

**IMMUNOCHEMICAL INVESTIGATION OF UV-INDUCED DNA
DAMAGE**

**by
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ABSTRACT

To understand the role of UV-induced DNA lesions in biological processes such as mutagenesis and carcinogenesis, the quantitation of DNA damage in cells is essential. In this thesis, immunochemical and chromatographic techniques were employed to assess the relative contribution of direct/indirect mechanisms to DNA damage in a number of model systems by measuring lesions associated with each mechanism. Efforts to produce polyclonal and monoclonal antibodies to oxidative DNA damage suggested that profiles of antigenicity and immunogenicity arise dependent on the method of reactive oxygen species (ROS) production. Furthermore, an antiserum, raised against DNA damaged by an established ROS-generating system, was shown to recognise direct UV damage and was characterised with a previously developed antiserum to UV-damaged DNA. Competitive enzyme-linked immunosorbent assay (ELISA) demonstrated the main epitopes recognised to be dimerised adjacent thymines, suggested to be cyclobutadithymine, in conjunction with a flanking sequence, specific to each antiserum. A selective and sensitive, novel capillary gas chromatography-mass spectrometry (GC-MS) assay was developed for the quantitation of *cis-syn* cyclobutadithymine. Employing a dimer-containing DNA standard, calibrated by GC-MS, the limits of detection for each antiserum were found to be 0.9 and 1.9 fmol, compared with 20-50 fmol for GC-MS. Such results confirm these antisera to be a sensitive approach for determining levels of UV damage in DNA, supported by their successful demonstration of cyclobutadithymine formation in naked DNA following UVC-irradiation. No discernible binding was seen to UVA-irradiated DNA, which was confirmed immunohistochemically in fixed and embedded sections of human skin. The qualitative induction of, dimers following UVB-, and ROS lesions, following UVA-irradiation of cultured human keratinocytes, was shown using an antiserum to cyclobutadithymine and a novel affinity technique for oxidised purines. Application of an antibody to 8-oxodG and a novel antiserum to ROS-modified DNA, allowed quantitative ELISA measurements of dimeric and oxidative DNA changes to be made, following irradiation of keratinocytes. It was concluded that oxidative DNA damage, along with cyclobutadithymine, represents an appreciable contribution to total damage following UVC irradiation and that oxidative processes, with little contribution by direct damage, represent the major mechanism responsible for UVA-induced DNA damage.

*To my parents, Chris and Sonja,
and to Emily.*

For their love and support - the greatest encouragement, from the beginning.

“The first step to knowledge is to know we’re ignorant.”

- Lord David Cecil

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ABBREVIATIONS

| | |
|-------------------|---|
| (6-4)PP | Pyrimidine-pyrimidone (6-4)photoproducts |
| 8-oxoA | 8-oxoadenine |
| 8-oxodG | 8-oxo-2' deoxyguanosine |
| 8-oxoG | 8-oxoguanine |
| 8-oxoGR | 8-oxoguanosine |
| AMCA | 7-amino-4 methyl coumarin-3-acetic acid |
| AP | Apurinic-apyrimidinic site |
| ASH | Ascorbate/hydrogen peroxide |
| BCC | Basal cell carcinoma |
| BSTFA | N,O-bis(trimethylsilyl)trifluoroacetamide |
| CAT | Catalase |
| CC | Cytosine-cytosine |
| CE | Capillary electrophoresis |
| CPD | Cyclobutane pyrimidine dimers |
| CT | Cytosine-thymine |
| DAD | Diode array detection |
| dG | Deoxyguanosine |
| dGMP | 2' deoxyguanosine 5' monophosphate |
| DMEM | Dulbecco's modified eagles medium |
| DNA | Deoxyribonucleic acid |
| ECD | Electrochemical detection |
| EDTA | Ethylene diamine tetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| FACS | Fluorescence activated cell sorting |
| FADH ₂ | Flavin adenine dinucleotide (reduced) |
| FapyAde | 4,6-diamino-5-formamidopyrimidine |
| FapyGua | 2,6 diamine-4-hydroxy-5-formamidopyrimidine |
| FCS | Foetal calf serum |
| FEH | Fe ²⁺ /H ₂ O ₂ |
| FITC | Fluorescein isothiocyanate |
| fpg protein | Formamidopyrimidine-DNA glycosylase |
| G ^{•+} | Guanine radical cation |
| GC-MS | Gas chromatography-mass spectrometry |

| | |
|------------------------|---|
| GSH | Glutathione |
| HBSS | Hanks balanced salt solution |
| HPLC | High performance liquid chromatography |
| IC₅₀ | Concentration of competitor giving 50% inhibition |
| MB | Methylene blue |
| MED | Minimal erythematol dose |
| MM | Malignant melanoma |
| MPG | Mammalian N-methylpurine-DNA-glycosylase |
| MSC | Melanoma skin cancer |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| NER | Nucleotide excision repair |
| NGS | Normal goat serum |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PEG | Polyethylene glycol |
| PMNS | Polymorphonuclear leukocytes |
| RA | Rheumatoid arthritis |
| RIA | Radioimmunoassay |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SCC | Squamous cell carcinoma |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| Sens | Sensitiser |
| SIM | Selected ion monitoring |
| SLE | Systemic lupus erythematosus |
| SOD | Superoxide dismutase |
| SP | Spore photoproduct |
| SSB | Single-stranded DNA binding protein |
| T⁻ | Thymine radical anion |
| T4 endo V | T4 endonuclease V |
| TC | Thymine-cytosine |

| | |
|--------|-----------------------------------|
| TG | Thymine glycol |
| TT | Thymine-thymine |
| UV | Ultraviolet |
| UV DNA | Ultraviolet modified DNA |
| UVA | Ultraviolet A (315-400nm) |
| UVB | Ultraviolet B (280-315nm) |
| UVC | Ultraviolet C (100-280nm) |
| UVH | UVC/H ₂ O ₂ |
| XP | Xeroderma pigmentosum |

DESCRIPTION OF IMMUNOCHEMICAL AND AFFINITY PROBES USED

| Probe | Description |
|---------------------|---|
| Ab529 | A polyclonal antiserum, raised against DNA irradiated with UVC and UVA combined. Characterised as recognising cyclobutane thymine dimers in conjunction with either a 3' or a 5' pyrimidine, specifically, TTT and TTC (Herbert <i>et al.</i> , 1994). |
| X18 | also known as UVC/H ₂ O ₂ polyclonal antiserum. Raised against DNA treated with UVC and H ₂ O ₂ . Characterised as recognising cyclobutane thymine dimers in conjunction with either a 3' or 5' cytosine, favouring the sequence containing a 5' cytosine (Chapter 5 and Cooke <i>et al.</i> , see appendix III). |
| Ab532 | also known as anti-ROS DNA antiserum. A polyclonal antiserum, raised against DNA treated with ascorbate and hydrogen peroxide. Currently undergoing characterisation, but appears to recognise ROS modifications of DNA currently postulated to be glyoxal-DNA adducts (Mistry <i>et al.</i> in preparation). |
| Avidin | Reported to detect 8-oxoguanine, 8-oxodeoxyguanosine, 8-oxoadenine and 8-oxodeoxyadenosine (Thomas, 1996 - Patent application; Clarke <i>et al.</i> , in preparation). |
| α -ssDNA Ab | Commercial monoclonal antibody to single-stranded DNA. |
| α -8-oxodGAb | Commercial monoclonal antibody to 8-oxodeoxyguanosine from Genox Corp. |

CHAPTER ONE

INTRODUCTION

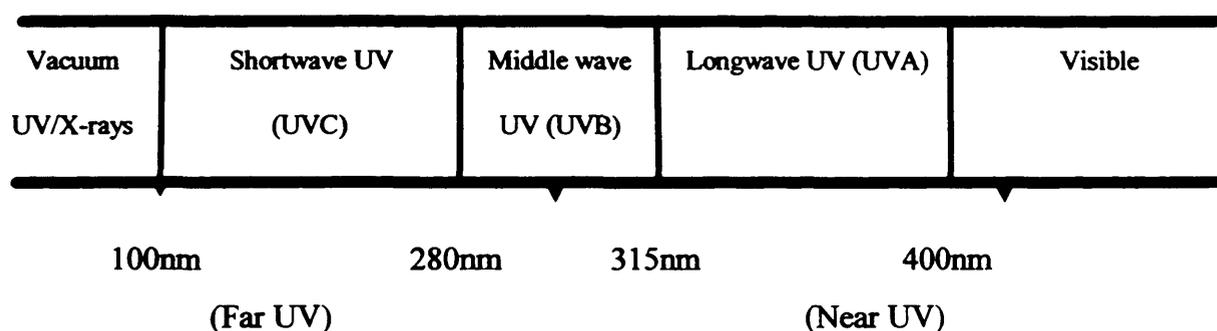
“Generally the theories we believe we call facts, and the facts we disbelieve we call theories.”

- Felix Cohen

1.0 ULTRAVIOLET RADIATION

Ultraviolet radiation (UV) is a part of the electromagnetic spectrum between X-rays and visible light (Figure 1.1). The photobiological designations of the Commission Internationale de l'Eclairage (CIE, International Commission on Illumination) have been adopted here to define the approximate spectral regions in which most relevant biological interactions occur.

Figure. 1.1. UV region of the electromagnetic spectrum, illustrating approximate wavebands for UVA, B and C.



It is important to note that these wave-band designations do not confer fine dividing lines below which an effect is present and above which the effect does not occur. In humans, the skin is the organ of the body with greatest exposure to UV and it is here that the majority of the deleterious effects of UV are seen.

1.1 THE SKIN

The skin is principally divided into three main areas; epidermis, reticular dermis and a layer of subcutaneous fat. The epidermis is then further divided into four areas (Thody and Friedmann, 1986) (Figure 1.2) and it is this region which is primarily of interest in the context of ultraviolet light.

There is considerable variation in the thickness of the epidermis and its overlying corneal layer, depending on the body site (Valanvanidis, 1994). The superficial epidermal cells, or keratinocytes, are continually exfoliated from the surface and replaced by cells which arise from mitotic activity in the basal layer. Generation of cells from the basal layer successively

displaces the cells above. As they move upwards, keratin accumulates in their interior until it largely replaces all the metabolically active cytoplasm. The cell dies and the nucleus, along with other organelles, disappears. The granular cell layer marks the boundary between the inner metabolically active strata and the outermost dead layer. The cell is finally shed (Uitto *et al.*, 1986). This sequence of changes is referred to as cytomorphosis of the keratinocyte, a process similar, in many ways, to apoptosis (McCall and Cohen, 1991).

1.1.1 CYTOMORPHOSIS

The first event which occurs in the process of keratinocyte differentiation is the loss of ability to proliferate, at a point just before or as the cell leaves the basal layer. Biosynthesis (RNA and proteins) continues throughout the spinous layer with the cell accumulating mass and enlarging (Green, 1979). On reaching the granular layer, protein and RNA synthesis arrest, cells become abnormally permeable, allowing entrance of Ca^{2+} and perhaps $\text{TGF}\beta$, which has been shown to be the primary factor that induces terminal differentiation of normal epithelial cells in culture (Green, 1979). The rise in intracellular Ca^{2+} activates certain endonucleases, producing DNA fragmentation (McCall and Cohen, 1991), as well as cross-linking transglutaminases, of which involucrin appears to be a substrate, producing a protein envelope (Simon and Green, 1988).

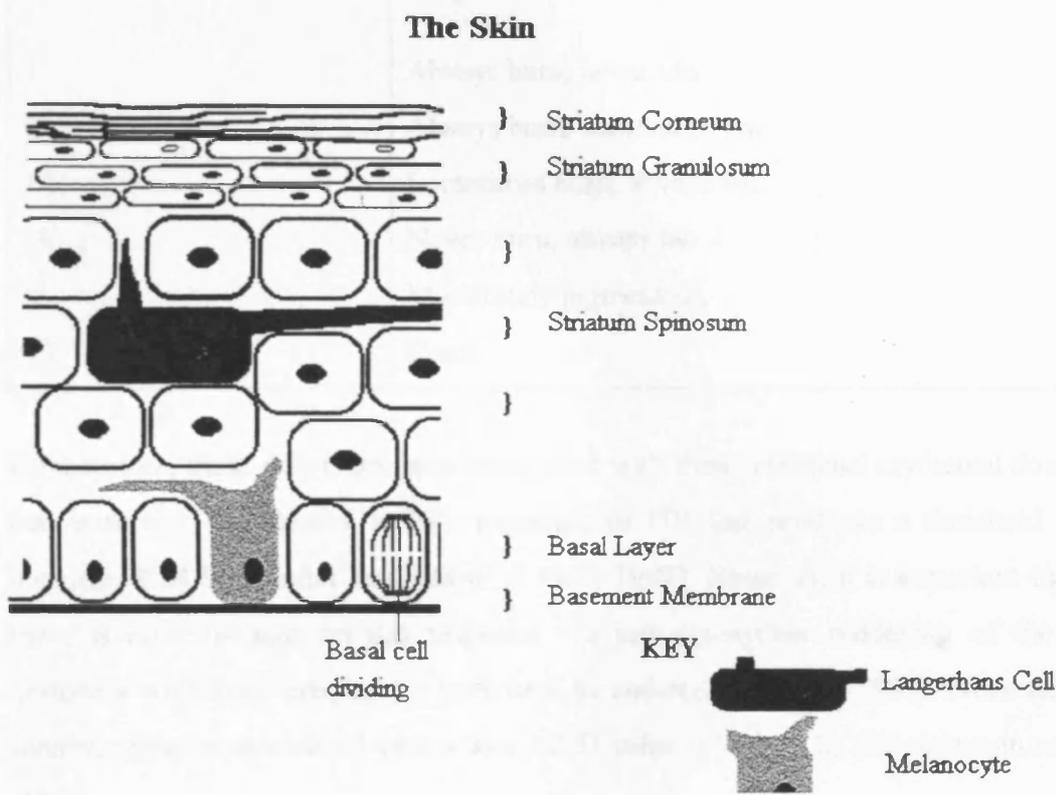


Figure 1.2. A schematic drawing of the epidermis illustrating the different layers of and the major cell types.

1.1.2 EFFECTS OF UV ON SKIN

Upon UV irradiation of the skin, individuals appear to respond in different ways, some being more liable to develop malignancy than others (Frain-Bell, 1985). This response has been broadly attributed to a classification of skin type (Table 1.1).

Table 1.1. Classification of skin types.

| Skin type | Characteristics |
|-----------|----------------------------|
| I | Always burn, never tan |
| II | Always burn, sometimes tan |
| III | Sometimes burn, always tan |
| IV | Never burn, always tan |
| V | Moderately pigmented |
| VI | Black |

Furthermore, these skin types have associated with them a minimal erythemal dose (MED), that is to say, the “lowest radiant exposure of UV that produces a threshold erythema response 8-24 hours after irradiation” (IARC, 1992). However, it is important to note that there is no consensus on this response - a just perceptible reddening of the skin and erythema with sharp margins are both used as end-points (IARC, 1992). Nonetheless, poor tanning ability is associated with a low MED value. (Table 1.2) (Hönigsmann and Thody, 1986).

Table 1.2. Comparison of MED for UVA and UVB with skin type.

| Skin type | UVA (broad spectrum) | UVB (broad spectrum) |
|-----------|--------------------------|--------------------------|
| I | 10-30 Jcm ⁻² | 10-30 mJcm ⁻² |
| II | 20-40 Jcm ⁻² | 20-40 mJcm ⁻² |
| III | 30-60 Jcm ⁻² | 20-50 mJcm ⁻² |
| IV | 40-100 Jcm ⁻² | 40-60 mJcm ⁻² |

1.1.3 UV AND SKIN CANCER

The UV region of the solar spectrum has been speculated as being an aetiological factor, if not the primary cause of numerous dermatological disorders which include skin ageing (Farr

and Friedmann, 1986), solar keratosis (Seywright, 1992), malignant melanoma (MM - Setlow *et al.*, 1993), basal cell carcinoma (BCC - Hunter *et al.*, 1990) and squamous cell carcinoma (SCC - Kricker *et al.*, 1994).

1.1.3.1 CARCINOGENESIS

It is clear that mammals would not be viable organisms were a single mutation in a particular gene sufficient to convert a healthy cell to a tumour cell (Alberts *et al.*, 1989). Multiple factors are involved, a theory supported by studies of tumour progression, where a slight defect in a cell's behaviour gradually leads to a tumour cell. This gave rise to the theory of multiple stages of neoplasia, an example of which is demonstrated by the process of chemical-induced skin carcinogenesis. It was shown that, whilst chronic, topical administration of a chemical known to be a skin carcinogen (e.g. benzpyrene) failed to produce tumours, during the lifespan of an animal; repeated application of a non-carcinogenic, irritant (in this case croton oil) following a single dose of carcinogen, produced benign tumours in a few weeks (Taussig, 1989). The conclusion was that the croton oil promoted a process initiated by the benzpyrene. Croton oil is highly irritating to the skin and causes hyperplasia of the skin cells. This, it is believed, makes it an important co-carcinogen. Two stages of events were hypothesised to have occurred, initiation and promotion, this is a rather simplified theory of carcinogenesis and a third stage has been added to explain the advance from benign lesions to malignant, namely, progression (Taussig, 1989).

1.1.3.2 MALIGNANT MELANOMA (MM)

MM is a life-threatening tumour, whose incidence, often in young and middle aged adults, is increasing. UV is an important aetiological factor and it is short, sharp bursts of UV, with associated sunburn at an early age, which are thought to be important (Seywright, 1992). Shown to be most at risk are skin type I individuals, who possess a number of pre-existing naevi (moles). The origins of most melanomas lie with the basal layer of the epidermis, where spread may occur in all directions (radial growth phase), including through into the dermis (vertical growth phase) where the opportunity to metastasise exists (Seywright, 1992). It is important to note that there are four clinicopathological subtypes of melanocytic tumour, not all of which are malignant (Seywright, 1992).

1.1.3.3 BASAL CELL CARCINOMA (BCC)

BCC is the most frequent malignant skin tumour, occurring mostly in the elderly, presenting at almost any sites, except palms and soles of feet. UV is again an important aetiological factor for this type of skin cancer and, in contrast to MM, the greater the cumulative exposure, the higher the risk of developing either BCC or squamous cell carcinomas (Seywright, 1992). The typical BCC begins as a small nodule, the centre of which breaks down and ulcerates, developing a characteristic “raised, rolled translucent or pearly border” (Seywright, 1992). The BCC appears histologically, as islands of cells, bordered by basal-like cells arranged as a well-defined columnar layer. The tumour appears to arise, in many cases, from the basal layer of the epidermis, invading the underlying dermis (Seywright, 1992).

1.1.3.4 SQUAMOUS CELL CARCINOMA (SCC)

SCC is the second most common malignant skin tumour. As with BCC, UV is an important aetiological factor, in particular with respect to prolonged exposure to excessive sun. The histological appearance of these tumours are of well-differentiated cells with abundant keratin formation, plus an apparent multicentric origin (Seywright, 1992). Generally, these tumours possess a good prognosis with a metastatic rate of 3%.

Associated with this tumour is a premalignant epidermal lesion, known as actinic (solar, senile) keratosis (AK), whose changes range from mild dysplasia to carcinoma *in situ* (Seywright, 1992). These lesions display much of the signs of sun damage, namely loss of elasticity, atrophy, hyperkeratosis and irregular pigmentation. There are studies linking the development of SCC from this lesion, suggesting that anywhere from 1-20% of individuals with AK will develop SCC (Seywright, 1992). Hori *et al.* (1992) proposed that repair deficiencies in AK may allow persistence of damage within cells, introducing AK as a process in multistage carcinogenesis.

1.2 FREE RADICALS

Free radicals are defined as any chemical moiety capable of existing with a lone electron in an orbital *i.e.* an unpaired electron (denoted as \cdot). It is this facet which makes free radicals more reactive than non-radicals, for orbital pairing of electrons increases stability. In order to share the same orbital the electrons must adopt equal but opposite spin ($\uparrow\downarrow$). Molecular oxygen possesses two unpaired electrons in two different orbitals [$(\uparrow)(\uparrow)$].

1.2.1 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are oxygen containing molecules which may be radical, for example, superoxide ($\cdot\text{O}_2^-$) and hydroxyl radicals ($\cdot\text{OH}$) or non-radical, for example hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$).

1.3 OXIDATIVE STRESS

Oxidative stress has been described as a “disturbance in the prooxidant-antioxidant balance in favour of the former” (Sies, 1991). Aerobic organisms are subjected to a variety of situations, including UV, in which oxidative stress is generated (Table 1.3).

Table 1.3. Examples of situations in which cellular oxidative stress is seen (Cerruti, 1985; Fuchs, 1992, 1993; Loft *et al.* 1995).

| |
|---|
| Hyperbaric oxygen |
| Ionising radiation |
| Modulators of the cytochrome P-450 electron transport chain |
| peroxisome proliferators |
| inhibitors of antioxidant defence system |
| membrane-active agents |
| ultraviolet light |
| ischaemia-reperfusion |
| metabolism of endogenous and xenobiotic substances |
| disruption of mitochondrial function |

An inevitable consequence of this oxidative stress is the production of reactive oxygen species including some oxygen-centred free radicals such as the superoxide radical and the hydroxyl radical. These damaging molecules may interact with cellular components leading

to potentially serious repercussions for the cell as a whole. The ability of ROS to structurally modify cellular components, activate cytoplasmic/nuclear signal transduction pathways, modulate genes and protein expression and alter DNA polymerase activity (Wiseman *et al.* 1995) has led to the implication of their involvement in a variety of pathological (Blake and Winyard, 1995) and degenerative (Halliwell, 1989; Wolff *et al.*, 1986) conditions including inflammation (Frenkel *et al.*, 1993), carcinogenesis (Kensler and Taffe, 1986; Frenkel, 1992), ageing (Bunker, 1992; Barnett and King, 1995) and autoimmunity (Lunec *et al.*, 1994). An important target for ROS within the cell is DNA, resulting in a broad range of products, including base and sugar modifications, covalent crosslinks with proteins and single- and double-strand breaks (Ward *et al.*, 1987). The ultraviolet radiation (UV) in sunlight is an important environmental insult and also induces oxidative stress (skin disorders in which ROS are suspected of playing a role are shown in Table 1.4).

Table 1.4. Some dermatological disorders that may involve ROS (Fuch, 1993)

| |
|--|
| Autoimmune symptoms (e.g. "butterfly rash" of SLE) |
| Skin cancer |
| Frostbite |
| Skin transplantation - reoxygenation injury |
| Skin wrinkling due to cigarette smoke |
| Photosensitising drugs/products |
| Contact dermatitis |
| Skin inflammation |
| Porphyria |
| Psoriasis |
| Thermal injury |
| UV-induced skin damage |
| Skin damage due to ionising radiation |

1.4 UV-MEDIATED DNA DAMAGE

Absorption by stratospheric ozone prevents the UVC range reaching the Earth's surface, allowing only UVB and UVA through to interact with terrestrial systems. DNA directly absorbs high energy, middlewave UV light leading to direct damage; however, lower energy, longwave UV light is poorly absorbed. It has been suggested that the photophysical processes following the absorption of a long wavelength photon by DNA may vary from those after the absorption of a short wavelength photon (Sutherland and Griffin, 1981). Therefore the mechanism of DNA damage by long wave, UVA is indirect and must involve some intermediate, such as ROS or free radicals.

1.4.1 DIRECT UV-INDUCED DNA DAMAGE

Predominantly the skin is affected by UV exposure, detrimental effects of which include those described in 1.1.3 (page 5), with the incidence of BCC, SCC and malignant melanoma showing an increase amongst the general population (Cascinelli and Marchesini, 1989; MacKie, 1993; Arlett *et al.* 1993; Potten *et al.*, 1993). Epidemiological and clinical studies have implicated UV radiation in sunlight as the primary aetiological agent in human skin cancer (Nataraj *et al.*, 1995). Molecular epidemiology has further suggested a possible link between UV-induced DNA lesions, mutagenesis (Tornaletti *et al.*, 1993; Tornaletti and Pfeiffer, 1994) and skin carcinogenesis (Dumaz, *et al.*, 1994). Potentially important targets for such damage are proto-oncogenes and tumour suppressor genes (Tornaletti *et al.*, 1993) such as *ras* and *p53*. Squamous cell carcinoma and melanocarcinoma have been found to have a mutation in the *p53* tumour suppressor gene and the *N-ras* gene respectively (Brash *et al.* 1991; van't Veer *et al.* 1989). The mutations frequently show CC→TT double base changes and a highly frequent C→T substitution at dipyrimidine sites which are considered to be UV specific "signatures" (Nataraj *et al.*, 1995).

A number of lesions are generated by the exposure of DNA to far or middle UV light (i.e. 280-315nm) and alterations in the earth's stratospheric ozone layer is likely to lead to variation in the frequency and type of damage (Freeman *et al.*, 1989). The major photoproducts are the cyclobutane pyrimidine dimers (CPD), formed between adjacent pyrimidine bases, consisting of thymine-thymine (TT) dimers as major products with smaller amounts of thymine-cytosine (TC), less still, cytosine-thymine (CT) and least of all, cytosine-cytosine (CC) (Cadet and Vigny, 1990; Smith, 1977b; Smith and Taylor, 1993; Spikes, 1983). Other types of DNA damage include formation of pyrimidine (6-4)

pyrimidone photoproducts (6-4 PP), cytosine photohydrates (Cadet and Vigny, 1990; Ananthaswamy and Pierceall, 1990), thymine glycol, single strand breaks, alkali-labile bonds, DNA-DNA and DNA-protein cross-links (Ahmed *et al.*, 1993). Whilst there is ongoing debate within the literature as to the relative biological importance of the formation of (6-4) photoproducts (Brash, 1988) and CPDs (Kim *et al.*, 1995; Douki *et al.*, 1995; Qin *et al.*, 1994), it has been proposed that CPDs may have lethal (Tornaletti *et al.*, 1993), mutagenic (Tornaletti and Peiffer, 1994; Brash *et al.*, 1991), or carcinogenic (Alcalay *et al.*, 1990) consequences if unrepaired. The overall yield of (6-4) photoproduct in DNA is reported to be anything from 3 to 10 times less than the cyclobutane-type pyrimidine dimer (Rahn, 1979; Wood, 1989), however, the relative levels of these two lesions in DNA seems unlikely to account exclusively for the biological effect of UV. Hutchinson (1987), in his review of UV mutagenesis, argued that data used to implicate (6-4)PP as being responsible for most UV mutations was derived from a model, not consistent with one in which over two thirds of mutations are CPD derived. Furthermore, Hutchinson (1987) stated the most convincing data implicating CPD, to be from photoreactivation experiments in which enzymatic photoreactivation of CPD resulted in a decreased mutation rate. Brash (1988) concluded that whilst (6-4) photoproducts and cyclobutane-type pyrimidine dimers are both major mutagenic lesions, the relative importance of the lesion varies between *Escherichia coli*. and human cells, the (6-4) photoproduct being more important in the former and the cyclobutane-type pyrimidine dimer in the latter. Additionally, it appears that location of the dipyrimidine site and local conformation at the damaged site also contribute to its consequences (Kim *et al.*, 1995). UV-induced lesions may lead to mutations during DNA repair, these mutations are believed to be due to translesion synthesis or failure of repair (Dumaz *et al.*, 1993). This repair has been shown not to be at uniform rates throughout the genome (Lan and Smerdon, 1985) and is even strand specific (Mullenders *et al.*, 1993). However, the importance of the events that follow the initial damage and end with tumour formation are not yet fully understood.

Until recently, the molecular importance of UV damage to DNA has centred solely on the direct formation of products such as cyclobutane-type pyrimidine dimers and pyrimidone(6-4)pyrimidine photoproducts (Cadet and Vigny, 1990), due to the strong absorption of far UV by DNA. Increasingly, however, UV-induced free radical damage has been shown to be important as near UV is shown to have deleterious effects too, in fact, van Weelden *et al.* (1988) concluded tanning with UVA to have carcinogenic risks in the same magnitude as

tanning with UVB. Berg *et al.* (1995a) showed that equally cytotoxic or mutagenic doses of UVB or UVA do not induce equal amounts of CPD suggesting the role of lesions other than CPDs and speculated on the role of ROS in the lethal and carcinogenic potential of UVA.

1.4.2 INDIRECT UV-MEDIATED DNA DAMAGE

Visible light and near UV have been shown to possess cytotoxic (Masini *et al.*, 1994), genotoxic (Pflaum *et al.*, 1994), mutagenic and carcinogenic (Runger *et al.* 1995) properties. It was formerly thought that near UV gives rise to lesions exclusively from oxygen-centred radicals, however, there would appear to exist two possible mechanisms for the formation of these lesions, one involving reactive oxygen species, the other being molecular oxygen-independent.

1.4.2.1 GENERATION OF ACTIVE OXYGEN INTERMEDIATES BY UV

Absorption of UV by many cellular components, such as tryptophan (McCormick *et al.*, 1976), flavins, quinones, porphyrins (Ito *et al.*, 1993), NADH and NADPH (Czochralska, 1984; Cunningham *et al.*, 1985a, 1985b), which act as chromophores, leads to the production of active oxygen intermediates, with the potential to generate lesions in DNA. These reactions are known as type I photosensitisation reactions. Here, the endogenous sensitizer molecule, raised to a triplet state (denoted $^3\text{Sens}$), reacts directly with a biomolecule by electron (or hydrogen atom) transfer to give a free radical form of the biomolecule (Spikes, 1983), ultimately generating H_2O_2 and superoxide (see Table 1.5).

Table 1.5. Generation of active oxygen species by photosensitisation (Spikes, 1983)

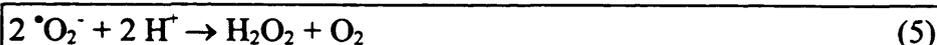
| | |
|---|-------------------------|
| $^3\text{Sens} + \text{R} \rightarrow \text{}^*\text{Sens} + \text{}^*\text{R}$ or $\text{}^*\text{SensH} + \text{}^*\text{R}$ | (1) |
| Further reactions involving the free radical form of the photosensitiser and molecular oxygen may lead to the formation of hydrogen peroxide: | |
| $\text{}^*\text{SensH} + \text{}^*\text{SensH} \rightarrow \text{Sens} + \text{SensH}_2$ | (2) |
| $\text{SensH}_2 + \text{}^3\text{O}_2 \rightarrow \text{Sens} + \text{H}_2\text{O}_2$ | (3) |
| and superoxide (Epe, 1993): | |
| $\text{}^*\text{Sens} + \text{}^3\text{O}_2 \rightarrow \text{Sens} + \text{}^*\text{O}_2$ | (electron transfer - 4) |

Where R = biomolecule, Sens = sensitiser molecule, * denotes an unpaired electron (free radical, $h\nu$ = irradiation, * denotes an excited state, $^3\text{O}_2$ = ground state oxygen, $^1\text{O}_2$ = singlet oxygen)

It has been hypothesised that hydrogen peroxide may be responsible for the cytotoxicity to mammalian cells following near UV irradiation in the presence of the photosensitiser, riboflavin (Sato *et al.*, 1995). Compared with the hydroxyl radical, hydrogen peroxide is relatively stable within the cell, reportedly penetrating membranes (Frimer *et al.*, 1983) and diffusing away from the site of generation; in this way a possible mechanism exists whereby hydrogen peroxide formed within the cytosol of mitochondrial cellular compartments may realise nuclear DNA damage.

1.4.2.2 DNA DAMAGE BY REACTIVE OXYGEN SPECIES; HYDROXYL RADICAL

Not all reactive oxygen species damage DNA directly (Halliwell, 1993). For example, hydrogen peroxide and the superoxide radical may initiate DNA damage by interaction with transition metal ion chelates, in particular iron and copper, in the metal-catalysed Haber-Weiss reaction, producing hydroxyl radicals (see Table 1.6)(Halliwell, 1991).

Table 1.6. Generation of the hydroxyl radical by interaction with metal ions (M^+)

Overall the Haber-Weiss reaction gives:



If the oxidised metal in this equation is Fe^{2+} the process is known as the Fenton reaction:



This reaction occurs at a faster rate if the metal ions are reduced (Fe^{2+} , Cu^+), therefore reducing agents, such as superoxide and ascorbate, can accelerate the production of hydroxyl radicals by maintaining the catalytic metal ions in a reduced form (Halliwell, 1991). It has been suggested that the copper ion plays an important role in the maintenance of nuclear matrix organisation and DNA folding via the stabilisation of the association between DNA and topoisomerase II (Chiu *et al.*, 1995). The copper ion's close association with DNA (Burkitt, 1994) may explain why most oxidative DNA damage is via the hydroxyl radical. The rapid reactivity of the hydroxyl radical, near its site of generation and histone protein protection (Enright *et al.* 1996) have promoted the theory of site-specific damage. This is further supported by the *in vitro* findings that copper-catalysed Haber-Weiss reactions in DNA cause more damage than with iron catalysis (Dizdaroglu, 1991c; Aruoma *et al.*, 1991). Availability of these ions is carefully controlled to minimise levels of free intra- and extracellular ions. Not only does this have an antioxidant function but it also allows $\cdot O_2^-$ and H_2O_2 to perform useful metabolic roles as intra- and intercellular signalling without the risk of $\cdot OH$ formation (Halliwell and Cross, 1994). Due to the rapid reactivity of the hydroxyl radical, minimising the availability and the location of metal ions are important factors in the generation and consequence of $\cdot OH$.

Many types of lesion are induced by hydroxyl radical attack on DNA, including strand breakages and covalent crosslinks. However, recently interest has focussed on base

damages due to their mutagenic potential and the possibility that they may act as biomarkers for oxidative damage. Over twenty different products are formed by hydroxyl radical attack on the bases in DNA, the principal products include 8-oxoadenine (8-oxoA), cytosine glycol, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-oxoguanine (8-oxoG), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol, TG) and 4,6-diamino-5-formamidopyrimidine (FapyAde)(Blakely *et al.*, 1990; Dizdaroglu, 1986a, 1991a). Examples of such lesions are represented in Figure 1.3.

1.4.2.3 DNA DAMAGE BY REACTIVE OXYGEN SPECIES; SINGLET OXYGEN

Photochemical reactions which lead to the transfer of excitation energy from excited photosensitisers (photoexcitation) to ground state oxygen ($^3\text{O}_2$) may generate electronically excited molecular oxygen (singlet oxygen, $^1\text{O}_2$) known as type II reactions (Martin and Burch, 1990)(Table 1.7).

Table 1.7. Generation of singlet oxygen by photoexcitation (Epe, 1993).

| | |
|---|------------------------|
| $\text{Sens} \xrightarrow{h\nu} ^1\text{Sens}^*$ | (excitation - 9) |
| $^1\text{Sens}^* \rightarrow ^3\text{Sens}^*$ | (spin conversion - 10) |
| $^3\text{Sens}^* + ^3\text{O}_2 \rightarrow ^1\text{Sens} + ^1\text{O}_2^*$ | (energy transfer - 11) |

Photosensitisers such as methylene blue, thiazin, phthalocyanine and riboflavin have all been reported to produce 8-oxodG in DNA, seemingly via singlet oxygen (Yamamoto *et al.*, 1992). However, 8-oxodG may not be the final product of DNA photooxidation. The photooxidation of 8-oxodG by singlet oxygen in a cell free system has been shown to occur three times faster than dG, leading, it is speculated, to further decomposition products of 8-oxodG which may also possess a mutagenic potential (Buchko *et al.*, 1995). This is supported by Mohammad and Morrison, (1996) who showed that 2'-deoxyguanosine 5'-monophosphate (dGMP) itself could photosensitise the formation of singlet oxygen, following irradiation with UVB. Additionally, dGMP/singlet oxygen photoproducts were also shown to sensitise the further formation of singlet oxygen, producing, what the authors describe as an additional source of UVB-induced lesions to the genome.

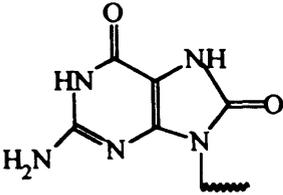
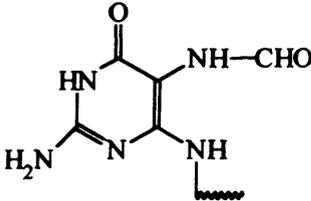
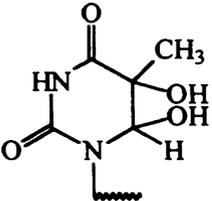
| BASE LESION | SOURCE OF FORMATION | PROPERTIES |
|---|---|----------------------|
|  | Hydroxyl radical, singlet oxygen, ionizing radiation, UV. | Miscoding, mutagenic |
| 8-Oxoguanine | | |
|  | Hydroxyl radical, ionizing radiation, UV. | Replicative block |
| Formamidopyrimidine (FapyGua) | | |
|  | Hydroxyl radical, ionizing radiation, UV. | Replicative block |
| Thymine glycol | | |

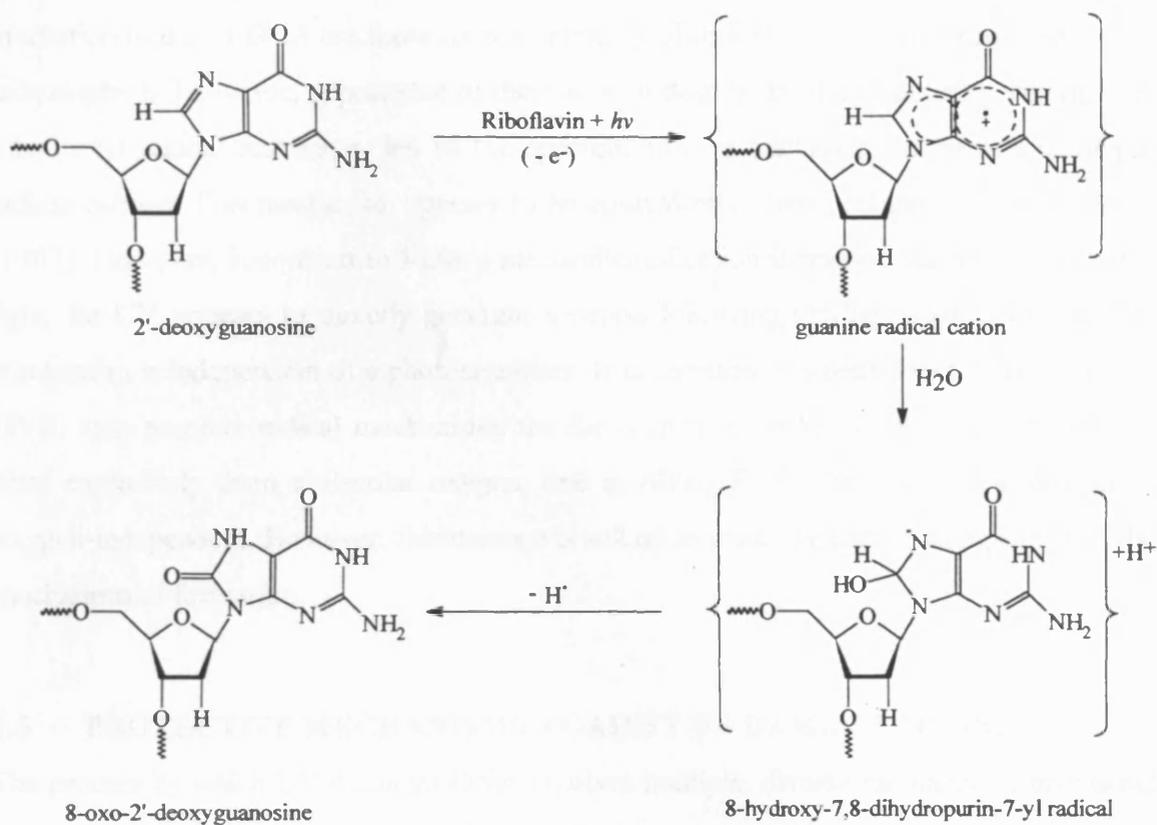
Figure 1.3. Representative oxidative DNA base lesions (from Demple and Harrison, 1994).

Clearly the cellular significance of lesions produced via this route would need to be compared to those via other established pathways such as hydroxyl radicals and direct absorption of UVB by pyrimidines and the consequential dimer formation.

Singlet oxygen can also be produced by dark reactions (chemiexcitation) e.g. by lipid peroxidation and the thermal decomposition of endoperoxides (Cadet *et al.*, 1994). Once formed in the vicinity of DNA, singlet oxygen selectively reacts with the guanine moiety in nucleosides forming predominantly 8-oxoG, but also the ring-opened derivative of guanine (FapyGua), single strand breaks and DNA-protein cross-links (Epe, 1991).

1.4.2.4 MOLECULAR OXYGEN-INDEPENDENT DNA DAMAGE INDUCED BY UV

Larson *et al.* (1992) suggested that the role of hydroxyl radical in the formation of DNA damage may be overemphasised, discussing the possibility that DNA-bound, UV-absorbing metal complexes may act as chromophores capable of generating DNA damage via free radicals derived from their ligands. Kasai *et al.* (1992) demonstrated the formation of 8-oxo-2'-deoxyguanosine (8-oxodG) within DNA, in the presence of riboflavin, via a molecular oxygen independent pathway, namely the formation of a guanine radical cation (Scheme 1.1), making it a type I photosensitiser reaction as described in Section 1.4.2.1. A radical cation is essentially a one-electron-oxidised species. Guanine, being the most easily oxidised of the nucleic acid bases, will invariably become the site for an electron-loss centre created in a system containing the four nucleic acid bases and consequentially form the guanine radical cation ($G^{\bullet+}$) (Steenken, 1989). The term cation does not indicate the radical carries a positive charge, merely that the precursor or "pristine" radical did (Steenken, 1989). Despite the great quantum energy differences, $G^{\bullet+}$ and anionic free radicals of thymine ($T^{\bullet-}$) are formed, in a photoionisation reaction, following UV (320nm) irradiation of DNA, much the same as γ -irradiation (Gräslund *et al.* 1979). Furthermore such radical species can also be formed via mechanisms involving photosensitisers (as in the case described by Kasai *et al.* 1992, above) reported by Hélène *et al.* (1966) and Sevilla *et al.* (1989). (For a more exhaustive review of such mechanisms see Steenken, 1989). The findings of Kasai *et al.* (1992) suggest the occurrence of the same intermediates in the formation of 8-oxodG whether by $\bullet\text{OH}$ or radical cations (Cadet *et al.*, 1993).



Scheme 1.1. Suggested mechanism for the formation of 8-oxo-2'-deoxyguanosine within DNA following irradiation with visible light in the presence of riboflavin (Kasai *et al.*, 1992).

More recently, Doetsch *et al.* (1995) demonstrated that the products of UVC and UVB irradiation of naked DNA are those more commonly observed as a consequence of hydroxyl radical attack. However, appearance of these lesions despite the use of dimethyl sulphoxide, a hydroxyl radical scavenger, led to the speculation of a damaging mechanism involving radical cations. This mechanism appears to be equivalent to that postulated by Kasai *et al.* (1992). However, in contrast to Kasai's mechanism of cation formation via riboflavin/visible light, far UV appears to directly generate a cation following irradiation of DNA, i.e. this mechanism is independent of a photosensitiser. It is therefore suggested that there exists for UVR, two possible radical mechanisms for the formation of lesions formerly thought to arise exclusively from molecular oxygen, one involving ROS, the other being molecular oxygen-independent. However, this damage is still of an oxidative nature, irrespective of the mechanism of formation.

1.5 PROTECTIVE MECHANISMS AGAINST UV DAMAGE TO DNA

The process by which UV damages DNA involves multiple, diverse mechanisms, producing a spectrum of different lesions. As the skin is the organ which receives the greatest UV exposure, many systems exist to prevent or minimise the damage induced, some of which are unique to the skin.

1.5.1 PROTECTION FROM DIRECT DNA DAMAGE

Skin penetration by far UV is limited, not progressing beyond the epidermis, largely due to absorption by the stratum corneum (Bruls *et al.* 1984) and epidermal pigments, such as melanin, which is more pronounced towards the shorter end of the spectrum (Kobayashi *et al.*, 1993), although this latter point seems to be a contentious issue (Kollias and Baquer, 1988). It would appear that damage to DNA and/or its repair actually enhances melanogenesis (Eller, 1996). The high absorption of UV by DNA would suggest that the condensed, or pyknotic, DNA of stratified keratinocytes also provides some protection to the most important keratinocytes of the basal layer.

The development of sunscreens, designed to minimise the effective doses of UV absorbed by the skin, has supported endogenous protective mechanisms and allowed greater sun exposure by individuals. However, classification of the protectivity of these sunscreens has been considered controversial, suggesting that the end-point measured not to be one of greatest biological significance (Lunec *et al.*, 1994).

1.5.2 PROTECTION FROM INDIRECT DNA DAMAGE

Alteration of the intensity and wavelength distribution of UV by epidermal protectants means that it is mainly near UV, which penetrates beyond the epidermis into the deeper layers of the dermis. The subsequent generation of potentially damaging agents such as ROS following near UV irradiation, has led to the evolution of elaborate enzymatic defence mechanisms, examples of which include superoxide dismutase (SOD), catalase (Degan *et al.*, 1991) and glutathione peroxidase (Tyrrell and Pidoux, 1986), which provides protection against lipid hydroperoxides (Stocker and Frei, 1991).

Hydrogen peroxide is generated by the dismutation of superoxide:



The hydrogen peroxide is then further acted upon by catalase to give water:



Non-enzymic defences include low molecular weight endogenous antioxidants such as vitamin C (L-ascorbic acid), vitamin E (tocopherols, of which the α -form predominates in most species) (Chow, 1988), glutathione (Moysan *et al.*, 1994), uric acid, bilirubin, ubiquinol and β -carotene (Degan *et al.*, 1991). Fischer-Nielsen *et al.* (1993), following demonstration that pre-incubation of cultured cells with ascorbate prior to UV ($\lambda = 240\text{-}580\text{nm}$) irradiation significantly reduced levels of 8-oxodG, concluded that ascorbate acts as an intracellular, and more specifically intranuclear antioxidant. This finding strongly supports the results of Fraga *et al.* (1991), who showed ascorbate to protect DNA from endogenous oxidative stresses. α -Tocopherol too, has been clearly shown to possess an inhibitory effect on the generation of ROS (van Staden *et al.* 1993). The process by which ascorbate or α -tocopherol will eliminate the free radical involves radical/antioxidant interaction and subsequent generation of a corresponding ascorbate or tocopheroxyl (vitamin E) radical, which is far less reactive (Cheeseman, 1993). However, this results in depletion of the antioxidant, for example, UVB irradiation of hairless mice was shown to cause a significant inhibition of both enzymic and non-enzymic defences (Fuchs *et al.*,

1989). Furthermore, solar UV light has been shown to generate vitamin E radicals, which are scavenged by other antioxidants, recycling the vitamin E, but depleting other antioxidants such as ascorbate (Kagan *et al.*, 1992).

The importance of transition metal ions to the formation of hydroxyl radicals is clear and therefore their availability is carefully controlled, primarily by sequestration, to minimise levels of free intra- and extracellular ions. Sequestration of transition metal ions has an antioxidant function reducing the risk of hydroxyl radical formation. However, oxidative stress itself, causes the release of sequestered iron allowing it to participate in free radical reactions (Zastawny *et al.*, 1995).

Certain conditions, such as UV, may result in these protective systems being overwhelmed, disturbing the balance between pro- and antioxidants, allowing increased levels of superoxide radicals and hydrogen peroxide, leading to cellular damage (Beehler *et al.*, 1992). Despite such elaborate defence mechanisms damage does occur in DNA, hence the evolution of DNA repair systems.

1.6 CONSEQUENCES OF UV-MEDIATED DNA DAMAGE - REPAIR OR MUTATION?

Although the major direct products are unique to far UV, there are many sources of reactive oxygen species and free radicals capable of initiating damage similar to that seen with near UV. These range from xenobiotics, where metabolism utilises cellular glutathione, therefore altering the normal pro-oxidant/antioxidant cellular balance, to those insults where free radical production is a direct consequence of their biochemical activity e.g. the redox cycling herbicide paraquat. Whilst the potentially most detrimental, long term repercussion for damaged DNA is mutation, the cellular consequences for a particular lesion are complex, depending on its mutagenicity and lethality. The variety of sources of damage to DNA and the requirement to preserve DNA integrity for continued successful cellular survival has necessitated the existence of systems to maintain fully functional DNA. Three principle DNA repair mechanisms occur; direct, base excision and nucleotide excision (Sancar, 1995).

1.6.1 REPAIR OF DIRECT DAMAGE TO DNA

The ability for damaged DNA to be repaired is a vital defence against insults which may lead to carcinogenesis, degenerative diseases and ageing (Bohr and Anson, 1995). Irradiation of DNA induces lesions which transiently inhibit replication (Spivak and Hanawalt, 1992). The CPD, a major class of far UV-induced DNA lesions, has been shown to be mutagenic and carcinogenic. It is therefore imperative that this lesion does not persist in the DNA. Three mechanisms have been described by which this lesion is removed in mammalian cells, (i) photoreactivation (ii) nucleotide excision repair (Sutherland *et al.*, 1980) and a third poorly understood mechanism of (iii) post-replication repair.

(i) Photoreactivation (direct repair)

CPDs are enzymically reversed to monomers by a DNA photolyase which binds to the damaged DNA. Following the absorption of a near UV and visible wavelength (blue light) photon, the enzyme transfers an electron, from the excited cofactor, flavin adenine dinucleotide (FADH₂), at the active site, splitting the cyclobutane ring (Sancar 1995). Initially, this enzyme was reported to be absent from the cells of placental mammals (Smith, 1977a; Cleaver 1966; Chao, 1993; Kato *et al.*, 1994) although it has subsequently been demonstrated in human cells (Sutherland, 1974; Harm, 1980, Roza *et al.*, 1991), including white blood cells (Sutherland and Bennett, 1995). However, perhaps of most biological relevance, the photorepair (PR) of CPD has been detected *in vivo* in human skin upon illumination with visible light (D'Ambrosio *et al.*, 1981). Sutherland and Sutherland (1975) suggested that a possible reason for the difficulty in detecting photoreactivation in human cells is its action spectrum of 300-600nm, with a maximum near 400nm. This enzyme utilises longer wavelengths than the much studied yeast homologue, leading to problems with "safelights" and the resultant detection difficulties. Tanew *et al.* (1993) found that using a pure UVA source (λ_{max} 372nm - within the above action spectrum) irradiation of cultured human fibroblasts did not cause photoreactivation of pyrimidine dimers. Despite this, Tanew *et al.*, (1993) used an optimised condition for PR, highlighting an unexplained culture medium phenomenon and also reviewed the ongoing debate within the literature as to the existence of PR within human cells.

Recently, the reversal of non-adjacent pyrimidine dimers, which occur only in single-stranded DNA formed transiently during replication, recombination and transcription, has been shown by DNA photolyase (Kim and Sancar, 1995). A distinct photolyase has also been reported for the reversal of (6-4)PPs (Kim *et al.*, 1994).

(ii) Nucleotide Excision Repair (NER)

This is the major DNA repair system in all species and is the sole mechanism for bulky adducts (Sancar, 1995). Although direct UV damage largely produces damage generally described as bulky adducts, distinct NER pathways have been shown to exist for CPDs and (6-4)PPs (Galloway *et al.*, 1994). The process is essentially the same in both pro- and eukaryotes; involving a multiprotein subunit, ATP-dependent excision nuclease (excinuclease) which makes both 5' and 3' incisions and removes an oligomer containing the damage (Svoboda *et al.*, 1993) described in detail by Mu *et al.* (1996). Suppression of repair by the use of DNA topoisomerase inhibitors, suggests the involvement of topoisomerases in the organisation of the DNA prior to incision (Thielmann *et al.*, 1993). Lesion type and sequence context can alter the sites of incision, however generally, human excinuclease removes the lesion in a 24-29 mer and *E.coli* (known as (A)BC excinuclease) in a 12-13 mer (Huang *et al.*, 1992). The resulting single-stranded gap is covered by a single-stranded DNA binding protein (SSB) to protect against non-specific nucleases and possibly recruits and enhances the effect of DNA polymerases (Coverly *et al.*, 1991). A DNA polymerase (Pol) is then stimulated by proliferating cell nuclear antigen (PCNA) to perform resynthesis and fill in the gap (Nichols and Sancar, 1992), using the unmodified strand as a template and sealed by a DNA ligase (Sancar and Tang, 1993). The proficiency of this system may be associated with the phase of the cell cycle (in replicating cells) (Berg *et al.*, 1995) and/or state of stimulation (for example in lymphocytes) (Freeman and Ryan, 1988). Barret *et al.* (1995) showed that activation of lymphocytes increased repair synthesis proportional to the percentage of cells in the S-phase of the cell cycle. This suggests perhaps, some connection between NER and DNA replication. An explanation for which may involve; recognition of specific, difficult to remove lesions, active genes possessing a more relaxed chromatin structure and a localisation of repair apparatus in the transcribed regions leading to swifter assembly of the repair complex (Tanaka and Wood, 1994).

Freyer *et al.* (1995) report an additional eukaryotic excision repair pathway for the processing of both CPDs and 6-4 PPs in the yeast *Saccharomyces pombe* whereby an endonuclease both recognises and cleaves immediately adjacent to the lesion, not dissimilar to the prokaryotic enzyme T4 endonuclease V (T4 endo V) (Nakabeppu *et al.*, 1982). However, in contrast to T4 endo V, this reported path does not possess a glycosylase and apurinic/apyrimidinic step. T4 endo V acts by first breaking the glycosidic bond of the 5'

component of the dimer. The second step involves cleavage of the phosphodiester backbone, 3' to the resultant apyrimidinic site, by β -elimination. T4 endo V was reported to have a substrate specificity predominantly for *cis-syn* cyclobutane pyrimidine dimers, although some activity towards the *trans-syn* isomer has been detected (Latham and Lloyd, 1994). Evidence has been provided by Dizdaroglu *et al.* (1996) that hydroxyl radical-induced monomeric products of DNA could also act as substrate for this enzyme.

The multiplicity of proteins involved in NER has produced a versatile and discerning mechanism to remove DNA modifications. However, this system is affected by ageing, as demonstrated by Roth *et al.* (1989), leading to a decreased ability to repair, at least certain, UV induced lesions. Failure of a part of this repair machinery may have serious consequences, as seen in the recessive disease xeroderma pigmentosum (XP). This disorder is characterised by major increases in both melanoma and non-melanoma skin tumours, correlating with a marked deficiency in nucleotide excision repair capacity (Cleaver and Trosko, 1970) which leads to distinct CC \rightarrow TT transitions due entirely to the UVB portion of the solar spectrum (Sage *et al.*, 1996). Other symptoms of XP include neurological abnormalities (Nagai *et al.* 1994), for which ROS have been implicated as being a contributing factor (Sato *et al.*, 1993).

There exists seven genetic complementation groups (A to G) corresponding to seven gene products whose impairment, to various degrees, in the early stages of NER produces the clinical symptoms of XP (Lehmann *et al.* 1975). Much work has been performed to identify and characterise the faulty factors, whose deficiencies range from an inability to recognise damage (Jones and Wood, 1993; Payne and Chu, 1994) to a failure of incision (Parrish *et al.*, 1992). Other pathological conditions arise from defective NER, such as trichothiodystrophy (TTD) and Cockayne's Syndrome (Kraemer *et al.*, 1994), but do not share XP's predisposition to skin cancer, although some sun sensitivity is displayed (Lehmann *et al.* 1989). Eveno *et al.* (1995) demonstrated reduced repair of CPD amongst the sun sensitive individuals with TTD, but normal levels of repair in non-photosensitive patients, a feature similar to that seen in Cockayne's Syndrome (Barrett *et al.*, 1991), from which it may be inferred that CPD are involved in photosensitivity but may not play such a major role in photocarcinogenicity. Further to this, Eveno *et al.* (1995) hypothesised that it may be unrepaired 6-4 PP which play a significant role in the process leading to skin carcinogenesis in XP. Moreover, Treiber *et al.* (1993) identified the 6-4 PP lesion to be a

principle target for a protein which recognises UV-damaged DNA. A defect in the ability of XP NER to remove an, as yet unidentified, class of ROS-induced lesions, has been hypothesised to contribute to carcinogenicity and neurodegeneration (Satoh *et al.*, 1993) through an accumulation of damage (Wei *et al.*, 1993). However, Runger *et al.* (1995) found a reduced capability to repair ROS lesions in only XP-C, a complementation group not normally associated with neurological abnormalities.

(iii) Post-replication repair

This process, reported to occur in human cells (Cleaver and Thomas, 1969; Fujiwara and Kondo, 1972), allows progression of replication without removal of the lesion and assumed to resemble bacterial recombinational repair, although the exact mechanism responsible for this has not been elucidated (Smith, 1977a). In *E. coli* at least, it appears that the damage is bypassed during replication, leaving a gap of several hundred nucleotides, the missing section on the complementary DNA strand is subsequently replaced by DNA polymerase (Myles and Sancar, 1989). Strictly speaking this is not a repair mechanism, as the lesion remains. This does, however, allow maintenance of the duplex and the lesion remains a target for NER, or if the lesion is in a non-essential gene it may be diluted out by repeated replication and cell division. Replication is clearly a crucial period for the cell and the bypass of CPD to allow replication is certainly mutagenic if not corrected (Thomas and Kunkel, 1993; Nelson *et al.* 1996). Interestingly, a group of XP variants have been identified which show normal levels of excision repair but defective post-replication repair (Lehmann *et al.* 1975).

1.6.2 REPAIR OF INDIRECT DAMAGE TO DNA

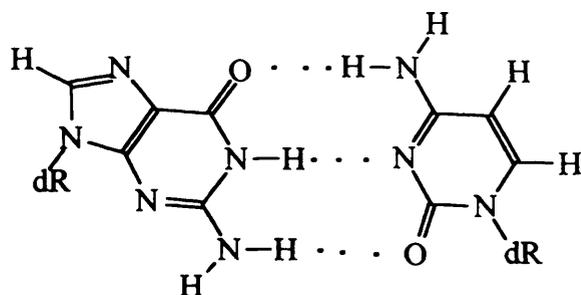
(i) *Base-excision repair*

Base excision repair is largely responsible for the removal of non-bulky base adducts. In this process, a DNA glycosylase removes the modified base leaving an apurinic-apyrimidinic (AP) site (AP-deoxyribose), which is subsequently removed by two AP endonucleases which incise 3' (AP lyase) and 5' (AP hydrolase) to the AP site (Sancar, 1995). The resultant gap is then filled by a DNA polymerase. A number of glycosylases have been identified, which include hydroxymethyluracil DNA glycosylase, thymine glycol DNA glycosylase and 8-oxoguanine DNA glycosylase (Dempfle and Harrison, 1994)

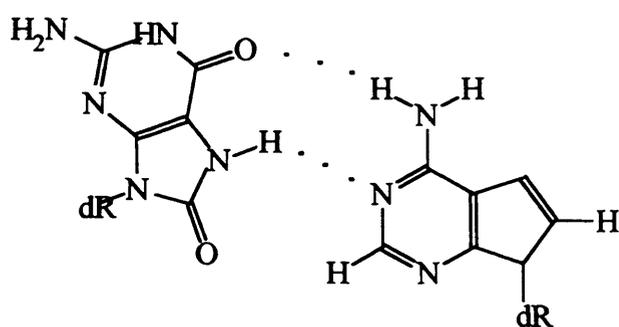
8-oxoguanine DNA glycosylase

A common product of oxidative stress is the DNA lesion 8-oxo-2'-deoxyguanosine (Halliwell, 1993), of which the 6,8-diketo form predominates under physiological conditions (Culp *et al.*, 1989). The lesion has a demonstrated mutagenic potential due to a loss of base pairing specificity, misreading of adjacent pyrimidines (Kuchino *et al.*, 1987), or insertion of cytosine or adenine opposite the lesion (Shibutani *et al.*, 1991). Mutations arising from 8-oxoguanine include G→T (Wood *et al.*, 1990) and A→C substitutions (Cheng *et al.*, 1992). Mis-pairing of 8-oxoG with adenine, appears to be possible due to the predominance of the energetically favoured *syn* conformation of 8-oxodG, whereas in the *anti* form pairing with dC is possible (see Figure 1.4) (Lipscomb *et al.* 1995). It may be that these structural alterations are involved in the recognition and subsequent repair of this lesion and yet 8-oxoadenine is reported to be at least one order of magnitude less mutagenic than 8-oxoG, in *E. coli* (Wood, 1992). This capacity for mutation provides 8-oxoG, along with other ROS-derived lesions, with a potentially important role in carcinogenesis (Floyd, 1990; Kasai and Nishimura, 1991; Jaruga *et al.*, 1994). It is therefore imperative that this lesion does not persist in the DNA; to achieve this end there exists five systems (Grollman *et al.* 1993):

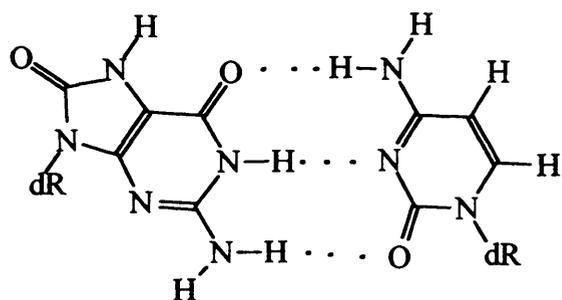
(i) The A→C mutation was shown to occur following misincorporation of 8-oxodGTP, from the nucleotide pool, opposite dA in DNA (Mo *et al.* 1992). The existence of a protein, 8-oxo-7,8-dihydroguanosine triphosphatase, (8-oxodeoxyguanosine triphosphatase, or 8-oxo-dGTPase) detected in human tissue, (a homologue of *E. coli* Mut T protein) (Maki and Sekiguchi, 1992) which hydrolyses the damaged triphosphate to the monophosphate (Wani and D'Ambrosio, 1995) assigns considerable importance to this mechanism for base lesion appearance in DNA.



dG (*anti*) pairing deoxycytidine (*anti*)



8-oxodG (*syn*) mispairing deoxyadenosine (*anti*)



8-oxodG (*anti*) pairing deoxycytidine (*anti*)

Figure 1.4. Base pairing of 8-oxodeoxyguanosine (from Grollman and Moriya, 1993)

(ii) Early studies of 8-oxoguanine DNA glycosylase were in *E. coli* where it was identified as Fapy glycosylase (or the Fpg protein from the *fpg* gene also known as the *mut M* gene) for removing formamido-pyrimidines (Czeczot *et al.*, 1991). Tchou *et al.* (1991) suggested that, on comparison of activity, bacterial Fapy glycosylase and mammalian 8-oxoguanine DNA glycosylase were identical. The substrate specificity of this enzyme has been further extended to include singlet oxygen damaged DNA (Müller *et al.*, 1990) (predominantly 8-oxoguanine) and also an appreciable amount of 8-oxoadenine (Boiteux *et al.*, 1992). The mechanism for this repair has been shown to involve two stages, glycolytic removal of the damaged base followed by a lyase activity towards the apurinic site leaving a strand break (Tchou and Grollman, 1993). Bessho *et al.* (1993a) reported the existence of two human 8-oxoG repair enzymes, one being 8-oxoG glycosylase (which in contrast to Mut M does not have a lyase activity) and an 8-oxoG endonuclease (which does not possess a glycosylase activity), although the activity of the former enzyme may be identical to mammalian N-methylpurine-DNA-glycosylase (Nash *et al.*, 1996).

van der Kemp *et al.* (1996) describe the cloning of a *Saccharomyces cerevisiae* gene named *OGG1*, a glycosylase/lyase (Ogg1) which acts on 8-oxoguanine when paired opposite cytosine. Nash *et al.* (1996) further this by reporting a second 8-oxoguanine glycosylase/lyase (tentatively designated Ogg2) which possesses a substrate preference for 8-oxoguanine paired opposite guanine. A gene, thought to be the human homologue of *OGG1*, was cloned by Arai *et al.* (1997). Expression this gene suppressed endogenous mutation in an *Escherichia coli* Mut M and Mut Y mutant and was shown to be ubiquitously expressed in a variety of human organs (Arai *et al.*, 1997).

(iii) Mammalian N-methylpurine-DNA-glycosylase (MPG), in addition to its main substrate of N-alkylpurine, removes 8-oxoguanine by glycosylase action (Bessho *et al.* 1993b).

(iv) First demonstrated by Holmes *et al.* (1990) and later characterised by McGoldrick *et al.* (1995), MYH, the mammalian homologue of Mut Y in *E. coli*, removes the mismatched, but otherwise undamaged, adenine opposite an 8-oxoguanine by a glycosylase action.

(v) NER, discussed below.

(ii) Nucleotide excision repair

NER acts as a “back-up” system for base excision in the repair of oxidative lesions (Huang *et al.*, 1994). Removal of these non-bulky lesions would appear to occur due to the non-specific binding of the NER mechanism recognition subunits to DNA (Huang *et al.*, 1994). This binding causes conformational changes in the DNA at the site which, if already conformationally altered due to a lesion, produces a higher affinity interaction and a more long-lived complex (Sancar and Tang, 1993). The result of which becomes an excinuclease target. Thus a process exists in mammalian cells (Chung *et al.*, 1991) whereby oxidative lesions which induce conformational changes and base mismatches may be recognised and removed by NER (Huang *et al.*, 1994).

1.6.3 URINARY 8-OXodG - A POSSIBLE BIOMARKER OF OXIDATIVE STRESS?

The appearance of 8-oxodG, a possible repair product of oxidatively damaged DNA, in human urine follows its removal from DNA by repair protein(s) as described above. It has therefore been proposed that urinary levels of this lesion can be used to non-invasively monitor *in vivo* oxidative stress (Cundy *et al.*, 1989; Simic, 1992), assuming an appropriate method of analysis exists (see section 1.8.2). However, it is important to note that urinary levels of any oxidative lesion rely on a balance between factors such as; the generation of damaging radicals, antioxidant defences, repair activities and renal excretion of damage products (Lagorio *et al.*, 1994). The quantitation of 8-oxodG in urine has been used to assay *in vivo* oxidative DNA damage (Shigenaga *et al.*, 1989). These workers demonstrated a correlation between levels of urinary 8-oxodG and species-specific metabolic rate and longevity, consistent with the hypothesis of a steady-state level of oxidative damage. Furthermore, this lesion and presumably others, has been shown to accumulate in tissue in an age-dependent manner, perhaps due to either a decrease in repair/antioxidant defence efficiency (Roth *et al.* 1989) or an increased rate of oxidant production (Fraga *et al.*, 1990). Urinary 8-oxodG has been examined in relation to smoking, gender and body mass index (Loft *et al.* 1992). The general finding, supported in a review by Loft *et al.* (1993) was that metabolic rate appeared to be a factor accounting for inter-species and subject variations in 8-oxodG excretion. Smoking increased the urinary levels of the lesion, but perhaps most surprisingly dietary intake of antioxidants were not associated with 8-oxodG excretion (Loft *et al.* 1992), although further investigation of this postulate was recommended (Loft *et al.* 1993). Suzuki *et al.* (1995) showed an increase in the urinary excretion of 8-oxoG following exposure of rats and humans to reactive oxygen-generating substances (2-

nitropropane, paraquat and hydroquinone, for the rats and car exhaust for the humans). The authors, whilst accepting that RNA and diet may contribute to urinary 8-oxoG levels, declared this lesion may nonetheless be used as a biomarker of DNA oxidation. Furthermore, its appearance in urine at considerably higher levels than 8-oxodG and adsorption to a strong cation exchange resin make it easier to detect. Despite the support of Suzuki *et al.* (1995) for 8-oxoG, the lesion of choice as a biomarker of oxidative DNA damage is 8-oxodG. The full relevance of this lesion to the assessment of *in vivo* oxidative damage to DNA, is not clear as it may also arise from the oxidation of free guanine deoxynucleotides in the cellular nucleotide pool (Cheng *et al.*, 1992; Maki and Sekiguchi, 1992). Nevertheless, urinary 8-oxodG remains a marker of *in vivo* oxidative stress.

It has been shown that individuals with malignant disease (breast, colon, lymphoma and teratoma) have a significantly higher excretion of 8-oxodG compared to healthy controls, hypothesised to be due to increased oxidative damage (Tagesson *et al.*, 1992, 1995). However, this increase may largely be due to the therapy which the patients are undergoing (Sangeetha *et al.*, 1990). Elevated levels of urinary 8-oxodG and therefore DNA damage, as determined by high performance liquid chromatography, have been proposed as an explanation for the higher incidence of malignancy in patients with cystic fibrosis (Brown *et al.*, 1995). Thus far, few studies have attempted to correlate increased urinary oxidative biomarkers with disease, perhaps due to a possible multifactorial explanation for elevated levels of oxidative stress in disease, or the drawbacks of analytical techniques available (discussed in Section 1.8.2). Nonetheless, cancer is just one disease in which oxidative damage to DNA has been postulated to play an aetiological role (Ames *et al.*, 1995). It would therefore be pertinent to examine levels of oxidative DNA biomarkers in other conditions, with which oxidative stress have been associated, such as the inflammatory, autoimmune disease, systemic lupus erythematosus (SLE) (Lunec *et al.*, 1994).

1.7 AUTOANTIBODIES AND SYSTEMIC LUPUS ERYTHEMATOSUS

Modification of DNA can render it more immunogenic (Seaman *et al.*, 1965). Native DNA has been reported to be a weak immunogen (Tan and Stoughton, 1969a) and yet in certain pathological conditions, for example the autoimmune disease systemic lupus erythematosus (SLE), autoantibodies to multiple nuclear antigens, including DNA and histones, are formed. Blount *et al.* (1991) postulated that DNA damaged by ROS may affect the

development of autoimmune diseases, such as SLE. The process by which DNA is damaged in chronic inflammatory diseases, may involve the release of ROS intermediates from phagocytic cells, their passage through cell membranes and finally reaction with DNA (Bashir *et al.* 1993). The hypothesis that ROS modification of DNA is involved in the development of autoantibodies in SLE has been supported by the enhanced reactivity of SLE anti-DNA antibodies to ROS-denatured DNA (Blount *et al.* 1989; Blount *et al.* 1990). The data of Alam *et al.* (1993) concurs, providing further evidence of the avidity of SLE sera for ROS-modified DNA. A further study, using UVC/H₂O₂ to produce a ROS-modified DNA antigen, showed high antigenic recognition by SLE sera, but also high immunogenicity following modification (Ara, *et al.* 1993).

The mechanisms by which UV can induce ROS formation have been discussed earlier. It is therefore interesting to note that UV photosensitivity is a common clinical feature of SLE, with sunlight either precipitating cutaneous LE or aggravating systemic LE (Morison, 1983). The spectrum for this reactivity has been shown to be in the range 230-320nm (Gilliam, 1987). Although this, rather confusingly, includes wavelengths not reaching the earth's surface, it does largely relate to UVB. It is pertinent therefore, to postulate that photoproduct formation or radical modification of DNA, along with immune modulation (Vermeer and Hurks, 1994) are factors, either in the induction or exacerbation, of an autoimmune response in SLE. The ubiquitous nature of free radicals may account for the broad range of possible aetiological factors in SLE, such as UV and pharmacological agents, through a common mechanism of action.

1.8 DETECTION OF UV-MEDIATED DNA DAMAGE

The proposed involvement of DNA damage in UV-associated pathologies has meant that the ability to detect and quantify levels is an advantage in understanding the pathogenesis of the condition.

Nuclear levels of direct DNA damage products have been examined to assess exposure and repair efficiency (Fritz and Smerdon, 1995; Aboussekhra and Wood, 1994). In addition, specific lesions such as cyclobutane pyrimidine dimers have been proposed as unique biomarkers (van Praag *et al.*, 1993). Whilst the extremely short *in vivo* half lives of ROS have largely precluded their direct measurement, their reactivity with other biomolecules has been exploited to demonstrate their formation. In the case of electron spin resonance (ESR)

techniques, spin traps are used, whereby organic compounds are used to rapidly trap radicals, forming more persistent radicals (spin adducts) allowing detection (Janzen, 1984; Rozen and Rauckman, 1984). The nucleus has been identified as a sensitive site for ROS action and therefore DNA modifications have been proposed as markers of ROS production. Specific lesions have been identified and assessed for their potential as biomarkers not only for ROS production but also for the cellular redox status (discussed in Section 1.8.2).

