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TECHNICAL PROGRESS REPORT

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AN IMMUNOCHEMICAL APPROACH TO THE STUDY OF DNA DAMAGE AND REPAIR

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GENERAL OBJECTIVES

The overall objective of this project has been to develop immunochemical methods to quantitate unique DNA base damages in order to facilitate studies on radiation-induced damage production and repair. Specifically, we have been using antibodies raised to damaged bases to quantitate unique lesions in model systems in order to evaluate their potential biological consequences. Our approach has been to synthesize modified nucleotides or nucleosides, conjugate them to protein carriers, and use the conjugates as immunogens in rabbits or to prepare monoclonal antibodies. We have been studying damages that are stable radiolysis products found in X-irradiated DNA and thus of potential biological consequence. Our aim is to build an *in vitro* and *in vivo* data base on the interactions between model DNA lesions and such cellular enzymes as DNA polymerases and repair endonucleases. Initial studies have focused on pyrimidine ring saturation products (thymine glycol and dihydrothymine), products resulting from ring fragmentation or base loss (urea, ß-ureidoisobutyric acid, abasic sites), 7-hydro-8-oxopurines, and more recently, cytosine radiolysis products. These modified bases serve as useful models for examining the potential lethal and/or mutagenic (carcinogenic) effects of the products of DNA radiolysis.

PROGRESS SINCE LAST SUBMISSION

1. Antibody Development

The purpose of this project, since its inception, has been to produce antibodies to unique modified DNA bases and to develop immunochemical assays to quantitate these lesions. We have been successful for thymine glycol, dihydrothymine, abasic sites, and the 7-hydro-8-oxopurines. As shall be described in Section 2B of the Progress Report, these reagents have been extremely useful to us in both our *in vitro* and *in vivo* biological studies of damage consequence.

A. Thymine Ring Saturation Products

Thymine ring saturation products are common products of DNA radiolysis and are the most studied. A summary of the processing of thymine glycol and dihydrothymine is given in Section 2B of the Progress Report. We elicited both poly- and monoclonal antibodies to the thymine glycol hapten. To do this, thymidine glycol monophosphate was synthesized, conjugated by the carbodiimide method (8) to bovine serum albumin (BSA), and used as an immunogen. The antibodies elicited had high affinity and specificity as measured by both immunoprecipitation with thymidine glycol monophosphate conjugated to rabbit serum albumin (RSA) and by enzyme immunoassay using either the conjugate or DNA oxidized by osmium tetroxide. Characterization of the polyclonal antibody was published during the prior funding period (Rajagopalan, Melamede, Laspia, Erlanger and Wallace in Radiation Research, 97:499, 1984); the monoclonal antibody was characterized during the current period (Chen, Hubbard, Ide, Wallace and Erlanger in Radiation Research, 124:131, 1990).

In both the direct and competitive assays, antibodies to thymine glycol react at a femtomole level of sensitivity with osmium tetroxide-treated DNA containing *cis*-thymine glycols as well as with DNA X-irradiated *in vitro*. Details of the quantitative studies can be found in Hubbard, Huang, Laspia, Ide, Erlanger, and Wallace in Radiation Research, 118:257, 1989.

To produce antibodies to dihydrothymine, 5,6-dihydrothymidine monophosphate was synthesized by catalytic hydrogenation of thymidine monophosphate, extensively purified by anion exchange high performance liquid chromatography, conjugated to BSA using the carbodiimide method (8) (a detailed description of this procedure is given on page 7, Section 1D of the Progress Report), 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 the femtomole level. 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. These studies were described in Hubbard, Ide, Erlanger and Wallace in Biochemistry, 28:4382, 1989.

B. Abasic Sites

Abasic (apurinic, apyrimidinic, AP) sites are the most common cellular DNA lesion (41). 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 the removal of a base from DNA (42). The reagent chosen was 0-4-nitrobenzylhydroxylamine (NBHA). Monoclonal antibodies specific for this moiety were raised by immunizing with a conjugate of BSA and the O-4-nitrobenzylhydroxylamine of deoxyribose-5'-phosphate. The antibody was specific for the NBHA residue as demonstrated by hapten inhibition, with IC_{50} values for 5'-phosphodeoxyribosyl-NBHA, deoxyribosyl-NBHA, ribosyl-NBHA and NBHA of 0.3 μM, 5 μM and 7 μM, respectively. Other haptens examined, including O-benzylhydroxylamine, 5'-phosphodeoxyribosyl-, deoxyribosyl-, and ribosyl-benzylhydroxylamine, showed no inhibition even at 1 mM. The antibody showed high specificity for NBHA-modified AP sites in DNA and exhibited no cross reactivity with normal DNA bases, otherwise-modified DNA bases or unmodified AP sites. Using a direct ELISA assay, the antibody detected 1 AP site (after NBHA-modification) per 10,000 base-pairs or approximately 10 femtomoles of AP sites in DNA. DNA lesions were detectable in ^{60}Co γ-irradiated DNA at a dose as low as 10 rad (0.1 Gy) and the production of antibody detectable sites was proportional to the γ-ray dose. This work was reported by Chen, Kubo, Ide, Erlanger, Wallace and Kow in Mutation Research, 273:253, 1992.

We have used the same principle to develop a sensitive chemical assay for quantitating abasic sites. Biotin-conjugated 0-carboxymethylhydroxylamine (aldehyde-reactive probe, ARP), that reacts specifically and quantitatively with the aldehyde group of abasic sites in DNA, was synthesized. The abasic site in DNA can be tagged with biotin and then the number of biotin-tagged abasic sites can be determined using horseradish peroxidase-conjugated avidin-biotin complex in an ELISA-like assay.

The ARP directly detects abasic sites in exposed DNA. This has been done in two experimental formats: 1) DNA was 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 (43), was exposed to an agent that produces AP sites using a suspension of the bacterial strain. Using this assay, we were able to detect generation of abasic sites in *E. coli* cells produced by 0.5 μg/ml methyl methanesulfonate (MMS). We calculate that the ARP is able to detect 1 abasic site per 2 million bases on 100 nanograms of DNA. This work has been described by Kubo, Ide, Wallace and Kow in Biochemistry, 31:3703, 1992.

C. 7-Hydro-8-oxopurines

The deoxyribose derivatives of 7-hydro-8-oxoadenine (8-oxoA) and 7-hydro-8-oxoguanine (8-oxoG) have been found as products of the radiolysis of nucleosides and DNA (2,44,45). Furthermore, it has recently been shown that 8-oxoG is an important mutagenic lesion (28-30) being subject to several DNA repair and mutagenic avoidance systems (46-48). To prepare antigens, nucleoside derivatives of 8-oxoA and 8-oxoG were synthesized according to the method of Ikehara *et al.* (49). For the synthesis of 8-oxoA, 8-bromoadenosine, which is commercially available (Sigma), was refluxed in acetic anhydride in the presence of sodium acetate. The resulting N-acetyl-8-oxoadenosine was incubated in concentrated ammonia at room temperature to remove the acetyl group. After removing acetamide by chloroform extraction, 7-hydro-8-oxoadenosine was recrystallized from water. 7-Hydro-8-oxoguanosine was prepared by essentially the same procedure as 8-oxoA except that 8-bromoguanosine was synthesized by bromination of guanosine (50).

