Bistranded Oxidized Purine Damage Clusters: Induced in DNA by Long-Wavelength Ultraviolet (290−400 nm) Radiation?†

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ABSTRACT: Bistranded clustered DNA damages involving oxidized bases, abasic sites, and strand breaks are produced by ionizing radiation and radiomimetic drugs, but it was not known whether they can be formed by other agents, e.g., nonionizing radiation. UV radiation produces clusters of cyclobutyl pyrimidine dimers, photoproducts that occur individually in high yield. Since long-wavelength UV (290−400 nm) radiation induces oxidized bases, abasic sites, and strand breaks at low yields, we tested whether it also produces clusters containing these lesions. We exposed supercoiled pUC18 DNA to UV radiation with wavelengths of >290 nm (UVB plus UVA radiation), and assessed the induction of bistranded clustered oxidized purine and abasic clusters, as recognized by *Escherichia coli* Fpg protein and *E. coli* Nfo protein (endonuclease IV), respectively, as well as double-strand breaks. These three classes of bistranded clusters were detected, albeit at very low yields (37 Fpg-OxyPurine clusters Gbp^{-1} kJ^{-1} m^2, 8.1 double-strand breaks Gbp^{-1} kJ^{-1} m^2, and 3.4 Nfo-abasic clusters Gbp^{-1} kJ^{-1} m^2). Thus, these bistranded OxyPurine clusters, abasic clusters, and double-strand breaks are not uniquely induced by ionizing radiation and radiomimetic drugs, but their level of production by UVB and UVA radiation is negligible compared to the levels of frequent photoproducts such as pyrimidine dimers.

Exposure of double-stranded DNA in solution or in cells to ionizing radiation induces bistranded clustered damage, two or more closely spaced oxidized bases, abasic sites, or strand breaks on opposing DNA strands (*1*−*3*). Such clustered damages are thought to be important radiation-induced sites, as their attempted repair could produce doubl-strand breaks (DSBs)[1] in addition to those induced directly by radiation (*4*−*7*). In fact, Blaisdell and Wallace recently showed that repair-proficient *Escherichia coli*, but not glycosylase-deficient cells, generate additional double-strand breaks during incubation after X irradiation (*8*).

A critical question is whether such ionizing radiation-induced DNA alterations are uniquely induced by ionizing radiation, or if they could be induced by normal oxidative metabolism or by environmental agents such as nonionizing radiation. Strong evidence indicates that short-wavelength UV (UVC, 180−290 nm), which is not found in the solar spectrum at the earth's surface, can induce bifilar clusters of intrastrand cyclobutyl pyrimidine dimers (CPDs) (*9*, *10*). Further, oxidized bases have been shown to be induced in DNA in solution and in cells by UV radiation (*11*−*15*). However, CPDs are formed with high efficiency by UV, in contrast to oxidized bases, abasic sites, and strand breaks, known components of ionizing radiation-induced clustered damages (*3*, *16*).

We therefore tested whether UV radiation that does reach the surface of the earth, UVB (290−320 nm) and UVA (320−400 nm), induces double-strand breaks and bistranded clustered damages of oxidized purines. To preclude the participation of pre-existing single-strand breaks in the measured clusters, we used a supercoiled DNA as a test system, since it contains no strand breaks. The data show that UVB and UVA radiation induces single-strand breaks, double-strand breaks, oxidized purine clusters (as recognized by the *E. coli* Fpg protein), and abasic clusters (recognized by the *E. coli* Nfo protein) in DNA in solution, albeit at extremely low yields compared to other UV-induced damages such as CPDs. Taking into account yearly doses to humans in the US of erythemal UV (*17*), shielding of DNA in living skin cells by overlying keratinized cells (*18*), and reduced production of oxidized bases in cells versus DNA in solution (*13*), we estimate that, in an average cell in human skin, environmental UV radiation would produce ∼13 bistranded Fpg-OxyPurine clusters per cell per year, 2.4 double-strand breaks per cell per year, and 1 bistranded Nfo-abasic cluster per cell per year. In comparison to the yearly

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[1] Abbreviations: Ab, abasic; CPD, cyclobutyl pyrimidine dimer; DSB, double-strand break; fapy, 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine; Gb(p), gigabase (pair), 10^9 bases (pairs); LIN, linear DNA molecules; MED, minimal erythemal dose; Mb(p), megabase (pairs), 10^6 bases (pairs); REL, relaxed (nicked circular) DNA; SC, supercoiled DNA; UVA, 320−400 nm radiation; UVB, 290−320 nm radiation; UVC, 180−290 nm radiation.