1.8.1 METHODS FOR THE DETECTION OF DIRECT UV DAMAGE

Cyclobutane thymine dimers exist in several isomeric forms (Figure 1.5). The *cis-syn* TT isomer has been shown to predominate in UV-irradiated DNA (Kovacs, *et al.*, 1993), making it a potential biomarker of far UV exposure to cells. The detection and accurate quantitation of *cis-syn* TT within DNA is therefore essential. Several methods have been developed to accomplish this. Generally, methods which allow detection and quantitation of direct UV damage involve enzymatic or chemical release of the lesion from DNA, followed by separation of modified bases by chromatography prior to detection. One of the earliest studies reported relied on formic acid digestion of radioactively pre-labelled DNA from cells irradiated with UVC in conjunction with two dimensional paper chromatography (Carrier and Setlow, 1971). However, this may be superseded by the introduction of assays utilising ³²P-postlabelling (Randerath *et al.*, 1985).

1.8.1.1 ³²P-POSTLABELLING

The use of radiolabels introduces a high degree of sensitivity to an assay. The application, by Bykov *et al.* (1995), of ³²P-postlabelling to the analysis of thymine dimers has enabled detection of dimers at the fmol level. However, the technique can only gain specificity for cyclobutane-type pyrimidine dimers following separate treatment of samples with T4 endonuclease V. Furthermore, TT dimers cannot be distinguished from TC, although due to the labelling technique requiring trinucleotides, AT=T can be differentiated from TT=T and TT=C (Bykov *et al.*, 1995).

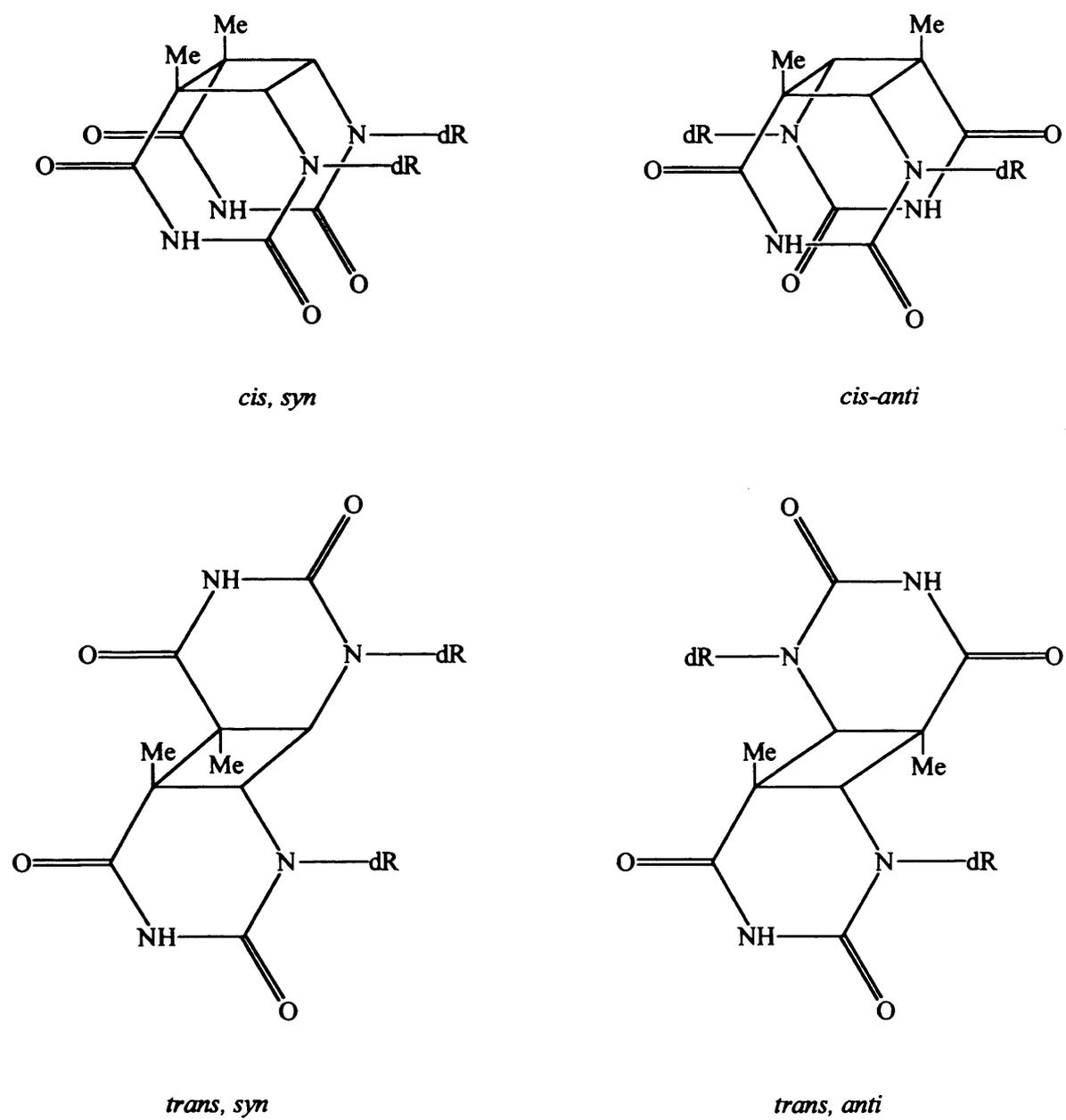


Figure 1.5. The four possible stereoconfigurations of cyclobutadithymine.

1.8.1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography has been used for the separation of the four thymine-thymine cyclobutane dimers, using UV detection (Cadet, *et al.* 1980). However, these were not derived from UV-irradiated DNA. Snyder *et al.* (1989) used HPLC in the analysis of thymidine-thymidine cyclobutane dimer repair in UVC-irradiated cells, containing radioactively pre-labelled DNA. Other UV-induced dimeric photoproducts have been assessed by HPLC, including the spore photoproduct (SP) (Sun *et al.* 1994), thymine-uracil (TC) dimers (Love and Friedberg, 1982) and three of the four possible pyrimidine-containing (6-4)PP (Douki *et al.* 1995). Quantitation of the SP again required radiolabelled DNA in the form of ^3H -thymidine, to gain sensitivity, as did the method for TC dimers; whereas the (6-4)PP was quantitated using fluorescence detection. This latter method provided a limit of detection of around 10pmol in UVC-irradiated DNA. Reversed-phase HPLC has also been utilised to separate short ($n=2-4$) thymine dimer-containing oligodeoxyribonucleotides - the ultimate objective of which would be their application as models in mutation and repair studies (Demidov and Potaman, 1984). Identification of the dimer type contained within the oligomer was reported to be possible by detection at several different UV wavelengths - quantitation was not attempted.

1.8.1.3 ENZYME-BASED ASSAYS

The discovery of lesion-specific enzymes, e.g. T4 endonuclease V (Yasuda and Sekiguchi, 1970) and the UV-endonuclease from *Micrococcus luteus* (Carrier and Setlow, 1970), which are reported to specifically nick DNA at cyclobutane pyrimidine dimers (TT, TC/CT and CC, Haseltine *et al.* 1980), were exploited in another form of assay, as a means of quantitating levels of lesion as single strand breaks, for example, using alkaline sucrose gradients (Sutherland *et al.* 1983) or alkaline agarose gel electrophoresis (Sutherland and Shih, 1983; Freeman *et al.*, 1986).

(i) *T4 endonuclease V / Micrococcus luteus endonuclease*

The method of Sutherland and Shih (1983) provided sensitive detection of dimers in nonradioactive DNA, after isolation, by enzymically treating the DNA and then separating the samples according to molecular weight on alkaline agarose gels. Comparison of migration distances with molecular weight standards allows the number of dimers per DNA molecule to be calculated. The enzyme used is a 18-kDa protein, known as *M. luteus* UV endonuclease, with possible homology to the 16-kDa endonuclease V from bacteriophage

T4, with a substrate preference for thymine-containing dimers over cytosine-containing dimers (under conditions of substrate excess, Piersen *et al.*, 1995). The reliance of the above technique on the activity of *M. luteus* endonuclease is perhaps an important source of error. Firstly, the single-strand “nicking” of the enzyme may be incomplete. Secondly, the activities of T4 endo V and *M. luteus* are very similar (Friedberg and King, 1971; Haseltine *et al.* 1980), with the two enzymes being surmised as identical by Dizdaroglu *et al.* (1996). Such a finding is crucial, when it is considered that the extract from *M. luteus* may not be limited solely to cyclobutane pyrimidine dimers (Setlow and Carrier, 1973), suggested as a result of its ability to make single-strand nicks in γ -irradiated DNA. However, the nature of the lesion was not identified, but the possibility it may have been γ radiation-induced CPD was ruled out (Setlow and Carrier, 1973). Furthermore, it was shown, by GC-MS, that T4 endo V’s repertoire of substrate includes FapyAde, described as a major UV radiation- and hydroxyl radical-induced lesion in DNA, perhaps identifying the lesion repaired in the γ -irradiated DNA of Setlow and Carrier (1973). Such a finding may have relevance to the report of Piersen *et al.* (1995) who firstly, describe that the *M. luteus* endonuclease protein is actually a 31-32-kDa protein and that the 18kDa protein, established in the literature, is actually a contaminant. Secondly, and with perhaps most relevance to the findings of Setlow and Carrier (1973) and Dizdaroglu *et al.* (1996), they described strong sequence similarities between the *pdg* (pyrimidine dimer glycosylase) gene product (*M. luteus* endonuclease) and *E. coli* DNA repair proteins, endonuclease II and MutY. However, no substrate overlap was noted (Piersen *et al.*, 1995), it therefore may be surmised that the similarity is associated with the similarities of the enzymes in their N-glycosylase/AP lyase modes of action. The situation is clearly not straightforward, with doubt being cast on the specificity of *M. luteus* endonuclease and T4 endo V, dependant on its purification. This detracts from the specificity of assays utilising these enzymes, such as the method of Kastan *et al.* (1995) which combined nicking with chromatography and fluorimetric DNA analysis.

Nevertheless, alkaline gel electrophoresis is described, by B. Sutherland (personal communication), to be a remarkably sensitive technique, giving exact agreement with values obtained by alkaline sucrose gradient methods. The strength of this method is its applicability to determining strand breaks in small quantities of DNA (30-50ng), not suitable for radioactive labelling, with a sensitivity limit of approximately 3 breaks/ 10^9 Da.

(ii) Non UV-specific endonucleases

Kinley *et al.* (1995) used alkaline elution, a variation on alkaline agarose gel electrophoresis, to detect strand breaks and alkali labile sites (SSB) in DNA induced either directly, by UVA, or indirectly, by UVB as a consequence of *in vivo* cellular repair. Clearly this method possesses very little lesion specificity compared to the above techniques, although the authors report good sensitivity, detecting SSB induced in skin samples following irradiation *in vivo* at a dose as low as 0.5 kJ/m² UV. However, the UV source used was broad spectrum, incorporating much UVB compared to UVA, making the issue of possible sources of SSB more complex.

The enzyme, T4 DNA polymerase, possesses a 3' → 5' exonuclease activity in the absence of deoxynucleotide triphosphate. Liu and Smerdon (1995) applied the inability of this enzyme to process cyclobutane dimers and (6-4)PP to quantitate levels of these lesions in UV irradiated DNA. The digested DNA was then bound to a membrane by a slot-blot apparatus, prior to hybridisation with a radioactive DNA probe, which allowed quantitation. A sensitivity study was performed, but the results were only described in terms of dose of UVC, not number of lesions detectable. Furthermore, to quantitate the number of (6-4)PP, all the CPD had to be first photoreversed with *E.coli* photolyase. Finally, not only are all forms of CPD (and this includes TT, CT/TC and CC) detected, but so are other bulky DNA adducts, such as cisplatin, which may be more likely to be present in biological samples.

1.8.1.4 GAS-CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

Capillary GC-MS using internal standards labelled with stable isotopes has been successfully applied to the study of several modified base lesions induced by reactive oxygen species (ROS) damage to DNA (Dizdaroglu, 1993, 1994; Djuric *et al.*, 1991; Hamberg and Zhang, 1995; Teixeira *et al.*, 1995 - see Section 1.8.2.2). Recent work (Doetsch *et al.*, 1995) has utilised GC-MS to study ROS-type base modifications in naked DNA following UV irradiation. However, pyrimidine dimers were not examined. Gas chromatography has been used previously for efficient separation of pyrimidine dimers as their corresponding trimethylsilyl derivatives (Fahr, 1973) and therefore the potential for the development of a GC-MS assay clearly exists.

The general techniques on which the above methods rely all have associated technical difficulties such as incomplete hydrolysis and enzyme specificity, although the potential for

artefactual direct damage is more easily overcome. However, the techniques do preclude the cellular localisation of damage products. There is therefore a need to develop immuno-type techniques in order to achieve this end.

1.8.1.5 IMMUNO-DETECTION OF DIRECT DAMAGE

Native DNA is not immunogenic *per se* (Plescia *et al.*, 1964a), but becomes so when denatured and complexed with a carrier, such as methylated bovine serum albumin, producing antibodies which recognise oligonucleotides and single-stranded DNA (Plescia, 1964b). Following on from this, early studies showed the potential for antibodies to be raised towards modified DNA (Seaman *et al.*, 1965). Certain modifications, for example, irradiation with far UV, increase immunogenicity greatly and the resulting antiserum is then applicable to the detection of UV-induced lesions in DNA (Natali and Tan, 1971). On this basis, polyclonal and monoclonal antibodies have been developed to specific UV lesions (reviewed in Herbert *et al.* 1994), allowing recognition and quantitation, although the latter largely relies on the use of radioisotopic labelling in a radioimmunoassay. Seaman *et al.* (1972), using a polyclonal antiserum, raised as described above, in a competitive radioimmunoassay (RIA), demonstrated the appearance of thymine dimers in irradiated bacterial and mammalian cells. Identification of the antigenic determinant responsible for the antiserum's binding was determined by competitive RIA, a principle used by Eggset *et al.* (1987) and applied to ELISA for the characterisation of UV antibodies which were subsequently shown to recognise (6-4)PP. Herbert *et al.* (1994) raised polyclonal antibodies to UV-irradiated DNA which, following characterisation by ELISA, appear to recognise thymine-thymine dimers in association with a third pyrimidine. Wani *et al.* (1987) developed an immunoslot blot technique to obtain sufficient sensitivity so as to detect levels of thymine dimers in non-radiolabelled cellular DNA at sub-lethal UV doses.

The immunochemical detection of UV-induced DNA in human skin irradiated *in vivo* was first reported by Tan and Stoughton (1969), using a polyclonal antiserum and immunofluorescence detection. Subsequent studies have developed the technique further, for example, using monoclonal antibodies, but fluorescent detection has largely remained consistent (Muramatsu *et al.*, 1992; Potten *et al.*, 1993). Roza *et al.*, (1991) used immunofluorescence microscopy with a monoclonal antibody, coupled with computer imaging and analysis, to detect photorepair in human epidermis which had been UV exposed. Hori *et al.* (1992) used a polyclonal antiserum in immunoblotting, along with

fluorescent immunohistochemistry, to demonstrate unremoved UV damage in the DNA of biopsies from patients suffering from actinic keratosis. Additionally, it was determined that this damage was of the cyclobutane pyrimidine dimer type. Quantitation by immunofluorescence microscopy is reported to introduce large errors due to variations in fluorescence per cell as a result of cross-sectioning the tissue and the relative low numbers of cells measured (Berg *et al.* 1993). It is therefore desirable to quantify fluorescence in large numbers of intact nuclei. Flow cytometry, a technique which allows measurement, for example, of fluorescently labelled, individual cells as they flow past a laser detection point, was shown to be applicable to the analysis of cells irradiated *in vitro* and *in vivo* (Berg *et al.* 1993). Furthermore, counterstaining of the nuclei enabled stage of the cell cycle to be determined also.

The capacity of the immunodetection approach for sensitive quantitation and importantly, localisation, distinguishes it from other methods for monitoring direct UV damage. Consequentially, it has many applications, from the detection of lesions in cells exposed to natural sunlight (Clingen *et al.* 1995a) to the assessment of sunscreens (van Praag *et al.*, 1993) and DNA repair in cells (Mitchell *et al.* 1985) and tissue (Vink *et al.* 1994).

1.8.2 METHODS FOR THE DETECTION OF INDIRECT UV DAMAGE

Increased free radical production and the resulting damage is present in most, if not all, human diseases (Fuchs, 1993). The significance of their contribution to pathology can vary between conditions, making specific assays that measure free radical activity and damage important. In order to show that ROS are important for the disease of study it is necessary to establish the following:

- ROS are formed at the site of injury.
- the time course of their formation is such that they could play a role in disease pathology.
- removal of ROS or prevention of their formation has beneficial effects
- direct application of ROS at concentrations found *in vivo* reproduces most or all the injury.

It is therefore crucial to develop analytical methods for assessing ROS, so that the above criteria may be satisfied when investigating conditions in which ROS are implicated.

1.8.2.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

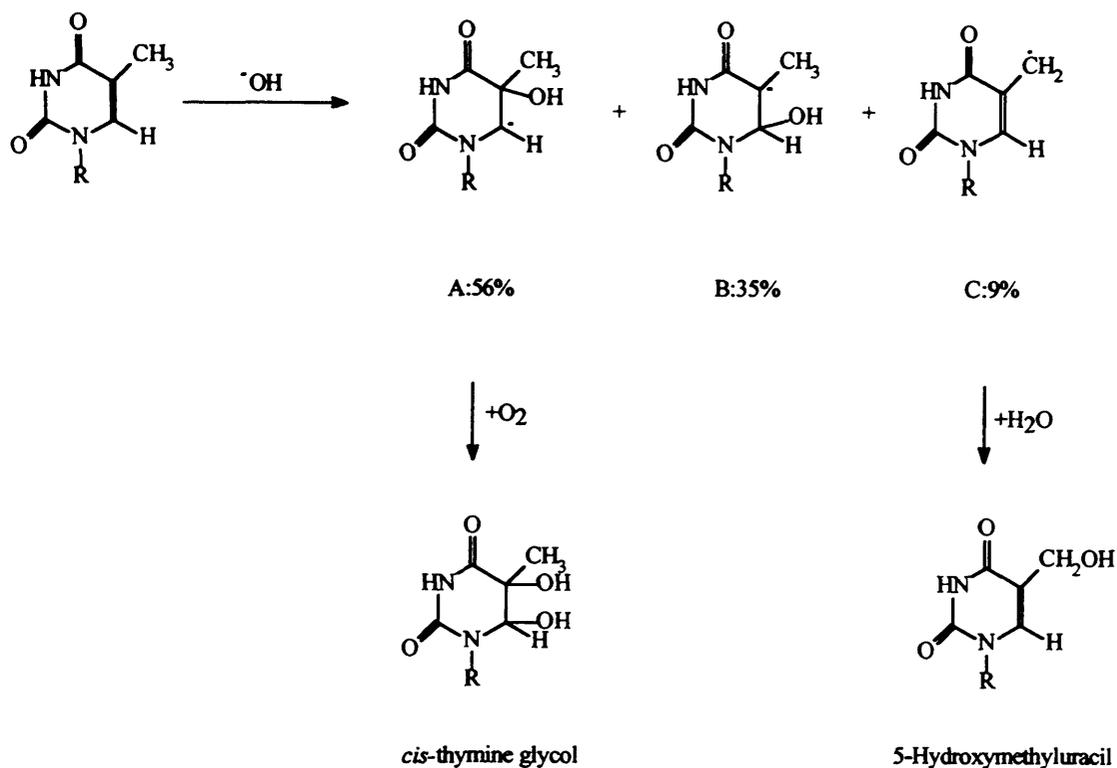
One of the first ROS-induced DNA lesions to be studied and found significant, was thymine glycol (Hariharan and Cerruti, 1974). Frenkel *et al.* (1981) used high performance liquid chromatography with UV detection (HPLC-UV) to identify and quantify thymine glycol in γ -irradiated DNA. Scheme 1.2. portrays the formation of thymine glycol in DNA following interaction with the hydroxyl radical. The thymine radical "C", whilst only a minor product, is a precursor of 5-hydroxymethyluracil an oxidation product detected both in DNA (Frenkel, 1985) and urine (Bergtold *et al.* 1988).

Thymine glycol is a relatively sensitive lesion to formation (resulting from 50% of the thymine/hydroxyl radical interactions) compared to 8-oxoguanine (only 25% of all products of guanine/hydroxyl radical interactions) and yet 8-oxo-2'-deoxyguanosine predominates over thymidine glycol in both mouse and human urine (Bergtold *et al.* 1988). Reasons for this are believed to include the relative instability of the thymine glycol, resulting in its decomposition, site specific hydroxyl radical formation (largely in guanine-rich regions) and selectivity in the occurrence or repair of damage (Bergtold *et al.* 1988). As a consequence, the sensitivity of the HPLC-UV assay when applied to thymine glycol detection in urine, appeared to be a problem (Cathcart *et al.*, 1984). Therefore quantitation required, radiolabelled thymine and, for example, large doses of radiation to induce detectable levels of thymine glycol (Cathcart *et al.*, 1984).

The issue of sensitivity associated with the detection of low levels of oxidative DNA damage such as thymine glycol was overcome by the measurement of 8-oxoguanine in its deoxynucleoside form, 8-oxo-2'-deoxyguanosine in DNA digests; this was achieved by HPLC with electrochemical detection (HPLC-EC) (Floyd *et al.*, 1986). In this procedure DNA is first extracted from cells or tissues and then enzymatically digested to yield free deoxynucleosides which are separated by reversed-phase HPLC; 8-oxo-2'-deoxyguanosine is measured sensitively by electrochemical detection, which provides 1000 times the sensitivity of UV detection (Bergtold *et al.* 1988). In the same HPLC run, native deoxyguanosine can be measured by UV absorbance in order to provide a reference. The HPLC-EC method has subsequently been applied to measure 8-oxodG in urine, employing a prepurification step, namely, solid phase clean-up of the urine (Shigenaga, *et al.* 1990) or immunoaffinity clean-up (Shigenaga, *et al.* 1994), or column coupling (Tagesson *et al.*, 1992). Recently, a modification of the above assay has been reported, in which formic acid

is utilised to yield 8-oxoG as a free base from DNA (Herbert *et al.* 1996). Previously, detection of this lesion proved difficult in the presence of high levels of endogenous guanine. The conversion of guanine to xanthine, by guanase (EC 3.5.4.3), eliminated the interference, facilitating detection of 8-oxoG. The authors further suggested the use of xanthine to provide the reference for quantitation of the DNA. Most recently, quantitation of DNA in samples has been by the measurement of guanine, necessitating an additional sample run, without the use of guanase (Herbert *et al.*, 1996). This extends the analysis time, but prevents any possible contribution to xanthine from RNA.

Capillary electrophoretic determinations of 8-oxo-2'-deoxyguanosine (Guarnieri *et al.*, 1994) and 8-oxoguanine (Poon *et al.*, 1995) have been described, but currently lacks the sensitivity required for DNA extracted from cells.



Scheme 1.2. Interaction of $\cdot\text{OH}$ with thymine, illustrating three possible sites of reaction and the resulting intermediates. Only “A” leads to the formation of thymine glycol (Breen and Murphy, 1995).

1.8.2.2 GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GC-MS)

Methods such as GC-MS produce a specific profile of damage effectively allowing characterisation of hydroxyl radical attack according to the range and levels of DNA base products formed (Gajewski *et al.*, 1990). Additionally, it has been shown that relative levels of these modifications vary depending on the generating system (see Table 1.8. derived from Spencer *et al.*, 1994).

Table 1.8. Damage to DNA bases by various ROS generating systems.

| Base product (nmol/mg) ¹ | DNA treatment | | | | |
|-------------------------------------|---------------|------------------------------------|-------------------------------------|--|--|
| | untreated DNA | DNA + Cu ²⁺ + ascorbate | DNA + H ₂ O ₂ | DNA + Cu ²⁺ + H ₂ O ₂ | DNA + Cu ²⁺ + H ₂ O ₂ + ascorbate |
| 8-oxoG | 0.177 | 1.554 | 0.198 | 0.531 | 55.640 |
| 8-oxoA | 0.095 | 0.517 | 0.198 | 0.604 | 8.076 |
| FapyGua | 0.682 | 2.319 | 0.561 | 0.659 | 6.972 |
| FapyAde | 0.146 | 0.518 | 0.178 | 0.471 | 2.082 |

The above work was repeated in the presence of Fe³⁺ ions instead of Cu²⁺, which although promoting oxidative DNA damage, it did so to a lesser extent compared to Cu²⁺. This supports the findings of Dizdaroglu *et al.* (1991c) and Aruoma *et al.* (1991) described in Section 1.4.2.2.

GC-MS methods have been used to quantitate 8-oxoguanine as the free base (Dizdaroglu, 1993, 1994). The facility has been enhanced by the ability to quantitate guanine in DNA, providing the ratio between G and 8-oxoG (Hamberg and Zhang, 1995). The GC-MS assay does appear to be quite versatile, being applied for the detection of 8-oxodG in urine and extracted organ DNA (Teixeira *et al.* 1995). Fewer groups use GC-MS than HPLC as the equipment is more expensive and technically more demanding making routine use arduous. HPLC and GC-MS have been compared (as reviewed by Halliwell and Dizdaroglu, 1992, Halliwell, 1993). Generally it is not the exact sensitivity of the technique that is important,

¹ (nmol damaged base/mg DNA)

moreover the ability to determine “background” levels in untreated, native DNA, or in the DNA from unstressed cells. It appears that levels of 8-oxoguanine are higher when determined by the GC-MS technique. In freshly isolated cells, baseline values of between 2 to 11 fold higher have been reported by GC-MS when compared to HPLC. A number of factors within the procedures may account for these discrepancies; these include (i) incomplete enzymolysis in the presence of DNA which has been oxidatively modified, (ii) sub-optimal enzymatic hydrolysis of DNA and (iii) the balance between incomplete depurination and the formation of artefacts by formic acid hydrolysis of DNA and derivatisation prior to GC-MS. Hamberg *et al.* (1995) examined the levels of 8-oxoG in native, commercial calf thymus DNA under a number of derivatisation conditions and compared these values in the literature. They concluded high temperature derivatisation, even in an anoxic environment, leads to a significant increase in 8-oxoG, when compared values obtained by HPLC. This agrees with the findings of Nackerdien *et al.* (1992) that formic acid hydrolysis provides no significant contribution to oxidation of free guanine to 8-oxoG. Development, by the authors, of a room temperature derivatisation method reduced background levels to a range in agreement with HPLC (4-9 8-oxoG/10⁵ dG). Douki *et al.* (1996b) showed that adenine, cytosine, thymine and thymidine all may give rise to oxidation products during derivatisation. Their solution to this problem was HPLC pre-purification of the hydrolysates, to remove the precursors of base oxidation, prior to derivatisation. This approach proved to be successful, bringing the background 8-oxoG level to agreement with HPLC. Enrichment is the approach adopted by Ravanat *et al.* (1995) to remove the overestimation by GC-MS. This group used an immunoaffinity column of monoclonal antibodies raised to 8-oxoG, as described in Sections 1.8.2.1 and 1.8.2.4.

The background levels of 8-oxoG in cellular DNA, measured either by GC-MS (0.9-3.1 8-oxoG/10⁵ G) (Olinski *et al.*, 1992) or HPLC (0.5-2.0 8-oxoG/10⁵ G) (Floyd, 1990) are approaching each technique’s limit of detection. This makes the prevention of artefactual guanine oxidation vital. Claycamp (1992) demonstrated phenol-based DNA extraction methods sensitise DNA to subsequent oxidation, a finding supported by Finnegan *et al.* (1996), who also demonstrated no detectable induction by a pronase E extraction. Herbert *et al.* (1996) attempted to address some of the issues regarding artefact formation by the development of the HPLC-EC assay described in section 1.8.2.1. The authors acknowledge that further work is required before it can be established that this method is an improvement on the HPLC assay for 8-oxodG, or the GC-MS assay for 8-oxoG. It is envisaged that this

technique would also locate the source of discrepancies between the values obtained by GC-MS and HPLC.

To date, GC-MS has rarely been used to examine the oxidative damage products of DNA following UV irradiation. Doetsch *et al.* (1995) described the formation of oxidative base lesions in calf thymus DNA following irradiation both with UVB and UVC. This work showed that formamido lesions were generated in the most appreciable amounts, particularly by UVC and the increase to be dose-responsive. Less attention was paid to the formation of 8-oxoG, an increase being measured following only a single dose of UVC. Importantly, through using dimethyl sulphoxide, a hydroxyl radical scavenger, it was concluded that the oxidative damage caused was by a mechanism which was hydroxyl radical-independent.

1.8.2.3 ³²P - POSTLABELLING

A number of other approaches to the determination of 8-oxoguanine in DNA have been described. ³²P-postlabelling procedures provide the potential for detection of a broad range of damage products, such as thymidine glycol (Hegi *et al.*, 1989), and 8-oxodG (Rosier and van Peteghem, 1988; Lutgerink *et al.*, 1992). However, the techniques are very time-consuming and cumbersome, involving DNA purification, digestion with enzymes, enrichment of the 8-oxodeoxynucleotide, addition of a radiolabelled phosphate group and thin layer chromatography (Carmichael *et al.*, 1992). The time-span for analysis is in the order of days. A method has also been described using the postlabelling principle to attach a fluorescent label to 8-oxodG which is then quantified using HPLC with a conventional fluorescence detector, avoiding the use of radiolabels (Sharma *et al.*, 1990).

1.8.2.4 IMMUNO-DETECTION OF INDIRECT DAMAGE

Using antibodies raised to photooxidised DNA, Seaman *et al.* (1966) demonstrated reactivity of this antiserum to photooxidised DNA in a dose-responsive manner. Additionally, they showed inhibition of this reactivity by photooxidised dGMP and concluded that the antigenicity of the modified DNA is largely due to altered guanine residues. Clearly the potential exists to develop assays, using antibodies to oxidised DNA. West *et al.* (1982a) used a polyclonal antiserum to specifically detect low levels of *cis* thymine glycol in the DNA of γ -irradiated *E. coli* by radioimmunoassay (RIA). Further to this, monoclonal antibodies have been produced to thymine glycol which allowed sensitive

detection of this lesion in γ -irradiated and H_2O_2 -treated DNA, by enzyme-linked immunosorbent assay (ELISA) (Leadon and Hanawalt, 1983; Hubbard *et al.*, 1989). RIAs using antibodies raised to 8-oxoadenine have also been developed (West *et al.* 1982b). Degan *et al.* (1991) produced high affinity, polyclonal antibodies to 8-oxo-2'-deoxyguanosine, providing an improved urine clean-up method by solid phase extraction, to reduce the complexity of the HPLC-EC chromatogram. This antiserum was also used to quantitate the 8-oxodG in enzymatic hydrolysates of DNA, by competitive RIA, showing excellent correlation with HPLC-EC analysis. Comparable limits of detection for 8-oxodG were also observed with the two techniques (63 fmol RIA; ~50 fmol HPLC-EC). Clearly such antibodies represent an improvement in the method for urinary 8-oxodG detection, as well as a respectable alternative to HPLC-EC method for the detection of 8-oxodG in DNA. This principle was extended by Park *et al.* (1992), using a monoclonal antibody column to enrich for 8-oxo-2'-deoxyguanosine from urine, blood plasma and culture medium. The authors describe this approach to be an improvement on solid-phase and polyclonal extraction methods. However, they also acknowledge the resulting antibody's limitations in the context of immunohistochemistry, competitive RIA and ELISA, due to its higher cross-reactivity with deoxyguanosine than the polyclonal. Direct quantitation of 8-oxodG in DNA with a polyclonal antiserum by immunoslot blot has been performed (Musarrat and Wani, 1994), although this necessitated the extraction of DNA from treated cells prior to analysis. Nonetheless, Musarrat and Wani (1994) demonstrated the applicability of such immunoassays to the detection of base modifications in very small DNA samples - nanogram amounts were immobilised. The strength of such techniques rely largely on the amplification step, by which levels of antibody binding are recognised and amplified by an enzyme/substrate process. Yin *et al.* (1995) used a monoclonal affinity column to isolate 8-oxodG from extracted DNA hydrolysates prior to HPLC. Additionally, the antibody was used to quantitate 8-oxodG, in extracted DNA, by ELISA but following immunoaffinity purification. This was necessary due to a cross-reactivity of the antibody with native deoxyguanosine. Again these values correlated well with those obtained by HPLC-EC, although the absolute values are higher than those determined by HPLC (1.1 - six-fold). As reported earlier, extraction of DNA can produce artefactual damage and therefore the development of methods for direct *in situ* demonstration of oxidative damage to DNA is very desirable. Yarborough *et al.* (1996) reported the successful application of the monoclonal antibody, developed by Yin *et al.* (1995) to the immunocytochemical detection and semi-quantitation of 8-oxodG in aflatoxin B₁-treated cells and frozen tissue

sections, following denaturation of the DNA. This method possesses the potential for application in numerous studies to assess oxidative damage.

THE PROBLEM

It is well established that the ultraviolet wavelengths, contained within solar radiation, have numerous effects following interaction with cellular systems. Such effects are the result of photophysical events following absorption of a UV photon. Properties inherent to UV photons include the relationship between wavelength and energy, with shorter wavelength photons possessing greater energy. Therefore as wavelength changes, so does energy and as a result, so does the nature of the photophysical processes post-UV absorption. In general terms, these processes can give rise to either direct or indirect damage, examples of which include cyclobutane dimers and 8-oxodeoxyguanosine, respectively.

Solar radiation has been implicated as a major aetiological agent in such forms of skin carcinogenesis as malignant melanoma, basal and squamous cell carcinoma. Much of the literature has examined the effects of direct damage products on cell mutation and cytotoxicity. However, more recently there is increasingly compelling evidence to suggest that longer wavelengths and indirect damage may also play an important role. These longer wavelengths represent approximately 95% of the total UV contained in sunlight reaching the earth's surface and have been shown to be as carcinogenic as some of the shorter wavelengths. Despite this, little has been done to establish the relative contribution of each type of damage to total damage in various DNA-containing systems, either from broad band UV sources or solar radiation. This may, in part, be due to the difficulty associated with sensitively measuring established markers of each damage type in a single assay run. Therefore the development of assays which allow such measurements to be made is vital. Once established, assessment of, not only relative contribution to damage, but also mechanisms involved in DNA damage, would be possible. This may in future facilitate a greater understanding of the consequences of UV irradiation along with the implications for carcinogenesis.

OBJECTIVE AND AIMS

The major objective of this thesis was to assess, principally by immunochemical means, the comparative induction of direct and indirect DNA damage in naked DNA and ultimately in cultured human cells, following irradiation with either UVA or UVC. From this, the relative importance of each damage type with respect to the consequences of cellular irradiation, may be implied.

Essential to the success of this objective was the development of methods for the analysis of damage to DNA following UV irradiation, applicable to naked DNA, cultured human keratinocytes and skin biopsies from human subjects. In order to achieve this objective the following experimental aims were defined:

1. To produce polyclonal antibodies against DNA containing thymine-thymine cyclobutane dimers and to subsequently establish the specificity and sensitivity of the antibodies.
2. To establish a robust gas chromatography-mass spectrometry (GC-MS) assay to specifically and sensitively detect absolute levels of thymine-thymine cyclobutane dimers in DNA.
3. To utilise GC-MS analysis (#2) in order to quantify the number of lesions in a DNA standard to be used in the assessment of an antibody binding assay (ELISA).
4. To utilise polyclonal and monoclonal antibodies against direct and indirect types of DNA damage to detect such lesions in various model systems, including human cultured cells, following irradiation with either UVA or UVC.

CHAPTER TWO

MATERIALS AND METHODS

“There nearly always is method in madness. It’s what drives men mad, being methodical.”

- GK. Chesterton.

2.1 MATERIALS

GENERAL MATERIALS

Calf thymus DNA was from Calbiochem (Nottingham, UK) and only batches with an A_{260}/A_{280} ratio greater than or equal to 1.8 were used. Dialysis tubing with a 12-14kD cut-off was from Scientific Industries International Inc. Ltd (Loughborough, UK). Chromacol CV2 tubes were from Chromacol Ltd, Welwyn Garden City, UK. The fused silica RTX-5 column (15m x 0.25mm i.d.) coated with cross-linked 5% phenylmethysiloxane (film thickness 0.25 μ m) was from Restex Corporation purchased from Thames Chromatography, Maidenhead, UK). Helium carrier gas was from British Oxygen Company. Nitrocellulose filters and the Bio-Dot apparatus were both from BioRad (Hemel Hempstead, UK). Putative photoprotective materials were commercial prototypes from Tansafe Ltd, UK. The protein A insolubilised on Sepharose CL-4B was obtained from Sigma (Sigma-Aldrich Chemical Company).

GENERAL CHEMICALS

All chemicals were purchased as the highest purity. The following reagents were obtained from Sigma (Sigma-Aldrich Chemical Company, Poole, UK): methylene blue; Tris base; sodium chloride; Magnesium chloride; sodium acetate; ethylenediamine tetra-acetic acid; potassium phosphate; hydrogen peroxide (30% solution); phosphate buffered saline (PBS, 0.01M, pH 7.4; was prepared from the tablet form); ascorbic acid; guanosine; sodium phosphate; keyhole limpet haemocyanin; sodium periodate; sodium carbonate; sodium borohydride; acetone (Analar grade); α -tocopherol; glycine; Trizma hydrochloride and thymine.

Fisher Scientific Ltd (Loughborough, UK) was the source of the ethanol (HPLC grade); methanol (HPLC grade); xylene.

Charcoal powder; hydrochloric acid were from BDH laboratory supplies, Poole, UK.

Ferric sulphate, the source of the Fe^{2+} ions, was obtained from Aldrich Chemical Company (Gillingham, UK) as was the internal standard for 8-oxoguanine, 2,6-diaminopurine (DAP); 8-oxoguanine and formic acid (99%).

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and silylation grade acetonitrile were from Pierce and Warriner (Chester, UK). Thymine- $\alpha,\alpha,\alpha,6\text{-}^2H_4$ was obtained from MSD Isotopes (Montreal, Canada). Isolation of the thymine dimer precipitate was achieved by a cellulose nitrate membrane (Whatman International Ltd, Maidstone UK). Oligomers

containing solely two, four, ten or twelve thymines were obtained from Alta Bioscience (School of Biochemistry, University of Birmingham, Birmingham, UK). Thymine-containing oligomers of specified sequence were synthesised by the Protein and Nucleic Acid Section (Centre for Mechanisms of Human Toxicity, University of Leicester, Leicester, UK).

A Vickrad ^{60}Co source provided the gamma irradiation of DNA.

EQUIPMENT

A UVC lamp (Anderman Company, East Molesey, UK), with a maximum output (λ_{max}) at 254nm, $10.5\mu\text{W}/\text{cm}^2$ (at 1m), was the source of UVC irradiation. UVA ($\lambda_{\text{max}} = 350\text{nm}$) and UVB ($\lambda_{\text{max}} = 302$) irradiations were performed by Model-UVL-56 and Model-UVM-57, Chromatovue lamps from Knight Optical Technologies, Leatherhead, Surrey, UK., as was the Optical Radiometer. The UVA lamp had been modified by the addition of a 4mm thick glass filter, so as to remove any UVB, although later analysis showed this modification to be redundant. 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). Spectral characteristics of the UV lamps and radiometers are shown in Appendix II. It was noted that the UVB lamp contained contribution from UVC and UVA regions of the spectrum making it unsuitable for use in experiments where quantitative comparisons of product formation are made between UVB and UVA are made. However, it was used for the qualitative induction of cyclobutane thymine dimers in keratinocytes and remains described as UVB* (Chapter Six). The spectral characteristics of the UV lamps were analysed using a 0.1nm resolution Spectrapro 275 monochromator (holographic grating - 1200grooves/mm) (Acton Research Corporation, Acton, MA, USA), in conjunction with a S20 Cathode, photomultiplier tube (Acton Research Corp.) coupled to a Labdata chart recorder (Labdata Instrument Services Ltd, Surrey, UK). The traces are reproduced in Appendix II (page 315). In order for the resultant traces to remain within the range of the chart recorder, the photomultiplier voltage was set at 350V for analysis of the UVA lamp, 290V for the UVB lamp and 250V for the UVC lamp. For this reason the y-axis is labelled relative radiation intensity and therefore peak heights cannot be used for comparison of intensity between UV sources. Furthermore, the linear scale has limitations, with low level contaminating peaks not being detectable at the given photomultiplier tube voltage.

* Indicates not a pure UVB source

Freeze drying was performed on a Lyoprep-3000 (IEC, Dunstable, UK). Assessment of protein content was made on a Lambda 2, UV/VIS Spectrometer (Perkin Elmer Ltd Beaconsfield, UK). Spectrophotometric measurements were made on a Beckman DU7000 spectrophotometer. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson). The gas chromatography-mass spectrometry was performed using a Perkin-Elmer autosystem GC interfaced with a Perkin-Elmer Q-mass 910 quadrupole mass spectrometer.

IMMUNOCHEMICALS

The anti-ROS DNA antiserum was raised against DNA modified by ascorbate and hydrogen peroxide (Division of Chemical Pathology, University of Leicester - manuscript in progress). Peroxidase-labelled avidin was from Sigma. the monoclonal antibody against single-stranded DNA was from Serotec (Oxford, UK). Biotinylated goat anti-rabbit immunoglobulin and peroxidase-labelled streptavidin/biotin complex were all from DAKO. Fluorescein isothiocyanate (FITC) -labelled goat anti-rabbit secondary antibody was obtained from Sigma and 7-amino-4 methyl coumarin-3-acetic acid (AMCA) -labelled streptavidin was from Vector Laboratories (Peterborough, UK). The IgG fraction of antiserum 529 was prepared on a protein A column by Mark Cappell, Division of Chemical Pathology, University of Leicester. Monoclonal mouse anti-human p53 protein (clone DO-7) was from DAKO. FITC labelled avidin was obtained from Sigma. Both the fluoresceinated goat anti-rabbit IgG (whole molecule) and fluoresceinated goat anti-mouse IgG (Fc-specific) were obtained from Sigma, as was the propidium iodide nuclear counterstain. The monoclonal anti-8-oxodeoxyguanosine antibody was obtained as part of a kit for measuring 8-oxodG in biological fluids from Genox Corp. (Baltimore, USA).

Demonstration of peroxidase activity utilised diaminobenzidine from Sigma. Counterstaining was achieved using 0.1% Mayer's Haematoxylin from Sigma. The resinous mountant used was XAM (BDH). Vectorshield (Vector Laboratories, Peterborough) was the aqueous mountant used for the fluorescent labels.

ENZYME PREPARATIONS

Bovine pancreas deoxyribonuclease I (EC 3.1.21.1, Type IV, 1750Kunits/mg protein), *Neurospora crassa* endonuclease (EC 3.1.30.1, 238 units/mg protein), *Staphylococcus*

aureus micrococcal endonuclease (EC 3.1.3.1, 195 units/mg protein), phosphodiesterase (EC 3.1.4.1 from *Crotalus atrox* venom, Type VII, 0.18 units/mg protein) and *Escherichia coli* alkaline phosphatase (EC 3.1.3.1, Type III-S, 10.5 units/mg protein) were all from Sigma. *Streptomyces griseus* protease Type XIV (EC 3.4.24.31) was used to reveal antigenic sites. Pronase E (*Streptomyces griseus* protease Type XXV, EC 3.4.24.31), RNase (EC 3.1.27.5 from bovine pancreas, Type XII-A, 100Kunits/mg protein) and buffer chemicals, all used during the DNA extractions were from Sigma.

GUANASE ASSAY

8-Oxoguanine (2-amino-6,8-dihydroxypurine) was obtained from Aldrich Chemicals, Poole, UK. The enzyme guanase (guanine deaminase, guanine aminohydrolase, from rabbit liver, EC 3.5.4.3) was purchased from Sigma.

HPLC SYSTEM I - ANALYTICAL HPLC

HPLC system I consisted of a Beckman Programmable Solvent Module model 126, an Autosampler 507, an Analogue Interface Module 406, an EG&G electrochemical detector model 400 (Princeton Applied research, Princeton, USA) and a model 168 diode array detector (DAD). The HPLC was controlled and the data analysed using Beckman System Gold Software (all: Beckman Instruments, High Wycombe, UK).

3 μ m ODS Hypersil column (100mm x 4.6mm i.d.) was from Shandon Scientific Ltd (Runcorn, UK).

HPLC SYSTEM II

Analysis of the crude preparation was performed on a Gilson 712 reversed-phase HPLC system with ECD and diode array detection (DAD).

HPLC SYSTEM III - PREPARATIVE HPLC

The preparative HPLC system consisted of a Gilson (Gilson Medival Electronics Ltd) 306 pump, 806 Manometric module, 811c dynamic mixer, 712 HPLC controller and a Ranin Dynamax UV1 Absorbance detector. The column used was a 25cm Phase Sep, SpherisORB ODS2 column (20mm internal diameter).

IMMUNISATIONS

Methylated bovine serum albumin (BSA) was obtained from Sigma, and the adjuvant, Titermax was from Stratech Scientific Ltd (Luton, UK). Freund's Complete Adjuvant was from Sigma.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

96 well Nunc, Immuno Maxisorp ELISA plates were from Life Technologies Ltd (Paisley, Scotland). Dried skimmed milk, was provided by Tesco Stores Ltd (Cheshunt, UK). Tween 20 was from Sigma. The secondary antibody, peroxidase-labelled goat anti-rabbit immunoglobulins A, M and G, was from DAKO Ltd (High Wycombe, UK). The sulphuric acid used to stop the peroxidase reaction was from Sigma.

MOUSE MONOCLONAL ANTIBODY PRODUCTION

Complete medium consisted of RPMI 1640 (Gibco BRL, Life Technologies) containing; 1M HEPES buffer (Gibco), 200mM Glutamax (L-alanyl-L-glutamine - Gibco), 0.1M sodium pyruvate (Sigma), 100 units/ml penicillin and 100µg/ml streptomycin (Sigma). Both the 125µm mesh and a solution of Tris NH₄Cl were obtained from Sigma. T80 NS0 mouse myeloma cell line was from the ECACC (#85110503). Polyethylene glycol (PEG) and foetal calf serum (FCS - Hybri-max) were from Sigma. Selection medium consisted of complete medium, FCS, 1x HAT media supplement (Hybri-max, Sigma) and 10x hybridoma enhancing supplement (Hybri-max, Sigma).

CLINICAL SAMPLES

In order to test the antigenicity of ROS DNA for SLE serum, serum samples were collected from patients attending the outpatients department at Leicester General Hospital and who satisfied revised criteria for the classification of SLE proposed by the American Rheumatism Association (Tan *et al.*, 1982). The peroxidase-conjugated goat anti-human IgG antibody was obtained from Sigma.

Sections of human normal skin, basal cell carcinoma and naevus were all provided by Dr Scott Saunders, Dept. Pathology, University of Birmingham.

Sections of biopsies taken from UVB irradiated volunteers were obtained and processed by Dr. Alistair Robson, Dept. Pathology, University of Leicester.

KERATINOCYTE CULTURE

Human SV40 immortalised RHT keratinocytes (passage #101) were a gift from Professor Irene Leigh, Department of Dermatology, London Hospital Medical College. Medium (RM+) (Life Technologies Ltd) consisted of DMEM containing L-glutamax, 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 100U/ml, streptomycin 100µg/ml from Flow Laboratories, Irvine, UK) and mitogens (see appendix I). Hank's Balanced Salt Solution (HBSS) was from Gibco. RM- contained DMEM and Ham's F12 (3:1) without foetal calf serum and mitogens. Tissue culture plastics were from Becton Dickinson Labware (Plymouth, UK), apart from the Nunc Lab-Tek eight well chamber slides (Life Technologies Ltd). All biochemicals used were from Sigma, except normal goat serum (NGS) (DAKO); and acetone, methanol and ethanol (Fisher Scientific)

2.2 METHODS

Methods are described according to the Chapter in which they appear.

2.3 IMMUNOGENICITY AND ANTIGENICITY OF MODIFIED DNA - POSSIBLE IMPLICATIONS FOR SYSTEMIC LUPUS ERYTHEMATOSUS

2.3.1 DEVELOPMENT OF POLYCLONAL ANTIBODIES TO ROS MODIFIED DNA

PREPARATION OF METHYLENE BLUE TREATED DNA (MB DNA)

The procedure was based on the method of Seaman *et al.* (1966) and Floyd *et al.* (1989). Briefly, calf thymus DNA (0.5mg/ml in water, final concentration) was irradiated in the presence of methylene blue (optimised at 20µg/ml, final concentration in 0.1M Tris, pH 8.5) on ice, shielded from the white light source (described in the Material and Methods) by 0.5cm of water, at a distance of 4cm. After irradiation, solid sodium chloride was added to the DNA solution to a final concentration of 1M and the DNA precipitated, on ice, by the addition of ethanol until a precipitate formed. The DNA was then removed and dissolved in a minimum volume of deionised water. This was repeated twice to remove all traces of methylene blue. A DNA solution incubated with methylene blue but not exposed to light, and an untreated DNA solution acted as controls.

DETERMINATION OF LEVELS OF 8-OXO-2'DEOXYGUANOSINE IN DNA

Enzymatic DNA hydrolysis

Magnesium chloride (0.3M in distilled water) was added to the DNA samples to give a final concentration of 10mM. The solutions were then heated for three minutes at 100°C prior to rapid cooling on ice. The samples were then incubated at 37°C overnight in the presence of DNase I (0.1mg/mg DNA) and endonuclease (0.14units/µg DNA). Following adjustment of the pH to 8.0 with Tris base (1M), phosphodiesterase (0.04units/mg DNA) and alkaline phosphatase (1 unit/mg DNA) were added and the samples incubated overnight at 37°C. The pH was readjusted to 7.0 with 1N HCl and the samples stored at -20°C until analysis as described below.

Analytical HPLC - the deoxynucleoside assay

The amount of 8-oxodeoxyguanosine present was determined by reversed-phase HPLC (system I). The mobile phase was 50mM sodium acetate buffer pH 5.1 containing 1mM ethylenediamine tetra-acetic acid and 5% (v/v) methanol, at a flow rate of 1ml/min. Injections of 50µl per sample were made. The 8-oxo 2' deoxyguanosine (8-oxodG) internal

standard was at a concentration of 50nM on column, the deoxyguanosine standard was at a concentration of 50 μ M on column. Deoxyguanosine was determined by ultraviolet detection (254nm). 8-oxodG was determined by electrochemical detection at 0.6V working potential as versus a Ag/AgCl reference electrode. The results were expressed in terms of percentage and moles of 8-oxodG per mole of deoxyguanosine in the sample.

DETERMINATION OF LEVELS OF 8-OXOGUANINE IN DNA

Formic acid hydrolysis

An aliquot of DNA (20 μ g) was diluted with 200 μ l water and then treated with 350 μ l of formic acid (60% v/v, final concentration) in sealed and evacuated vacuum hydrolysis tubes, at 140°C for 30 minutes. The sample was then freeze-dried, prior to reconstitution in 500 μ l of ultrapure water. The sample was then halved. To one sample, NaOH (100mM) was added dropwise, to give a pH of 8.0-8.5. To this sample was further added 15 μ l guanase (1mg guanase reconstituted in 500 μ l water - 1.7mU guanase/20 μ g DNA) and the sample incubated at 37°C for one hour.

Analytical HPLC - the guanase assay

Injections of the DNA hydrolysates were made with and without the enzyme onto HPLC I, as described previously in section 2.1. The mobile phase consisted of 40mM potassium phosphate, 1mM EDTA, 1% (v/v) methanol, pH 5.0; with a flow rate of 1ml/min. Typically 50 μ l injections were made. Both guanine and 8-oxoguanine were identified by co-injection of authentic standards and quantitation achieved by plotting a standard curve of peak height against concentration.

PREPARATION OF LOW DOSE GAMMA IRRADIATED DNA

A 1mg/ml solution of calf thymus DNA was gamma irradiated at a dose of approximately 4.95Gy by a ^{60}Co source (at 0.48 Grays/sec).

PREPARATION OF HIGH DOSE GAMMA IRRADIATED DNA

A 1mg/ml solution of calf thymus DNA was gamma irradiated at a dose of approximately 275Gy by a ^{60}Co source (at 0.48 Grays/sec).

IMMUNISATION PROTOCOL #1

Reference bleeds were obtained prior to immunisation.

The damaged DNA solutions were boiled for 10 minutes and then cooled rapidly on ice prior to conjugation with a 1% solution of methylated BSA to produce a 500µg/ml solution of both DNA and BSA. This conjugate was then homogenised 1:1 with the adjuvant, Titermax. The rabbits (n=6; 2 per DNA preparation) were immunised with 50µl of immunogen in each hind quadricep. Test bleeds were performed after 14 and 28 days and assayed by enzyme-linked immunosorbent assay (ELISA).

ENZYME-LINKED IMMUNOSORBENT ASSAY (GENERAL PROTOCOL)

Double stranded, DNA, damaged as described above, (50µg/ml), 50µl/well, was bound to a 96 well ELISA plate by incubation, in a humidified environment at 37°C for one hour, after which the plate was washed three times with PBS. Free sites were then blocked by incubation with 150µl/well 4% (w/v) dried skimmed milk in PBS (4% milk/PBS) for one hour at 37°C in a humidified environment and the wells were then washed with PBS. The test antiserum, 50µl/well, was used at a dilution of 1/10, 1/100 and 1/1000 in 4% milk/PBS and incubated for one hour as described above. Following washing three times with PBS containing 0.05% (v/v) Tween 20, the secondary antibody, a peroxidase-labelled goat anti-rabbit immunoglobulins (IgA, IgM and IgG) at a 1/2000 dilution in milk PBS, was applied (50µl/well). After the plate had been incubated as previously described, and washed with PBS/Tween 20, the substrate solution, orthophenylenediamine (0.5mg/ml in 0.05M phosphate-citrate, pH 5.0 and containing 0.03% w/v sodium perborate) was added (50µl/well) and incubated for 15 minutes at room temperature. The reaction was stopped using 25µl/well 2M H₂SO₄. The resulting absorbance was read at 492nm, using a plate reader. The final data included correction for background values.

PREPARATION OF Fe²⁺ AND HYDROGEN PEROXIDE MODIFIED DNA (FEH-DNA)

To a solution of 1mg/ml calf thymus DNA in deionised water, was added 525µM ferric sulphate and hydrogen peroxide (3030µM) and incubated at room temperature for 30 minutes in 0.01M PBS, according to the method of Alam *et al.* (1993). At the end of incubation, reaction products were dialysed overnight at 4°C against PBS. Native and DNA treated with either hydrogen peroxide or ferrous ions alone served as controls.

PREPARATION OF UVC/HYDROGEN PEROXIDE MODIFIED DNA (UVH-DNA)

An aqueous solution (1mg/ml) of calf thymus DNA was irradiated under UVC light for 30 minutes at room temperature in the presence of hydrogen peroxide (15.1mM), as described previously (Ara *et al.* 1993). Excess hydrogen peroxide was then removed by dialysis against 0.01M phosphate-buffered saline, pH 7.4 (PBS) overnight at 4°C. Native DNA and DNA exposed to hydrogen peroxide or UVC alone were used as the appropriate controls.

IMMUNISATION PROTOCOL #2

Reference bleeds were obtained from male, New Zealand white rabbits prior to immunisation. Damaged DNA solutions were boiled for 10 minutes and then cooled rapidly on ice to yield single stranded DNA, prior to conjugation with a 1% solution of methylated BSA. This conjugate was then homogenised 1:1 with Freund's Complete Adjuvant. The rabbits (n=4; 2 per each of the 2 DNA preparations) were immunised with 1ml of immunogen subcutaneously at multiple sites. The animals were boosted intravenously with conjugate alone, in saline, four weeks later. Test bleeds were performed 10 days post-boost. The animals were sacrificed and the sera collected when a sufficient titre of antibody had been obtained. The latter was assessed by enzyme-linked immunosorbent assay (as described above).

Successful antisera were then characterised as described in the following section.

COMPETITIVE ELISA

A competitive ELISA was performed for the polyclonal antiserum, arising from the immunisation with UVH-DNA initially, as described below.

The method was essentially as previously described (Herbert *et al.* 1994). 96 well plates were coated with double stranded, ROS-modified DNA (50µg/ml in PBS) by incubation at 37°C in a humidified chamber for one hour. Free sites were then blocked by incubation of 4% milk/PBS for one hour at 37°C in a humidified chamber. Anti-ROS-DNA serum, diluted to 1:100 in 4% milk/PBS, was mixed with increasing amounts of variously treated, double stranded DNAs and then incubated in the wells for one hour at 37°C. Detection of bound antibody was achieved as described for the non-competitive ELISA.

The initial results from this assay required that further characterisation be performed by competitive ELISA, UVC-modified DNA as the solid phase antigen and various thymine-containing oligomers as the competitors.

ANTIBODY PURIFICATION ON PROTEIN A COLUMNS

Preparation of the protein A column

Purification of Ab529, for FACS analysis, was performed by Mark Cappell, Division of Chemical Pathology, University of Leicester.

The protein A column was prepared as follows; a small amount of glass wool was placed at the base of a 1 ml syringe. 0.125g (dry weight) of the protein A/Sepharose conjugate was swollen with ultra pure water until it formed a slurry. This was then introduced into the syringe, whereupon it formed a gel. This was then washed with ultrapure water.

Purification of the antiserum

The pH of the crude antibody preparation was adjusted to 8.0 by adding 1/10 volume of 1.0M Tris (pH 8.0). The antibody solution was then passed through the protein A column. The column was subsequently washed with 10 column volumes of 100mM Tris (pH 8.0) and then 10 column volumes of 10mM Tris (pH 8.0). Bound antibody was then eluted from the column using 100mM glycine (pH 3.0) in 500 μ l volumes and collected in 1ml Eppendorf tubes containing 50 μ l 1M Tris (pH 8.0). The tubes were then gently mixed to return the pH to neutral. The protein-containing fractions were then identified spectrophotometrically by absorbance at 280nm.

2.3.2 DEVELOPMENT OF MONOCLONAL ANTIBODIES TO ROS MODIFIED DNA

The UVC/H₂O₂ DNA immunogen, used for monoclonal production, was prepared as described earlier (Section 2.3.1).

PREPARATION OF ASCORBATE/HYDROGEN PEROXIDE MODIFIED DNA (ASH-DNA)

Site specific \cdot OH attack of DNA, utilising endogenous metal ions was performed using hydrogen peroxide and ascorbate. An aqueous solution (0.5mg/ml) of calf thymus DNA was incubated with 200 μ M H₂O₂ and 10mM ascorbate for one hour at 37°C. This was followed by overnight dialysis against deionised water at 4°C. Native, hydrogen peroxide treated and ascorbate treated DNA acted as controls.

IMMUNISATION PROTOCOL FOR MONOCLONAL ANTISERA TO DAMAGED DNA

Female, 6 week old mice were immunised according to the following protocol. Reference bleeds were obtained prior to immunisation. The damaged DNA solutions (UVC/H₂O₂, and ascorbate/H₂O₂) were boiled for 10 minutes and then cooled rapidly on ice to yield single stranded DNA, prior to conjugation with a 1% solution of methylated BSA. This conjugate was then homogenised 1:1 with Freund's Complete Adjuvant. The mice (n=6; 3 per each of the 2 DNA preparations) were immunised with 200µl immunogen mix subcutaneously at multiple sites. The animals were boosted subcutaneously with 50µg immunogen in 100µl sterile saline seven days later. Test bleeds were performed 7 days post-boost. The animals were sacrificed, three days after a final intraperitoneal boost, and spleen and sera collected when a sufficient titre of antibody had been obtained. The latter was assessed by enzyme-linked immunosorbent assay. The spleen was collected into a bijoux containing 5ml of medium (see Materials). The medium used contained additives described in the Materials, but remained free from foetal calf serum until the fusion was completed.

MOUSE MONOCLONAL PRODUCTION

The preparation of an immortalised hybridoma cell line was performed by Julie Chamberlain, Cell Culture Facility, Centre for Mechanisms of Human Toxicity, University of Leicester, Leicester.

The surface of a 125µm mesh was washed with a little medium (as above) prior to the spleen being forced through with the plunger of a 10ml syringe. The cells were washed with medium and counted following Tris NH₄Cl lysis of the red cells. The spleen and NS0 cells were mixed at a ratio of 4:1 and pelleted at 300g. NS0 cells are an established mouse, fusion partner cell line. The medium was removed, the pellet loosened and 800µL of PEG at 41°C was added slowly over a minute stirring continuously. Medium was then used to dilute the PEG, stirring continuously. The cells were then centrifuged and resuspended in RPMI supplemented with FCS to 15% at 2x10⁶ spleen cells per ml. These cells were then plated out, 1ml per well, in a 24 well plate and incubated at 37°C in 5% CO₂. After 24 hours, selection medium was added and the cultures fed as required over the next 2-3 weeks with selection medium. As the wells approached confluence the supernatants were tested and the wells re-fed

Screening of supernatants

The medium supernatants were tested by ELISA (2.3.1), with damaged DNA appropriate to the immunogen, as the solid phase antigen. Neat culture medium was used as a negative control and final whole serum as a positive control.

Dilution cloning

1ml of cells were harvested from a positive well and the cells diluted to 10 cells per ml. The cells were then plated out onto a 96 well plate at 100 μ l per well, i.e. 1 cell per well. To the wells was then added a further 100 μ l of medium and incubated at 37°C in 5% CO₂. At day six the wells were monitored for single colonies and fed with fresh medium. Testing occurred around day 9-10, with extra medium being added the day before. Three to six positives were chosen and expanded prior to isotyping and freezing.

2.3.3 DEVELOPMENT OF POLY- AND MONOCLONAL ANTIBODIES TO 8-OXODEOXYGUANOSINE

Preparation of 8-oxoguanosine

The ribonucleoside immunogen, 8-oxo-guanosine (8-oxoGR), was synthesised by a method based on that of the Udenfriend system (Kasai *et al.* 1984). Guanosine (1g) was dissolved in 1560ml of 0.13M sodium phosphate buffer (pH 6.8) in a two litre flat bottomed flask. 140ml of 0.1M ascorbic acid, 65ml of 0.1M EDTA and 13ml of 0.1M FeSO₄ were then added successively. Oxygen gas was then bubbled through the solution at 37°C for three hours, in the dark. The pH was then adjusted to 3.7 with 1M HCl. 10g of charcoal powder was then added with stirring. The mixture was then poured into a glass column (1m long, 2cm diameter) with a sintered glass filter and allowed to settle. The column was drained and the residue washed with ultrapure water. The material adsorbed to the charcoal was then eluted with aqueous acetone (500ml, 1:1 v/v). The resulting eluent was then freeze-dried. The crude preparation was then dissolved in a minimum volume of HPLC grade methanol.

Initial analytical HPLC of crude 8-oxoguanosine preparation

Analysis of the crude preparation was performed on HPLC system II (Section 2.1, Materials), with ECD and diode array detection (DAD). The mobile phase was 50mM potassium phosphate and 10% methanol at pH 5.5 used at a flow rate of 1ml/min. 40 μ L samples were injected onto a 25cm Phase Sep ODS2, C-18 column (4.6mm internal

diameter). Following confirmation of a successful synthesis, preparative HPLC was performed to isolate the 8-oxoguanosine (8-oxoGR).

Preparative HPLC of crude 8-oxoguanosine preparation

Preparative HPLC was performed by system III (Section 2.1, Materials). Typically 2500 μ L injections were made onto a fully endcapped, 25cm Phase Sep, SpherisORB ODS2 column (20mm internal diameter). The mobile phase was 50mM potassium phosphate and 10% methanol at pH 5.5 at a flow rate of 8ml/min. 4ml fractions were collected every half minute. Collected fractions of the peak eluting just after the main peak of guanosine were examined spectrophotometrically to confirm the presence of 8-oxoguanosine. The collected samples were then pooled and re-run on the above HPLC system with 2.5% methanol in ultrapure water as the mobile phase to purify the sample from the salts.

A sample of the collected fraction was sent for confirmatory electrospray mass-spectrometry by Dr Gavain Sweetman, Biomonitoring Group, MRC, Centre for Mechanisms of Human Toxicity, University of Leicester, Leicester.

Periodate linkage of 8-oxoguanosine and guanosine to protein

The isolated 8-oxoGR was then linked either to keyhole limpet haemocyanin or bovine serum albumin which were to act as protein carriers based on the method of Erlanger *et al.* (1964). The applicability of the periodate linkage method used requires that the ribonucleoside derivative of the base lesion be available, hence the use of guanosine as the starting material. The use of two carrier proteins allowed one to be used as immunogen and the other as ELISA antigen for testing the immune response, minimising the risk of detecting antibodies displaying cross-reactivity to carrier protein.

8-oxoguanosine (3mg) was dissolved in 100 μ L of 0.1M sodium periodate. This solution was kept on ice for 15 minutes whilst the protein (BSA or KLH - 5mg in 400 μ L NaCl) was added dropwise. The pH was then adjusted to 8.5-9.0 with 0.15M Na₂CO₃ (pH 12.0) and the solution stirred for 20 minutes. A solution of 0.15g sodium borohydride in 10.0ml ultra pure water was then added and the solution left for 18 hours. There then followed extensive dialysis against PBS at 4°C. The solution was then freeze dried and reconstituted in ultrapure water to 5mg/ml protein. Guanosine was also linked to BSA by the above protocol.

Immunisation protocol for monoclonal antisera to 8-oxoguanosine

Female, 6 week old mice were immunised according to the following protocol. Reference bleeds were obtained prior to immunisation. The 8-oxoguanosine/KLH conjugate was then homogenised 1:1 with Freund's Complete Adjuvant. The mice (n=4) were immunised with 50µL immunogen mix subcutaneously at multiple sites. The animals were boosted subcutaneously with 50µg immunogen in 100µL sterile saline four weeks later. Test bleeds were performed 10 days post-boost. The animals were sacrificed, three days after a final intra peritoneal boost, and spleen and sera collected when the appropriate titre of antibody had been obtained. The latter was assessed by enzyme-linked immunosorbent assay. The spleen was collected into a bijoux containing 5ml of medium.

Mouse monoclonal production - 8-oxoguanosine

Monoclonal production was performed as described earlier in section 2.3.2 by Core Tissue Culture Facilities (Centre for Mechanisms of Human Toxicity, University of Leicester, Leicester, UK). Screening was performed as in section 2.3.1 only with 8-oxoguanosine/BSA conjugate as the solid phase antigen. In later screenings this was replaced by double and single stranded methylene blue damaged DNA as it appeared to possess a greater number of antigenic determinants. Double and single stranded native DNA was also included. Dilution cloning was carried out as described earlier (section 2.3.2).

Mouse monoclonal production - UVH and ASH DNA

Monoclonal production was performed as described earlier in section 2.3.2 by Dr. Paul Butler, Division of Chemical Pathology (University of Leicester, Glenfield General Hospital, Leicester, UK). Screening was performed as in section 2.3.1 only with UVH or ASH as the solid phase antigen.

2.3.4 ANTIGENICITY OF ROS-MODIFIED DNA FOR SLE SERA

Preparation of FEH DNA

As described above (Section 2.3.1).

Enzyme-linked immunosorbent assay

Avidity of serum anti-DNA antibodies, diluted 1:100, for UV treated DNA was detected by ELISA, essentially as described above. The following modifications were made; the solid phase antigen was FEH modified DNA, with native DNA as a control and blocking was performed with 1% BSA. ASH treated DNA acted as a positive control. Peroxidase-conjugated goat anti-human IgG antibody, diluted 1 in 5000, was used to detect the autoantibodies.

2.4 APPLICATION OF GC-MS FOR THE EVALUATION OF DAMAGE TO DNA FOLLOWING UV IRRADIATION

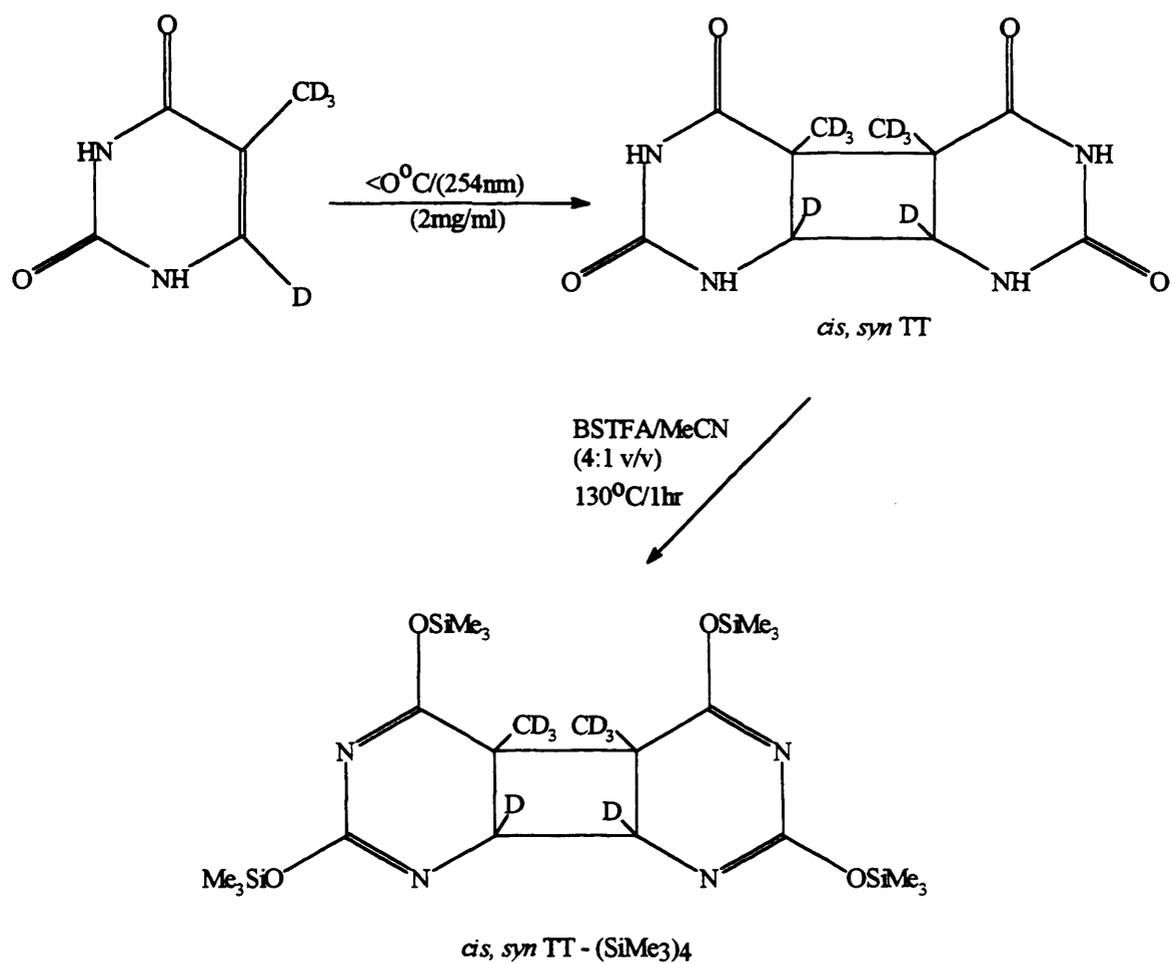
GC-MS assays already exist for the evaluation of oxidative DNA damage induced by UV light (Doetsch *et al.*, 1995), but not the most abundant lesion, the *cis-syn* cyclobutane thymine dimer. An assay was developed in collaboration with Dr Ian Podmore (Division of Chemical Pathology, University of Leicester), using stable isotope-dilution mass spectrometry, to address this point.

2.4.1 PREPARATION OF CYCLOBUTANE THYMINE DIMERS (*CIS-SYN* CYCLOBUTADITHYMINE)

The method used was based on that of Wang (1961). A frozen, aqueous solution of thymine (2mg/ml) was UV irradiated on solid CO₂ using a UVC lamp, at a distance of 4cm for 5 hours (maximum output at 254nm of 10.5μW/cm² at a distance of 1m). The solution was then thawed and the resultant precipitate isolated by filtration onto a 0.45μm cellulose nitrate membrane and thoroughly dried in a dessicator. Using thymine- $\alpha,\alpha,\alpha,6\text{-}^2\text{H}_4$, the deuterated internal standard (*cis-syn* cyclobutadithymine-²H₈) was prepared in the same manner (Scheme 2.1).

2.4.2 ASSESSMENT OF DIMER PURITY BY HPLC

The purity of the dimers was checked by high performance liquid chromatography (HPLC). Isocratic reversed-phase HPLC was performed using system I (Section 2.2, Materials), onto which 50μl injections were made. The mobile phase consisted of 10mM potassium phosphate buffer, pH 5.1 in 2.5% (v/v) methanol at a flow rate of 1ml/min. UV detection of the dimer was carried out at 230nm and diode array analysis, over the range 190-350nm, was used for peak identification. Using a standard, HPLC analysis with UV detection was also carried out at 260nm to assess possible thymine impurity. Both labelled and non-labelled dimers were found to be free of thymine.



