Oxidative Damage to DNA: Do We Have a Reliable Biomarker?

Andrew R. Collins,[1] Mária Dušinská,[2] Catherine M. Gedik,[1] and Rudolf Štětina[3]

[1]Rowett Research Institute, Aberdeen, Scotland, United Kingdom; [2]Institute of Preventive and Clinical Medicine, Bratislava, Slovak Republic; [3]Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Olešnice, Czech Republic

Oxidized bases in DNA can be measured directly by high-performance liquid chromatography (HPLC). 7,8-Dihydro-8-oxo-guanine (8-OHgua), as the most abundant oxidation product, is often regarded as an indicator of oxidative stress. Estimates of endogenous 8-OHgua levels in human lymphocyte DNA are between 2 and 8 for every 10^5 unaltered bases—a high frequency in view of the potential mutagenicity of this base alteration and of the presence of an effective base excision repair pathway in eukaryotic cells. An alternative approach to the measurement of oxidized bases makes use of repair endonucleases with appropriate lesion specificities—endonuclease III, for oxidized pyrimidines and formamidopyrimidine glycosylase for 8-OHgua. These enzymes introduce breaks at sites of damage in DNA. The comet assay (single cell gel electrophoresis) can then be used to detect the DNA breaks. This modified comet assay, like other enzyme-linked DNA breakage assays, gives a value for endogenous oxidative base damage that is more than 10-fold lower than most estimates from HPLC. It is possible that HPLC-based estimates are artificially high because oxidation of guanine occurs during isolation, storage, or hydrolysis of DNA. Using a revised DNA isolation procedure designed to decrease *in vitro* oxidation, we have obtained results for 8-OHgua concentrations in human lymphocytes that are closer to the figures obtained by the comet assay. It is still an open question whether 8-OHgua, measured by HPLC or by the comet assay, is a valid marker for oxidative damage. — Environ Health Perspect 104(Suppl 3):465–469 (1996)

Key words: DNA damage; 8-OH guanine; comet assay; DNA repair enzymes

Introduction

Oxidative damage to DNA, whether induced exogenously by radiation or chemical agents or endogenously by free radicals released during normal respiration, is generally regarded as a significant contributory cause of cancer. The evidence for this statement arises at several different levels of investigation. First, at the level of epidemiology, there are many reports of correlations between a high consumption of fruit and vegetables, or of specific dietary antioxidants (vitamin C, carotenoids, vitamin E), and a relatively low incidence of several cancers (*1–4*). Ames (*5*) proposed that dietary antioxidants in general protect against cancer by removing reactive oxygen species before they have a chance to cause damage to biological molecules.

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Address correspondence to Dr. A.R. Collins, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K. Telephone: 44-1224-716634. Fax: 44-1224-716622. E-mail: a.collins@rri.sari.ac.uk

Abbreviations used: 8-OHgua, 7,8-dihydro-8-oxo-guanine; 8-OHdG, 7,8-dihydro-8-oxo-deoxyguanosine; GC-MS, gas chromatography with mass spectrometry; HPLC-EC, high-performance liquid chromatography with electrochemical detection; AP site, apurinic/apyrimidinic site; FPG, formamidopyrimidine glycosylase; PBS, phosphate-buffered saline; SCGE, single cell gel electrophoresis; DAPI, 4,6-diamidino-2-phenylindole; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SSC, 0.15M NaCl/0.015 M trisodium citrate, pH 7.0; UV, ultraviolet; dG, deoxyguanosine.

At the molecular level, the spectrum of oxidation products formed in DNA includes strand breaks, base-less sugars or AP (apurinic/apyrimidinic) sites, and oxidized bases (*6*); within the last group, attention has been focused on 7,8-dihydro-8-oxo-guanine (8-OHgua) as a major product with a clear mutagenic potential. The base-pairing specificity of 8-OHgua is not as strict as that of guanine itself, and in *in vitro* replication with bacterial and eukaryotic DNA polymerases, there is significant incorporation of adenine opposite guanine in the daughter strand (*7*).

