Free Rad. Res., Vol. 33, pp. 369–381
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Immunochemical Quantitation of UV-induced Oxidative and Dimeric DNA Damage to Human Keratinocytes

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Accepted by Prof. B. Halliwell

(Received 10 August 1999; In revised form 28 February 2000)

There is growing evidence to suggest that solar radiation-induced, oxidative DNA damage may play an important role in skin carcinogenesis. Numerous methods have been developed to sensitively quantitate 8-oxo-2′deoxyguanosine (8-oxodG), a recognised biomarker of oxidative DNA damage. Immunoassays may represent a means by which the limitations of many techniques, principally derived from DNA extraction and sample workup, may be overcome. We report the evaluation of probes to thymine dimers and oxidative damage in UV-irradiated cells and the DNA derived therefrom. Thymine dimers were most readily recognised, irrespective of whether *in situ* in cells or in extracted DNA. However, using antibody-based detection the more subtle oxidative modifications required extraction and, in the case of 8-oxodG, denaturation of the DNA prior to successful recognition. In contrast, a recently described novel probe for 8-oxodG detection showed strong recognition in cells, although appearing unsuitable for use with extracted DNA. The probes were subsequently applied to examine the relative induction of lesions in cells following UV irradiation. Guanine-glyoxal lesions predominated over thymine dimers subsequent to UVB irradiation, whereas whilst oxidative lesions increased significantly following UVA irradiation, no induction of thymine dimers was seen. These data support the emerging importance of oxidative DNA damage in UV-induced carcinogenesis.

Keywords: ELISA, UV, 8-oxo-2′deoxyguanosine, DNA, antibodies, thymine dimers

Abbreviations: ELISA, enzyme-linked immunosorbant assay; UV, ultraviolet radiation; 8-oxodG, 8-oxo-2′deoxyguanosine; CPD, cyclobutane pyrimidine dimers; (6-4)PP, pyrimidine(6-4)pyrimidone photoproducts; HPLC-EC, high performance liquid chromatography with electrochemical detection; GC-MS, gas chromatography-mass spectrometry; FITC, fluoroscein isothiocyanate; ROS, reactive oxygen species

INTRODUCTION

The ultraviolet (UV) region of the solar spectrum has been speculated to be the primary aetiological factor in skin carcinogenesis.[1,2] Such a finding is supported by molecular epidemiology

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which has linked UV-induced DNA lesions with mutation[3,4] and ultimately skin cancer.[5] The formation of direct damage products such as cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidone photoproducts ((6-4)PP)[6] arises subsequent to the absorption of high energy, short wave UV by DNA.[7] Until recently, the importance of UV-induced DNA damage has focused on these direct products. However, the role of indirect, oxidative DNA damage, generated by UV-induced reactive oxygen species, is gaining importance due to the physio-chemical consequences of UVB- and UVA-irradiation,[8–10] the latter representing a major proportion of the solar UV spectrum. Indeed, oxidative damage to DNA causes mutagenesis[11] and is strongly implicated in carcinogenesis,[12] suggesting a possible role in skin cancer development. A principal product and certainly the most studied consequence of oxidative attack on DNA, is the lesion 8-oxo-2′deoxyguanosine (8-oxodG). The level of 8-oxodG in DNA is recognised as a biomarker of oxidative stress,[13] with its accumulation postulated to have a predictive significance for certain types of cancer.[14] On this basis, the sensitive assessment of 8-oxodG, in addition to dimeric lesions, following UV irradiation, is essential.

Principally, two techniques are used for 8-oxodG measurement. High performance liquid chromatography with electrochemical detection (HPLC-EC) is a sensitive approach to the detection of this lesion in enzymatically digested DNA,[15] with gas chromatography-mass spectrometry (GC-MS) being capable of detecting the base equivalent (8-oxoguanine, 8-oxoG) in formic acid digests of DNA.[16] However, both techniques have limitations, which include; a significant potential for artefactual oxidation of the DNA through sample workup (DNA extraction and hydrolysis), the requirement for appreciable amounts of DNA for analysis (μg amounts) and the inability to localise the damage *in situ*. Immunoassays have the potential to address these issues. Although several reports of polyclonal and monoclonal antibodies to 8-oxodG have been published[17,18] (reviewed by Herbert and Lunec[19]), their recognition of 8-oxodG in DNA has, for the most part, been either limited or impossible. Whilst reasons for this have been discussed elsewhere,[20] a clear exception does exist.[21] More recently a technique has been described, whereby avidin was utilised to detect 8-oxodG and potentially 8-oxodA, in DNA and cells exposed to various reactive oxygen species (ROS)-generating systems.[22,23] We report the evaluation of this and other immunochemical techniques for the detection and quantitation of oxidative and dimeric lesions, in various model systems following UV exposure.

MATERIALS AND METHODS

UV Sources and Exposure

A UVC lamp (Anderman Company, East Molesey, UK), with a maximum output (λ_{max}) at 254 nm, 10.5 μW/cm^2 (at 1 m), was the source of UVC irradiation. Broad-band UVA ($\lambda_{max} = 366$ nm) and UVB ($\lambda_{max} = 302$) irradiations were performed by Model-UVL-56 and Model-UVM-57, Chromatovue lamps from Knight Optical Technologies, Leatherhead, Surrey, UK. Spectroradiometric analysis (Department of Chemistry, University of Leicester) showed the UVA and UVC sources to be pure (320–392 nm and 254 nm, respectively). Although the UVB source showed contamination from UVA and UVC regions this was judged to be minimal and of no consequence to the study of DNA damage probe applicability. Measurements of irradiance were performed using sensors calibrated and provided by Knight Optical Technologies, appropriate for the waveband under study: UVA (MP-136 sensor), UVB (MP-131) and UVC (MP-125), in conjunction with the optical radiometer (MP100) (all from Knight Optical Technologies).

DNA Damage Probes

Antiserum 529, shown to recognise thymine dimers was raised against UVC-irradiated DNA.[24] The anti-ROS DNA antiserum (antiserum 532) was raised against DNA modified by ascorbate and hydrogen peroxide (H_2O_2) and fully characterised elsewhere.[25] Both peroxidase-labelled avidin and fluorescein isothiocyanate (FITC)-labelled avidin were from Sigma. The monoclonal anti-8-oxo-2′deoxyguanosine antibody (MAb N45.1) was obtained as part of a kit for measuring 8-oxodG in biological fluids (Genox Corp., Baltimore, USA).

Source of Human Keratinocytes

Human SV40 immortalised RHT keratinocytes (passage #101) were a gift from Professor Irene Leigh, Department of Dermatology, London Hospital Medical College, London, UK.