Scheme 2.1. Preparation of cyclobutane thymine dimers (*cis-syn* cyclobutadithymine).

2.4.3 PREPARATION OF TRIMETHYLSILYL (TMS) ETHER DERIVATIVES

Aqueous solutions (20 μ M) of *cis-syn* cyclobutadithymine (TT- 2 H₀) and *cis-syn* cyclobutadithymine- 2 H₈ (TT- 2 H₈) were prepared in 2L volumetric flasks, covered with aluminium foil to minimise any possible photoreversion to monomeric thymine, by stirring for several hours at room temperature. Aliquots (3ml) were placed in pre-silanised tubes and lyophilised overnight in a freeze-drier. The samples were then capped with PTFE under a nitrogen atmosphere and derivatised using 100 μ l of a BSTFA/acetonitrile (4:1 v/v) mixture heated at 130°C for 60 minutes.

2.4.4 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

For identification of the trimethylsilyl (TMS) ether derivatives, 1 μ l of sample was injected into the GC injection port using the split mode (split ratio 20:1). GC-MS in the total ion scan mode was performed using a Perkin-Elmer Autosystem GC interfaced with a Perkin-Elmer Q-mass 910 quadrupole mass spectrometer. The injection port and GC-MS transfer line were kept at 250°C and 280°C respectively. Separations were carried out on an RTX-5 fused silica capillary column. Helium (CP grade) was the carrier gas with a constant flow rate of 1ml/min. The column temperature was increased from 120°C to 250°C at 10°/min after an initial 2 minutes at 120°C, subsequently, the temperature was increased from 250°C to 280°C at 30°/min and then kept at 280°C for 2 mins.

2.4.5 UV IRRADIATION OF DNA

A solution of calf thymus DNA was prepared in 0.01M PBS, pH 7.4, at a concentration of 1mg/ml. 1ml aliquots (2mm depth) were irradiated at room temperature with the UVC lamp used previously (λ_{max} = 254nm, maximum output 10.5 μ W/cm² at a distance of 1m). Irradiations were carried out at a distance of 4cm and the dose received calculated based on measurement by the sensor (MP-125) in conjunction with the radiometer (MP-100), for a dose range of 0-3500Jm⁻² (i.e. 0 to 120s). The amount of DNA for each sample was determined spectrophotometrically (Beckman DU-7000) at 260nm (A_{260} of 1 = 50 μ g DNA/ml). 2nmol of TT- 2 H₈ was added as an internal standard to each aliquot containing 100 μ g DNA and the samples were subsequently lyophilised.

2.4.6 CHEMICAL HYDROLYSIS AND DERIVATISATION OF DNA

It has been shown previously that *cis-syn* cyclobutadithymine is released from DNA by chemical hydrolysis using formic acid (Carrier and Setlow, 1972; Cadet *et al.* 1983). In this

study DNA (100 μ g) was treated with 0.5ml formic acid (60%v/v) in evacuated and sealed tubes for 45 minutes at 140°C. Samples were then transferred to silanised tubes and derivatisation was performed using 100 μ L of a mixture of BSTFA/acetonitrile (4:1 v/v) heated at 130°C for 60 minutes.

2.4.8 ANALYSIS OF UV IRRADIATED DNA

Derivatised samples (1 μ l) of UV-irradiated DNA (\approx 1 μ g) were auto-injected with the injection port in splitless mode. In addition, separate samples (1 μ l) of a mixture of derivatised labelled and non-labelled dimer were injected for generation of a calibration curve. All other GC parameters i.e. oven, transfer line, injector temperatures and column conditions were as described earlier (Section 2.4.4). Mass spectral detection was carried out with the instrument in the selected ion monitoring (SIM) mode for increased sensitivity.

2.4.9 QUANTITATIVE DETERMINATION OF 8-OXOGUANINE BY GC-MS

8-oxoguanine levels in DNA following UVC irradiation were determined for comparison to the values obtained for cyclobutane thymine dimers.

GC-MS methodology

The DNA analysed was obtained following irradiation as described in Section 2.4.5. 2,6-diaminopurine (DAP) was used as the internal standard and 8-oxoguanine was the analyte under investigation. Concentrations of DAP and 8-oxoG were determined spectrophotometrically (λ_{max} 283nm) as described by Hamberg and Zhang (1995) and used to produce a calibration curve. The methods for hydrolysis and derivatisation were essentially as above (Section 2.4.6) with the following modifications; hydrolysis was performed at 140°C for 45mins and derivatisation was at 110°C for one hour.

2.4.9 ELISA-BASED INVESTIGATION OF UVC-INDUCED DNA DAMAGE

Detection of damage by avidin, Ab529 and an anti-single-stranded antibody

The DNA used for analysis was irradiated as described in Section 2.3.1. This DNA (50 μ l per well at 50 μ g/ml in PBS) was then bound either to a Nunc 96-well ELISA plate, by incubation for one hour; or to a multiscreen-HV 96-well plate, by vacuum, following incubation for ten minutes at room temperature. The DNA on each plate was then probed by four affinity techniques:

Avidin assay

Blocking was performed by incubation with 1% gelatin (100µl/well, heat dissolved) for one hour at 37°C. This was pulled through the membrane under vacuum, prior to washing three times with 0.05% Tween in 0.01M PBS (PBST). Avidin-peroxidase conjugate (1mg/ml, diluted 1:500 in PBS) was added 50µl/well and incubated for one hour at 37°C. The membrane was then washed five times with PBST. The peroxidase substrate was made up as described previously (Section 2.3.1) and incubated (50µl/well) for ten minutes in the dark at room temperature. 25µl/well of 2M sulphuric acid was used to stop the reaction and the solutions in the wells transferred to a 96-well ELISA plate where the absorbances were read, at 492nm, on an Anthos 2001 plate reader.

Ab529

Ab529, diluted 1/2000 (in 4% skimmed milk in PBS) was used to probe the damaged DNA on both plates using the ELISA protocol described in Section 2.3.1.

Anti-single-stranded monoclonal antibody

The antibody, diluted 1/1000 (in 4% skimmed milk in PBS), was used to probe the damaged DNA according to the ELISA protocol described in Section 2.3.1. The primary difference was that the secondary antibody was a peroxidase-labelled, goat anti-mouse immunoglobulins (IgG, IgA, IgM).

Anti-ROS damaged DNA antibody

An experimental polyclonal antiserum, raised to ascorbate/H₂O₂ DNA within the Division of Chemical Pathology, has been tentatively characterised as to recognising ROS DNA. This antiserum was used diluted 1/100 (in 4% skimmed milk in PBS) and used in the ELISA protocol described Section 2.3.5.

2.5 CHARACTERISATION OF ANTIBODIES TO UV-INDUCED DNA DAMAGE AND LIMITS OF DETECTION

2.5.1 IMMUNOCHEMICAL INVESTIGATION OF UV IRRADIATED DNA

Preparation of UVC- and UVB- and UVA-modified DNA (UVC, UVB and UVA DNA)

UVC DNA, used for investigation by GC-MS and ELISA, was irradiated as described previously (Chapter 2.4). In essence, 1ml aliquots were irradiated at distance of 4cm from the UVC lamp (λ_{max} 254nm) for a dose range of 0-300 seconds (i.e. 0-8700 Jm⁻²), at room temperature. UVB and UVA irradiations were of 1ml aliquots over an equivalent dose range as for the UVC-treated samples. Additional doses of UVC were required and the doses of 116, 175, 233 and 291 Jm⁻² were included. The amount of DNA in each sample was determined spectrophotometrically (Beckman DU-7000) at 260nm (A_{260} of 1 = 50 μ g DNA/ml).

Characterisation of antisera by competitive ELISA

Aqueous solutions (1mg/ml in ultrapure water) of calf thymus DNA (1ml aliquots) were irradiated, on ice, under a UVC light for 30 minutes at a distance of 6cm. Synthetic oligonucleotides were irradiated in the same manner to that described for DNA above.

The method was essentially as previously described by Herbert *et al.* (1994). Briefly, 96 well plates were coated with double stranded, UVC modified DNA (50 μ g/ml in PBS) by incubation at 37°C in a humidified chamber for one hour. Free sites were then blocked by incubation with 4% milk/PBS for one hour at 37°C in a humidified chamber. The UVC/H₂O₂ antiserum or Ab529, diluted to 1:100 and 1:2000 respectively (the latter having been determined as optimal by Herbert *et al.* 1994), in 4% milk/PBS, was mixed with increasing amounts of variously treated, double stranded DNAs, or oligonucleotides and then incubated in the wells for one hour at 37°C. Detection of bound antibody was achieved as described for the non-competitive ELISA. Percentage inhibition of antibody binding to the solid phase antigen (UVC DNA) by competitive inhibition was calculated as:

$$\% \text{ inhibition} = \left(1 - \left(\frac{A_{492} - \text{No primary}}{\text{No competitor} - \text{No primary}} \right) \right) \times 100$$

Photoisomerisation of (6-4) photoproducts by UVB

(6-4) photoproducts are most effectively isomerised to the Dewar isomer by irradiation with UV wavelengths which lie between 310 and 320nm (Mitchell and Rosenstein, 1987). Therefore, to establish the nature of the lesion primarily detected by these antisera the following protocol was performed. UVB (λ_{max} 302nm) irradiation of DNA, previously irradiated with UVC (8700 Jm^{-2}) was performed at doses of 0-1000 Jm^{-2} .

2.5.1.4 Determination of antigen in DNA (ELISA)

The pertinent double stranded, damaged DNA under investigation ($50\mu\text{g/ml}$), $50\mu\text{l/well}$, was bound to an ELISA plate (96 well, Immuno plate Maxisorp, Nunc) by incubation, in a humidified environment at 37°C for one hour. The ELISA was then performed essentially as described in Section 2.3.1. In this case the test antisera were applied $50\mu\text{l/well}$, either at a dilution of 1/100 (X18), or 1/2000 (Ab529) in 4% milk/PBS and incubated for one hour as described previously.

Determination of the limit of detection

A solution of DNA (1mg/ml) was irradiated with a dose of 436 Jm^{-2} UVC, to induce a known amount of dimer, as determined by GC-MS. Doubling dilutions of this DNA were made across an ELISA plate, with a starting concentration of $50\mu\text{g/ml}$ (PBS). Previous attempts to dilute in 4% milk/PBS were unsuccessful and no antiserum binding was seen. Investigation of this lead to the conclusion that the milk was competing with the DNA to bind to the plate, therefore PBS was used. ELISA detection of damage was made using the UVC/ H_2O_2 antiserum and Ab529 at dilutions of 1:100 and 1:2000 respectively, following the method described above.

Investigation of UV irradiated DNA by GC-MS

Immunochemical investigation of the UVC irradiated DNA was compared with analysis by gas chromatography-mass spectrometry. The GC-MS method was as described in Section 2.4.4.

2.6 INVESTIGATION OF UV-INDUCED DNA DAMAGE IN A KERATINOCYTE CELL LINE AND BIOPSIES FROM HUMAN SUBJECTS

2.6.1 GENERAL KERATINOCYTE CULTURE PROTOCOL

Human immortalised RHT keratinocytes were grown to confluence in RM+ (Chapter 2.1 - Materials) and routinely passaged every 3-4 days as required. The cells were maintained in 250ml tissue culture flasks under a humidified atmosphere of 95% air, 5% CO₂ at 37°C.

Cell counting

Cell number was determined by the Trypan Blue assay. Briefly, a 20µL volume of cell suspension was mixed with an equal volume of Trypan Blue (0.4% w/v in 0.8% w/v sodium chloride, 0.06% w/v dipotassium hydrogen phosphate). The resultant mix was applied to a haemocytometer and the cells excluding the dye were deemed to be viable and counted under a x40 microscope objective. Total cell number were calculated thus:

$$\text{Viable cells per ml} = \text{viable cells counted} \times 10^4 \times \text{dilution factor.}$$

2.6.2 QUALITATIVE DETECTION OF UVB-INDUCED DAMAGE IN KERATINOCYTES

Cells on Chamber Slides

Human immortalised RHT keratinocytes (passage #101) were grown to confluence in RM+ and routinely passaged every 3-4 days. At 50% confluence the RHT cells were seeded in eight well chamber slides and left overnight until they were 60-80% confluent.

Pre-loading with α -tocopherol

The putative protective effect of α -tocopherol against UVA-induced ROS (Clement-Lacroix *et al.*, 1996) was assessed by the addition of a 200µM solution of α -tocopherol (final concentration) to the medium of selected wells 24 hours 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), 1, 2 and 3J/cm² UVA, using a long wave UV Blak Ray lamp or 0, 13.5, 27 and 108mJ/cm² using the UVB* lamp. Control wells on the same slide were sham irradiated. Fresh medium was then replaced to the chambers and the cells incubated for 1, 6 or 24 hours at which points the

slides were removed and the cells fixed by the addition of 1:1 methanol/acetone and left at 4 °C for at least ten minutes. Following fixation the cells were allowed to air-dry and stored at 4°C.

Immunocytochemical staining

Non-specific binding was blocked by incubation of the chamber slides with 2% normal goat serum in 0.01M PBS, following rehydration in PBS. Detection of thymine-thymine CPD, following UVB irradiation, was performed by the incubation of cells with Ab529 diluted 1/5000 in 2% normal goat serum (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 fluorescein isothiocyanate (FITC)-labelled avidin was also investigated. The avidin-FITC conjugate (1mg/ml), applied at a 1 in 1000 dilution in 2% normal goat serum in 0.01PBS overnight at 4°C. All chamber slides were washed in 0.01M PBS prior to a final wash in deionised water prior to the slide being allowed to air-dry then mounted in Vectorshield.

2.6.3 QUANTITATIVE ELISA DETECTION OF DNA DAMAGE FROM UV IRRADIATED KERATINOCYTES

Cells were cultured as described above (Chapter 2.6.1) until eight 250ml flasks were confluent. The cells were subsequently seeded into eight Nunc Delta Petri dishes and grown to near confluence. Each dish represented a single UVB dose and, following removal of medium and washing with Hank's Balanced Salt Solution, was then irradiated, on ice, with doses between 0 and 21.6Jcm⁻² (UVA). Following irradiation, the cells were lifted from the flask using TE, which was then neutralised with an equal volume of medium, and pelleted at 400g for ten minutes at 4°C. The supernatants were discarded and the DNA was then extracted using pronase E extraction (see below).

Pronase E DNA extraction

The cells were resuspended in 5-10ml PBS and the pelleted at 700g for 15 minutes. The cells were then resuspended in 1.75ml of ice-cold Buffer 1 (5mM trisodium citrate, 20mM NaCl pH 6.5). 2ml of Buffer 2 (20mM Trizma base, 20mM EDTA, 1.5% sarkosyl, pH 8.5) was then added with vigorous mixing, finally 250µg of RNase A in RNase buffer A (50mM Tris-HCl, 10mM EDTA, 10mM NaCl, pH 6.0) was added, prior to incubation at 37°C for one hour. 2mg of Pronase E in 0.5ml Buffer 1 was added and incubated overnight. 2ml of

Buffer 3 was added along with 0.5ml 7.5M ammonium acetate and mixed by inversion after each addition. Finally the DNA was precipitated by the addition of 18ml ice cold ethanol. The DNA was then spooled onto a pipette hook and washed in 70% and then 100% ethanol for ten minutes each. Residual ethanol was removed by a stream of nitrogen.

ELISA of extracted DNA

The dried DNA pellets were rehydrated in 1ml of ultra-pure water and the DNA content determined by the absorbance at 260nm. The DNA was bound to the 96 well ELISA plate at a concentration of 50µg/ml in PBS. The rest of the protocol was performed essentially as described previously (Chapter 2.3.5). The primary antisera used were Ab529 (diluted 1/5000), the UVC/H₂O₂ antiserum (diluted 1/100) and an anti-ROS damaged DNA polyclonal antiserum (Division of Chemical Pathology - manuscript in preparation), diluted 1/100. Additional probes were used for the UVA experiment; peroxidase-labelled avidin (1/500) and a commercial anti-8-oxodG monoclonal antibody (prepared by full reconstitution in buffer as advised by the manufacturer). All dilutions were in 4% milk/PBS.

2.6.4 EXAMINATION OF UVA-INDUCED DAMAGE IN KERATINOCYTES BY FLOW CYTOMETRY

Immunostaining of cells in culture with Ab529, following UVA irradiation has been previously shown by the Division of Chemical Pathology. The involvement of p53 protein along with dose response and stage of cell cycle have not been established. The following methods were adapted and developed for these specific experimental applications. This included modifications of cell culture procedures, maximisation of cell fixation, optimisation of flow cytometric conditions (including cell concentration, primary and secondary antibody titers).

Culture Medium

Medium (RM+) consisted of DMEM containing L-glutamax, Ham's F12 (Imperial) (3:1), 10% heat inactivated foetal calf serum (FCS) and mitogens (see appendix I).

Medium (RM-) used for cell synchronisation experiments contained DMEM and Ham's F12 alone.

Cell Culture and irradiation

Human immortalised RHT keratinocytes were grown to confluence in RM+ and routinely passaged every 3-4 days. At various passage numbers cells were taken and frozen in 10% dimethyl sulphoxide (DMSO) plus 40% RM+, plus 50% FCS in liquid nitrogen. The RHT cells were seeded in Petri dishes at 2×10^6 cells per dish and left overnight until they were 60-80% confluent. Cell synchronisation was achieved by culturing in RM- (see Chapter 2.1) for 48 hours (serum deprivation). Complete medium was reintroduced to cells and, after rinsing with Hank's Balanced Salt Solution (HBSS), they were irradiated at room temperature with 0 (control) 0.5 or 1 J/cm^2 UVA, using a long wave UV Blak Ray lamp; 0, 1, 2, 3, and 24 hours post-reintroduction of complete medium. The cells were incubated with 2ml of 0.02% EDTA in PBS at 37°C for 30 minutes and then 200 μl of Tris-EDTA ($\times 10$ TE) buffer was added for 10 minutes at 37°C . A threefold volume of 2% foetal calf serum in PBS was then added, prior to filtration of the cells through a 25 μm gauge needle to obtain a single cell suspension. The cells were pelleted at 400g for 10 minutes at 4°C , before the cell number was estimated and the cells being fixed by the addition of 1:1 methanol/acetone, followed by agitation and incubation at 4°C for at least ten minutes.

Immunostaining

It was hoped that it would be possible to determine Ab529 and anti-p53 binding in the same sample, however, appropriately labelled secondary antibodies were not available, meaning the secondary antibodies for both primary antibodies were to be FITC-labelled. This meant the quantitation of binding of the two probes had to be carried out separately, effectively doubling the number of experiments carried out.

For immunofluorescence staining, the fixed cells were harvested by centrifugation before resuspension in 250 μl of incubation buffer A (0.01M PBS, 20mM EDTA, 0.5% Tween 20, 10% normal goat serum). The primary antibodies; 529 (IgG fraction) or monoclonal mouse anti-human p53 protein were added at a dilution of 1 in 100 in 0.1% NGS/PBS and

incubated overnight at 4°C. The cells were then washed twice with 20mM EDTA in 0.01M PBS, before resuspension in 250µl incubation buffer A and addition of the appropriate fluoresceinated (either goat anti-rabbit IgG, for 529, or goat anti-mouse IgG, Fc-specific, for p53) secondary antibody at 1 in 80 or 1 in 160 respectively. Following incubation for one hour on ice, the cells were then washed twice with PBS/EDTA and finally resuspended in 800µl sterile PBS. To this was added 100µl of propidium iodide solution (100µg/ml in sterile water) and 100µl of RNase solution (1mg/ml in sterile water) and analysis performed following incubation for 15 minutes at 37°C. The samples were kept on ice during analysis.

Flow Cytometric Analysis

Quantitation of the percentage of DNA damage and p53 protein present, as determined by the amount of antibody binding, was performed using a FACScan flow cytometer. Cells with increased green fluorescence, compared to controls, due to antibody binding, were judged to be UVA-affected. The nuclear counterstain, propidium iodide, enable cell cycle analyses to be performed based on the DNA content per cell (Creamer, 1992).

2.6.5 IMMUNOHISTOCHEMICAL DETECTION OF UV-INDUCED DNA DAMAGE

Frozen sections of normal skin and formalin-fixed, paraffin-embedded sections of breast tissue were obtained from the Breast Cancer Research Unit, Department of Pathology, University of Leicester, Glenfield Hospital. The polyclonal anti-UV damaged DNA rabbit antiserum was Ab529.

Pre-treatment to irradiation.

Frozen sections were fixed for ten minutes in cold (-20°C) Analar grade acetone, allowed to dry and then equilibrated in 0.01M phosphate buffered saline (PBS) pH 7.2, prior to irradiation on ice, covered in PBS. Formalin-fixed, paraffin-embedded sections were dewaxed in xylene and then rehydrated by immersion in a series of 99% and 95% ethanol solutions, diluted with distilled water, prior to rinsing in distilled water and equilibrating in PBS.

PRE-TREATMENT TO DETECTION.

UV Irradiation

The sections on slides were irradiated on ice for either 15, 30 or 60 minutes using UVA and UVC combined (using the Anderman lamp described in section 2.1), at a distance of 6cm giving a dose of 4.2, 8.4, 16.9Jcm⁻² (UVA) and 2.6, 5.2, 10.5Jcm⁻² (UVC).

Post-irradiation, protease digestion of formalin-fixed tissues

Sections were immersed in warmed (37°C) PBS for five minutes prior to being placed in 0.05% protease type XIV solution for five minutes at 37°C. The sections were then washed in tap water and equilibrated in PBS.

Blocking endogenous peroxidase

Fixed, frozen sections were immersed in 0.3% (w/v) solution of hydrogen peroxide in distilled water for fifteen minutes at room temperature, then rinsed in PBS. Formalin-fixed, paraffin-embedded sections were immersed in a 2% (w/v) solution of hydrogen peroxide for 30 minutes at room temperature.

DETECTION

Peroxidase-labelled streptavidin/biotin method

The slides were drained and excess buffer blotted from around the sections. They were then covered with 20% normal goat serum in PBS, then incubated in a covered chamber for ten minutes at room temperature. Without rinsing, the slides were drained, excess serum removed from around the sections and the primary antibody (Ab529) was added, diluted 1 in 2000 in 2% normal goat serum in PBS. The slides were then incubated in a covered chamber for thirty minutes at room temperature. Following two consecutive five minute washes in PBS, the slides were drained of excess buffer and covered in biotinylated goat anti-rabbit immunoglobulin diluted 1 in 1000 in 2% normal goat serum in PBS. After a thirty minute incubation in a covered chamber, the slides were once again washed twice for five minutes, before being covered in peroxidase labelled streptavidin /biotin complex (made following the accompanying instructions contained within the kit). A thirty minute incubation in a covered chamber was followed by two five minute washes in PBS and one in distilled water.

Peroxidase activity was demonstrated as follows:

the slides were drained and covered in freshly prepared 0.5ml 0.5% diaminobenzidine in 9.5ml PBS activated by 3% hydrogen peroxide, and incubated five to fifteen minutes in a covered chamber. The slides were then washed in tap water for five minutes and then counterstained with Mayer's Haematoxylin for ten seconds, then washed again in running tap water prior to dehydration (by immersion in a series of 95% and 99% ethanol solutions, then finally xylene) and mounting in resinous mountant.

Fluorescein isothiocyanate (FITC) labelled secondary antibody

This fluorescent label has an excitation wavelength of 495nm, and emits at 515nm.

Following irradiation the sections were incubated for ten minutes in a blocking solution of 20% normal goat serum in PBS in a humid chamber at room temperature. The tissue was then incubated with the primary antibody, at a dilution of 1 in 2000, for two hours at room temperature. The sections were washed in 0.01M PBS and then incubated with the FITC labelled goat anti-rabbit secondary, diluted 1 in 80 with 2% normal goat serum/PBS, in the dark at room temperature. Following washing in PBS the sections were mounted and sealed.

7-amino-4 methyl coumarin-3-acetic acid (AMCA) labelled streptavidin

This fluorescent label has an excitation wavelength of 350nm, and emits at 450nm.

The slides were drained and excess buffer removed from around the sections. They were then covered with 20% normal goat serum in PBS, then incubated in a covered chamber for ten minutes at room temperature. Without rinsing, the slides were drained, excess serum removed from around the sections and the primary antibody was added, diluted 1 in 2000 with 2% normal goat serum in PBS. The slides were then incubated in a covered chamber for thirty minutes at room temperature (or overnight at 4°C). Following two consecutive five minute washes in PBS, the slides were drained of excess buffer and covered in biotinylated goat anti-rabbit immunoglobulin diluted 1 in 1000 in 2% normal goat serum in PBS. After a thirty minute incubation in a covered chamber, the slides were once again washed twice for five minutes, before being covered in AMCA labelled streptavidin diluted 1 in 50 with 2% normal goat serum/PBS. A thirty minute incubation in a covered chamber in the dark was followed by two five minute washes in PBS and one in distilled water. The sections were then mounted and sealed.

2.6.6 IMMUNOHISTOCHEMICAL DETECTION OF CYCLOBUTANE THYMINE DIMERS IN SKIN BIOPSIES OF HUMAN SUBJECTS IRRADIATED WITH UVB

Source of tissue

Sections of skin biopsies from a kinetic study were obtained by Dr. Alistair Robson (Dept. Pathology, University of Leicester) from the Department of Dermatology, University Hospital of Wales, Cardiff. Full details of the irradiations, biopsy procedure, fixation etc. are described in Anstey *et al.* (1996). Briefly, two MED UVB (300nm +/- 10nm), from a 100W xenon arc lamp coupled to an Oriel Corporation grating monochromator, were delivered to the buttock skin of six human volunteers. Post-irradiation, 4mm punch biopsies were taken 5, 8, 24 and 48 hours later, along with adjacent, unirradiated, control skin. The samples were then formalin fixed and mounted in paraffin wax.

Immunohistochemical staining

The sections were dewaxed, rehydrated and stained, within the Dept. of Pathology, according to the method described in Chapter 2.6, utilising Ab529 in conjunction with a peroxidase-labelled, streptavidin/biotin detection system.

CHAPTER THREE

IMMUNOGENICITY AND ANTIGENICITY OF MODIFIED DNA

- POSSIBLE IMPLICATIONS FOR SYSTEMIC LUPUS

ERYTHEMATOSUS.

3.1 IMMUNOGENICITY AND ANTIGENICITY OF ROS-DAMAGED DNA - POSSIBLE IMPLICATIONS FOR SYSTEMIC LUPUS ERYTHEMATOSUS.

INTRODUCTION

It is well established that UV light can induce damage to DNA both by direct absorption, producing largely cyclobutane dimers and (6-4) photoproducts (Mitchell *et al.*, 1989), and indirectly, via radical intermediates leading to oxidative products (Rahn, 1979). A polyclonal antiserum, characterised as recognising sequence-specific thymine-thymine dimers (Herbert *et al.*, 1994), had been raised to investigate direct damage. Antisera to DNA lesions represent the basis of a diversity of assays which allow quantitation and localisation of damage to be achieved without the need for expensive equipment or extensive sample preparation (the latter being associated with the difficulties of artefact formation). For this reason, the development of antibodies to oxidative DNA damage is highly desirable.

The approach to developing antibodies which recognise oxidative damage has broadly followed two routes; (i) the development of polyclonal antisera to DNA modified with a multiplicity of lesion types, or (ii) monoclonal antibody production to specific lesions. The literature reports numerous methods for the induction of ROS damage to DNA (ROS DNA), including iron (II) sulphate (Alam, 1993), ascorbate (Blount, 1990) or UVC (Ara, 1993), all in conjunction with hydrogen peroxide. Polyclonal and monoclonal antibodies have been raised to oxidative lesions such as photooxidised dGMP (Seaman *et al.*, 1966), a process later shown to yield 8-oxoG (Floyd *et al.*, 1989), to thymine glycol (West *et al.*, 1982a; Leadon and Hanawalt, 1983) and to 8-oxoA (West *et al.*, 1982b). However, the oxidative DNA lesion of choice for monoclonal production has been 8-oxo-2'-deoxyguanosine (8-oxodG), proposed to be a biomarker of oxidative DNA damage. This approach for monoclonals, first successfully reported by Park *et al.* (1992), necessitates the synthesis of the modified guanine nucleoside and conjugation with a carrier protein prior to immunisation.

With the exception of Degan *et al.* (1991), the development of polyclonal antisera to ROS DNA has largely been without application to the quantitation of DNA damage. However, the investigation of these antibodies to modified DNA and their reactivity with native DNA

has supported the postulate that ROS DNA may be involved in the pathogenesis of autoimmune diseases such as SLE (Ara and Ali, 1993; Cooke *et al.*, 1997).

AIM

To produce suitable antisera for the immunochemical detection of DNA damage, not only within naked DNA, but also cells and tissues.

METHODS

(Described fully in Chapter Two, page 55)

Generation of antibodies to ROS DNA.

Antigen preparations:

Methylene blue and white light damaged DNA (MB DNA)

DNA was incubated with methylene blue and irradiated with white light (Floyd *et al.* 1989), a system which produces almost exclusively 8-oxodG (Floyd *et al.*, 1989). Calf thymus DNA (1mg/ml in ultra pure water) was irradiated with white light, on ice, in the presence of methylene blue, for 24 hours. The methylene blue was removed by repeatedly precipitating and dissolving the DNA.

Gamma irradiated DNA

DNA was irradiated with gamma radiation (Dizdaroglu *et al.* 1986). A solution of calf thymus DNA, in a 50ml Universal™ container on ice, was irradiated by a ⁶⁰Co (VICKRAD) source (at 0.48 Grays/sec) to give a biologically relevant dose of 4.95Gy. A further sample of DNA was gamma irradiated, although at a much higher dose. A solution of calf thymus DNA, in a 50ml Universal™ on ice, was irradiated by a ⁶⁰Co (VICKRAD) source (at 0.48 Grays/sec) to give a dose of 275Gy.

Immunisation Protocol #1 - polyclonal

The damaged DNA was electrostatically conjugated to bovine serum albumin (BSA) and homogenised with Titermax adjuvant, prior to immunisation of the rabbits in each hind quadricep. Test bleeds were performed every 14 days until a sufficient titre of polyclonal antiserum reacting to MB DNA, had been reached.

Fe²⁺ and hydrogen peroxide damaged DNA (FEH DNA)

Hydroxyl radical damaged DNA, using Fenton chemistry was prepared according to the method of Alam *et al.* (1993). Calf thymus DNA was incubated for thirty minutes, at room temperature, in the presence of hydrogen peroxide and ferrous ions. At the end of the incubation the reaction products were exhaustively dialysed against PBS.

Hydrogen peroxide and UVC damaged DNA (UVH DNA)

This procedure is an established ROS generating system and has previously been used to successfully raised a polyclonal antiserum to ROS-modified DNA (Ara *et al.* 1993). Calf thymus DNA was irradiated with UVC in the presence of hydrogen peroxide. Excess hydrogen peroxide was removed by extensive dialysis against PBS.

Immunisation protocol #2 - polyclonal

The damaged DNA was conjugated to bovine serum albumin (BSA) and homogenised with Freund's Complete Adjuvant prior to subcutaneous immunisation (see Materials and Methods section 2.3.9). The rabbits were boosted with the antigen in saline via the intra-venous route, four weeks later.

Ascorbate and hydrogen peroxide damaged DNA (ASH DNA)

Calf thymus DNA was incubated with a combination of hydrogen peroxide and ascorbate for one hour at 37°C. Excess hydrogen peroxide was removed by extensive dialysis against PBS.

Immunisation protocol #3 monoclonal

Procedure for the immunisation of mice leading to monoclonal production is detailed in Chapter 2.3.2. Briefly, the mice were initially immunised subcutaneously, with subsequent immunisations via the intra-venous route. Test bleeds were performed seven days post-boost and when a suitable response had been achieved, the animals were sacrificed three days after a final intra-peritoneal boost.

Generation of Antibodies to 8-oxodeoxyguanosine

Production of the immunogen, 8-oxoguanosine (8-oxoGR - where R indicates the ribonucleoside moiety) was achieved by modification of native guanosine by the 'Udenfriend System' (iron, ascorbate, EDTA and oxygen - to generate ROS) as described in Chapter 2.2. 8-oxoGR is known to be electrochemically active (Floyd *et al.*, 1986) and this was used to initially establish whether the synthesis was successful. The electrochemically active peak, with a retention time of 9.5 minutes (Figure 3.1) suggested that 8-oxoGR had been produced. Preparative HPLC was performed on the product and 4ml fractions, corresponding to the peak of interest (Figure 3.2), were collected by a fraction collector, every 30 seconds. To confirm that the fractions collected did contain 8-oxoGR, all were assessed spectrophotometrically for the characteristic 8-oxoGR spectrum (Figure 3.3 represents an example of this spectrum, obtained by HPLC with diode array detection, for comparison with the collected fractions). In order to test that removal of the salts derived from the mobile phase was possible by HPLC, a sample was run on an analytical column (Figure 3.4); this was successfully achieved and clean-up of the pooled, collected fractions was then performed on the preparative system. Further evidence for the identity of the peak collected was again provided by UV spectral analysis of the pooled, desalted fractions. The diode array of these fractions showed absorbance peaks of guanine hydroxylated at the C-8 position (Figure 3.3). Electrospray mass spectrometry (EMS) was performed by Dr. Gavain Sweetman (Biomonitoring Group, Centre for Mechanisms of Human Toxicity, Leicester), the results of which indicated the peak of interest at M_w 322 to be hydroxylated guanosine (8-oxoGR) which had gained both H^+ and Na^+ , as part of the EMS assay (Figure 3.5). Taken together, the single peak collected by preparative HPLC following spectrophotometric analysis, compared to an authentic standard and coupled with the electrospray mass chromatogram strongly suggested that 8-oxoguanosine had been produced.

The isolated 8-oxoGR was then linked by the periodate method (Erlanger and Beiser, 1964) either to keyhole limpet haemocyanin (KLH), or bovine serum albumin (BSA) (see Materials and Methods section 2.3.16.4) which were to act as protein carriers (Scheme 3.1). The applicability of the periodate linkage method used requires that the ribonucleoside derivative of the base lesion be available, hence the use of guanosine as the starting material. The use of two carrier proteins allowed one to be used as immunogen and the other as the

ELISA solid phase antigen for testing the immune response, eliminating the risk of detecting antibodies displaying cross-reactivity to the carrier protein.

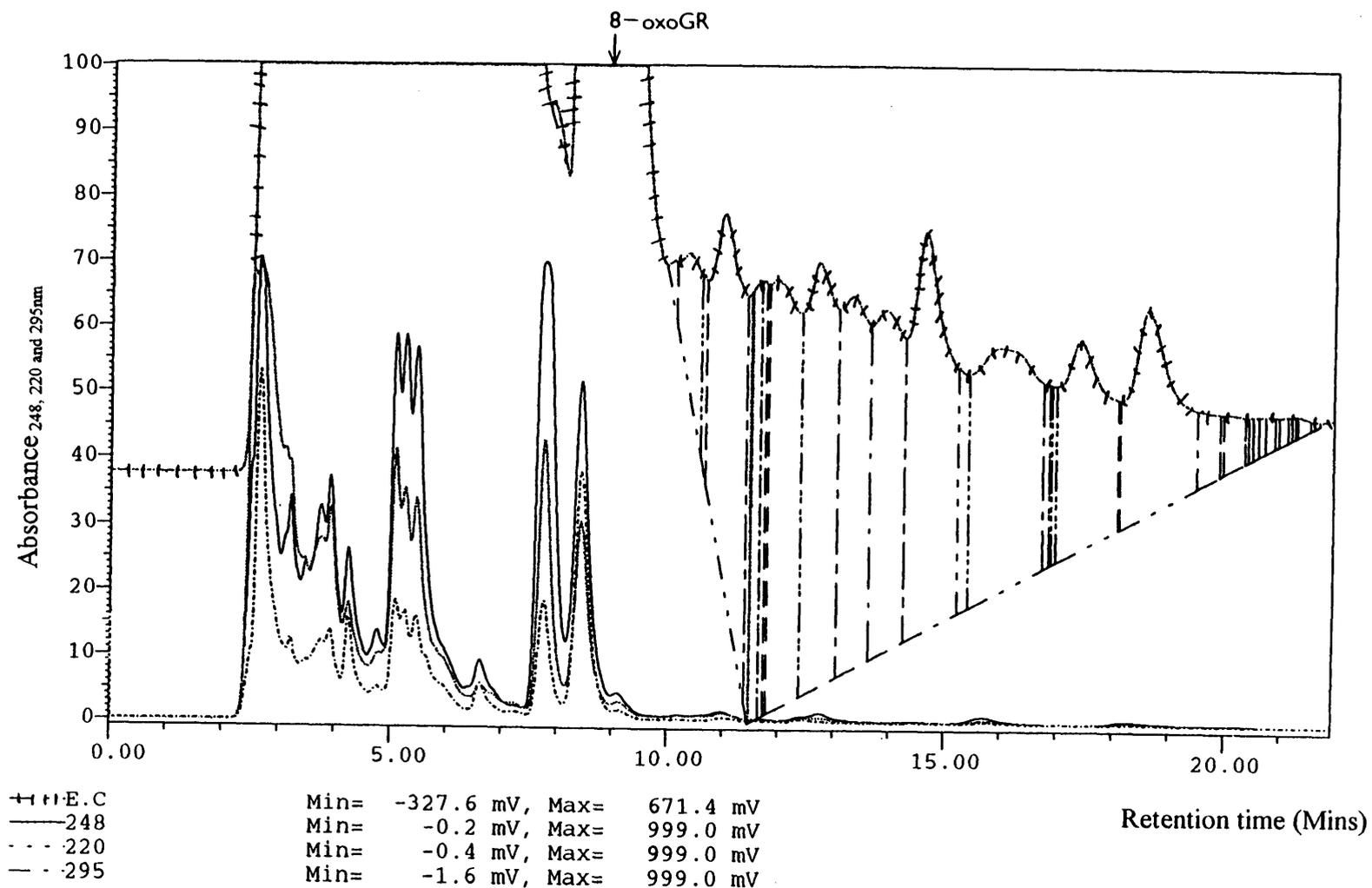


Figure 3.1. Representative analytical HPLC chromatogram of the whole crude preparation of 8-oxoGR synthesised by the Udenfriend method. Injected onto a Phase Sep ODS2 column (250x4.6mm i.d). Mobile phase consisted of 50mM KH_2PO_4 , 10.0% MeOH, pH5.5, with a flow rate of 1ml/min. UV detection was performed at 248, 220 and 295nm see System II, section 2.3.16.2). Electrochemical detection (EC) was at an applied potential of +1.0V versus an Ag/AgCl electrode. The electrochemical peak at 9.5 minutes represents 8-oxoGR.

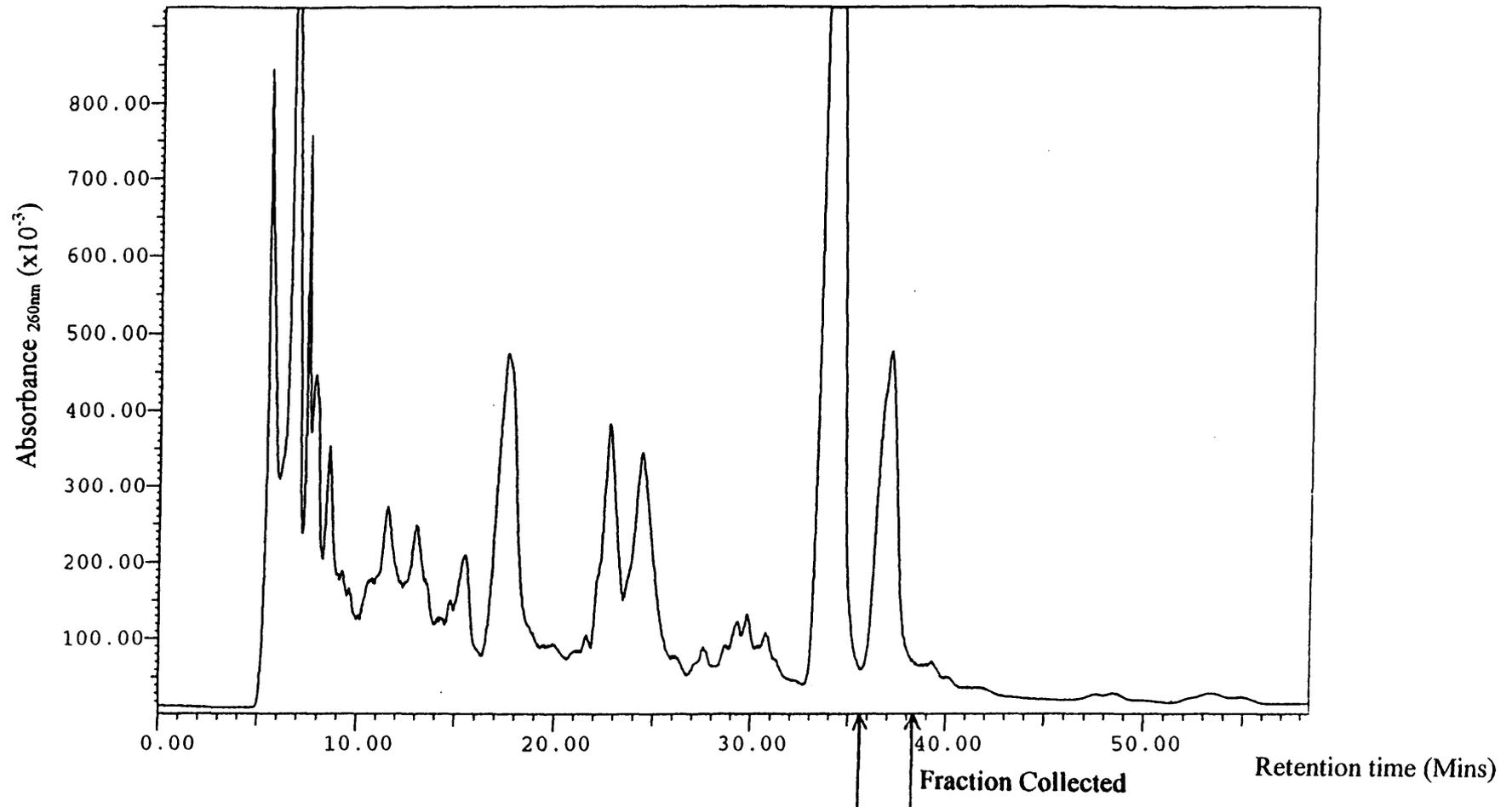


Figure 3.2. Representative preparative HPLC chromatogram of a 1000 μ L injection of the whole crude preparation of 8-oxoGR by the Udenfriend method onto a Phase Sep, ODS2 column (250x20mm i.d). Mobile phase consisted of 50mM KH_2PO_4 , 10.0% MeOH, pH 5.5, with a flow rate of 8ml/min. UV detection was performed at 260nm (see System III, section 2.3.16.3). The fraction corresponding to 8-oxoGR was collected.

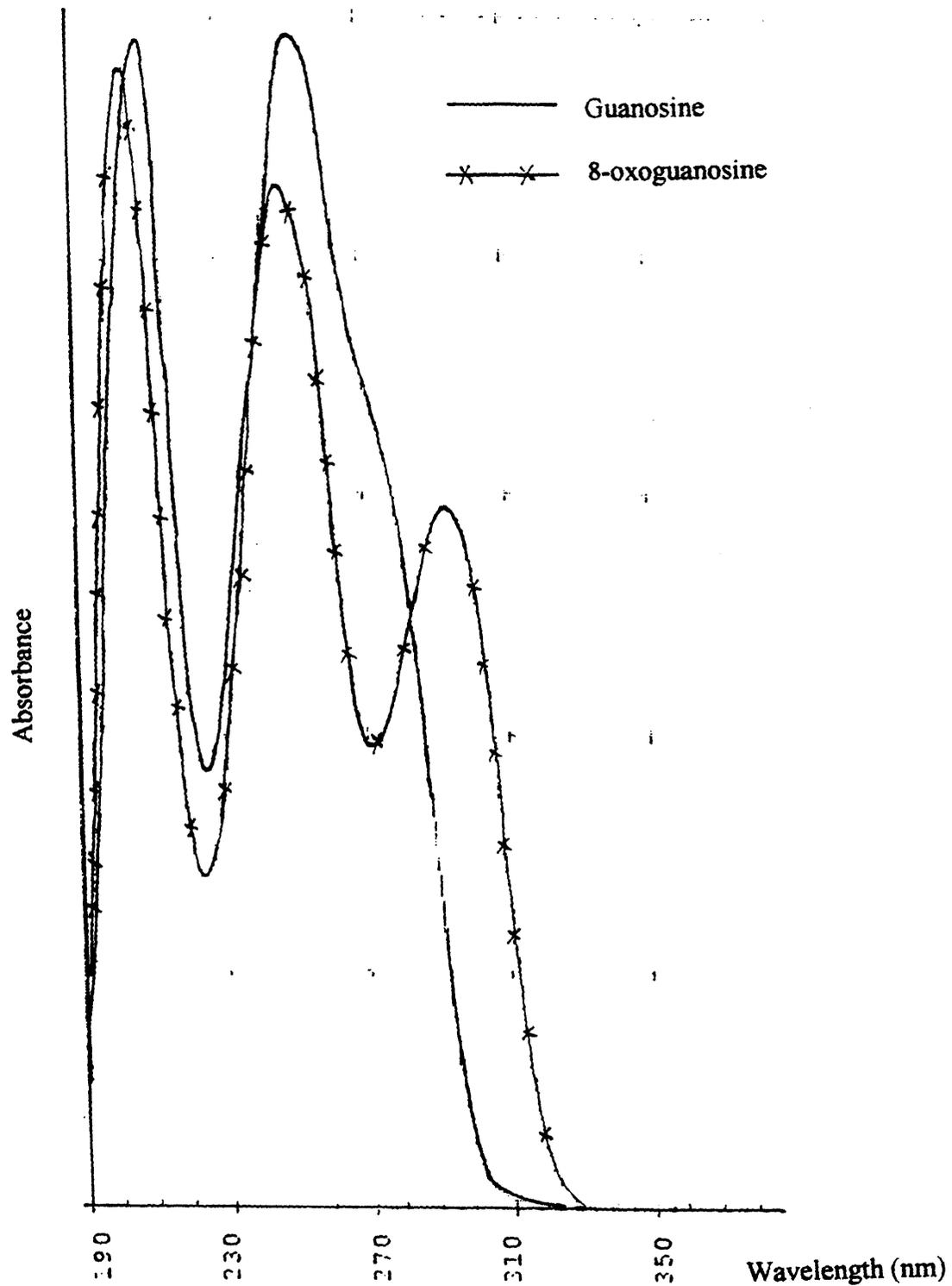


Figure 3.3. Representative UV spectrophotometric scans (performed by HPLC system II) comparing putative 8-oxoguanosine to guanosine and showing the novel peak at 295nm in 8-oxoguanosine.

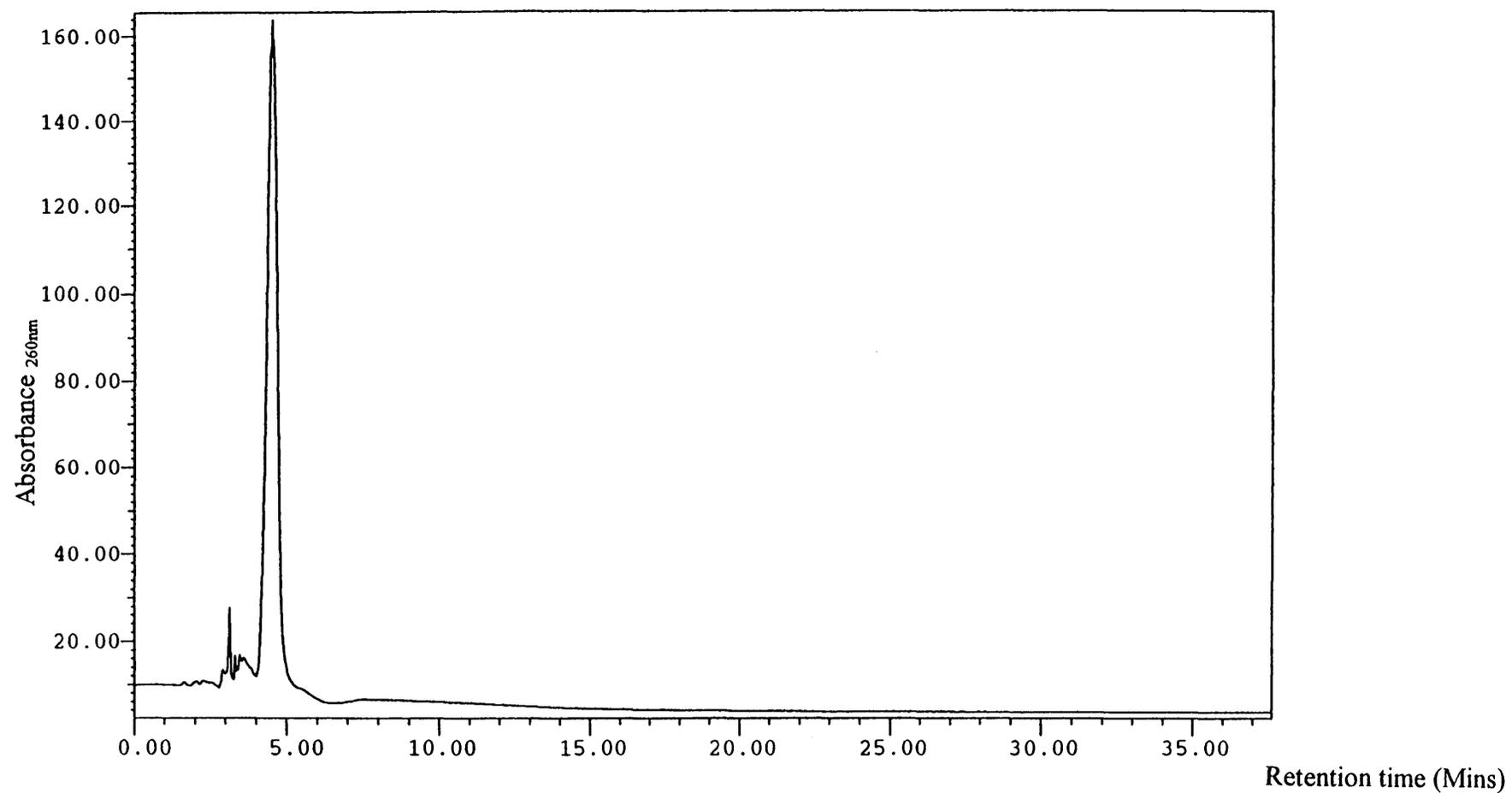


Figure 3.4. Representative analytical HPLC chromatogram of a 40 μ L test injection of the pooled, 8-oxoGR fractions onto a 25cm Phase Sep, ODS2, C-18 column to remove salts from the previous HPLC run. Mobile phase consisted of 2.5% v/v MeOH, with a flow rate of 1ml/min. UV detection was performed at 260nm.

8-oxoGuo 85-90
MARU1P 75 (0.777) Sb (0.20.00); Sm (Mu, 2x1.00); Cm (45:102-1:15)

19-Jun-1995
Scan E5+
1.61e6

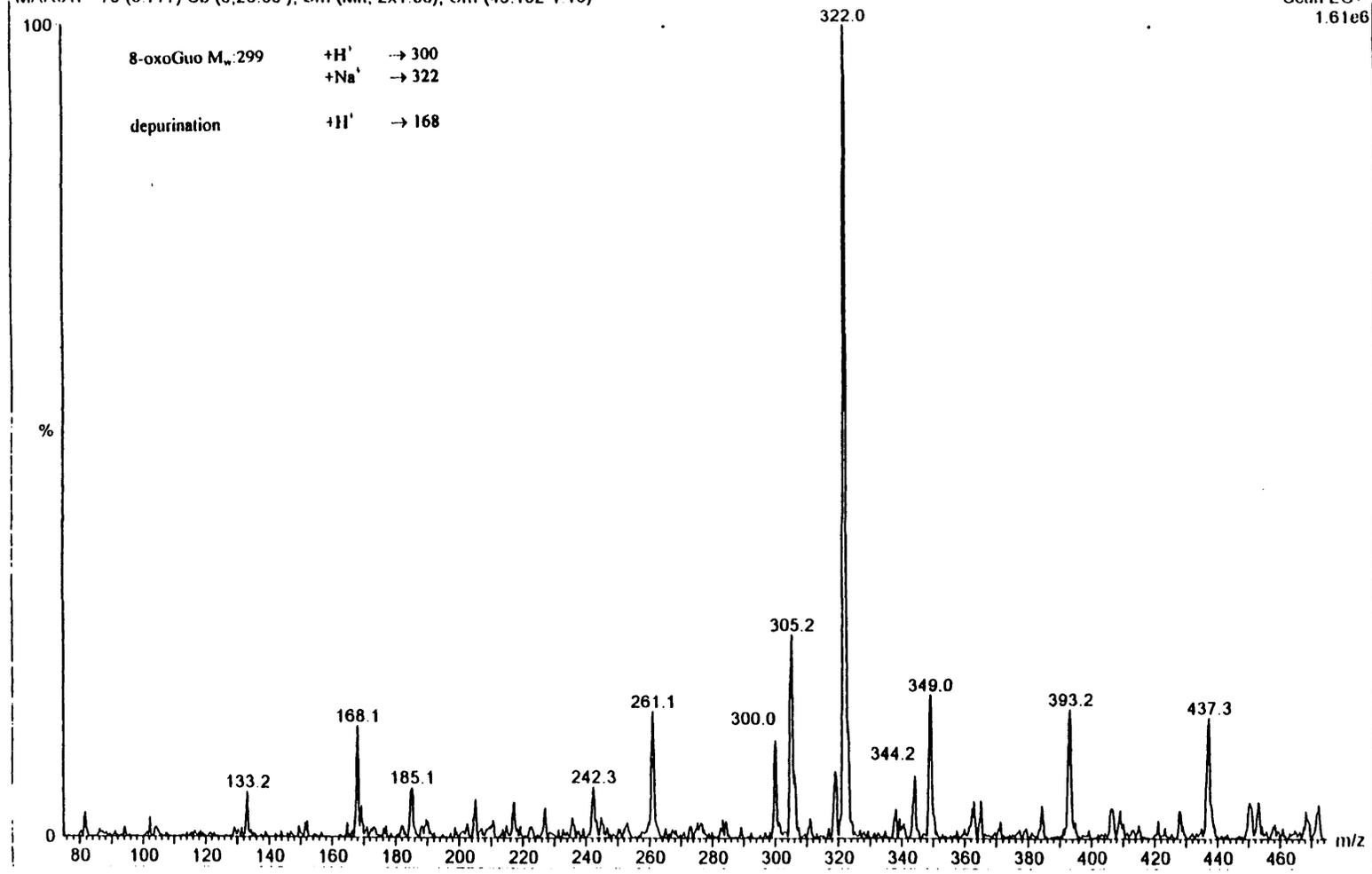
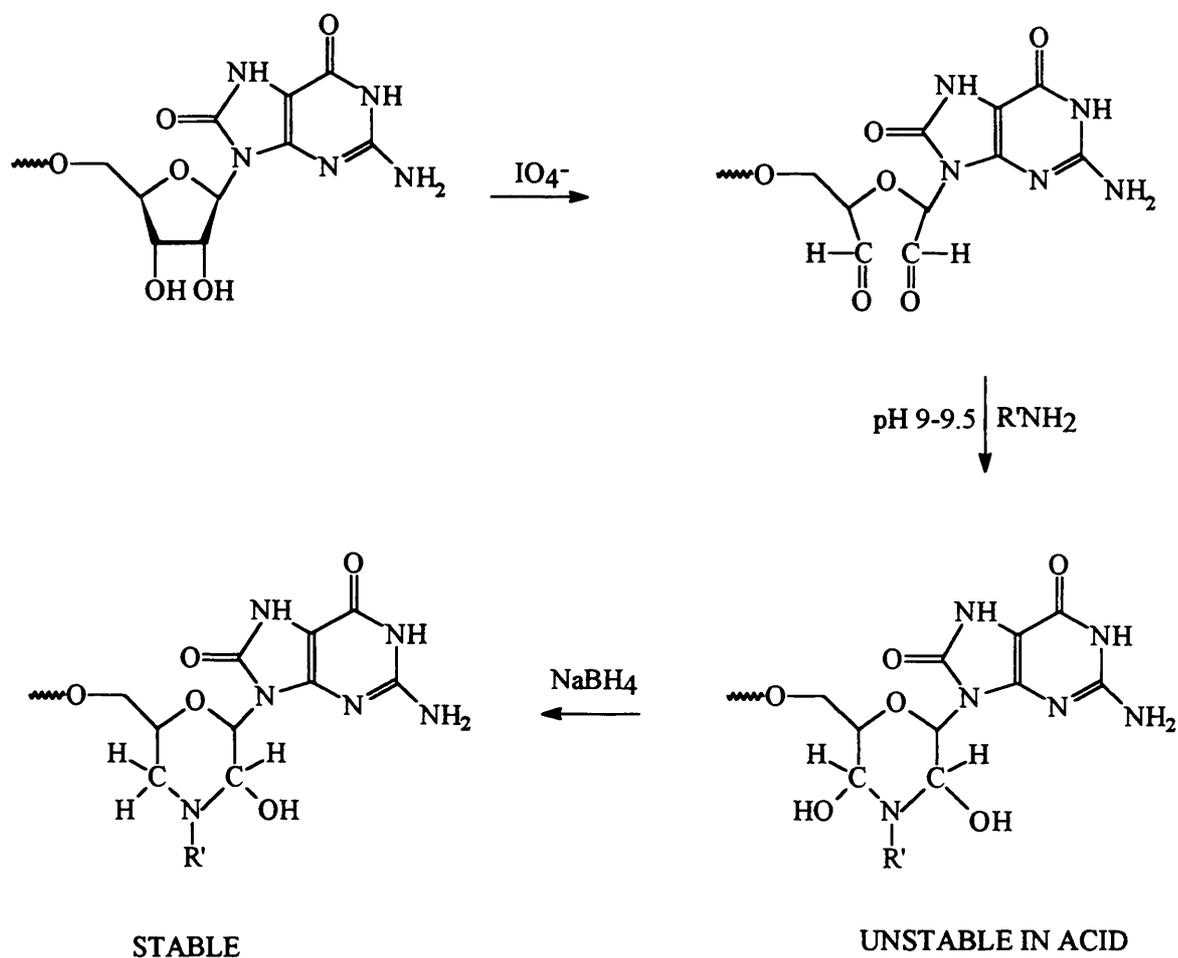


Figure 3.5. Representative electrospray mass chromatogram of the purified collected fraction. The peak of interest is M_w 322, demonstrating that the fraction contains hydroxylated guanosine (8-oxoGR).



R' - protein backbone; i.e the N is supplied by the lysine residues

Scheme 3.1. Conjugation of 8-oxoguanosine to protein, based on the method of Erlanger and Beiser, 1964.

Following preparation of the immunogen, mice (n=4) for monoclonal antibodies and rabbits (n=2) for polyclonal antibodies, were immunised with the 8-oxoGR/KLH conjugate. Methylene blue-modified DNA, which contains predominantly 8-oxodG, was used as the solid phase antigen as it was deemed to possess more antigenic determinant sites than the 8-oxoGR/BSA conjugate (description of how this was achieved is detailed in the "immunogenicity of 8-oxoguanosine" section, page 103). When a suitable titre, of immunoglobulin binding to MB DNA, had been achieved the mouse was exsanguinated and the spleen removed. Following passage of the spleen through a membrane, the resulting single cells were mixed with myeloma cells in order to allow fusion of the two cell types to occur and thereby immortalise the spleen cells. Subsequent to this fusion, the resulting hybridomas were plated into multiple wells and the clones of interest selected by ELISA testing of the supernatant from the well.

Antigenicity of ROS DNA by SLE serum

Serum, from a patient displaying high titres for anti-DNA antibodies and satisfying the revised criteria for the classification of SLE proposed by the American Rheumatism Association (Tan *et al.*, 1982), was collected and used in the ELISA described below.

ELISA

Avidity of serum anti-DNA antibodies, at various dilutions, for iron/hydrogen peroxide-treated DNA was detected by ELISA, as described in Chapter 2 (page 57), Materials and Methods. Briefly, the solid phase antigen was FEH modified DNA, with native DNA as a control, blocking was performed with 1% bovine serum albumin (Sigma). ASH treated DNA acted as a positive control. Peroxidase-conjugated goat anti-human IgG antibody, diluted 1 in 5000, (Sigma) was used to detect the autoantibodies.

RESULTS

Immunogenicity of MB DNA

Modification of DNA by methylene blue in conjunction with white light (MB DNA) yielded one 8-oxodG lesion in every 100 dGs, as determined by HPLC-ECD, following enzymatic digestion (see Materials and Methods, section 2.3.1, page 55). Calculation of this value was by integration of the dG and 8-oxodG peaks from the MB DNA sample and quantitation by comparison with their corresponding standards. The peak of interest was that detected electrochemically at a retention time of 7.45 minutes (Figure 3.6). Comparison with literature values for 8-oxodG in commercial calf thymus DNA, determined by HPLC-ECD (22 ± 2 , 8-oxodG/ 10^5 dG, Floyd *et al.*, 1989) suggested the MB DNA to be extensively modified. Despite the level of modification, subsequent immunisation of two experimental animals with MB DNA produced antiserum binding to MB DNA of 0.15 and 0.105 absorbance units, compared to pre-immune values of 0.08 and 0.05, respectively (Figure 3.7). It was judged that this experimental system had failed to elicit a sufficient immune response.

Immunogenicity of gamma irradiated DNA

Gamma irradiation is reported to be a potent source of \cdot OH via radiolysis of water. However, immunisation with DNA, gamma irradiated at levels known to induce damage (4.95 and 275Gy), failed to induce an appreciable immune response, with no discrimination between native and modified DNA.

Comparison of immunogenicity with level of DNA oxidative modification

On the basis that, despite its apparently extensive oxidative modification, MB DNA did not appear particularly immunogenic in rabbits, it was clearly necessary to examine the role of oxidative damage in eliciting an antibody response. A novel HPLC procedure for the detection of 8-oxoguanine has been developed within the Division of Chemical Pathology (Herbert *et al.*, 1996) and it was this which was used to determine levels of lesion. Levels of modification (nmol 8-oxoG/mg DNA) are represented for each type of modified DNA in Figure 3.9.

There would appear to be some disparity between the two HPLC methods when measuring levels of oxidative damage to guanine moieties, as evidenced by the different values for MB DNA. Using the enzyme digestion to release the deoxynucleoside, a value of one 8-oxodG

lesion in every 100 dGs was determined, however, formic acid hydrolysis, which gives rise to base release, yielded 2.8 oxoGs per 100 Gs. Possible reasons for such a difference are discussed later. Also noted was a high level of modification for commercial calf thymus DNA (nDNA) compared to other literature values; 992 8-oxoG/10⁵G, versus 22+/-2, 8-oxodG/10⁵dG (Floyd *et al.*, 1989) - a 45-fold difference. However, these values are derived from HPLC methods which utilise very different methods of DNA hydrolysis. Therefore, the guanase assay may have more similarity with GC-MS values (7.8nmol 8-oxoG/mg DNA versus 0.5-1.0 nmol 8-oxoG/mg DNA; Halliwell, 1993 - a 7.8-fold difference). Comparison between level of oxidative modification, as determined by 8-oxoG formation, and immunogenicity, is shown in Table 3.1.

Table 3.1. Comparison of oxidative modification and immunogenicity

| Nature of modification | Level of modification (8-oxoG/100 G) | Immunogenicity |
|------------------------|--------------------------------------|-----------------------------|
| MB DNA | 2.8 | Poor (Figure 3.8) |
| CASH | 6.2 | Not tested |
| UVH | 1.92 | Good (Figure 3.10) |
| γ 4.95 Grays | 0.4 | Poor (Figure 3.9) |
| γ 275 Grays | 2.4 | Poor (Figure 3.9) |
| FEH | 1.4 | Poor (Figure 3.12 and 3.13) |
| nDNA | 0.9 | Not tested |

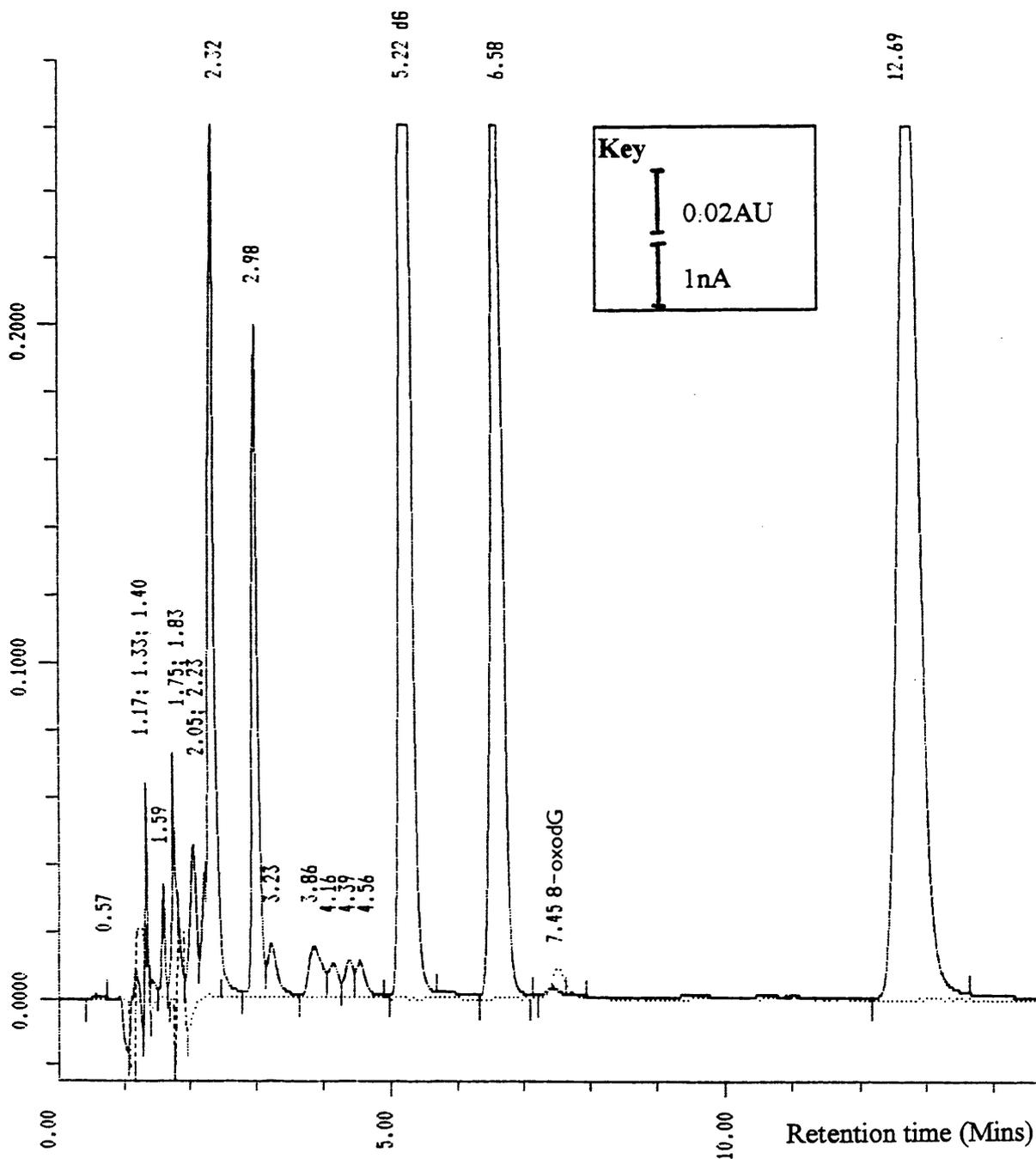


Figure 3.6. Representative analytical HPLC chromatogram of a 50 μ l sample of enzymically digested MB DNA analysed on a Hypersil ODS Phenomenex (3 μ m) column (150x4.6mm i.d). Mobile phase consisted of 50mM sodium acetate, 1mM EDTA, 5.0% MeOH, pH 5.5, with a flow rate of 1ml/min. UV detection was performed at 260nm (represented by —). Electrochemical detection (represented by) was at an applied potential of +0.6V. The electrochemical peak at 7.45 minutes is 8-oxodG

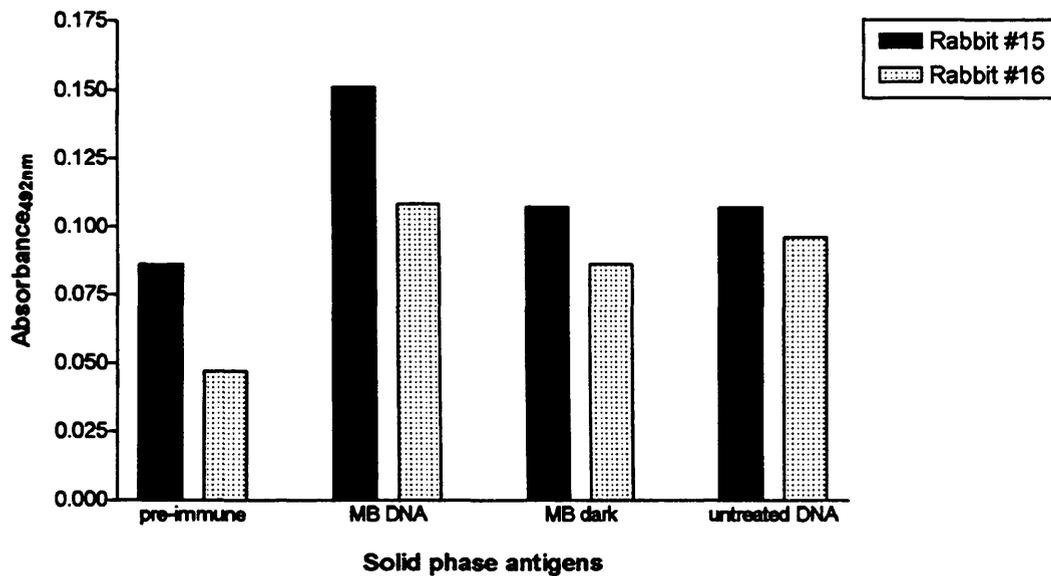


Figure 3.7. Binding of antibodies in whole serum, diluted 1/100, from final bleeds of two rabbits (#15, #16), to untreated DNA, DNA exposed to methylene blue, but kept from light (MB dark) and DNA exposed to methylene blue and white light (MB DNA) were used as controls. Comparative binding of pre-immune sera to MB DNA is also illustrated. Values represent the mean (SEM) of quadruplicate determinations.

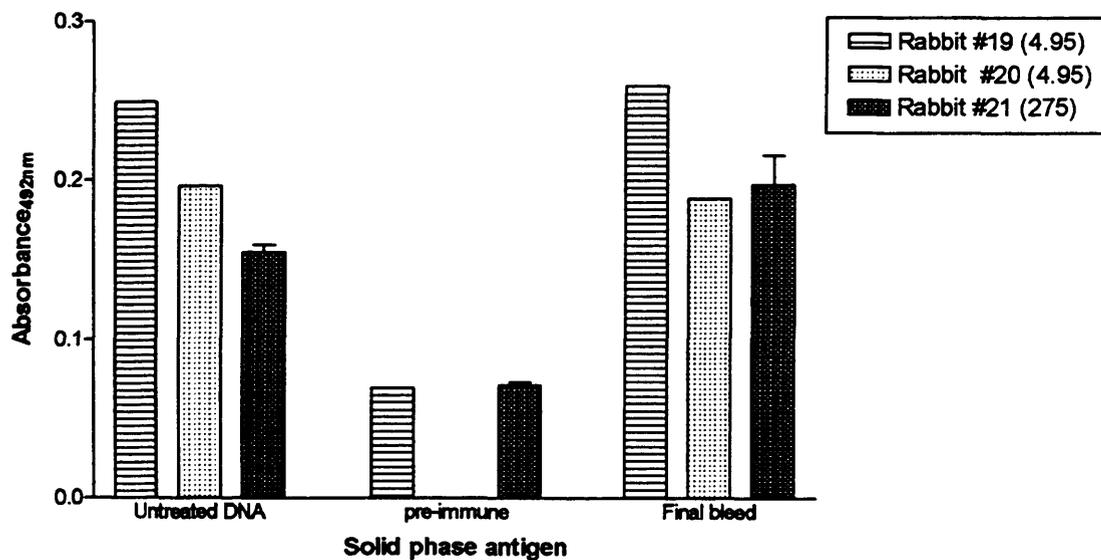


Figure 3.8. ELISA binding of antibodies in whole serum diluted 1/100 against native and γ -irradiated DNA (4.95 or 275Gy). Comparative binding of pre-immune serum to irradiated DNA is also illustrated. Values represent the mean (SEM) of quadruplicate values. Pre-immune serum values for #20 were not obtained.