In view of its potential importance and the relative ease with which it can be measured, 8-OHgua (or the nucleoside, 7,8-dihydro-8-oxo-deoxyguanosine [8-OHdG]) has come to be commonly regarded as a good marker of oxidative damage (*8,9*). 8-OHdG can be measured in hydrolyzed DNA from lymphocytes or tissues where it represents the steady state that exists between the input of damage and its repair by cellular processes. An alternative approach is to assay oxidation products excreted in urine on the assumption that these products arise from excision repair occurring in the cells. 8-OHgua and 7,8-dihydro-8-oxo-guanosine are abundant in urine, but they may originate in cellular RNA as well as in DNA, and they may be derived from nucleic acids in the diet (*10*). In contrast, 8-OHdG occurs specifically in DNA and is apparently not absorbed through the gut. It therefore appears to be a reasonable marker for oxidative DNA damage because (assuming a steady state) the rate of output of 8-OHdG by repair should balance the rate of input of damage. There is a question concerning the origin of 8-OHdG in urine because the nucleoside is not a product of the established base excision repair pathway, which simply excises damaged bases—in this case 8-OHgua. A novel pathway that does remove the nucleoside (*11*) may account for release of 8-OHdG. However, Lindahl (*12*) has pointed out the possibility that DNA breakdown products from dead cells may undergo oxidation in the kidneys, so measurement of 8-OHdG in urine may be only a poor indicator of oxidative stress in the organism.

Notwithstanding these caveats, numerous studies have been published with measurements of 8-OHdG in DNA or urine of human subjects. Urinary 8-OHdG was found to correlate with metabolic rate and

with smoking (*13,14*). Therapeutic doses of ionizing radiation led to severalfold increases in 8-OHdG in leukocytes (*15*); however, others have reported no correlation of 8-OHdG in DNA with smoking or with age (*16*).

Widely different methods have been used to measure the steady-state concentration of oxidative base damage in DNA. Gas chromatography with mass spectrometric detection (GC-MS) (*9*) and high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC) (*8*) have the advantage of specificity, since different oxidation products can be identified; however, they require relatively large quantities of starting material. ^{32}P-postlabeling has only recently been applied to oxidation products from DNA (*17*); it is a sensitive method, but unequivocal identification of oxidation products can present problems. Three other techniques make use of repair endonucleases with specificity for oxidative base damage to make breaks in DNA at the sites of damage. These breaks are then measured by alkaline elution (*18*), a modified nick translation assay (*19*), or modified single cell gel electrophoresis (SCGE; the comet assay) (*20*).

To date, the chemical methods of analysis have given values for 8-OHgua concentrations in DNA of human cells that are far in excess of the values obtained in any of the DNA breakage-based assays—tens of thousands of oxidized bases per cell compared with a few hundred. We report here the results of experiments designed to eliminate possible artifacts that might account for these discrepancies. We have used both the direct method of measuring 8-OHdG by HPLC and the indirect method of SCGE combined with repair enzymes. Formamidopyrimidine glycosylase (FPG) removes 8-OHgua, leaving AP sites that are converted to strand breaks by the associated AP endonuclease activity. An analogous enzyme, endonuclease III, creates strand breaks at sites of oxidized pyrimidines.

Methods

Cell Culture and Treatment with DNA-damaging Agents

Transformed human epithelial (HeLa) cells were routinely cultured as monolayers in Glasgow-modified Eagle's Minimal Essential Medium (ICN Flow, Thame, England) with 5% fetal calf serum, 5% newborn calf serum, penicillin, and streptomycin at 37°C in a 5% CO_2 atmosphere. Oxidative damage was introduced by replacing the medium with phosphate-buffered saline (PBS) containing H_2O_2 at the required concentration. After 5 min on ice, the H_2O_2 solution was removed, and cells were either processed for SCGE immediately or first incubated at 37°C in medium to allow rejoining of strand breaks.

The standard method of centrifugation over a layer of Ficoll-based separation medium was used to isolate human lymphocytes from finger-prick samples of blood from volunteers (who had given their informed consent). The lymphocytes were X-irradiated on ice after embedding in agarose, but before lysis. Radiation was delivered by a TUR-250 X-ray generator operating at 200 kW and 15 mA without filtration. The dose rate was 1.38 Gy/min.

