Human white blood cells contain cyclobutyl pyrimidine dimer photolyase

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ABSTRACT Although enzymatic photoreactivation of cyclobutyl pyrimidine dimers in DNA is present in almost all organisms, its presence in placental mammals is controversial. We tested human white blood cells for photolyase by using three defined DNAs (supercoiled pET-2, nonsupercoiled bacteriophage λ , and a defined-sequence 287-bp oligonucleotide), two dimer-specific endonucleases (T4 endonuclease V and UV endonuclease from *Micrococcus luteus*), and three assay methods. We show that human white blood cells contain photolyase that can photorepair pyrimidine dimers in defined supercoiled and linear DNAs and in a 287-bp oligonucleotide and that human photolyase is active on genomic DNA in intact human cells.

Evaluating the impact of sunlight exposure on human health—as well as that of possible increases in environmental ultraviolet B (290–320 nm) resulting from stratospheric ozone depletion—requires knowledge of the ability of human tissues to repair UV-induced DNA damage (1). Cyclobutyl pyrimidine dimers (CPDs) are a major class of mutagenic (2) and oncogenic (3, 4) UV-induced damage. CPDs are repaired by photoreactivation (PR), in which a single light-dependent enzyme (photolyase) reverses CPDs to monomers.

Although PR was initially reported to be absent in cultured human cells (5), Sutherland (6), Sutherland *et al.* (7), and Harm (8, 9) demonstrated photolyase activity in cells from placental mammals, including humans. Results on PR enzyme activity in human tissues also differ: although Cook and McGrath (10) were unable to find photolyase activity in mammalian tissues, Sutherland *et al.* (7), Harm (8, 9), and Ogut *et al.* (11) measured its activity in a variety of human fetal, neonatal, and adult tissues, including white blood cells (WBCs). While human photorepair activity had been measured in cells and in cell extracts by using standard chromatographic methods (12, 13), most experiments yielding positive results used assay methods—bacterial transformation (8, 9) and nuclease digestion (6)—that might have reflected light-dependent alteration of other lesions. This possibility would have been consistent with the failure of Li *et al.* (14) to find PR enzyme activity in human WBCs by using CPD-specific assays.

We used CPD-specific methods and defined DNAs to test PR activity in human WBC extracts and cellular dimer PR in intact human WBCs. Human WBC extracts photorepair CPDs in supercoiled and nonsupercoiled DNA and specific CPDs in a defined sequence oligonucleotide. Further, intact human WBCs carry out photorepair of CPDs in genomic DNA.

MATERIALS AND METHODS

WBCs. Peripheral blood (≈ 5 ml) from healthy humans (with informed consent), collected in a heparinized tube, was mixed with one-third volume of sterile 3% (wt/vol) gelatin (Sigma, Type 1) in phosphate-buffered saline (PBS = 0.17 M NaCl/3.4

FIG. 1. (A) Photorepair of pyrimidine dimers in supercoiled DNA by human WBC extract. Supercoiled pET-2 DNA (10 ng, ≈ 1 CPD per molecule) was incubated with human WBC extract ($6\text{--}60 \times 10^{-7}$ CE per CPD; 37°C , 15 min), exposed to light ($\circ$) or kept in the dark ($\bullet$) for 60 min, and then treated with *M. luteus* UV endonuclease. Sterile stop mixture (0.54% SDS/0.014% bromophenol blue/50% glycerol) was added, and samples were electrophoresed [0.7% LE agarose (FMC) gel in TAE (40 mM Tris/20 mM acetate/20 mM EDTA/0.18 M NaCl, pH 8); Bio-Rad MiniSub Cell, 50 V, 1 hr, buffer recirculation, chilled bath]. Gels were stained with ethidium bromide (1 $\mu\text{g}/\text{ml}$) and destained. A quantitative electronic image was obtained (23), and the fractions of supercoiled and cleaved DNA molecules were determined (24). (B) Photorepair of CPDs in λ DNA by human WBC extract. DNA (50 ng, ≈ 130 CPDs per Mb) was incubated (37°C , 15 min) with human extract (2×10^3 CE, 1.6×10^{-7} CE per CPD) and then exposed to light. Samples were divided; one part was treated with *M. luteus* UV endonuclease and the other was incubated with buffer. DNAs were denatured by 0.5 M NaOH/0.125% bromocresol green/50% glycerol (alkaline stop mixture) and electrophoresed along with molecular length standards [λ , 48.5 kb; T7, 40 kb; and a *Bgl* I digest of T7 (22.5, 13.5, and 4 kb), 0.4% alkaline LE agarose gel; Bio-Rad MiniSub Cell, 2 mM EDTA/30 mM NaOH; 2 hr, 30 V, buffer recirculation]. Gels were neutralized (0.1 M Tris-HCl, pH 8), stained with ethidium, and destained, and a quantitative electronic image was obtained. DNA profiles of samples and length-standard lanes were obtained, and a dispersion function (molecular length vs. migration position) was constructed from molecular length standards. Number average molecular length, L_n , of each DNA distribution and lesion frequencies were calculated (25) from replicate gels. Small symbols, individual data; large symbols, average values; error bars, SD.