To produce the immunogens, 7-hydro-8-oxoadenosine and 7-hydro-8-oxoguanosine were conjugated to BSA and RSA by the method of Erlanger and Beiser (6). This involves the periodate cleavage of the ribose ring, conjugation to primary 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 antibodies to 8-oxoA and 8-oxoG precipitated the homologous antigens in an Ouchterlony gel diffusion test and no cross-reactivity was observed with either antibody towards adenosine, thymidine, guanosine or thymidine monophosphate conjugates. The antibodies exhibited the same specificity for the homologous conjugate in an ELISA assay.

Antibody specificity was also examined by hapten inhibition of antibody reactivity with homologous conjugates using an ELISA assay. For anti 8-oxoA the IC_{50} for 8-oxoadenosine was 8 μM. 8-Bromoadenosine, adenosine, guanosine and inosine did not

inhibit even at concentrations of 1.25 mM. Similarly, the antibody to 8-oxoG was highly specific for the eliciting hapten, with the IC_{50} for 8-oxoguanosine being 0.1 μM. 8-Methoxyguanosine also inhibited the reaction but 7-hydro-8-oxoguanosine was about 500 times more effective. 8-Bromoguanosine, guanosine, adenosine, thymidine glycol monophosphate or 7-hydro-8-oxoadenosine did not inhibit at concentrations up to 100 mM. Furthermore, antibodies to 8-oxoA only reacted with irradiated poly dA and not to irradiated or unirradiated homopolymers containing dC, dA or dT. We were unable to obtain consistent results with poly dG, probably because it aggregates in solution. Thus, both antibodies appear to be highly specific for the eliciting antigen exhibiting little cross-reactivity to related unirradiated and irradiated bases.

The X-ray dose response of the production of 8-oxoA in poly dA irradiated in phosphate buffer was also determined. Here adducts were easily detectable at 2.5 Gy. In order to standardize the ELISA signal, the amount of 8-oxoA produced in irradiated poly dA was analyzed by HPLC. First the polynucleotide was digested to completion with P1 nuclease and the 5' phosphates removed by *E. coli* alkaline phosphatase. The resulting nucleosides were analyzed by HPLC and detectable quantities of 2'-deoxy-7-hydro-8-oxoadenosine were measured at doses of 22.5 gray. These data were then used to quantify the number of 8-oxoA residues produced per 1000 nucleotides of irradiated poly dA using an ELISA assay. Similarly the number of 8-oxoG adducts was quantified by HPLC and electrochemical detection. The results of this work has been submitted for publication to Radiation Research (Ide, Kow, Chen, Erlanger and Wallace).

D. Antibodies to 5-hydroxyuracil and 5-hydroxycytosine

Both 2'-deoxy-5-hydroxycytidine and 2'-deoxy-5-hydroxyuridine are major products of deoxycytidine oxidative damage in DNA (27) and are potentially important premutagenic lesions. In order to prepare antibodies to these lesions, 2'-deoxy-5-hydroxycytidine-5'-phosphate ($dC^{5OH}MP$) and 2'-deoxy-5-hydroxyuridine-5'-phosphate ($dU^{5OH}MP$) were synthesized.

$dC^{5OH}MP$ was prepared from dCMP by a method similar to one previously described for the conversion of cytidine to 5-hydroxycytidine (51). $dC^{5OH}MP$ was obtained by the action of bromine on dCMP in aqueous medium followed by 2,4,6-collidine treatment. After ether extraction of 2,4,6-collidine, the reaction mixture was loaded on to a DEAE-Sephadex A-25 column. The nucleotide fraction was eluted from the column with a linear gradient of triethylammonium bicarbonate (TEAB) buffer (0 to 0.8 M, pH 7,8). TEAB was removed by repeated evaporation with ethanol at 50° C. The product obtained was analyzed by FPLC using a Mono Q column and a C18 column coupled with an electrochemical detector. The elution peaks were monitored by simultaneous UV absorption at 270 nm (λ_{max} for dCMP at neutral pH) and 290 nm (λ_{max} for $dC^{5OH}MP$ at neutral pH) and electrochemical detection. The peak corresponding to $dC^{5OH}MP$ was identified by its UV spectrum and oxidation potential value (27,51). The conversion of dCMP to $dC^{5OH}MP$ was found to be more then 98%. The amount of the initial dCMP and $dU^{5OH}MP$ (the deamination product of $dC^{5OH}MP$) in the final mixture was less then 1%.

$dU^{5OH}MP$ was prepared in the same way as described above for $dC^{5OH}MP$, but using pyridine instead of 2,4,6-collidine (52). After column chromatography on DEAE Sephadex A-25, the nucleotide fraction contained two major products: $dU^{5OH}MP$ (about 50-55%) and 5-bromo-dUMP (about 20-25%). $dU^{5OH}MP$ was further purified by FPLC using a Mono Q column. The elution buffers were 0.005 M NaCl, 20 mM Tris-HCl, pH 7.5 (buffer A) and 0.9 M NaCl, 20mM Tris-HCl, pH 7.5 (buffer B). The components were separated by elution with a gradient of 0 to 25% B over 72 minutes. The elution peaks were monitored by UV absorption at 260 nm (λ_{max} for dUMP at neutral pH) and 290 nm (λ_{max} for $dU^{5OH}UMP$ at neutral pH). The peak corresponding to $dU^{5OH}MP$ was identified by its UV spectrum (52) and further confirmed by its oxidation potential value (27). Pure $dU^{5OH}MP$ was desalted by loading on to a DEAE-Sephadex A-25 column and elution with 0.4 M TEAB buffer. TEAB was removed by the evaporation with ethanol at 50° C. The $dU^{5OH}MP$ obtained was homogenous in analytical FPLC and HPLC runs.

To prepare antigens, $dC^{5OH}MP$ and $dU^{5OH}MP$ were covalently attached to BSA and RSA through the 5'-phosphate group using the water-soluble carbodiimide conjugation procedure (8). The mixture of BSA (or RSA) and $dC^{5OH}MP$ (or $dU^{5OH}MP$) in water was incubated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide at room temperature for 24 hours. The solution was then dialyzed against 10 mM Tris-HCl, pH 7.6, followed by dialysis against distilled water. The remaining products were lyophilized.

2. Synthesis of Nucleoside Triphosphates

Since a major part of our DOE initiative over the years has been the development of antibodies to DNA damages and thus the synthesis of appropriate haptens, it is the DOE effort that has provided the underpinning for all the nucleotide chemistry in the laboratory. When nucleoside monophosphates were synthesized for conjugation to protein carriers, the nucleoside triphosphates were synthesized to use as substrates for DNA polymerases. The pairing properties of the lesion in the *de novo* strand provides information as to the potential mutagenicity of a particular damage. Furthermore, the ability of polymerases to introduce defined damages into DNA allows us to examine their interactions with biologically important proteins *in vitro* and in cells.