Single Cell Gel Electrophoresis

The comet assay was based on the method of Singh et al. (*21*) with several modifications. A layer of 85µl of 1% standard agarose (Gibco BRL, Paisley, Scotland) in PBS was set on a microscope slide and overlaid with 85 µl of 1% low-melting-point agarose (Gibco BRL) in PBS in which cells were suspended at 37°C. The cells were lysed by immersing the slides in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl, pH 10, 1% Triton X-100 for 1 hr; this treatment leaves residual nuclei, or nucleoids, embedded in the gel. After lysis, slides were washed three times with enzyme buffer [0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) KOH, 0.2 mg/ml bovine serum albumin, pH 8.0] and treated with FPG or endonuclease III (see below) before placing in an electrophoresis tank in 0.3 M NaOH, 1 mM Na_2EDTA for 40 min. Electrophoresis was then carried out at 25 V and approximately 300 mA for 30 min. After staining with 4,6-diamidino-2-phenylindole (DAPI), each slide was viewed by fluorescence microscopy and the degree of damage in the nucleoids was assessed visually. Each of 100 nucleoids, or comets, was assigned a score from 0 to 4, depending on the fraction of DNA pulled out into the tail under the influence of the electric field. The overall score for each slide was therefore between 0 (undamaged) and 400 (maximally damaged). In some experiments, an alternative method of quantitation was used, based on image analysis with Komet 3.0 software (Kinetic Imaging Ltd., Liverpool, England). The arbitrary units assigned by visual scoring are closely related to the tail intensity as measured by computer image analysis (*22*).

Lesion-specific Enzymes

Enzymes were isolated from over-producing bacterial strains for FPG and endonuclease III, kindly provided by S. Boiteux (Institut Gustave-Roussy, Villejuif, France) and R. Cunningham (Department of Biological Sciences, State University of New York, Albany, NY), respectively.

Isolation and Hydrolysis of DNA

The method developed for the isolation and hydrolysis of DNA from lymphocytes will be described in full elsewhere (CM Gedik and AR Collins, in preparation). Briefly, lymphocytes were lysed with sodium dodecyl sulfate and digested with proteinase K (Boehringer Mannheim, Lewes, England) at 55°C for 3 hr. Addition of NaCl to 1.5 M to precipitate residual peptides (at room temperature) was followed by centrifugation. DNA was precipitated from the supernatant by addition of 2 volumes of ethanol at –20°C. The pellet of DNA was redissolved in a 0.01×SSC (0.15M NaCl/0.015 M trisodium citrate, pH 7.0) buffer with 1 mM EDTA, incubated for 1 hr at 37°C with ribonucleases IIIA and T1 (Sigma, Poole, England), and reprecipitated with ethanol. The DNA was redissolved in 40 mM Tris-HCl, pH 8.5, degassed in a sonicating water bath, and stored under N_2 at –20°C. DNA was hydrolyzed with deoxyribonuclease I, alkaline phosphatase, and phosphodiesterases I and II (all from Boehringer Mannheim) in the presence of Mg^{++} for 2 hr at 37°C, following the method of Richter et al. (23). It was then degassed and stored under N_2 at –20°C.

HPLC Analysis of 7,8-Dihydro-8-oxo-deoxyguanosine

The DNA hydrolysate was analyzed on a 15×0.46 cm Apex C18 3 µm column (Jones, Hengoed, Wales) with a 2×0.4 cm guard column containing Perisorb RP18 (Anachem, Luton, England). The mobile phase was 50 mM potassium phosphate, pH 5.5, with 7.5% methanol and the flow rate was 0.8 ml/min. Deoxyguanosine (dG) was measured with a Gilson Holochrome UV detector set at 254 nm; 8-OHdG detection required an electrochemical detector (Coulochem 5100H) with a 5021 conditioning cell and 5010 analytical cell.