mM KCl/10.1 mM Na₂HPO₄/1.8 mM KH₂PO₄, pH 7.4), and sedimented by gravity (37°C , 45 min, then 22°C , 15 min). The supernatant was centrifuged ($1100 \times g$, 5 min) and WBCs were resuspended in 15 ml of PBS and rinsed. Cell concentrations were determined and cells were suspended in ice-cold HPR buffer (20 mM KPO₄, pH 7.2/0.1 mM EDTA/0.1 mM dithiothreitol) or PBS.

Extracts. WBCs in ice-cold HPR buffer ($1\text{--}4 \times 10^8$ cells per ml) were sonicated (Kontes; power setting 6–7; $0\text{--}4^\circ\text{C}$, 45 sec),

Abbreviations: CPD, cyclobutyl pyrimidine dimers; PR, photorepair; WBC, white blood cell; CE, cell equivalent, extract from one WBC; Mb, megabase(s).

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FIG. 2. Photorepair by human WBC extract of specific pyrimidine dimers in a ³²P-5'-end-labeled 287-bp oligomer with ≈ 1 CPD per molecule. Oligomer was incubated with buffer or with human WBC extract ($1-10 \times 10^4$ CE, 1×10^{-7} CE per CPD; 37°C, 15 min), and then one part was kept in the dark and the other was exposed to light for 90 min. DNAs were treated with dimer-specific endonuclease; purified by phenol extraction, ethanol precipitation, and vacuum drying; resuspended in H₂O; and then mixed with 95% deionized formamide/20 mM EDTA/0.05% xylene cyanol/0.05% bromophenol blue/0.25% SDS. Equal quantities of radioactivity from companion light and dark samples were applied to sequencing gels [8% polyacrylamide/8 M urea in 1× TBE (90 mM Tris borate/1 mM EDTA, pH 8)] and electrophoresed [1200 V, ≈ 2 hr; upper buffer reservoir, 0.5× TBE; lower, 1× TBE]. Gels were mounted on Whatman 3MM paper and dried, and a digital image was obtained using a Molecular Dynamics PhosphorImager. Total radioactivity in each lane and in dimer bands was computed by determining the total radioactivity within rectangles of equivalent size on the image with Molecular Dynamics IMAGEQUANT. (A) (Left) Electronic image of companion samples of oligonucleotide mixed with 4×10^4 CE of human extract and, after light (lane L) or dark (lane D) treatment, digested with T4 endonuclease V. (Right) Enlargements of the three dimer bands marked at left: CC, nt 42-43; CT, nt 55-56; CC, nt 66-67. (B)

protein concentration was determined, and ice-cold sterile glycerol was added to 50% (vol/vol). Extracts were stored at -20°C and handled in red light. For PR activity assays, extract was diluted into freshly prepared HPR buffer containing 1 mM dithiothreitol and acetylated bovine serum albumin (New England Biolabs; 100 $\mu\text{g}/\text{ml}$). Enzyme specific activities were calculated by using protein mass [determined by a micro Lowry method (15)] and by using cell equivalents (CEs; 1 CE = extract from 1 WBC), which gave highly reproducible values among replicate preparations.