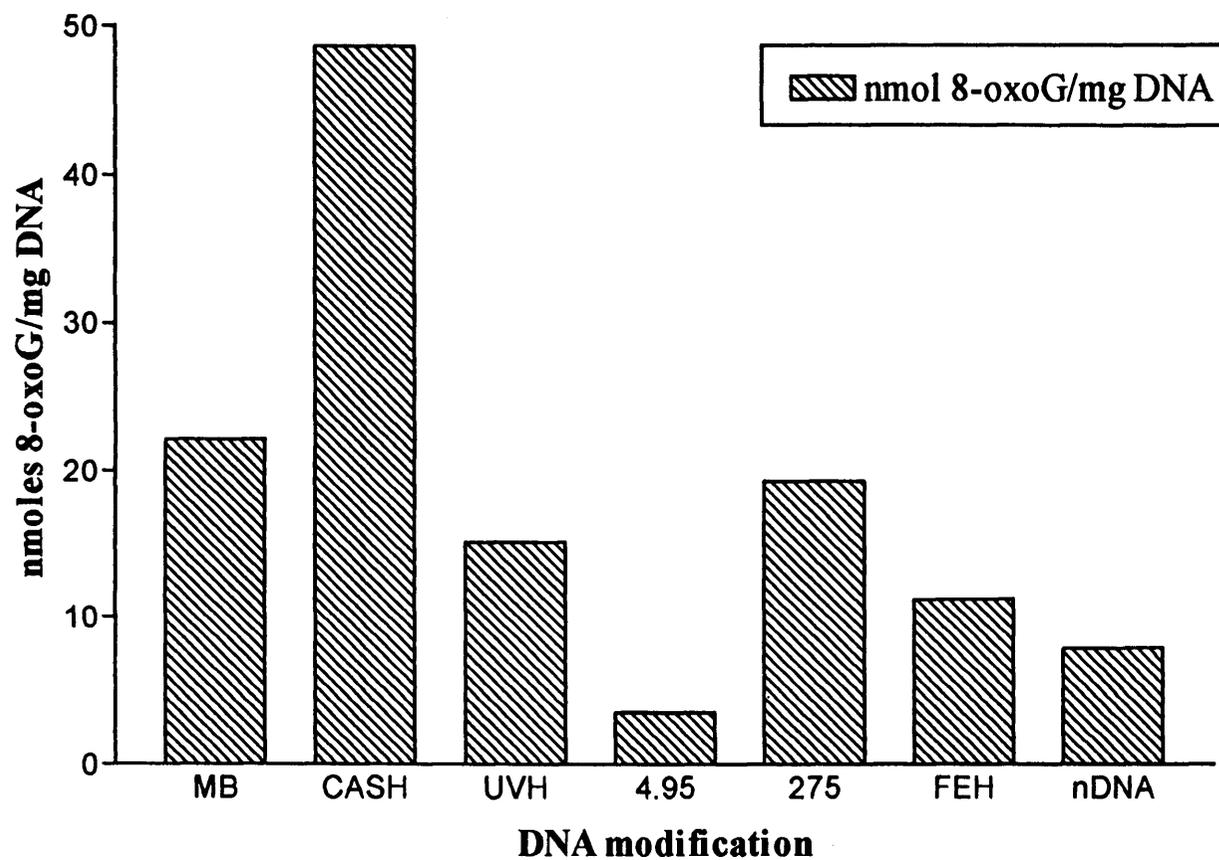


Figure 3.9. Levels of 8-oxoguanine in native (nDNA) DNA and DNA modified by various ROS-generating systems (from left to right: methylene blue; copper/ascorbate/H₂O₂; UVC/H₂O₂; gamma radiation (Grays) and iron/H₂O₂.

Immunogenicity of UVH DNA

Modification of DNA by hydrogen peroxide in conjunction with UVC (UVH DNA) yielded one 8-oxodG lesion in every 1000 dGs, as determined by HPLC-EC (using the same method as described for MB DNA in Chapter 2.2). Following booster injections with UVH DNA an immunogenic response was elicited, such that at a 1:10 dilution of the serum from one of the rabbits, the ELISA absorbance with the UVH as antigen was 1.65 (Figure 3.10). In comparison, the binding of pre-immune serum to the same antigen was very low (corrected absorbance 0.2). Binding to native DNA was also very low (corrected absorbance 0.3). Slightly increased binding (0.3 absorbance units), compared to native DNA, was noted for H₂O₂ DNA and this difference was consistent at 1/100 and 1/1000 dilutions. However, a high degree of binding was noted for DNA treated with UVC alone (corrected absorbance 1.6; Figure 3.10) which was only marginally less than that for UVH DNA at both 1/10 and 1/100. At 1/1000, binding to UVH DNA was similar (0.2) to that of UVC DNA (0.2). The similarity in antigenic response towards UVH DNA and UVC DNA required that the specificity of this serum be further investigated.

Antigenic specificity of antiserum raised against UVH DNA

In order to discriminate between the binding of the antiserum to UVH DNA and UVC DNA a competition ELISA was established. Using UVH DNA as the solid-phase antigen and a working antiserum dilution of 1:100, UVH-modified, double-stranded DNA was an effective inhibitor ($IC_{50} < 1.95 \mu\text{g/ml}$; Figure 3.11). The concentration of antigen giving 50% inhibition (IC_{50}) is an index of antigenicity of a compound, and the lower the value the better the inhibitor. Therefore this ELISA system was used to investigate the specificity of the polyclonal antiserum by comparing the ability of putative antigens to compete for binding with the antiserum. UVC modified double-stranded DNA (UVC DNA) was a slightly less effective inhibitor than UVH DNA ($IC_{50} \sim 2.1 \mu\text{g/ml}$; Figure 3.11). There appeared to be a demonstrable difference (Figure 3.11) between the avidity of the antiserum for UVH DNA and UVC DNA at all concentrations of competing antigen, although the data suggested that the majority of the response may be to shared antigenic sites.

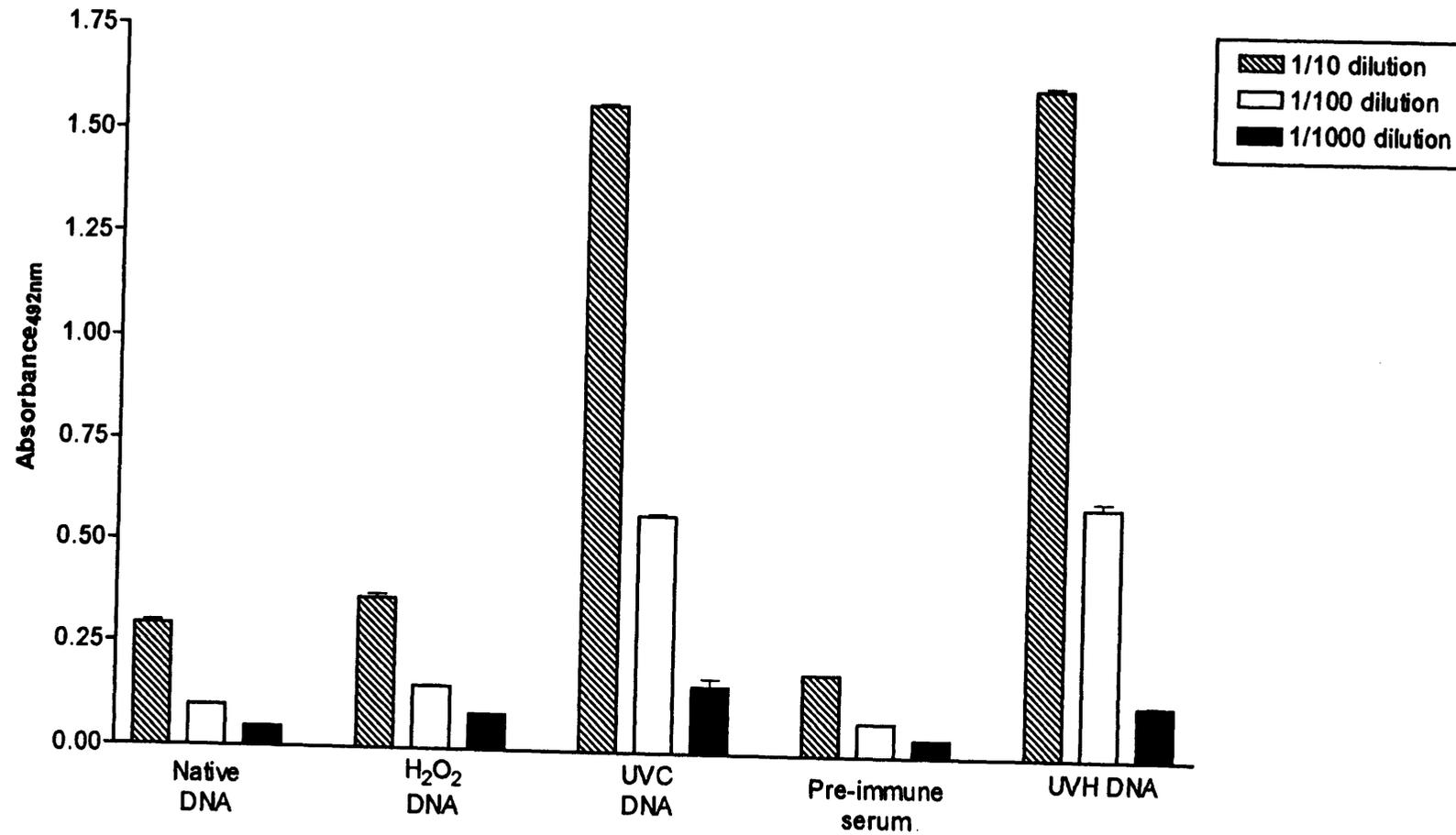


Figure 3.10. Direct ELISA binding of anti-UVH antibodies in whole rabbit antiserum to modified and native DNA. Comparison is made with direct antibody binding in pre-immune serum to UVH DNA. Results shown were derived from a single animal. Values represent the mean (SEM) of duplicate determinations per dilution.

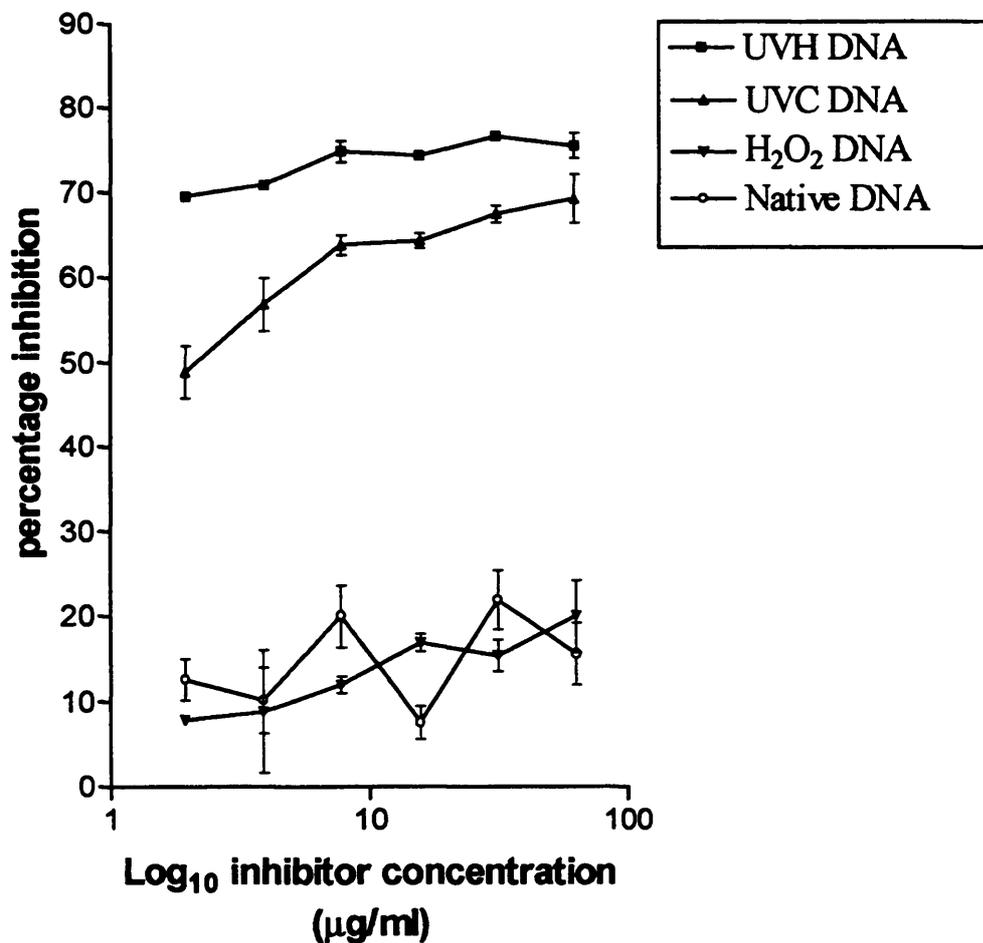


Figure 3.11. Inhibition of binding by native, UVH, UVC and H₂O₂-modified DNA of rabbit immunoglobulins in serum to UVH DNA. Maximum inhibition (100%) was that observed in the absence of solid phase antigen (UVH DNA). Zero percent inhibition was that in the absence of competitors. Values represent the mean (SEM) of three determinations per concentration.

Comparison of the percentage inhibition by UVH and UVC DNA, at a concentration of 1.95µg/ml showed a statistically significant difference ($p < 0.01$), indicating this to be a real effect. Poor inhibition was observed using either H₂O₂ DNA or native DNA as competitor, requiring calculation of maximum inhibition (26% and 21% respectively for an inhibitor concentration of 250µg/ml; Figure 3.11). Further characterisation of this antiserum is presented in Chapter 5.

Immunogenicity of FEH DNA

Modification of DNA by the ROS generating system, iron/H₂O₂, yielded one 8-oxodG lesion in every 100 dGs, the same value as for MB DNA. This value was obtained by the nucleoside assay (Chapter 2.3.1) and again is much higher than literature values for commercial calf thymus DNA (22±2 8-oxodG/10⁵dG; Floyd *et al.*, 1989), suggesting extensive modification of the DNA had occurred. After a booster injection with FEH DNA, a weaker response than for UVH DNA was noted, such that at a 1:10 dilution of the serum from one animal (#13), the corrected ELISA absorbance with the FEH DNA as antigen was 0.2 (Figure 3.12). A similar response was also seen for the other animal (#14, Figure 3.13). With respect to #13, the binding of pre-immune serum to the same antigen (FEH DNA) was low (corrected absorbance 0.1). Binding to native DNA was 0.2 (corrected absorbance) which was similar to the binding to FEH DNA. This suggested that a response had developed to FEH DNA, in two experimental animals, with production of antibodies that appeared to recognise native double-stranded DNA with an avidity close to that for FEH DNA.

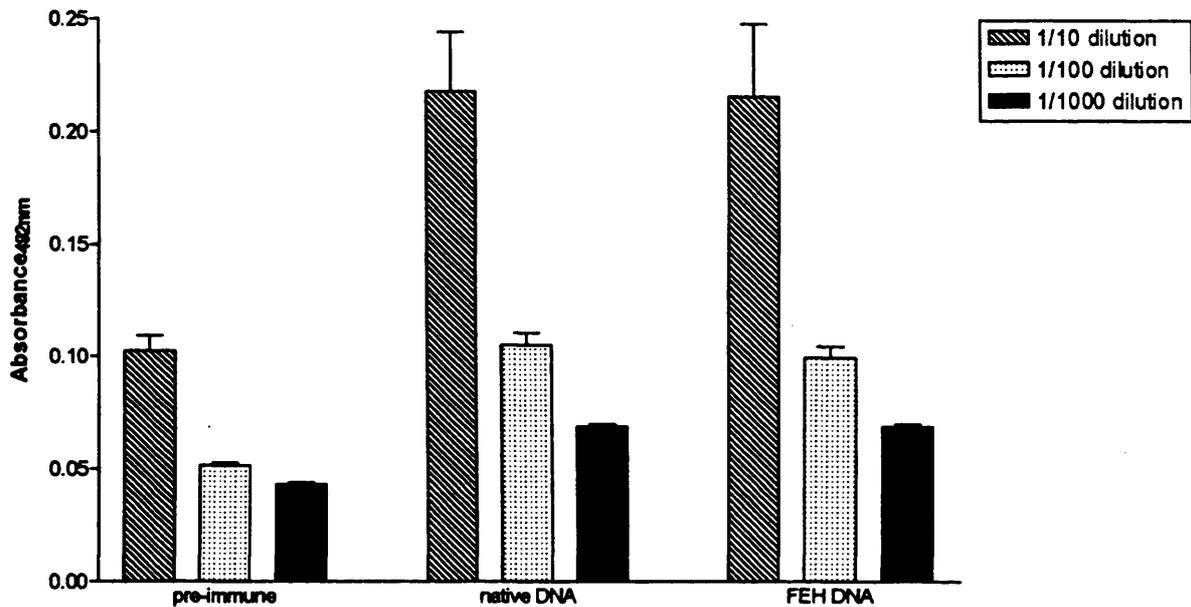


Figure 3.12. Direct ELISA binding of rabbit anti-FEH DNA antibodies in serum to modified and native DNA and direct binding of antibodies in pre-immune serum to FEH DNA. Values were derived from a single animal (#13). Values represent the mean (SEM) of four determinations per dilution.

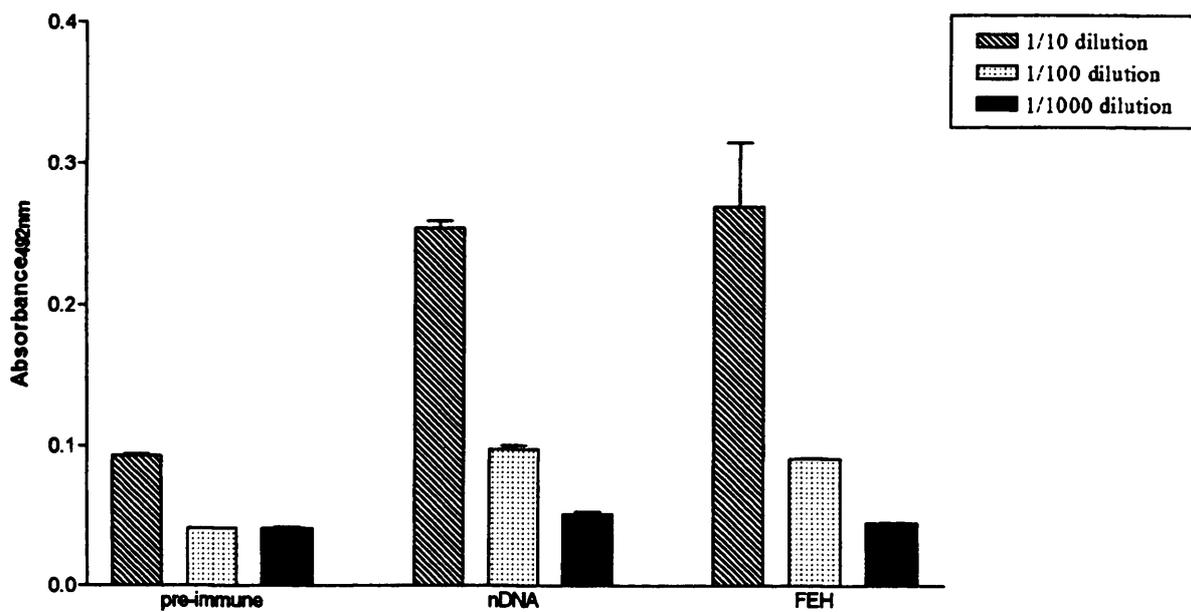


Figure 3.13. Direct ELISA binding of rabbit anti-FEH DNA antibodies in serum to modified and native DNA and direct binding of antibodies in pre-immune serum to FEH DNA. Values were derived from a single animal (#14). Values represent the mean (SEM) of two determinations per dilution.

Immunogenicity of 8-oxoguanosine - examination of the polyclonal response

The polyclonal response to the 8-oxoguanosine/KLH conjugate (8-oxoKLH) in two rabbits was examined, in order to test the suitability for progression towards a monoclonal approach. A sufficient titre is required to be seen before a monoclonal approach may be taken and therefore the titres following immunisation were assessed. A titre is the dilution of antiserum required to give 50% maximum binding and acts as an index of the antibody levels contained within the serum. Here, the titre is calculated by ELISA, using doubling-dilutions of the antiserum against the solid phase antigen, native or MB DNA (represented by Figures 3.14a and 3.14b). It is important to note that when calculating the titre a sigmoidal curve should be fitted to the data, not the points merely connected as shown here.

Following booster injections with 8-oxoKLH an immunogenic response was noted over pre-immune, which improved over the first four to five weeks, achieving a maximum titre of 1/548 in one animal (#199), against MB DNA (Figure 3.15). MB DNA was used as the solid phase antigen as it was deemed to possess more antigenic sites than the prepared 8-oxoguanosine/BSA conjugate (8-oxoBSA). This was on the basis that, given an equal concentration of DNA, for MB DNA, or protein, for 8-oxoBSA, test antisera to 8-oxodG bound more strongly to MB DNA, than 8-oxoBSA. It was subsequently shown that comparison of the response to MB DNA, with native DNA, displayed a striking similarity, also seen with the other animal (Figure 3.16). It was noted that in both animals preferential binding to native or MB DNA could vary over the immunisation time course. For example, at month 3, for animal #199, the titre towards MB DNA was 1/548 and to native DNA, 1/506. However, at month 7 both of these values had fallen to 1/258. Likewise in animal #200, at month 5, the titre towards MB DNA was 1/426 and to native DNA, 1/497. Again, by month 7 these values had dropped to 1/92 and 1/96, respectively. Clearly the maximum titre, in both animals, had been achieved during this immunisation schedule, although subsequent immunisations, instead of increasing the titre further, merely resulted in a loss of response. Nonetheless, a response which recognised DNA treated with methylene blue and white light had been achieved and thus warranted progression to a monoclonal system in order to select the clone producing the antibodies which could differentiate between native and MB DNA.

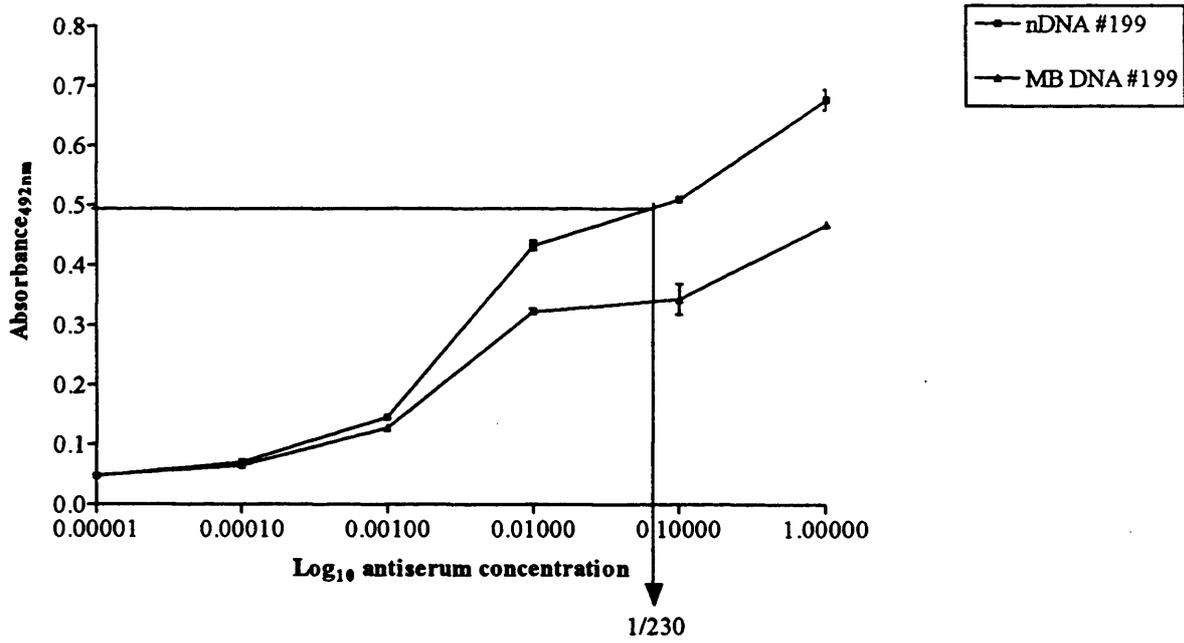


Figure 3.14a. Representative figure by which antiserum titres are calculated. Titre is the concentration of antiserum which gives half maximum absorbance, the example here is for animal #199, against MB DNA. Data shown is for experimental animal #199, and illustrates antiserum binding to double-stranded native and MBDNA.

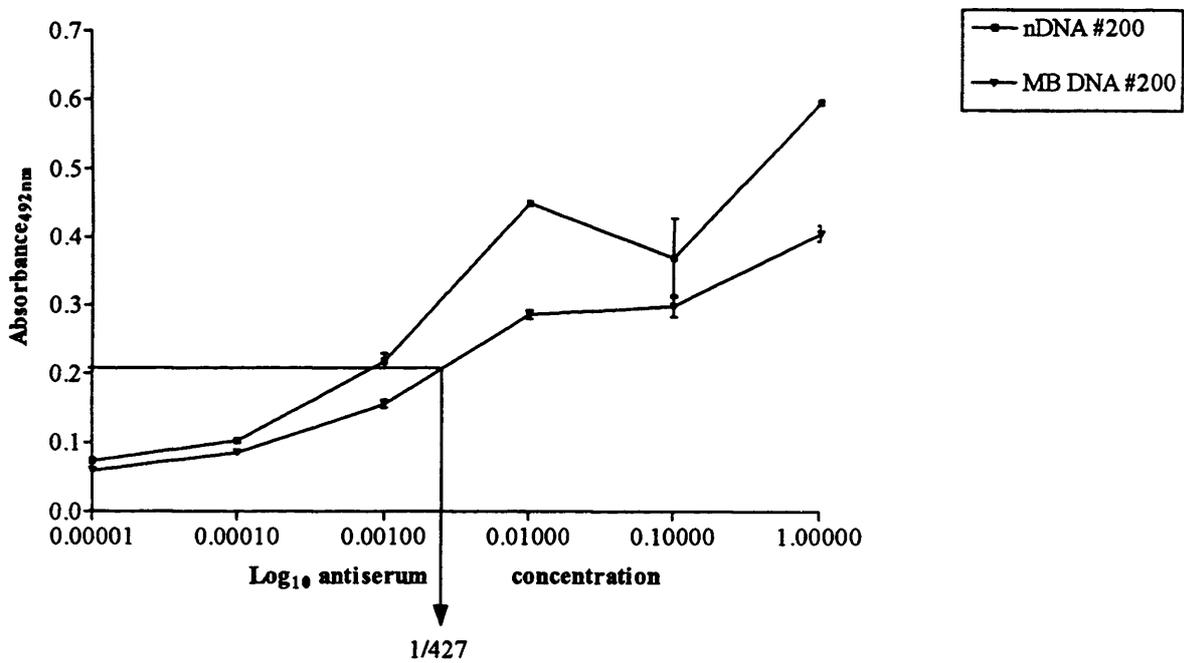


Figure 3.14b. Representative figure by which antiserum titres are calculated. Titre is the concentration of antiserum which gives half maximum absorbance, the example here is for animal #200, against MB DNA. Data shown is for experimental animal #200, and illustrates antiserum binding to double-stranded native and MBDNA.

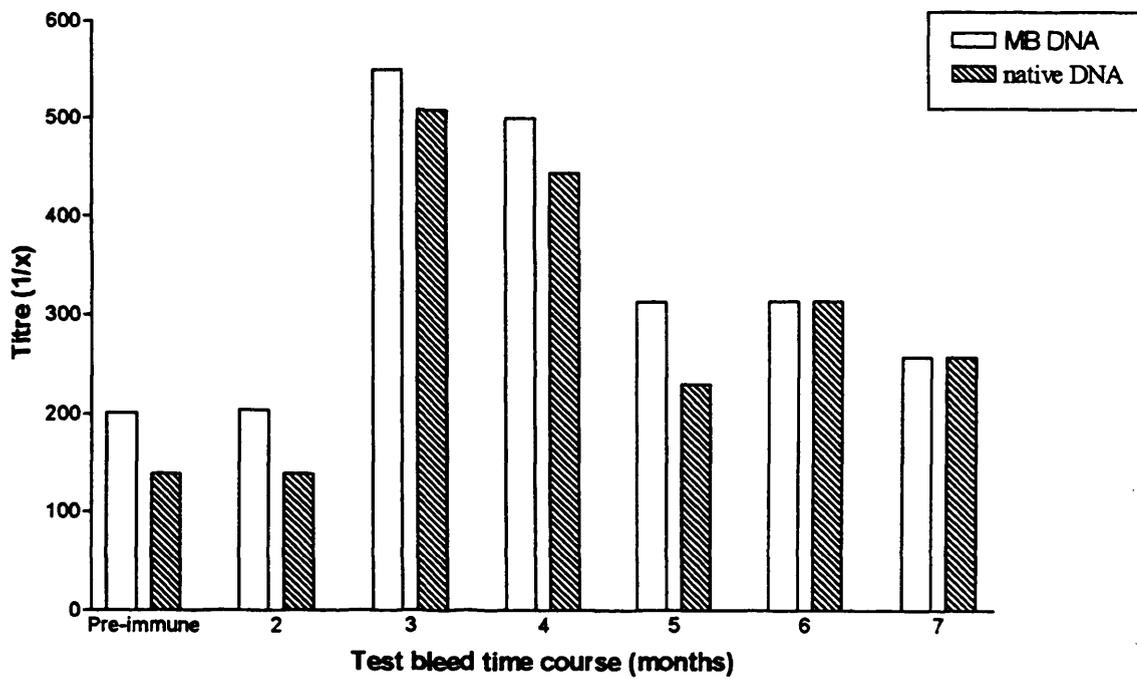


Figure 3.15. Variation in antiserum titre against double-stranded native and MB DNA as solid phase antigens with time in a single experimental animal (#199) following successive monthly boosts with 8-oxoguanosine conjugated to keyhole limpet haemocyanin.

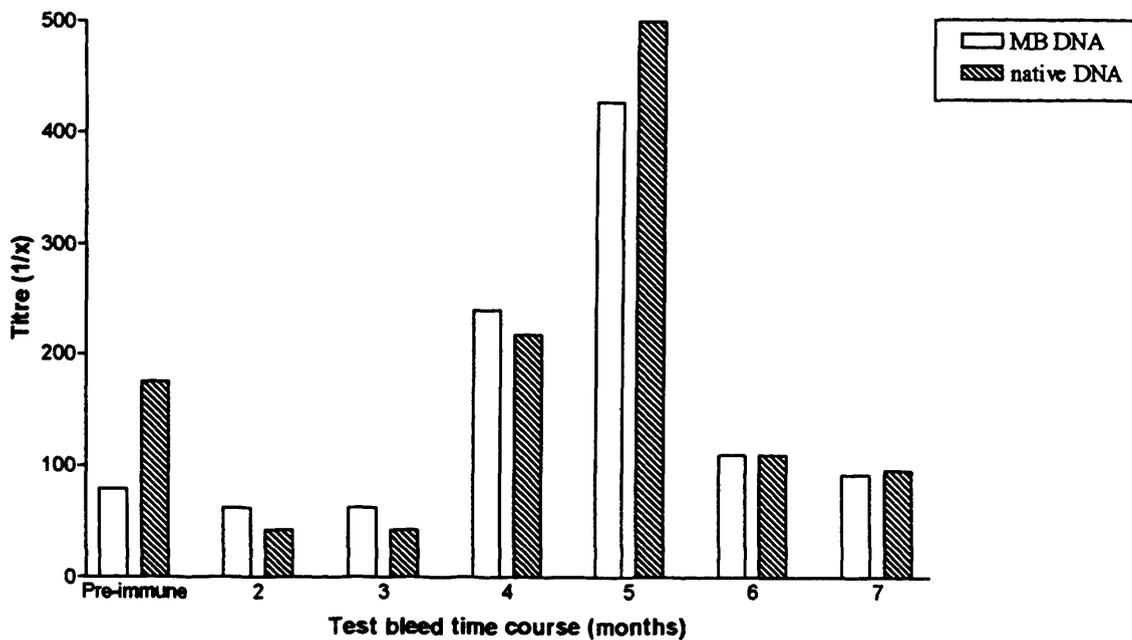


Figure 3.16. Variation in antiserum titre against double-stranded native and MB DNA as solid phase antigens with time in a single experimental animal (#200) following successive monthly boosts with 8-oxoguanosine conjugated to keyhole limpet haemocyanin.

Immunogenicity of 8-oxoguanosine - examination of the monoclonal response

Following booster injections with 8-oxoKLH an immune response was produced in all four mice, against double-stranded MB DNA, compared to pre-immune (Figure 3.17). From these results a slight anomaly was evident, absorbances for the 1/1000 dilution, post boost, were greater than the 1/100 values. Furthermore, the 1/10 preimmune values were greater than their corresponding 1/10, post boost values - this may have been due to a large proportion of non-specific antibody binding which became more specific, but represented a smaller proportion of total antibody, following immunisation. Although both points defied full explanation, the most important feature, greater post boost binding at 1/100 and 1/1000, was clear. A repeat of this experiment, using the 8-oxoBSA conjugate as the solid phase antigen did not show the same anomaly, with respect to the higher 1/1000 post boost binding compared to 1/100 (Figure 3.18). Additionally, it was noted that the dilution-matched differences between preimmune and post boost were not as great as with MB DNA as the solid phase antigen. This was thought to perhaps be due to differences in relative numbers of antigenic sites on each solid phase antigen. Animal #4, appearing to be responding most favourably to immunisation, was selected to enter the monoclonal process. However a successful fusion did not occur, which was thought to be associated with a highly fibrous spleen. Animal #3 was subsequently selected and the nature of its polyclonal response was examined in more detail.

Examination of the polyclonal response in mouse #3 demonstrated only a slight discrimination between MB and native double-stranded DNA at all dilutions of the antiserum (Figure 3.19). However, denaturation of the DNA, by boiling and rapid cooling, allowed the demonstration of an at least two-fold recognition of MB over native DNA, at all dilutions of the antiserum (Figure 3.20). On obtaining a suitable titre, this mouse (#3) was chosen to enter the monoclonal process, as described in Chapter 2.2. Briefly, the mouse was boosted via the intra peritoneal route with the 8-oxo/BSA conjugate in saline three days prior to removal of the spleen. Mainly B-cells are retrieved following passage of the spleen through a mesh and these are immortalised by fusion with a myeloma cell line (NS0). The resultant hybridomas are cultured and then screened by ELISA for immunoglobulin production.

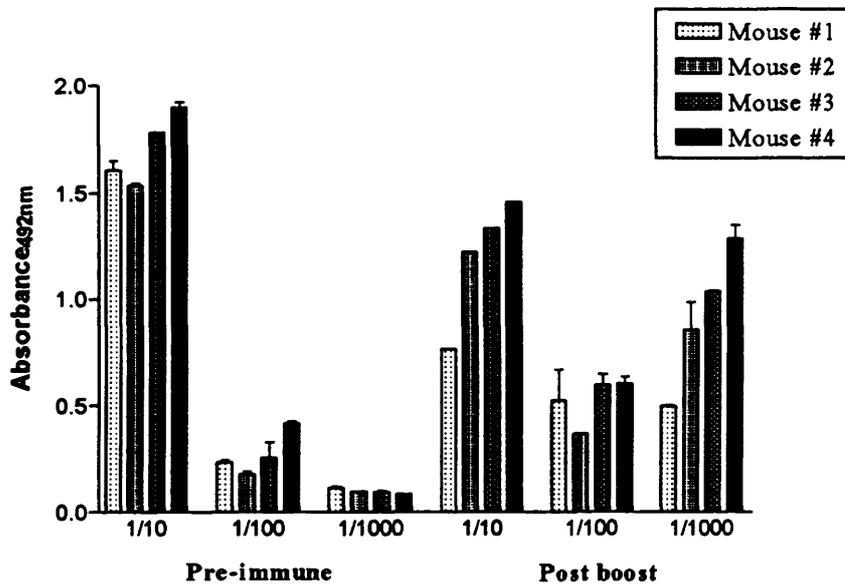


Figure 3.17. Direct ELISA binding of mouse antisera to MB DNA. Values are derived from four animals and represent the mean (SEM) of three determinations per dilution.

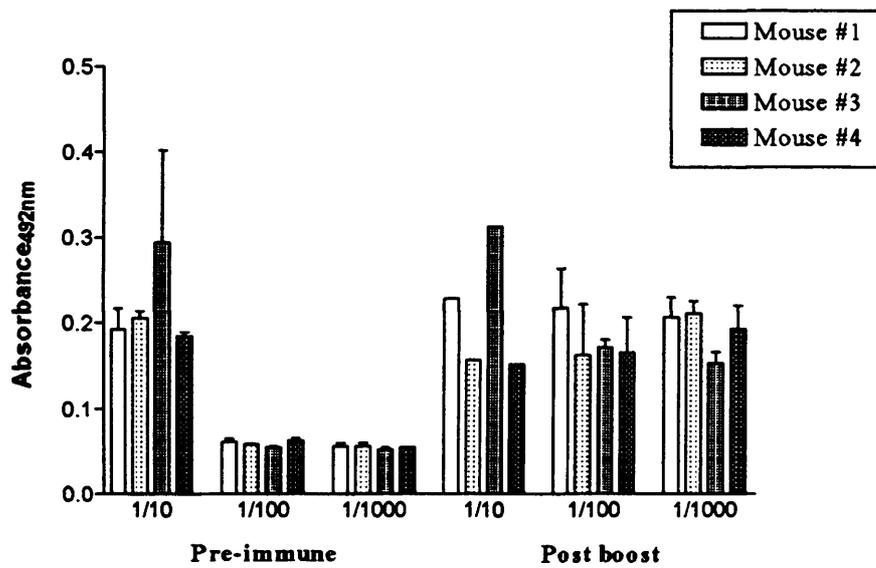


Figure 3.18. Direct ELISA binding of mouse antisera to 8-oxoguanosine/BSA. Values are derived from four animals and represent the mean (SEM) of three determinations per dilution.

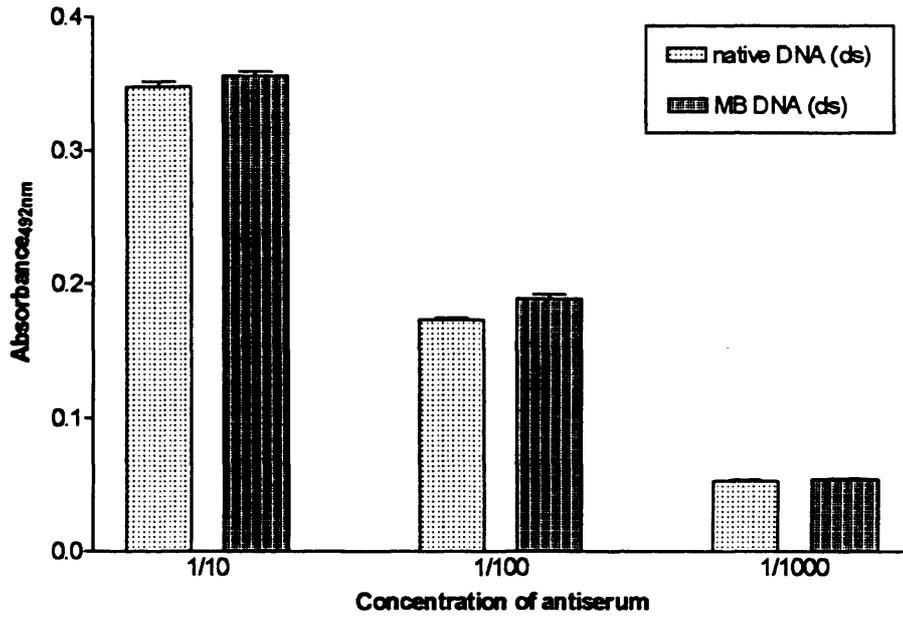


Figure 3.19. Representative direct ELISA binding of antiserum from a single mouse (#3), immunised with 8-oxoguanosine conjugated to keyhole limpet haemocyanin, to double-stranded native and methylene blue treated DNA. Values represent the mean (SEM) of triplicate determinations per dilution.

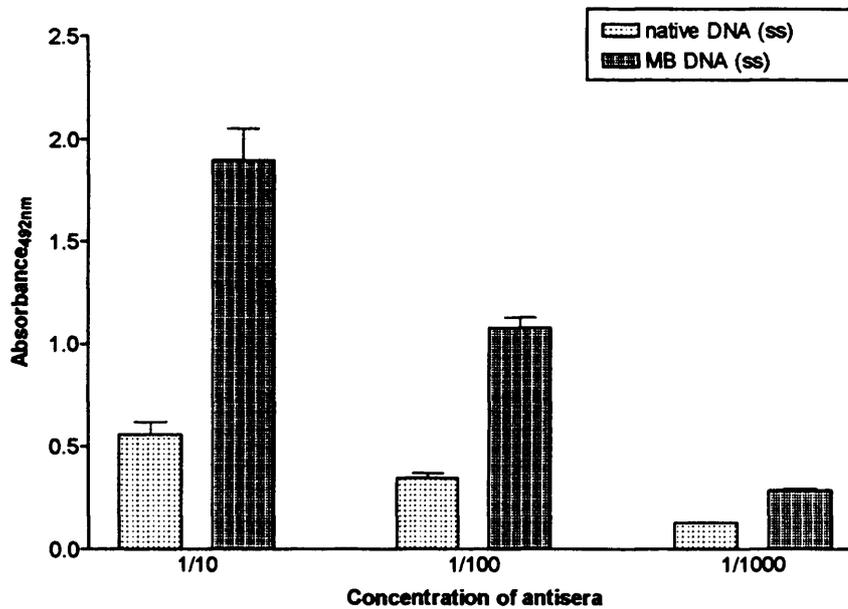


Figure 3.20. Representative direct ELISA binding of antiserum from a single mouse (#3), immunised with 8-oxoguanosine conjugated to keyhole limpet haemocyanin, to single-stranded native and methylene blue treated DNA. Values represent the mean (SEM) of triplicate determinations per dilution.

Initial screening of mixed colony cultures revealed one culture (denoted 3/8) to be producing a response over the medium control. This was subsequently cloned, producing three secreting clones (denoted 3/8/1, 3/8/2 and 3/8/3)(Figure 3.21). Clone 3/8/1 appeared to be producing the best response to 8-oxoguanosine/BSA compared to the medium control. This clone was determined to be monoclonal as a result of the serial dilution process adopted during its culturing, however, confirmation of this by an isotyping kit was not performed. Further screening of this clone showed no recognition of double-stranded MB DNA over native (Figure 3.22), as had been seen with the polyclonal sera. However, denaturation of the double-stranded MB DNA resulted in a two-fold increase in binding of 3/8/1 immunoglobulin (Figure 3.23). Additionally, use of the 8-oxoGR/BSA conjugate also resulted in an increase in binding over double-stranded MB DNA. Binding to native DNA, irrespective of whether it was denatured or not, was two-fold lower than to single-stranded MB DNA.

Immunogenicity of UVH and ASH DNA - examination of the monoclonal response

The polyclonal responses to UVH DNA, in three mice, are shown in Figures 3.24 and 3.25. These results reproduce exactly those seen previously with UVH DNA (Figure 3.10), with the antiserum showing greater recognition of UVC-modified DNA than UVH DNA. These results are most apparent for a 1/1000 dilution of the antisera (Figure 3.25). Again, some recognition of DNA modified by hydrogen peroxide alone (H_2O_2 DNA) was seen, suggesting the antiserum to contain some recognition of ROS-modified DNA.

Following sacrifice the spleens were obtained from all three mice and treated as described previously. Only three hybridomas were produced, all being from mouse number three and denoted F3/9, F3/50 and F3/29. Screening of supernatants from these three hybridomas was performed, the results of which are shown in Figure 3.26. Most striking is the response of F3/9 towards native DNA, over UVH DNA, contrasting with the polyclonal results. Cloning of F3/9 produced four clones, denoted F3/9/4, F3/9/7, F3/9/10 and F3/9/11, all of which, when screened against native and UVH DNA, displayed strong preferential recognition of native, with high absorbance values (Figure 3.27).

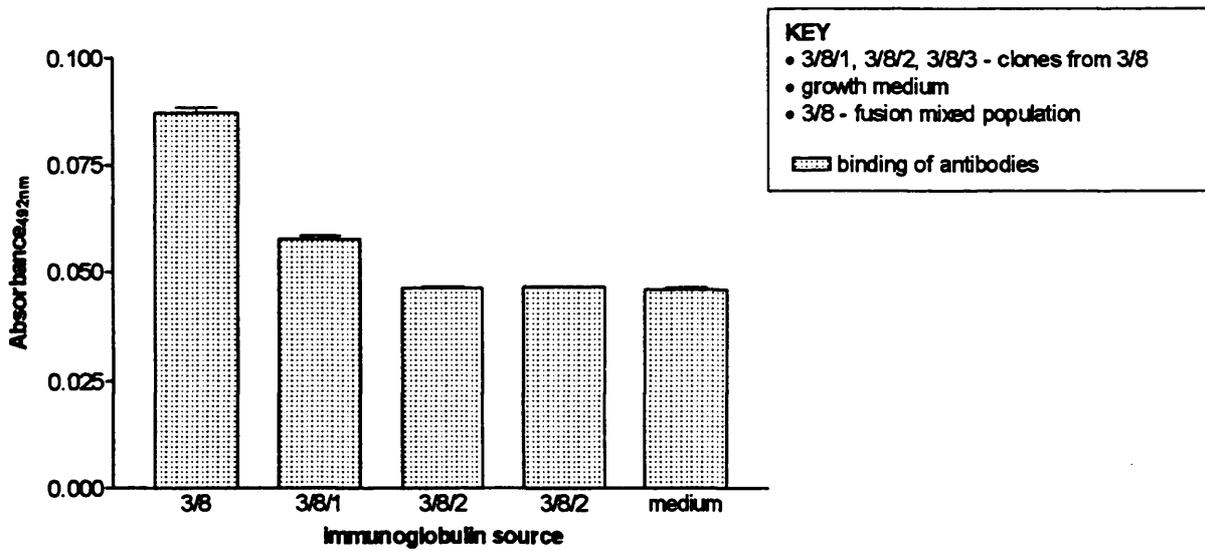


Fig 3.21. Direct ELISA binding of antibodies from a representative fusion and subsequent cloning, compared to values derived from culture medium. Solid phase antigen is 8-oxoguanosine/BSA conjugate. Values represent the mean (SEM) of quadruplicate determinations.

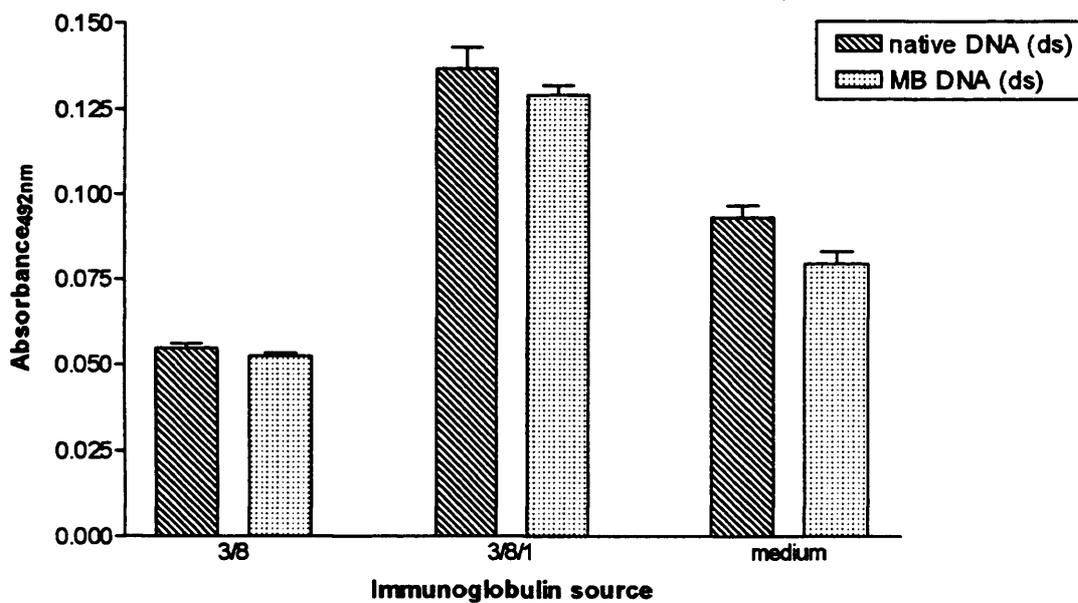


Figure 3.22. Direct ELISA binding of immunoglobulin from clone 3/8/1 compared to binding from mixed population culture 3/8 and culture medium against double-stranded (ds) native and MB DNA. Values represent the mean (SEM) of multiple determinations (n=5).

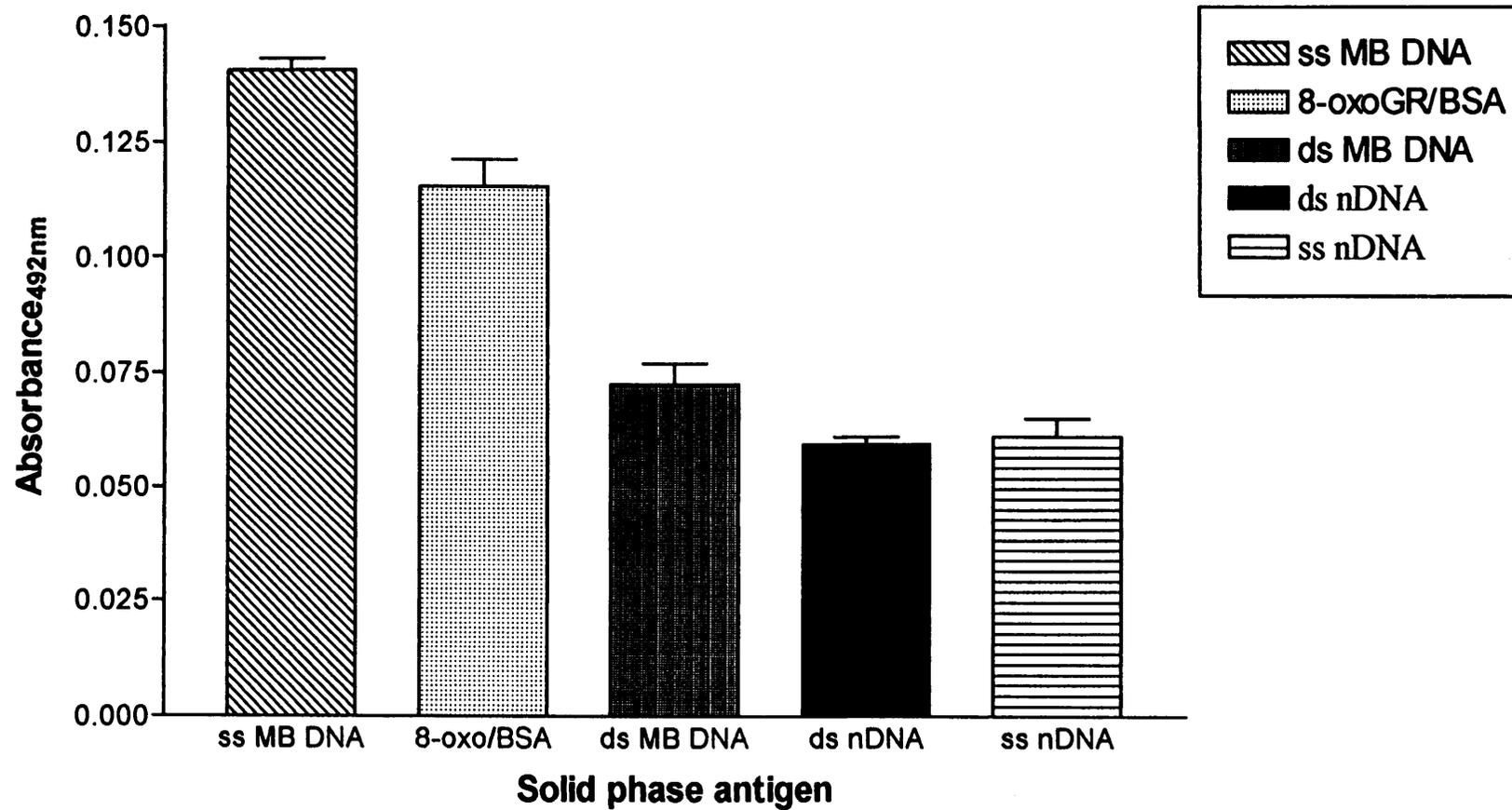


Figure 3.23. Direct ELISA binding of immunoglobulin from clone 3/8/1 to single-stranded (ss) and double-stranded (ds) methylene blue modified DNA, native DNA and the 8-oxoguanosine/BSA conjugate (8-oxoGR/BSA). Values represent the mean (SEM) of duplicate determinations.

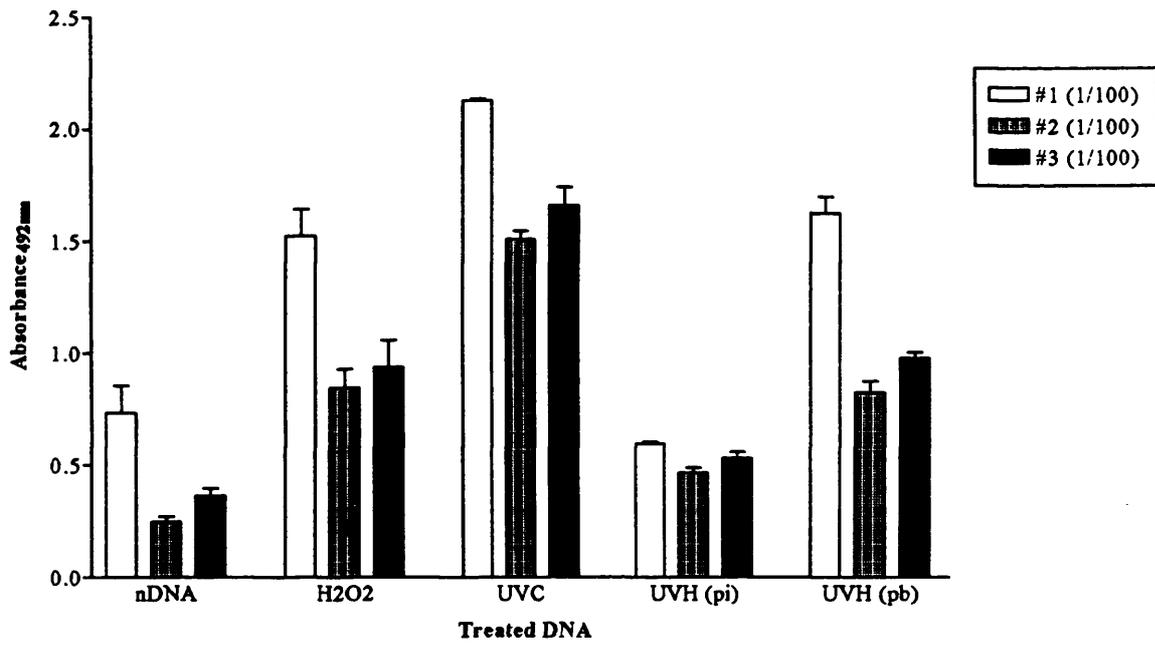


Figure 3.24. ELISA results illustrating the relative affinities exhibited by the polyclonal antiserum, diluted (1/100), of three mice immunised with the UVH/H₂O₂ (UVH) immunogen. Binding of pre-immune (pi) and post boost (pb) antisera to UVH DNA is also shown. Values represent the mean and range of two determinations.

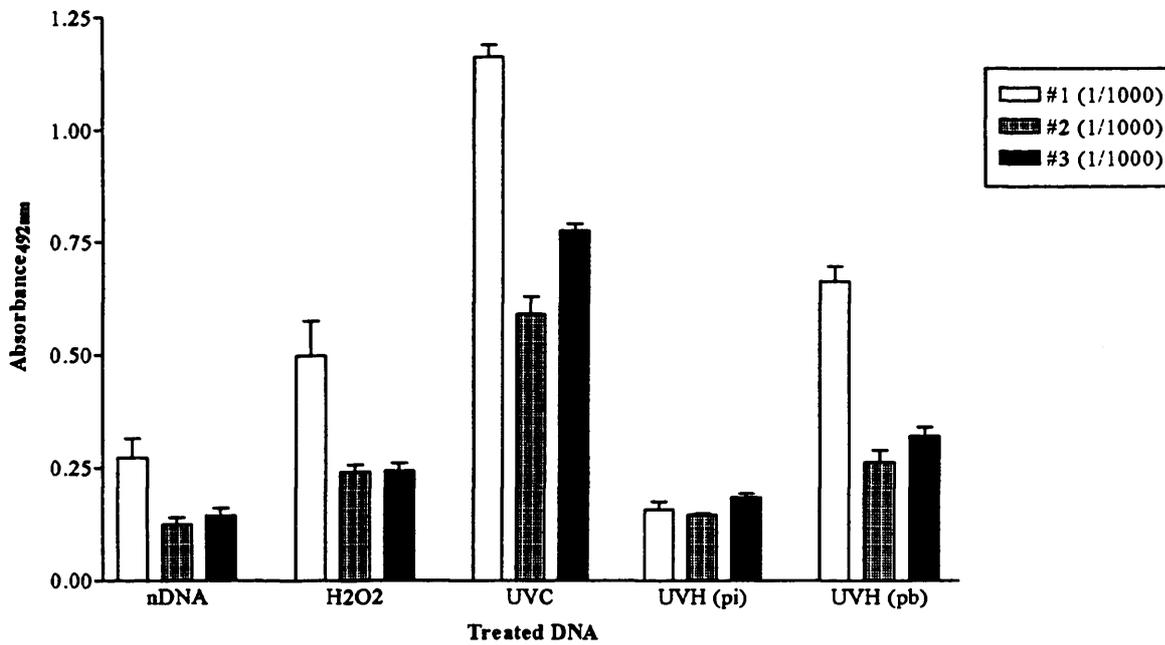


Figure 3.25. ELISA results illustrating the relative affinities exhibited by the polyclonal antiserum, diluted (1/1000), of three mice immunised with the UVH/H₂O₂ (UVH) immunogen. Binding of pre-immune (pi) and post boost (pb) antisera to UVH DNA is also shown. Values represent the mean and range of two determinations.

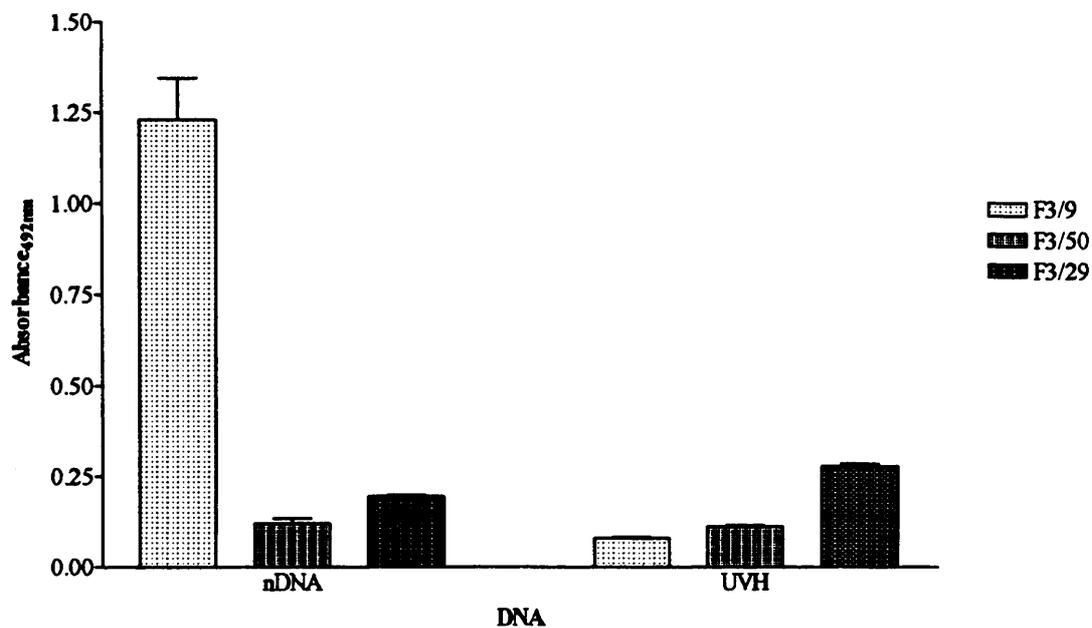


Figure 3.26. ELISA binding of antibodies in the supernatants from three mixed fusion populations to native and UVC/H₂O₂ (UVH) treated DNA. Fusions are derived from mouse number 3, immunised with UVH DNA. Values represent the mean (SEM) of three determinations and are corrected for the control (NSO) binding.

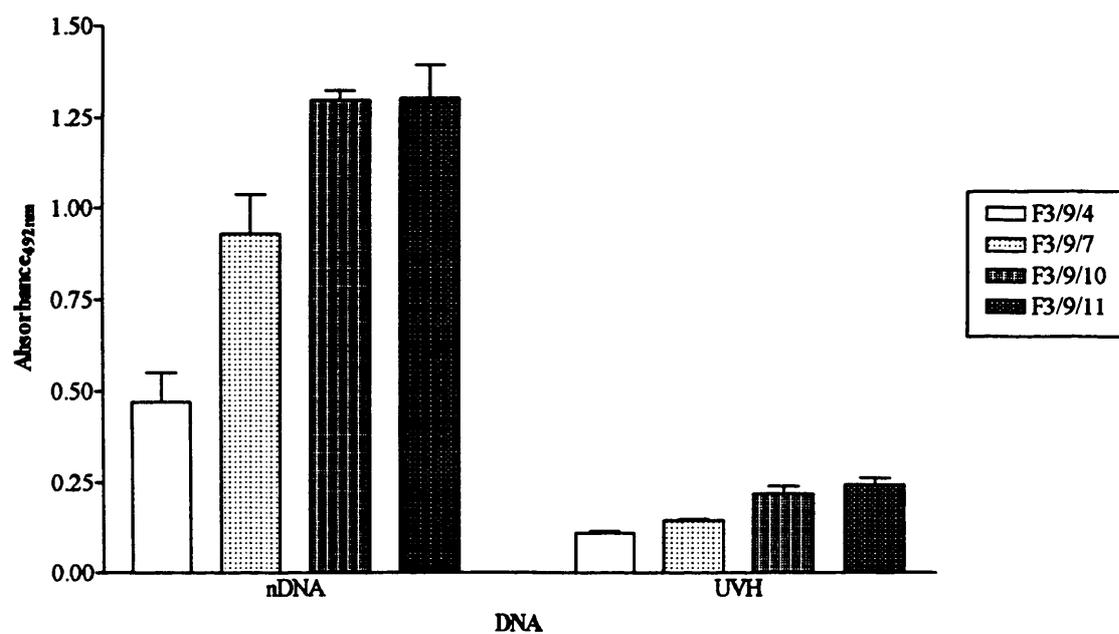


Figure 3.27. ELISA binding of antibodies in the supernatants of four clones from the mixed fusion population F3/9, to native and UVC/H₂O₂ (UVH) treated DNA. Values represent the mean (SEM) of three determinations and are corrected for the control (NSO) binding.

Figures 3.28-3.31 illustrate initial screening studies to characterise binding of antibody from the above clones. F3/9/11 proved to be of most interest and also possessed the greatest response to native DNA. From these results it would appear that F3/9/11 recognises an antigenic moiety present in single-stranded native DNA and double-stranded ASH DNA. In contrast to 3/8/1 it does not recognise MB DNA, irrespective of denaturation. Further characterisation of this response will need to be performed as part of future work.

It was seen that for the UVH DNA, the polyclonal responses in three animals bore no resemblance to the monoclonal response. For this reason, test bleeds of mice immunised with ASH DNA (n=3) were not performed. The subsequent attempts to produce viable, secreting hybridomas proved to be unsuccessful, although the reason for this was unclear.

Recognition of FEH DNA by SLE serum

The antisera, produced by immunisation of experimental animals with FEH DNA, demonstrated cross-reactivity between FEH and native DNA (Figures 3.12 and 3.13, page 102). Cross-reactivity, of SLE sera, between ROS and native DNA has previously been noted (Blount *et al.*, 1990). It was therefore pertinent to examine the avidity of SLE serum for FEH DNA, the results of which are shown in Figure 3.32. The serum, containing high titres of anti-DNA antibodies, displayed equal binding to both native and modified DNA, at all dilutions of the serum.

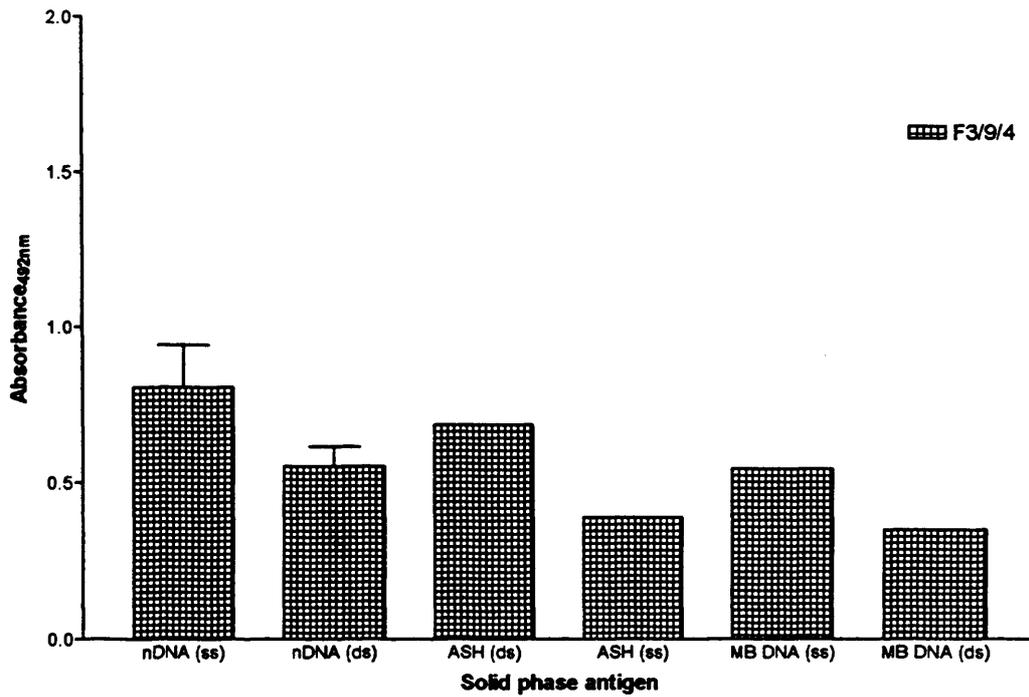


Figure 3.28. Binding of immunoglobulin in supernatant from clone F3/9/4 to single-stranded (ss) or double-stranded (ds), native (nDNA), ascorbate/hydrogen peroxide (ASH) or methylene blue (MB) DNA.

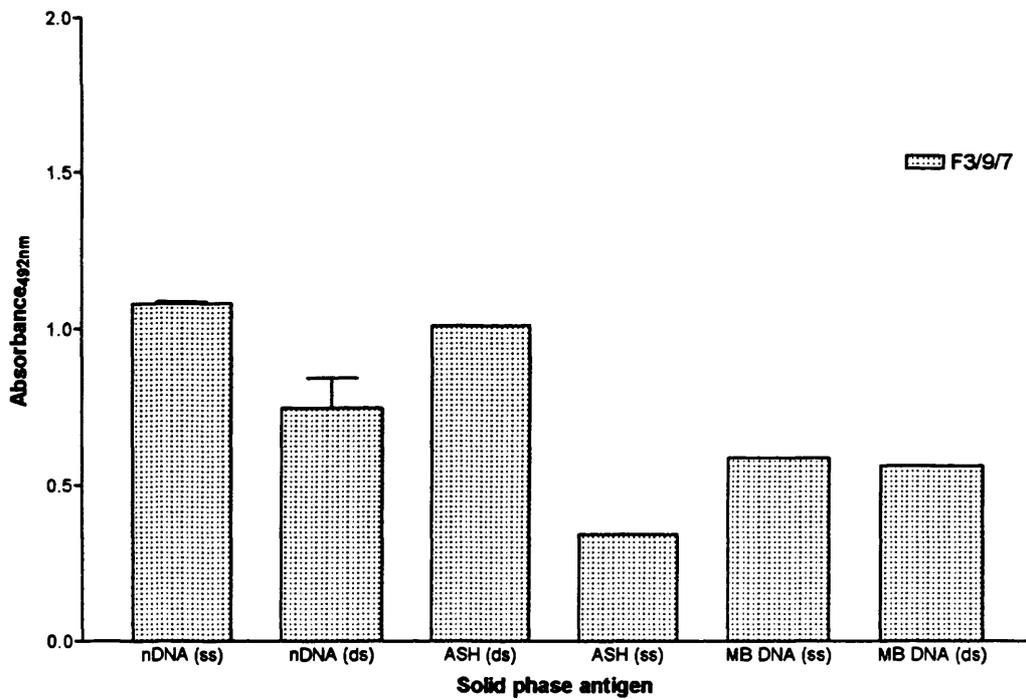


Figure 3.29. Binding of immunoglobulin in supernatant from clone F3/9/7 to single-stranded (ss) or double-stranded (ds), native (nDNA), ascorbate/hydrogen peroxide (ASH) or methylene blue (MB) DNA.

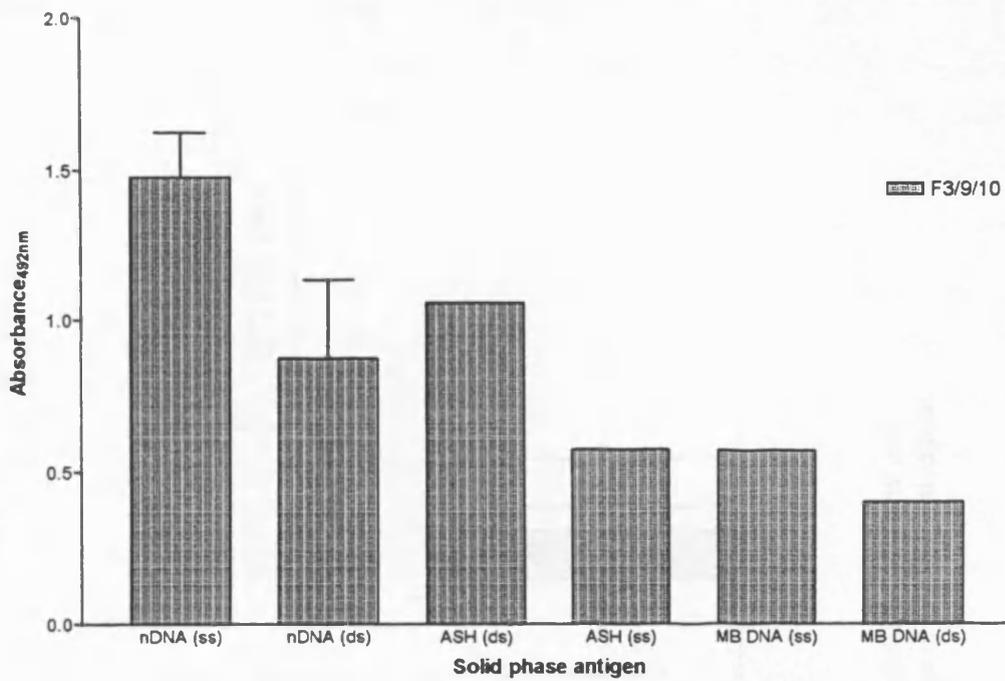


Figure 3.30. Binding of immunoglobulin in supernatant from clone F3/9/10 to single-stranded (ss) or double-stranded (ds), native (nDNA), ascorbate/hydrogen peroxide (ASH) or methylene blue (MB) DNA.