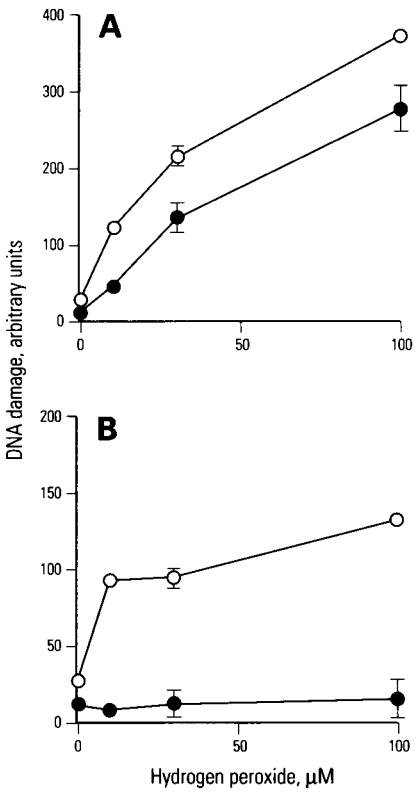

Figure 1. The use of FPG to reveal base oxidation in the comet assay. HeLa cells were treated with H_2O_2 and assayed for DNA damage either immediately (*A*) or after a 2-hr incubation (*B*). DNA strand breaks were detected with the standard comet assay (●). To detect 8-OHdG, the DNA was digested (in the gel) with FPG for 30 min before electrophoresis (○). Quantitation was by visual examination of 100 DAPI-stained fluorescent comet images per slide. Bars indicate SEM from three experiments.

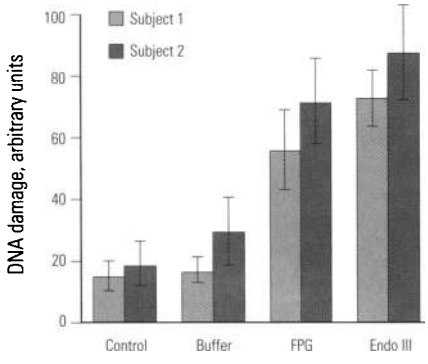

Figure 2. Human lymphocytes assessed for endogenous oxidative damage with the comet assay. Lymphocytes from two subjects, without any *in vitro* treatment with damaging agent, were embedded in agarose, lysed, and electrophoresed immediately (control) or after 30-min incubation with reaction buffer alone, with FPG, or with endonuclease III (Endo III). Comets were scored visually. Bars indicate SEM from five experiments carried out on lymphocytes from blood samples taken on different days.

Results

The Comet Assay: Detection of Strand Breaks and 7,8-Dihydro-8-oxo-deoxyguanosine

The comet assay, as generally used, detects strand breaks by virtue of their ability to relax supercoils of DNA and to allow DNA loops to extend to form a comet tail when an electric field is applied. The intensity of DNA in the tail (measured with a fluorescent stain—in this case DAPI) reflects the DNA break frequency. H_2O_2 treatment causes dose-dependent strand breakage (Figure 1A). If the DNA is digested with the repair enzyme FPG after lysis of cells embedded in the gel, extra breaks are seen. These represent sites of endonucleolytic attack at 8-OHdG, which is the principal substrate for FPG (*24*). Because of the high level of strand breaks, the discrimination of extra enzyme sites is rather imprecise. However, when HeLa cells are incubated after H_2O_2 treatment, strand breaks are quickly rejoined (*22*). By 2 hr, they are at background level and oxidized purines (which are only slowly repaired) can be more accurately determined (Figure 1B). For example, strand breaks induced by 30 μM H_2O_2 (Figure 1A) amount to 136 units, while the additional FPG-sensitive sites (Figure 1B) are represented by 83 units.

We have measured the levels of oxidized bases in the DNA of human lymphocytes from two subjects using FPG and also endonuclease III, whose substrate is oxidized pyrimidines. The level of strand breaks measured simply after lysis of agarose-embedded cells is very low (Figure 2), and incubation for 30 min with buffer alone does not significantly increase the damage seen. However, inclusion of FPG or endonuclease III has a substantial effect, increasing the damage index up to 4-fold. (In other experiments [not shown], we found that inclusion of both FPG and endonuclease III together had an additive effect, which is expected if they are detecting different classes of lesion.)

The units used to express damage in Figures 1 and 2 are arbitrary units derived from a visual assessment of the relative intensity of comet tails. A more objective computer-based image analysis has also been employed using Komet 3.0 software. Table 1 shows results from a study of DNA damage in healthy males (smokers and nonsmokers; not significantly different in this study) from 40 to 60 years of age. The mean values are given for strand breaks (present on incubation without enzyme), FPG-sensitive sites, and endonuclease III-sensitive sites, expressed as percent of DNA in the tail and as tail moment (the product of relative tail intensity and length).

