also induce strand breaks. Thus it seemed desirable to develop an immunochemical method to measure this important lesion. Our strategy consisted of eliciting an antibody specific for a reagent that reacts with the aldehyde group that is formed as a result of depurination of DNA (74). The reagent chosen was O-4-nitrobenzylhydroxylamine. Monoclonal antibodies specific for this moiety were raised by immunizing with a conjugate of BSA and the O-4-nitrobenzylhydroxylamine of deoxyribose-5'-phosphate as described above for thymine glycol. Screening was done by an ELISA using O-4-nitrobenzylhydroxylamine deoxyribose-5'-phosphate conjugated to RSA. Of 40 wells tested, 10 were positive. The positive supernatants were subsequently assayed by ELISA for binding to depurinated calf thymus DNA (200 sites per 10,000 bp) treated with O-4-nitrobenzylhydroxylamine. Of the 10 positive lines, one reacted with the apurinic-DNA, as well as with native calf thymus DNA, both treated with O-4-nitrobenzylhydroxylamine. Two clones bound treated apurinic DNA only. The latter were subcloned twice by limiting dilution. Four clones were isolated and culture supernatants were collected. Ascites were prepared from them as well.

Using the direct ELISA assay, as few as 1 abasic sites per 20,000 bp could be detected. The O-4-nitrobenzoxylamine derivative of deoxyribose-5'-phosphate was the best inhibitor of the reaction, about 20 times more effective than O-4-nitrobenzylhydroxylamine. There was about a two-fold increase in immunoreactivity with the modified abasic site in denatured versus native DNA. The results of these studies are in preparation for Radiation Research (Chen et al).

We have used the same principle to develop a sensitive chemical assay for quantitating abasic sites. We have synthesized a chemical compound, biotin-conjugated O-carboxymethylhydroxylamine (aldehyde-reactive probe, ARP), that reacts specifically and quantitatively with the aldehyde group of abasic sites in DNA. Therefore, we can tag the abasic site in the DNA with biotin and then determine the number of biotin-tagged abasic sites using horseradish peroxidase-conjugated avidin-biotin complex in an ELISA-like assay.

The abasic site (ABS) test directly detects abasic sites in exposed DNA. This can be done in two experimental formats: 1) DNA is directly exposed to the agent in vitro and the number of abasic sites produced determined. 2) An E. coli mutant defective in AP endonucleases essential for base excision repair (75), is exposed to an agent that produces AP sites using a suspension of the bacterial strain. Using this assay, we are able to detect generation of abasic sites by in E. coli cells produced by 0.5 μg/ml methyl methanesulfonate (MMS). We calculate that the ABS test is able to detect 1 abasic site per 2 million bases on 100 nanograms of DNA. The results of these studies are in preparation for PNAS (Kow et al) pending filing a patent application.

D. Preparation of Antibodies to 8-Hydroxyadenine and 8-Hydroxyguanine

The deoxyribose derivatives of 8-hydroxyadenine and 8-hydroxyguanine have been found as products of the radiolysis of nucleosides and DNA (76,77). We have prepared the nucleoside derivatives according to the method of Ikehara et al (78). For the synthesis of 8-hydroxyadenosine, 8-bromoadenosine, which is commercially available, was refluxed in acetic anhydride in the presence of sodium acetate. The resulting N^6-acetyl-8-hydroxyadenosine was incubated in concentrated ammonia at room temperature to remove the acetyl group. After removing acetamide by chloroform extraction, 8-hydroxyadenosine was recrystallized from water. 8-Hydroxyguanosine was prepared by essentially the same procedure as 8-hydroxyadenosine except that 8-bromoguanosine was synthesized by bromination of guanosine (79).

To produce the immunogens, 8-hydroxyadenosine and 8-hydroxyguanosine were conjugated to BSA and RSA by the method of Erlanger and Beiser (5). This involves the periodate cleavage of the ribose ring, conjugation to free amino groups in the albumin, reduction of the Schiff base and stabilization of the conjugate by sodium borohydride. The number of haptenic groups was determined by UV absorbance. Rabbits were immunized with the BSA conjugates and polyclonal antibodies were produced.

The antibody to 8-hydroxyadenine has a very high titer, giving a strong signal with the RSA conjugate at a 1:10,000 dilution. The IC_{50} for 8-hydroxyadenosine was 20 μM. No inhibition was observed with 8-bromadenosine, adenosine, guanosine or inosine at levels of 1.25 mM. This method has already been successfully used by West et al (20) to elicit antibodies to 8-hydroxyadenosine. Unfortunately, due to a refrigerator failure, these antibodies are no longer available (J. Ward, personal

communication). Rabbits have been immunized with the 8-hydroxyguanosine conjugate and we are currently awaiting the results from the first test bleed.