A. Nucleoside Triphosphates of Ring Saturation Products

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. This work was reported in the prior funding period (Ide et al. Biochemistry, 26:964, 1987)

The ability of dihydrothymidine and thymidine glycol 5'-triphosphates to serve as substrates for different DNA polymerases was investigated. DHdTTP was an efficient (~ 10% of TTP) substrate for *E. coli* DNA polymerase I, but not for T4 DNA polymerase or

avian myeloblastosis virus reverse transcriptase. Thymidine glycol 5'-triphosphate was not an efficient substrate for any polymerase used. Furthermore, their ability 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. This work was also published during the prior funding period (Ide and Wallace in Nucleic Acids Res., 16:11339, 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 model substrates and in X-irradiated DNA

B. Nucleoside Triphosphates of 8-Oxopurines

2'-deoxy-7-hydro-8-oxoadenosine 5'-triphosphate (8-oxodATP) was prepared from 2'-deoxy-7-hydro-8-oxoadenosine 5'-monphosphate (8-oxodAMP) using the method of Hoard and Ott (53) by the imidazolid activation of the phosphomonoester group of 8-oxodAMP followed by treatment with excess tetrabutylammonium pyrophosphate. The final reaction mixture was analyzed by FPLC under neutral pH conditions (0.005 M NaCl, 20 mM Tris-HCl, pH 7.5 - buffer A; 0.9 M NaCl, 20 mM Tris-HCl, pH 7.5 - buffer B) using a Mono Q column. The FPLC estimated conversion of 8-oxodAMP to 8-oxodATP was about 40-50%. FPLC purified 8-oxodATP was desalted on a C-18 column using water as the eluent (the retention time for 8-oxodATP was about 3.5-4 minutes).

2'-deoxy-7-hydro-8-oxoguanosine 5'-triphosphate (8-oxodGTP) was prepared from dGTP using a modification of the method of Kasai and Nishimura (54). dGTP was hydroxylated at the position C-8 by ascorbic acid in the presence of oxygen (O_2) and Fe^{2+} ions in 0.1 M phosphate buffer pH 6.8 at 37° C in the dark. The fraction of the reaction mixture containing nucleoside triphosphates was purified by ion-exchange chromatography on DEAE-Sephadex A-25 in TEAB gradient buffer (0 to 1.2 M, pH 7.8). After the removal of TEAB, 8-oxodGTP was purified by HPLC on a C-18 column using 10 mM sodium acetate, 3.5 mM sodium citrate buffer pH 5.1 containing 5% of methanol as the eluent. Under these conditions, the retention times of dGTP and 8-oxodGTP were 3.2 and 4.5 minutes, respectively. The elution peak corresponding to 8-oxodGTP was identified by its UV spectrum and oxidation potential value (54). The conversion of dGTP to 8-oxodG was about 10% by HPLC estimation. The peak of 8-oxodG was concentrated and desalted on a C-18 column using water as a eluent. For enzyme-catalyzed incorporation into DNA, only freshly prepared 8-oxodATP and 8-oxodGTP were used.

To examine whether 8-oxodATP and 8-oxodGTP would serve as substrates for various DNA polymerases, an M13 mp18 DNA template was annealed to a ^{32}P-labeled 17-

member primer ("-40" or "-20" primer). Four combinations of three normal nucleoside triphosphates (ATC, GTC , GAC and GAT) were used plus 8-oxodATP or 8-oxodGTP. We showed that Sequenase (Ver. 2.0), Klenow and Klenow (exonuclease free) polymerases incorporate 8-oxodATP only instead of normal dATP (pairing with T) with an efficiency of about 3-5% (dATP - 100%). Incorporation was more efficient if the concentration of 8-oxodATP was raised to 150-200 μM (the concentration of normal dNTP's was 40 μM). Slowdown of replication was observed when DNA polymerases encountered runs of "T" on the template suggesting that the 8-oxoA residue (or 8-oxoA run) on the 3'-end of the primer is not a good substrate for elongation.

Similarly we found that 8-oxodGTP could be incorporated into DNA by all three DNA polymerases instead of normal G (with an efficiency of about 10-12%), and instead of T (with an efficiency of about 2-4%). A manuscript describing these studies is in preparation for Biochemistry.

C. Nucleoside Triphosphates of 5-hydroxycytosine and 5-hydroxyuracil

$dC^{5OH}TP$ was prepared by the action of bromine on dCTP in aqueous medium followed by 2,4,6-collidine treatment as described in Section 1D of the Progress Report for $dC^{5OH}MP$. The reaction mixture was separated on a DEAE-Sephadex A-25 column with TEAB linear gradient (0 to 1.5 M, pH 7.8). The major peak, corresponding to the fraction of nucleoside triphosphates, was desalted by repeated evaporation with ethanol and analyzed by FPLC in neutral pH-conditions using a Mono Q column. $dC^{5OH}TP$ was found to be the major product (75-80%) in the mixture analyzed. $dC^{50H}TP$ was further confirmed by alkaline phosphatase treatment and analysis on a C-18 column coupled with an electrochemical detector. The products of $dC^{5OH}TP$ decay, $dC^{5OH}MP$ and $dC^{5OH}DP$, were also found (4-6% and 11-15% respectively). Under the chromatography conditions used, the initial dCTP was well separated from $dC^{5OH}TP$ (the retention times being 19.3 and 24.0 minutes, respectively). The amount of dCTP in the mixture analyzed was less then 1%. For incorporation into DNA, FPLC-purified $dC^{5OH}TP$ will be used immediately after separation.

$dU^{5OH}TP$ was synthesized by the action of bromine on dUTP in aqueous medium followed by pyridine treatment as described in Section 1D for $dU^{5OH}MP$. After separation of the nucleoside triphosphate fraction by ion-exchange chromatography on DEAE-Sephadex A-25, and the removal of TEAB buffer, the reaction mixture was analyzed by FPLC in neutral pH-conditions using a Mono Q column. $dU^{5OH}TP$ was found to be the major product in the mixture analyzed (about 50-60%). $dU^{50H}TP$ was further confirmed by alkaline phosphatase treatment and analysis on a C-18 column coupled with an electrochemical detector. As in the case of $dC^{5OH}TP$, for incorporation into DNA, FPLC-purified $dU^{5OH}TP$ will be used immediately.