In order to relate these units to DNA break frequencies and thus to be able to estimate numbers of oxidized bases, lymphocytes were X-irradiated on ice after embedding in agarose and then lysed and electrophoresed. Figure 3 shows the increase in DNA breakage with X-ray dose as indicated by image analysis using Komet 3.0. As well as indicating the range of sensitivity of the comet assay and its linearity, this figure can be used as a calibration curve because the yield of DNA breaks/alkali-labile sites per unit dose has been established by other means (*25*).

7,8-Dihydro-8-oxo-deoxyguanosine Measured by HPLC

We have used a method of DNA isolation that avoids phenol-chloroform, as it has been suggested that these reagents can induce spurious oxidation of DNA or sensitize DNA to future oxidative attack (*9,26*). Figure 4 gives the results of analysis of the 8-OHdG content of lymphocyte DNA from 25 healthy nonsmoking males aged between 40 and 60 years. 8-OHdG is related to unoxidized dG by measuring UV absorbance (for dG) as well as the electrochemical signal (for 8-OHdG).

Table 1. Endogenous oxidative DNA damage assessed in lymphocyte samples from 25 men (aged 40–60 years).

Digestion conditions	Buffer	Endonuclease III	FPG
% DNA in tail	8.80 ± 0.89	13.70 ± 0.89	14.30 ± 0.90
DNA breaks/10^9 daltons	0.18	0.40	0.43
Tail moment	4.05 ± 0.79	11.37 ± 1.70	11.61 ± 1.51
DNA breaks/10^9 daltons	0.36	0.65	0.66

The comet assay was run with or without FPG or endonuclease III digestion before electrophoresis. Comets were analyzed using Komet 3.0, which provides various parameters including the percent of DNA in the tail and the tail moment. Mean values of these parameters from 25 comets per subject were calculated, and the population means derived from these figures are shown here (±SE). DNA break frequencies are estimated using Figure 3 as a calibration curve.

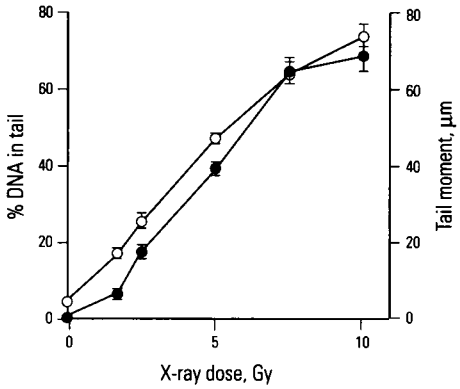

Figure 3. X-ray calibration of comet assay. Lymphocytes were embedded in agarose and X-irradiated with a range of doses before lysis and electrophoresis. Comets were quantitated using image analysis (Komet 3.0). The percent of DNA in the tail (○) and the tail moment (●) are plotted here (means of results from duplicate slides). The DNA breakage conversion factor is 0.31 breaks per 10^9 daltons per Gy (*25*).

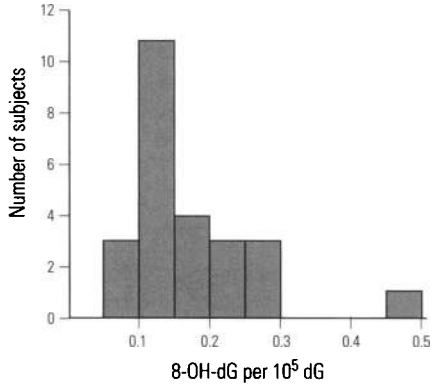

Figure 4. Distribution of values of 8-OHdG concentration in DNA of lymphocytes from 25 male nonsmokers aged 40–60 years. 8-OHdG was determined in two samples of hydrolyzed DNA taken from a single blood sample.

Discussion

Physicochemical methods, HPLC-EC and GC-MS, have been used for the measurement of 8-OHgua in DNA treated with free radical-generating agents such as ionizing radiation and H_2O_2; for several years it has been apparent that, with comparable treatments, values obtained by GC-MS are consistently higher than those from HPLC. Whether HPLC underestimates or GC-MS overestimates 8-OHgua has been unclear, but recently Ravenat et al. (*27*) carried out a careful comparative study applying HPLC-EC and GC-MS to identical DNA samples. The initial 50-fold discrepancy between the two methods was shown to result from oxidation of guanine occurring during processing for GC-MS. The yield of 8-OHgua from guanine increased linearly with the period allowed for the derivatization reaction. If 8-OHgua was first purified by HPLC before derivatization (so that no gua was available for oxidation), the values determined by HPLC-EC and by GC-MS were comparable.