DNAs. Supercoiled pET-2 plasmid DNA (16), exposed to 254-nm UV (10 J/m²), contained ≈ 1 CPD per DNA molecule. N⁶-Methyladenine-free bacteriophage λ DNA (New England Biolabs) was exposed to 254-nm UV (20 J/m²), producing ≈ 13 CPDs per λ molecule. A ³²P-end-labeled 287-bp oligonucleotide was prepared by polymerase chain reaction using one 5'-³²P-labeled primer (³²PATGCGTCCGCGCTAGA) and one nonradioactive primer (CACTATCGACTACGC-GATCA) to amplify the 337- to 623-nt region of pET-3 [gift of A. Rosenberg (17)], gel-purified, located by contact radiography, extracted, ethanol-precipitated, suspended in 20 mM potassium phosphate (pH 7.4), and then exposed to 254-nm UV (550 J/m²) (≈ 1 CPD per molecule).

UV and Visible Light. The 254-nm UV was measured with a Jagger meter (18) calibrated vs. a Molecron (Sunnyvale, CA) radiometer. Visible PR light from a 150-W spot passed through a heat-reflecting filter (Edmund Scientific, Barrington, NJ) to remove infrared radiation [in some cases, a Hoya Optics (Fremont, CA) L42 filter removed UV of less than ≈ 400 nm] and then through the bottom of a Pyrex water bath at $\approx 35^\circ\text{C}$, in which samples were suspended. Red room lighting (General Electric, 25W, Sign Service Group) prevented uncontrolled photorepair (19, 20).

RESULTS

For unambiguous determination of PR enzyme activity, we used three defined DNAs with known CPD frequencies, two CPD-specific enzymes, and three separation modes. PR light was in the wavelength range of the human and marsupial PR action spectra (19, 20) filtered to exclude radiation inducing CPDs (21, 22).

Photorepair of Pyrimidine Dimers in Supercoiled DNA by Human WBC Extract. Photorepair of dimers in supercoiled DNA by the human WBC enzyme removed potential sites for UV endonuclease, preventing conversion of supercoils to relaxed DNAs: Fig. 1A shows that in the light human WBC enzyme effectively repaired dimers, while in the dark the dimer frequency was unchanged. Treatment of DNA with extract in the light or dark, without UV endonuclease treatment, did not affect the fraction of supercoiled molecules. Activity was inactivated by heat and reduced $\approx 70\%$ by brief trypsin treatment.

Activity of Photorepair Enzyme in Human WBC Extracts on Dimers in λ DNA. To determine the rate of human photolyase repair in DNA containing multiple CPDs, we measured the initial PR rate in λ DNA containing ≈ 13 CPDs per DNA (25). Fig. 1B shows that human photolyase rapidly photorepaired CPDs in such DNA at an initial rate of $\approx 3 \times 10^4$ CPDs per CE per min, ≈ 30 times faster than in supercoiled DNA containing ≈ 1 CPD.

Human WBC Photorepair at Specific Pyrimidine Dimer Sites. We determined human photolyase activity on specific CPDs in a 5'-³²P-end-labeled 287-mer containing ≈ 1 CPD. Gel electrophoresis of oligomer treated with *Micrococcus luteus* UV endonuclease or T4 endonuclease V produces a

Differences in fraction of radioactivity in dark vs. light in the C-T dimer at positions 55-56 (digestion by *M. luteus* UV endonuclease, □; by T4 endonuclease V, ■).

FIG. 3. Cellular photorepair of pyrimidine dimers in genomic DNA. Human WBCs were exposed to 254-nm UV to give ≈ 8 CPDs per Mb, kept in the dark or exposed to light, harvested at increasing times after UV, and embedded in agarose plugs, and DNA was isolated by proteinase K digestion (27). Companion plugs were incubated at 37°C with or without UV endonuclease; reactions were terminated by adding alkaline stop mixture (30 min, 37°C). DNAs were electrophoresed [0.4% alkaline agarose gel using unidirectional pulsed field (15 V/cm; 0.3-s pulse, 10-s interpulse; 16 hr, 10°C with buffer recirculation)] (23) along with molecular length standards (T4, 170 kb; λ , 48.5 kb; T7, 40 kb; a *Bgl* I digest of T7, 22.5, 13.5, and 4 kb). Gels were neutralized, stained with ethidium, and destained. An electronic image