3. Applications

A. Biological Consequences of Model DNA Damages

In order to determine the biological consequences of individual damages for the purpose of risk assessment, we have employed the strategy of selectively introducing unique

lesions of interest into viral DNA molecules. The number of damages introduced is measured by the immunochemical assay. The damage-containing DNA is then examined for its ability to serve as a substrate for repair enzymes and polymerases *in vitro* and for processing *in vivo*. Using a number of endpoints, we have thus far compared the properties of four modified thymine residues, thymine glycol, urea, dihydrothymine, and ß-ureidoisobutyric acid, to sites of base loss (abasic sites). The results of the studies undertaken during this project period have been published as journal articles (Laspia and Wallace in Journal of Molecular Biology 207:53, 1989; Ide, Petrullo, Hatahet and Wallace in Journal of Biological Chemistry, 266:1469, 1989; Evans, Maccabee, Hatahet, Courcelle, Bockrath, Ide and Wallace in Mutation Research, in press) as well as in a number of book chapters. An article describing the mutagenic potential of the ring fragmentation products is in preparation for the Journal of Molecular Biology.

i. Thymine Ring Saturation Products

We have used thymine glycol and dihydrothymine as representative ring saturation products resulting from free radical interaction with DNA pyrimidines. Thymine glycol has been selectively introduced into DNA by osmium tetroxide oxidation and dihydrothymine (DHT) by virtue of its being a substrate for DNA polymerases *in vitro* and *in vivo*.

As a point of departure for predicting the biological consequences of defined DNA lesions, the interaction between the lesion on the template strand with various DNA polymerases was examined. If the lesion in the template is a block to DNA synthesis, termination bands should appear in its vicinity. When single-stranded DNA containing thymine glycol was examined for its ability to serve as a template for *E. coli* DNA polymerase I, we (55) and others (56-58) observed strong termination bands indicating that thymine glycol is a block to DNA synthesis. The termination bands were found primarily opposite the thymine glycol lesion suggesting that thymine glycol retains the ability to base pair. These data suggested that thymine glycol would be lethal, and if the block to replication is released, that it would not be a potent mutagenic lesion. In contrast to thymine glycol, when the template contained dihydrothymine, no termination bands were observed (59). Thus DHT does not appear to be a blocking lesion. These and other studies showed that dihydrothymine also pairs with A. However, since dihydrothymine is so efficiently bypassed, a low frequency mispair event could result in substantial mutation induction.

When thymine glycol and dihydrothymine were introduced individually into single-stranded biologically active DNA, thymine glycol was a lethal lesion having an inactivation efficiency of 1, whereas dihydrothymine was not (60-62). Thus the *in vitro* studies predicted the *in vivo* results. Furthermore, when mutation induction was measured in SOS-induced hosts using f1-K12 hybrid DNA containing an *E. coli* target gene, thymine glycol (62) and dihydrothymine (61) were found to be inefficient as mutagenic lesions, suggesting that *in vivo*, as *in vitro*, they primarily code for A. Thus we might generalize that saturation of the 5-6 double bond of pyrimidines in and of itself does not alter the interaction of the base with DNA polymerase nor the ability of the lesion to base pair. However, if the addition product is a glycol, where the OH groups protrude into the major groove, the lesion retains its base pairing characteristics, but becomes an effective block to DNA polymerases.

ii. Pyrimidine Ring Fragmentation Products

Urea N-glycosides and ß-ureidoisobutyric acid (UBA) residues were used as models for pyrimidine ring fragmentation products. These are the alkali hydrolysis products of thymine glycol and dihydrothymine, respectively. The number of urea and UBA residues per DNA molecule was determined by antibody reactivity directed against the original thymine glycol and dihydrothymine lesions. We showed that the ring fragmentation products urea and ß-ureidoisobutyric acid, as well as abasic sites, are strong blocks to DNA polymerases *in vitro* (55,59) and lethal *in vivo* having an inactivation efficiency of 1 (59-61). When the survival of biologically active single-stranded DNA containing urea or abasic sites was examined in UV-induced *E. coli* cells where the block to replication is released, no increase in survival (Weigle Reactivation) was observed (60,61), suggesting inefficient bypass of these lesions. This is in contrast to thymine glycol-containing DNA, where the release of the replication block by SOS-induction of the cells greatly enhances survival of the phage (60-62). Interestingly, the survival of phage DNA containing UBA residues was slightly (about two fold) enhanced in induced compared to uninduced cells.

The rate of mutation induction by a particular lesion should depend on the frequency of bypass of that lesion, as well as the specificity for misinsertion opposite the lesion. Thus, lesions that are bypassed more efficiently, providing they are mis- or non-coding, would be expected to give rise to a higher mutation frequency.

In the case of abasic sites, bypass efficiency is extremely low even in induced cells. However, it has been shown *in vitro* (63-65) and *in vivo* (66,67) that adenine (followed by guanine) is preferentially inserted opposite this damage. If the abasic sites are derived from purines, this would lead to mutation (as has been observed); although, if they are derived from T, insertion of an A would not be mutagenic. Urea residues are also very poorly bypassed as measured by Weigle Reactivation (60,61). However, because they have some structure, and also the potential to form a base pair, then even though they are derived from T, they might show an increase in mutation frequency over T-derived abasic sites, especially if A were not totally preferred for insertion. UBA lesions are more efficiently bypassed than either abasic sites or urea residues in SOS-induced cells (59), and so, using the above argument, would be expected to be better premutagenic lesions than either of these. Again however, this would depend on potential hydrogen bonding capabilities of UBA with adenine, the correct base, versus an incorrect base.

To test this idea, the ability of randomly introduced AP, urea, and UBA lesions to cause mutations was examined in a forward mutation assay, using a single-stranded phage vector with a *lacI* target. Single stranded DNA was used to circumvent DNA repair processing and to ensure that interactions with DNA polymerases would determine the mutagenic outcome. We found urea residues to be potent premutagenic lesions, and in fact, the mutation frequency observed was approximately the same as with AP sites derived from purines. This was rather surprising in light of the fact that urea is derived from T, and with the known preference for A misinsertions opposite abasic sites and presumably other non-instructive lesions, we had expected that mutation induction by urea residues would be much lower than that from purine-derived AP sites. The fact that mutation induction by urea is similar to that for abasic sites, suggests that either urea is bypassed more efficiently, or that

it directs base misinsertion, possibly by the formation of a single hydrogen bond. We have recently measured mutation induction by AP sites derived from T. Mutation frequency in this case is very low in induced cells rising at most to two fold above background. This confirms the "A-rule" for abasic sites but suggests that the situation is quite different for fragmentation products such as urea.