There is little information on normal levels of 8-OHgua (induced by endogenous oxidative attack) in the DNA of human lymphocytes. The results from this study, taking precautions to avoid oxidation during DNA isolation, are considerably lower than others (Table 2). 0.17 8-OHgua per 10^5 gua (our figure) corresponds to 1.3 per 10^9 daltons of DNA. Given a DNA content of 4×10^{12} daltons per cell, there are about 5,000 8-OHgua residues per cell.

The alternative approach, based on the use of lesion-specific enzymes to introduce DNA breaks at sites of damage, has been incorporated into three different methods. Czene and Harms-Ringdal (*19*) developed a nick-translation method to estimate DNA break frequencies; incorporation of ^{35}S-deoxycytidine triphosphate by *Escherichia coli* DNA pol I indicates the presence of breaks. The assay is calibrated with plasmid DNA in which the frequency of breaks is measured directly by a relaxation assay. Gamma irradiation of cultured human fibroblasts induced FPG-sensitive sites as well as direct strand breaks in a ratio of approximately 1:2. The background level of FPG-sensitive lesions was about 500 per cell. Alkaline elution was used by Epe and Hegler (*18*) to measure lesions sensitive to several repair endonucleases including FPG. After H_2O_2 treatment of L1210 mouse leukemia cells, they found approximately equal numbers of single strand breaks and base modifications.

We previously used the comet assay to detect endonuclease III-sensitive sites (i.e., oxidized pyrimidines) in lymphocytes and estimated that there were a few hundred per cell (*28*). Our calibration of the assay at that time was indirect and approximate. We can now attempt a more precise calculation of the frequency of FPG sites and endonuclease III-sensitive sites using Figure 3. From the curve relating tail DNA to break frequency, we estimate the background level of strand breaks to be 0.18 per 10^9 daltons; the extra sites revealed with endonuclease III and FPG amount to 0.22 and 0.25 per 10^9 daltons, respectively. The curve for tail moment gives corresponding values of 0.36, 0.29, and 0.30 per 10^9 daltons. These figures correspond to about a thousand endonuclease III sites per cell and a similar number of FPG sites. Recent experiments using alkaline elution to measure DNA breaks have indicated about 250 FPG sites per 10^9 base pairs (B Epe, personal communication), equivalent to 1,500 per cell; thus, two distinct approaches give very similar estimates of the level of oxidative base damage in human cells.

Table 2. 8-OHdG in human lymphoctyes measured by HPLC-EC.

Study group	8-OHdG/10^5 dG	References
Healthy volunteers	7.6 ± 1.5	(*29*)
Swimmers	3.46 ± 1.8	(*30*)
Runners	2.32 ± 1.24	(*30*)
Healthy males	0.17 ± 0.1	Present study

Results are mean ± SD.

A potential problem with the use of enzymes is that they may not detect all the potential substrate. In the case of the comet assay, we optimized the reaction conditions for FPG (*31*). Confirmation that the enzyme is working effectively comes from the experiment in Figure 1. It can be seen that, at different doses of H_2O_2, the yield of FPG-sensitive sites (Figure 1B) is similar to the yield of strand breaks (Figure 1A); this is in accord with the work of Czene and Harms-Ringdahl (*19*) and Epe and Hegler (*18*) discussed above and also with direct analysis of the spectrum of lesions in DNA after oxidative attack (*32*).

With the revised DNA isolation procedure, we appear to have eliminated a major oxidation artifact. The estimated 8-OHgua content of DNA is still several times greater than that indicated by the comet assay. It appears to us that *a priori* the bias must be toward the lower end of the range of values for endogenous damage because it seems unlikely that the mammalian cell, with a complement of appropriate repair enzymes, would tolerate a substantial burden of DNA damage of a kind that is known to be potentially mutagenic. The chemical analytical methods, which involve lengthy DNA isolation and hydrolysis procedures, must be considered as more prone to artifact than the simpler enzymic methods. Both approaches, however, have been applied in population studies and have revealed significant correlations between oxidized bases and aspects of oxidative stress such as radiotherapy, smoking, and antioxidant status (*15,33*). Thus, while we have elucidated the enigma, the discrepancy between different experimental approaches remains to be fully resolved.

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