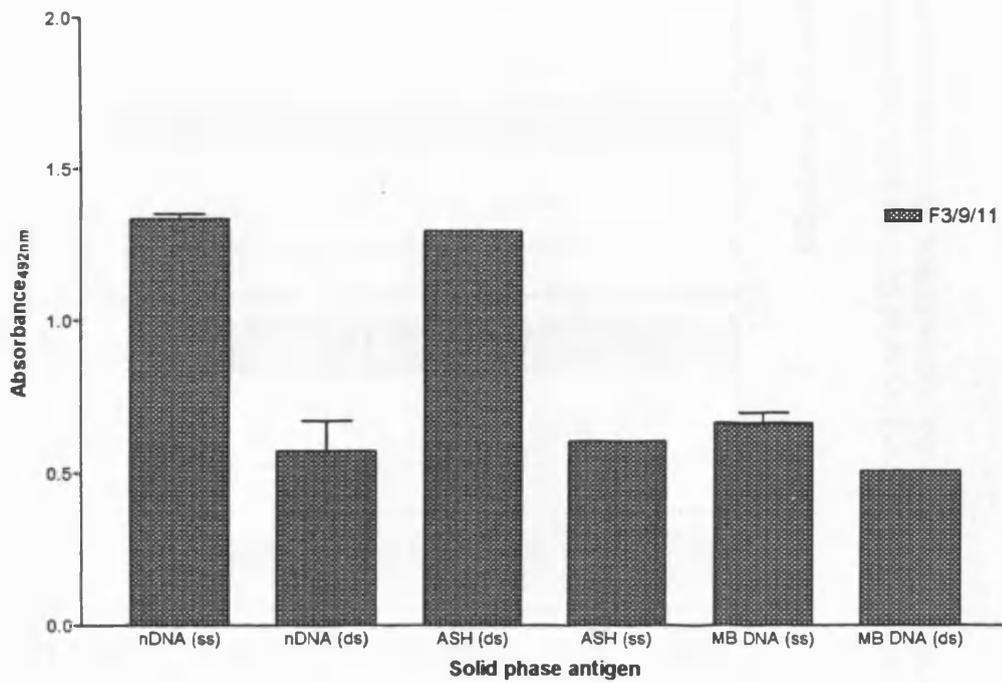


Figure 3.31. Binding of immunoglobulin in supernatant from clone F3/9/11 to single-stranded (ss) or double-stranded (ds), native (nDNA), ascorbate/hydrogen peroxide (ASH) or methylene blue (MB) DNA.

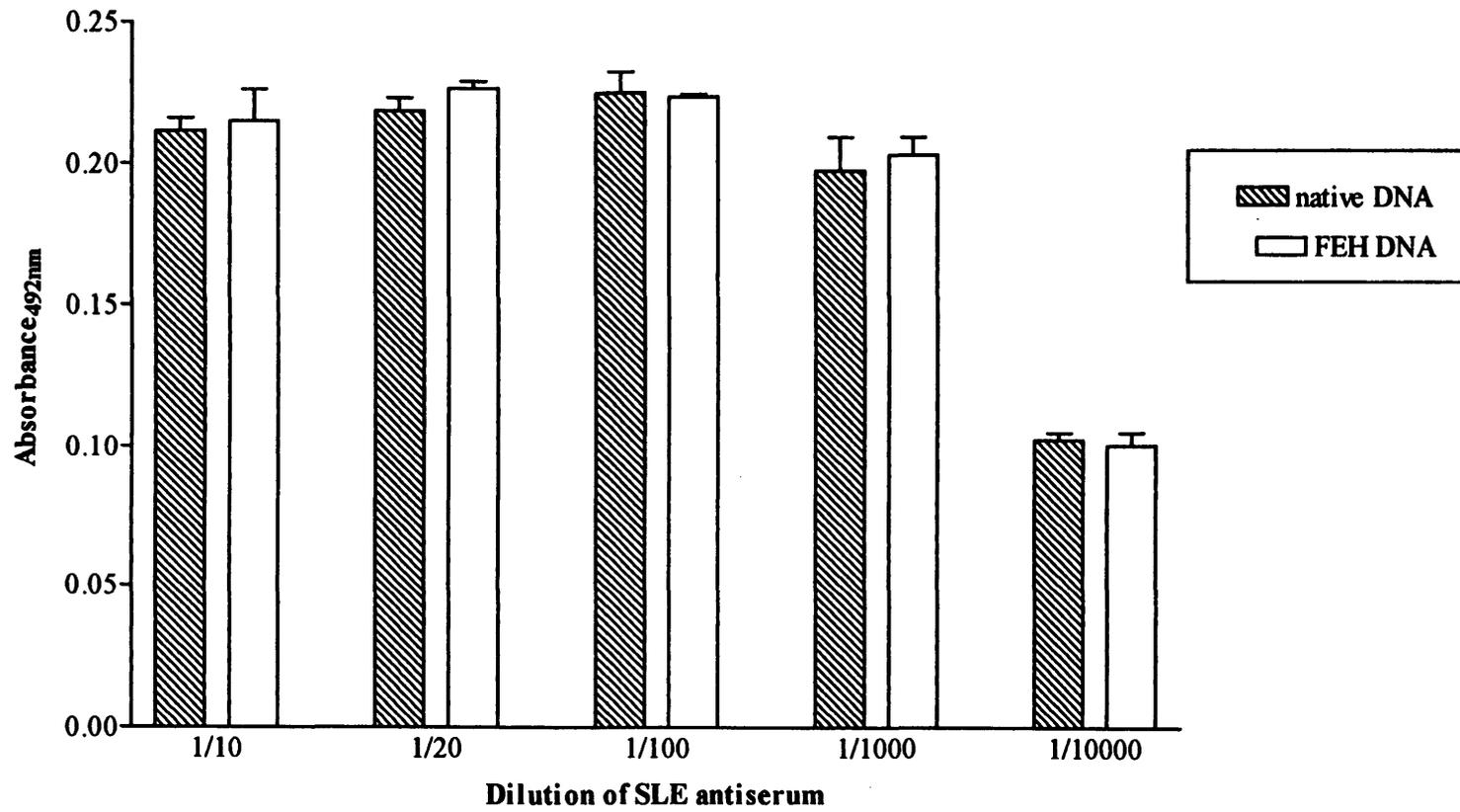


Figure 3.32. Direct binding of SLE anti-DNA IgG antibodies in whole antiserum, to native and FEH-modified double-stranded DNA. Values represent the mean and range of two determinations.

Summary of antisera and antibodies produced

The production of polyclonal antisera to methylene blue (MB) and γ -irradiated DNA proved to be unsuccessful. It is speculated that possible explanations for this may include insufficient modification of the DNA, particularly in the case of the low dose γ -irradiated DNA, although the MB DNA was shown to contain 1% of the total deoxyguanines as 8-oxodG. The choice of Titermax as an adjuvant, may also have affected the response, although this is reported to be an effective alternative to Complete Freund's in many situations (Bennet *et al.*, 1992). The DNA irradiated with a high dose of γ radiation may have been severely damaged, to the extent that very little intact DNA structure remained, presenting a poor immunogen, despite the presence of BSA. The iron/hydrogen peroxide system (FEH) did not produce a strong immunological response towards the immunogen, suggested by the low binding to FEH DNA in ELISA, although an explanation for this was unclear, as extensive oxidative modification was noted. Furthermore, cross-reactivity with native, unmodified DNA was also seen, not an attribute for a useful antiserum to DNA damage. This feature was an important finding with ramifications for the postulated role of ROS DNA in the pathogenesis of the disease systemic lupus erythematosus, fully explained in the discussion, below. Although used as ROS generating system, with a view to producing an antiserum to ROS-modified DNA, a serendipitous finding with UVC/hydrogen peroxide, suggested most of the antiserum's response to be towards UV damage, the implications of which are fully discussed later.

A literature precedent (Degan *et al.*, 1991) and the polyclonal results of immunisation with MB DNA suggested that immunisation with a specific oxidative DNA lesion to be a pertinent approach to generating antibodies to ROS-damaged DNA. Both polyclonal and monoclonal approaches were adopted. A modest polyclonal response ($\sim 1/550$ and $\sim 1/500$) was elicited in both experimental animals, however, this response displayed no specificity for MB DNA compared to native. The monoclonal approach appeared to be more successful resulting in a clone (3/8/1) secreting immunoglobulin which, whilst not discriminating between double-stranded native and MB DNA, demonstrated a two-fold greater recognition of single-stranded MB DNA over likewise denatured native DNA. This clone was concluded to have been unstable after prolonged culturing resulting in a loss of discriminatory immunoglobulin binding to native and MB DNA. Subsequent attempts at

cloning with spleens from mice #1 and 2 were not successful, perhaps due to the fusion conditions not being optimised.

Good polyclonal responses were seen in all three mice immunised with UVH DNA. The splenocytes of only one mouse out of three successfully produced hybridomas. Most surprisingly, based on the polyclonal results, the binding of antibodies in the supernatants was predominantly towards native DNA over UVH. This was more apparent after cloning, with F3/9/11, showing the strongest response. Initial screens of the antibodies produced from the clones suggested that they all recognise similar antigens, although to different extents.

The attempt to produce monoclonal antibodies to ASH DNA proved unsuccessful, highlighting the unpredictability of the cloning process.

DISCUSSION

This work was undertaken to produce an immunochemical tool for the detection of UV-induced ROS damaged DNA. The ROS-generating systems used all had precedents as such within the literature (Blount *et al.*, 1989; Alam *et al.*, 1993; Ara and Ali, 1993), although for some this was the first time they had been used to modify DNA prior to immunisation.

The failure of MB DNA to produce a suitable immune response in rabbits was concluded to be due to the adjuvant, Titermax, recommended by Biomedical Services Unit (University of Leicester) for its low toxicity, although Freund's had previously been used by the Division of Chemical Pathology. Reversion to Freund's for the remainder of the immunisation protocol improved the immune responses seen.

Comparison between immunogenicity of modified DNA and degree of oxidative modification suggested there to be no correlation between the two. Highly modified DNA did not appear any more immunogenic than minimally modified. Initially, this would suggest that the DNA lesion, 8-oxoG, is not immunogenic. Clearly, from the work in both rabbits and mice with 8-oxoGR, this is not true. Therefore it would appear that the presentation of the immunogen to the immune system is important. Extensive oxidative modification to DNA would likely result in many strand breaks which may give rise to fragmentation and loss of the DNA when preparing for immunisation and hence a poor immunogen. The

immunogenicity of UVH DNA, as discussed later, with appreciable oxidative modification, is believed to be largely due to other DNA lesions.

Immunisation of rabbits with the 8-oxoguanosine/KLH conjugate elicited an immune response which rose with subsequent boosts, achieving titres of $\sim 1/550$ and $\sim 1/500$ in the two animals. Loss of this response may have been due to the induction of tolerance to the immunogen in the animals, although such a regime was reported to be successful for the generation of a polyclonal antiserum by Degan *et al.* (1991), whose whole protocol of monthly immunisations lasted one year. Cross-reactivity of the antisera with native double-stranded DNA was also noted, although this occurred to various degrees during the immunisation schedule. This is perhaps not surprising as the modified nucleoside is structurally different from the native form only in a carbonyl group at the C-8 position - resulting in antisera which are raised to 8-oxoGR recognising dG. Some cross-reactivity with the native base and nucleoside has also been noted in the competition RIAs of Degan *et al.* (1991) and, to a lesser extent, Park *et al.* (1992) with their poly- and monoclonal antibodies, respectively. (See table below.)

| Inhibitor | I_{50} (Moles)* |
|-----------|-----------------------|
| 8-oxodG | 5.0×10^{-13} |
| 8-oxoGR | 2.0×10^{-12} |
| 8-oxoG | 8.5×10^{-12} |
| dG | 3.0×10^{-9} |
| Guanine | 2.9×10^{-9} |

(adapted from Park *et al.* 1992)

Assuming the polyclonal antiserum generated above, possessed, for example, the discrimination of the antiserum of Degan *et al.* (1991), the combination of an approximately 10000-fold discrimination between the native and modified form of dG along with the MB DNA used as the solid phase antigen only having one modified dG in every hundred (as determined by HPLC-EC) is very likely to result in cross-reactivity with native DNA.

* Moles of inhibitor which produces 50% inhibition.

A similar result was seen in the polyclonal response of mice, although further investigation of this response demonstrated how rendering both the native and MB DNA single-stranded seemingly unmasked the antigenic site recognised, resulting in a marked increase in discrimination. This property was successfully maintained through the cloning process and was apparent in the binding of immunoglobulins from clone 3/8/1. These data would suggest that the response is largely to the lesion itself, although double strandedness allows the lesion to adopt a conformation whereby the antiserum may still partially recognise the site.

In DNA, guanine normally adopts the keto form with an *anti* glycosidic conformation, resulting in base-pairing with cytosine. The 6,8-diketo form of 8-oxodG is energetically favoured (Aida and Nishimura, 1987) and is prone to adopting the *syn* glycosidic conformation which possesses the ability to distort the DNA making normal base-pairing unsuitable (Culp *et al.* 1989). Whilst pairing with dC may still occur, this conformation of 8-oxodG makes mis-pairing with dA and dT possible. Any resultant structural changes to the DNA are subsequently located near the site of modification (Oda *et al.* 1991). However, most work on this subject has been to establish the mechanisms by which mis-pairing occurs during replication and why proof-reading/error-correcting DNA polymerases are unable to recognise and repair these lesions (Kouchakdjian *et al.*, 1991; McAuley-Hecht *et al.*, 1994) and not the effects the formation of this lesion has on DNA structure. Despite this, the literature suggests that some conformational changes to the DNA helix may occur following formation of the lesion. Taken together, these data suggest that the monomeric 8-oxoGR in the immunogen is free to adopt the *syn* glycosidic conformation and as a consequence it is this with which the immune system is presented. Within double-stranded DNA the lesion is unlikely to possess such a conformation due to it being held in place (in the *anti* conformation) by base-pairing with cytosine. Larger conformational changes of the DNA duplex may be introduced by the torsion exerted as the 8-oxodG attempts to swap to the energetic favourability of the *syn* conformation - leading to limited exposure of the lesion and some recognition by the antisera. However, rendering the DNA single-stranded allows the 8-oxodG to adopt the *syn* conformation as in the immunogen. It would therefore appear that the combination of the *anti* to *syn* conformational change, along with the modification at the C-8 position maybe responsible for the two-fold increase in recognition of 8-oxodG over dG in single-stranded DNA. A factor which may explain the failure of antisera to detect 8-oxodG in DNA, is steric hindrance, that is to say, the coiling and super-coiling of

the DNA. This may physically prevent the antibody access to the lesion, despite being antigenic for the antibody whilst *in situ* in double-stranded DNA.

The strong response of the UVH clones to native DNA, over UVH DNA was a surprising result and although full characterisation of the clones remains to be future work, an initial study showed some equally interesting results. The greater binding to single-stranded (ss) native DNA than double-stranded (ds) would suggest an anti-ss DNA antibody. However, good recognition of ds ASH DNA was seen and rendering this ss seemed to destroy the antigen, causing a reduction in binding. Some recognition of ss MB DNA over ds was seen, but not as seen with F3/8/1. This result requires further study to clarify the nature of the antigen recognised.

Reactive oxygen species (ROS) are implicated in the inflammatory, autoimmune, connective tissue disease, systemic lupus erythematosus (SLE), particularly in respect of processes leading to the formation of pathological anti-DNA antibodies (Blount *et al.*, 1989, 1990). Exposure to ROS increases the immunogenicity of DNA, as seen above, furthermore, it also increases the antigenicity of DNA for SLE antibodies (Blount *et al.*, 1989, 1990, Ara and Ali, 1993). The affinity SLE anti-DNA antibodies have for native and modified DNA support the postulate that ROS modification of DNA may be involved in the pathogenesis of SLE (Blount *et al.*, 1991). Previous studies (Blount *et al.*, 1990) have indicated that DNA modified by ASH is highly antigenic for SLE sera. The immunogenicity of ASH DNA has also been examined and found to be strong (approximately eight-fold, Cooke *et al.*, 1997). However, the comparative binding of the ASH antiserum to native double-stranded DNA as antigen was very low. In order to investigate the role UV-induced radical modifications of DNA has in the pathogenesis of and photosensitivity associated with SLE, the antigenicity of UV damaged DNA for SLE sera was studied by Cooke *et al.* (1997). The data in this manuscript indicated that DNA modified by either UVA, UVB or UVC was antigenic for SLE sera. However, the binding of these three antigens was shown to be not significantly different from binding to native DNA. ASH DNA acted as a positive control with a marked increase in binding over native DNA. To further elucidate the role ROS-induced DNA lesions have in the pathogenesis of SLE, the antigenicity of ROS damaged DNA for SLE sera was examined by Cooke *et al.* (1997a) (see Appendix). The level of IgG-class, anti-ROS DNA antibodies in SLE sera was determined by ELISA. Recognition by SLE sera of double stranded (ds) DNA, modified by ascorbate in the presence of

hydrogen peroxide (0.327 +/- 0.030), increased to a statistically significant level compared to native double-stranded DNA (0.206 +/- 0.030), as calculated by a Student's t-test ($p < 0.05$). However, rendering the ASH-DNA single stranded (ss) actually reduced binding when compared to ss native DNA. Additionally, binding of SLE sera was found to be significantly greater ($p < 0.05$) to ds methylene blue treated DNA than to ds native DNA. SLE sera were found to bind equally well to both denatured native and MB-DNA

The findings of Cooke *et al.* (1997 and 1997a), described above, showed that rendering ASH DNA, which is preferentially recognised to native by SLE sera, single-stranded, significantly reduced its recognition by SLE sera, compared to single-stranded native DNA. This suggests that denaturation destroys the epitope induced in DNA by ASH systems. Furthermore, SLE sera showed significantly greater binding to double-stranded MB DNA than to double-stranded native DNA possibly due to the presence of 8-oxodG, which has already been postulated to be involved in the pathogenesis of SLE (Lunec *et al.*, 1994). Characterisation of experimentally induced antisera, recognising single- and double-stranded DNA, has suggested guanine residues play an important role in antigen recognition (Munns *et al.*, 1982; Stollar *et al.*, 1986). Denaturation of the DNA has also been reported to be a prerequisite to preferential binding for these and some SLE antisera (Stollar *et al.*, 1986). Additionally, Sanford *et al.* (1990) noted that the glycosidic conformation of the guanine residues involved in the binding sites of two anti-Z-DNA antibodies was in fact *syn*, not the native *anti*. Bepalov *et al.* (1996) raised recombinant phage Fab fragments (fragment antigen binding) recognising 8-oxoG, which although not binding to DNA, did display structural homology with two monoclonal antibodies to native single-stranded DNA. Taken together, these data support the antigen-driven hypothesis (Blount *et al.*, 1991; Cooke *et al.*, 1997) for the formation of anti-DNA antibodies in SLE. The data further suggests that whilst native DNA is not immunogenic, damage to DNA may alter native bases both structurally and conformationally which increases their immunogenicity, the resultant antiserum subsequently recognising native bases and therefore DNA in addition to the modified forms.

The data presented here supports the evidence that hydroxyl radical generating systems can alter the immunogenicity and antigenicity of native DNA. However, some conclusions from this study are in direct contrast to those of Ara *et al.* (1993) in which hydrogen peroxide and UV (254nm) were used as a hydroxyl radical generating system. Ara *et al.* (1993)

stated that immunisation with DNA damaged by UVH resulted in antibodies with reactivity to ROS DNA. Hydroxyl radicals produce a variety of DNA damage products (Dizdaroglu, 1991a). Additionally, it is maintained that the UV component of the UVH system would primarily induce UV specific damage such as pyrimidine dimers (Fuchs, 1993; Ahmed *et al.*, 1993; Potten *et al.*, 1993). In the previous study of ROS-DNA antibodies by Ara *et al.* (1993) the data from the appropriate controls were not shown in order to discriminate between antibodies produced to different components of the DNA damage. From this study, it is suggested that this process leads to two principle forms of damage, $\cdot\text{OH}$ -derived and direct UVC-derived lesions. Furthermore, this data presented here and in Cooke *et al.* (1997) suggests that the majority of the immune response was to the UV-induced dimer lesions, rather than ROS lesions. That is to say, UVC-induced dimer lesions are more immunogenic. Previous studies (Natali *et al.*, 1971; Eggset *et al.*, 1987; Herbert *et al.* 1994) have clearly demonstrated the immunogenicity of such photoproducts. UVC-modified DNA has been shown to elicit a strong antibody response in laboratory animals. The resulting antibodies generally have minimal reactivity with native DNA (Herbert *et al.*, 1994; Natali *et al.*, 1971; Tan, 1968) although some cross reactivity has been reported (Levine *et al.*, 1966). Using UVC, the predominant lesions induced are the cyclobutane pyrimidine dimer and the pyrimidine (6-4) pyrimidone photoproduct (Mitchell *et al.*, 1989) and antibodies have been raised specifically to these lesions (Mori *et al.*, 1991). However, other non-ROS, radical-derived products, such as formamidopyrimidines have been shown to occur, but at frequencies well below that of dimer lesions (Doetsch *et al.*, 1995). The immunogenicity of UVH DNA is perhaps due to the nature of the bipyrimidine changes. Base alterations (West *et al.*, 1982a,b; Prevost *et al.*, 1990; Bonfanti *et al.*, 1990) and conformational changes (Ali *et al.*, 1985) have both been shown to influence immunogenicity. Alternatively, it could be simply that UVC produces more lesions than $\cdot\text{OH}$ and therefore increases the overall number of available antigenic determinants on the DNA.

There was a clear difference between the reactivity of the antisera to UVH DNA as antigen and to DNA damaged with UVC alone. This discrimination may be due to the reactivity of the antisera to $\cdot\text{OH}$ damage. In contrast to the findings of Ara *et al.* (1992 and 1993), minimal cross-reactivity with native DNA was demonstrated, consistent with other reports for a UVC antibody response (Herbert *et al.*, 1994; Natali *et al.*, 1971; Tan, 1968). The antiserum appeared to recognise H_2O_2 DNA marginally better than native DNA, implying

some modification had occurred by the action of H₂O₂ alone. It is speculated here that endogenous metal ions associated with the DNA, are able to facilitate Fenton-type reactions and the subsequent production of hydroxyl radical in close proximity (site specific) to the DNA, since H₂O₂ alone does not damage DNA (Schraufstatter *et al.*, 1988; Halliwell, 1993). A small portion of the antibodies present in the antiserum would therefore appear to recognise [•]OH modified DNA.

Alam *et al.* (1993) also utilised Fe²⁺ and hydrogen peroxide, via a Fenton-type reaction, to produce hydroxyl radicals. This system is more specific compared to UVH, inasmuch as the products are almost exclusively hydroxyl radical derived (Blakely *et al.*, 1990; Zastawny *et al.*, 1995). In the study of immunogenicity, presented here, the difference between binding of pre-immune serum and the antiserum to modified DNA again demonstrates that the action of the hydroxyl radical renders native DNA more immunogenic. Most significantly, this antiserum recognises native double-stranded DNA, in agreement with the findings of Alam *et al.* (1993). SLE sera also display this dual reactivity for ROS-modified and native DNA (Blount *et al.*, 1991; Ara *et al.* 1992; Frenkel *et al.*, 1993) and consequently a mechanism for antigen-driven autoantibody formation has been developed (Blount *et al.*, 1991). This theory is further supported by the data shown here in which antibodies in SLE serum show equal binding to native and the oxidatively-modified FEH DNA. This suggests that modification of non-immunogenic, native DNA, by the FEH system, increases its immunogenicity, giving rise to an antiserum which cross reacts with native and modified DNA, as SLE serum may.

The hydrogen peroxide/ascorbate (ASH) system used by Blount *et al.* (1990) and Cooke *et al.* (1997) was developed on the basis that reducing agents such as ascorbate potentiate the Fenton reaction by reducing metal ions and enabling further catalysis. Furthermore, this system is preferable to the UVH method, as only hydroxyl radical-induced products are formed. The DNA damage resulting from this mechanism clearly produced a better immunogen, demonstrating a stronger antibody response, than FEH. The hydroxyl radical is understood to form very similar patterns of base modification irrespective of the mechanism by which they are derived (Dizdaroglu *et al.* 1991c; Aruoma *et al.*, 1991). However, Spencer *et al.* (1994) have shown that the relative levels of these modifications vary depending on the generating system. Therefore the difference between FEH and ASH systems may be due to the effective action of ascorbate, maintaining the metal ions in a

reduced state and allowing a greater number of hydroxyl radicals and therefore lesions to be produced. Alternatively, some products, which are particularly immunogenic, may predominate with the ascorbate method. This in itself, is good evidence that variation exists within the “fingerprint” of hydroxyl radical damage, according to the method of production. An important contrast with the FEH method is the lack of cross-reactivity of the ASH antiserum with native DNA, which is consistent with the results of the UVH system. Clearly this is important when the goal is to produce an antiserum with useful applications including the detection of damaged DNA regions within largely native DNA.

The FEH results show the possibility for induction of anti-native DNA antibodies by a ROS-DNA antigen. The data of Cooke *et al.* (1997), using UV as a relevant environmental ROS-generating system with proven SLE exacerbating potential, showed that there was no preferential binding of SLE sera to UV-modified double stranded DNA compared to native double stranded DNA. This demonstrates that, whilst DNA irradiated with far UV is strongly immunogenic in rabbits (Herbert *et al.*, 1994) and native DNA is not (Tan and Stoughton, 1969), it is no more antigenic than native DNA for SLE sera. Far UV has recently been reported (Doetsch *et al.*, 1995) to generate products other than dimers; such products are amongst those also observed as a consequence of $\cdot\text{OH}$ attack, for example, FapyGua and FapyAde. Therefore some recognition of these lesions by SLE sera may be expected in UVC DNA. However an increase in binding was not seen. This suggested that these far UV-derived radical lesions are either below the level of detection by ELISA, or not important antigens recognised by SLE sera. Many modifications of DNA are immunogenic and some may also be recognised by SLE antisera (Blount *et al.*, 1989; Blount *et al.*, 1991; Frenkel *et al.*, 1993; Moinuddin and Ali, 1994), producing the proposal that autoantibodies arise *in vivo* because the subsequent immune response to ROS-modified DNA may display cross-reactivity with native double stranded DNA (Blount *et al.*, 1990). This is supported by the hypothesis of Diamond *et al.* (1992) that the persistence of anti-DNA antibodies in SLE patients, despite systems to suppress self-recognition, suggests that the response is driven by an antigen which resembles native DNA, in conjunction with a large number of somatic mutations in the complementarity determining region within B cells. ASH generated, ROS modified DNA is a potential antigen for such a system. However, whilst Cooke *et al.* (1997) demonstrated ASH DNA to be immunogenic in rabbits and Blount *et al.* (1990) have demonstrated its antigenicity for SLE sera, Cooke *et al.* (1997) failed to demonstrate antibodies in the antiserum raised to ASH-modified DNA, which react

with native DNA. The immunogenicity of FEH DNA has been established here, along with the ability of antisera raised to DNA modified by such a system to cross-react with native DNA, unlike ASH DNA. Such a finding also makes FEH DNA a potential antigen for the process described above, a hypothesis supported by the finding here that high titre anti-DNA antibodies in SLE serum show equal affinities for both native and FEH DNA.

It has been speculated here that photoproduct formation or radical modification in DNA may be associated with UV photosensitivity in SLE patients *in vivo*. Apoptosis is thought to play a central role in the pathogenesis of SLE. An increased rate of apoptosis, correlating to disease activity, has been demonstrated *in vitro* in lymphocytes from SLE patients (Emlen *et al.*, 1994). This may account for the passage of immunoproliferative and autoimmunogenic nucleohistones (Bell *et al.*, 1990) into the systemic circulation, allowing the immune system interface with this potential antigen. A primed immune system, by previous exposure to modified DNA, coupled with UV-driven, apoptosis-derived autoantigen exposure (Casciola-Rosen *et al.*, 1994) may induce or exacerbate disease activity in SLE. However, the data discussed here do not support the theory that far UV-induced lesions are responsible, by rendering DNA more immunogenic, for the exacerbation of SLE by sunlight. Becker *et al.* (1989) also showed that SLE sera did not bind to UVB (290-320nm) irradiated DNA, suggesting that the immunogenic changes induced by UVB are not responsible for the development of autoantibodies. The data of Cooke *et al.* (1997) would also suggest that this is true for near UV. Other workers have also failed to demonstrate a difference in binding of SLE anti-DNA antibodies to native and UVA irradiated DNA (Becker *et al.*, 1989). However, this is perhaps due to the absence of an appropriate cellular chromophore or photosensitiser that would be present *in vivo* and give rise to radical species. It is for this reason that the involvement of UVA-derived ROS cannot be discounted, although the principle photosensitiser involved has yet to be identified. Utilised due to their SLE properties, cultured MRL murine spleen cells have been shown to exhibit UVA (320-400nm) photosensitivity in the form of an increased susceptibility to UV-induced damage and a limited capacity for unscheduled DNA repair synthesis (Golan and Borel, 1984). Emerit *et al.* (1981) implicated $^{\bullet}\text{O}_2$ or other reactive oxygen species to be involved in the clastogenic photosensitivity of SLE lymphocytes. The detection of 8-oxodeoxyguanosine, a marker of oxidative damage, in the immune complex-derived DNA of SLE (Lunec *et al.*, 1994) reinforces the evidence that ROS may be involved in SLE. It is therefore suggested that UVA in the presence of an endogenous photosensitiser, may lead

to ROS production, the consequential modification of cellular systems and finally, the clinical features of photosensitivity in SLE. This warrants further investigation.

Taken together, this study demonstrates that dependent on the method of generation, hydroxyl radical generating systems can produce DNA modifications with different degrees of immunogenicity and spectra of antigenicity. The strong immunogenic potential of UVC-modified DNA has been confirmed and antisera which largely recognise this form of damage have been produced, although within the antisera there would appear to remain a fraction which recognises oxidatively modified DNA. From a series of immunisations with putative ROS DNA, only one, FEH DNA, has produced an immune response displaying cross-recognition with native double-stranded DNA.

3.2 CHAPTER THREE SUMMARY

Reactive oxygen species (ROS) are implicated in the inflammatory, autoimmune, connective tissue disease, systemic lupus erythematosus (SLE), particularly in respect of processes leading to the formation of pathological anti-DNA antibodies. Exposure to ROS increases the antigenicity of DNA for SLE antibodies, but data on the immunogenicity of ROS-DNA are not conclusive. In this study, the immunogenicity in rabbits and mice, of DNA modified by hydroxyl radical generating systems has been examined, with the objective of achieving an antiserum which recognises such modifications in the DNA of UV irradiated cells. Modification of DNA by both ROS and far UV dramatically increased its immunogenicity; with the Fe^{2+} and H_2O_2 system resulting in antibodies which recognised both native and modified DNA, a finding with possible implications for SLE. Initial screening of the antiserum generated by the UVC/ H_2O_2 system revealed that, contrary to a literature report, the majority of the antiserum's response was to UVC-, rather than ROS-induced damage. Further investigation of this antiserum will be carried out in later chapters. The aim of immunisation of mice with 8-oxoguanosine, coupled to a carrier protein, was to produce monoclonal antibodies specific for the oxidative DNA lesion 8-oxodeoxyguanosine (8-oxodG). Antibodies from a single clone were shown to successfully discriminate between native and oxidatively modified DNA, although only when denatured, nevertheless a property elusive to many previously reported monoclonals to 8-oxodG.

The data contained in this chapter suggested that not only do different profiles of antigenicity and immunogenicity arise dependent on the method of ROS production, although not necessarily dependent on the level of modification, but also that ROS-DNA may be a factor in antigen-driven immune complex formation in SLE.

CHAPTER FOUR

APPLICATION OF GC-MS TO THE EVALUATION OF DAMAGE TO DNA FOLLOWING UV IRRADIATION: COMPARISON WITH ANTIBODY MEASUREMENT

4.1 NOVEL GC-MS ASSAY FOR THE DETERMINATION OF CYCLOBUTANE THYMINE DIMERS IN DNA.

INTRODUCTION

Methods utilising gas chromatography-mass spectrometry (GC-MS) to analyse oxidative DNA damage have been well established within the literature (reviewed in Dizdaroglu, 1991a), resulting in an expansion of knowledge concerning the action of oxidants, such as the hydroxyl radical and the subsequent products (Blakely *et al.*, 1990; Dizdaroglu *et al.*, 1991; Aruoma, *et al.*, 1991; Zastawny *et al.*, 1995). Previously, ionising radiation and hydrogen peroxide-based methods have been largely the oxidative insults of choice (Gajewski *et al.*, 1990; Spencer *et al.*, 1994). However, recently UV has been investigated by GC-MS (Doetsch *et al.*, 1995), although once again only oxidative damage was examined. UV has been shown to induce indirect, oxidative and direct, non-oxidative damage to DNA. Such non-oxidative lesions include the cyclobutane thymine dimer and the (6-4) photoproducts which are reported to possibly be important in UV-induced skin cancer (Dumaz *et al.*, 1994). This makes the development of GC-MS assays for the detection of such damage in human cells and biological fluids highly desirable. Initially, the assay must be shown to work for native DNA, irradiated *in vitro*, only then can judgements be made as to its suitability for assessing damage from *in vivo* systems. On the basis that GC-MS methods for the investigation of oxidative stress have successfully quantitated damage in DNA (Dizdaroglu *et al.*, 1986; Ravanat 1995), cells (Dizdaroglu *et al.*, 1991; Zastawny *et al.*, 1995), tissues (Olinski *et al.*, 1992) and urine (Teixeira *et al.*, 1995), an assay for direct UV photoproducts would likewise be expected to be applicable.

AIM

The aim of this work was to develop an assay to specifically measure the *cis-syn* cyclobutane thymine-thymine dimer (*cis-syn* cyclobutadithymine) in DNA using GC-MS. Such an assay would allow the determination of dimers in DNA standards, facilitating calibration of antibodies to such lesions. Additionally, incorporation of this assay with existing GC-MS assays for oxidative damage may allow examination of a spectrum of UV-induced DNA base products. This assay was conceived in collaboration with Dr. Ian Podmore, Division of Chemical Pathology, University of Leicester.

METHODS

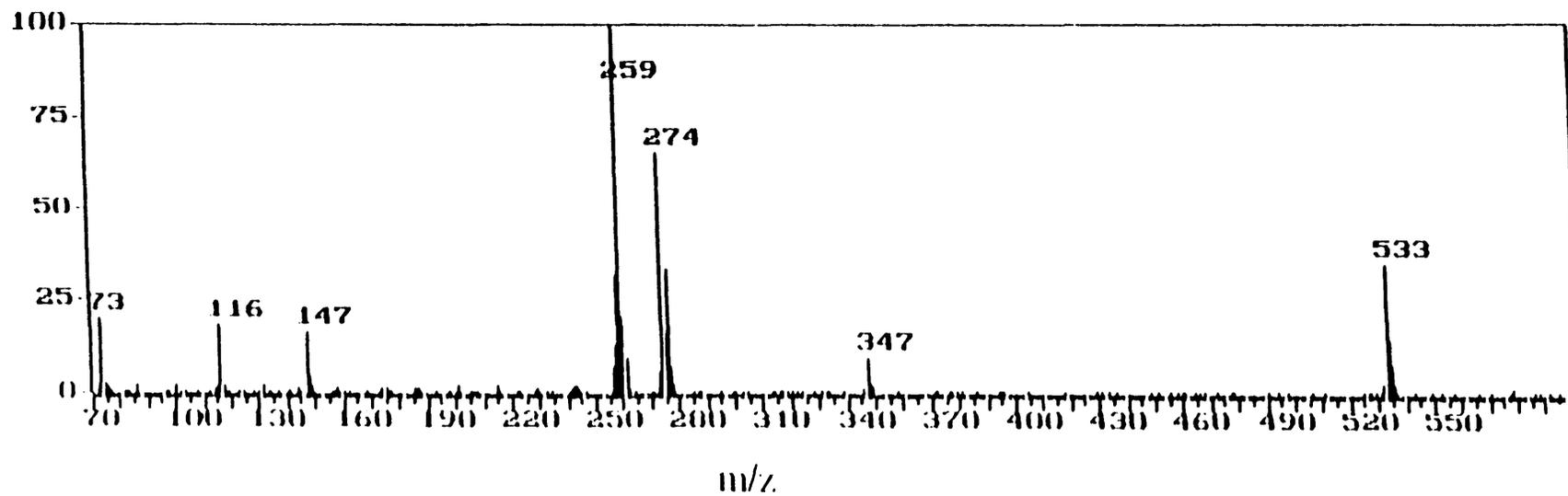
Quantitation of the *cis-syn* cyclobutadithymine was achieved by the use of an internal standard in the form of a stable ^2H -labelled analogue of the cyclobutane thymine dimer. Both isotopically labelled and non-labelled dimers were prepared directly from their corresponding monomers (see Chapter 2.2, page 65). Each was identified as their trimethylsilyl ether derivative by GC-MS. Calibration plots were obtained for known quantities of both non-labelled analyte and internal standard. On the basis that formic acid hydrolysis of UV irradiated DNA yields only the *cis-syn* isomer of the cyclobutane thymine dimer (Weinblum and Johns, 1966), hydrolysates of DNA, irradiated with increasing doses of UVC, were derivatised and analysed by GC-MS in single ion monitoring mode. (See Materials and Methods, Chapter 2.1 and 2.2, pages 51 and 67, for full methodological details.)

RESULTS AND DISCUSSION

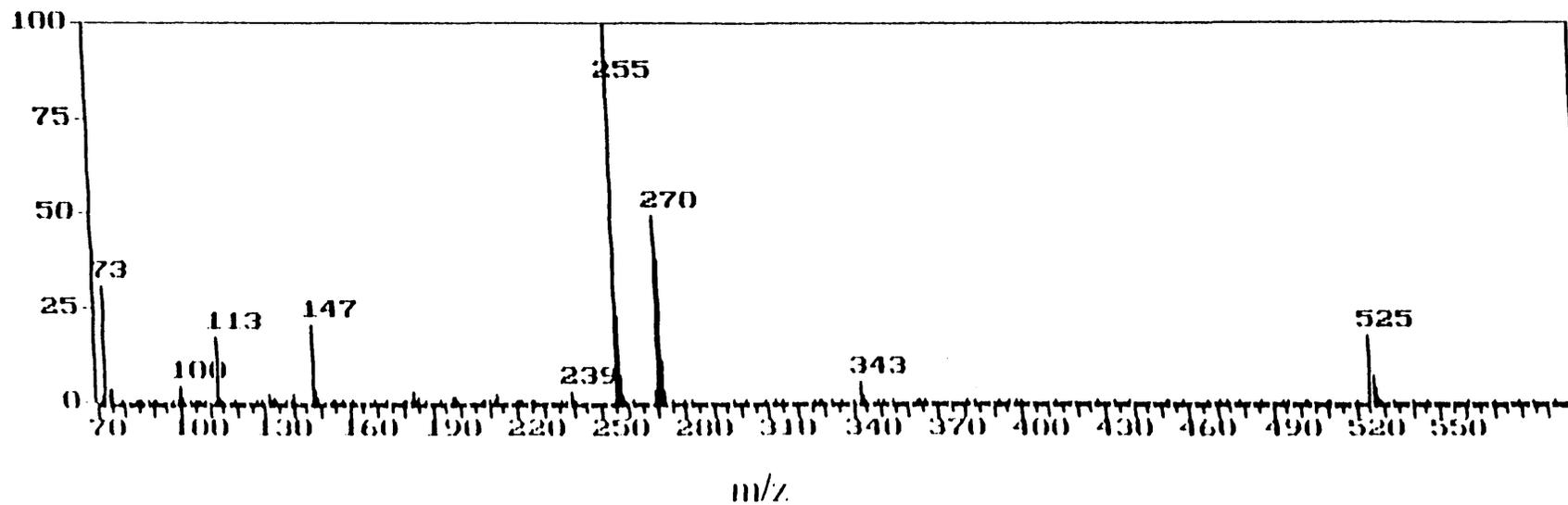
The identification of TMS ether derivatives of *cis-syn* cyclobutadithymine

Electron-impact (EI) mass spectra of the trimethylsilylated dimer and its stable isotope analogue were obtained by GC-MS in total ion monitoring mode (Figure 4.1). They clearly demonstrate formation of the tetrakis-TMS derivative i.e. addition of trimethylsilyl groups to all available carbonyl oxygen atoms (Scheme 4.1). On impact, high energy electrons (70eV) induce ionisation of individual molecules and although the molecular ion (M^+) is not apparent, a fragment consistent with the loss of a methyl radical from it $(\text{M} - \text{CH}_3)^+$ can be seen. For *cis-syn* cyclobutadithymine-(SiMe_3)₄ the $(\text{M} - 15)^+$ ion, observed in EI mass spectra of TMS derivatives of a variety of purine and pyrimidine bases (Dizdaroglu, 1994; White *et al.*, 1971) appears at m/z 525. As expected a shift of 8 m/z units is produced for the corresponding deuterated analogue, confirmation of the synthesis of the labelled standard. The ion at m/z 270 (Figure 4.1b) is that of derivatised monomeric thymine i.e. $(\text{M}^*/2)$. The strong tendency for the radical-cation (molecular ion) of these dimers to form monomers has been shown previously (Fenselau, 1976; Pouwels *et al.*, 1995) and is further confirmed here by the intensity of both this ion and the corresponding ion at m/z 274 for the deuterated analogue (Figure 4.1a). Indeed, comparison of the mass spectrum in Figure 4.1b to that of the TMS ether derivative of thymine [thymine-(SiMe_3)₂] (White *et al.*, 1971) reveals the production of identical ions at m/z 270 and below, giving clear indication of fragmentation of dimer to monomeric thymine (Scheme 4.1).

Relative Abundance

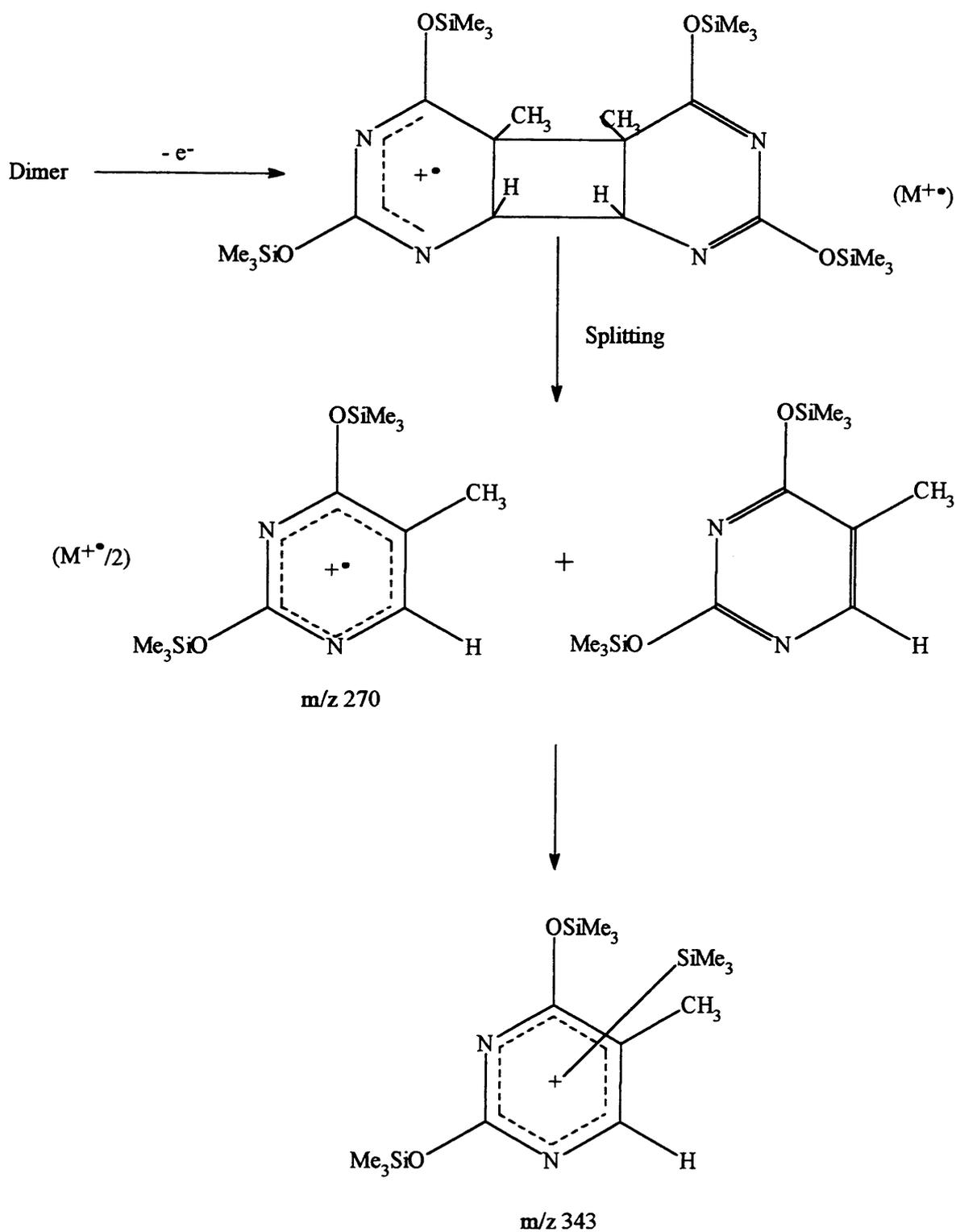


(a)



(b)

Figure 4.1. EI-mass spectra of TMS derivatives of (a) *cis-syn* cyclobutadithimine-²H₈ and (b) *cis-syn* cyclobutadithimine-²H₀.



Scheme 4.1. Formation of the tetrakis-TMS derivatives of *cis-syn* cyclobutadithimine.

The most abundant ion at m/z 255 is generated as a result of loss of a methyl radical from the monomer $[(M^+/2)-15]$ and has a counterpart at m/z 259 for the $^2\text{H}_8$ compound. Ions at m/z 73 and 147 in Figure 4.1 are common fragments of Me_3Si derivatives and serve no diagnostic purpose (White *et al.*, 1971), although the former may help explain the ions at m/z 347 and 343 (Figures 4.1a and b respectively). Since $4m/z$ units separate these species, it is reasonable to conclude that they contain carbon atoms at 5 and 6 positions of the pyrimidine ring. Furthermore, their m/z ratio exceeds that of monomeric thymine (or thymine- $^2\text{H}_4$) and by 73 units in each case. Therefore, it is proposed that these ions are as a result of a 'net' addition of SiMe_3 to monomeric thymine - which in itself is generated by initial splitting of the dimer (Scheme 4.1). Finally, White *et al.* (1971) suggested that the ion at m/z 113 (Figure 4.1b) was due to the fragment $(\text{Me}_2\text{SiOCCCH}_3)^+$. This proposal is supported in our study whereby an ion at m/z 116 is generated in the mass spectrum of the $^2\text{H}_8$ analogue, i.e. $(\text{Me}_2\text{SiOCCCD}_3)^+$ (where $\text{D} = ^2\text{H}_8$).

The gas chromatographic retention time of the Me_3Si derivative of *cis-syn* cyclobutadithymine was found to be almost identical to that of the ^2H -labelled analogue (Figure 4.2). The slight resolution observed (0.07 min) is indicative of an isotope effect on the elution behaviour of the labelled compound (Dizdaroglu, 1994). The TMS derivatives of both compounds were found to elute at approximately 11.0 min under the chromatographic conditions used. In addition, it should be noted that the TMS derivative of a monomeric thymine standard was found to elute at approximately 2.5 min. The absence of ion current at such a retention time for both the dimers is thereby further evidence of their purity.

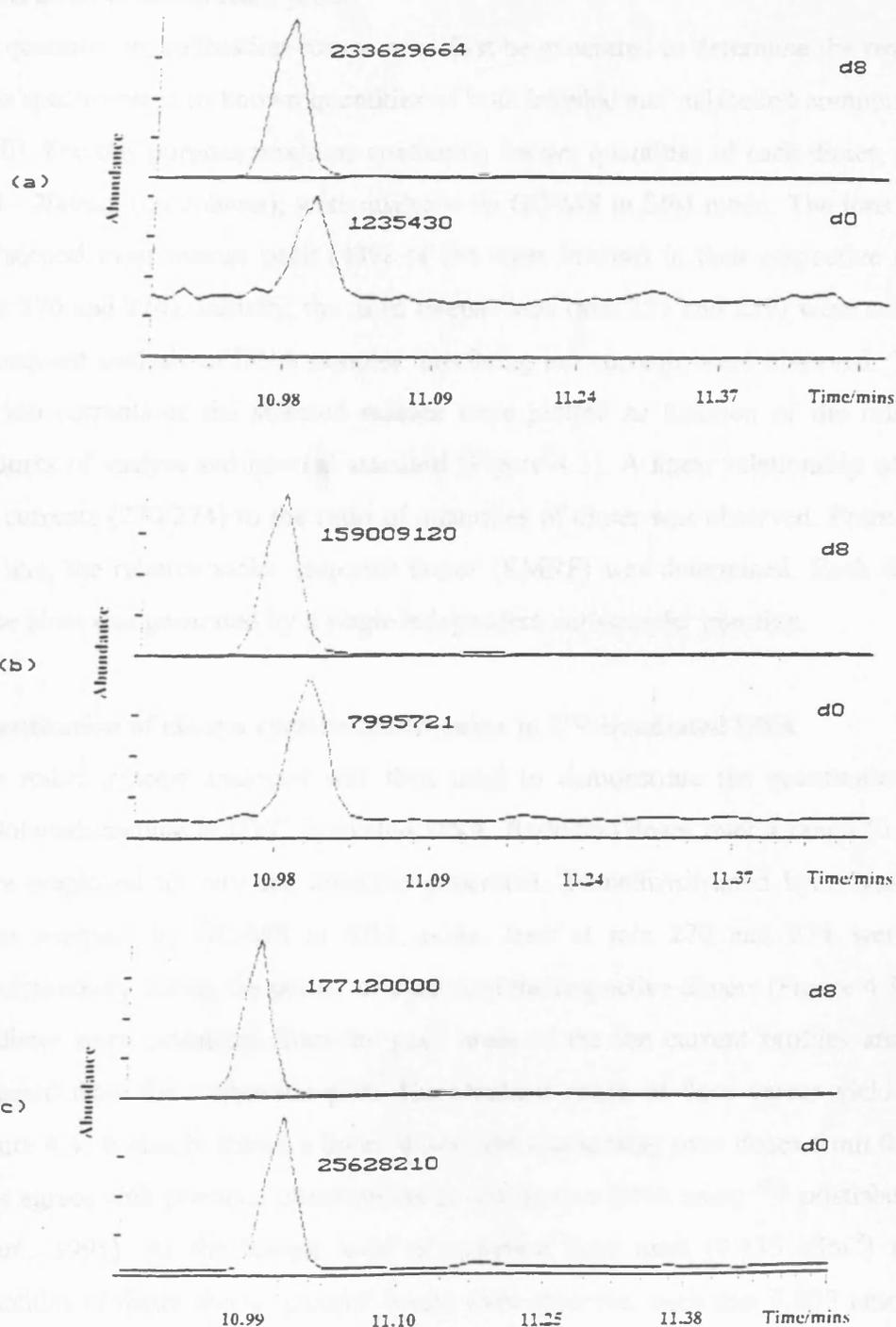


Figure 4.2. Selected ion monitoring profiles of (a) an unirradiated DNA sample; (b) UVC-irradiated DNA sample (dose = 435J/m^2); (c) a UVC-irradiated DNA sample (dose = 1305J/m^2). All DNA samples were spiked with internal standard (where d8 represents $^2\text{H}_8$). Ions were monitored with m/z 270 and m/z 274 corresponding to TMS derivatives of *cis-syn* cyclobutadithymine- $^2\text{H}_0$ (d0) and *cis-syn* cyclobutadithymine- $^2\text{H}_8$ (dimer and internal standard respectively). The peak area values shown are in arbitrary values.

Generation of calibration plots

For quantitation, calibration curves must first be generated to determine the response of the mass spectrometer to known quantities of both labelled and unlabelled compound (Watson, 1990). For this purpose mixtures containing known quantities of each dimer, over a range of 0 - 20pmol (on column), were analysed by GC-MS in SIM mode. The ions chosen gave the second most intense peak (43% of the most intense) in their respective mass spectra (m/z 270 and 274). Initially, the most intense ions (m/z 255 and 259) were selected but on subsequent analysis of DNA samples interfering ion currents were observed. The ratios of the ion currents at the selected masses were plotted as function of the ratios of molar amounts of analyte and internal standard (Figure 4.3). A linear relationship of the ratio of ion currents (270/274) to the ratio of quantities of dimer was observed. From the slope of the line, the relative molar response factor (RMRF) was determined. Each data point on these plots was generated by a single independent autosampler injection.

Quantitation of *cis-syn* cyclobutadithymine in UV-irradiated DNA

The stable isotope analogue was then used to demonstrate the quantitation of *cis-syn* cyclobutadithymine in UVC irradiated DNA. Radiation doses over a range (0 to 3.5kJm^{-2}) were employed to vary the amounts generated. Trimethylsilylated hydrolysates of DNA were analysed by GC-MS in SIM mode. Ions at m/z 270 and 274 were monitored simultaneously during the period of elution of the respective dimers (Figure 4.3). Quantities of dimer were calculated from the peak areas of the ion current profiles and the RMRF obtained from the calibration plot. The resultant graph of dose versus yield is shown in Figure 4.4. It clearly shows a linear dose-yield relationship over doses from 0 to 3.5kJm^{-2} . This agrees with previous observations in calf thymus DNA using ^{32}P -postlabelling (Bykov *et al.*, 1995). At the lowest level of radiation dose used (0.435kJm^{-2}) elevations in quantities of dimer above 'control' levels were observed, such that 0.805nmol/mg (DNA) ± 0.1 ($n=3$) of dimer were detected. Control levels of dimer in commercially available calf thymus DNA were found to be 0.09nmol/mg DNA (equivalent to 3 lesions per 10^5 bases).

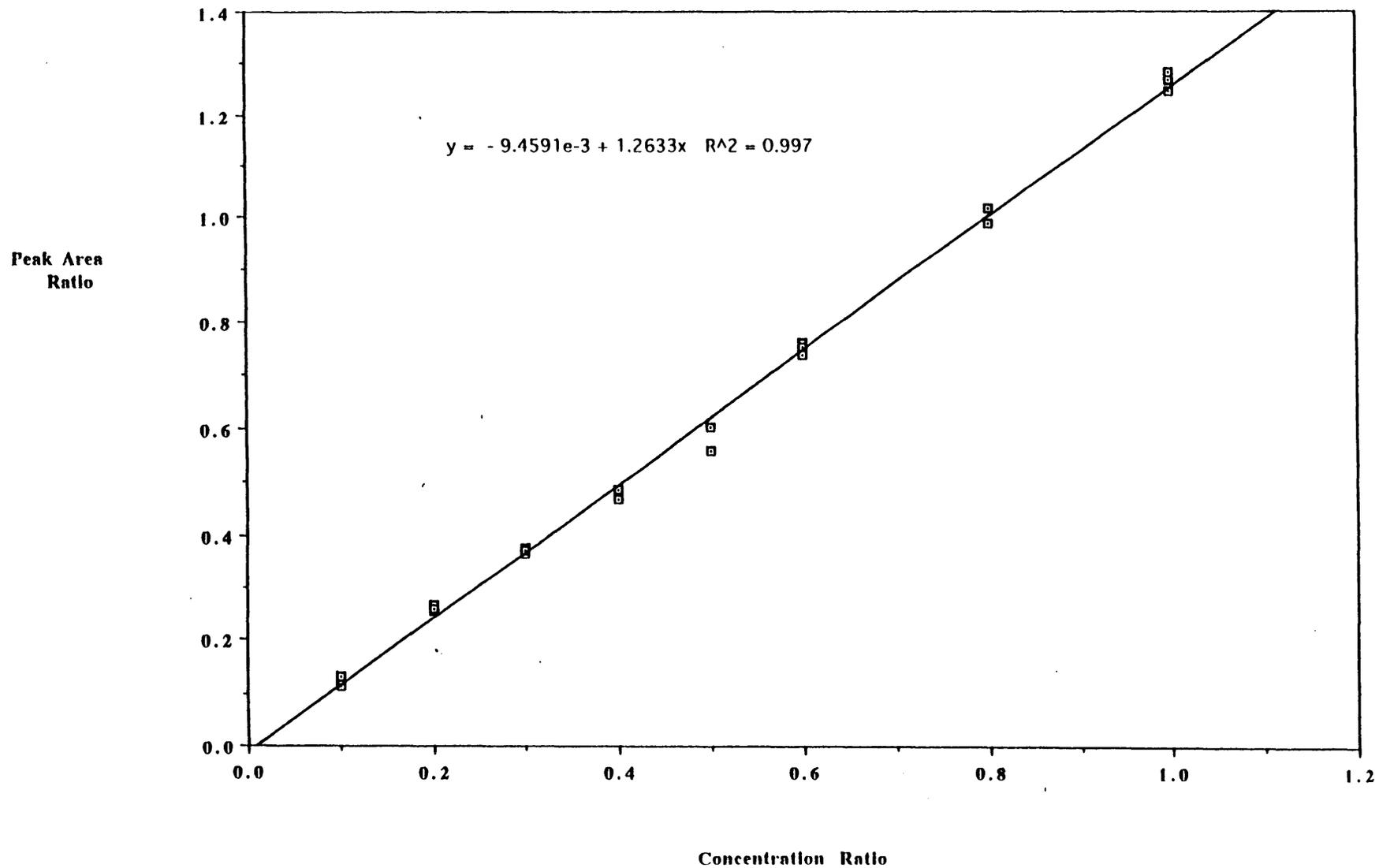


Figure 4.3. Calibration plot of peak area ratio (m/z [270/274]) versus concentration ratio for *cis-syn* cyclobutadithimine. A ratio of 1.0 \equiv 20pmol of dimer injected on column. Each data point represents a single independent sample injection.

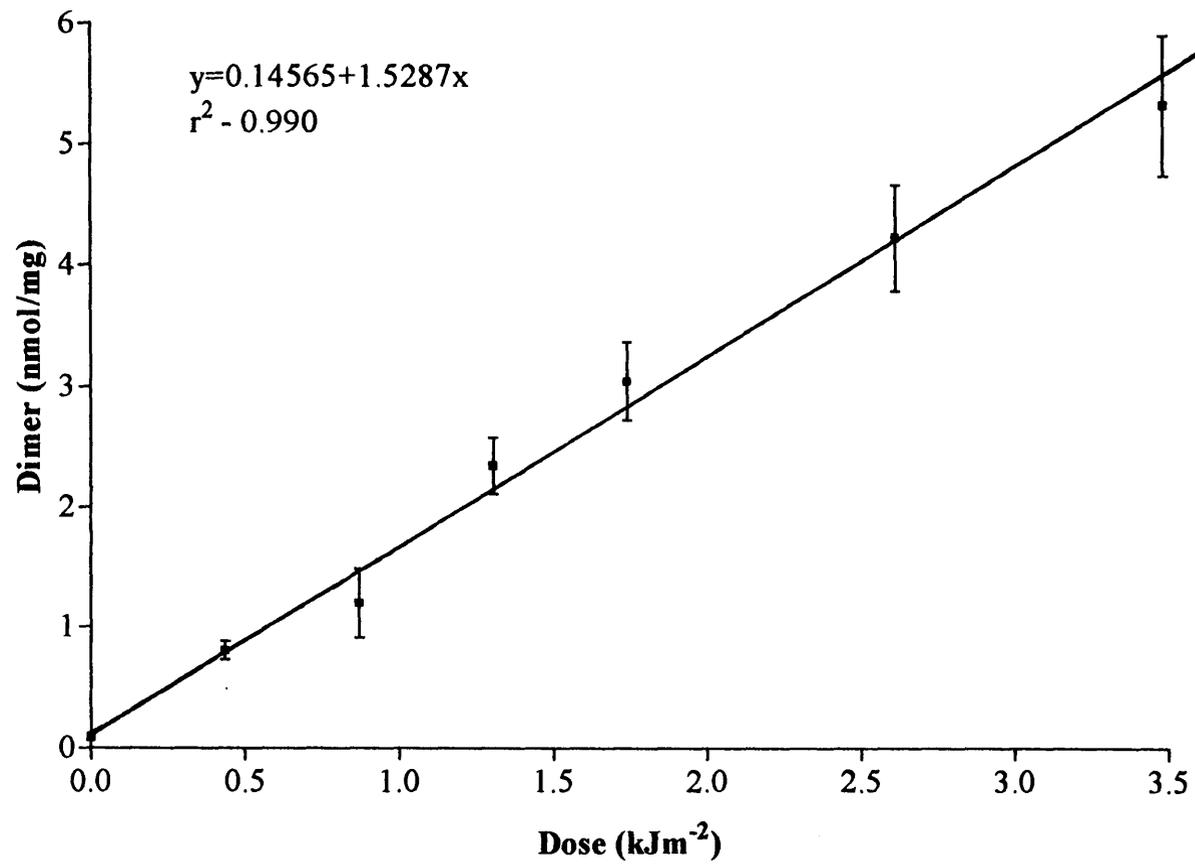


Figure 4.4. Dose-yield plot of *cis-syn* cyclobutadithymine. The DNA samples were UV irradiated (254nm) at the indicated doses. Each point on the graph is the mean value of three independent autosampler injections and the corresponding standard errors are represented by the error bars shown.

In order to test for the possibility of artefactual production of dimer during sample handling separate equimolar samples of thymine (500pmoles; approximately equivalent to the amount present in DNA samples) were subjected to either (a) hydrolysis (60% formic acid, 140°C, 45min) and derivatisation (BSTFA/acetonitrile, 4:1 v/v, 130°C, 1 hour), or (b) derivatisation alone. Subsequent to formic acid hydrolysis and prior to derivatisation samples were spiked with known amounts of isotopically-labelled dimer. Levels of thymine dimer were then assessed with GC-MS in SIM mode as described earlier. No dimer was detected from samples subjected to conditions described in either (a) or (b). It was therefore concluded that no dimer is produced from free thymine base under our experimental conditions. In addition to a possible artefactual contribution from the handling procedures to observed control levels of dimer, there remains the possibility of a contribution from interfering MS peaks from DNA samples. In order to assess this, derivatised samples of both *cis,syn*-cyclobutadithymine and a hydrolysate of UVC-irradiated DNA were subjected to analysis by GC-MS-SIM. Four ions (m/z 255, 270, 343, 525) were monitored simultaneously and their peak area ratios calculated. Comparison of these ratios for UVC-irradiated DNA revealed the ions at m/z 270, 343 and 525 to be in the exact proportion to the standard demonstrating the lack of interference from ion currents due to DNA at these masses.

A limit of detection study was performed with the fragment ion at m/z 270, using decreasing amounts of internal standard injected and subsequently detected and quantitated using a signal to noise ratio of 3:1. These results suggested that as low as 20-50fmol dimer/ μ L injection can be achieved (i.e. equivalent to 0.02-0.05nmol/mg DNA or 6-16 lesions per 10^6 bases). In a parallel study within the Division of Chemical Pathology, for the ROS-induced lesion, 8-oxoguanine, a limit of detection was found of 0.01nmol/mg DNA (3 lesions per 10^6 bases): although a difference of 2-5 fold in sensitivity this is nevertheless a highly sensitive analytical assay for the dimer. In addition, the value reported here, of \sim 5nmol dimer/mg DNA for a UV dose of 3.5kJm^{-2} is greater than that reported by Bykov *et al.*, (1995) using a ^{32}P -postlabelling method, suggesting that methods using enzymatic digestion to trinucleotides by may be incomplete. Despite this the authors report that the postlabelling method also has a fmol sensitivity, comparable to the GC-MS assay. However, the method of Bykov *et al.*, (1995) fails to discriminate between trinucleotides containing

TT=T dimers and those containing TT=C, whereas the GC-MS method described above is specific for T=T.

Methods not requiring any radioactivity, particularly radioactive DNA, are likely to be more useful for routine use and amenable to a greater variety of sample sources, *i.e. ex vivo*. An example of such a method is that described by Freeman *et al.* (1986) which utilises the DNA nicking ability of *M. luteus*, described in Chapter One (page 35), in conjunction with alkaline gel electrophoresis. This method requires 50ng of DNA from which a detection limit of 1 dimer per 5×10^6 bases can be achieved. Comparison of the alkaline gel electrophoresis method with the GC-MS method shows a lower limit of detection for the former (0.2 dimers/ 10^6 bases *versus* 6-16 dimers/ 10^6 bases, respectively). Additionally, the alkaline gel electrophoresis method obtains this value from 50ng of sample, whereas the GC-MS assay analyses 1000ng of material. Freeman *et al.* (1986) describe the gel electrophoresis method as an improvement over previous alkaline elution techniques which detected one lesion in 1×10^7 bases, but required 2000ng DNA. From this it can be concluded that the GC-MS technique possesses a limit of detection at least 30 times higher than the gel electrophoresis technique and requires more material, although not as much as previous alkaline elution techniques. Absolute identification of the lesion can be provided by GC-MS, particularly relevant in consideration of the substrate specificity of *M. luteus* (Chapter One, page 35) and the potential to incorporate the measurement of other lesions in the same assay run also exists (Chapter 4.2). Many techniques for measuring dimers cannot express lesions induced or limits of detection in terms of number of lesions, or do so, for example, in terms of "enzyme sensitive sites/ 10^6 D" (Kasten *et al.*, 1995) or "photoproducts per plasmid (Liu and Smerdon, 1995), largely precluding them from comparison with techniques described above. However, some groups have calibrated their antibodies, in terms of limit of detection and as suggested by Wani *et al.* (1987) may represent a reasonable alternative to agarose gel electrophoresis. For example, in the case of the antibodies used in this thesis, they were shown to be capable of detecting 0.2-0.5 lesions per 10^6 bases with the potential for utilising only nanogram amounts of DNA (see Chapter Five).

Although the detrimental effects of UV have been known for some time (Deering, 1961), possible depletion of stratospheric ozone and the consequential increase in solar radiation on the earth's surface (Jokela *et al.*, 1995) has drawn further attention to the importance of

studying UV, its effects and consequences. To reiterate, the aim of the present study was to develop a sensitive assay for the detection and accurate quantitation of *cis-syn* cyclobutadithymine, a major lesion generated by UV light in DNA, using capillary GC-MS. Accurate quantitation was achieved by the synthesis and use of an isotopically labelled internal standard which allows for any chemical or physical degradation of the analyte. The 'clean' synthesis of both analyte and internal standard was easily performed by UV-irradiation of a frozen aqueous solution of monomer, generating little or no by-products.

4.2 COMPARISON BY GC-MS OF LEVELS OF OXIDATIVE DAMAGE AND CYCLOBUTANE DIMERS IN DNA, FOLLOWING UVC IRRADIATION

INTRODUCTION

Oxidative DNA damage has been proposed to play an important role in a number of pathological processes, including carcinogenesis (Kensler *et al.*, 1986; Frenkel *et al.*, 1992). Previously, the UV-induced DNA products most associated with mutation and carcinogenesis have been the cyclobutane pyrimidine dimer and the (6-4)PP. More recently, attention has turned to UV-induced oxidative lesions and their role in the cellular events following UV irradiation has been examined (Berg *et al.*, 1995). The wavelengths most associated with oxidative damage *in vivo* are those of the UVA range; however, the mechanism for formation of such lesions has been reported to be indirect, involving a sensitiser, as DNA does not absorb at these wavelengths (Sutherland *et al.*, 1981). Doetsch *et al.*, 1995 demonstrated, by GC-MS, the formation of monomeric base damage, of the type seen following oxidative attack, following far UV irradiation of plasmid DNA. However, direct comparison with the induction of CPD was not performed and values were simply related to a literature value which was not obtained by GC-MS. It would therefore be of value to compare levels of both oxidative and non-oxidative lesions in UV-irradiated DNA by the same method, as the literature suggests that all such lesions may have an effect subsequent to their formation, perhaps even acting synergistically.

AIM

To apply GC-MS methods for determining absolute amounts of dimeric and oxidative lesions to DNA irradiated with UVC and subsequently compare the levels of damage.

METHOD

The method was essentially as in Section 4.1.3., although conditions of hydrolysis and derivatisation were slightly modified, by hydrolysis being at 140°C for 45min and derivatisation being at 110°C for one hour. The internal standard utilised was 2,6-diaminopurine as 8-oxoguanine was the analyte to be investigated. In order to establish the response of the mass spectrometer and allow quantitation, an 8-oxoguanine calibration plot was generated in a similar way to that for *cis,syn* cyclobutadithymine. For this purpose, quantities of 8-oxoG (over the range 250fmol - 4pmol, on column) and DAP (4pmol, on column) were analysed by GC-MS in SIM mode. The ions corresponding to *m/z* 440.2 and

m/z 351.1 were monitored for 8-oxoG and DAP respectively. The ratios of ion currents at the selected masses were plotted as a function of the ratios of molar amounts of analyte and internal standard. A linear relationship of the peak area ratio of ion currents (m/z [440.1/351.1]) to the ratio of quantities of 8-oxoG to DAP were observed. From the slope of the line the RMRF was determined.

RESULTS

Measurement of oxidative damage in UV irradiated DNA by GC-MS

Using 8-oxoguanine (8-oxoG) as a marker of oxidative DNA damage (Shigenaga, 1991), levels of both this and *cis,syn* cyclobutadithymine were assessed by GC-MS in the same samples. The calibration plot for 8-oxoG is represented by Figure 4.2.0. The results for cyclobutadithymine from Figure 4.4 were replotted to illustrate linearity up to 3.5kJm^{-2} and the plateau effect starting to appear at doses greater than 3.5kJm^{-2} (Figure 4.2.1). Figure 4.2.1 illustrates the comparative dose-yield plot of 8-oxoG and cyclobutadithymine in the dose range 0- 8.7kJm^{-2} . The unirradiated control sample appeared to possess higher than expected levels of 8-oxoG (2.248nmol/mg , equivalent to 716 8-oxoG per 10^6 DNA bases). Literature values for 8-oxoG in commercial calf thymus DNA, determined by GC-MS are 159-318 per 10^6 DNA bases (Halliwell and Aruoma, 1993). A considerable increase over the control amount of 8-oxoG was observed at the dose range from the lowest dose 0.435kJm^{-2} to the highest dose, 8.7kJm^{-2} corresponding to 3.45 and 8.43nmol/mg . Between 0 and 3.5kJm^{-2} there was a linear dose-response relationship, reaching a plateau following 3.5kJm^{-2} , the same dose at which the cyclobutadithymine dose-response begins to lose its linearity. Clearly, 8-oxoG was formed in DNA following UVC irradiation.

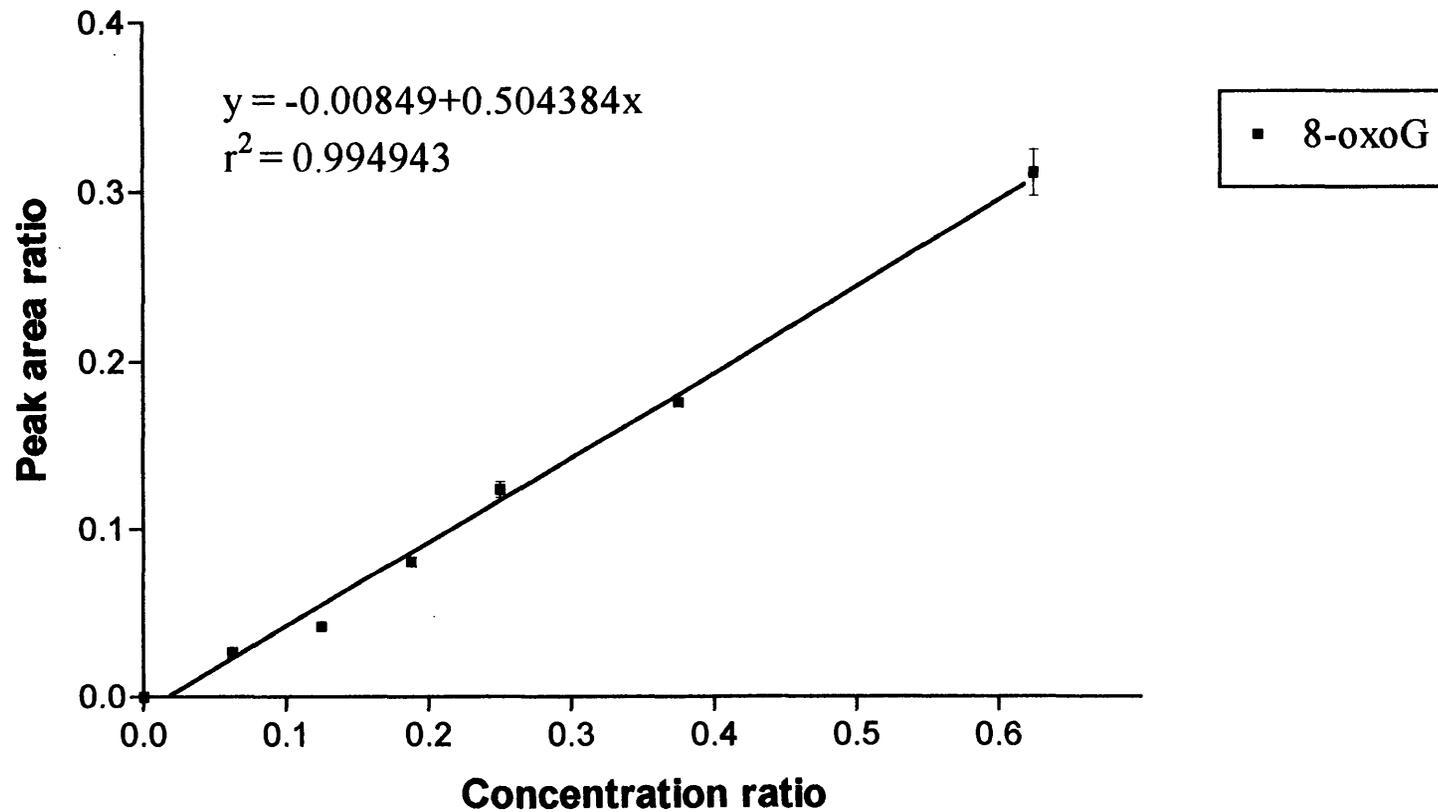


Figure 4.2.0. Calibration plot of peak area ratio *versus* concentration ratio for 8-oxoguanine. Each data point represents the mean (SEM) of three independent autosampler injections.