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level of pyrimidine dimers (approximately 2.4−16 × 10^9 CPD per cell per year), the levels of clustered Fpg-OxyPurine and Nfo-abasic damages are extremely low. Thus, while bistranded oxidized purine and abasic clusters are not induced solely by ionizing radiation, in assessments of DNA damage in human skin, they would be negligible.

EXPERIMENTAL PROCEDURES

UV Source and Plasmid DNA Irradiation. UV radiation was obtained from Westinghouse FS20 lamps emitting broad-spectrum UV (Westinghouse Electric, Lamp Division, Bloomfield, NJ); the lamp was filtered by a UV-30 filter (Hoya Optics, Fremont, CA) to remove UVC radiation (wavelengths of <290 nm). The dose rate was determined to be 38 J m^{-2} s^{-1} by a Jagger meter (*19*) calibrated versus a Molectron PR200 radiometer (Molectron Detector, Inc., Portland, OR).

pUC18 DNA (2686 bp; from Bayou Biolabs, Harahan, LA) was diluted to a concentration of 100 ng/μL in 20 mM potassium phosphate buffer (pH 7.4). It was then transferred into a quartz capillary (Charles Supper Co., Natick, MA), and the capillary was sealed tightly with Parafilm to prevent evaporation during irradiation. The capillary was placed on a glass Petri dish on ice and exposed to UV radiation. A sample of DNA solution was removed from the capillary at increasing doses, and was mixed with an equal amount of HEPES/KOH buffer solution [140 mM HEPES/KOH (pH 7.6), 200 mM KCl, and 2 mM EDTA] to give a final DNA concentration of 50 ng/μL. The maximum exposure time was 32 h.

Fpg Protein and Nfo Protein Treatment. Irradiated DNAs and unirradiated control DNA in 70 mM HEPES/KOH (pH 7.6), 100 mM KCl, 1 mM EDTA containing 1 mM DTT, and 50 ng/μL bovine serum albumin were treated with sufficient *E. coli* Fpg protein or Nfo protein to cleave at all available sites for the respective enzyme. *E. coli* Fpg was purified by the methods of Zharkov et al. and Rabow et al. (*20*, *21*) and contained a single protein band on Coomassie Blue-stained gels. Fpg protein substrates include 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-5-formamidopyrimidine (FapyGua), 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (MethylFapyGua), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), some abasic sites, C8-oxoadenine, and, to a lesser extent, other modified purines (*22*−*26*). The Fpg protein excises some pyrimidine-derived lesions present as single lesions in synthetic oligonucleotides (*25*, *27*). However, the Fpg protein was shown not to excise pyrimidine-derived lesions in damaged DNA (*23*, *28*). The *E. coli* Nfo protein was purified to homogeneity from the overexpression plasmid pET24-Eco-Nfo (the kind gift of B. Demple, Harvard University, Cambridge, MA) in *E. coli* B834DE8 (the kind gift of W. Studier, Brookhaven National Laboratory) by the method of Levin et al. (*29*). Substrates for the Nfo protein include regular and oxidized abasic sites (*30*). Recent data indicate that the Nfo protein cleaves synthetic oligonucleotides at sites of some oxidized bases (*31*). However, since several enzymes have been shown to cleave oligonucleotides containing a single modified species, but exhibit no activity toward the same lesion in irradiated DNA containing multiple modified sites (*32*), it is not clear whether Nfo can cleave pyrimidine-derived lesions in irradiated DNA. The reaction mixtures were incubated at 37 °C for 1 h. To remove protein and prevent possible interference with gel electrophoresis, the mixtures were subsequently incubated with EDTA (0.1 M) and proteinase K (1.33 mg/mL) at 37 °C overnight.