Keratinocyte Culture Conditions

Medium (RM+) (Life Technologies Ltd) consisted of DMEM containing L-glutamax (L-alanyl-L-glutamine), Ham's Nutrient Mixture F12 (Ham's F12), (Imperial Laboratories Ltd, Andover, UK) at a ratio of 3 : 1, 10% heat inactivated foetal calf serum (Sigma) penicillin-G-streptomycin (penicillin 100 U/mL, streptomycin 100 μg/mL from Flow Laboratories, Irvine, UK) and mitogens. Hank's Balanced Salt Solution (HBSS) was from Gibco.

Cells on Chamber Slides

Human RHT keratinocytes were grown to confluence in RM+ and routinely passaged every 3–4 days. At 50% confluence the RHT cells were seeded in Nunc Lab-Tek eight well chamber slides (Life Technologies Ltd) and left overnight until they were 60–80% confluent.

Pre-loading with α-Tocopherol

The putative protective effect of α-tocopherol (Sigma) against UVA-induced ROS[26] was assessed by the addition of a 200 μM solution of α-tocopherol (final concentration) to the medium of selected wells 24 h prior to treatment. Medium containing an equal volume of solvent (ethanol) was added to the control cultures.

UV Irradiation Prior to Immunocytostaining

The cells were irradiated on ice, following removal of medium, with 0 (control), 10, 20 and 30 kJ/m^2 UVA, using a long wave UV Blak Ray lamp or 0, 0.135, 0.27 and 1.08 kJ/m^2 using the UVB lamp. Control wells on the same slide were sham irradiated. Following treatment, the slides were removed and the cells fixed by the addition of 1 : 1 methanol/acetone and left at 4°C for at least 10 min. Following fixation the cells were allowed to air-dry prior to immunostaining.

Immunocytochemical Staining

Non-specific binding was blocked by incubation of the chamber slides with 2% normal goat serum (NGS) in 0.01 M PBS, following rehydration in PBS. Detection of thymine–thymine dimers, following UVB irradiation, was performed by the incubation of cells with antiserum 529 diluted 1/5000 in 2% NGS. A FITC-labelled goat anti-rabbit secondary antibody, diluted 1 in 200 in NGS, was used to localise binding of the primary. The potential for UVA-induced oxidative purine lesions to be detected using FITC-labelled avidin, as described by Struthers *et al.*,[23] was also investigated. The avidin–FITC conjugate (1 mg/mL) was applied at a 1 in 1000 dilution in 2% NGS in 0.01 M PBS overnight at 4°C. All chamber slides were washed in 0.01 M PBS prior to a final wash in deionised water prior to the slide being allowed to air-dry then mounted in Vectorshield (Vector Laboratories, Peterborough, UK).

Cell-based ELISA

Peripheral blood mononuclear cells (PBMC) were isolated from whole human blood using Histopaque 1077, as instructed by the manufacturer. Once isolated, the cells were diluted to a concentration of 1×10^5/mL and added to a 96 well Nunc Immuno Maxisorp, ELISA plate (Life Technologies Ltd., Paisley, Scotland). UV irradiation of the PBMC was performed over the range 0–1.17 kJ/m^2 (for UVC, UVB and UVA), prior to overnight fixation with 100% methanol at 4°C. Immunostaining was performed using antiserum 529, antiserum 532, avidin-peroxidase and MAb N45.1, essentially as described for the ELISA of extracted DNA. Washing of plates coated with cells was performed by hand, not with an automated platewasher.

Culture of Keratinocytes and Treatment Conditions for Quantitative Assessment

Cells were cultured as described above, seeded into eight Nunc Delta Petri dishes and grown to near confluence. Each dish represented a single treatment/dose and, following removal of medium and washing with HBSS, was then irradiated, on ice, with doses between either 0 and 216 kJ/m^2 (UVA) or 0 and 0.8 kJ/m^2 (UVB). Treatment of cells with H_2O_2 was performed by incubation in medium containing increasing concentrations of H_2O_2 (0–40 mM) for 1 h. Following treatment, the cells were lifted from the flask using TE, which was then neutralised with an equal volume of medium and pelleted at 400*g* for 10 min at 4°C. The supernatants were discarded and the DNA was then extracted using pronase E extraction.

Pronase E DNA Extraction

The cells were resuspended in 5–10 mL PBS and then pelleted at 700*g* for 15 min. The cells were then resuspended in 1.75 mL of ice-cold Buffer 1 (5 mM trisodium citrate, 20 mM NaCl pH 6.5). Two mL of Buffer 2 (20 mM Trizma base, 20 mM EDTA, 1.5% sarkosyl, pH 8.5) was then added with vigorous mixing, finally 250 µg of RNase A in RNase buffer A (50 mM Tris–HCl, 10 mM EDTA, 10 mM NaCl, pH 6.0) was added, prior to incubation at 37°C for 1 h. Two mg of Pronase E in 0.5 mL Buffer 1 was added and incubated overnight. Two mL of Buffer 3 (10 mM Tris–HCl, 10 mM EDTA, pH 7.5) was added along with 0.5 mL of 7.5 M ammonium acetate and mixed by inversion after each addition. Finally the DNA was precipitated by the addition of 18 mL ice-cold ethanol. The DNA was then spooled onto a pipette hook and washed in 70% and then 100% ethanol for 10 min each. Residual ethanol was removed by a stream of nitrogen. The dried DNA pellets were rehydrated in 1 mL of ultra-pure water and the DNA content determined by the absorbance at 260 nm.

Enzyme-linked Immunosorbant Assay

Double-stranded (or single-stranded) DNA (50 µg/mL), 50 µl/well, was bound to a 96 well Nunc Immuno Maxisorp, ELISA plate (Life Technologies Ltd., Paisley, Scotland) by incubation, in a humidified environment at 37°C for 1 h, after which the plate was washed 3 times with PBS. Free sites were then blocked by incubation with 150 µl/well 4% (w/v) dried skimmed milk (Tesco Stores Ltd., Cheshunt, UK) in PBS (4% milk/PBS) for 1 h at 37°C in a humidified environment and the wells were then washed with PBS. The test antiserum, 50 µl/well, was used diluted in 4% milk/PBS (antiserum 529 – 1/5000; antiserum 532 – 1/100; avidin–peroxidase – 1 mg/mL; MAb N45.1 – prepared by full reconstitution in buffer as advised by the manufacturer) and incubated for 1 h as described above. Following washing 3 times with PBS containing 0.05% (v/v) Tween 20 (Sigma), the appropriate secondary antibody was applied (50 µl/well). After the plate had been incubated as previously described, and washed with PBS/Tween 20, the substrate solution, orthophenylenediamine (0.5 mg/mL in 0.05 M

phosphate–citrate, pH 5.0 and containing 0.03% w/v sodium perborate) was added (50 µl/well) and incubated for 15 min at room temperature. The reaction was stopped using 25 µl/well 2 M H_2SO_4. The resulting absorbance was read at 492 nm, using a plate reader. The final data included correction for background values.