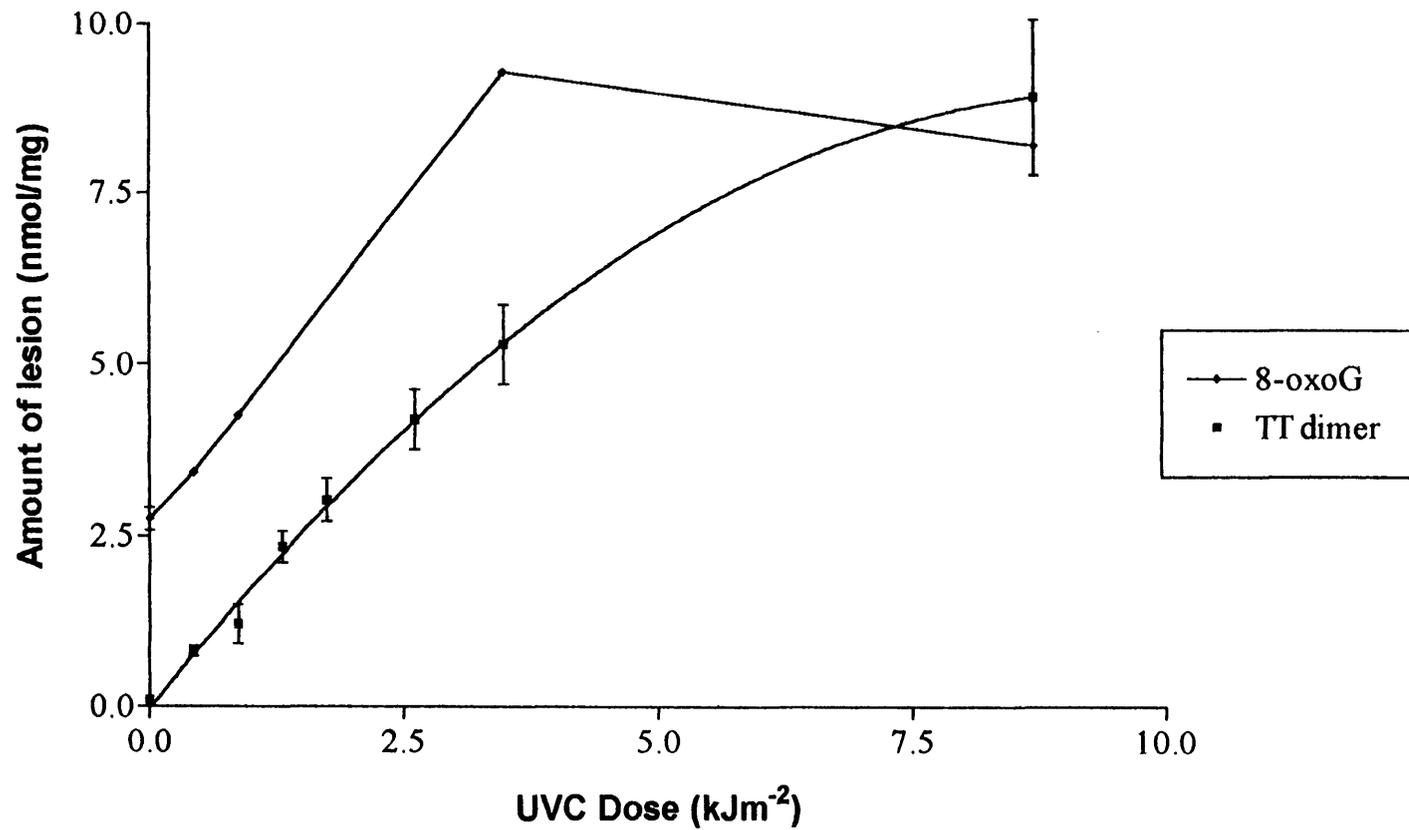


Figure 4.2.1. Dose-yield plot, obtained by GC-MS of *cis-syn* cyclobutadithymine and 8-oxoguanine (8-oxoG). The DNA samples were UV-irradiated (254nm) at the indicated doses. Each point on the graph is the mean value of three independent autosampler injections and the corresponding standard errors are represented by the error bars shown.

DISCUSSION

Oxidative damage, of the type seen with hydroxyl radicals, has been shown to also occur in a buffered aqueous solution of DNA upon UVC irradiation, yielding in greatest amounts; 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 5-hydroxy-5,6-dihydrothymine (5-OH-5,6-diHThy) (Doetsch *et al.*, 1995). In the work of Doetsch *et al.* (1995), the relative induction of oxidative products versus cyclobutane pyrimidine dimers (CPD) could only be compared by reference to the formation of CPD estimated by Mitchell *et al.* (1991), at particular UV doses. Herein is described, using GC-MS, the relative induction of 8-oxoG, a relevant marker of oxidative damage, and cyclobutane thymine dimers in DNA following UV irradiation. The results show that 8-oxoG levels increase appreciably over and above the control value, despite being unexpectedly high. The shape of the curve immediately suggests that all the available guanines have been modified when the plateau is reached. It is unlikely that all have been converted to 8-oxoG, and the induction of other products has been described (Doetsch *et al.*, 1995) which suggests that other guanine-derived lesions are preferentially formed. Indeed, it may be that once formed, the subsequent irradiation of 8-oxoG leads to the formation of other products with this reaction favouring the formation of these secondary products. Sources of artefactual damage in the GC-MS method have been discussed within the literature (Halliwell, 1993) and centre on the formation of oxidative damage during hydrolysis and derivatisation. The author cites a reference (Halliwell and Dizdaroglu, 1992) in which it is claimed that formic acid hydrolysis does not contribute to 8-oxoG levels in DNA. High temperature derivatisation (140°C/30min), in particular, has been shown to induce an approximately eight-fold increase in 8-oxoG compared to room temperature methods (Hamberg and Zhang, 1995). However, the development of such derivatisation methods was not published at the time of the above investigation. Nevertheless, the relative increase in the levels of 8-oxoG are significant, irrespective of artefactual contribution. Development of a GC-MS assay whereby both oxidative and dimeric photoproducts may be monitored in a single run would be represent a great advantage over existing methods and provide investigators with a powerful tool for DNA damage analysis. However, a major drawback to the achievement of this aim is the poor derivatising ability of pyrimidines by the room temperature method, so crucial for minimising artefactual damage to purines (Hamberg and Zhang, 1995) and pyrimidines (Douki *et al.* 1996a). A procedure which may allow successful derivatisation of pyrimidines with no appearance of artefactual damage is HPLC pre-purification of the damaged DNA

hydrolysates to remove all native bases, as performed by Douki *et al.* (1996b). This adds another step to the workup which increases analysis time, but at present this seems to be the best solution.

The method for the quantitation of *cis-syn* cyclobutadithymine in DNA exploited the availability of a stable, isotopically labelled internal standard. Labelled internal standards were first adopted in the analysis of oxidative damage, replacing, in the case of 8-oxoguanine, DAP with isotopically-labelled 8-oxoguanine. Standards of this nature have a number advantages, primarily associated with the generation of calibration curves. Addition of the standard will compensate for loss during the sample workup and chromatography. The latter is an important source of error, with possible adsorption onto the injection line and column. Despite a mass difference, the same chromatographic properties are possessed as the analyte, excepting a slight isotope effect. These factors combined maintain the same relative ratios, producing better calibration curves. An isotopically-labelled 8-oxoguanine was unavailable to the Division of Chemical Pathology at the time of this experimentation.

It is perhaps noteworthy that the source of DNA used by Doetsch *et al.* (1995) was plasmid and the source used here was calf thymus. Plasmid DNA does not possess all the higher structural attributes of eukaryotic DNA (Stryer, 1988). It is unclear as to whether this would produce less base damage in the calf thymus DNA than in the plasmid DNA, for a given dose of UV. Direct comparison between the results for 8-oxoG, shown here and those of Doetsch *et al.* (1995) was not possible due to methodological differences, notably in hydrolysis and derivatisation; 60% formic acid for 45 mins at 140°C and a one hour derivatisation at 110°C (described above), versus 60% formic acid for 30 mins at 140°C and a thirty minute derivatisation at 120°C (Doetsch *et al.* 1995) and the use of isotopically-labelled internal standards (Doetsch *et al.* 1995). However, approximately, in percentage terms for a dose of 10kJm⁻² UVC Doetsch *et al.* (1995) noted a 68% increase in 8-oxodG over control, whereas a 31% increase was noted in the results above. This discrepancy may be due to the 31% value being derived from Figure 4.2.1, an irradiation of 10kJm⁻² have not been carried out. Expression of both values in relative percentage terms corrected for any methodological differences. This would suggest that some experimental system differences exist, such as the one described above.

It is also worth noting that the range of doses over which the dose-response plots were linear were the same, both for 8-oxoG and cyclobutadithymine ($0-3.5\text{kJm}^{-2}$). This suggested that above this dose either the linear response of the instrument had been exceeded, or all the unmodified base was becoming modified (a theory discussed again in Chapter 5). Doetsch *et al.* (1995) reported the examination of 8-oxoG levels at only one dose of UV (10kJm^{-2}) with comparison to the unirradiated control. This meant the plateauing effect was not seen for this lesion, although it was apparent for other lesions studied, in particular FapyAde, where a plateau began around 3.5 to 5kJm^{-2} .

Doetsch *et al.* (1995) provided evidence to suggest that a hydroxyl radical-independent mechanism, *i.e.* base radicals, was responsible for the damage induction (see Scheme 1.1., page 18, Chapter One). In light of this, any possible effects due to DNA source differences should not have occurred, although the evidence excluding hydroxyl radicals in this damaging system is discussed further in Section 4.3, page 155.

The increases in 8-oxoG seen in DNA following UV irradiation may have biological significance, for 8-oxoG has been demonstrated to possess a mutagenic potential (Wood, 1990; Shibutani, 1991; Cheng, 1992) and the role of UV induced oxidative damage has recently become of increasing interest (Stewart *et al.*, 1996; Bertling *et al.*, 1996; Rosen *et al.*, 1996). It is perhaps pertinent to speculate that 8-oxoG, in addition to other oxidative lesions, may be responsible for UV-induced mutations at non-dipyrimidine sites. Potentially important genomic targets for such damage are proto-oncogenes and tumour suppressor genes (Tornaletti *et al.*, 1993), such as *ras* and the *p53* genes. The *p53* mutations in UV-induced skin cancers have been frequently reported to possess C→T and CC→TT mutations at dipyrimidine sites, which were described as unique “signatures” of UV by Nataraj, 1995. As a consequence, the *p53* gene has been selected as a model to study the occurrence of photoproducts at specific sequences following UV radiation (Dumaz, 1994). However, oxidative damage has also been shown to produce CC→TT mutations (Reid, 1992), possibly contributing to the total number induced by non-oxidative lesions. Yet mutations in skin tumours have only been found where UV signature mutations (C→T) could affect the amino acid coded for (Ziegler, 1993) suggesting the C→T mutation to be the true UV signature. Nonetheless, the involvement of UV-mediated oxidative damage has

been suggested as a potentially important cofactor or promoter of UV photocarcinogenesis (Beehler, 1992).

4.3 QUANTITATIVE DETERMINATION OF OXIDATIVE DAMAGE TO DNA, BY ELISA-BASED TECHNOLOGY

INTRODUCTION

As described previously (Chapter 1, page 10 - Introduction) UV irradiation can result in a number of different damage products, ranging from base modifications to DNA-protein crosslinks. It is therefore desirable to possess techniques whereby such DNA alterations can be examined in naked DNA, cells, tissues and biological fluids. The development and application of various, broadly described, "affinity" techniques to non-dimer damage is still very new and may reveal valuable information as the mechanisms of damage induction and its localisation (Degan *et al.*, 1991; Park, EM. *et al.*, 1992; Yarborough *et al.*, 1996; Hattori *et al.*, 1997). Chapter Three describes the difficulties in developing antibodies to specific lesions such as 8-oxo-2'-deoxyguanosine. However, adopting the approach of producing antisera to ROS damaged DNA, in a more general sense, has met with success within the Division of Chemical Pathology. An antiserum has been developed to ascorbate/H₂O₂ damaged DNA and has been broadly classified as an anti-ROS DNA antiserum (manuscript in preparation). Furthermore, a novel affinity technique for the detection of 8-oxoguanosine (8-oxoGR) has also recently been developed within the Division of Chemical Pathology (Thomas, 1996; Clarke *et al.*, 1997). This method exploits the serendipitous finding that avidin, a 70-kd protein in egg white (Stryer, 1988) bound to the nuclei of UVA irradiated, cultured, differentiated IMR32 neuroblastoma cells. The conclusion that avidin bound to 8-oxodeoxyguanosine followed structural comparisons between biotin and DNA components (Thomas, 1996). However, the description that it specifically recognises 8-oxoguanine along with its nucleoside and deoxynucleoside derivatives (Clarke *et al.* in preparation) would appear rather speculative. Whilst avidin does appear to possess specificity for the modified form of guanine over the unmodified form, competitors of the type used to characterise the epitopes recognised by poly- and monoclonal antibodies to 8-oxodG (Degan *et al.*, 1991; Park, EM. *et al.*, 1992) have not been utilised. Such competitive ELISAs may reveal a broader range of possible epitopes. Although 8-oxodG may not be the sole epitope, the demonstration of avidin binding to DNA exposed to free radical generating systems both *in vitro* and *ex vivo* (Clarke *et al.*, in preparation) supports the development of avidin binding as a probe of oxidative damage to DNA.

AIM

To assess the modifications to naked DNA following UVC irradiation by the use of three probes which recognise reported markers of DNA damage. Furthermore, Ab529 will be used as a positive control for UV-induced DNA damage.

METHOD

The methods were as described in Chapter 2.4.8. ELISAs were performed on both 96-well ELISA plates and 96-well membrane plates, using Ab529 (diluted 1/2000), peroxidase-labelled avidin (diluted 1/500), the anti-ROS DNA antiserum (1/100) and a commercial monoclonal antibody to single-stranded DNA (1/1000). The full methodology is described in Chapter 2.4.8, Materials and Methods.

Analyses of variance were performed by GraphPad Prism, version 2.0.

RESULTS

Solid phase antigen bound to a 96-well ELISA plate

Figure 4.3.1 illustrates the binding of the anti-ROS DNA antiserum to UVC irradiated DNA, the solid phase antigen on a standard 96-well ELISA plate. This binding produced a clear dose response, achieving a plateau at about 2kJm^{-2} . The results for 8-oxoguanine from Figure 4.2.1 were replotted for comparison with the binding of the anti-ROS DNA antiserum and some similarity in shape is shared between the two graphs.

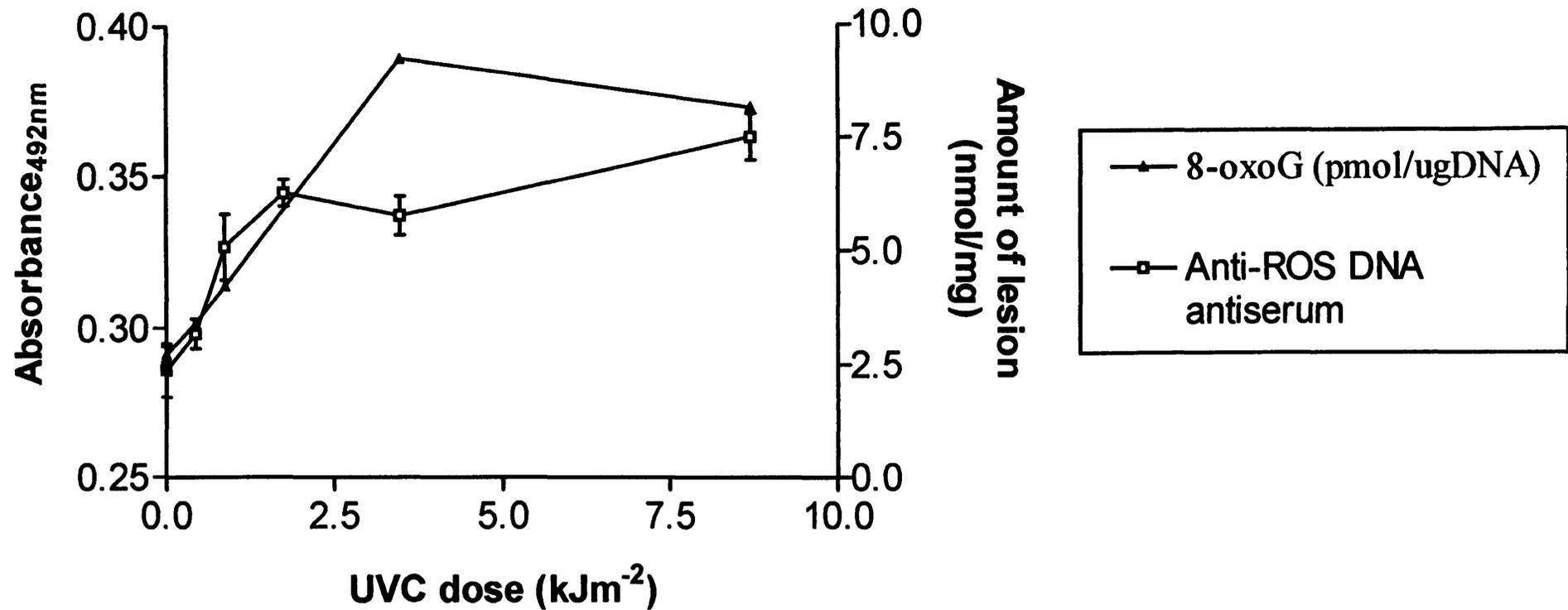


Figure 4.3.1. ELISA binding of polyclonal anti-ROS DNA antiserum to UVC irradiated calf thymus DNA. Error bars indicate the mean (SEM) of four determinations. Also shown, levels of 8-oxoguanine in the same DNA samples, determined by GC-MS. Values represent the mean (SEM) of three independent autosampler injections.

As expected, good binding to UVC DNA was seen with Ab529 (Figure 4.3.2) giving a near maximal absorbance following the first dose of irradiation (0.435kJm^{-2}). An increase in avidin binding, over the control, was seen, although this was judged not to be statistically significant ($P>0.05$). There was no significant increase in binding of the antibody to single-stranded DNA ($P>0.05$).

Solid phase antigen immobilised on a 96-well membrane plate

Using the membrane plate, again good binding was seen with Ab529 although with lower absorbances (Figure 4.3.3). There was a discernible increase ($P<0.05$) in binding of Ab529 to the irradiated over the unirradiated sample, with no overlap of error bars. However, a slightly different pattern of staining was apparent compared to that seen in Figure 4.3.2. A statistically significant increase in avidin binding to the irradiated sample was calculated to have occurred ($P<0.05$), despite overlapping error bars making visual determination difficult. There was also a statistical increase ($P<0.05$) in binding of the anti-single-stranded DNA antibody with dose, despite some error bars displaying large variation.

DISCUSSION

The binding of Ab529 acted as a positive control indicating DNA to be present and UV irradiated. The antiserum described as recognising changes to DNA following exposure to ROS (ROS DNA), displayed dose-responsive, if not high, binding to the UVC DNA. As discussed earlier (Section 4.2), UVC can induce damage to DNA commonly seen formed via the hydroxyl radical. Doetsch *et al.* (1995) suggested that the formation of such oxidative lesions as 8-oxodG was via a hydroxyl radical-independent route, namely radical cations, although this has yet to be confirmed. Nonetheless, this data supports the evidence that the antiserum recognises oxidative modifications to DNA, although the similarity in shape of the 8-oxoG and anti-ROS DNA antiserum cannot be taken to imply they recognise the same moiety. Failure of this anti-ROS DNA antiserum to detect post-UVB modifications to DNA suggests that either the lesion detected is not induced by UVB or that it is not present in levels sufficient for detection.

An increased binding of avidin to DNA, following UVC irradiation, was shown to occur, although only when the membrane plate, solid phase support was used.

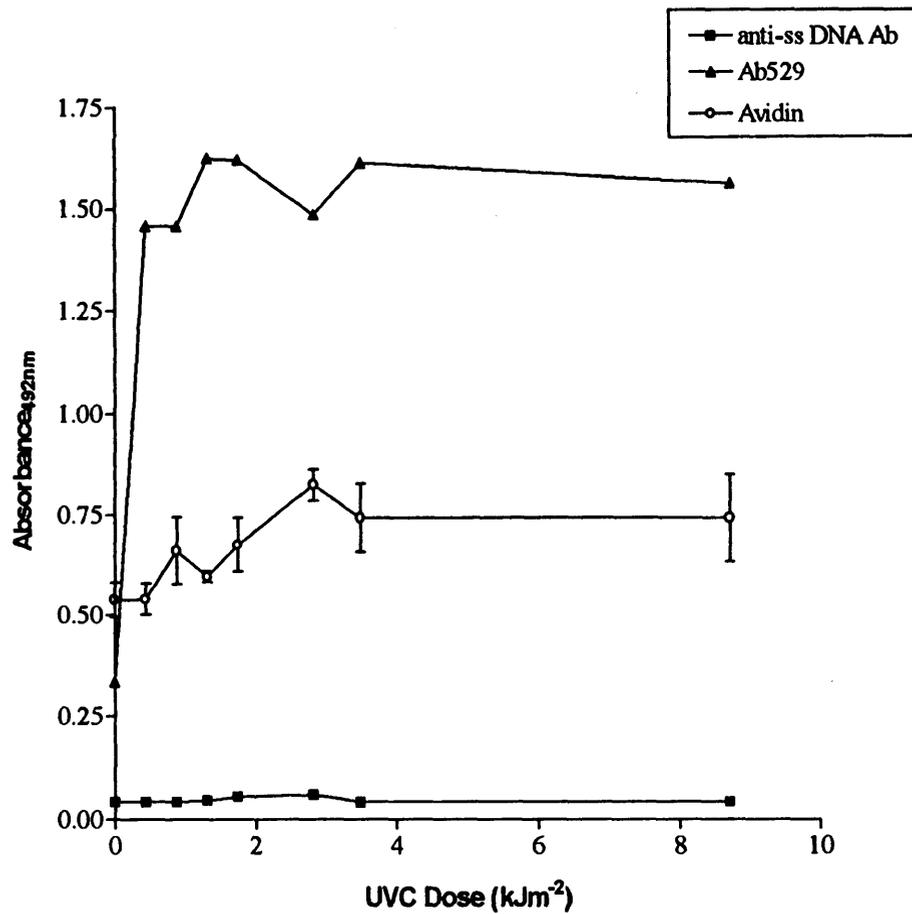


Figure 4.3.2. ELISA binding of UVC-induced DNA damage either by Ab529 (n = 1 determination), directly peroxidase-labelled avidin (n = 3), or a monoclonal antibody against single-stranded DNA (anti-ss DNA Ab) (n = 2). The damaged DNA is bound to a standard 96-well ELISA plate.

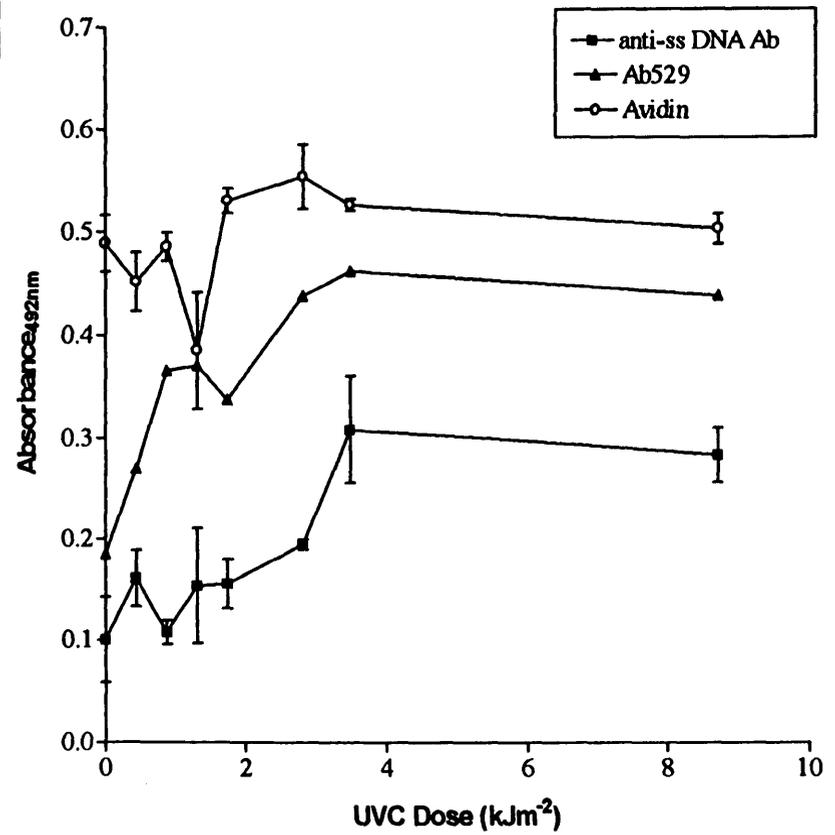


Figure 4.3.3. ELISA binding of UVC-induced DNA damage either by Ab529 (n = 1), directly peroxidase-labelled avidin (n = 3), or a monoclonal antibody against single-stranded DNA (anti-ss DNA Ab) (n = 2). The damaged DNA is bound to a 96-well membrane plate.

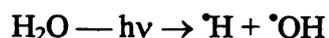
The GC-MS data indicated that significant levels of 8-oxoG to be present (Figure 4.2.1) and therefore the binding of avidin would have been expected to increase appreciably. This was not the case with either form of solid phase support, the increase being barely discernible due to large error bars. A surprisingly poor result was obtained with avidin and the membrane plate, which is the method described by Clarke *et al.* (1997), although this did produce a statistically significant result, whereas the 96-well ELISA plate did not.

Double-strandedness has been shown to impede the recognition of 8-oxodG by experimental antibodies to this lesion (Chapter 3) and this may have been a factor here. No significant increase in avidin binding was noted when using the 96-well plate and double-stranded UVC DNA. However, the use of paraformaldehyde, in conjunction with the membrane plate, was utilised both to bind the DNA and reduce its double-strandedness according to the protocol of Clarke *et al.* (1997) and here a significant increase was seen. This would suggest that a degree of DNA denaturation allowed the avidin access to the lesion. Again, conformation appears to have affected damage recognition by an experimental DNA probe. An explanation of this effect is made for the single-stranded data later in this chapter. These results have highlighted the effects that the choice of solid phase support and DNA manipulations carried out prior to assessment, may have on an assay.

Despite the low increases in binding and poor error bars, an increase in avidin binding and by implication, an increase in 8-oxodG, supported by GC-MS data, was seen following UVC irradiation. This may be seen to add supportive evidence for the use of avidin as a probe of oxidative damage. Whilst the *in vitro*, cellular data of Clarke *et al.* (manuscript in preparation) appears convincing, the competitive work and damaged DNA studies are less so and specific binding cannot be implied purely on structural studies. The data obtained from UVC DNA presented here, in conjunction with that of Clarke *et al.* would suggest that the assay format is less favourable for naked DNA, appearing to be better in cells - an approach to be investigated in Chapter 7.2.

A statistically significant increase in binding of the anti-single-stranded antibody was seen with the membrane plate indicating that UVC irradiation gives rise to regions of single-strandedness. Blount *et al.* (1992) found an increase in binding of anti-single-stranded antisera to DNA irradiated with a combination of 366nm and 254nm UV, although γ -irradiation produced a greater effect. Hydroxyl radicals are known to produce DNA single-

strand breaks indiscriminately in regions not protected by proteins (Chiu *et al.*, 1995). Blount *et al.* (1992) described the formation of hydroxyl radicals by UV as a consequence of H_2O_2 , produced in solution by UV (McCord and Fridovich, 1973):



which in the presence of oxygen, $\cdot\text{H}$ gives rise to $\cdot\text{O}_2^-$:



which can give lead to H_2O_2 production and may subsequently be split by UV to produce $\cdot\text{OH}$. The reaction of two $\cdot\text{OH}$, also gives rise to H_2O_2 . It was then implied that these radicals were responsible for exposing the antigenic determinants recognised by the anti-single-stranded antisera i.e. hydroxyl radicals produced single-strand breaks which lead to denaturation of the DNA. This may well be an explanation for the results presented above. However, whilst McCord and Fridovich (1973) do not state the λ_{max} of their UV source, they do include references which describe water absorbing strongly wavelengths shorter than 200nm. The question arising from this is, can the irradiation of water by a combination of 366nm and 245nm UV, give rise to significant levels of hydrogen peroxide and therefore hydroxyl radicals? On the basis of the information in the McCord and Fridovich (1973) reference and coupled with the findings of Doetsch *et al.* (1995) which suggested a hydroxyl radical-independent damaging mechanism to be acting on the DNA during irradiation, it would appear unlikely, at least with respect to the formation of 8-oxodG. A hydroxyl radical-independent mechanism for the formation of strand breaks has also been proposed. This mechanism is reported to involve DNA sugar radicals, formed via hydroxyl radical, or perhaps more importantly base radical precursors, which give rise to strand breakage and base release (Von Sonntag 1984).

The use of a hydroxyl radical scavenger, DMSO, as used by Doetsch *et al.* (1995), does not exclude the production of hydroxyl radicals, as DNA-bound metal ions at specific sites may not be readily accessible to some scavengers and chelators (Halliwell and Gutteridge, 1990). From this it may be reasoned that whilst 8-oxodG can be formed by UVC, independently of hydroxyl radicals, strand breaks can still occur via hydroxyl radicals, assuming hydrogen

peroxide is formed on the UV irradiation of water. This perhaps is due to a metal ion, probably iron, associated with the DNA backbone, (Enright *et al.*, 1992, whereas endogenous, bound copper primarily mediates base modification, Drouin *et al.*, 1996), interacting with hydrogen peroxide and producing site-specific damage. Recent work by Toyokuni *et al.* (1996) has suggested that oxidative modification of DNA bases and strand breakage to be directly interrelated, postulating that they are two different end points of a common process. Clearly the damaging mechanism(s) are complex and further work needs to be employed, for example the use of $^{18}\text{O}_2$ -labelled water could confirm the involvement of guanine radical cations in the formation of 8-oxodG by UVC radiation. Conversely, the generation of hydrogen peroxide by the photolysis of water, following irradiation by UVA and UVC combined, may be assayed for according to McCord and Fridovich (1973). The basis of this assay was the monitoring of changes in absorbance at 550nm, the consequence of cytochrome *c* reduction. If $^{\bullet}\text{O}_2^-$ was formed by the photolysis of water, it would be able to reduce cytochrome *c*, furthermore, the identity of $^{\bullet}\text{O}_2^-$ would be confirmed by the use of superoxide dismutase.

The discrepancy of anti-single-stranded antibody binding between the membrane and 96-well ELISA plates may be explained by the different processes by which the two plates immobilise DNA. The ELISA plate relies solely on electrostatic attraction to bind DNA and whilst single-stranded DNA can be bound, if the DNA is sufficiently fragmented into short runs of deoxynucleotides they may not possess enough total charge to remain bound during subsequent washes. In contrast, the membrane plate binds DNA not only via electrostatic attraction, but also by physically holding the DNA based on molecular weight and pore size of the membrane when the vacuum is applied. The addition of paraformaldehyde is expected to enhance this effect by cross-linking the DNA to the membrane. As a consequence the membrane may retain more of the small fragments. Use of antibodies to single-stranded DNA in assays for damage are not common within the literature. However, on the basis of the conclusion reached by Toyokuni *et al.*, (1996), regarding the association between 8-oxodG and strand breaks, it would be expected that such immunoassays may be a possible alternative to those involving specific DNA lesions. Using a method similar to that of Van der Schans *et al.* (1989), Timmerman *et al.*, (1995) were required to introduce an alkali unwinding stage to their protocol, in order to achieve recognition of single stranded DNA in white blood cells by a monoclonal antibody. This entails the incubation of alkali-treated whole blood in 96-well plates, with which the antibody has been pre-coated. Single-stranded

DNA fragments remain bound to the plate during subsequent washes and are detected with an alkaline phosphatase-labelled, anti-single stranded DNA antibody conjugate.

Immunochemical/affinity techniques such as these, once optimised for each application, represent a simple way by which DNA damage can be rapidly assessed and comparative information can be easily obtained.

4.4 CHAPTER FOUR SUMMARY

In order to understand the role of UV-induced DNA lesions in biological processes such as mutagenesis and carcinogenesis, it is essential to detect and quantify DNA damage in cells. A novel and both highly selective and sensitive assay using capillary gas chromatography combined with mass spectrometry for the detection and accurate quantitation of a major product of UV-induced DNA damage (*cis-syn* cyclobutadithymine), was presented in this chapter. Quantitation of the cyclobutane thymine dimer was achieved by the use of an internal standard in the form of a stable ^2H -labelled analogue. Both isotopically labelled and non-labelled dimer were prepared directly from their corresponding monomers. Each was identified as their trimethylsilyl ether derivative by GC-MS. Calibration plots were obtained for known quantities of both non-labelled analyte and internal standard. Quantitation of *cis-syn* cyclobutadithymine was demonstrated in DNA exposed to UVC radiation over a dose range of 0 to 3500Jm^{-2} . Under the conditions used, the limit of detection was found to be 20-50fmol on column (equivalent to 0.02-0.05nmol dimer per mg DNA). The results of the present study indicate that capillary GC-MS is an ideally suited technique for selective and sensitive quantification of *cis-syn* cyclobutadithymine in DNA and hence UV-induced DNA damage. Introduction of this assay with a pre-existing GC-MS method for 8-oxoguanine allowed the examination of both lesions in the same sample of UV irradiated DNA, although differences in derivatisation methods prevented both lesions being detected simultaneously. This represents the first time both lesions have been examined by GC-MS and revealed that their induction occurs in a dose-responsive manner.

The DNA, UV irradiated as described above, was also assayed by ELISA-based technologies for oxidative modifications, using techniques described in the literature (anti-single-stranded DNA antibodies) and unique to the Division of Chemical Pathology (anti-ROS DNA antiserum and avidin) in order to test the applicability of these probes to UV-induced damage. Increases were seen with all probes in response to increasing doses of UV radiation, although some discrepancies were noted depending on the format of the assay used, comparison was between 96-well membrane plates and standard ELISA plates. Despite this, such techniques, with the appropriate assay format, do appear amenable to the detection and quantitation of damage to naked DNA.

CHAPTER FIVE

CHARACTERISATION OF ANTIBODIES TO UV-INDUCED DNA DAMAGE AND LIMITS OF DETECTION.

INTRODUCTION

The established methods of detecting both oxidative and non-oxidative, UV-induced DNA damage, such as HPLC-EC (Floyd, 1986), ³²P-postlabelling (Lutgerink, 1992; Bykov *et al.*, 1995) and alkaline agarose gel electrophoresis (Sutherland *et al.*, 1983) have all allowed the quantitation of damage in UV irradiated DNA. However, such methods possess the inherent disadvantage in that they require the extraction of DNA prior to quantitation, furthermore, the potential for artefactual, in particular oxidative, damage to arise from such processing increases (Finnegan *et al.*, 1996).

In 1966, Levine *et al.* (1966) produced a polyclonal antiserum to UV-induced DNA photoproducts by immunisation of a rabbit with UV-irradiated DNA complexed with methylated bovine serum albumin, the principle of which had first been demonstrated by Seaman *et al.* (1966) for yielding antibodies to photooxidised DNA. It was subsequently shown, by inhibition experiments, that this antiserum appeared to be recognising thymine-containing photoproducts. Using such an antiserum as this in a radioimmunoassay, Seaman *et al.* (1972) demonstrated the induction and repair of thymine dimers in the DNA of UV irradiated bacterial and mammalian cells. Since then monoclonal antibodies to specific lesions such as the cyclobutane dimer (Roza *et al.*, 1988) and (6-4) photoproduct (Mori *et al.*, 1988) have been developed and characterised by ELISA-based inhibition experiments (described by Eggset *et al.*, 1987). Although, ELISA methods have been used to quantitate levels of damage in irradiated calf thymus DNA (Wani *et al.*, 1984), quantitation of absolute levels of lesion has only been achieved by immunoslot blot (Wani *et al.*, 1987). In order to achieve this DNA standards containing a known amount of lesion, as determined by the action of *Micrococcus luteus* UV-endonuclease, were used to calibrate binding. This also appears to be the only report in which limits of detection were determined experimentally, rather than based on the assumption of a theoretical rate of lesion induction (Mori *et al.*, 1991).

AIM

The aim of this work was (i) to characterise the antigenic sites recognised by the antiserum raised to UVH DNA described in Chapter 3 and (ii) identify the nature of

the lesion involving two adjacent pyrimidines, recognised by Ab529. (iii) Subsequently, to determine the limits of detection of both antisera for their antigens in DNA.

METHOD

A competitive ELISA using various oligonucleotides (see Chapter Two, page 71, Materials and Methods) was established to determine the main antigenic site recognised by the antiserum. Photoisomerisation of (6-4) photoproducts, present in DNA irradiated with 254nm UV, was also used to identify the nature of the thymine-containing lesion recognised by the antisera. Recognition of lesions in double stranded DNA was demonstrated in ELISA by using DNA irradiated with UVC. A novel experimental system, utilising the GC-MS assay of Chapter Four, was employed to ascertain the limits of detection for these two antisera, using a DNA standard containing a known amount of dimer.

Statistical analyses were performed by GraphPad Prism, version 2.0.

RESULTS

Investigation of the antiserum raised against UVC/hydrogen peroxide treated DNA

Previously the strong immunogenic effect of UVC/hydrogen peroxide treated DNA was noted (Chapter 3). Subsequently it was demonstrated by ELISA, using an anti-rabbit IgG secondary antibody, that a large IgG response had been achieved (Figure 5.0). To allow characterisation of the antiserum raised against UVC/hydrogen peroxide treated DNA, a competitive ELISA was instituted. UVC DNA was used as the solid phase antigen on the basis of the data in Chapter Three (Figures 3.10 and 3.11), which showed that the majority of the antiserum response to be towards DNA changes induced by UVC. With UVC DNA as the solid-phase antigen and an antiserum working dilution of 1:100, UVC double-stranded DNA (UVC DNA) was shown to be a most effective inhibitor with an $IC_{50} < 0.09 \mu\text{g/ml}$ (Figure 5.1). The IC_{50} is the concentration of antigen giving 50% inhibition, providing an index of antigenicity of a compound. The lower the value, the better the inhibitor. By comparing the ability of

putative antigens to compete for binding with the polyclonal antiserum, this ELISA system was used to further investigate the specificity of the antiserum. No competition was demonstrated by unirradiated DNA (Table 5.1).

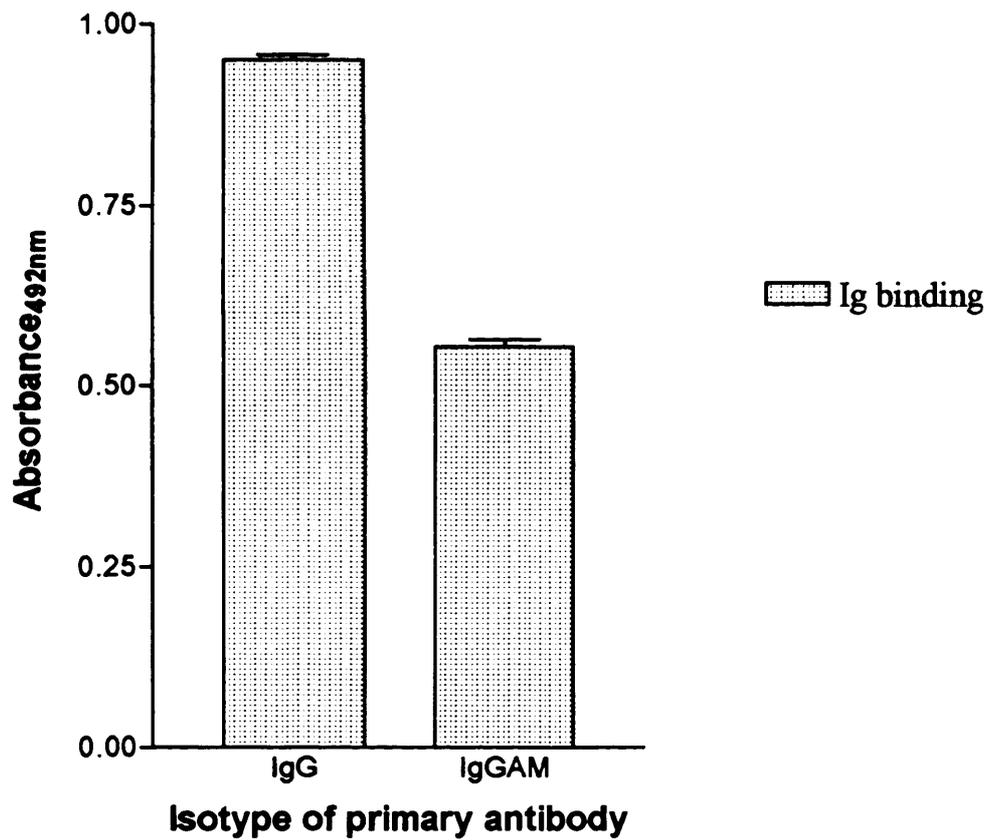


Figure 5.0. Recognition of primary UVC/H₂O₂ antiserum (X18) by isotype-specific secondary antiserum by ELISA. Values represent the mean (SEM) of quadruplicate determinations.

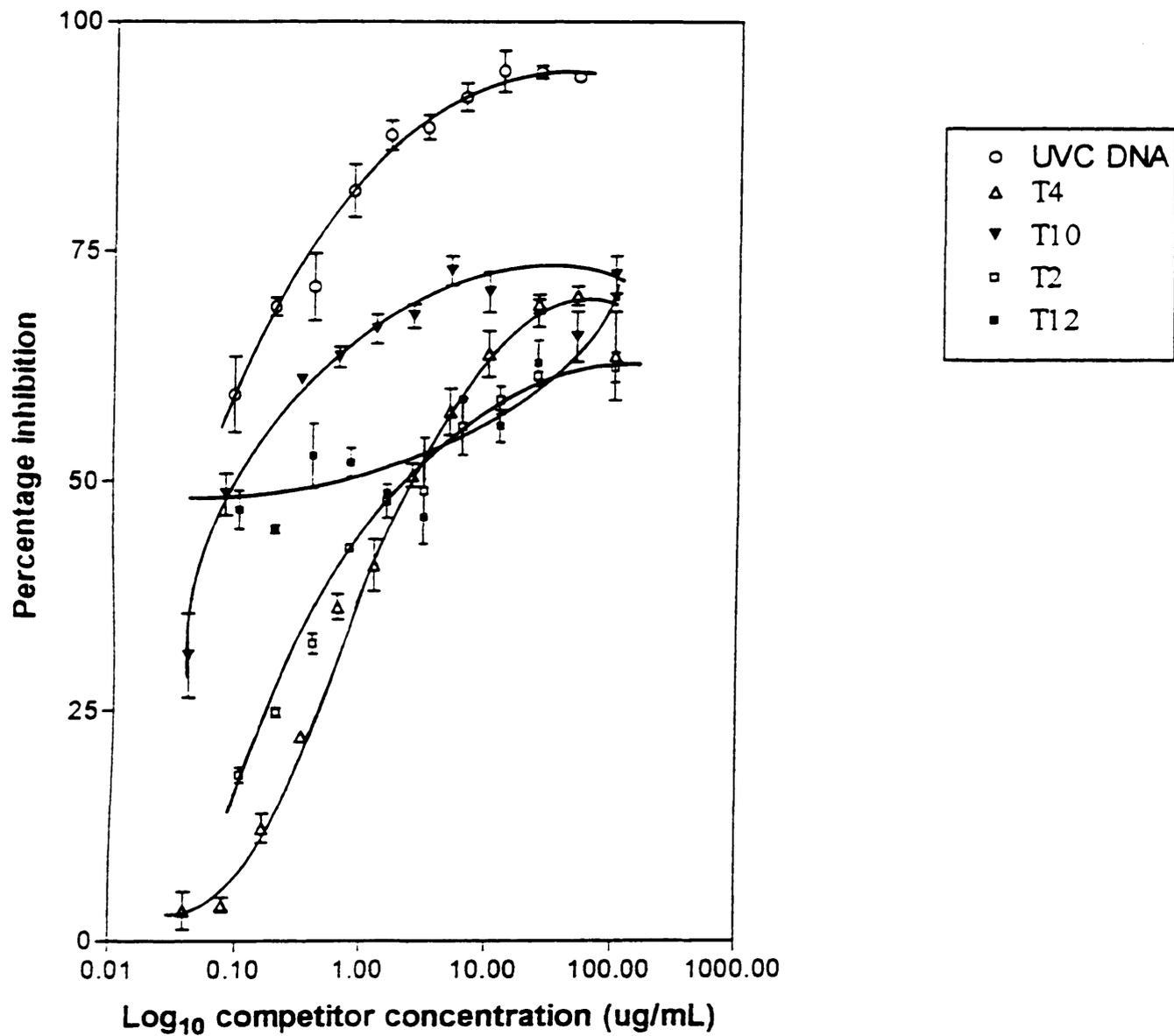


Figure 5.1. Inhibition of ELISA binding of UVC/H₂O₂ antiserum to UVC-DNA by different UV-treated polythymidylate chains of increasing length. Maximum inhibition (100%) was that observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean of three determinations per concentration.

Inhibition by UVC irradiated polymers of thymine

It has been previously shown that UV irradiated polymers of thymine nucleotides to be crucial in the recognition of UV-DNA by an antiserum raised to UV-damaged DNA (Herbert *et al.*, 1994), suggesting thymine dimers to perhaps be responsible for the antigenicity of UVC/hydrogen peroxide treated DNA. As a consequence, the ability of the antiserum to recognise free thymine base dimers, that is to say dimers without a polymer of other bases, was examined. These were synthesised and unequivocally identified as described in the Materials and Methods section (page 66). However, this preparation of dimers did not inhibit the binding of the polyclonal UVC/H₂O₂ antiserum (Table 5.1).

UV-irradiated polythymidylate [poly(dT)] was a very effective inhibitor with an IC₅₀ of <0.09 µg/ml (Table 5.1). Thymine-containing oligonucleotides of various lengths were used in competitive ELISA to further identify the epitopes recognised by the antiserum. Un-irradiated thymidylate oligonucleotides were found to be extremely poor inhibitors (IC₅₀ >50µg/ml). Generally, inhibition increased with increasing irradiated oligonucleotide length following irradiation (Figure 5.1), being most evident between poly(T₄) and poly(T₁₀) (a difference in IC₅₀ of 2.129µg/ml) (Table 5.1). Irradiation of DNA with UVC yields a number of different products of which pyrimidine (6-4) pyrimidone (6-4)PP and cyclobutane dimers appear to predominate (Cadet and Vigny, 1990). To investigate the nature of the lesions recognised by both our antisera, DNA, irradiated with a dose of 254nm known to create maximal binding of both antisera, was exposed to subsequent doses from a longer wavelength UV source, which has been shown to photoisomerise the (6-4)PP to Dewar isomers, leaving the cyclobutane dimer unaffected (Patrick, 1970; Ikenaga and Jagger, 1971; Mitchell and Rosenstein, 1987) (Figure 5.2). Although not monochromatic, this source possesses a significant output in the range 305-340nm, effective for photoisomerisation (Mitchell and Rosenstein, 1987). DNA, treated as described, has previously been used to identify the type of dimer associated with the antigenic site (Mori *et al.*, 1988). The consistent level of binding observed with both the UVC/H₂O₂ antiserum and Ab529 suggests cyclobutane dimers rather than (6-4)PP to participate in the epitopes recognised by both antisera (Figure 5.2).

Sequence specificity

To further investigate the hypothesis that the epitope being recognised was larger than a single thymine dimer, an additional group of oligonucleotides was studied. The basis of these oligonucleotides was a septamer or octamer containing A and T residues in various sequences. Poorest inhibition was noted by an irradiated octamer of alternating A and T residues ($IC_{50} > 50\mu\text{g/ml}$; Table 5.1), demonstrating the minimum requirement for two adjacent thymines, or at least pyrimidines. The sequence T-C-T surrounded by A residues in a septamer, showed relatively poor, although detectable, inhibition ($IC_{50} = 50\mu\text{g/ml}$; Figure 5.3), suggesting that C-T dimers are not important epitopes for the antiserum. An octamer of alternating T-T and A-A sequences gave moderate inhibition ($IC_{50} = 29.6\mu\text{g/ml}$), indicating that dimerisation of thymine alone was not sufficient to produce effective inhibition of the antiserum. Comparison of this with the inhibition provided by T-T-T, surrounded by A residues ($IC_{50} = 15.1\mu\text{g/ml}$) indicated a minimum of two adjacent thymines in conjunction with a third thymine, or perhaps pyrimidine to be crucial for the epitope. A further appreciable increase in inhibition was demonstrated using a sequence of four thymines flanked by adenine residues ($IC_{50} = 2.7\mu\text{g/ml}$; Figure 5.3).

Table 5.1. Index of inhibition by a variety of oligonucleotide and modified DNA competitors as determined by competitive ELISA with the UVC/H₂O₂ antiserum (X18). Unirradiated oligonucleotides were not inhibitory.

| Oligonucleotide competitor | IC ₅₀ (µg/ml) |
|---|--------------------------|
| UVC DNA | <0.09 |
| Unirradiated DNA | >50.0 |
| Cyclobutane thymine dimer | >50.0 |
| T ₂ | 3.144 |
| T ₄ | 2.219 |
| T ₁₀ | 0.09 |
| T ₁₂ | 0.304 |
| TATATATA | >50.0 |
| TTAATTAA | All UV irradiated 29.873 |
| AATTTAA | 15.087 |
| AATTTTAA | 2.735 |
| poly(dT) | <0.09 |
| poly(dC) | >50.0 |
| poly(dG) | 42.923 |
| Methylene blue DNA | >100.0 |
| Ascorbate/H ₂ O ₂ | >100.0 |
| iron/H ₂ O ₂ | >100.0 |
| 8-oxoguanosine/KLH | >100.0 |

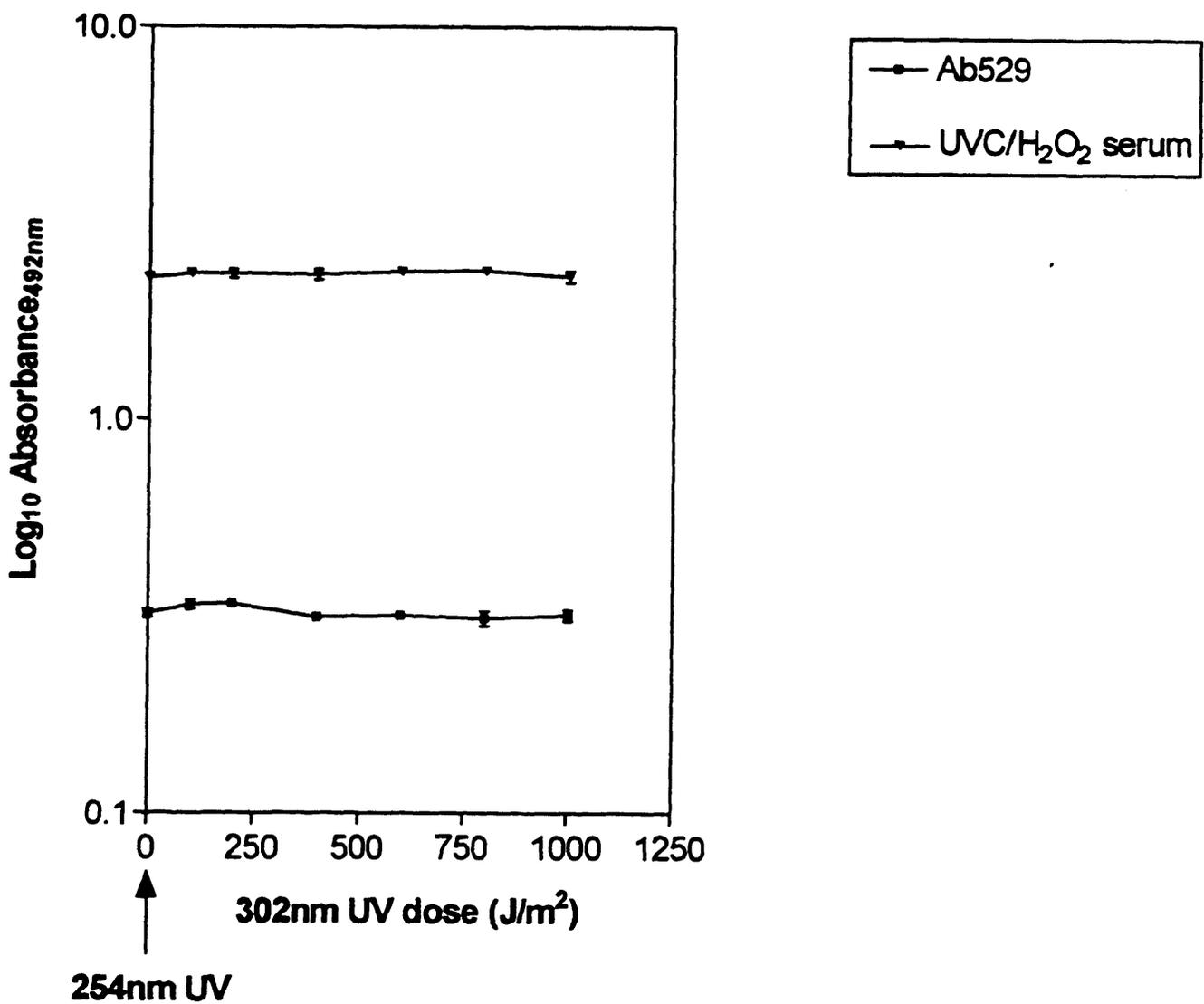


Figure 5.2. The binding abilities of 529 and UVC/H₂O₂ antisera to DNA damage induced by 254nm UV radiation (8751 Jm⁻²) and alteration of binding abilities after subsequent irradiation of the DNA with 302nm UV. 529 and UVC/H₂O₂ antisera were diluted 1/2000 and 1/100 respectively. The points represent the mean of three determinations per dose.

Having identified the minimum epitope requirement of -TTPyr- (or -PyrTT-) further oligonucleotides were synthesised to investigate the possibility of substituting the 5' or 3' thymine with a cytosine. Replacement with cytosine as described above proved to be a more effective inhibitor than the original AATTTAA sequence which had an IC_{50} of $9.4\mu\text{g/ml}$ (Figure 5.4). Furthermore the inhibition was greatest when the T-T sequence was flanked by a 5' C residue (AACTTAA) ($IC_{50} = 0.345\ \mu\text{g/ml}$) instead of a 3' C (AATTCAA) (Figure 5.4) ($IC_{50} = 1.052\ \mu\text{g/ml}$), having an IC_{50} showing at least a 3-fold greater effectiveness. Substitution of C between the two thymines provided minimal inhibition as shown in Figure 5.3.

ELISA detection of oxidative DNA damage products

Although the data in Figures 3.10 and 3.11 (Chapter Three, pages 99 and 100) suggested that the majority of the antibody response to be towards UVC DNA, some recognition of the UVH DNA was shown. The nature the modifications contained within UVH DNA were unknown, although oxidatively modified guanines were shown to be present (Chapter Three). On the basis that both ROS and UVC can give rise to the most common guanine lesion, 8-oxoG, UV irradiated polyG was investigated as a possible competitor. Inhibition was demonstrated to occur, although very poor ($IC_{50} = 42.9\mu\text{g/ml}$; Table 5.1). Subsequent oxidatively modified inhibitors were investigated, including ascorbate/hydrogen peroxide modified-, iron/hydrogen peroxide modified- and methylene blue/white light modified DNA, in addition to the 8-oxoguanosine/KLH conjugate. However, no competition was seen, even with concentrations of inhibitor up to $100\mu\text{g/ml}$ ($IC_{50} > 100\mu\text{g/ml}$; Table 5.1).

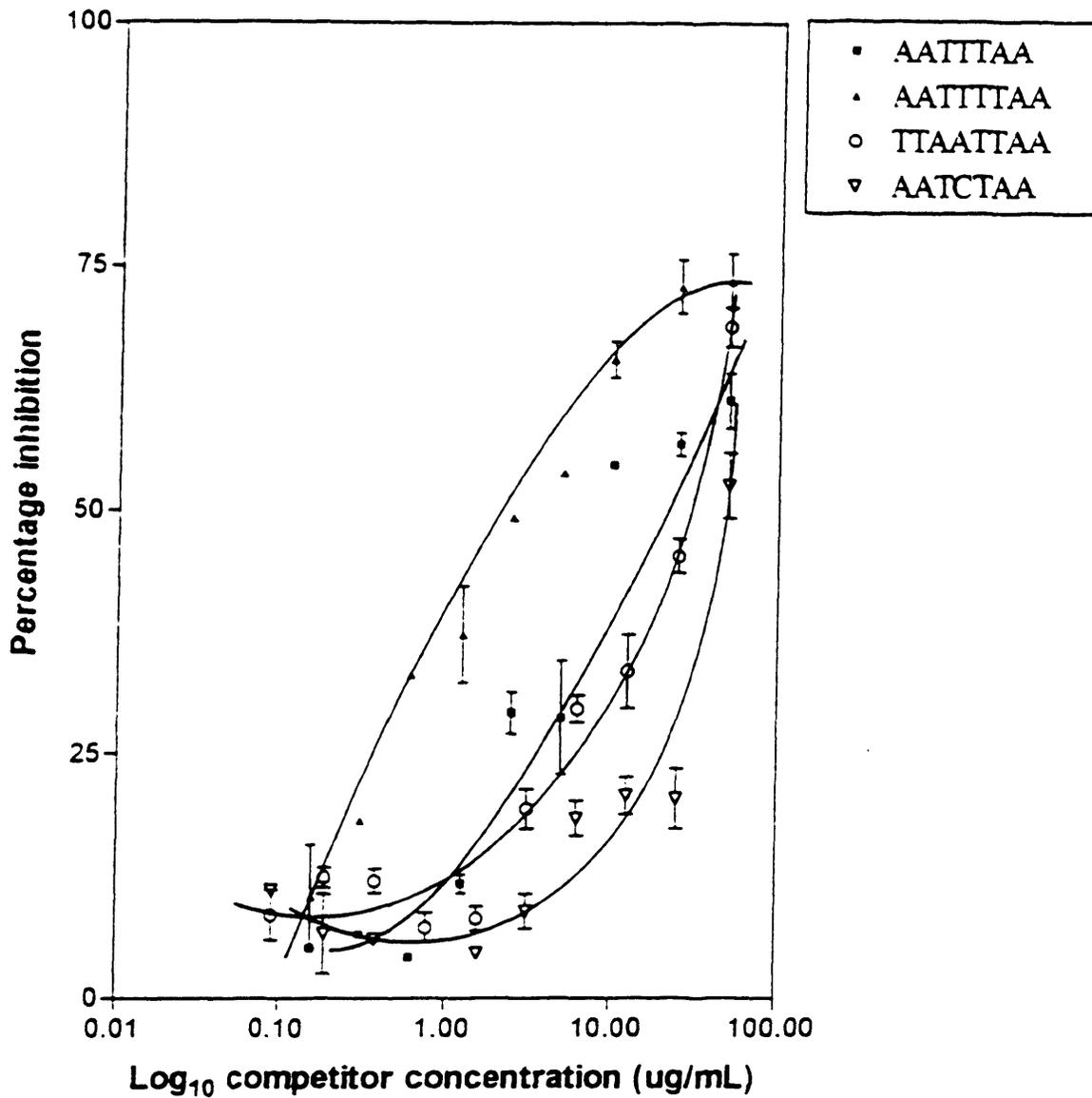


Figure 5.3. Inhibition of ELISA binding of UVC/H₂O₂ antiserum to UVC-DNA by UV-treated oligonucleotides of different sequence. Maximum inhibition (100%) was that observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean of three determinations per concentration.

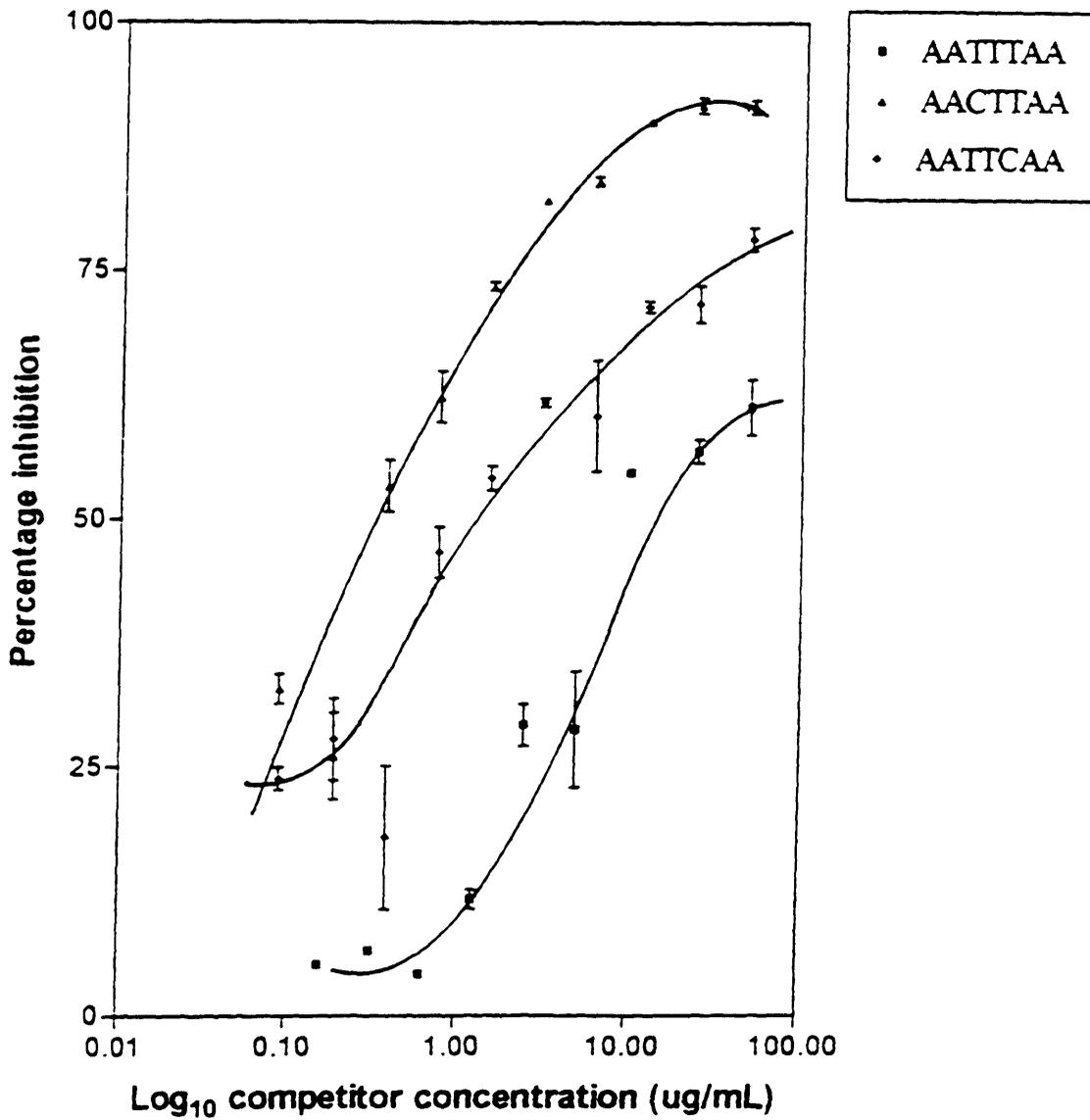


Figure 5.4. Inhibition of ELISA binding of UVC/H₂O₂ antiserum to UVC-DNA by UV-treated septanucleotides of different sequence. Maximum inhibition (100%) was that observed in the absence of solid-phase antigen (UVC-DNA). Values represent the mean of three determinations per concentration.

Assessment of UV irradiated DNA by ELISA

Two antisera were utilised for this study, raised by either using DNA irradiated with UVA and UVC (Ab529), or DNA irradiated with UVC in the presence of hydrogen peroxide (UVC/H₂O₂ antiserum), as immunogens. Both antisera have now been characterised as recognising cyclobutane thymine dimers in DNA, each with distinct sequence specificities. To further support the evidence presented as characterisation, the ability of the antisera to bind UV irradiated DNA was investigated. Additional dosed samples of irradiated DNA were included in the ELISA assay with the UVC DNA from the GC-MS experiment as binding of both antibodies was shown to saturate after 0.875 kJm⁻². Figure 5.5 (a) shows the binding of Ab529 to DNA irradiated with UVC. At the lowest level of UVC irradiation used (0.116 kJm⁻²) the level of binding increased appreciably and significantly ($P < 0.0001$) over control levels in the unirradiated sample, showing saturation around the 0.875 kJm⁻² dose. Represented by Figure 5.5 (b) is the binding of the UVC/H₂O₂ antiserum to DNA irradiated with UVC. This plot of antiserum binding exhibited marked similarity to those seen with Ab529, although with lower absorbances. Also the increase in binding, following a single dose of UVC, appeared greater for Ab529 than the UVC/H₂O₂ antiserum. Investigation of antisera binding to DNA following UVA demonstrated no detectable increase in binding with dose, compared to the unirradiated sample.

Quantitation of cyclobutane thymine dimers in UV irradiated DNA by GC-MS

Stable isotope-dilution mass spectrometry was used to accurately determine the levels of *cis-syn* cyclobutane thymine dimers produced in DNA following irradiation with various doses of UVC. The purpose of this was to provide a known quantity of dimer, in DNA, for assessment by antibody binding. The graph of dose versus yield demonstrated the linear relationship over the lower portion of the dose range of 0 to 3.5 kJm⁻², as described previously (Chapter 4). However, at the maximum dose the curve started to deviate from this relationship, seeming to approach a plateau (these data are included in Figures 5.5 a and b).

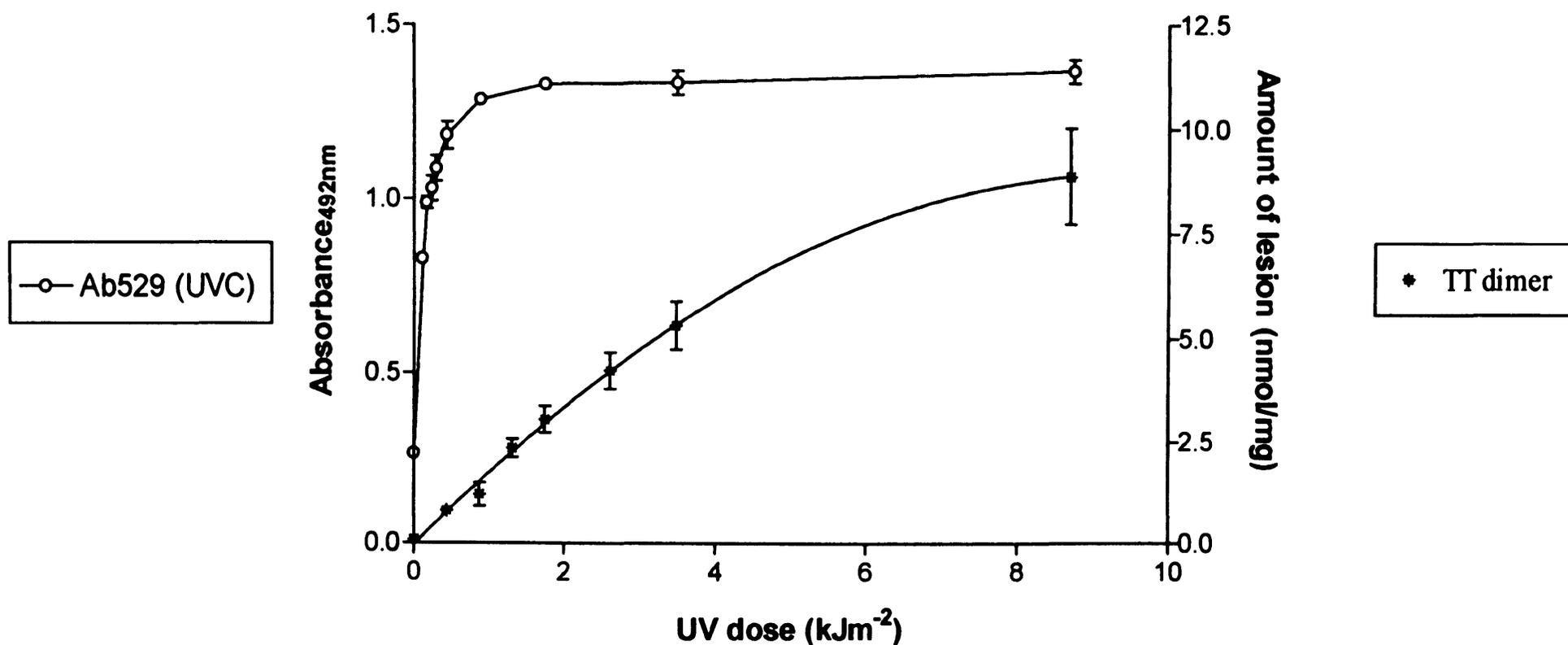


Figure 5.5 (a). ELISA results for the induction of cyclobutane thymine-thymine dimers within specific sequence foci of DNA irradiated with increasing doses of UVC, detected by Ab529 (diluted 1/2000). Each point shows the mean of three determinations. Also shown, dose-yield plot of *cis-syn* cyclobutadithymine (TT dimer), in UVC irradiated DNA, as determined by GC-MS. Values represent the mean and standard deviation of three independent autosampler injections.