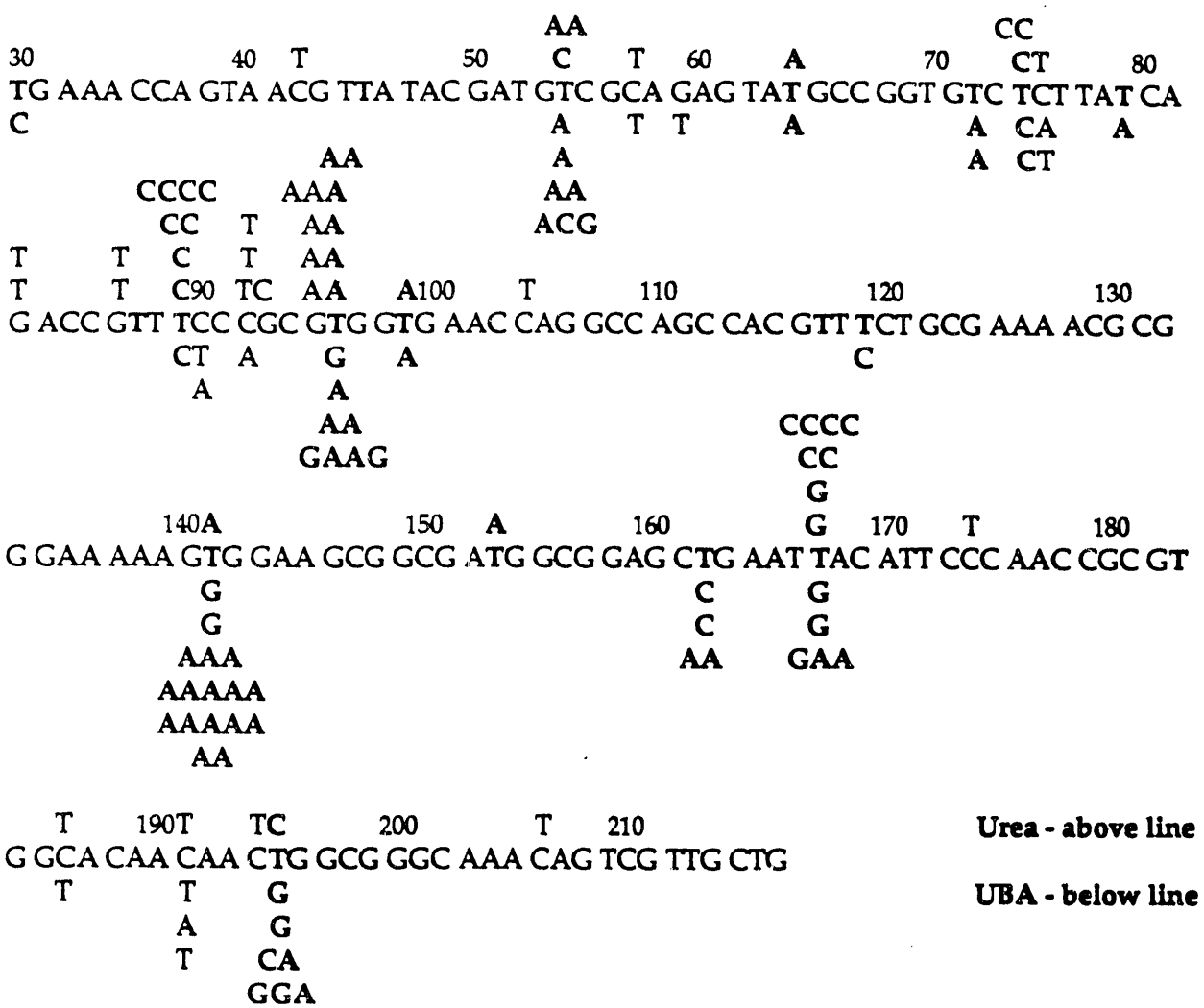
Of all the apparently "non-coding" damages, UBA was the most effective pre-mutagenic lesion, being 5 or 6 fold more potent than an apurinic site or a urea glycoside. Since the UBA lesion is bypassed as measured by Weigle Reactivation, it is tempting to speculate that the reason for its effectiveness as a mutagenic lesion is due to an increased efficiency of bypass. This could be the result of more effective base stacking, since UBA lesions have a greater mass than urea and abasic sites, but it could also be due to an increase in incorrect pairing via potential hydrogen bond formation.

When urea and UBA mutants in the i^{-d} region of *lacI* were sequenced, an interesting result was obtained. In the case of urea, 62% of the changes were T → C transitions, suggesting misinsertion of G opposite the urea site. In contrast, 62% of the UBA mutants were T → A transversions suggesting T insertion opposite the UBA site (See Figure 1). Again, A insertions would not be detected since they would not give rise to mutants. In the studies described here, we have introduced a particular lesion into all possible T sites. We know this because the *in vitro* studies with DNA polymerases show termination bands at all putative T sites in the template strand. Thus, within some small margin of error, all 19 mutable T sites in the i^{-d} region (68) have an equal probability of containing the damage in question. Thus, because we are using single stranded DNA, the mutation spectrum observed here for unique lesions at 19 possible sites allows us to focus not only on lesion structure, but at the same time, sequence context effects as they relate to polymerase-directed misinsertion opposite a lesion.

For the two lesions discussed here, urea and UBA, the most frequently misinserted base was different for both lesions, and different hot spots were observed (Figure 1). For example, at site 89, a urea hotspot, there was only one UBA mutation observed while at site 141, a UBA hotspot, there was only one urea mutation observed. There could be two potential but not mutually exclusive reasons for these observations: 1) It could be that at site 89, UBA always pairs with A while urea pairs with A (no mutations) or G which leads to the T → C transition hot spot. In contrast, at site 141, urea might always pair with A while UBA pairs with A (no mutation) or T leading to the observed T → A transversion hot spot. 2) At site 89, UBA might always be a lethal blocking lesion even in induced cells thus leading to no observed mutations, while at site 141, urea could always be blocking.

Using the Silicon Graphics IRIS 4D 340 computer modeling facility which was funded by the Department of Energy, we have done both energy minimization and molecular dynamics calculations for both sites 89 and 141 with normal base pairs, all mispairs, and the UBA and urea lesions, again in all combinations. Canonical B-DNA 13-mers of the sequences were constructed with each site as the central base using INSIGHT II (Biosym Corp., San Diego, CA), and energy minimized using DISCOVER (Biosym) with AMBER force fields and potentials (69,70). Partial changes for AP sites, urea and UBA were calculated using MOPAC and atom types were assigned from the AMBER all-atom set. All

Distribution and Type of Base Substitutions Seen in *lac* I^{-d} DNA Containing Either β–Ureidoisobutyric Acid or Urea Lesions



structures were energy minimized to an RMS derivative of > 0.001 kcal/A° and the resultant structures displayed using INSIGHT. To assess the range of conformational space available to these structures, 50 picosecond molecular dynamics simulations have been undertaken using DISCOVER with AMBER parameters for each of the energy minimized structures. These simulations include 2,000 steps of equilibration and 50,000 dynamics steps at 300° K. The results of these simulations are being analyzed using the INSIGHT ANALYSIS routines.