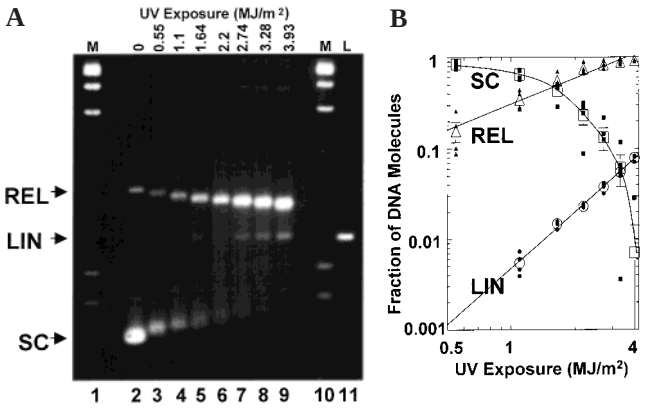

FIGURE 1: (A) Electronic image of a neutral agarose electrophoretic gel containing pUC18 DNA exposed to 0−3.93 MJ/m^2 UVB and UVA radiation (lanes 2−9): SC, supercoiled pUC18; LIN, linear pUC18; REL, nicked circular pUC18; lanes 1, 10, and M, positional DNA standards (*Hin*dIII digest of λ); and lanes 11 and L, pUC18 DNA linearized by *Eco*RI digestion. (B) Quantitative analysis of UV-induced strand breaks in pUC18 DNA: (□ and ■) supercoiled, (△ and ▲) nicked circles, and (○ and ●) linear. Small filled symbols represent individual data points from independent experiments. Large empty symbols represent averages. Error bars represent standard errors of the means. In some cases, the errors are smaller than the symbols for the averages. Lines for induction of linear and nicked circular molecules are least-squares fit to the averages; the curve for supercoiled DNA levels was fit by eye to the average points.

Gel Electrophoresis and Analysis. The DNA samples were mixed with a neutral stop solution containing 0.125% bromophenol blue and 0.5% sodium lauryl sulfate in 50% glycerol and then electrophoresed in 2% (w/v) agarose (SeaKem LE; FMC, Rockland, ME) in Tris-acetate buffer (pH 8) at 15 V for 24 h. Positional markers were a *Hin*dIII digest of λ DNA (23.1, 9.4, 6.5, 4.4, 2.3, 2, and 0.56 kbp).

After electrophoresis, the gel was stained with ethidium bromide (1 μg/mL) for 15 min and then destained with distilled water for 2 h. An electronic image was obtained from a charge-coupled device-based system that gives a linear response to ethidium fluorescence over at least 3 orders of magnitude (*33*, *34*). The relative quantities of the supercoiled, relaxed, and linear molecules within each lane were calculated by integrating the "area" of each band, and using a factor of 1.4 to correct for the lower level of binding of ethidium to supercoiled molecules than to other conformers (*35*). Fpg protein-sensitive sites were identified by subtracting the fraction of DNA present in a specific form after enzyme treatment from that present in the same DNA sample before enzyme treatment.

Hazardous Procedures. Since UVA and UVB radiation are hazardous to eyes and skin, UV-opaque goggles and skin protection were used.

RESULTS

We first determined whether UV induced strand breaks in supercoiled DNA under our conditions. Figure 1 shows a representative agarose gel containing pUC18 DNA exposed to 0−3.9 MJ/m^2 of UV radiation. The gel clearly shows that the supercoiled molecules (SC) disappear, while the level