ladder of bands, each terminating at a specific dimer (26). Fig. 2A Left contains a phosphor image of radioactivity in UV-irradiated oligonucleotide treated with human extract and kept in the dark or exposed to light for 90 min. Photorepair of a specific CPD removed an endonuclease site, decreasing the radioactivity in that band (and correspondingly increasing the intact 287-mer). Although PR at many sites was evident from comparison of the lanes, three well-separated CPD bands (T-T at positions 42-43, C-T at positions 55-56, and C-C at positions 66-67) were quantitated; enlargements of these bands in light and dark samples are also shown (Fig. 2A Right). The PR enzyme was active toward all dimer types examined. Fig. 2B shows results for the C-T dimer at positions 55-56 for digestion with the *M. luteus* UV endonuclease or with T4 endonuclease V.

Photorepair of Pyrimidine Dimers in Intact Human WBCs.

Human WBCs were exposed to UV, producing 8 CPDs per megabase (Mb) ($\approx 10\%$ of a minimal erythral dose to skin, well within the biological range), and incubated in the dark or exposed to light, and DNA were electrophoresed on alkaline gels. The resulting DNA profiles show that in the light, cells photorepaired dimers, but during 40 min in the dark they removed CPDs slowly (Fig. 3).

DISCUSSION

Our results demonstrate photolyase activity in human WBCs. All assays gave consistent, positive results during an experimental period of over a year. Since studies by others have yielded differing results on photolyase in mammalian cells, it is important to delineate factors contributing to positive results: (i) We handled cell extracts in subdued light and stored them unfrozen. We could not obtain active human photolyase from WBCs stored at -80°C by the method of Li *et al.* (14). (ii) We optimized storage and assay conditions [phosphate buffer, low ionic strength (6), and assay temperature at $35-37^\circ\text{C}$] for human PR enzyme. The assay conditions (Tris buffer at 23°C) of Li *et al.* (14) may have contributed to their failure to detect human photolyase. (iii) As substrates we used purified defined DNAs with known dimer frequencies or—for intact cells—genomic DNA. (iv) To preclude recognition of other lesions by partially purified *M. luteus* UV endonuclease, we also used cloned purified dimer-specific T4 endonuclease V, which yielded similar results. (v) To ensure that positive results were not unique to one assay method, we used three assays with different analysis approaches and separation methods. (vi) For PR we used light within the range of the mammalian PR action spectrum filtered to remove radiation less than ≈ 400 nm, since UV as long as 365 nm induces CPDs (21, 22, 28). Since PR experiments use high-intensity light for long times, CPDs may be induced during illumination, decreasing or obscuring PR entirely. Medium or long wavelength UV radiation can induce other biologically significant lesions that reduce biological PR or could be mistaken for dimers in non-CPD-specific assays. The “white-light effect” of Harm (8, 9), which interfered with detection of PR, and the PR of survival of murine cells in N_2 but not in O_2 (29) might have resulted from induction of oxidative damages. Illumination of

was obtained, and the L_n values of each DNA population and CPD frequencies were determined (25). (A–C) DNA profiles from pairs of samples. Solid lines, samples treated with *M. luteus* UV endonuclease; dotted lines, without endonuclease treatment. (A) UV, harvest immediately. (B) UV, 40 min of visible light. (C) UV, 40 min of dark. Arrows at top of A show peak positions of molecular length standard DNAs (length in kb). L_n values and CPD frequencies were calculated from duplicate independent gels. (D) Dimers remaining versus time in light ($\circ$) and in dark ($\bullet$). Small symbols, individual values; large symbols, average values; error bars, SD.

mammalian cells in culture medium could induce damages obscuring photorepair. The light intensity can determine the PR rate: by using high-intensity light, D'Ambrosio *et al.* (30, 31) observed a higher PR rate in human skin *in situ* than Sutherland *et al.* (32), who used lower intensity.

Adequate negative controls for damage induction by PR light and successful positive PR controls are necessary. Repair-competent organisms are not good negative controls, since they may repair damage induced during illumination (thus yielding no net damage), and it may thus appear that no damage was induced. Unshielded DNA in a UV-transparent buffer is an adequate negative control (33). Positive controls using PR-competent organisms are useful [but see Cleaver (5)]. In addition, competing dimer repair systems must be minimized. Thus Wagner *et al.* (34) and Henderson (35) readily detected PR of viral production in excision-deficient but not in excision-proficient human cells.