Avidin Assay

This was performed as described by Struthers *et al.*[23] Briefly, extracted DNA (100 µl per well at 100 µg/mL in water) was bound to a multi-screen-HV 96-well plate, by vacuum, following fixation with 4% paraformaldehyde in PBS. Blocking was performed by incubation with 1% gelatin (w/v in PBS). This was pulled through the membrane under vacuum, prior to washing 3 times with 0.01 M PBS. Avidin–peroxidase conjugate (1 mg/mL, diluted 1:500 in PBS) was added 50 µl/well and incubated for 1 h at 37°C. The membrane was then washed with PBS prior to addition of the substrate (orthophenylenediamine, 0.5 mg/mL in 0.05 M phosphate–citrate, pH 5.0, containing 0.03% w/v sodium perborate). 25 µl/well of 2 M sulphuric acid was used to stop the reaction and the absorbances of the solutions read at 492 nm on an Anthos 2001 plate reader.

RESULTS

Immunocytochemical Detection of Oxidative DNA Damage and Thymine Dimers in Keratinocytes Following UVB- or UVA-irradiation

UVB-irradiation

Figure 1A represents green fluorescence, positive staining for thymine dimers within the nuclei of human keratinocytes, post-irradiation with 1.08 kJ/m^2 UVB, using antiserum 529. This clearly contrasts with the absence of binding to the unirradiated control (Figure 1B), although

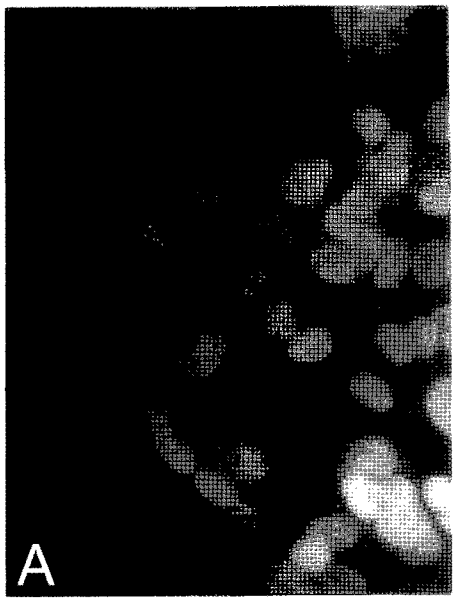


FIGURE 1A RHT keratinocytes were irradiated with 1.08 kJ/cm^2 UVB and processed as described in the Materials and Methods section prior to indirect immunofluorescent staining with anti-thymine dimer antibodies, utilising an FITC-labelled secondary. (×400 magnification.) Intense staining was noted using fluorescence microscopy, appearing localised in the nucleus. (See Color plate I at the end of this issue.)

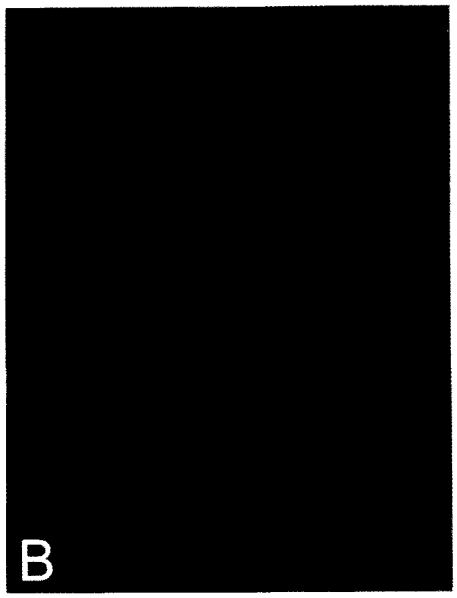
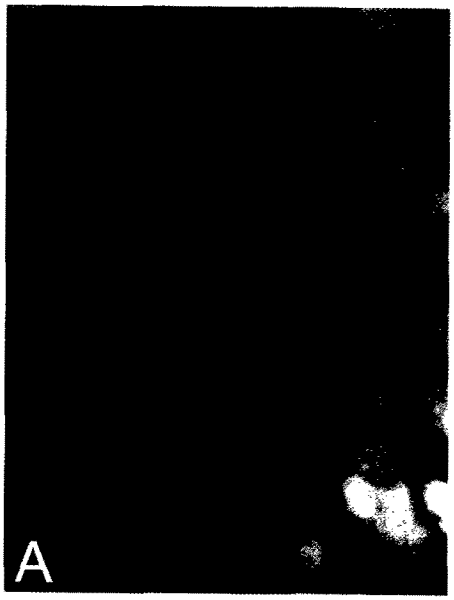
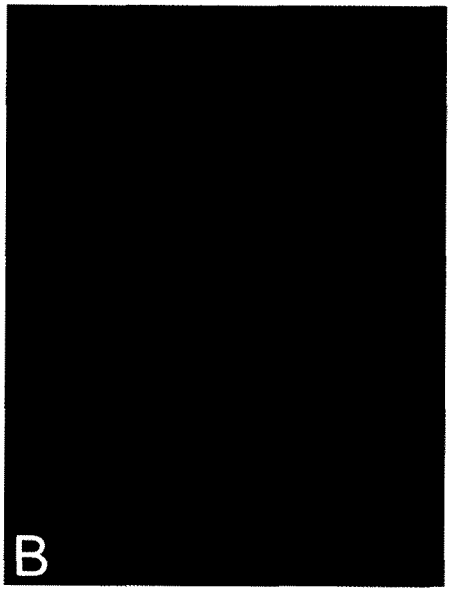


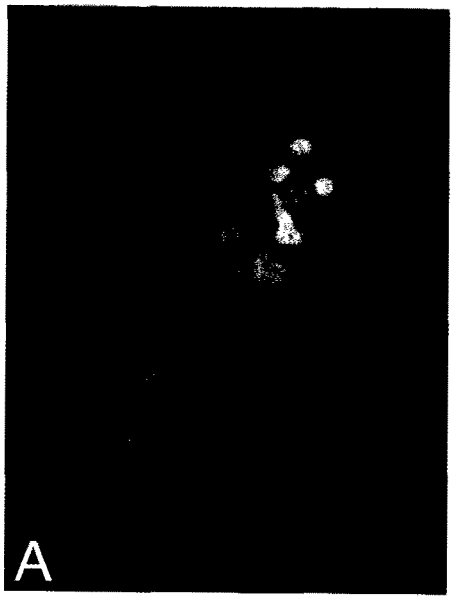
FIGURE 1B Unirradiated RHT keratinocytes processed as described for Figure 1A. (×400 magnification.) No staining of thymine dimers was apparent. Weak background cytoplasmic autofluorescence is visible. (See Color plate II at the end of this issue.)