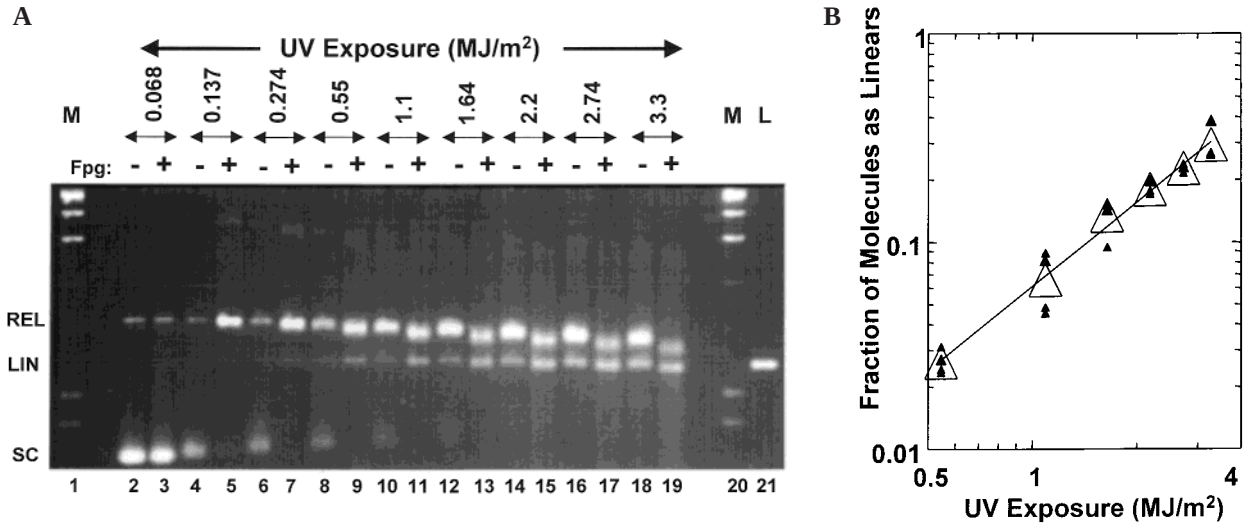

FIGURE 2: (A) Electronic image of a neutral agarose electrophoretic gel containing pUC18 DNA exposed to UV; data are in pairs of increasing doses, with the first of each pair not treated (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18) and the second treated with the Fpg protein (lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19): SC, supercoiled pUC18; LIN, linear pUC18; REL, nicked circular pUC18; lanes 1, 20, and M, positional DNA standards (*Hin*dIII digest of λ); and lanes 21 and L, pUC18 linearized by *Eco*RI. (B) Quantitative analysis of UV-induced bistranded Fpg-OxyPurine clusters in pUC18 DNA. The data represent the level of linear molecules produced by Fpg protein cleavage in excess of those produced directly by UV radiation. Small filled symbols represent individual data points from independent experiments. Large empty symbols represent averages. The line represents a linear least-squares fit to the averages. Error bars represent standard errors of the mean; in some cases, the error is smaller than the symbol and is not shown.

of relaxed molecules (REL) increases strikingly as a function of increasing dose. Linear molecules (LIN) are evident at the higher levels of exposure. Induction of a single-strand break in a supercoiled molecule produces a circular molecule with a single nick (relaxed DNA). The relaxed population may also include molecules with multiple single-strand breaks, as long as the breaks are not located on opposing strands at a spacing close enough to produce a double-strand break. The linear population could result from the production of a double-strand break in the circular molecule, either by simultaneous scission of both strands at sites sufficiently close to produce a double-strand break (SC to LIN or REL to LIN) or by sequential production of two single-strand breaks, with the induction of the second strand break near a pre-existing break in the relaxed DNA converting it to a linear molecule. The alterations in mobility at a high UV dose result from modification of the DNA tertiary structure, including unwinding of supercoils due to pyrimidine dimer formation (*36*−*39*). Figure 1B shows quantitative data from gels such as that shown in Figure 1A. Figure 1 clearly shows that UV radiation induces single-strand breaks (producing relaxed molecules) and double-strand breaks (producing linear molecules) in supercoiled DNA molecules.

UV radiation is known to produce oxidized bases, and strong evidence identifies 8-oxodeoxyguanosine as one of these bases (*13*, *14*, *40*, *41*). We therefore asked if UV radiation could induce damage clusters containing oxidized bases, as ionizing radiation does. To test for damage clusters involving oxidized purines, we used the *E. coli* Fpg protein, a glycosylase with strong lyase activity (*22*). Its action at an oxidized purine results in release of the oxidized base (through its glycosylase activity), and cleavage of the DNA backbone through β,δ elimination (*42*). If a DNA molecule contains an OxyPurine cluster (at least two closely spaced Fpg-recognized lesions on opposing strands, or an Fpg site vs a single-strand break), treatment with the Fpg protein would produce a de novo DSB (in addition to those produced by UV), resulting in the production of additional linear molecules. Such clusters are termed Fpg-"OxyPurine" clusters, to denote the enzyme that recognized them and the major type of lesion recognized by that enzyme.

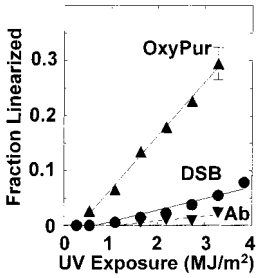

FIGURE 3: Induction of bistranded Fpg-OxyPurine clusters, double-strand breaks, and bistranded Nfo-abasic clusters by UV radiation: (▲) Fpg-OxyPurine clusters, (●) double-strand breaks, and (▼) Nfo-abasic clusters. Lines are linear least-squares fits for for OxyPurine and abasic clusters and a second-degree polynomial for DSBs.