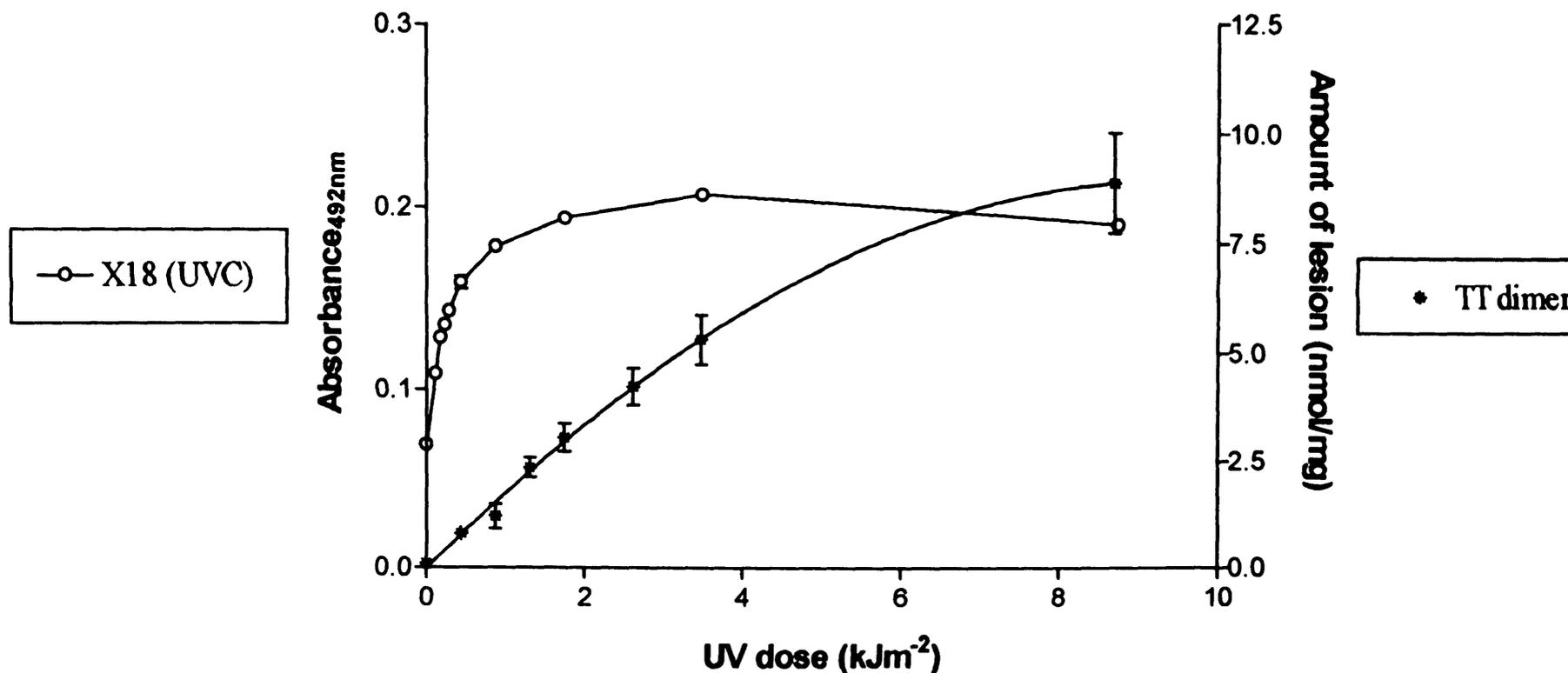


Figure 5.5 (b). ELISA results for the induction of cyclobutane thymine-thymine dimers within specific sequence foci of DNA irradiated with increasing doses of UVC, detected by AbX18 (diluted 1/100). Each point shows the mean of three determinations. Also shown, dose-yield plot of *cis-syn* cyclobutadithymine (TT dimer) in UVC irradiated DNA, as determined by GC-MS. Values represent the mean and standard deviation of three independent autosampler injections.

Antiserum limits of immunodetection

On the basis that the levels of *cis-syn* cyclobutane thymine dimer contained in a sample of DNA irradiated with 437 Jm^{-2} could be accurately quantitated by GC-MS, doubling dilutions of this DNA sample were performed for assessment by ELISA. Binding of the UVC/H₂O₂ antiserum and Ab529 was still apparent with between 2.441 and 1.220ng of DNA respectively, on the plate (assuming 100% binding of DNA to the plate). Data provided by GC-MS indicated there to be 0.805 nmol of dimer/mg of DNA. Dimers within the antiserum's appropriate sequence specificity, would be expected to be present in fewer numbers than the total number of dimers. Using these data, the limits of detection possessed by the UVC/H₂O₂ antiserum and Ab529 were calculated to be 1.9 and 0.9fmol (approximately 5 and 2 lesions per 1×10^7 bases), respectively, values similar to those reported by Mori *et al.* (1991) with their monoclonal antibodies and the immunoslot blot assay of Wani *et al.* (1987).

DISCUSSION

The immunogenicity of UVC/hydrogen peroxide treated DNA has previously been noted (Chapter 3, page 98; Cooke *et al.*, 1997). It was concluded that this was largely due to modifications induced by the UV component of the damaging system, agreeing with other observations reporting the immunogenicity of UV DNA (Herbert *et al.*, 1994; Natali and Tan, 1971; Eggset *et al.*, 1987). However, full characterisation of the antiserum was not demonstrated, only implying there to exist both a UV and a ROS component.

In this study, it was shown that, for an antiserum raised to UVC/hydrogen peroxide treated DNA, there existed a strong competition by solely thymine containing, UV-irradiated oligonucleotides. Furthermore, this competition displayed increasing effectiveness with increasing chain length, in agreement with the findings of Levine *et al.* (1966). No inhibition was demonstrated using the UV irradiated TATATATA oligonucleotide, despite there being evidence for UV-induced dimerisation between non-adjacent thymines (Love and Minton, 1992). This may be explained by the process of such dimerisation only occurring in single-stranded DNA polymers (Nguyen and Minton, 1988), whereas our A-T oligomer, possessing complementary strands, was

likely to have annealed. Concurring with Natali and Tan (1971), who concluded the antigenic determinants of UV DNA to be thymidine-associated photoproducts, inhibition with polydeoxycytidylic acid did not occur. Furthermore, the photoproduct involved in the antigenic site was shown to be cyclobutane thymine dimers rather than (6-4)PP. The data using T residues flanked by adenines, showed unequivocally the minimum antigen to be a run of three thymines. This would suggest that the dimer confers conformational changes on the DNA polymer which accounts for the increased immunogenicity and it is a combination of these changes, along with the lesion itself, which the antiserum recognises. This finding is supported by other studies (reviewed in Cadet *et al.*, 1985) and that free cyclobutane thymine dimer failed to show any inhibition in the competitive ELISA studies shown here. Furthermore, one flanking thymine, 5' or 3', of the dimer may be successfully replaced with a cytosine. The observations in this thesis indicated that inhibition increased according to location of the third pyrimidine relative to the dimer (CTT > TTC > TTT), with CTT having an IC_{50} three times lower than TTC. These findings are perhaps surprising when it is considered that in a defined sequence of DNA, the relative induction of dimers for TT and CT/TC sequences was ~2:1 (Umlas *et al.*, 1985). Previously it has been noted that the induction of cyclobutane dimers is strongly influenced by the sequence of neighbouring bases, in particular the 5' base. Mitchell *et al.* (1992) showed that a pyrimidine 5' to a dipyrimidine site enhanced the potential for dimerisation. Therefore the difference in recognition seen between the CTT and TTC sequences may be accounted for by dimer being more readily formed in the CTT sequence due to the 5' pyrimidine, leading to greater overall numbers of dimer being present. However, Mitchell *et al.* (1992) states that the 5' pyrimidines of CTT and TTT would yield more dimer than TTC, therefore if binding was dependant solely on dimer yield greater inhibition would be seen with TTT rather than TTC. The competitive studies described here do not support this and substantiate the hypothesis for sequence specificity. Furthermore, Mitchell *et al.* (1992) did not compare the effect of the 5' pyrimidine being a thymine or a cytosine, which may further affect the potential for dimerisation. On this basis, our data suggested that the 5' cytosine affects binding of the antibody, either by increasing dimerisation of the thymines or is inherently linked as part of the epitope. Matsunaga *et al.* (1990) produced a monoclonal antibody to (6-4)PP which

they described to possess sequence specificity. However, this referred to the recognition of dTpdT preferentially to dTpdC, with no reference to the flanking sequences.

A polyclonal antiserum which recognises thymine containing dimers possessing sequence specificity consisting of TTT and TTC (Ab529) has been described, using DNA irradiated with UVC and UVA as the immunogen (Herbert *et al.*, 1994). This sequence specificity contrasts to that recognised by the antiserum raised against UVC and hydrogen peroxide, as described in this thesis, which showed preferential binding to CTT sequences. In both cases, the immunogen used had not been previously used to generate an antiserum to UV damage and furthermore sequence specificity had not been described outside this laboratory. Therefore the role of the generating system in producing these sequence specificities requires further investigation.

Antibodies possessing the ability to recognise specific sequences, albeit following UV irradiation, may have potential application in molecular biology where sequence probes are required. For example, UV signature mutations (CC→TT and C→T) in the *p53* gene have been reported to occur predominantly at dipyrimidine sites (Dumaz *et al.*, 1993; Molès *et al.*, 1993), which are also more slowly repaired than other areas of the gene (Tournaletti, 1994). This has further implicated dipyrimidine photoproducts as being oncogenic along with suggesting a role which the *p53* gene may play in UV carcinogenesis (Brash *et al.*, 1991). Utilising the above antibodies, the potential exists, following UV irradiation, to recognise mutated sequences particularly when flanked by a pyrimidine on either side.

At doses of UVC, lower than 1kJm^{-2} , linear induction curves of cyclobutane thymine dimers in DNA were obtained using a direct ELISA with the polyclonal antisera UVC/H₂O₂ (X18) and Ab529. Both plots show a rapid increase in absorbance at low doses of UVC reaching a plateau at only 875Jm^{-2} this is in contrast to the binding of the monoclonal antibodies of Mori *et al.* (1991) and Mizuno *et al.* (1991), the binding of which are still increasing even at 1000Jm^{-2} . The shape of these induction curves suggest that either all potential sites for lesions had been detected, or the threshold of

the ELISA assay had been reached, or the antisera had bound to all available sites, steric inhibition preventing access to other sites. The first explanation is clearly the most unlikely. This suggests that the latter two explanations may be more important, particularly with consideration of the results derived from GC-MS analysis. These results show a plateau was not approached until over 6kJm^{-2} which, due to the nature of the assay hydrolysing DNA to free bases, is likely to be a more realistic description of the induction of dimers. Indeed, such an effect can be seen from the results of Bykov *et al.* (1995), a technique which utilised enzymolysis of the DNA to free deoxynucleotides. It is therefore likely that saturation of the ELISA assay, or steric inhibition of the antibodies, either by antibodies already bound to the DNA or due to the coiling of the irradiated DNA may result in a plateau being reached at doses lower than those for the GC-MS assay. Dimersation of all the available adjacent thymines would appear to be the simplest explanation for the plateau effect seen with the GC-MS data. An optional explanation for why this plateau is reached may involve the position of the steady-state between formation of the thymine dimer and monomerisation, on irradiation, which varies dramatically with wavelength (Johns *et al.*, 1962). On this basis, it appeared that UVC rapidly reached an equilibrium of dimer formation and monomerisation, favouring the former. It was noted that Ab529 produced a greater increase in binding following an initial dose of UVC than the UVC/H₂O₂ antiserum, suggesting a greater sensitivity which, coupled with its more economical working dilution, justified its continued use in this thesis.

Induction of pyrimidine dimers in cells exposed to UVA has been reported, although 365nm is the longest wavelength at which they have been detected (Tyrrell, 1973). Freeman *et al.* (1986) initially claimed to have detected pyrimidine dimers in DNA from cells irradiated with 385nm and 405nm UV, however a subsequent publication (Hacham *et al.*, 1990) concluded these wavelengths to be ineffective for dimer induction. The failure of the antisera described here to bind DNA irradiated with UVA is perhaps not surprising as DNA absorbs UVA weakly (Sutherland and Griffin, 1981). This suggests that an, as yet unelucidated, mechanism, perhaps involving cellular sensitisers, but contrasting with that for UVB and UVC (Ellison and Childs, 1981), is responsible for dimer production by UVA (Peak *et al.* 1984). However, if this is the

case the wavelength cut-off for this effect must lie between 365nm and 385nm. Oxidative damage to the DNA of cells irradiated with UVA is also reported to occur (Tyrrell, 1985; Keyse and Tyrrell, 1989; Applegate and Frenk, 1995), although in the absence of a photosensitiser this process is unlikely to take place. The UVC/H₂O₂ antiserum was raised against DNA modified by a system reported to generate reactive oxygen species (ROS) and it has previously speculated that a proportion of the antiserum may recognise oxidative alterations to DNA (Chapter 3), although the apparent generation of an antiserum to UV-irradiated DNA was clearly a serendipitous finding. This theory was supported by the low, but demonstrable, inhibition of UVH antiserum by UVC irradiated poly(dG) (Table 5.1). Further investigation of this phenomenon, using other types of ROS-modified DNA as competitors, failed to show any inhibition; 50% was not achieved even at 100µg/ml competitor (Table 5.1), resulting in the conclusion that the effect of such a portion of the antiserum was negligible.

The ELISA technique was shown to be extremely sensitive when compared with the GC-MS method for dimers. GC-MS sensitivity was determined to be 20-50 fmol (0.02-0.05 nmol/mg DNA), or 6-15 lesions per 10⁶ bases. Antibody limits of detection were determined by ELISA, based on levels of dimer in UV irradiated DNA as quantified by GC-MS. For antisera 529 and UVC/H₂O₂ it was indicated that levels as low as 0.9 and 1.9 fmol of cyclobutane dimers were detectable, values that are comparable to those obtained by Mori *et al.* (1991) for their antibodies to dimers. These detection limits are theoretical, as the GC-MS provides the total number of dimers in DNA, whereas the antisera show specificities for dimers within a sequence context and not all the DNA may be binding to the plate. Therefore the actual limits are likely to be lower. Comparison of GC-MS and ELISA would suggest ELISA to possess a limit of detection 10-25 times lower than that of GC-MS (see page 141, Chapter Four). This sensitivity is partly derived from the enzyme/substrate amplification step, allowing very low levels of damage to produce a signal. GC-MS, however, allows absolute quantitation along with positive identification of lesions within DNA. Furthermore, whereas GC-MS requires approximately 40µg of DNA per sample, here, dimers have been detected by ELISA in as little DNA as 1.22ng

reflecting the difference in sensitivity. This represents the first time that the limits of detection of antisera to thymine dimers have been determined experimentally using GC-MS. Previously, the limits have been determined relying on the assumption of a theoretical rate of lesion induction (Mori *et al.*, 1991); this was achieved by calculating the number of dimers present in DNA following the lowest detectable dose of UV, based on the assumption that the induction rate of dimers is 0.003% of total thymines per Jm^{-2} . Mori *et al.* (1991) report a detection limit of their monoclonal antibody to cyclobutane thymine dimers of 0.28fmol, they further assert that this is almost the same as the limit for the monoclonal of Wani *et al.* (1987) which was determined using a dimer-containing DNA standard, calibrated using an *M. luteus* UV endonuclease-based assay. Combined, these reports of detection limits suggest that, not only is the method developed here for determining such limits acceptable, but so are the limits for the polyclonal antisera which have been characterised.

Using the equation derived from the GC-MS assay ($y = 0.14565 + 1.5287x$, Chapter Four) as a reference and excepting the conditions of sequence specificity, the binding to DNA irradiated with 0.116kJm^{-2} corresponds to 9.6 dimers per 10^6 bases, or a total of 0.0008nmol of dimer in 2.5 μg DNA (equivalent to 250 cells - on the basis that 1 cell contains 10pg DNA). Again, from the GC-MS data it is possible to calculate the number of dimers/ 10^6 bases/ Jm^{-2} using the equation described above. The intercept must not be included as this represents the background number of lesions in an unirradiated sample and does not influence the rate of induction. Therefore, substitution of 0.001kJm^{-2} (1Jm^{-2}) into the equation produces a value of 0.147nmol/mg, equivalent to 5 cyclobutane thymine dimers per 10^7 bases per Jm^{-2} , or 2 cyclobutane thymine dimers per 10^6 thymines per Jm^{-2} (0.0002% of thymines per Jm^{-2}). Comparison of this with the 0.003% value of Mori *et al.* (1991) shows a factor of ten difference, however because Mori *et al.* describe this as this being from unpublished data and do not describe the process by which this value is derived, reliable comparison of the two values cannot be made.

Taken together, these data shows the establishment and characterisation of two polyclonal antisera, each recognising cyclobutane thymine-thymine dimers, but

possessing two different sequence specificities. Application of these antisera and comparison with data by GC-MS has revealed findings concerning the induction and recognition of UV lesions in DNA by sequence-specific antisera in ELISA. Comparison of the two techniques has shown the ELISA to possess a high degree of sensitivity, making it a useful tool in the detection and quantitation of UV damage.

5.1 CHAPTER FIVE SUMMARY

Described here is the comprehensive characterisation of two antisera to UV-damaged DNA. Competitive ELISA demonstrated the main epitopes recognised to be dimerised adjacent thymines. Furthermore, recognition of this lesion was shown to be in conjunction with a flanking sequence, specific to each antiserum. The thymine lesion was subsequently shown to be a cyclobutane thymine-thymine dimer. Application of these unique antisera successfully demonstrated the induction of such lesions in DNA irradiated with UVC. No discernible binding to UVA irradiated DNA was shown. A novel experimental system was employed to ascertain the limits of detection for these two antisera. Using a DNA standard containing a known amount of dimer, the limits of detection were found to be 0.9 and 1.9 fmol. This was compared to a limit of 20-50 fmol using gas chromatography-mass spectrometry. The results of the present study indicate these antisera to be a sensitive approach for determining levels of UV damage in DNA and their sequence-specificity may indicate a potential use in mutagenesis studies.

CHAPTER SIX

IMMUNOCHEMICAL DETECTION OF UV-INDUCED DNA DAMAGE IN A KERATINOCYTE CELL LINE AND BIOPSIES FROM HUMAN SUBJECTS.

6.1 RATIONALE FOR USING RHT KERATINOCYTES

The skin is the area of the human body exposed to the greatest levels of UV radiation, making keratinocytes the cell type of choice for investigation of the effects of UV, as they represent the majority of cells within the epidermis. Also, basal and squamous cell skin cancers are keratinocyte in origin. Use of a SV40T transformed human keratinocyte cell line (RHT cells) was part of a logical progression of model systems in which the experimental antisera have been tested, which has already included calf thymus DNA (Chapters Four and Five). Cell culture methods were developed specifically for the following applications: chambered cell culture for multiple treatments, irradiation of mass cultures for damaged DNA isolation and finally flow cytometry, for sensitive *in situ* analysis of DNA damage in large numbers of cells.

6.2 QUALITATIVE DETECTION OF UV-INDUCED DNA DAMAGE IN KERATINOCYTES

INTRODUCTION

The incidence of basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma (MM) is showing an increase amongst the general population (Potten *et al.*, 1993). Indirect epidemiological evidence and depletion of the ozone layer (Kriker *et al.*, 1994) has focused research on the relationship between ultraviolet radiation (UVR) and skin tumourigenesis. UV irradiation of DNA has been reported to produce a number of photoproducts, both directly and indirectly (Rahn, 1979), as well as the activation of transduction pathways and the induction of specific genes (Bill and Tofilon, 1994). The formation of CPD in DNA irradiated with UVB, is well established (Urbach *et al.*, 1972). Reports that UVA irradiation of, not only bacteria (Tyrrell, 1973), but also human skin (Freeman *et al.*, 1987) gives rise to the induction of pyrimidine dimers lead to the examination here of a number of antisera binding in keratinocytes, post-UVA irradiation. On the basis that O'Neil *et al.* (1991) described reports in the literature which contain accounts of pyrimidine dimers occurring by free radical mechanisms, the putative effects of a free radical scavenger was also investigated. Furthermore, it is well established that UVA irradiation of cells induces oxidative stress (Fuchs and Packer, 1991) leading to damage of DNA and membranes (Tyrrell and Keyse, 1990).

Thomas (1996) described the binding of avidin to 8-oxodG within DNA which had been exposed to free radical generating systems and some evidence to support this has already been presented (Chapter 4). Therefore the binding of avidin to UVA irradiated keratinocytes was investigated along with the protective effects of vitamin E against oxidative stress-related damage.

AIM

Having established the formation and detection of cyclobutane pyrimidine dimers in naked DNA, the aim of this work was to further establish the formation of CPD in the DNA of cells irradiated with UVB[⊙] or UVA *in vitro*. Additionally, the use of avidin on cells irradiated with UVA was investigated, not only to further investigate potential applications for this probe, but also, if successful, to assess the induction of oxidative mechanisms.

METHOD

The RHT cells were cultured as described in Chapter 2.2 (Methods, page 77) onto chambered glass slides. Irradiations were of 0, 10, 20 and 30kJm⁻² UVA[⊙] (λ_{max} = 366nm) and 0, 0.135, 0.27 and 1.08kJm⁻² UVB[⊙] (λ_{max} = 302nm). The protective effects of the free radical scavenger, α -tocopherol, was tested by pre-loading the cells for 24 hours, prior to irradiation. Treatment of cells with 50 μ M hydrogen peroxide for one hour, known to induce oxidative damage acted as a positive control for the experiment. Methanol fixation of the cells preceded probing for damage. Binding of directly FITC-labelled avidin (final concentration 2 μ g/ml) and Ab529 (1/5000), localised by a FITC-labelled secondary antibody, was assessed qualitatively by microscopy.

RESULTS

Ab529

Figure 6.2.1 represents green, positive staining within the nuclei of human keratinocytes, post-irradiation with 1.08kJm⁻² UVB[⊙]. This clearly contrasts with the absence of binding to the unirradiated control (Figure 6.2.2). At x200 magnification this staining was more clearly observed, with some nuclei showing an intense (yellow) colour, indicating high levels of antibody binding (Figure 6.2.3). Again this contrasts with the poor binding to the unirradiated cells (Figure 6.2.4). Observation of the irradiated cells at x400 magnification

[⊙] For conversions see Appendix IIa

confirmed strict nuclear localisation of the staining (Figure 6.2.5), although some perinuclear fluorescence was apparent in the unirradiated cells at this magnification (Figure 6.2.6). An apparent high background fluorescence was seen in some of the slides and is believed to be due to poor washings, which were undertaken by hand and could vary from slide to slide. It was apparent as high background and did not affect the pattern of staining in either the positive or negative slides. Binding to irradiated cells was most apparent at the dose of 1.08kJm^{-2} and a discernible dose-effect was not clear, illustrating the difficulty of making quantitative determinations based on cellular fluorescence. No discernible binding was seen to cells irradiated with UVA (results identical to Figure 6.2.2).

Avidin

Figure 6.2.8 represents the binding of the avidin-FITC conjugate to keratinocytes irradiated with 30kJm^{-2} UVA and shows very intense staining, contrasting with Figure 6.2.9 which illustrates the poor binding of avidin to unirradiated cells. Some faint nuclear localisation was, however, seen in the unirradiated cells, in contrast to the perinuclear staining in the unirradiated cells probed with Ab529, which was only apparent at x400 magnification (see Figure 6.2.6). At x200 magnification the nuclear localisation was clearly apparent, as were the areas of high background described previously (Figure 6.2.10). This poor quality of background was more evident in Figure 6.2.11, the unirradiated control at x200 magnification and some binding of avidin may have occurred, although no binding comparable to that seen in Figure 6.2.10 was seen. The effect of hydrogen peroxide on keratinocytes was examined as model system for oxidative stress induction. Figure 6.2.11 illustrates the binding of avidin to hydrogen peroxide 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 hydrogen peroxide after a 24 hour pre-incubation with α -tocopherol (Figure 6.2.12), suggesting the staining due to avidin was caused by ROS. Some non-nuclear, non-specific staining was apparent, although this did not detract from the clear absence of nuclear staining, contrasting with Figure 6.2.11.

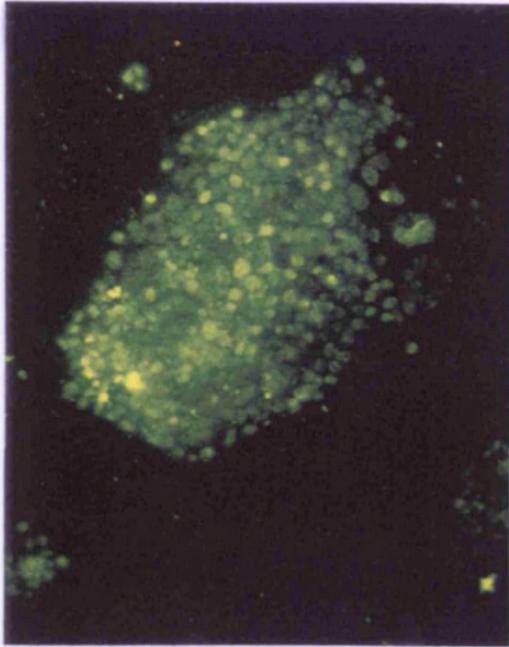


Figure 6.2.1. RHT keratinocytes (x100 magnification) irradiated with 1.08kJm^{-2} , using the UVB source described in Chapter Two and probed with Ab529 utilising a FITC labelled secondary. The green fluorescence indicates Ab529 binding to thymine CPD.



Figure 6.2.2. Unirradiated RHT keratinocytes (x100 magnification) probed with Ab529 utilising a FITC labelled secondary. No staining with Ab529 is apparent.

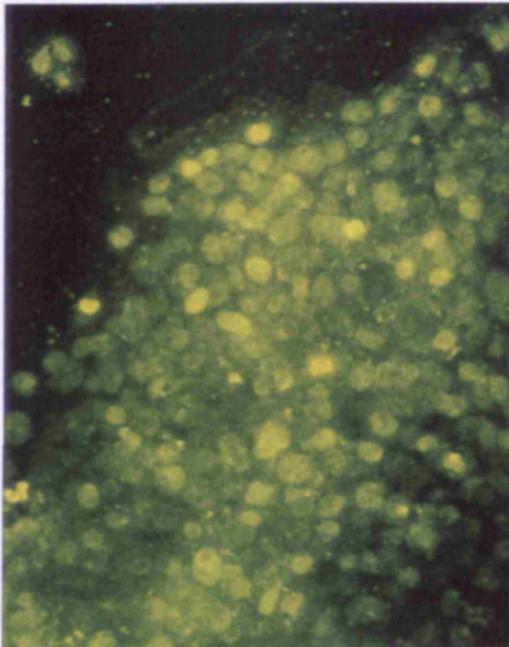


Figure 6.2.3. RHT keratinocytes (x200 magnification) irradiated with 1.08kJm^{-2} UVB and probed with Ab529 utilising a FITC labelled secondary. The green fluorescence indicates Ab529 binding to thymine CPD, although there is a clear variation in staining intensity.

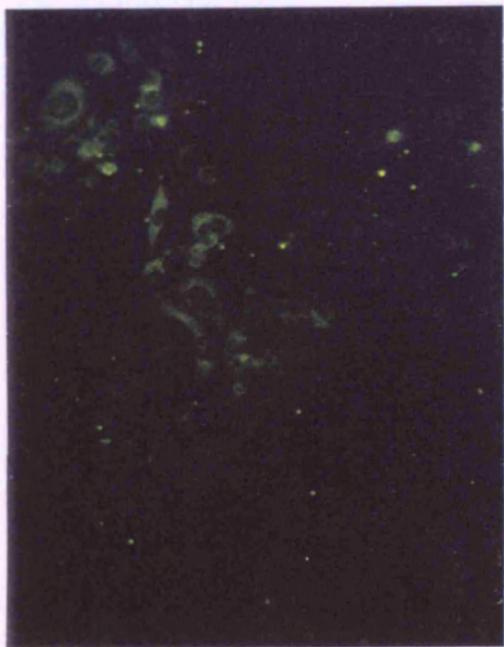


Figure 6.2.4. Unirradiated RHT keratinocytes (x200 magnification) probed with Ab529 utilising a FITC labelled secondary. No nuclear staining with Ab529 is apparent compared to Figure 6.2.3.

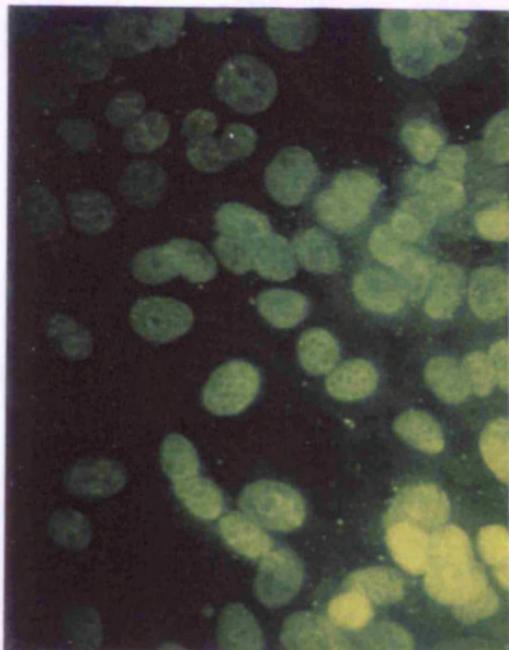


Figure 6.2.5. RHT keratinocytes (x400 magnification) irradiated with 1.08kJm^{-2} UVB and probed with Ab529 utilising a FITC labelled secondary. Strict nuclear localisation is achieved and some of the more intensely staining nuclei appear bright yellow.

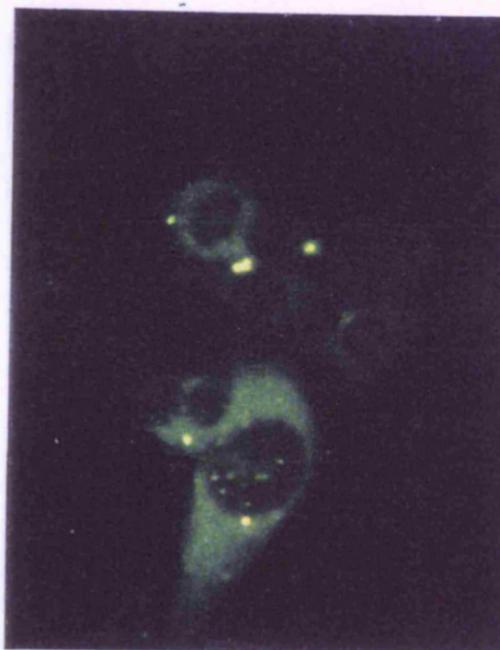


Figure 6.2.6. Unirradiated RHT keratinocytes (x400 magnification) probed with Ab529 utilising a FITC labelled secondary. Some perinuclear staining is apparent.

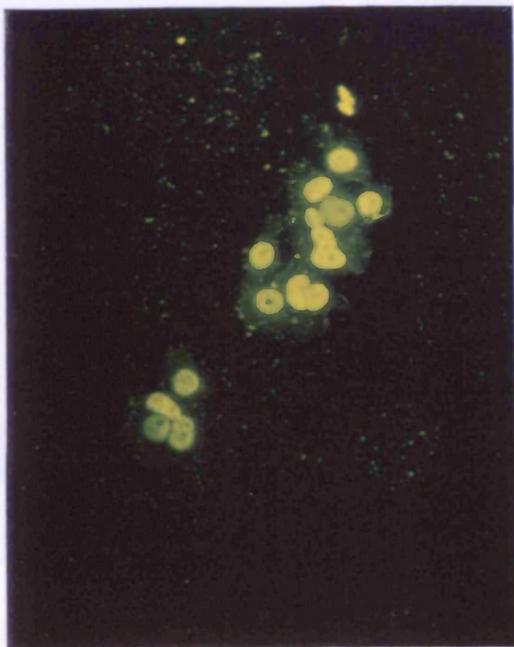


Figure 6.2.7. RHT keratinocytes (x200 magnification) irradiated with 30kJm^{-2} UVA and probed with directly FITC-labelled avidin. Strong nuclear staining is achieved.

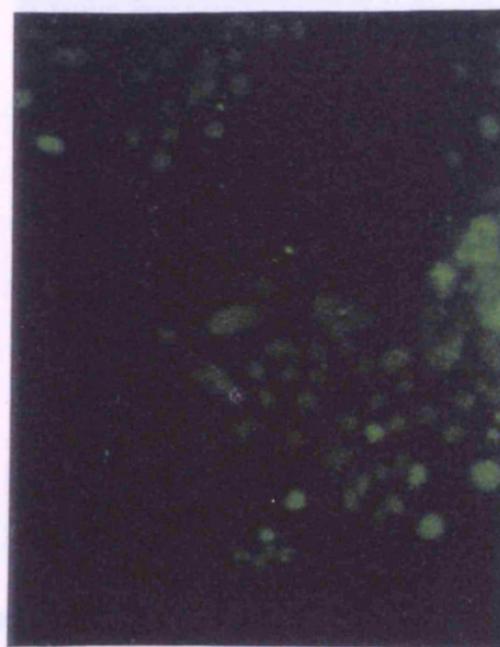


Figure 6.2.8. Unirradiated RHT keratinocytes (x100 magnification) probed with directly FITC-labelled avidin. There appears to be some faint nuclear staining.

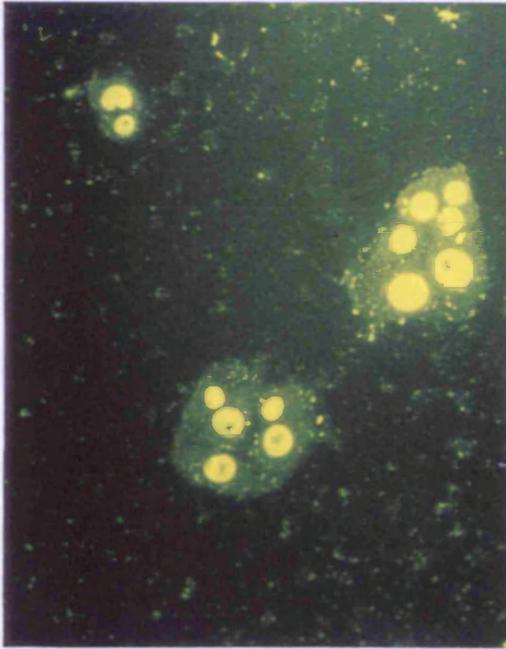


Figure 6.2.9. RHT keratinocytes (x200 magnification) irradiated with 30kJm^{-2} UVA and probed with directly FITC-labelled avidin. Strong nuclear staining is achieved, although a high background is evident.

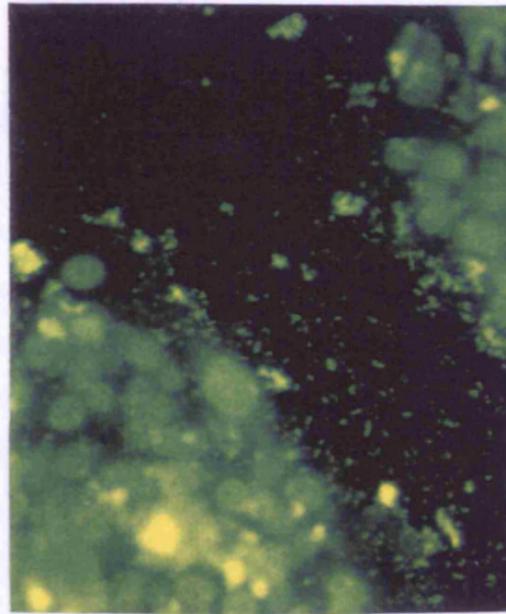


Figure 6.2.10. Unirradiated RHT keratinocytes (x200 magnification) probed with directly FITC-labelled avidin. There appears to be some faint nuclear staining, despite a high and non-specific background.

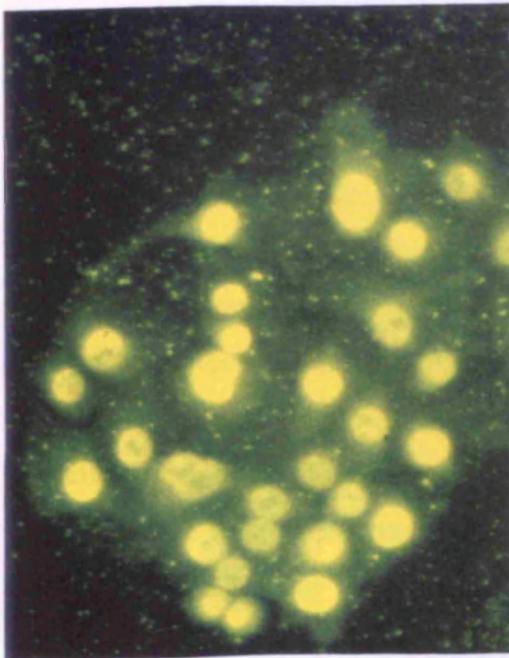


Figure 6.2.11. RHT keratinocytes (x200 magnification) treated with $50\mu\text{M}$ hydrogen peroxide for one hour and probed with directly FITC-labelled avidin. Strong nuclear staining is achieved, although a high background is again evident.

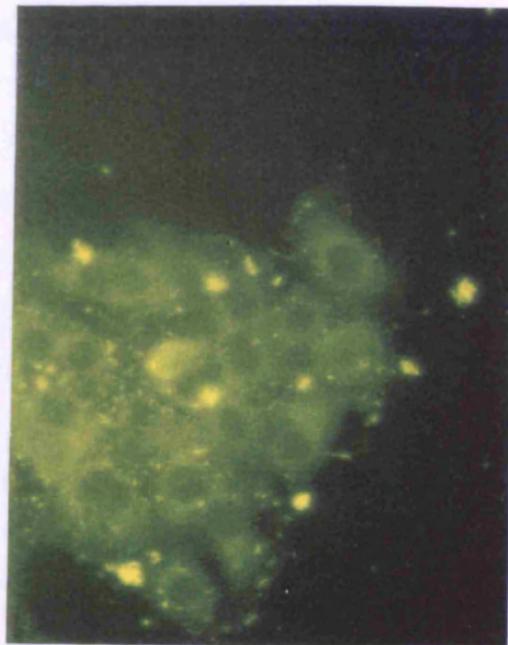


Figure 6.2.12. RHT keratinocytes (x200 magnification) treated with $50\mu\text{M}$ hydrogen peroxide for one hour following a 24 hour pre-incubation with $200\mu\text{M}$ α -tocopherol. The cells were then probed with directly FITC-labelled avidin. No nuclear staining is evident.

DISCUSSION

This study was to further elucidate the applicability of experimental DNA damage probes to the detection of thymine CPD in UVB^{*} treated cells and oxidative damage in cells treated with UVA or hydrogen peroxide. No data relating to dose/response or recovery from DNA damage was included in the results as it was impossible to reliably and objectively quantitate increases or decreases in fluorescence by eye. This emphasised the need for a method whereby increases in binding to damaged DNA may be quantitatively assessed. Induction of thymine CPD in UVB^{*} irradiated human keratinocytes was unequivocally shown to occur at a dose of 1.08kJm^{-2} UVB^{*}, using Ab529. 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 the human cultured cell type for the immunochemical demonstration of thymine CPD *in vivo* (Seaman *et al.*, 1972; Roza *et al.*, 1988; Mori *et al.*, 1991; Mizuno *et al.*, 1991), perhaps due, until recently, to the absence of a commercially available keratinocyte cell line. The demonstration here of pyrimidine dimer induction, in a keratinocyte cell line, using a unique sequence-specific antiserum is therefore novel. No discernible binding, post UVA irradiation, was seen with anti-thymine dimer antiserum (Ab529) despite its demonstrated sensitivity and a limit of detection of $<0.9\text{fmol}$ (Chapter 5). However, this 0.9fmol detection was achieved in 1220pg DNA, perhaps there is insufficient thymine in one cell to produce a detectable amount of dimer? Assuming there to be approximately 10pg DNA per cell and therefore approximately 2.5pg thymine per cell, 0.9pmol dimer is equivalent to 0.432pg dimer. Clearly there would, potentially, be enough thymine, if a sufficient dose of UV were to be given. Future work may be to utilise greater doses of UVA and investigate dimer formation, both by antiserum binding in cells and by GC-MS analysis of the extracted DNA from irradiated cells. It is noteworthy that some of the sensitivity of the antiserum must be due to the ELISA assay format and its signal amplification step. The previous reports of pyrimidine dimer detection following UVA irradiation (λ_{max} 365nm) of cells have utilised radioactivity (Tyrrell, 1973) or the specific enzyme-based assay of Sutherland and Shih (1983) to achieve detection. The apparent lack of binding seen here may be due largely to the low doses of UVA used. The sensitivity of the technique may also need to be addressed, by the utilisation of a different technique, such as ELISA or flow cytometry, which would, in addition, allow quantitative assessment.

Also shown here is the novel application of avidin to the demonstration of oxidative damage in keratinocytes following UVA irradiation or treatment with hydrogen peroxide. The differentiated neuronal cell line, IMR32, has previously been used to show the binding of avidin following UVA irradiation (Thomas *et al.*, 1994), although this is clearly not a 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 UVA or hydrogen peroxide, in keratinocytes supports the evidence for a radical-mediated mechanism of DNA damage. Further discussion of the use of avidin to detect UV-induced DNA lesions is in Chapter Eight.

Having established the occurrence of DNA damaging events subsequent to UV irradiation, it would be pertinent to examine quantitatively, the changes in binding with dose.

6.3 QUANTITATIVE ELISA DETECTION OF DNA DAMAGE FROM UV-IRRADIATED KERATINOCYTES

INTRODUCTION

Having qualitatively established that oxidative DNA damage is formed in keratinocytes irradiated with UVA, it was crucial to quantitate this damage with increasing dose, again by immunochemical means. It has been reported that UVA irradiation of keratinocytes may also induce oxidative DNA damage, such as 8-oxoG, as demonstrated by HPLC-ECD (Beehler *et al.*, 1992; Maccubbin *et al.*, 1995) and as a consequence alterations in the levels of ROS damaged DNA were investigated using the anti-ROS DNA antiserum described in Chapter 4. Avidin was used to confirm the results from Chapter Four that suggested this probe to be unsuitable for ELISA-format assays, whereas previous cellular work was more convincing (Thomas, 1996).

As discussed in section 6.2, the dose-effects of UVA radiation on keratinocytes were not discernible, although on the basis of the avidin results, oxidative DNA changes were occurring. Furthermore, the induction of thymine CPD by UVA could not be established. It was therefore necessary to examine the relative contributions of oxidative DNA damage and thymine CPD to DNA damage induced in keratinocytes by UVA and quantitate these changes relative to dose in an ELISA format.

Mori *et al.* (1991) irradiated human fibroblasts with what were described as “physiological” doses of 254nm UV prior to DNA extraction and damage quantitation by ELISA. As 254nm UV does not reach the earth, these doses cannot be described as physiological; however, the experiment did illustrate that the method worked and that DNA damage could be easily quantitated by ELISA. Peroxidase labelling in conjunction with a streptavidin/biotin amplification step gave sufficient sensitivity to detect CPD and (6-4)PP in DNA irradiated with doses as low as 5 Jm⁻². This is clearly an improvement in the report of detection of damage, in human cells, following doses of 20 Jm⁻² using a ³H-labelled antibody to (6-4)PP (Mori *et al.*, 1988). ELISA-based assays have been utilised for both lesion induction and repair studies (Hiramoto *et al.*, 1989), illustrating their versatility. Although *in situ* analysis may be preferable, the equipment required, such as laser cytometry (Mori *et al.*, 1990) or flow cytometry, would have been more costly and the technique more technically demanding. The advantage of using ELISAs is the low amounts of DNA

required. Wani *et al.* (1984) used an ELISA method to characterise their polyclonal antiserum to CPD, reporting it to recognise lesions in as little as 10ng DNA, following 5.0 Jm^{-2} 254nm UV, although for cellular work this group used flow cytometry, thereby avoiding the DNA extraction step.

ELISA methods seem eminently suitable for the detection and quantitation of oxidative damage in the extracted DNA of UV irradiated cells. This has, as far as can be determined, been limited only to cells irradiated with 254nm UVC not biologically relevant UVA.

AIM

To quantitate the induction of ROS damage to DNA in keratinocytes irradiated with UVA, using immunochemical probes in ELISA.

METHOD

Fully methodological details are contained in Chapter 2, Materials and Methods, Section 2.7.3, page 78. Briefly, keratinocytes were cultured in tissue culture flasks and then seeded into Petri dishes, whereupon, they were irradiated with biologically relevant doses of UVA. DNA from these cells was extracted using a Pronase E method and quantitated by A_{260} prior to damage quantitation by ELISA. The antisera used for damage quantitation were Ab529, AbX18 (UVC/H₂O₂) and the anti-ROS antiserum. Additionally for the examination of UVA-induced oxidative DNA damage, a commercial anti-8-oxodG monoclonal antibody and peroxidase-labelled avidin were also utilised.

RESULTS

UVA-irradiated keratinocytes

The ELISA binding of five probes to DNA damage was examined in the DNA of UVA-irradiated keratinocytes. Levels of binding for Ab532, the antiserum to ROS-induced DNA damage, increased markedly over the unirradiated control, in a clear dose-responsive manner (Figure 6.3.1). A maximal absorbance of 0.217 absorbance units was seen, following a dose of 144 kJm^{-2} (which is approximately $\frac{3}{4}$ 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 kJm^{-2} (less than $\frac{1}{3}$ MED for type II skin) with this antiserum. A similar pattern of binding was seen with antiserum X18, characterised as recognising thymine-thymine CPD, suggesting these lesions to be formed.

However, no increase was seen with Ab529, the more sensitive of the antisera recognising CPD, irrespective of dose. Increases were not seen either with the commercial anti-8-oxodG antibody or avidin. However, when the extracted DNA was denatured and probed with the anti-8-oxodG antibody, marked increases were seen (Figure 6.3.1).

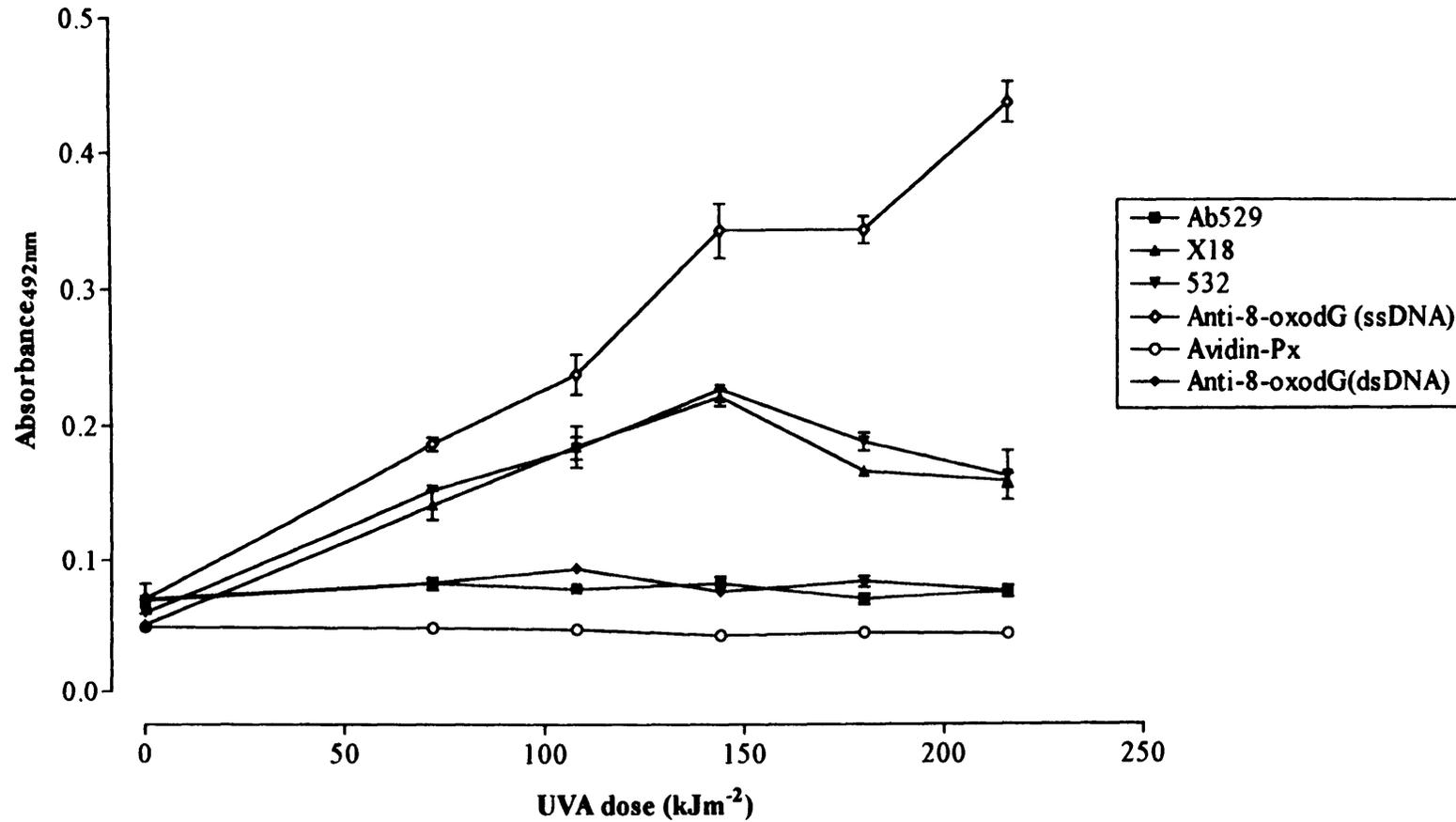


Figure 6.3.1. ELISA binding of antisera to double-stranded DNA extracted from UVA irradiated keratinocytes, with the exception of the anti-8-oxodG monoclonal antibody which is to DNA that has been rendered single-stranded. Values represent the mean and error bars the range, of two determinations.

DISCUSSION

The usefulness of antibodies in the detection of DNA damage has been discussed in Chapter One (Introduction). Demonstrated here is the applicability of antibodies to DNA damage in an assay, which is more cost-effective than 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 become sufficiently specific to be used for analysis. ELISA-based techniques require small amounts of DNA, for example, Wani *et al.* (1984) detected damage in DNA samples as small as 10ng, although higher levels allowed for greater sensitivity. DNA levels of 2.5µg were used in the assay reported here which, although high compared to Wani *et al.* (1984), are much lower than those required by GC-MS (100µg DNA per sample - Podmore *et al.*, 1996) or HPLC (100µg DNA per sample - Love and Friedberg, 1982). A development from ELISA assays was immunoslot blot (ISB), used by Wani *et al.* (1987) to study repair in irradiated human fibroblasts. Hori *et al.* (1992) also utilised immunoblotting to qualitatively demonstrate unrepaired CPD in actinic keratosis.

The results presented in Figure 6.3.1 suggest oxidative damage to DNA to predominate over the induction of CPD, in cells irradiated with UVA. This conclusion is reached despite the apparent increase in binding of antiserum X18. It is believed that, as discussed in Chapter 5, although the majority of this antiserum's response is to thymine CPD, there exists a component which recognises UV-modified guanines. On the basis of the findings of Doetsch *et al.* (1995), these modifications are speculated here to be 8-oxodG. This is supported, in part, by the finding that levels of 8-oxodG, as determined by HPLC-ECD, do increase in UVA-irradiated keratinocytes (Finnegan, 1996). This result suggests that affinity purification of this antiserum with UV-irradiated poly-dG would be useful. This component of the antiserum is unlikely to have made a major contribution to binding in previous experiments as levels of thymine CPD were always likely to be such that detection of these lesions predominated. It would appear unlikely that X18 is recognising thymine CPD in the DNA of this experiment as no recognition by Ab529 was seen. This polyclonal antiserum, deemed as the more sensitive (0.09pmol, Chapter 5) was used at 1/2000 in this assay to increase the likelihood of detecting CPD in any of the sequence contexts previously described (Herbert *et al.*, 1994). Use of Ab529 at 1/5000 with the UVB irradiated DNA was to maintain its stringency for thymine CPD with either a 5' or 3' pyrimidine. Thus it would have been expected for Ab529 to have detected any CPD present. An appreciable

increase in binding, to single-stranded DNA from UVA irradiated cells, was seen with the commercial anti-8-oxodG antibody. This would indicate, in conjunction with the results obtained by antiserum 532, that UVA irradiation of cells gives rise to immunochemically detectable levels of oxidative DNA products. No increases in binding were seen with either the anti-8-oxodG antibody or avidin when the DNA is double-stranded. It is suggested that this is due to assay format, particularly with avidin which has previously produced anomalous results when used in a ELISA format assay. Taken together, these results would suggest that whilst no detectable increases in CPD were seen, ROS-induced damage, and possibly, specifically 8-oxodG was formed following UVA irradiation at biologically relevant doses. At this point a question mark remains as to the ability of lesion-specific antisera to detect thymine CPD induced in the DNA of UVA-irradiated cells, perhaps a yet more sensitive technique is required?

Although the results demonstrated here do not provide values to the number of lesions present in DNA, the potential exists for such an ELISA to be developed. To this end, Roza *et al.* (1988) developed a competitive ELISA, utilising a reference DNA sample containing a known concentration of dimer, determined by HPLC. A similar approach for future work is intended here. Utilising the GC-MS assay (described in Chapter 5), DNA standards, containing a known amount of dimer, would be developed and a calibration curve for Ab529 established. Thus for a given absorbance from antiserum binding to sample DNA, containing an unknown amount of dimer, the number of dimers contained therein can be obtained from the calibration curve. DNA standards for antibody binding represent better standards than the isolated antigen as conformational influences are also accounted for. The GC-MS assay is a very sensitive technique for absolute quantitation of the specific lesion making it the method of choice for assessing the DNA standard. Comparison with the HPLC method of Roza *et al.* (1988) was not possible as experimental details were not given. Nevertheless, the data presented here is the first report of the simultaneous immunochemical detection of both dimer and ROS changes in the DNA of keratinocytes following biologically relevant doses of UVB.

Clearly, immunochemical detection techniques, whether they be ELISA or slot blot, represent economical and sensitive methods of analysis. Furthermore calibration of damage standards by a "benchmark" technique, such as GC-MS, will allow fully quantitative assessments of damage to be made. However, such techniques still require the extraction of

DNA from irradiated cells and whilst the problem of artefactual damage by sample handling may not be an issue for dimer damage, it is for oxidative lesions (Claycamp, 1992; Finnegan *et al.*, 1996). Furthermore, one of the advantages of utilising immunochemical techniques is the ability to detect damage *in situ*. One approach to addressing this point is flow cytometry.

6.4 EXAMINATION OF UVA-INDUCED DNA DAMAGE IN KERATINOCYTES BY FLOW CYTOMETRY

INTRODUCTION

Potentially important targets for damage from UV radiation are proto-oncogenes and tumour suppressor genes (Tornaletti *et al.*, 1993). The tumour suppressor gene, p53, via post-translational mechanisms, is a vital regulator of a G₁ cell cycle checkpoint (see Figure 6.4.0) and of apoptosis following DNA damage (Campbell *et al.* 1993), although the relationship between damage and elevation of p53 protein levels is unclear (Lane, 1992; Nelson and Kastan 1994). The term “cell cycle checkpoint” refers to an undefined point within a stage of the cell cycle at which progress may be halted.

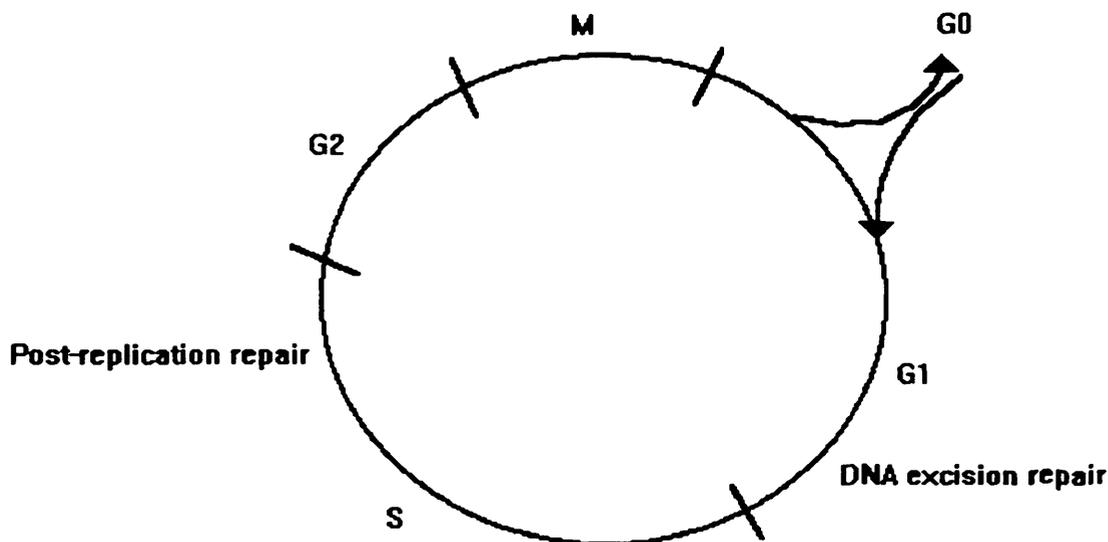


Figure 6.4.0. Phases in the cell cycle of a eukaryotic cell: M (mitosis); G₁ (gap 1, prior to DNA synthesis); S (period of DNA synthesis); and G₂ (gap 2, between DNA synthesis and mitosis). G₀ is the resting or quiescent state. Dividing lines segregate one stage of the cycle from the subsequent. (Adapted from Stryer, 1988.)

Impairment of the p53 system can result in serious consequences for the cell. Inactivation, either by mutation or functionally, of this gene contributes to a wide variety of human cancers (~50% according to Ozturk, 1994) and is of particular interest in cutaneous disorders (McNutt *et al.* 1994). Nelson and Kastan, (1994) indicated that p53 induction occurs after DNA strand breaks which can arise for a variety of reasons, base damage -

repair/replication, ionising radiation, radical species, DNA rearrangements and recombination events.

SCC and melanocarcinoma have been found to be associated with mutations in the p53 and the N-ras gene respectively (Brash *et al.* 1991; van't Veer *et al.* 1989). The mutations frequently show CC→TT double base changes and a highly frequent C→T substitution at dipyrimidine sites, the latter of which is considered to be UV specific (Brash *et al.* 1991). UV-induced lesions may lead to mutations during DNA repair, these mutations are believed to be due to translesion synthesis, failure of repair (Dumaz *et al.*, 1993) or, perhaps more importantly, slow repair (Tornaletti and Pfeiffer, 1994). This repair has been shown not to be at uniform rates throughout the genome (Lan and Smerdon, 1985) and is even strand specific (Mullenders *et al.*, 1993). Repair is clearly an important factor in the development of skin cancer for individuals with the hereditary autosomal recessive disease, xeroderma pigmentosum, who have deficient pyrimidine dimer repair and develop tumours in early life (van Praag *et al.* 1993; Runger *et al.* 1995). However, the importance of the events that follow the initial damage and end with tumour formation are not yet fully understood.

A proposed biomarker of UV exposure is cyclobutane pyrimidine dimer formation and the development of antisera specific for thymine-containing CPD has been described here (Chapter 5) and demonstrated in UVB-irradiated, cultured cells (Chapter 6.1). Whilst the induction of these lesions in naked DNA irradiated either with UVC or UVB has also been demonstrated, irradiation with UVA failed to induce changes recognised by the antisera. However, the literature contains reports of the appearance of such lesions in cells irradiated with UVA (Freeman *et al.*, 1987), although a tendency for the formation of more cytosine- rather than thymine-containing dimers was also reported (Ellison and Childs, 1981). In order to demonstrate whether thymine-thymine CPD were induced in the DNA of cells irradiated with UVA it was necessary to develop a sensitive assay. Previous work has shown flow cytometry to possess potential sensitivity, in addition to allowing multiple parameters, such as stage of cell cycle and binding of other antibodies, to be simultaneously assessed (Berg *et al.*, 1993).

AIM

The aim of the present work was to develop a flow cytometry-based assay whereby the induction of thymine-thymine cyclobutane dimers in UVA irradiated cells may be established. Additionally, the induction of DNA damage as indicated by p53 expression was examined in conjunction with the phase of cell cycle.

FLOW CYTOMETRIC ANALYSIS

Post-irradiation examination of thymine dimer induction, along with p53 protein expression and in conjunction with cell cycle stage had not been previously examined by FACS analysis, either within the literature or the Division of Chemical Pathology. For this reason, the final assay used required much development (full description of which is contained in Chapter 2.7.4).

Synchronisation of keratinocyte cell cycle

A synchronous population of cultured human RHT keratinocytes was established by serum deprivation, having been optimised and deemed more appropriate and efficient than incubation with hydroxyurea (Finnegan, 1996) a DNA synthesis inhibitor (Collins *et al.*, 1980). This was achieved by cell culture in medium lacking foetal calf serum and mitogens essential for normal culturing. Following serum deprivation for a period of 48 hours, the stage of the cell cycle was determined by FACScan analysis, following harvesting, fixation and staining of the cells with propidium iodide. It was subsequently shown that 48 hour serum deprivation lead to a reduction in the mean number of cells in s-phase to below 3%, compared with a control of 19%.

Optimisation of antibody concentrations for FACS analysis

Herbert *et al.* (1994) stated that the majority of antibody response to UV modified DNA was of the IgG class of immunoglobulin. Therefore, to limit non-specific binding due to an excess of immunoglobulin in the polyclonal antiserum, only the IgG portion of Ab529, prepared by Mark Cappell (Division of Chemical Pathology, University of Leicester), was to be used in the flow cytometric experiments.

Optimisation of antibody dilution was performed using the flow cytometer prior to experimentation, the appropriate controls included cells with/without antibody and cells with/without UV irradiation. The dilution used for the anti-p53 protein monoclonal

antibody was that recommended by the manufacturers (DAKO) for immunocytochemistry (1/100). The dilutions of the secondary antibodies were also as recommended by the manufacturers, 1/80 for the goat anti-rabbit (FITC labelled) and 1/160 for the goat anti-mouse IgG, Fc specific (again FITC labelled) immunoglobulins. As described in Chapter 2.7.4, because both secondary antibodies were FITC-labelled, quantitation of Ab529 and anti-p53 protein binding had to be carried out in separate samples, doubling the size of the experiment. The dilution of Ab529, however, was based on the recommendation by Carter and Meyer (1988) that the concentration of a monoclonal antibody for flow cytometry should be $1.0\mu\text{g protein}/10^6$ cells. Staining was achieved in irradiated cells and absent in unirradiated cells, therefore this concentration was deemed to be suitable for Ab529.

METHODS

Full details are contained within Chapter 2.7.4, page 79. Briefly, a synchronous population of human keratinocytes (RHT cells) were UVA irradiated ($\lambda_{\text{max}} = 366\text{nm}$) at either 0, 1, 2, 3, or 24 hours following reintroduction of complete medium and then probed with either antiserum 529 or an anti-p53 protein antibody and counterstained with propidium iodide. Quantitation of immunoglobulin binding and stage of the cell cycle was performed by a Becton Dickinson FACScan flow cytometer. The harvesting procedure of these adherent cells was lengthy and time consuming, therefore all time points were corrected by 50 minutes.

| Overview of experimental time course. | |
|--|--|
| Time | Procedure |
| - 48 hours | serum deprivation begins. |
| 0 hours | cells now synchronised, complete medium reintroduced and first sample of cells irradiated, then assayed. |
| +1 hour | second sample of cells irradiated, then assayed |
| + 2 hours | third sample of cells irradiated, then assayed |
| +3 hours | fourth sample of cells irradiated, then assayed |
| + 24 hours | fifth sample of cells irradiated, then assayed |

RESULTS

Variation in antibody binding

Figures 6.4.1 and 6.4.2 represent the levels of antibody (both Ab529 and anti-p53) binding in synchronised RHT cells treated with $0.5\text{J}/\text{cm}^2$ UVA compared to time following medium reintroduction (which occurred at zero time). Antiserum binding was expressed as percentage fluorescence relative to unirradiated cells. Also shown are the number of cells in each stage of the cell cycle, expressed as a percentage of the total number of cells. The levels of binding were assessed at 0, 1, 2, 3 and 24 hours post-reintroduction of complete medium and irradiation.

Following irradiation with $0.5\text{J}/\text{cm}^2$ and at one and two hours post-reintroduction of complete medium, levels of Ab529 binding had decreased from the time zero value of 114% (SEM = 37) to 92% (SEM = 30) and 66% (SEM = 15) (Figure 6.4.1). The corresponding p53 values showed an increase from the time zero (73%, SEM = 26) to 87 (SEM = 12) and 80% (SEM = 12) (6.4.2). Values for both Ab529 and anti-p53 binding were maximal at the three hour time point (139%, SEM = 33 and 129, SEM = 9 respectively). However, none of these values proved to be statistically significant. Twenty four hours following irradiation with $0.5\text{J}/\text{cm}^2$ UVA, binding of Ab529 and anti-p53 had reduced to 61% (SEM = 33) and 91% (SEM = 14).

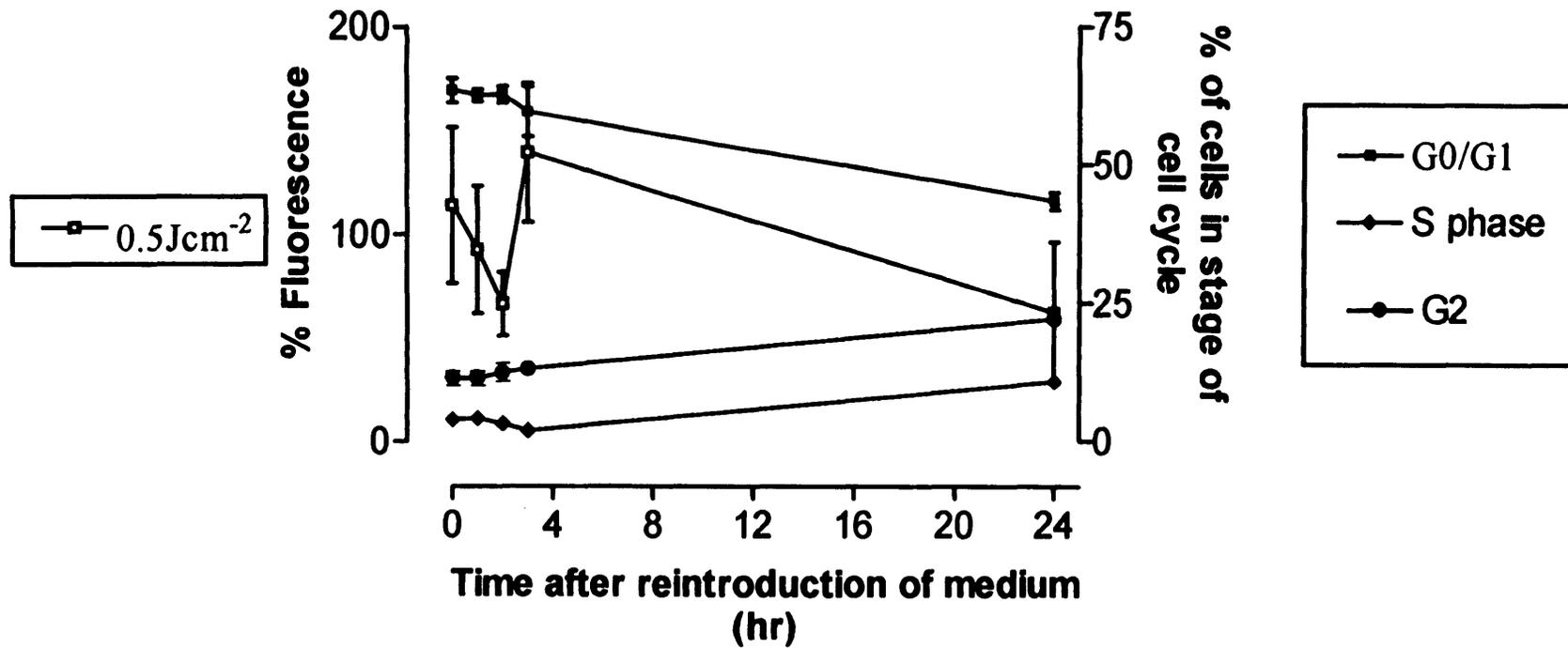


Figure 6.4.1. Percentage Ab529 binding in synchronised RHT cells treated with 0.5Jcm⁻² UVA after reintroduction of complete medium (each point represents the mean value of n=5 experiments with SEM bars). These values were normalised for the fluorescence of untreated cells at each sample time point.

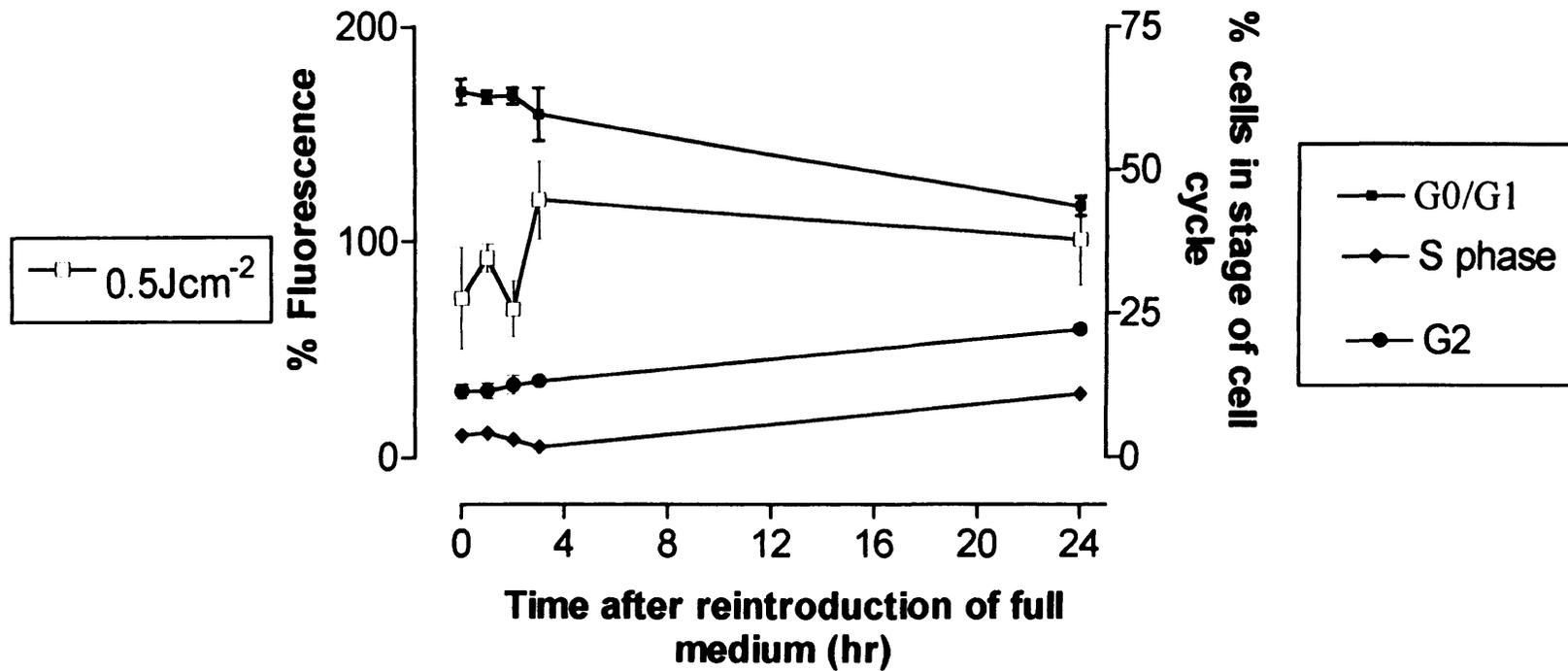


Figure 6.4.2. Percentage antibody binding to p53 protein in synchronised RHT cells treated with 0.5Jcm⁻² UVA after reintroduction of complete medium (each point represents the mean value of n=4 experiments with SEM bars). These values were normalised for the fluorescence of untreated cells at each sample time point.

Figures 6.4.3 and 6.4.4 represent the levels of Ab529 and anti-p53 protein binding in keratinocytes irradiated with 1Jcm^{-2} following reintroduction of complete medium. On irradiation and at one and two hours post-reintroduction, Ab529 binding had shown a small decrease from a time zero value of 105% (SEM = 74) to 96% (SEM = 29) and 77% (SEM = 17). In contrast, antibody binding to p53 protein showed a small increase over the time zero value (74%, SEM = 23) at the one hour time point (92%, SEM = 6), before returning to 77% (SEM = 12) at two hours. As with 0.5Jcm^{-2} , maximal binding of both antibodies was again achieved at the three hour time point, giving 127% (SEM = 34) for Ab529 and 119% (SEM = 18) for the anti-p53 protein. Following irradiation with 1Jcm^{-2} and 24 hours post-reintroduction of complete medium, binding of Ab529 had decreased to 89% (SEM = 25) from a zero time point value of 105% (SEM = 10); p53 binding had remained raised at 100% (SEM = 20) compared to zero (74%, SEM = 23). However, overall none of the points representing antiserum binding were calculated to be significantly different compared to the control (zero hours) time point when analysed by two-way analysis of variance.