Interestingly, when site 141 was energy minimized with the normal T→A base pair, a fair amount of distortion was observed in the propellar twist angles of the opposing bases and the base pairs on either side of the site and, as well, the bases at 141 were not co-planar. Thus, significant distortion of the 141 site appears to occur even with the normal base pairing. In contrast, no marked distortion was observed at site 89 which assumes the canonical B form conformation. In keeping with this observation, site 141 is a hot spot for spontaneous mutation in addition to UBA-induced mutation, while site 89 is not. When the two lesions were examined at site 89, the hot spot for urea mutagenesis, urea appears to form a single hydrogen bond with A. Although no hydrogen bonding was observed with G opposite the urea residue (which is the mutational event), the G is stacked well into the normal B form DNA. However, when UBA is modeled at site 89, the calculations show that UBA does not fit well opposite any of the bases, suggesting UBA might be a blocking lesion at this site. In contrast, at the 141 site, which is the spontaneous mutation site and the UBA hot spot. UBA forms a stable hydrogen bond with T. This combination appears to give the least structural distortion at this already distorted site. In contrast, no stable pairing or stacking was found with any of the bases opposite the urea residue, suggesting that urea might be blocking at this site. Thus, the energy minimization studies have begun to guide our thinking about how individual structurally diverse lesions behave in particular sequence contexts within which they are mutagenic.

B. Measurement of Damage in X-Irradiated DNA

Inspection of the literature shows that surprisingly little has been accomplished on quantitating individual lesions in X-irradiated DNA. Comparisons are difficult among the available data since DNA or cells are irradiated under a wide variety of conditions and often, no dose response is given. With the application of GC/MS to the radiation field, more information has become recently available.

As a point of departure for quantitating the production of X-ray-induced DNA lesions in cells, we have measured 8-oxoA, 8-oxoG, thymine glycol and ARP-reactive sites in f1 DNA irradiated in air, in dilute aqueous solution. The data in Figure 2 are a compilation of several sets using the ELISA. When these data are compared to those of Fuciarelli *et al* (71) with GC/MS using denatured calf thymus DNA irradiated in air, they are in reasonable agreement. At equal doses, we measure a several-fold greater production of the 7-hydro-8-oxopurines than these investigators and about the same amount of thymine glycol. It should be noted that the chemically synthesized standards might be different in addition to intrinsic variations in the methods themselves. We also compared these data

FIGURE 2

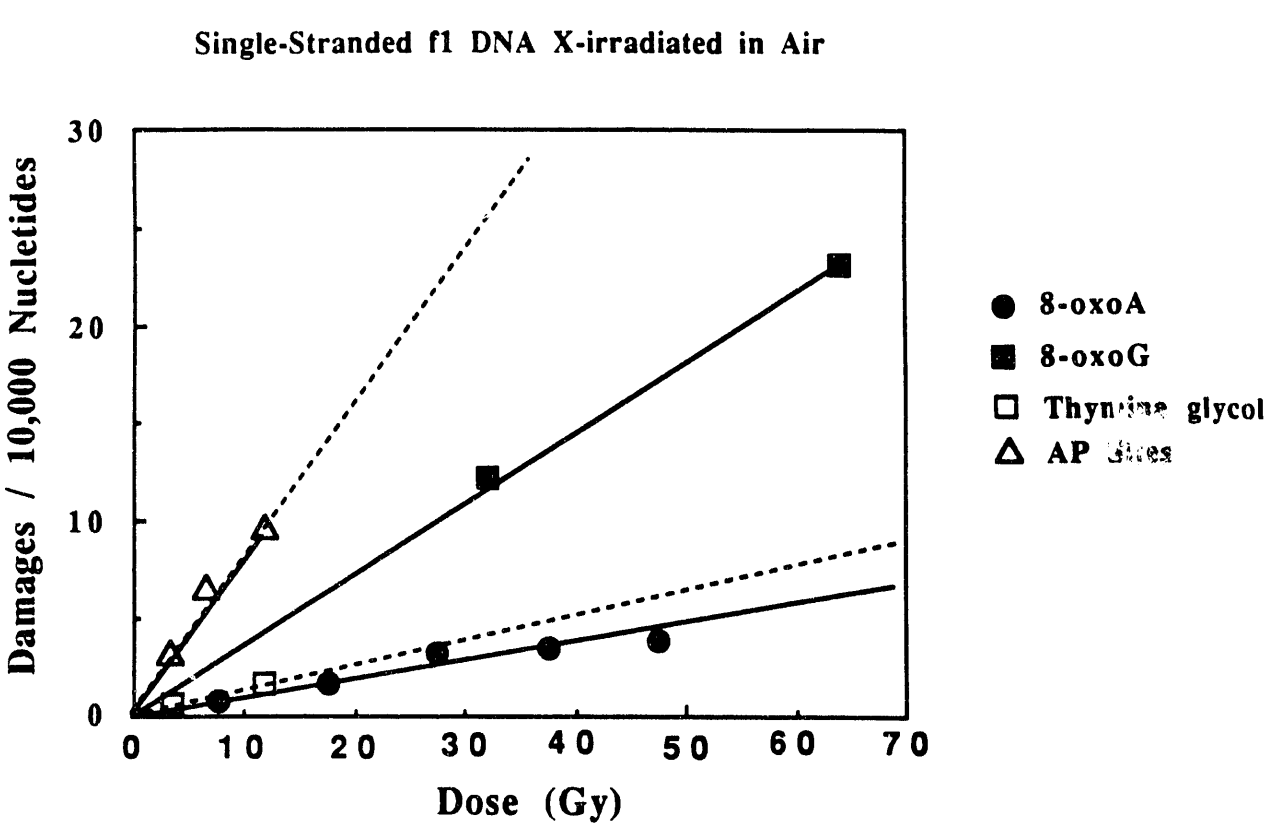
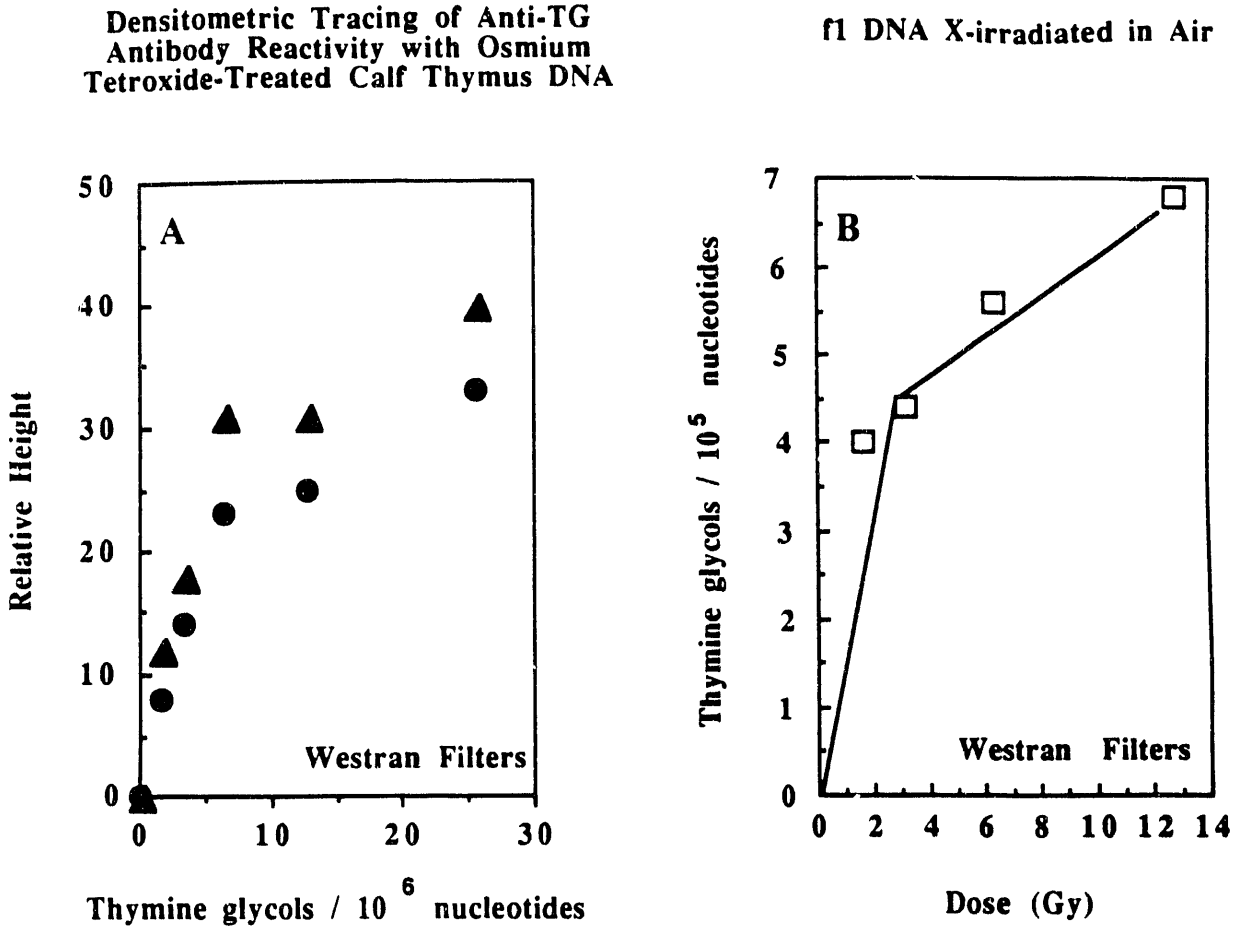