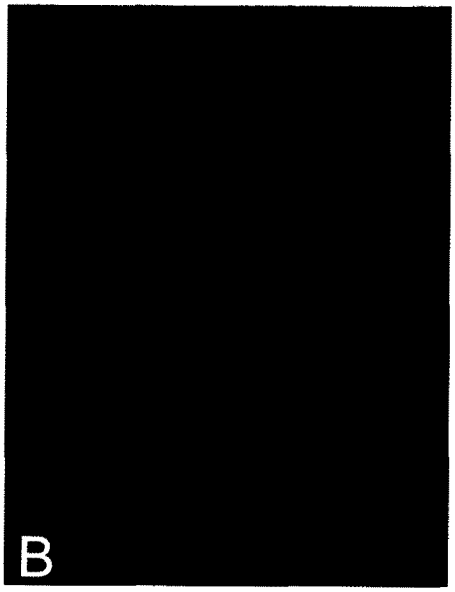
Color Plate I (see page 373, figure 1A) RHT keratinocytes were irradiated with 1.08 kJ/cm^2 UVB and processed as described in the Materials and Methods section prior to indirect immunofluorescent staining with anti-thymine dimer antibodies, utilising an FITC-labelled secondary. (×400 magnification.) Intense staining was noted using fluorescence microscopy, appearing localised in the nucleus.



Color Plate II (see page 373, figure 1B) Unirradiated RHT keratinocytes processed as described for Figure 1A. (×400 magnification.) No staining of thymine dimers was apparent. Weak background cytoplasmic autofluorescence is visible.



Color Plate III (see page 374, figure 2A) RHT keratinocytes were irradiated with 30 kJ/m^2 UVA and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) Intense staining, proposed to be oxidative DNA damage, was noted using fluorescence microscopy, localised in the nucleus.



Color Plate IV (see page 374, figure 2B) Unirradiated RHT keratinocytes processed as described for Figure 1A. (×200 magnification.) Some faint staining was apparent.

background, cytoplasmic autofluorescence was present. Background autofluorescence was seen in cells in the absence of primary or secondary antibody. Observation of the irradiated cells at ×400 magnification confirmed strong nuclear localisation of the staining. Binding to irradiated cells was most apparent at the dose of 1.08 kJ/m^2 although a dose-effect was not clear. No discernable binding of antiserum 529 was seen in cells irradiated with UVA (results identical to Figure 1B).

UVA-irradiation

Figure 2A represents the binding of the avidin–FITC conjugate to keratinocytes irradiated with 30 kJ/m^2 UVA and shows very intense, nuclear staining (×200 magnification), contrasting with Figure 2B which illustrates the negligible binding of avidin to unirradiated cells. The effect of H_2O_2 on keratinocytes was examined as model system for oxidative stress induction. Figure 3A illustrates the nuclear binding of avidin to H_2O_2 treated cells, which gave a similar intensity of staining to that seen with UVA. A lack of nuclear staining can clearly be seen in the cells treated with H_2O_2 after a 24 h pre-incubation with α-tocopherol (Figure 3B), suggesting the staining due to avidin was indeed caused by ROS. Cells, incubated with α-tocopherol, but not exposed to H_2O_2 appeared as in Figure 2B where there was no nuclear staining.

Quantitative Immunocytochemical Detection of DNA Damage in PBMC Following UV-irradiation

An aim of using immunochemical techniques for the quantitation of, particularly oxidative, DNA damage, is the prevention of artefact derived from extraction procedures. An ELISA-based assay for the quantitation of cellular antibody binding was therefore developed and used to test the applicability of our probes to the *in situ* detection of DNA damage in UV-irradiated cells.

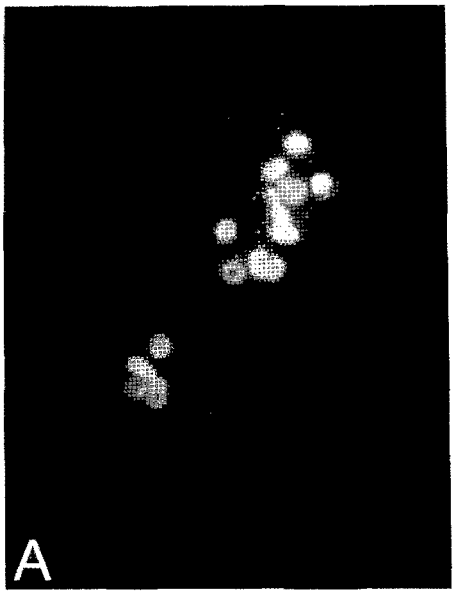


FIGURE 2A RHT keratinocytes were irradiated with 30 kJ/m^2 UVA and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) Intense staining, proposed to be oxidative DNA damage, was noted using fluorescence microscopy, localised in the nucleus. (See Color plate III at the end of this issue.)