E. Results with Other Conjugates

a. Urea residues

Urea residues are fragmentation products of the radiolysis of thymine and they have been shown to be both cytotoxic (51) and mutagenic (27). To prepare antibodies to urea glycosides, thymidine glycol nucleoside monophosphate was prepared by hydrolytic bromination of thymidine monophosphate and conjugated to BSA by the carbodiimide method (7). The conjugates were then hydrolyzed in alkali to cleave the thymine glycols (31,44) leaving urea residues attached to the BSA moiety. This was then used as an immunogen in rabbits. Although the antisera reacted with the urea conjugate in an ELISA assay, they reacted poorly with urea in DNA thus obviating their use for immunochemical quantitation of urea in oxidized or irradiated DNA. It is possible that the antigenic determinant of the urea moiety is too small to elicit a tightly binding antibody.

b. 5-Hydroxy-5-methylhydantoin

When DNA is X-irradiated under aerobic conditions in vitro, a predominant stable hydrolysis product of thymine found is 5-hydroxy-5-methylhydantoin. The presence of this product has been demonstrated either by mild acid hydrolysis of X-irradiated DNA (28) or by HPLC analysis of endonuclease III-digested X-irradiated DNA (29). [Endonuclease III releases 5-hydroxy-5-methylhydantoin in a glycosylic reaction (80)]. To prepare antibodies to 5-hydroxy-5-methylhydantoin, we chemically synthesized an antigenic analogue, 5-hydroxy-hydantoin-5-propionic acid, which was conjugated to BSA for use as an immunogen. Antibodies elicited to this conjugate reacted with RSA conjugates of the hapten in an ELISA assay but reacted poorly with DNA oxidized with $KMnO_4$ which has been shown to contain high levels of hydantoin (80). It is possible that because of the nature of the hapten-conjugate link, the antigenic determinants of the hapten in the immunogen were structurally different from the hydantoin in DNA.

2. Applications

A. Synthesis and Use of Nucleotide Haptens

5,6-Dihydrothymidine 5'-triphosphate (DHdTTP) was synthesized by catalytic hydrogenation of thymidine 5'-triphosphate (dTTP). Thymidine glycol 5'-triphosphate was prepared by bromination of dTTP followed by treatment with Ag_2O. The modified nucleotides were extensively purified by anion-exchange HPLC. Alkaline phosphatase digestion of DHdTTP and thymidine glycol 5'-triphosphate gave the expected products (5,6-dihydrothymidine and cis-thymidine glycol), the identities of which were confirmed by reverse-phase HPLC using authentic markers. HPLC analysis of the alkaline phosphatase-digested DHdTTP revealed that DHdTTP was a mixture of C5 diastereoisomers [(5S)-and (5R)-DHdTTP]. Despite the significant distortion of the pyrimidine ring in DHdTTP, it was incorporated in place of dTTP during primer elongation catalyzed by Escherichia coli DNA polymerase I Klenow fragment. The rate of incorporation of DHdTTP was about 10-25-fold lower than that of dTTP. On the other hand, thymidine glycol 5'-triphosphate, which also has a distorted pyrimidine ring, did not replace dTTP, and no elongation of the primer was observed. In order to study the preference of incorporation of the

diastereoisomers of DHdTTP into DNA, salmon testes DNA, activated by exonuclease III, was used as a template for DNA polymerase I Klenow fragment in the presence of [^{3}H]DHdTTP (S and R mixture) and normal nucleotides. After enzymatic digestion of the DNA to nucleosides, the products were analyzed by HPLC. The ratio of the isomers incorporated into DNA (S:R = 73:27) was virtually the same as that of the [^{3}H]DHdTTP substrates (S:R = 79:21). This result suggests that Escherichia coli DNA polymerase I uses both isomers of DHdTTP as substrates and that the overall efficiency of incorporation is primarily determined by the concentration of the isomers in the nucleotide pool. These studies were published in Biochemistry (Ide et al, 1987).