Figure 2A shows a representative gel from such an experiment. The lanes are in pairs (at increasing levels of UV exposures), with the first of each pair not treated with the Fpg protein and the second treated with the Fpg protein. The production of isolated Fgp-recognized sites is clearly shown by the production of relaxed molecules upon enzyme treatment of DNA irradiated with 0.27 MJ/m^2 of UVB and UVA. Linear molecules induced by Fpg protein treatment are clearly evident at UV exposures of ~0.55 MJ/m^2. Figure 2B shows quantitative data for the production of Fpg-OxyPurine clusters resulting from experiments such as that shown in Figure 2A.

Clearly, UV radiation alone induces double-strand breaks, and Fpg protein treatment of the UV-irradiated DNA produces additional linear molecules, the signature of clustered damages recognized by this enzyme (Figures 2 and 3). However, since the Fpg protein has a lyase activity that cleaves DNA at abasic sites, it was possible that the apparent

Fpg-OxyPurine clusters were actually clusters containing abasic sites rather than oxidized purines. We therefore tested the ability of the *E. coli* Nfo protein (endonuclease IV), which cleaves DNA principally at oxidized and regular abasic sites (*30*). The dotted line in Figure 3 shows that only an extremely low level of bistranded Nfo-abasic clusters was detected in the UV-irradiated DNA. Thus, abasic clusters cannot account for the Fpg-recognized clusters.

DISCUSSION

Lesions are induced in DNA by radiation throughout the UVC, UVB, and UVA ranges. UVC induces cyclobutyl pyrimidine dimers by direct photon absorption by the DNA bases, and oxidized bases are also produced by direct absorption of UVC radiation (*11*, *43*). UVB induces CPDs by direct absorption (*43*); however, the substantial (but not complete) reduction in yields of SSBs and Fpg-sensitive sites by Tris, an effective hydroxyl radical scavenger (*44*), suggests that both direct absorption and a radical-mediated mechanism could contribute to the formation of these lesions. UVA wavelengths as long as 365 nm induce CPDs in DNA in solution and in cells (*18*, *45*−*48*), but again, Tris reduces yields of SSBs and Fpg-sensitive sites, suggesting the possibility of multiple mechanisms of induction.

The energy of UV photons in the wavelength range of 290−400 nm is 4.3−3.1 eV. Since the light source was not a high-intensity laser, biphotonic excitation can be ruled out. Energies of 3 or 4 eV are insufficient for ionization of DNA, so radical cation intermediates are not involved. There is evidence that double lesions on the same strand are formed after reaction with a single reactive oxygen radical species (*49*). Box et al. speculated that an intermediate singly damaged species can undergo an intramolecular reaction in which a neighboring nucleotide residue is also damaged. The intermediate might well be a peroxyl radical, a common product from any organic compound in the presence of oxygen after UV or ionizing radiation. Milligan et al. showed that these compounds produce FPG-sensitive sites in DNA (*50*). A similar reaction might occur with reactive intermediates derived from excited states of DNA bases produced by absorbing 3 or 4 eV photons.

In addition to consideration of the mechanism of induction of the lesions constituting a bistranded cluster, it is important to know the number of events required to produce a cluster. High-level UV radiation exposures induce single- and double-strand breaks in DNA in solution (Figure 1). The slopes on the log−log plot of Figure 1B for production of single-strand breaks are 0.93 (curve fit coefficient of 0.98) and for double-strand breaks 2.07 (curve fit coefficient of 0.998), indicating that SSBs were formed by one event and DSBs by two independent events.