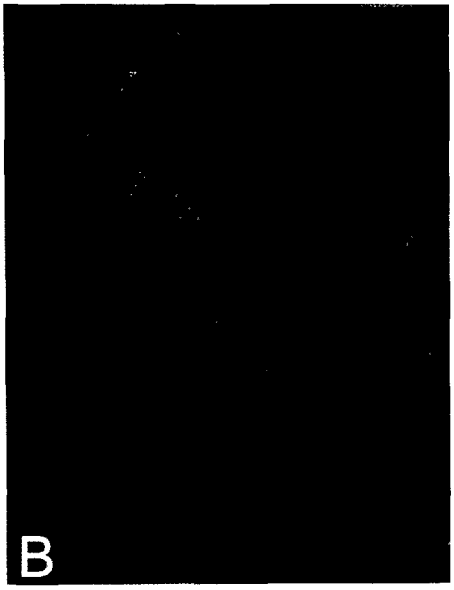
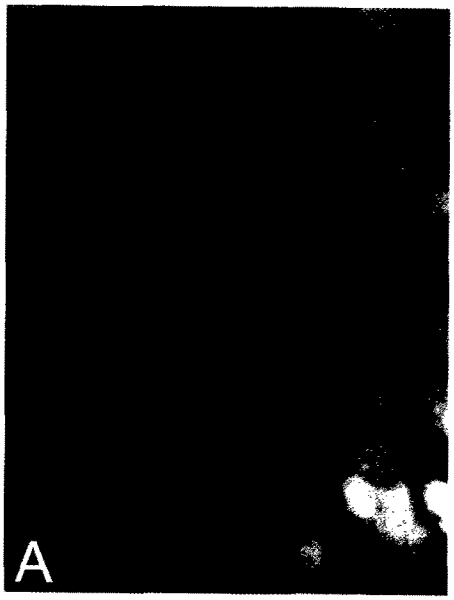
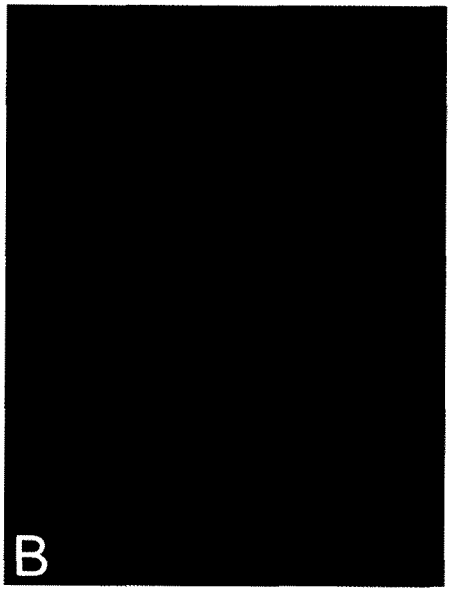


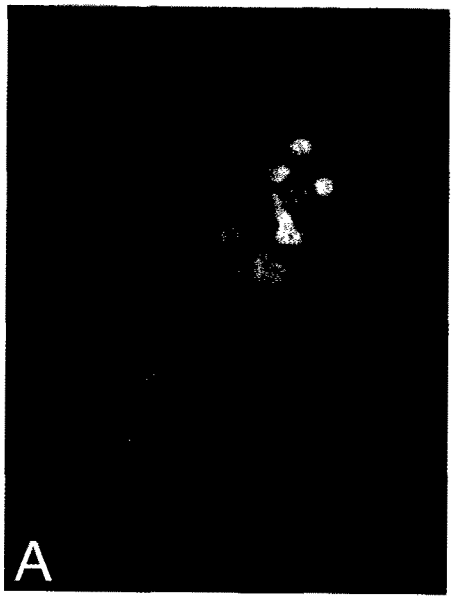
FIGURE 2B Unirradiated RHT keratinocytes processed as described for Figure 1A. (×200 magnification.) Some faint staining was apparent. (See Color plate IV at the end of this issue.)



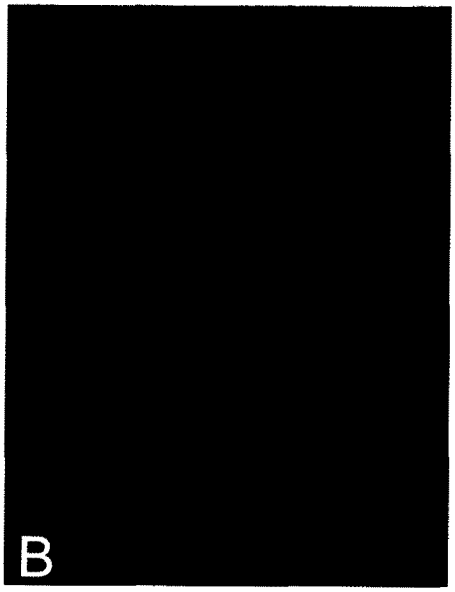
Color Plate I (see page 373, figure 1A) RHT keratinocytes were irradiated with 1.08 kJ/cm^2 UVB and processed as described in the Materials and Methods section prior to indirect immunofluorescent staining with anti-thymine dimer antibodies, utilising an FITC-labelled secondary. (×400 magnification.) Intense staining was noted using fluorescence microscopy, appearing localised in the nucleus.



Color Plate II (see page 373, figure 1B) Unirradiated RHT keratinocytes processed as described for Figure 1A. (×400 magnification.) No staining of thymine dimers was apparent. Weak background cytoplasmic autofluorescence is visible.



Color Plate III (see page 374, figure 2A) RHT keratinocytes were irradiated with 30 kJ/m^2 UVA and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) Intense staining, proposed to be oxidative DNA damage, was noted using fluorescence microscopy, localised in the nucleus.



Color Plate IV (see page 374, figure 2B) Unirradiated RHT keratinocytes processed as described for Figure 1A. (×200 magnification.) Some faint staining was apparent.

FIGURE 3A RHT keratinocytes were treated with 50 μM H_2O_2 for 1 h and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) Intense nuclear staining, proposed to be oxidative DNA damage, was noted using fluorescence microscopy. (B) RHT keratinocytes were treated with 50 μM H_2O_2 for 1 h following a 24 h pre-incubation with 200 μM α-tocopherol and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) No nuclear staining was noted. (See Color plate V at the end of this issue.)

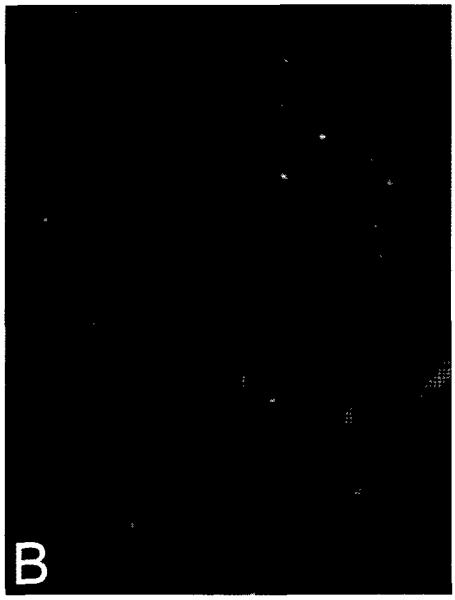


FIGURE 3B RHT keratinocytes were treated with 50 μM H_2O_2 for 1 h following a 24 h pre-incubation with 200 μM α-tocopherol and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) No nuclear staining was noted. (See Color plate VI at the end of this issue.)