The ability of dihydrothymidine and thymidine glycol 5'-triphosphates to serve as substrates for different DNA polymerases was investigated. Although DHdTTP but not thymidine glycol 5'-triphosphate was used as a substrate by E. coli DNA polymerase I, within the detection limit of the assay used, neither T4 DNA polymerase nor avian myeloblastosis virus reverse transcriptase used DHdTTP or thymidine glycol 5'-triphosphate as substrates. The ability of DHdTTP and thymidine glycol 5'-triphosphate to undergo enzyme-catalyzed turnover to the monophosphate paralleled their ability to serve as substrates for polymerization. These results, along with kinetic parameters for the incorporation of DHdTTP with Pol I, strongly suggest that the saturation of thymine C5-C6 bond and the substituent groups at C5 and C6 differentially exert effects on binding to DNA polymerases. DNA sequencing gel analysis of the polymerization products revealed that most single adenine sites were capable of templating DHdTTP; however, DNA synthesis was partially arrested at multiple adenine sites, suggesting that sequential incorporation of DHdTTP produced significant disorder in the primer terminus. These results suggest that incorporation, or lack thereof, of modified nucleoside triphosphates is not directly related to their ability to base pair but may be related to stacking interactions and/or initial binding to the polymerases. These data were published in Nucleic Acids Research (Ide and Wallace, 1988).

Using this approach we have been able to construct duplex DNA molecules containing dihydrothymine as standards for anti-dihydrothymine antibody reactions so that we can directly quantitate the production of this radiolysis product in X-irradiated DNA.

B. Antibodies to Thymine Glycol

a. In vitro studies.

Using polyclonal antibody to thymine glycol, we have quantitated the production of thymine glycols in oxidized and X-irradiated DNA. Escherichia coli, ØX-174 RFI, PM2, and M13 phage DNA containing thymine glycols introduced by OsO_4 oxidation were used as antigens in a direct ELISA assay. The number of thymine glycols per molecule was determined by reactivity with anti-thymine glycol antibody standardized either to the acetol fragment assay or to the number of Escherichia coli endonuclease III-sensitive sites. The number of thymine glycols was also determined in ØX-174 RF DNA X-irradiated in either phosphate or Tris buffer; in phosphate buffer, the antibody detected damage at the level of 40 Gy. The immunochemical assay was sensitive, specific, quantitative and independent of DNA structure. These results were published in Radiation Research (Hubbard et al, 1989).

In order to determine the contribution of thymine glycols to the spectrum of damages produced by X-rays, antibodies raised to thymine glycol were used to quantitate this lesion in X-irradiated DNA, and the number observed was compared to the number of acetol fragments and endonuclease III-sensitive sites. PM2 DNA was X-irradiated under a variety of conditions and in all cases, the majority of endonuclease III-sensitive sites measured were thymine ring-saturation products. In phosphate buffer, thymine glycols represented a small proportion of thymine ring-saturation products, while in Tris, all thymine ring-saturation products were thymine glycols. An hydroxyl radical scavenger, KI, more efficiently reduced the number of thymine ring-saturation products than endonuclease III-sensitive sites. The results of these studies are in preparation for Radiation Research (Hubbard et al).

b. In vivo studies.

We have utilized the anti-thymine glycol antibody to quantitate thymine glycols in phage transfecting DNA in order to assess the lethal and mutagenic consequences of this base lesion. The genetic requirements for the excision repair of thymine glycols, urea residues, and abasic sites were examined by measuring the survival in Escherichia coli mutants of ØX174 replicative form (RF) I transfecting DNA containing selectively introduced lesions. ØX RF I DNA containing thymine glycols was inactivated at a greater rate in mutants deficient in endonuclease III (nth) than in wild-type hosts, suggesting that endonuclease III is involved in the repair of thymine glycols in vivo. ØX RF I DNA containing thymine glycols was also inactivated at a greater rate in mutants that were deficient in both exonuclease III and endonuclease IV (xth nfo) than in wild-type hosts, suggesting that a class II AP endonuclease is required for the in vivo processing of thymine glycols. ØX duplex-transfecting DNA containing urea residues or AP sites was inactivated at a greater rate in xth nfo double mutants than in wild-type, but not in single-mutant hosts, suggesting that exonuclease III or endonuclease IV is required for the repair of these damages and that either activity can substitute for the other. These data are in agreement with the known in vitro substrate specificities of endonuclease III, exonuclease III, and endonuclease IV. The results of this work were published in Journal of Bacteriology (Laspia and Wallace, 1988).

It is interesting that thymine glycols have the same inactivation efficiency in this system as urea residues. This is similar to what we had previously observed in the PM2 system for thymine glycols and apurinic sites (50). Thus, this minor modified base, thymine glycol, and two-non-instructive lesions, urea residues and AP sites, all constitute replicative blocks to DNA synthesis in vitro (43-46,56) and have about the same inactivation efficiency in vivo.