Bistranded clustered damages recognized by the *E. coli* Fpg protein are also produced by UV radiation (Figure 2). Fpg protein substrates include modified purines as well as some abasic sites (*22*−*26*). As discussed above, the Fpg protein also cleaves pyrimidine-derived lesions as single sites in synthetic oligonucleotides (*25*) but fails to cleave such lesions in irradiated DNA containing many damages (*23*, *28*). Since the Fpg protein does recognize regular abasic sites (sites of base loss without additional alterations), it was possible that the clusters we measured were in fact abasic clusters. To test for the presence of abasic clusters, we treated UV-irradiated DNA with the *E. coli* Nfo protein (endonuclease IV) and looked for the production of linear molecules. The Nfo protein cleaves several types of abasic sites, including oxidized abasic sites (*30*, *51*). Figure 3 shows that there are very few bistranded clustered damages recognized by the Nfo protein, and thus, clusters of abasic sites (or of other substrates of the Nfo protein) are only minor contributors to the UV-induced Fpg-OxyPurine clustered damages. The OxyPurine clusters we measure are thus principally composed of oxidized purine clusters. Further, since Doetsch et al. showed by mass spectroscopic analysis that 254 nm radiation (UVC) produces 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-5-formamidopyrimidine (FapyGua) (*11*), and Kuluncsics et al. showed that UVB radiation and UVA radiation induce sites for the Fpg protein (*41*) (which recognizes both FapyAde and FapyGua), it is highly likely that the bistranded clusters we measure contain FapyAde and FapyGua. In addition, Douki et al. showed that UVB, UVA, and solar simulated light also produced 8-oxo-7,8-dihydrodeoxyguanosine (8-oxodGuo) (*52*), and thus, the UV-induced clusters may contain 8-oxodGua as well. UVB and UVA radiation may contribute differentially to production of specific clusters, as Kuluncsics et al. found that UVA irradiation of plasmid DNA on a glass support produced both Fpg- and Nfo-sensitive sites, whereas UVB did not produce significant levels of Nfo-sensitive sites (*41*).

The slope of the induction line for Fpg-OxyPurine clusters in Figure 2B is 1.35 (fitting coefficient of 0.997), suggesting that these clusters are not formed exclusively by single events (in contrast to single-strand breaks; see Figure 1). Frequent UV photoproducts such as CPDs can occur in bistranded clusters in bacteriophage DNA (*9*) and in mammalian cells (*10*, *53*). The dependence of CPD induction on dose was found to be in the range of 1−1.4 in different DNAs and cellular systems (*53*−*55*). Lam and Reynolds showed that the induction of CPD clusters depended on DNA sequence, with higher frequencies at sites in closely opposed runs of pyrimidines (*56*). Thus, base composition, or even the differential susceptibility of some base sequences to distortion by an initial lesion, may affect the induction of oxidized base clusters.

UV radiation clearly induces bistranded clustered damages containing oxidized purines. The yields we obtain correspond to 37 Fpg-OxyPurine clusters Gbp^{-1} kJ^{-1} m^2, 8.l DSBs Gbp^{-1} kJ^{-1} m^2, and 3.4 Nfo-abasic clusters Gbp^{-1} kJ^{-1} m^2. Therefore, neither oxidized purine clusters, double-strand breaks, nor abasic clusters are uniquely induced by ionizing radiation. We can ask whether they would occur in the skin of humans exposed to environmental UV radiation. The total erythemal UV doses have been calculated to be in the range of 22−33 kJ m^{-2} $year^{-1}$ for Americans (*17*). However, DNA in living cells in skin of average humans is shielded with respect to damage induction by a factor of 10−100 (depending on UV wavelength) by the overlying keratinized layer (*18*). Further, although oxidized bases are known to be formed in DNA in solution and in human cells (*13*, *14*, *40*), ∼40 times more 8-oxoguanine is formed by UVB and UVA radiation in DNA in solution than in human cells (*13*). Using an average erythemal UV dose of 25 kJ/m^2, a factor of 10 as a conservative estimate of shielding, and a factor of 40 for the reduction in the level of oxidized base production

from DNA in solution compared to DNA in human cells, we estimate that cells in human skin would be subjected to approximately 14 Fpg-OxyPurine clusters per cell per year, 3 DSBs per cell per year, and 1 Nfo-abasic cluster per cell per year. These are extremely low levels compared with those of pyrimidine dimers produced yearly [approximately 2.4−16 × 10^9 CPDs/cell year, based on ∼6 × 10^5 to ∼12 × 10^5 CPDs per cell induced by one minimal erythemal dose to human skin, 60 mJ/cm^2 as an average MED value, and ∼4000 MEDs per year (*17*)]. Such OxyPurine clusters and abasic clusters thus comprise a negligible background of damage in human skin.

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