Significant increases in antiserum 529 binding, in a dose-responsive manner, were seen to freshly isolated human lymphocytes irradiated with UVC ($p = 0.001$) and UVB ($p = 0.01$) (Figure 4). No changes in binding were noted following UVA irradiation over the equivalent doses. No changes in binding were seen with MAb N45.1, antiserum 532 and peroxidase-labelled avidin (data not shown).

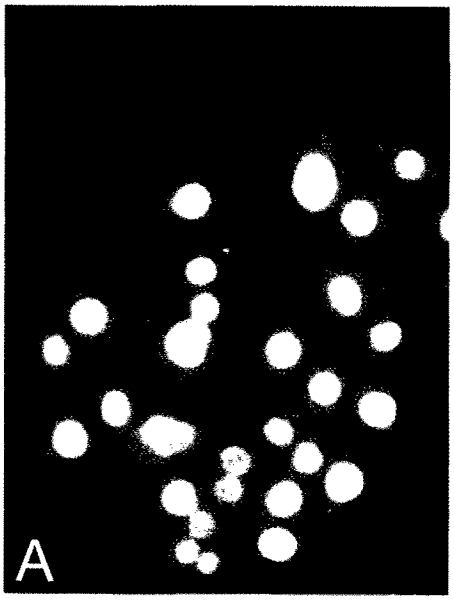
Quantitative Immunodetection of Oxidative Damage and CPD in Keratinocyte DNA Following UVB- or UVA-irradiation

UVB-irradiation

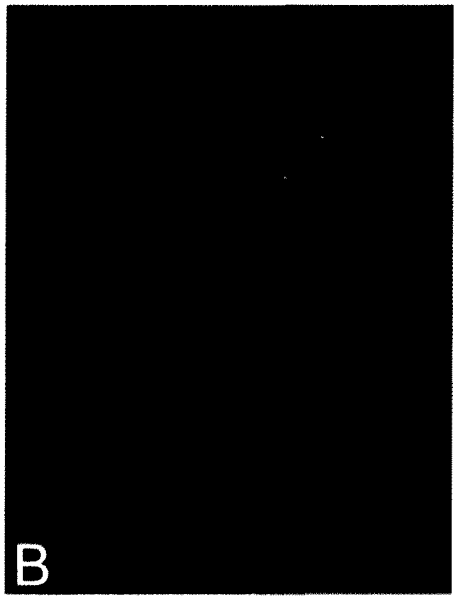
The ELISA binding of 3 DNA damage probes was examined in the extracted DNA of UVB-irradiated keratinocytes. Levels of binding for antiserum 532, raised to ascorbate/H_2O_2 modified DNA,[25] increased markedly over the unirradiated control, in a clear dose-responsive manner (Figure 5). An absorbance of 1.25 arbitrary absorbance units was seen, following a dose of 0.8 kJ/m^2 (approximately 2 MED for type II skin; $p < 0.01$). Binding was still apparent at the lowest dose of UV, 0.3 kJ/m^2 (less than 1 MED for type II skin; $p < 0.01$). A pattern of binding was seen with antiserum 529, which recognises thymine dimers,[24] such that no significant increase had occurred at 0.3 kJ/m^2, but binding increased, to reach statistical significance at subsequent doses. A maximum of 0.65 absorbance units was achieved at 0.8 kJ/m^2, at which point the rate of increase appeared to decline.

UVA-irradiation

The ELISA binding of 5 probes to DNA damage was examined in the DNA extracted from UVA-irradiated keratinocytes. Levels of binding for



Color Plate V (see page 375, figure 3A) RHT keratinocytes were treated with 50 μM H_2O_2 for 1 h and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) Intense nuclear staining, proposed to be oxidative DNA damage, was noted using fluorescence microscopy. (B) RHT keratinocytes were treated with 50 μM H_2O_2 for 1 h following a 24 h pre-incubation with 200 μM α-tocopherol and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) No nuclear staining was noted.



Color Plate VI (see page 375, figure 3B) RHT keratinocytes were treated with 50 μM H_2O_2 for 1 h following a 24 h pre-incubation with 200 μM α-tocopherol and processed as described in the Materials and Methods section prior to direct immunofluorescent staining with FITC-labelled avidin. (×200 magnification.) No nuclear staining was noted.

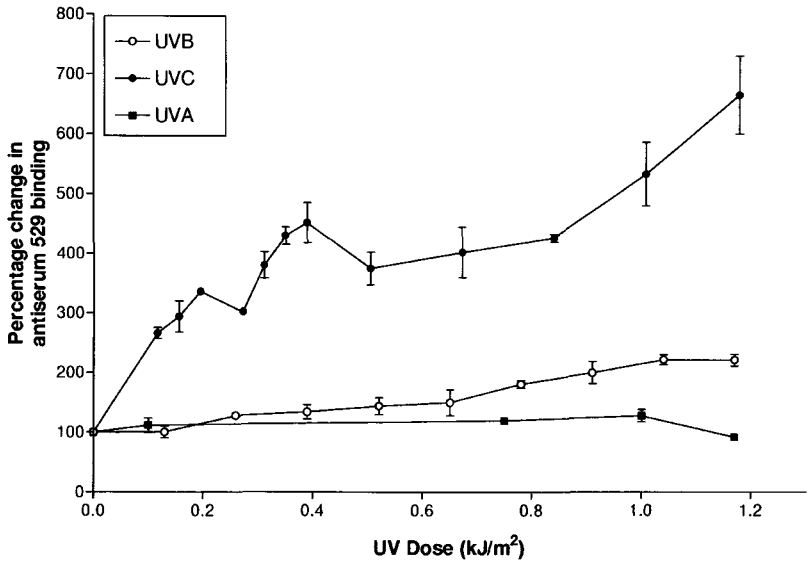


FIGURE 4 ELISA-based, immunocytochemical staining of UVC-, UVB- or UVA-irradiated isolated human peripheral blood mononuclear cells with antiserum 529, which recognises thymine dimers. Values represent the mean (SEM) of three determinations.