In collaborative study with Gene LeClerc at the University of Rochester, single stranded M13 DNA was oxidized with OsO_4, the number of thymine glycols quantitated by the ELISA assay, and the DNA used in a forward mutation assay. Inactivation of transfecting molecules showed that one lethal hit corresponded to 1.5 to 2.1 thymine glycols per phage DNA molecule in normal cells, whereas SOS induction reversed 60 to 80% of inactivating events. Forward mutations in the lacI and lacZ'(α) genes of f1 and M13 hybrid phage DNAs were induced in OsO_4-treated DNA in a dose-dependent manner, in both wild-type and umuC cells. Sequence analysis of hybrid phage mutants revealed that mutations occurred preferentially at cytosine sites rather than thymine sites, indicating that thymine glycols were not the principal pre-mutagenic lesions in the single-stranded DNA. A mutagenic specificity for C --> T transitions was confirmed by OsO_4-induced reversion of mutant lac phage.

It would appear therefore, that a minor oxidation product of C is responsible for the mutagenic response of osmium tetroxide. A likely candidate is 5-hydroxyuracil, a degradation product of the unstable cytosine glycol which has been found in OsO_4 oxidized DNA (81). 5-Hydroxyuracil should be a coding lesion leading to the observed transitions. Further support for this idea comes from the fact that osmium tetroxide-induced mutagenesis in M13 showed very little dependence on preinduction of the SOS response, whereas osmium tetroxide-induced lethality, as measured by reactivation, was greatly reduced by preinduction of the SOS system. The latter presumably results because of bypass of the thymine glycols which are replicative blocks (lethal) but not mutagenic. These studies were published in the Journal of Molecular Biology (Hayes et al, 1988).

ØX-174 RF 1 transfecting DNA containing thymine glycols quantitated by the anti-thymine glycol antibody, urea glycosides or abasic sites was used to study SOS processing of unique lesions in Escherichia coli. An increase in survival of damage-containing DNA in SOS-induced hosts (Weigle reactivation) was used as a measure of translesion bypass. As with single-stranded DNA, thymine glycols were very efficiently bypassed with some 65% of the lesions being reactivated in UV-induced hosts that were defective in the excision repair of this damage.

Genetic studies demonstrated that SOS processing of thymine glycols required the activated form of RecA as well as UmuC. Since thymine glycols have been shown to be blocks to replication but template an A, these data suggest that both the recA and umuDC gene products are required for the chain elongation step. In contrast to thymine glycols, no reactivation of ØX RF 1 DNA containing urea glycosides or AP sites was observed. These data suggest that SOS-induced increases in survival do not reflect an induced generalized repair system for all replication-blocking, lethal lesions but rather, that the efficiency of reactivation is lesion-dependent with non-instructive, potentially mutagenic lesions being inefficiently bypassed. Lastly, a substantial fraction, about 35%, of potentially lethal thymine glycols could be UV-reactivated in an umuC, lexA, recA, independent manner suggesting the existence of an as yet uncharacterized damage-inducible SOS-independent mode of thymine glycol repair. These studies were published in the Journal of Molecular Biology (Laspia and Wallace, 1989).

C. Antibodies to Dihydrothymine

a. In vitro studies.

Antibodies to dihydrothymine were used to quantitate the formation of dihydrothymine in DNA X-irradiated under N_2 (detection was at the femtomole level). 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^- (82). As a result, the primary reducing species are e^-_{aq} and CO_2^-. The conversion of thymine to dihydrothymine by e^-_{aq} is achieved by an electron adduct formed with thymine (83) followed by a subsequent protonation of the electron adduct. In the case of CO_2^-, a possible mechanism for the production of dihydrothymine is that of an electron transfer to yield a thymine radical anion (82), which is then protonated. The increase in reducing species in deaerated formate solutions is 1.8 times (82) 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 the denatured state, the increase was at least 2-fold 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 results of these studies were published in Biochemistry (Hubbard et al, 1989).