Single-Stranded f1 DNA X-irradiated in Air
Damages / 10,000 Nucletides
30
20
10
0
0
10
20
30
40
50
60
70
Dose (Gy)
8-oxoA
8-oxoG
Thymine glycol
AP Sites

FIGURE 3


Densitometric Tracing of Anti-TG Antibody Reactivity with Osmium Tetroxide-Treated Calf Thymus DNA
A
Relative Height
Thymine glycols / 10 6 nucleotides
Westran Filters
0
10
20
30
40
50
f1 DNA X-irradiated in Air
B
Thymine glycols / 10 5 nucleotides
Dose (Gy)
Westran Filters
0
1
2
3
4
5
6
7
0 2 4 6 8 10 12 14

with those obtained by ^{32}P-post labeling of thymine glycol in γ-irradiated duplex calf thymus DNA (72). Although we observe several fold more thymine glycol at the same dose using the immunochemical assay, here again, the irradiation conditions and DNA structure are not identical and these investigators observed very little dose response over two logs. All in all, the immunochemical data are in reasonable agreement with those reported by two other sensitive methods.

With regard to sensitivity, with GC/MS, the limit of detection reported for DNA irradiated *in vitro* was about 20 Gy (71), with postlabeling it was about the same (72). With the ELISA, we can easily measure 5-10 Gy lower. Furthermore, with GC/MS, an expensive piece of apparatus is required, large amounts of DNA are used for each sample, acid hydrolysis is necessary, and finally, derivitization of the lesion is necessary for detection. Immunochemical technology is sensitive, relatively simple and inexpensive and could be made available for widespread use.

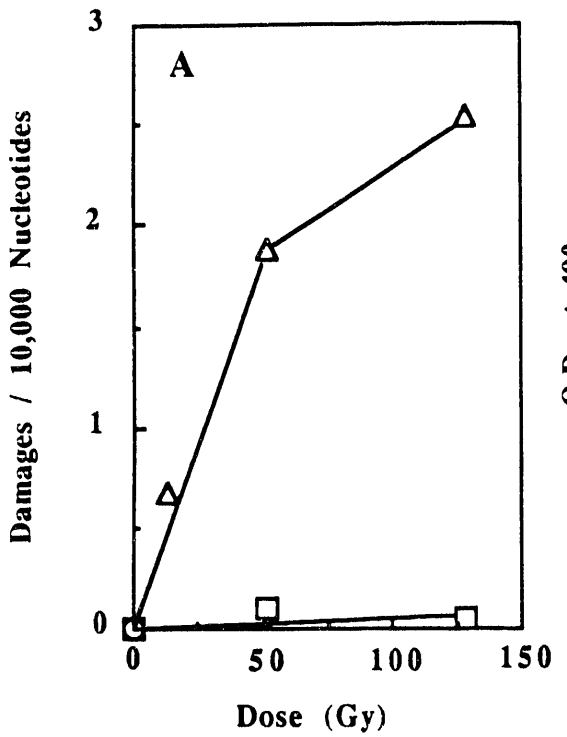
In order to increase the level of sensitivity of the immunochemical assay, we have used fluorescent and luminescent probes which unfortunately, although very sensitive, gave high backgrounds. We have also tried various types of filter papers as matrices for DNA absorption. Typically only about 20-80 ng of DNA bind to a well of an ELISA plate thus limiting the detection of low levels of damages. Paper matrices have a higher DNA binding capacity than plastic and bind small fragments of DNA, which are common after high radiation doses. However, there appears to be a much more limited linear range of signal detection than is found with the traditional plate method.

Figure 3A shows the results of a densitometer scan of immunochemical detection of thymine glycol in osmium tetroxide-oxidized DNA. Here 1 thymine glycol in 10^6 nucleotides can be detected. Figure 3B shows a preliminary experiment where the production of thymine glycol in f1 DNA X-irradiated in phosphate buffer in air is measured. The lowest dose shown here is one second of irradiation so the lack of linearity is likely due to an error in dose. In any case, low dose levels can be measured.