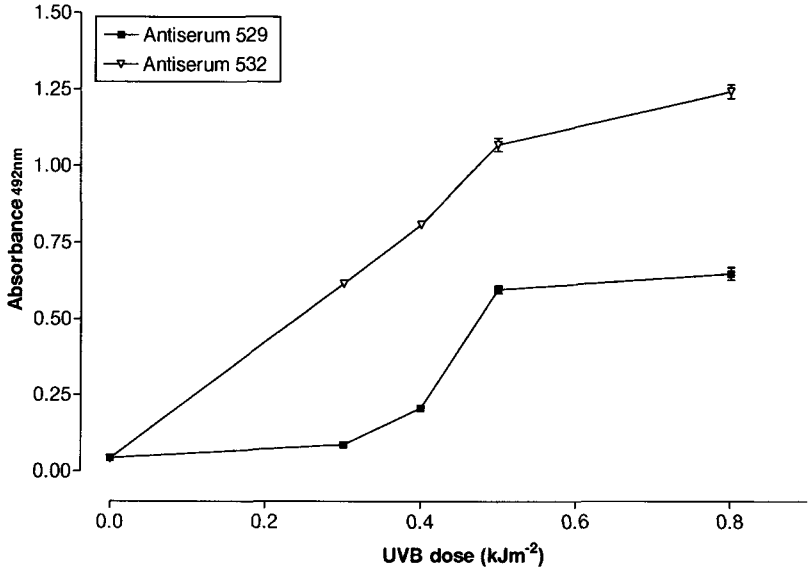


FIGURE 5 Direct ELISA binding of antisera to double-stranded DNA extracted from UVB irradiated human keratinocytes. Antiserum 529 has been shown to recognise thymine dimers, whereas antiserum 532 recognises reactive oxygen species-modified DNA. Values represent the means (SEM) of three determinations.

antiserum 532 increased markedly over the unirradiated control, in a clear dose-responsive manner (Figure 6). A maximal absorbance of 0.217 absorbance units was seen, following a dose of 144 kJ/m^2 (which is approximately 3/4 MED for type II skin). Binding of the antiserum subsequently appeared to decline with continued doses of radiation. Detection of damage was possible at the lowest dose of UV, 70 kJ/m^2 (less than 1/3 MED for type II skin) with this antiserum.

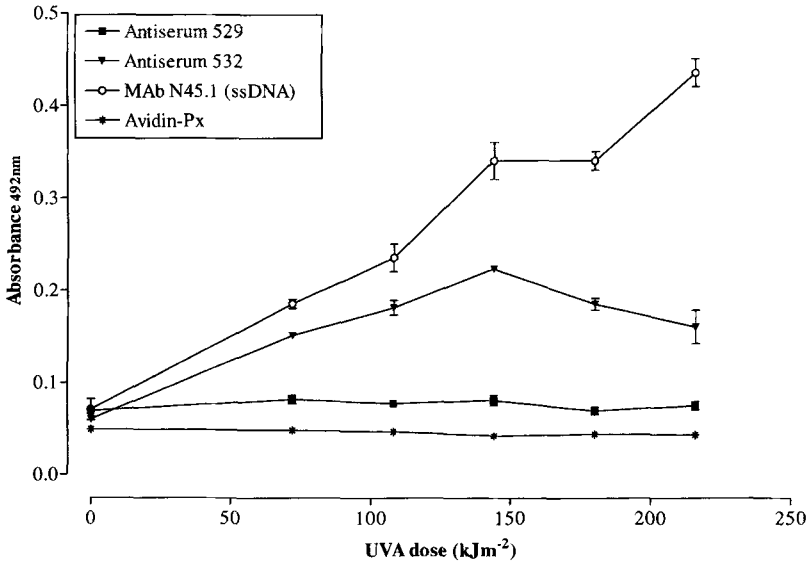


FIGURE 6 Direct ELISA binding of antisera to double-stranded DNA extracted from UVA irradiated keratinocytes, with the exception of MAb N45.1 binding which is to the same DNA which has been rendered single-stranded (ssDNA). Values represent the mean (SEM) of three determinations.

No increase was seen with antiserum 529, suggest that thymine dimers had not been formed. Increases were not seen either with MAb N45.1 or avidin. However, when the extracted DNA was denatured and probed with the anti-8-oxodG antibody, marked increases were seen (Figure 6). This was not the case, in our hands, for avidin, either in a standard ELISA format, or indeed the specific format reported previously.[23] Comparison of these results using extracted DNA with results derived from intact cells would suggest that the latter assay format is either unsuitable for N45.1 and antiserum 532, or extraction of DNA prior to ELISA analysis is required. Conversely, it would appear that whilst UVA- or H_2O_2-induced DNA damage in intact cells may be detected by fluorescently labelled avidin, detection of such lesions with peroxidase-labelled avidin cannot be performed upon DNA extracted from such treated cells.

Hydrogen Peroxide Treatment

The ELISA binding of 3 probes to DNA damage was examined in the DNA extracted from H_2O_2-treated keratinocytes. Levels of binding for MAb N45.1 increased significantly ($p = 0.02$) compared to the unirradiated control following incubation of cells with 5 mM H_2O_2, up to a maximal absorbance of 0.37 absorbance units, following incubation of cells with 40 mM H_2O_2 (Figure 7). Binding of antiserum 532 increased dose-responsively, in a manner similar to that seen with MAb N45.1 (Figure 7) achieving a maximal absorbance of 0.22 absorbance units at 40 mM H_2O_2. A significant increase in binding was also seen at the lowest dose, 5 mM H_2O_2 ($p = 0.002$). No increase in binding was seen with antiserum 529, suggesting that this probe does not detect oxidative modifications of DNA.

DISCUSSION

The shorter wavelengths of solar UV are potent inducers of dimeric lesions, such as CPD and (6-4)PP, with well documented consequences for the cell.[27] However, visible and near wavelengths predominate over the shorter

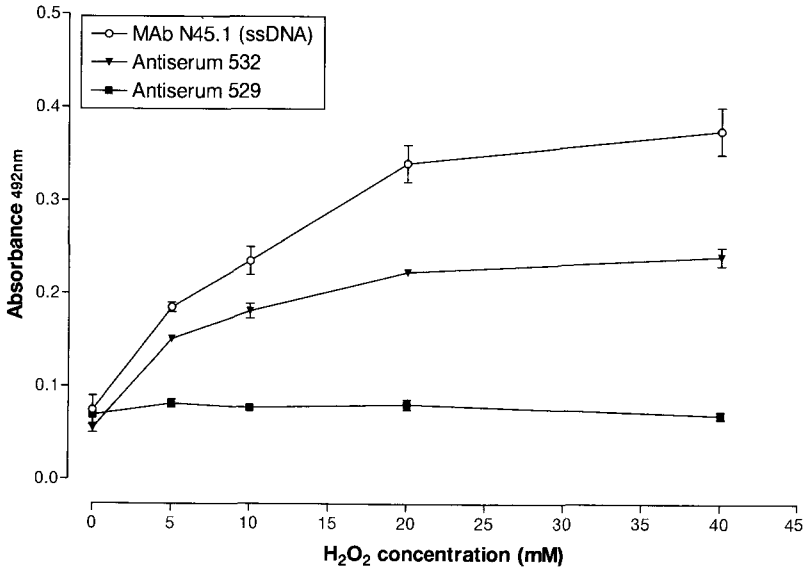


FIGURE 7 Direct ELISA binding of antisera to double-stranded DNA extracted from keratinocytes treated with increasing doses of H_2O_2 (0–40 mM), with the exception of MAb N45.1 binding which is to the same DNA which has been rendered single-stranded (ssDNA). Values represent the mean (SEM) of three determinations.