The anti-dihydrothymine antibody was also used in the study of processing of DNA base damages by DNA polymerases. Single-stranded f1 hybrid phage DNA containing dihydrothymine or β-ureidoisobutylic acid was used as a model for instructive or non-instructive base lesions, respectively. Hybrid phage (f1-K12) DNA containing dihydrothymine was prepared by in vivo incorporation of exogenous dihydrothymidine in DNA, while DNA containing β-ureidoisobutylic acid was prepared by mild alkali hydrolysis of dihydrothymine-containing f1-K12 DNA. To predict potential biological consequences, the interaction of DNA containing these lesions with DNA polymerases in vitro was examined. DNA synthesis was measured by ^{3}H-labeled nucleotide incorporation and DNA sequencing gel analysis of newly synthesized products showed that dihydrothymine in the template strand constituted only a weak block to in vitro DNA synthesis catalyzed by Escherichia coli DNA polymerase I Klenow fragment (Pol I), T4 DNA polymerase, and avian myeloblastosis virus (AMV) reverse transcriptase. In contrast, β-ureidoisobutylic acid constituted a very strong (probably absolute) replicative block in vitro. Although β-ureidoisobutylic acid is devoid of pyrimidine ring structure and is apparently non-instructive, a nucleotide was incorporated opposite the putative site by Pol I and AMV reverse transcriptase. With T4 DNA polymerase, that contains a highly active 3'-5' exonuclease, DNA synthesis was arrested almost exclusively one base prior to (3' to) the putative β-ureidoisobutylic acid site in the template strand. These data predict that dihydrothymine should be innocuous in vivo while the ring-open product, β-ureidoisobutylic acid should be cytotoxic. These data have been submitted for publication to the Journal of Molecular Biology (Ide et al).

b. In vivo studies.

Antibodies to dihydrothymine were 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 in 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 (84-86). 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 goes through the same salvage pathway as thymidine, 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. These studies were reported in Biochemistry (Hubbard et al, 1989).

We also found that exogenously added dihydrothymidine was incorporated into phage PM2 DNA in vivo. The maximum amount of dihydrothymidine incorporated was 1.38 per PM2 DNA molecule, which corresponds to 0.018% of thymine in PM2. Interestingly, the percentage of form I DNA in the DNA preparation and the total yield of DNA decreased with increasing amounts of dihydrothymidine added. It is likely that a presumptive DNA repair enzyme from the host (Altermonas espejiana) produced nicks at dihydrothymine sites in PM2 DNA so that the percentage of form I and the yield of PM2 DNA were reduced simultaneously. We also checked the

incorporation of dihydrothymidine analogues, thymidine glycol and deoxydihydrouridine. However, these compounds were not incorporated into PM2 DNA. These results were published in Anticarcinogenesis and Radiation Protection (Ide et al, 1987).

Lastly, the cytoxicity of dihydrothymine lesions was examined in single-stranded phage transfecting DNA. In keeping with the results obtained in vitro, dihydrothymine present in transfecting f1-K12 DNA did not constitute a significant inactivating lesion. However, it took about 0.9 β-ureidoisobutylic acid to inactivate transfecting f1-K12 DNA, suggesting that this lesion was an absolute replicative block in vivo. When host cells were UV-irradiated to induce the SOS-response, a slight increase (about 2-fold) in survival of transfecting f1-K12 phage DNA containing β-ureidoisobutylic acid was observed. These results were also included in the recent submission to the Journal of Molecular Biology (Ide et al).

D. Detection of Abasic Sites

Using heat/acid depurinated calf thymus DNA, both the O-carboxymethylhydroxylamine-biotin conjugate and the monoclonal antibody elicited to O-4-nitrobenzylhydroxylamine deoxyriboside 5'-monophosphate were able to detect 1 AP site per 20,000 bp. The ABS test also detected in vivo depurination induced by 0.5 μg/ml of methyl methanesulfonate (MMS) in wild type and an excision repair defective strain of E. coli. The rate of formation of abasic sites was linear in the DNA isolated from E. coli pretreated with MMS in the range between 0.5 and 10 μg/ml. Furthermore, using the ABS test, X-ray-induced damages were detected in vitro below 100 rad. By extrapolation of the dose response curve, we estimate that we can detect damage at a dose as low as 30 rad.

3. Professional Personnel

Susan S. Wallace, Ph.D., Professor, New York Medical College, Professor and Chair, University of Vermont, 15%, 12-1-88 to present.

Bernard F. Erlanger, Ph.D., Professor of Microbiology, Columbia University, College of Physicians and Surgeons, 10%, 5-15-86 to present.

Kihei Kubo, D.V.M., Postdoctoral Research Associate, New York Medical College, University of Vermont, 100%, 12-1-88 to 2-28-89.

S. Ramabhadian, Research Assistant, Columbia University, College of Physicians and Surgeons, 50%, 12-1-88 to present.

Bi-Xing Chen, Postdoctoral Research Associate, Columbia University, College of Physicians and Surgeons, 60%, 2-1-88 to present (salary supported by other funds).

Hiroshi Ide, Ph.D., Research Assistant Professor, New York Medical College and University of Vermont, 15%, 12-1-88 to 2-28-89 (salary supported by other sources); 25%, 3-1-89 to present.

Yoke Wah Kow, Ph.D., Research Assistant Professor, New York Medical College and University of Vermont, 10%, 12-1-88 to present (salary supported by other funds).

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