In order to measure the production of damage in DNA extracted from irradiated cells, we have had to take a number of precautions. A number of DNA lesions are labile, therefore heat and high pH in particular must be avoided if the damages are to be preserved intact on the DNA molecule. Additionally, phenol extraction and exposure to air introduce large amounts of oxidative damages into DNA (72A). Lastly, the cellular enzymes that remove the DNA lesions must be inactivated and/or the DNA must be extracted as soon as possible after irradiation so that the lesions are not removed before they can be detected. This has been more of a problem with *E. coli* than with mammalian cells, probably because the repair processing is very rapid. Figure 4A shows the production of ARP-reactive sites in X-irradiated heat-inactivated *E. coli* cells. At the mean lethal dose, 100 Gy, we detected 2 ARP sites per 10,000 nucleotides. We were unable to detect thymine glycol under the same conditions. Preliminary data (ELISA) on the production of 8-oxoG, 8-oxoA, thymine glycol and ARP-reactive sites in an X-irradiated human T cell line (HPB-ALL), obtained from the departmental Tissue Culture Facility, is shown in Figure 4B. All

FIGURE 4

Production of ARP-Reactive Sites in the DNA of Heat Inactivated X-irradiated *E, coli*



Production of DNA Damages in X-irradiated Human T Cells

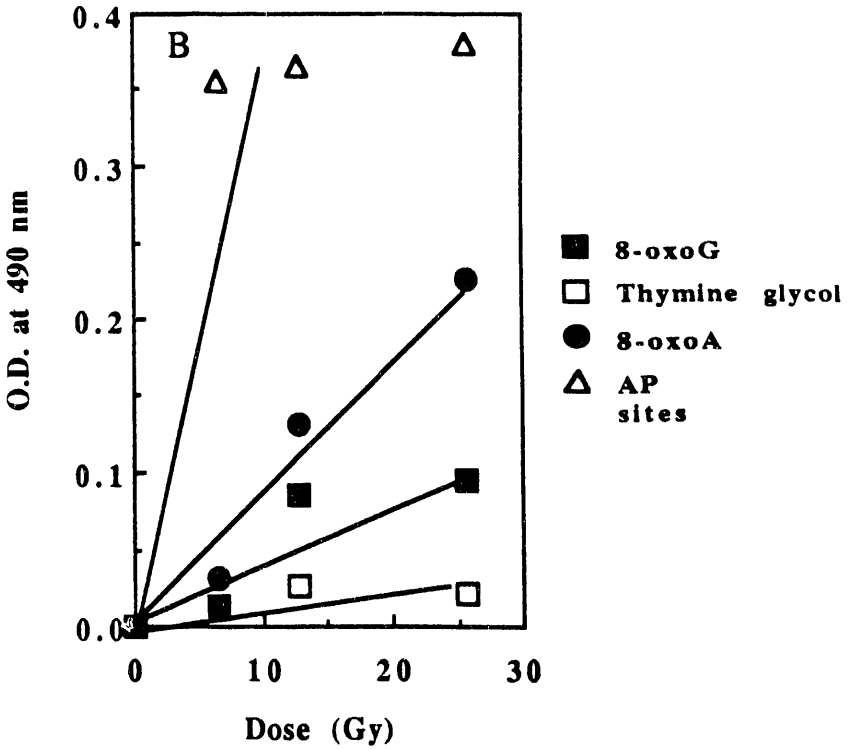
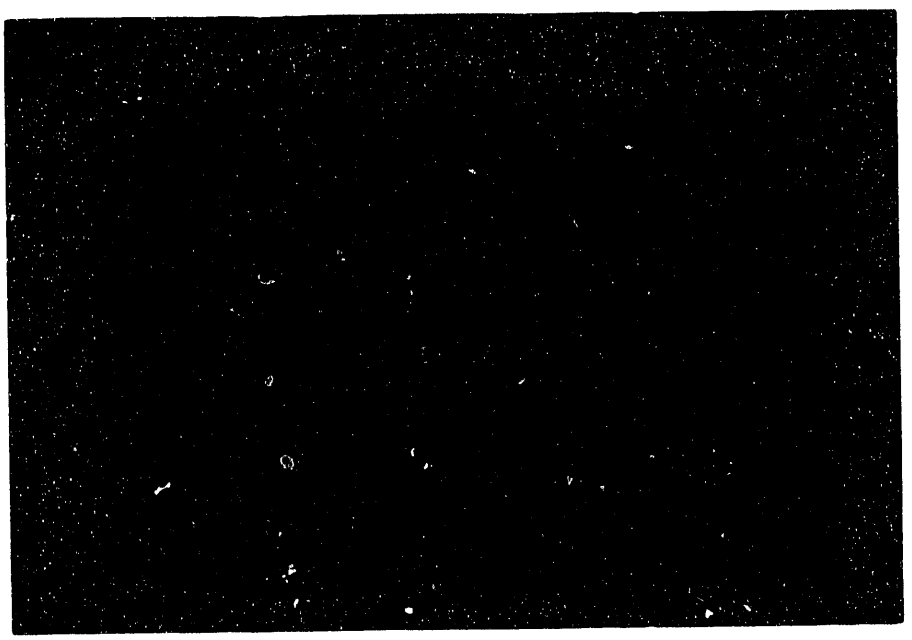


FIGURE 5

Immunofluorescence Anti 8-oxoA

A - no hydrogen peroxide

B - 0.16 mM hydrogen peroxide



4 lesions can be detected at doses of 5 Gy. As expected, ARP-reactive sites appeared to be produced at a 10-fold greater rate than the individual base lesions. Quantitating individual lesions in DNA extracted from X-irradiated human cells will be a major effort during the near-term.

C. *In Situ* Chromosomal Staining of Damaged DNA

We have recently used immunofluorescence to look for the presence of 8-oxoA in nuclear DNA of an H_2O_2-treated mouse T cell lymphoma line (EL-4). This line was initially chosen for study because it has a large nucleus. We looked for 8-oxoA because it has been shown by Dizdaroglu *et al.* (73) to be a major product of H_2O_2 treatment of mammalian cells. The cells were grown in IMDM-F12 medium containing 5% fetal calf serum overnight at 37° in Lab-Tak chamber slides. Then various concentrations of H_2O_2 were added and the cells were incubated at 37° for 60 hours. The cells were then fixed in 3.7% formaldehyde in PBS for 30 minutes, washed and then permeabilized by exposure to 1% Triton X-100 in PBS for 5 minutes. After washing, the cells were then treated with RNase (100 μg/ml) for 1 hour at 37°C. After washing with PBS, the cells were treated with proteinase K (10 μg/ml) for 10 minutes at 25°C. The cells were washed with PBS again, treated with 4 N HCI for 10 minutes at 25°C to remove remaining histones, and then incubated with 10% horse serum in PBS-0.1% Tween-20 for 30 minutes at 37°C to eliminate possible non-specific staining with antibody. The treated cells were incubated for 30 minutes at 25°C with a 1:20 dilution of anti-8-oxoA in PBS-0.1% Tween 20 - 10% horse serum, washed with PBS-Tween, treated with a 1/100 dilution of biotinylated goat anti-rabbit IgG (Sigma) and then after washing again with PBS-Tween, were incubated for 30 minutes at 25°C with FITC-goat anti-biotin (Sigma) at a dilution of 1:40 in PBS-Tween 20 - 10% horse serum.

The treated cells were then examined by ultraviolet microscopy and selected fields were photographed. Controls consisted of cells that were not exposed to hydrogen peroxide but were otherwise treated identically. The results of some preliminary experiments are shown in Figure 5.

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