PR in mammalian cells and tissues has been demonstrated by a variety of methods. Thin layer chromatography and nuclease-digestion analyses showed PR activity in human, bovine, and murine cell and tissue extracts as well as PR in cells (6, 7, 12, 13). By using a bacterial transformation assay, Harm (8, 9) found PR activity in cultured murine and human cells, and in human, feline, and bovine tissues. Viral reactivation assays also showed PR of UV-irradiated herpes virus in cultured human cells (34) and Epstein-Barr virus in human lymphocytes (35). Further, several groups showed CPD photorepair in human skin *in situ* (30–32).

Data indicating the absence of PR activity in some mammalian cells have also been reported. Such reports are reasonable, since it is unlikely that all mammalian tissues or cells express PR enzyme under all conditions. The activity and/or expression of PR activity is regulated in prokaryotes (36), in simple eukaryotes (37), and in complex organisms (10, 11, 38). Factors regulating PR activity or PR gene expression are not understood; in cultured human cells, medium composition affects PR activity level (12, 39). The evolutionary relationship of human PR enzyme to other photolyases is also not understood: the simple expectation of similar protein structure and thus nucleic acid sequence was not borne out by probing human genomic DNA or RNA transcripts with cloned photolyase sequences from the marsupial *Monodelphis domestica* (40), or a cDNA probe for *Potorous tridactylis* photolyase (38), suggesting that human photolyase is of convergent function but dissimilar structure to other photolyases.

There is apparently confusion on result of PR assays and characteristics of mammalian PR enzymes. For example, Li *et al.* (14) claimed that "one of the most sensitive assays for photolyase is the transformation assay. Harm [42] easily detected photolyase in marsupial cell extract by this assay, but was unable to detect the activity with extract from placental animals." Harm's work (42) included only studies of the marsupial *Potorous tridactylus*, but none on any placental mammal. Moreover, in other publications, using bacterial transformation, Harm (8, 9) demonstrated photolyase activity in cells and tissues of several placental mammals, including humans, mouse, ox, and cat. Li and colleagues (19) also claimed that the report of human PR enzyme activity produced by wavelengths >500 nm was unparalleled by any other photolyase; in fact, Chiang and Rupert (20) state that (for photoreactivation of the PtK-w mammalian cell line of the marsupial *Potorous tridactylus*) "maximum effectiveness occurs around 366 nm, but appreciable photoreactivation occurs at wavelengths as long as 546 nm." Thus, both this marsupial and the human enzyme can utilize light >500 nm in dimer photorepair.

By using optimized conditions, we find substantial levels of PR in WBCs. Can we predict *in situ* repair rates from *in vitro* data? Assays of supercoiled DNA (4.5 kbp) containing ≤ 1 CPD per DNA gave an initial reaction rate of ≈ 8500 CPDs per

min per CE, and in λ DNA (48.5 kbp) containing 13 CPDs per DNA, $\approx 30,000$ CPDs per min per CE, suggesting that the PR rate may depend on several factors, including intramolecular dimer frequency. To simulate CPD levels that WBCs encounter *in situ*, we used dimer frequencies <10 CPDs per Mb, obtaining a PR rate of ≈ 1000 per cell per min. In alfalfa seedlings, the PR and excision rates depend on the initial CPD frequency (43). Thus, the rate we measured may be a fraction of the maximum velocity, possibly obtained ($\approx 200,000$ CPDs per cell per min) by D'Ambrosio *et al.* (30, 31) in human skin, using an initial level of ≈ 80 CPDs per Mb and an intense PR lamp. At this rate, dimers induced by one minimal erythral dose (50–300 CPDs per Mb) would be repaired within minutes (30, 31). The level of PR activity in humans presumably differs among individuals. The roles of genotype or local or systemic skin UV exposure that may induce PR (44) or alter contributions of different repair paths to defense of skin against UV damage—including erythema, immune system effects, and skin cancer induction—are not known. It is clear that photorepair is present in human cells and carries out light-dependent repair of CPDs in extracts and in intact human cells.

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