wavelengths in the solar spectrum at sea level.[28] These lower energy, longer wavelengths have been shown to possess cytotoxic,[29] genotoxic,[30] mutagenic and carcinogenic properties.[31] It is therefore speculated that ROS, a well established consequence of UV irradiation of cells,[32] are responsible for these biological effects. Such findings, coupled with a proposed role in UV-induced carcinogenesis, make vital the ability to assess ROS-induced, in addition to dimeric, damage to cellular biomolecules.

This study examined the applicability of various DNA damage probes to the detection of thymine dimers and oxidative damage in cells, following UV insult. Induction of thymine dimers in UVB irradiated human keratinocytes was unequivocally shown to occur at a dose of 1.08 kJ/m^2, using antiserum 529. Some perinuclear and cytoplasmic fluorescence was apparent in the unirradiated cells at the highest magnification. As this was non-nuclear it was assumed to be possible cytoplasmic constituents weakly autofluorescing. Fibroblasts are predominantly the human cultured cell type for the immunochemical demonstration of thymine CPD *in vivo*.[33–36] The demonstration here of pyrimidine dimer induction, in a keratinocyte cell line, using a unique sequence-specific antiserum is therefore novel and more relevant to a disease context *i.e.* non-melanoma skin cancer. No discernible binding, post UVA irradiation, was seen with anti-thymine dimer antiserum (antiserum 529) despite its sensitivity and a limit of detection of < 0.9 fmol dimer (unpublished results). The previous reports of pyrimidine dimer detection following UVA irradiation (λ_{max} 365 nm) of cells have utilised radioactivity[37] or the specific enzyme-based assay of Sutherland and Shih[38] to achieve detection. The apparent lack of binding seen here may be due largely to the low, but biologically relevant, doses of UVA used.

Also shown here is the novel application of avidin to the demonstration of oxidative damage in keratinocytes following UVA irradiation or treatment with H_2O_2. The differentiated neuronal cell line, IMR32, has previously been used to show the binding of avidin following

UVA irradiation,[23] although no dose information was given, merely an exposure time. Again, keratinocytes are a more biologically relevant cell system in which to investigate the effects of UV. The utilisation of avidin as an experimental probe of oxidative damage in keratinocytes has not been previously shown. Furthermore the demonstration of α-tocopherol to ameliorate such damage by H_2O_2, in keratinocytes supports the evidence for a radical-mediated mechanism of DNA damage. In contrast to the immunocytochemistry, we were unable to demonstrate the applicability of avidin to extracted DNA, either in a standard ELISA assay, or in the assay described specifically for its use.[23]

Dose-responsive changes were seen in antiserum 529 binding to PBMC irradiated either with UVB or UVC, although no changes were seen with UVA. This is in agreement with the above results, demonstrating the versatility of antiserum 529 in a number of assay formats, although the nature of the lesion detected may be more responsible for its ease of detection. In the same system, no changes were seen with any of the remaining probes (antiserum 532, MAb N45.1 and avidin-peroxidase). This finding suggests that extraction of DNA may be required prior to quantitative analysis by these probes.

Quantitative increases in binding to extracted DNA were seen both with antiserum 529 and antiserum 532, demonstrating the generation of cyclobutane thymine dimers and ROS-induced lesions in human keratinocytes, following UVB irradiation. Increases in binding with these two antisera were seen at doses as low as $0.3\,kJ/m^2$, although they clearly have the potential to detect at even lower doses. Demonstrated here is their applicability in an assay, which is more cost-effective than other techniques, such as GC-MS, detecting damage at biologically relevant doses. Whilst antibodies may not possess the absolute, singular specificity of GC-MS, with thorough characterisation and the appropriate assay development they do possess the potential to reliably and reproducibly detect and quantitate DNA damage. ELISA-based techniques require small amounts of DNA, for example, Wani *et al.*[39] detected damage in DNA samples as small as 10 ng, although higher levels allowed for greater sensitivity. DNA levels of 2.5 µg were used in the assay reported here which, although high compared to Wani *et al.*,[39] are much lower than those required by GC-MS or HPLC (100 µg DNA per sample[40,41]). Our aim for immunochemical assays is to perform quantitative assessments of damage without the need for extraction, as such manipulations of DNA is likely to result in the artefactual production of oxidative damage.[42,43] On this basis, we developed a cell-based ELISA method to demonstrate dose-responsive damage induction. However, at present this technique remains suitable for antiserum 529 only.

The characterisation of the polyclonal antiserum 532 is reported by Mistry *et al.*[25] and describes the antigen as a product of metal-mediated, Fenton reaction, guanine-glyoxal. Clearly levels of this product increase appreciably following UVA and may have important consequences for the cell. A significant increase in binding, to single-stranded DNA from UVA irradiated cells, was seen with MAb N45.1. This would indicate that, in conjunction with the results obtained by antiserum 532, UVA irradiation of cells gives rise to immunochemically detectable levels of oxidative DNA lesions. Furthermore, this suggests 8-oxodG to be one of the major modified DNA products, following UVA irradiation of cells, predominating over CPD induction.

The quantitative induction of H_2O_2-induced DNA damage to keratinocytes was demonstrated immunochemically using antibodies to 8-oxodG and guanine-glyoxal adducts. The lack of recognition of such changes by antiserum 529 excluded oxidative DNA damage as a possible antigen whilst the specificity for UV-induced thymine dimers was supported.

Taken together, these results would suggest that whilst no detectable increases in CPD were seen, ROS-induced damage, and specifically

8-oxodG was formed following UVA irradiation of keratinocytes at biologically relevant doses. Furthermore, we have shown the applicability of several DNA damage probes to the investigation of UV-induced cellular changes, highlighting their importance as tools for elucidating the induction and consequences of DNA lesions.

Acknowledgements

The authors gratefully acknowledge financial assistance from the UK Ministry of Agriculture Fisheries and Food along with the Arthritis and Rheumatism Council. The assistance of Dr. Andy Ellis, Department of Chemistry, University of Leicester is also acknowledged.

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