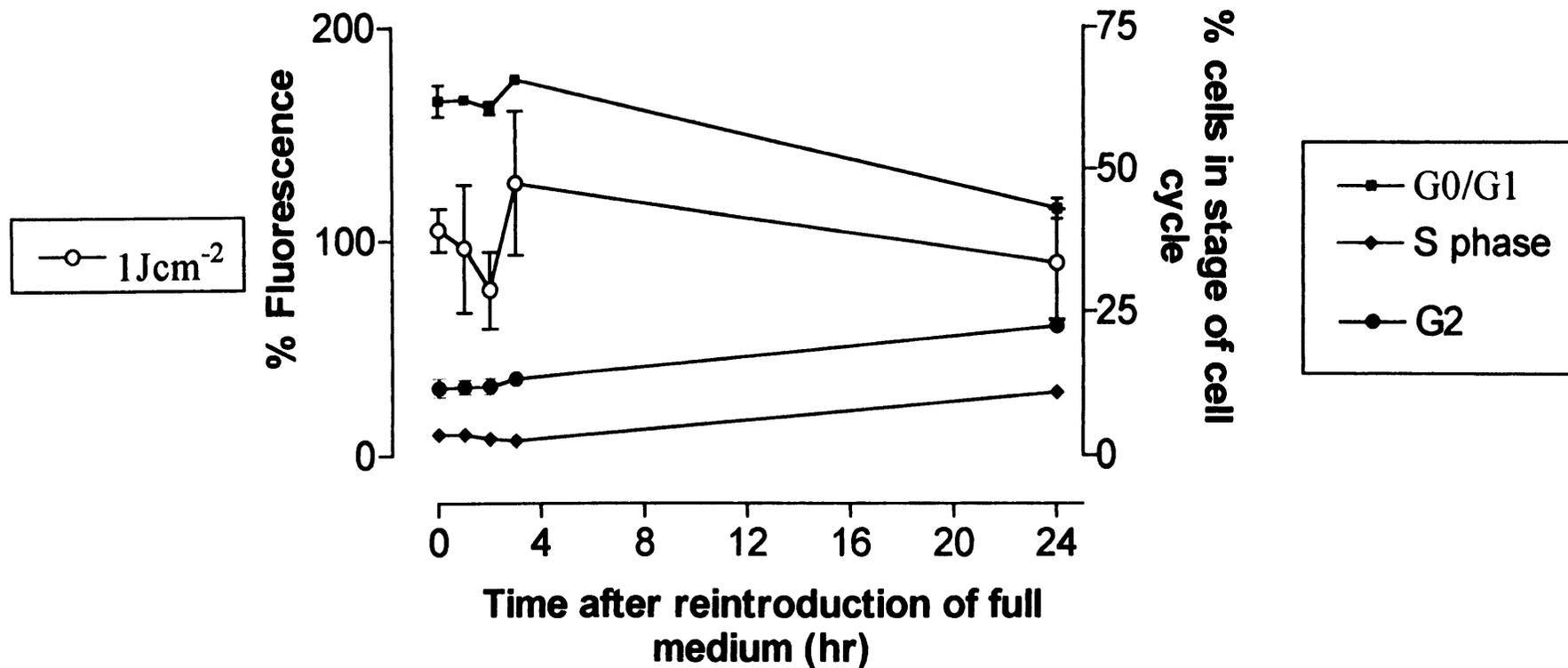


Figure 6.4.3. Percentage Ab529 binding in synchronised RHT cells treated with 1 Jcm⁻² UVA following reintroduction of complete medium (each point represents the mean value of n=3 experiments with SEM bars). These values were normalised for the fluorescence of untreated cells at each sample time point.

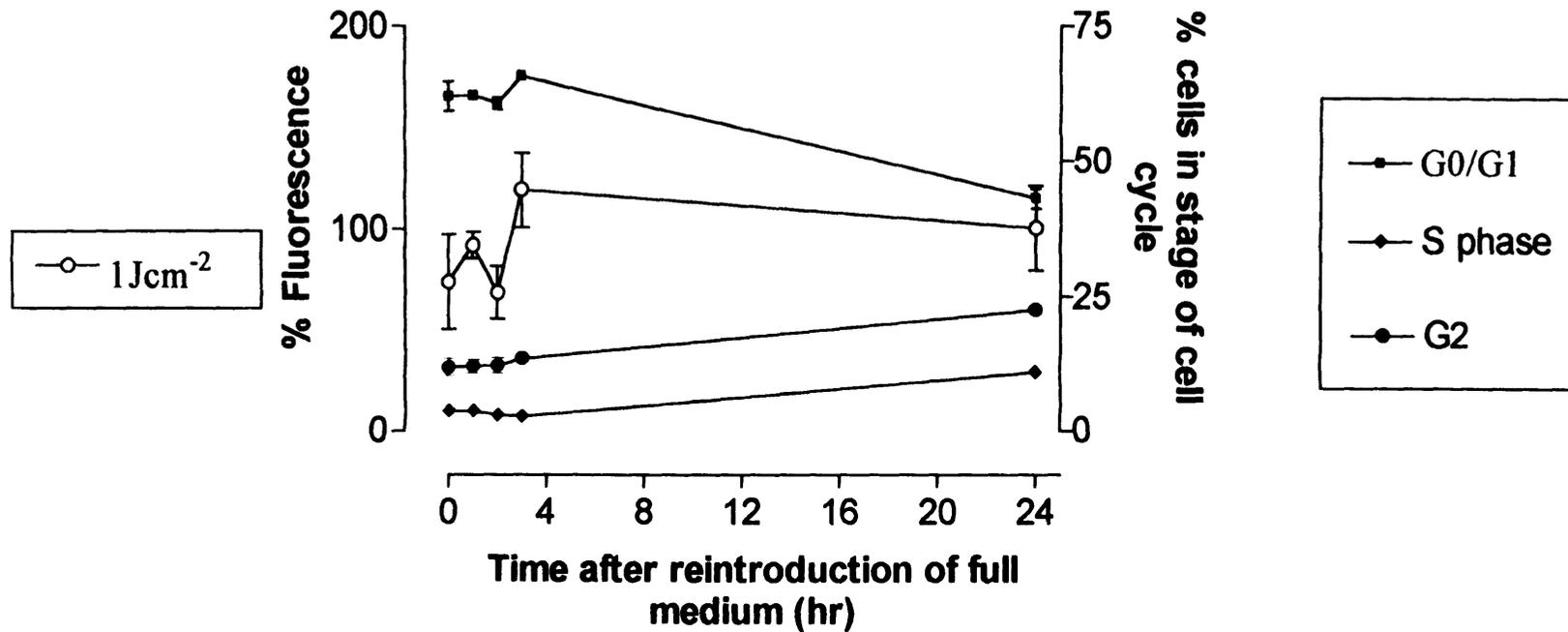


Figure 6.4.4. Percentage antibody binding to p53 protein in synchronised RHT cells treated with 1.0 Jcm^{-2} UVA after reintroduction of complete medium (each point represents the mean value of $n=3$ experiments with SEM bars). These values were normalised for the fluorescence of untreated cells at each sample time point.

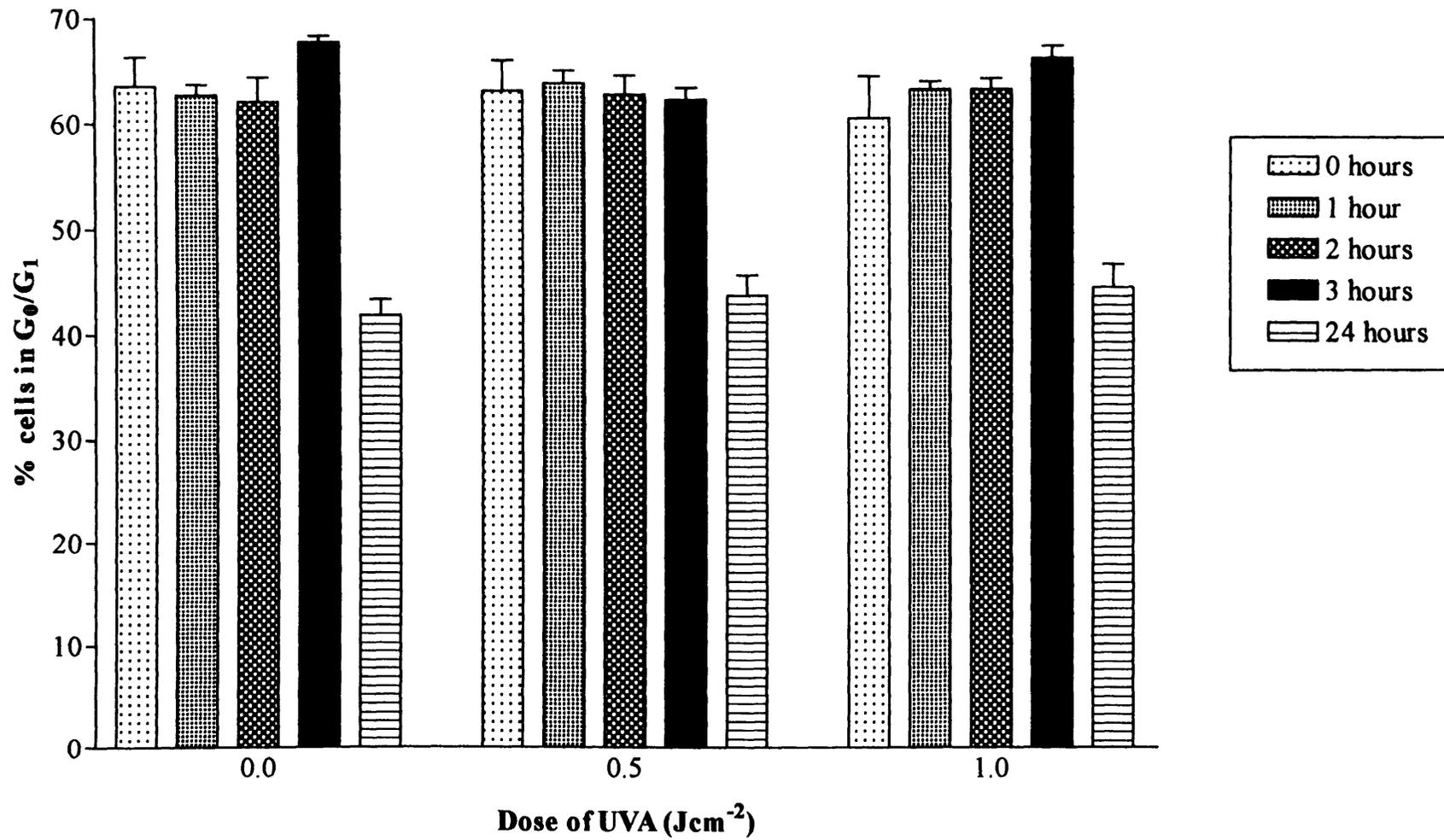


Figure 6.4.5. Effect of UVA irradiation on the percentage of cells in the G₀/G₁ phase of the cell cycle, 0, 1, 2, 3 and 24 hours post-reintroduction of complete medium. Values represent the mean (SEM) of 3 determinations.

Examination of percentage of cells in stage of cell cycle

The effect of UVA on the number of cells in the G_0/G_1 phase of the cell cycle was also studied (Figure 6.4.5) and shown to have no effect over all five time periods. However, the number of cells in G_0/G_1 did vary with time following reintroduction of complete medium (Figures 6.4.1 - 6.4.5). At time zero the percentage of cells in G_0/G_1 following 0, 0.5 or 1Jcm^{-2} was 63% (SEM = 2.8), 63% (SEM = 2.1) and 62% (SEM = 2.7) respectively. There was no significant difference between unirradiated and irradiated samples. A similar result was seen at one hour post-reintroduction of complete medium; 63% (SEM = 1), 63% (SEM = 1) and 62% (SEM = 0.7), for 0, 0.5 and 1Jcm^{-2} respectively. Again, the same result was observed at two hours for the doses 0, 0.5 and 1Jcm^{-2} ; 62% (SEM = 1.1), 61% (SEM = 1.4) and 61 (SEM = 1.1) for 0, 0.5 and 1Jcm^{-2} respectively.

However, the percentage of cells in G_0/G_1 three hours after reintroduction of complete medium at both the zero (68%, SEM = 0.6) and 1Jcm^{-2} (65%, SEM = 0.7) dose were significantly greater than at the zero ($p < 0.01$), 1 ($p < 0.01$) and 2 ($p < 0.01$) hour time points; as determined by a two-sample t-test. This is in contrast to the 0.5Jcm^{-2} dose, three hours post-reintroduction of complete medium, where the number of cells in G_0/G_1 was not significantly higher than at the corresponding 0, 1, or 2 hour time points (59%, SEM = 4.5). Moreover, the trend for 0.5Jcm^{-2} , appeared to be towards a decrease in cells in G_0/G_1 . The percentage of cells in the G_0/G_1 phase of the cell cycle 24 hours after reintroduction of complete medium was consistently between 40-45%, a significant reduction compared to the other time points (60-70%), irrespective of irradiation.

The peaks in binding for both Ab529 and anti-p53, at the three hour time points, noted in both Figure 6.4.3 and 6.4.4 coincided with a peak of cells in G_0/G_1 phase of the cell cycle when irradiated with 1Jcm^{-2} . This data could be taken to imply an increased sensitivity to UV in the cells in G_0/G_1 , three hours after reintroduction of complete medium and hence the increase in p53 protein.

In summary, whilst neither dose nor time affects binding of either antibody to a statistically significant extent, there would appear to be a trend, in all four experiments, for greater binding of both antibodies at the three hour time point which coincides with a significant increase in the number of cells in G_0/G_1 . There may exist, therefore, a relationship between stage of the cell cycle and sensitivity to irradiation.

DISCUSSION

Direct *in situ* immunofluorescent detection of UV-induced DNA damage has great advantages over methods where DNA has to be extracted e.g. HPLC or techniques utilising specific endonucleases. Due to its increasing biomedical applications (Creamer, 1992) and therefore prevalence of instruments, flow cytometry possesses advantages over immunofluorescent detection in tissue sections (Qin *et al.*, 1994) or cells in culture (Mori *et al.*, 1989) by not requiring specialised image analysis equipment and being able to analyse thousands of cells per sample.

Flow cytometric immunofluorescence assay has been applied to the quantitation of cyclobutane thymine dimers in UV irradiated: human skin fibroblasts (Berg *et al.*, 1993), extracorporeal blood mononuclear cells (Snopov *et al.*, 1995) and epidermal cells (Berg *et al.*, 1995). Berg *et al.* (1993 and 1995) also utilised the ability of flow cytometry to determine the stage of the cell cycle at irradiation. However, in all these studies the cells were irradiated either with UVB, or UVC, the effects of UVA alone were not examined. The purpose of this work was to establish Ab529 detection of UVA induced CPD, by flow cytometry. However, the results presented here appear equivocal due to a lack of statistical significance. The emphasis on formation by UVA may favour CC or CT rather than TT dimers, however, Tyrrell (1973) not only demonstrated TT dimers to be formed by 365nm UV, but furthermore, the ratio of TT to UT was five or six, compared to at 254nm, which gives 1.1 and 1.2. The action of formic acid, used in the DNA hydrolysis (Tyrrell, 1973), deaminates cytosine to uracil (Setlow and Carrier, 1966), although there will likely be a contribution from RNA and UT dimers formed therein. Ellison and Childs (1981) reported a window of 300-313nm UV where CT were induced at a higher rate than TT dimers

DNA absorbs UVA poorly (Sutherland and Griffin, 1981), suggesting mechanisms other than direct absorption account for the formation of CPD in cells irradiated with UVA. Peak *et al.* (1984) proposed that due to a poor absorption of UVA by DNA, a photosensitiser must be involved in the formation of dimers, although no mechanism was suggested. Presumably such a mechanism would involve some form of radical species/excited state. O'Neil *et al.* (1991) included a number of references (Norins *et al.*, 1962; Horio and Okamoto, 1987; Butler and Conway, 1953) in which, they claimed, contained descriptions

of free radical mechanism by which pyrimidine dimers form. However, examination of these references found no such explanation.

It may be possible that a mechanism similar to that whereby riboflavin sensitises the formation of 8-oxodG in DNA following irradiation with visible light (Kasai *et al.*, 1992), via a radical cation may exist. However, a radical intermediate in the formation of CPD has not been reported and this theory seems unlikely as both thymines must become radical anions and simultaneously form the cyclobutane ring. Moysan *et al.* (1991) reported the photosensitised formation of cyclobutane thymine dimers in DNA irradiated with UVA. The photosensitisers investigated were a group of psoralens known as pyridopsoralens, capable of inducing *cis-syn* cyclobutadithymines in significant amounts within DNA. In agreement with the data from Chapter 5, they found no dimerisation following the exposure of naked DNA to UVA alone. The mechanism proposed by Moysan *et al.* (1991) involves the transfer of energy from the photoexcited, long-lived, triplet state pyridopsoralen to ground state thymidine. The now triplet state thymine then reacts with an adjacent thymine in DNA. Furthermore this effect has been shown to occur in cells in association with the pyridopsoralens (Moysan *et al.*, 1991). Extension of this discussion could elucidate a mechanism by which the photosensitised formation of pyrimidine dimers in DNA may occur, namely by an endogenous pyridopsoralen analogue. Tyrrell (1973) saw a large induction of TT dimers compared to CT (UT post-hydrolysis), which could be explained by the higher energy triplet level of 2'-deoxycytidine than thymidine which impedes the energy transfer mechanism (Moysan *et al.*, 1991).

Not only was the induction of thymine CPD by UVA examined, but so was the effect of stage of the cell cycle. Collins *et al.* (1980) stated that mitotic and G₂ cells are less sensitive to UV-induced cytotoxicity than late G₁ and early S phase cells, suggesting that the effectiveness of UV to cause damage varies throughout the cell cycle, a theory supported, in part, by the data presented here. Further evidence that the amount of damage induced varies with stage of the cell cycle was provided by Berg *et al.* (1993), who noted a lower mean induction of dimers in the replicating G₂M cells than in nonreplicating G₀/G₁. This was hypothesised to be due either: (i) to the DNA of the G₂M cells being more condensed in compact mitotic chromosomes, or (ii) that the CPD detected by the antibody are not randomly distributed over the DNA but located on the surfaces of DNA clusters. Some evidence obtained by confocal microscopy adds support to theory (i), suggesting that CPD

detection by Ab529 is targeted predominantly towards the surface of an irradiated nucleus (Division of Chemical Pathology, unpublished results) although further work, such as electron microscopy would be of value.

The p53 protein has been shown to be responsible for transient G₁ arrest following UV irradiation, adopting a cell cycle “checkpoint” role (Lane, 1992; Enoch and Norbury, 1995), preventing damaged cells entering S phase and allowing more time for the DNA damage to be repaired (Inohara *et al.*, 1995) preventing possible mutagenesis. Molès *et al.* (1993) presented data showing that 48% of the basal cell carcinomas (BCC) tested presented a mutated p53 gene. Furthermore all were from sun exposed body sites, which implicated UV in the carcinogenesis.

There exists a relationship between UV-induced DNA damage and p53 induction (Nelson and Kastan, 1994), and the results presented here add support to this. Expression of the p53 protein has previously been shown to be induced in normal human keratinocytes following irradiation with UVA or UVB, but not in unirradiated cells (Hall *et al.*, 1993). Therefore, whilst not reaching statistical significance, the increase in p53 protein at the three hour time point may be concluded to be due to UVA irradiation, despite Hall *et al.* (1993) not seeing expression of p53 until over one hour post-irradiation. However, the possibility exists that the cells at G₀/G₁ are primed in some way for a p53 increase and this just coincides with the UV irradiation. A *caveat* must be made when comparing these two experiments as the RHT cell line used here was SV40 transformed, whereas the cells used by Hall *et al.* (1993) are essentially normal. The possible significance of this is unclear but viral transformation has been noted to alter cellular responses to genotoxic insult (Kaufmann and Kaufman, 1993). For example, infection of normal oral keratinocytes with human papillomavirus (HPV) results in the cells failing to accumulate p53 protein in their nuclei following UV irradiation (Gujuluva *et al.*, 1994).

Whilst they cannot be disregarded, the significance of the formation of pyrimidine dimers by UVA is unclear. Some groups have measured the induction of thymine dimers in the DNA of cells irradiated with UVA (Tyrrell, 1973; Freeman *et al.*, 1987) and whilst their contribution to cytotoxicity may be important (Tyrrell, 1973), their role in carcinogenicity does not appear to be so (Berg *et al.*, 1995a). It was shown that the levels of CPD are lower in cells irradiated with a combined dose of UVA and UVB than with, what was

described as, an equally carcinogenic dose of UVB alone (Berg *et al.*, 1995a). From this it was concluded that types of DNA damage, e.g. those that arise from oxidative stress (Tyrrell, 1991), other than thymine CPD were likely to contribute to UVA-induced skin carcinogenicity. Furthermore, other radical mediated processes occur following UVA irradiation of a cell, including lipid peroxidation, lipid and melanin radical generation and antioxidant depletion (Darr and Fridovich, 1994) all of which may influence the initiation or promotion of carcinogenesis. It is pertinent at this point to speculate that a number of damage lesions may act synergistically in carcinogenesis, although perhaps more importantly it is not the total number of lesions that are important but their location within the genome. This makes the development of methods which can assay for a number of lesions simultaneously, such as GC-MS; and gene-specific assays to localise damage, crucial to our understanding of the early events in carcinogenesis.

Although the post irradiation changes in immunoglobulin binding, for both Ab529 and anti-p53, are not statistically significant, the trend would suggest that antigenic sites, although possibly in low levels, are being produced. Lack of statistical significance arises from the variation in SEM for each data time point. These values are derived from three or five experiments and suggest that there exists some inter-experiment variation which may obscure a true picture. Previous experiments utilising flow cytometry in such a way only used duplicate experiments, minimising inter-experiment errors (Berg *et al.*, 1993). Nevertheless, closer studies at the period of 2-3 hours would benefit our understanding of the processes involved following UVA-induction of DNA damage.

The importance for the detection and localisation of dimers produced *in vivo* in the skin of individuals exposed to solar UV, which comprises both UVA and UVB, remains clear. Therefore, in the first instance, the *in vivo* induction of CPD by UVB was examined immunohistochemically with the novel antiserum Ab529.

6.5 CAN UV-INDUCED DNA DAMAGE BE DETECTED IN FIXED TISSUE SECTIONS BY IMMUNOHISTOCHEMISTRY WITH THESE ANTISERA?

INTRODUCTION

Despite the poor likelihood that pyrimidine dimers would be induced artefactually, by DNA extraction, their *in situ* detection remains desirable. Such a capacity would allow localisation of damage, not only within the cell but also the tissue as a whole. Immunohistochemical methods have been developed by other groups for the assessment of damage in *ex vivo* skin biopsy samples (Tan and Stoughton, 1969b; Potten *et al.*, 1993). This has allowed the induction of specific lesions to be studied including the cyclobutane dimer (Roza *et al.*, 1991), (6-4) photoproduct (Young *et al.*, 1996) and even 8-oxodG (Hattori-Nakakuki *et al.*, 1994). Furthermore, the kinetics of induction and repair of the dimer lesions has also been shown (Muramatsu *et al.*, 1992; Qin *et al.*, 1994).

AIM

To demonstrate the production of cyclobutane pyrimidine dimers in the DNA of fixed tissue sections, by Ab529, following irradiation of the whole section.

METHODS

Immunohistochemistry

UV-induced DNA damage was detected *in situ* in fixed tissue sections using antibody 529 (diluted 1/2000), following irradiation of sections with UVA and UVC combined or with UVB alone. Peroxidase detection was with a directly labelled secondary antibody, however for maximum sensitivity, fluorescent detection was performed using a biotinylated secondary antibody employing the use of a streptavidin/biotin complex detection system.

RESULTS

Frozen tissue sections - peroxidase label

Immunohistochemical staining was demonstrated in normal skin which had been UV irradiated *in vitro* with UVA and UVC combined to give a total dose of 6.8 J/cm² (Figure 6.5.1). There was clear staining of the nuclei in the epidermal layer, from the basal layer to the stratum granulosum, and in some areas of the dermis (Figure 6.5.1). Sections showed some blue nuclei due to counterstaining with Mayer's Haematoxylin. There was an absence of antibody staining in unirradiated sections (Figures 6.5.2 and 6.5.4), with only counterstaining being observed.



Figure 6.5.1. Transverse section of normal human skin irradiated with UVA and UVC to a dose of 4.2 and 2.6Jcm^{-2} respectively and subsequently probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label. The section shows visible staining in the keratinocytes of the epidermis (E) and cells in the dermis (D). (100 magnification.)



Figure 6.5.2. Transverse section of unirradiated normal human skin and subsequently probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label. This section represents a negative control and no staining is visible in keratinocytes of the epidermis (E) and cells in the dermis (D). (x200 magnification).

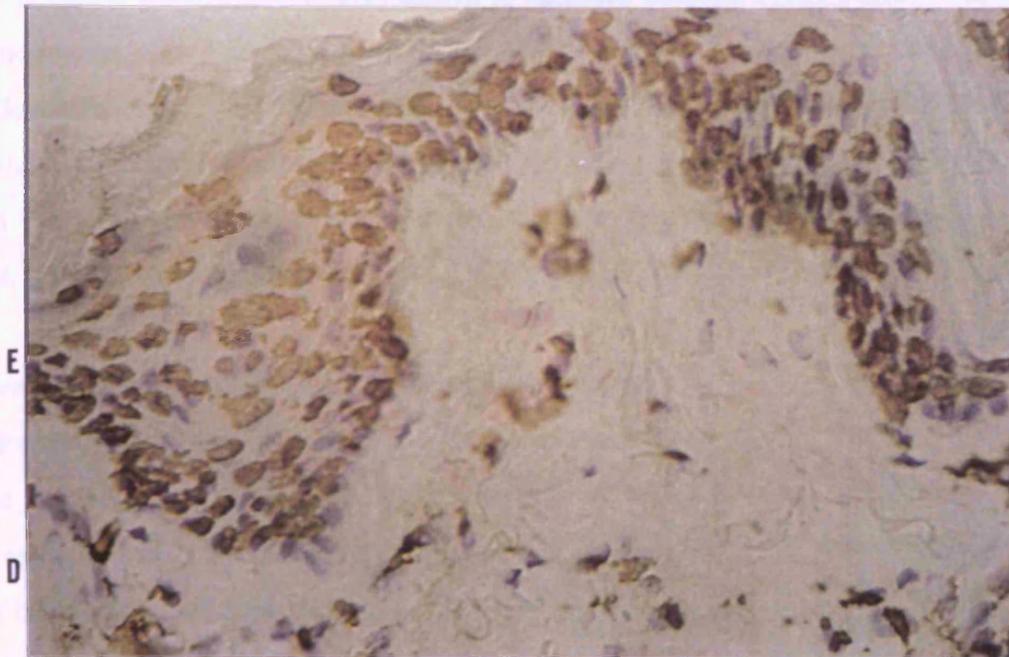


Figure 6.5.3. Transverse section of normal human skin irradiated with UVA and UVC to a dose of 4.2 and 2.6Jcm^{-2} respectively and subsequently probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label. The section shows visible staining in the keratinocytes of the epidermis (E) and cells in the dermis (D). (x400 magnification).

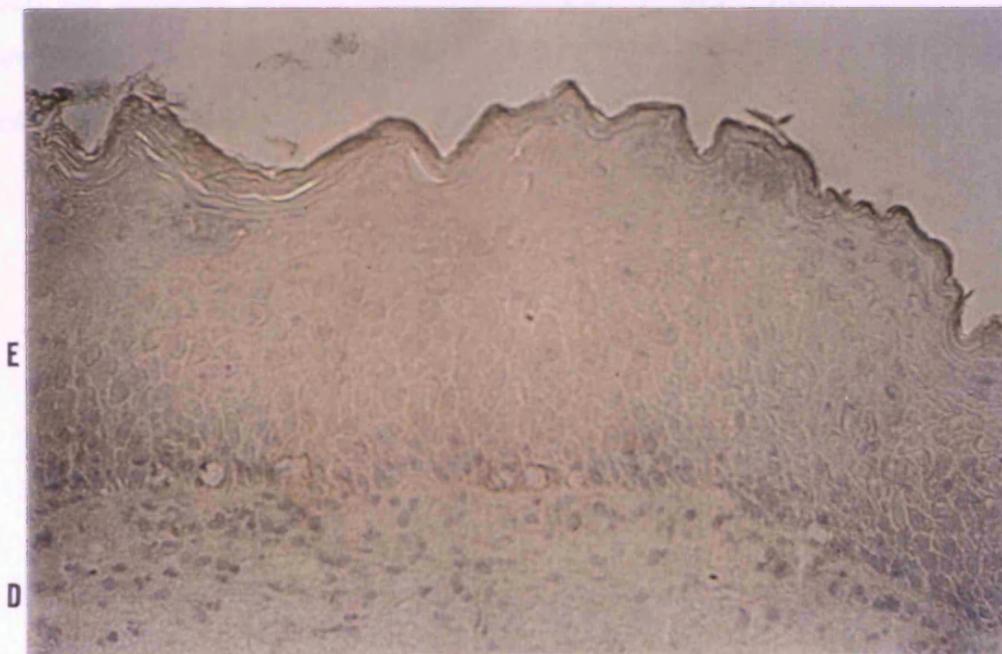


Figure 6.5.4. Transverse section of unirradiated normal human skin and subsequently probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label. This section represents a negative control for Figure 6.5.3. and no staining is visible in the keratinocytes of the epidermis (E) and cells in the dermis (D). (x400 magnification).

At higher magnification it was clear that specific nuclear localisation of antibody staining was obtained (Figure 6.5.3). The pattern of antibody staining within positive nuclei was not homogenous and there appeared to be some staining of the plasma membrane and the nuclear membrane itself seemed severely disrupted (Figure 6.5.5). Additionally, some nuclei of the basal layer in the irradiated sections did not appear to stain (Figure 6.5.5), a feature that was consistently observed. The pattern of binding did not alter in sections irradiated with UVC alone, however, no staining was seen following irradiation with UVA alone.

Formalin fixed, paraffin embedded tissue sections.

1. Peroxidase label.

The apparent lack of staining within the nuclei of cells in the basal layer led to the investigation of other tissues in order to reproduce the effect. A sub-population of cells in the basal layer are transcriptionally active, the stem cells, the remainder cells have not yet started to undergo cytomorphosis. It was suspected that the process of cytomorphosis may make damage in the nuclei more accessible to the antibodies, however as the keratinocytes are the only cell type which display this form of progression it was decided that cells undergoing apoptosis, a process similar to cytomorphosis, would be a suitable model. For this reason sections of human reactive lymph node were examined. As a supply of breast tissue was available, sections were also examined to allow comparison with the effects in skin. Figure 6.5.6 shows the application of the technique in breast tumour tissue. Nuclear localisation was observed following irradiation with UVA and UVC at a dose of 6.8 J/cm^2 .

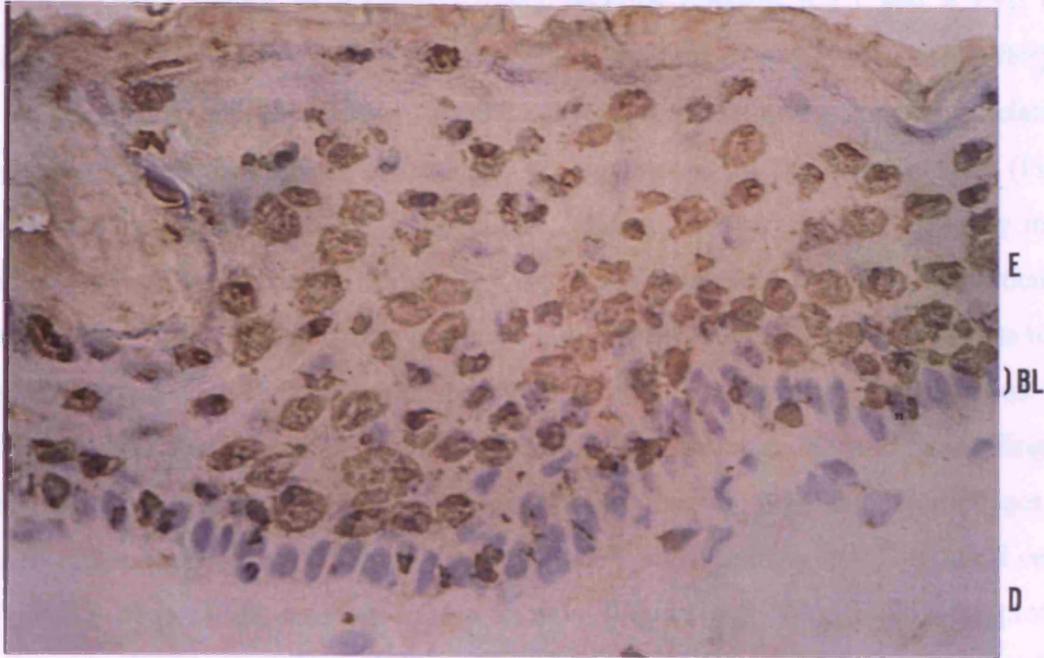


Figure 6.5.5. Transverse section of normal human skin irradiated with UVA and UVC to a dose of 4.2 and 2.6Jcm^{-2} respectively and subsequently probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label. The section illustrates the lack of staining within cells of the basal layer (BL) ($\times 400$ magnification). Epidermis denoted (E) and dermis (D)

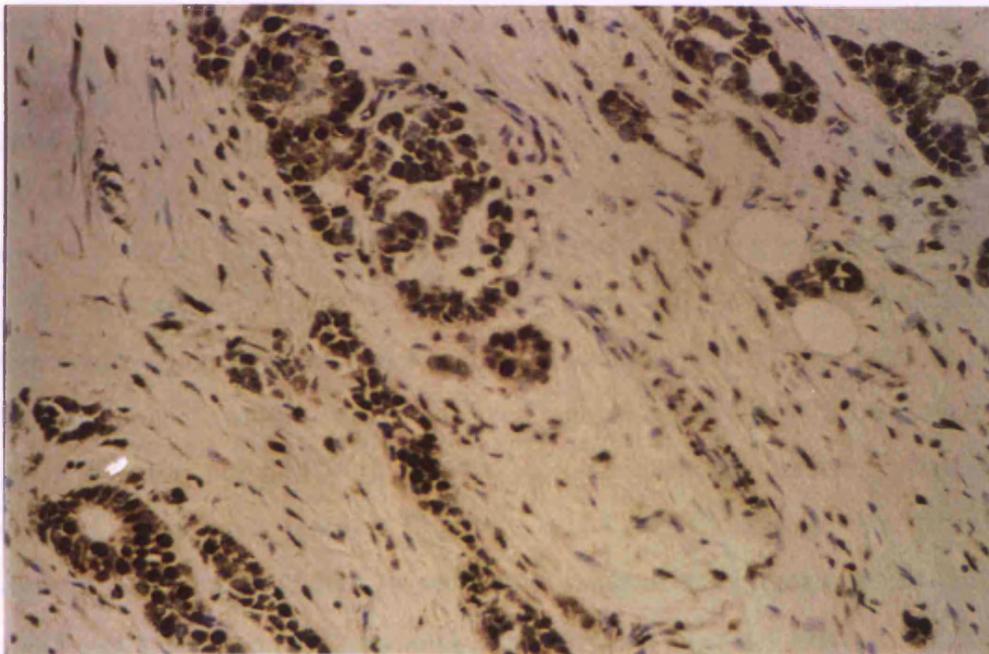


Figure 6.5.6. Transverse section of human breast tumour tissue irradiated with a dose of 4.2 and 2.6Jcm^{-2} UVA and UVC, respectively. All nuclei appear to show uniform staining when probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label ($\times 100$ magnification).

No staining was achieved in the unirradiated sections (Figures 6.5.7 and 6.5.9), even following protease treatment, a standard procedure for antigen retrieval (Naish, 1989). At x400 magnification strong staining of the nuclei could be seen, appearing relatively homogenous across the nucleus (Figure 6.5.8), contrasting to that seen in skin (Figure 6.5.5). Use of protease, revealed more nuclei staining and with greater intensity in the treated sections of breast tissue (Figure 6.5.10). This increase in staining was particularly apparent in the stromal areas. It was observed in a number of sections that the dense nuclei of immune-derived cells did not appear to stain as easily as other nuclei in the section. This was investigated further using reactive human lymph node sections. Surrounding the germinal centre of a secondary follicle, which contains dendritic cells and macrophages, is a mantle of lymphocytes. Post UV-irradiation produced good staining in the germinal centre, with varying degrees of staining in the mantle (Figure 6.5.11A). Following protease treatment a greater proportion of cells in the mantle stained along with increased intensity of the majority of nuclei throughout the section (Figure 6.5.11B) with successful antigen retrieval.

Sections showing UV-related pathology

The sections used were basal cell carcinoma, melanoma and naevi. No staining with Ab529 or an anti-p53 monoclonal antibody was demonstrated in any of the sections.

2. Use of an alternative fluorescent label

Tissue studies using the FITC label did show nuclear staining following UV irradiation, however, there was a large amount of autofluorescence resulting in sections which were not ideal for photography (Figure 6.5.12A). In areas with lower autofluorescence, some positive nuclei could be seen when irradiated sections were probed with Ab529 (Figure 6.5.12B). Most paraffin-embedded sections have little fluorescence in the excitation/emission conditions of AMCA, so this label was utilised. No binding was detected in unirradiated sections (Figure 6.5.13A). DNA damage was detected, using streptavidin labelled with AMCA, in sections of reactive human lymph node which had been irradiated with UVA and UVC at a dose of 6.8 J/cm^2 (Figure 6.5.13B).

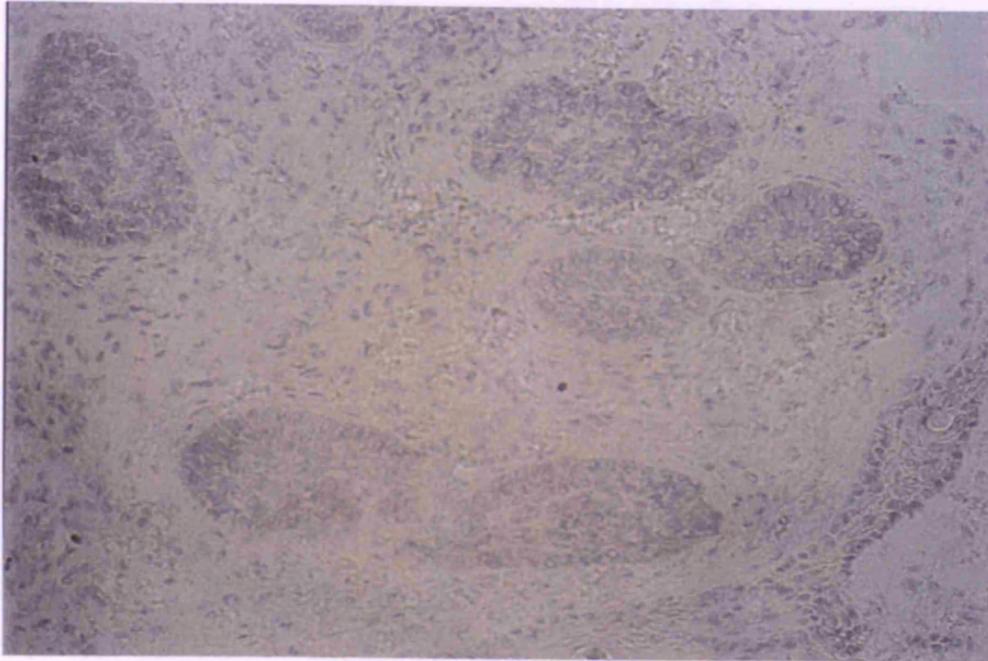


Figure 6.5.7. Transverse section of unirradiated human breast tumour tissue. This section displayed no staining when probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label and represents the negative control for Figure 6.5.6. (x100 magnification).

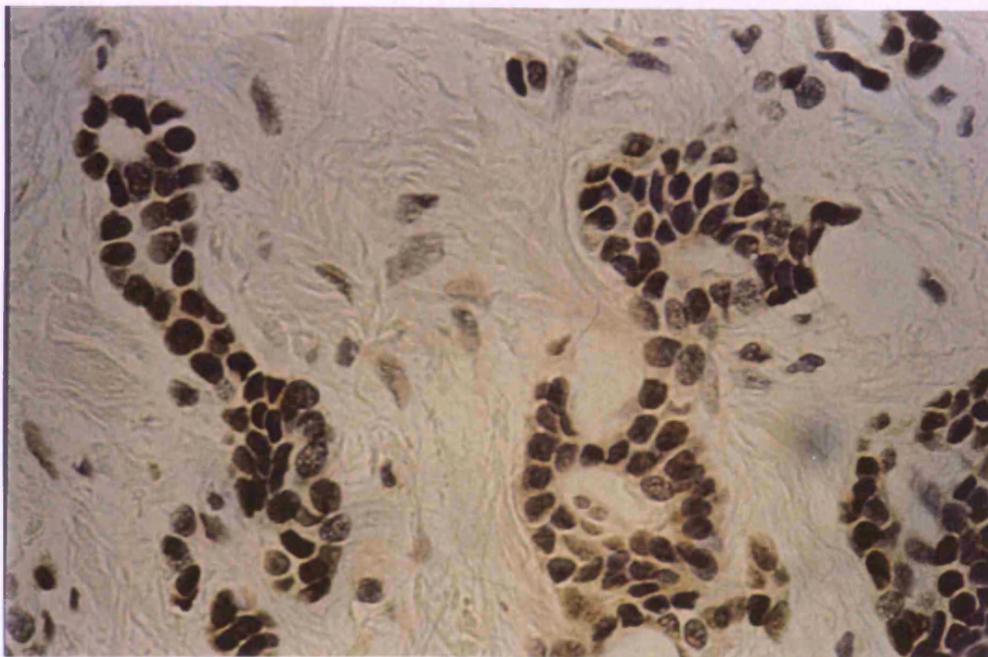


Figure 6.5.8. Transverse section of human breast tumour tissue irradiated with a combined dose of UVA and UVC (4.2 and 2.6Jcm⁻² respectively). All nuclei appear to be stained when probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label (x400 magnification).

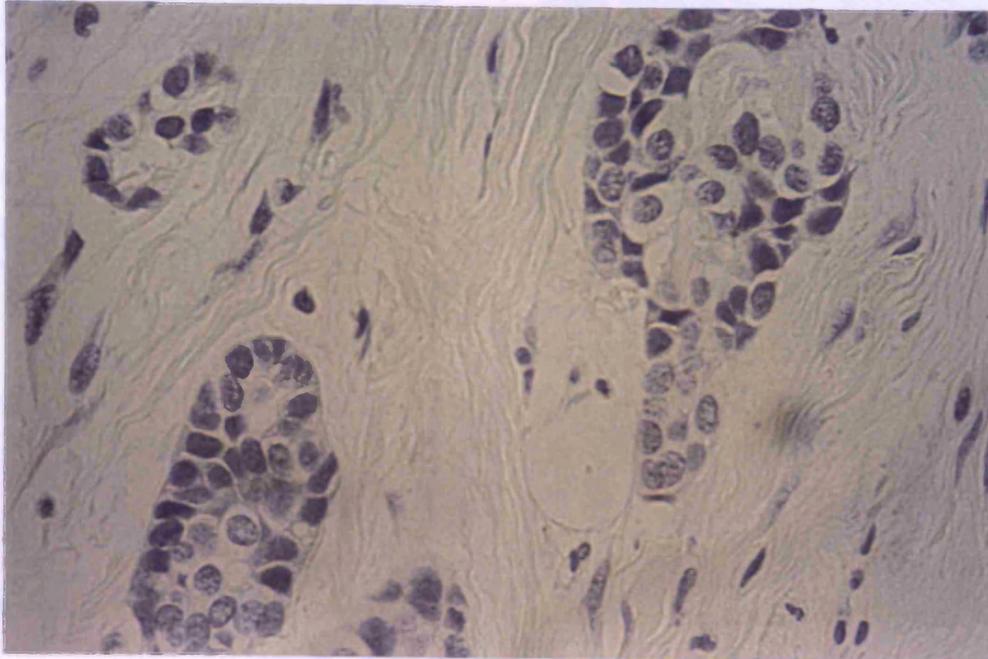


Figure 6.5.9. Transverse section of unirradiated human breast tumour tissue. This section displayed no staining when probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label and represents the negative control for Figure 6.5.8. (x400 magnification).

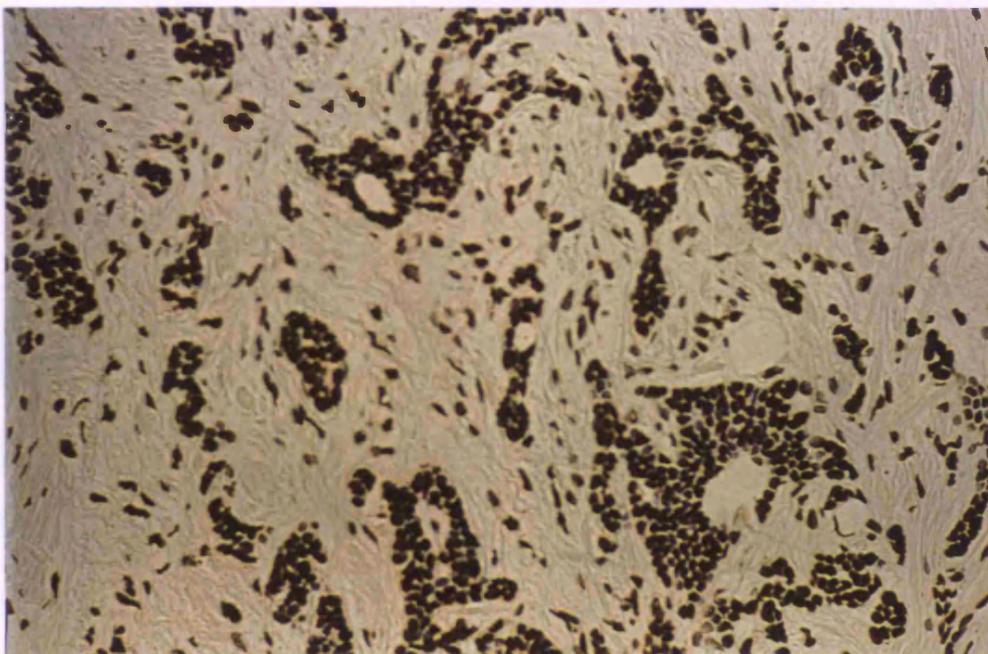


Figure 6.5.10. Transverse section of protease treated, human breast tumour tissue irradiated with a combined dose of UVA and UVC (4.2 and 2.6Jcm^{-2} respectively). All nuclei showed an increase in the intensity of staining when probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label (x400 magnification).

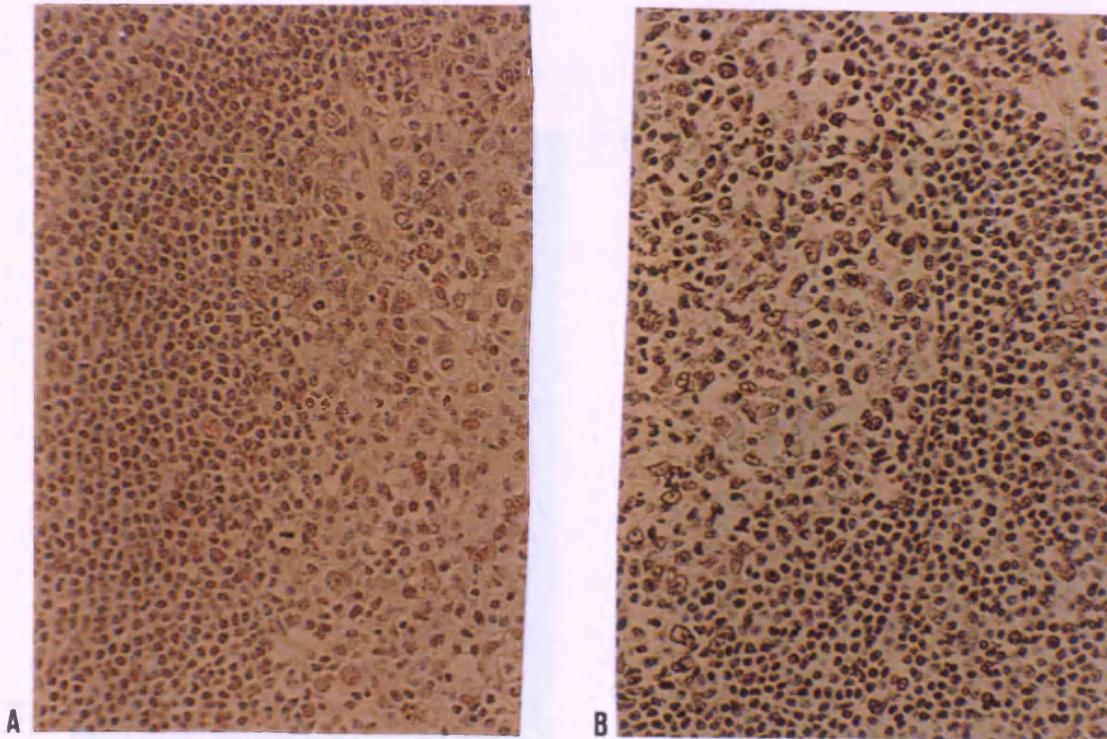


Figure 6.5.11. Effects of protease treatment compared in two transverse sections of irradiated (to a dose of 4.2 and 2.6Jcm⁻² UVA and UVC respectively) human reactive lymph node, when probed for cyclobutane thymine dimers with Ab529, localised with a peroxidase label. Section (A) is without protease, (B) is with protease. A clear increase in the intensity of staining was seen in (B). (x200 magnification).

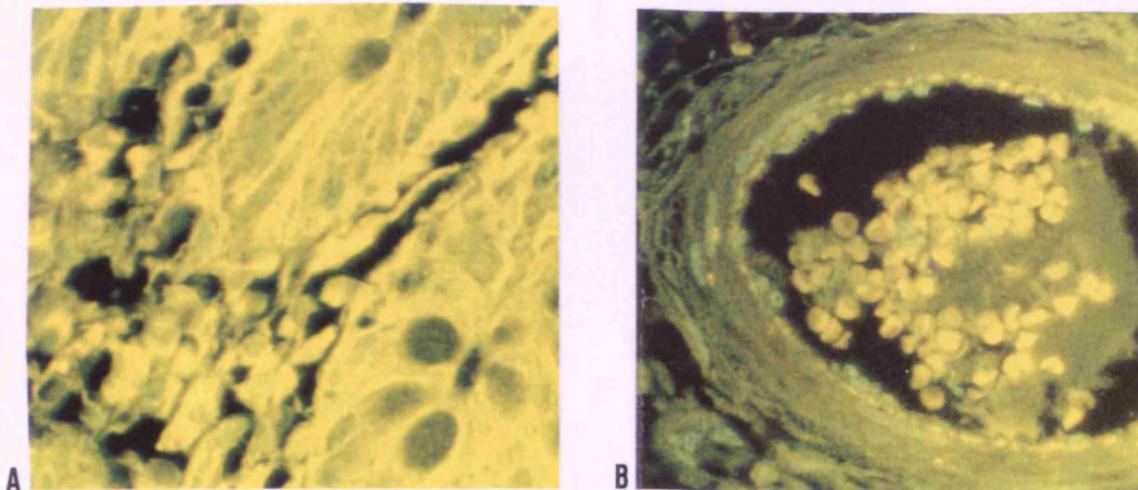


Figure 6.5.12. Transverse sections from areas of human colon viewed under fluorescence microscopy. Both illustrate the problem of autofluorescence. Section (A) is an unirradiated section from a glandular region of colon and no fluorescent nuclei are visible. Section (B) is a blood vessel of the colon, irradiated with a combined dose of UVA and UVC (4.2 and 2.6Jcm⁻² respectively). Some pale green staining of nuclei can be seen in this section which was probed for cyclobutane thymine dimers by Ab529, localised with a FITC label (x400 magnification).

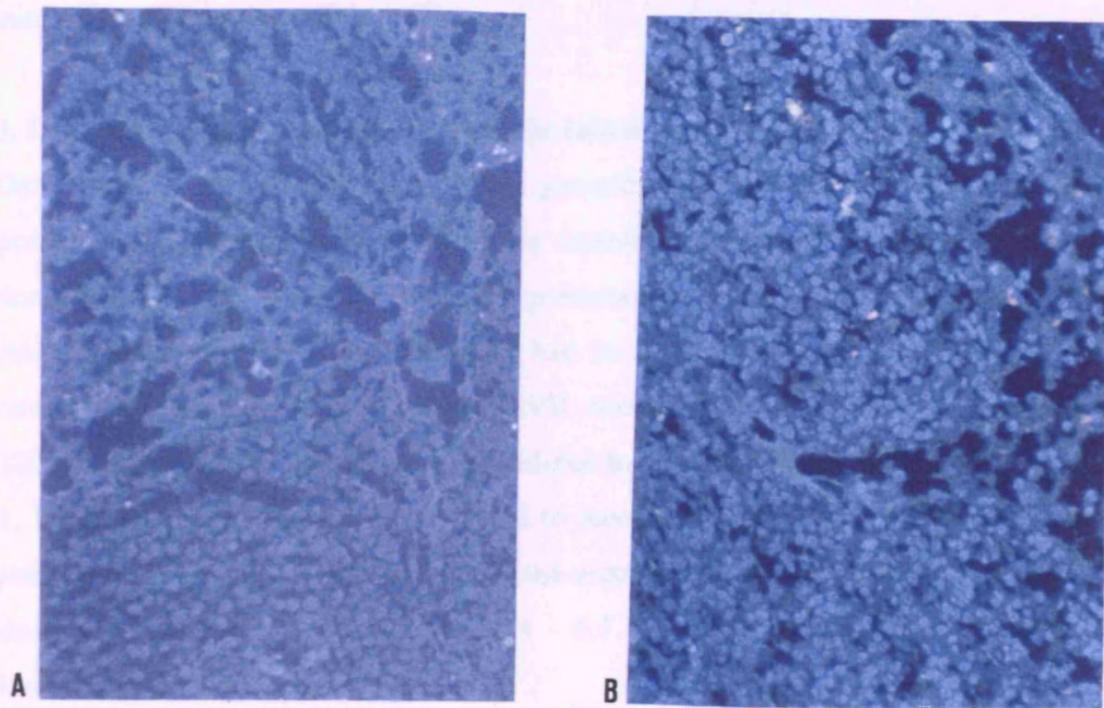


Figure 6.5.13. Use of an alternative fluorescent label with human reactive lymph node. The reduced autofluorescence is seen in both the (A) unirradiated and (B) irradiated (UVA and UVC, 4.2 and 2.6Jcm⁻² respectively). (x200 magnification).

Use of this label in sections of lymph node, provided clear, discrete localisation, with minimal background autofluorescence.

3. Demonstration of dose-response *in situ* following UVB irradiation

Data from the above work showed that peroxidase-based labelling, whilst simplest, also possesses suitable sensitivity to allow the establishment of a dose-response with various doses of UVB. This work was to act as a precursor for the sections derived from biopsies *in situ* irradiated with UVB and therefore had to involve what are considered biologically relevant doses; measured in mJcm^{-2} for UVB. Sections were irradiated with doses from 2 to 120mJ/cm^2 , where $10\text{-}30\text{ mJ/cm}^2$ is considered to be the MED for skin type I (see Chapter 1, Introduction). Protease was employed to increase the sensitivity. A marked increase in staining from 2 to 120mJ/cm^2 was seen (the sections irradiated with the lowest and highest doses are represented by Figures 6.5.14 - 6.5.16). It is noteworthy that staining at the lowest dose was still apparent.

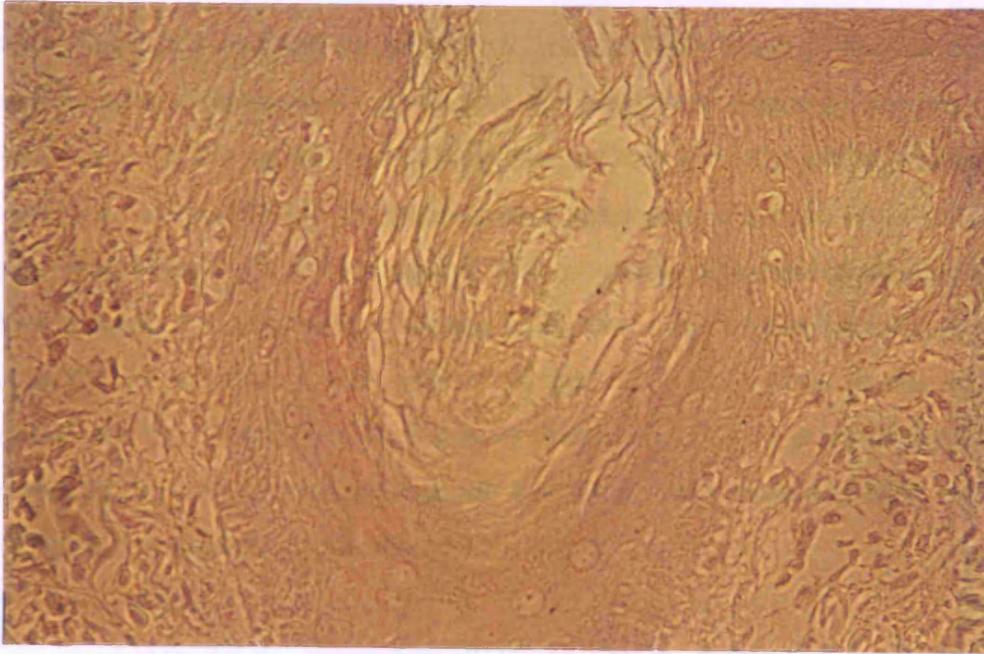


Figure 6.5.14. Section of unirradiated, normal human skin, probed with Ab529. No staining is visible. (x400 magnification.)

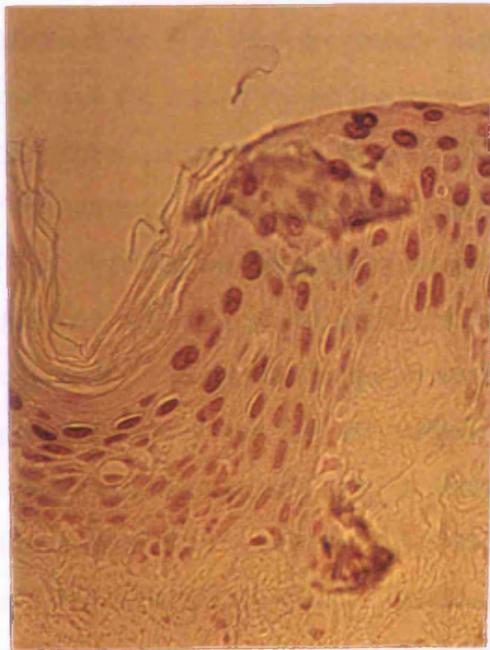


Figure 6.5.15. Section of normal human skin, irradiated with 2mJcm⁻² UVB and probed with Ab529. Staining of nuclei is clearly visible. (x400 magnification.)

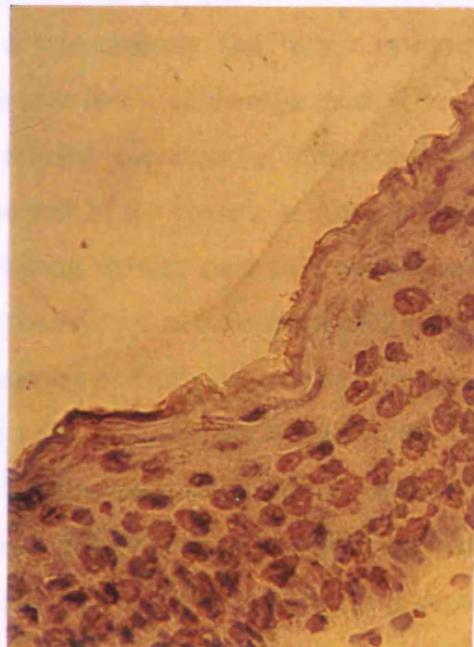


Figure 6.5.16. Section of normal human skin, irradiated with 120mJcm⁻² UVB and probed with Ab529. Strong staining of nuclei is clearly visible. (x400 magnification.)

Discussion

Described here is a method by which UV-induced DNA damage can be detected and localised in human tissue sections, whether the latter were frozen or formalin fixed. Full characterisation of this antiserum was described in Chapter 5. The antibody presents certain sequence specificity in DNA; the main epitope recognised consisted of dimerised adjacent thymines with either a 3' or 5' pyrimidine. The formation of thymine containing CPD in the DNA of sections irradiated with UVA and UVC was immunohistochemically demonstrated using Ab529. In addition, it was determined that the UVC portion of this radiation was responsible for the induction of such lesions. Failure of UVA to produce any modification detectable by the antiserum would suggest that merely the presence of DNA within cells is not sufficient for dimers to be produced by UVA.

The choice of three labels, peroxidase, FITC or AMCA, means that qualitative as well as quantitative assays may be carried out within a variety of sections. Some techniques, in particular using antinuclear antibodies, require DNA denaturation to produce results (Gonchoroff *et al.*, 1985; Gonchoroff *et al.*, 1986). Here, the use of protease increased the number and intensity of nuclei staining. This suggests that protease treatment reveals sites affected by UV, but previously inaccessible to the antibody and may consequentially increase the sensitivity of the assay where only low levels of damage exist. Use of this technique on irradiated, formalin fixed skin revealed a pattern of differential staining between cells of the epidermal basal layer and the rest of the epidermis. Areas of the basal layer displayed an apparent complete lack of staining. It was clear that the antisera was reaching cells deep in the section, ruling out any possibility that these basal cells are deep in the section and that inefficient dewaxing was hindering antibody penetrance. The two main possible reasons for this are, either some basal cells are not being damaged by UV for reason or reasons unknown, or the damage is in sites inaccessible to the antibody. The former explanation seems unlikely as the high doses of UVC will induce sufficient numbers of CPD such that they will be detected by the antiserum. The DNA within cells is associated with many proteins organised in nucleosomes, whereas the DNA used in the ELISA of Chapter 5 was spectrophotometrically determined to possess a relatively low protein content (A_{260}/A_{280} ratio ≥ 1.8 , see page 50, Materials). Therefore arrangement of the DNA by these nucleosome may alter both the location of the lesions and the ability of the antisera to reach the lesions. Detection of lesions in supra-basal cells was a consistent feature in the UV irradiated sections. An apparent lack of staining has been seen in other cells in the

section, particularly immune-derived cells, suggesting that the conformation of the DNA is important to either the formation or detection of damage. In these cells, protease treatment can reveal damage which was otherwise not visible. As progenitor cells make up the cells of the basal layer, and these cells are more likely to have a relaxed chromatin conformation due to transcription, it was reasoned that conformation may play a role in the explanation of this phenomenon. This was supported by the finding that chromatin conformation has been shown previously to alter the distribution (Gale, *et al.* 1987; Brown *et al.*, 1993) and formation of UV photoproducts (Schieferstein and Thoma, 1996).

Pyrimidine dimers are an important marker of UV-induced DNA damage *in vitro* and the detection of pyrimidine dimers in the precancerous forms of certain skin cancers would be an indication of a multistep process for UV-induced tumourigenesis. For example, the progression of solar keratosis to squamous cell carcinoma may be due to the accumulation or persistence of thymine dimers which lead to tumourigenic changes. However, there are conflicting reports as to whether such damage can be detected in biopsies from such patients (Hori *et al.*, 1992; Taguchi *et al.*, 1993) this is perhaps due to repair (Reusch *et al.* 1988) or the thymine dimer is not the lesion responsible for tumourigenic changes and its presence is therefore not relevant. Whether this is due to the differing sensitivities of the techniques used is unclear. The procedure described in this thesis may allow more accurate determination of the role played by pyrimidine dimers in precancerous and cancerous lesions.

Pyrimidine dimers containing at least one cytosine have been reported to have a greater oncogenic potential than thymine dimers (Brash *et al.*, 1991). This makes the raising of antisera to pyrimidine dimers containing cytosine also attractive. Application can then be made to the determination of comparative excision rates of pyrimidine dimers and other photoproducts from DNA. It appears that it is a combination of the lesion itself (where and how easily it is formed, for example) and whether it is slowly or rapidly repaired (Tornaletti *et al.*, 1993) that determines its mutagenicity.

The successful detection of lesions in normal skin sections irradiated with a range of UVB, which included doses notably below the MED for even type I skin, indicated the technique to be suitable for application to biopsies. However, it is vital to note that these sections were irradiated across the section, representing a cross-section of the epidermis and dermis.

Thus no penetration effects of the UV passing through the striatum corneum and granulosum would be introduced. Such effects would, however, would contribute to any staining seen in the biopsy samples.

6.6 IMMUNOHISTOCHEMICAL DETECTION OF CYCLOBUTANE THYMINE DIMERS IN SKIN BIOPSIES OF HUMAN SUBJECTS IRRADIATED WITH UVB

INTRODUCTION

The literature describes applications for antibodies to UV damage which include detection of damage in naked DNA (Natali and Tan, 1971), cells (Berg *et al.*, 1993) and tissues (Tan and Stoughton, 1969b). In this thesis, antibodies to UV DNA damage have been generated, characterised and successfully applied to the detection of lesions in cultured cells and the tissue of fixed sections. However, as UV-induced DNA photoproducts are understood to play an important role in skin carcinogenesis of sun-exposed sites, it is vital that lesions can be detected *in situ*, in skin following UV irradiation. Both formation and repair of such lesions have been studied *in vivo* and *in vitro* both by immunochemical and alternative methods (Eggset *et al.*, 1983; Reusch *et al.*, 1988; Mizuno *et al.*, 1991; Muramatsu *et al.*, 1992; Koehler *et al.*, 1996). From these references and ones contained therein, the general consensus appears to be that cyclobutane pyrimidine dimers (CPD) are repaired more slowly than (6-4) photoproducts, either because they are more easily formed by UVB, or their relative repair is less efficient (Muramatsu *et al.*, 1992). Nonetheless, 50% CPD have been reported to have been repaired one hour post-irradiation *in vivo* and *in vitro* in human keratinocytes, with less than 10% remaining 24 hours after irradiation (D'Ambrosio *et al.*, 1981a; Reusch *et al.*, 1988). Furthermore, not only has the repair of CPD, at least in mice, been shown to be from transcriptionally active genes (Ruven *et al.*, 1993), but also from the transcribed strand, preferentially (Ruven *et al.*, 1994). It has also been shown that individuals who have developed basal cell carcinomas have a decreased ability to repair CPD, compared to a control group (Alcalay *et al.*, 1990; Grossman and Wei, 1995). This was demonstrated by quantifying dimers contained in biopsy material taken from subjects at post-irradiation time intervals.

The cells in which the effects of lesion induction and repair are most crucial are the progenitor or stem cells of the basal layer. These cells receive some protection from UV radiation due to the absorption by supra-basal layers of keratinocytes (Bruls *et al.*, 1984). This was supported immunohistochemically by Eggset *et al.* (1983) who showed the outer layers of human epidermis, such as the striatum spinosum and striatum granulosum, were more heavily damaged by two MED UVB, compared to the basal layer. In order for such

studies to be performed by the antiserum described in this thesis, sections from biopsies of UV irradiated subjects were probed for positive staining.

AIM

To apply Ab529, using the immunohistochemical method developed in Chapter 6, to detect cyclobutane thymine dimers in the skin of volunteers exposed to biologically relevant doses of UVB.

METHOD

Samples were kindly provided by Dr Alistair Robson, Department of Pathology, University of Leicester. The samples were treated and mounted as described in Anstey *et al.* (1996). Briefly, six human volunteers were irradiated with 2 MED of UVB (λ_{max} 300nm) and skin biopsies taken 2, 4, 8, 24 and 48 hours post-irradiation. Control material was taken from adjacent non-irradiated skin. These specimens were 4mm punch biopsies, which were subsequently fixed in formalin and mounted in paraffin wax. Immunohistochemical staining, using Ab529 and a streptavidin/biotin amplification system, was performed as developed previously and fully described in the Materials and Methods (Chapter 2, Section 2.6.3, page 73).

RESULTS

Figure 6.6.1. was derived from a section of normal human skin, which illustrated the epidermis and dermis. The section demonstrated an absence of staining in a irradiated biopsy where the primary antibody (Ab529) has been omitted from the staining protocol. The nuclear counterstain, haematoxylin, stained all nuclei in both the epidermis and dermis a pale blue colour. Inclusion of Ab529 into the staining procedure produced the pattern of staining seen in Figure 6.6.2. The material from which this section was made was biopsied two hours post-irradiation and showed strong nuclear staining, particularly in the outermost stratum of the epidermis, stratum granulosum. A gradation of staining through the layers of the epidermis was seen, with staining of some cells in the dermis also occurring. The strong, dark brown staining of epidermal nuclei is even more apparent at x400 magnification, with localisation, in particular, around the periphery of the nuclei (Figure 6.6.3). A section from a biopsy, taken four hours post-irradiation (Figure 6.6.4.), showed markedly reduced levels of binding and fewer nuclei demonstrated the intense dark brown staining, in comparison with Figure 6.6.2. This result would suggest that some repair of

damage had occurred during the two and four hour post-UV, time periods. Interpretation of the results described above was made more complex by the results from the unirradiated control biopsy. Figure 6.6.5. illustrates binding of Ab529 to a section taken from an unirradiated subject. Although there was little defined nuclear staining, a high background was present throughout the epidermis, not seen in the other sections. Few nuclei stain heavily and their location appeared arbitrary within the section. Staining of dermal nuclei was also noted.

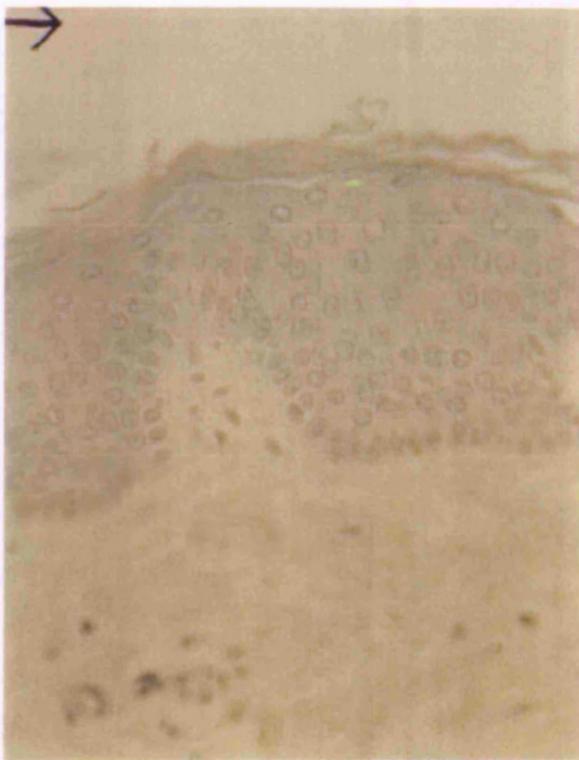


Figure 6.6.1. Section of UVB irradiated (2 MED), normal human skin from a biopsy taken two hours post-irradiation. The primary antiserum (Ab529) was omitted from the staining protocol and no peroxidase staining is visible. The blue nuclear counterstain is haematoxylin (x200 magnification).

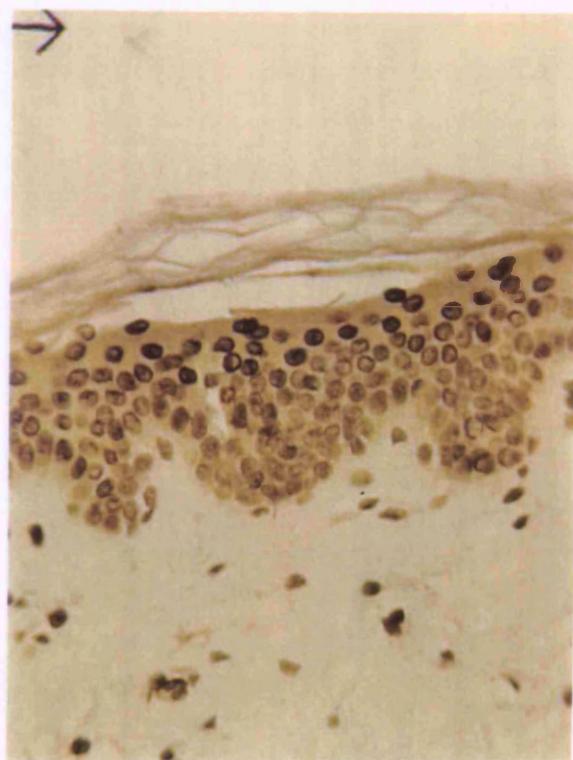


Figure 6.6.2. Section of UVB irradiated (2 MED), normal human skin, from a biopsy taken two hours post-irradiation, probed with Ab529, using a peroxidase-labelled secondary antibody. Staining of epidermal and dermal nuclei is evident, with some gradation of staining through the epidermis. (x200 magnification).

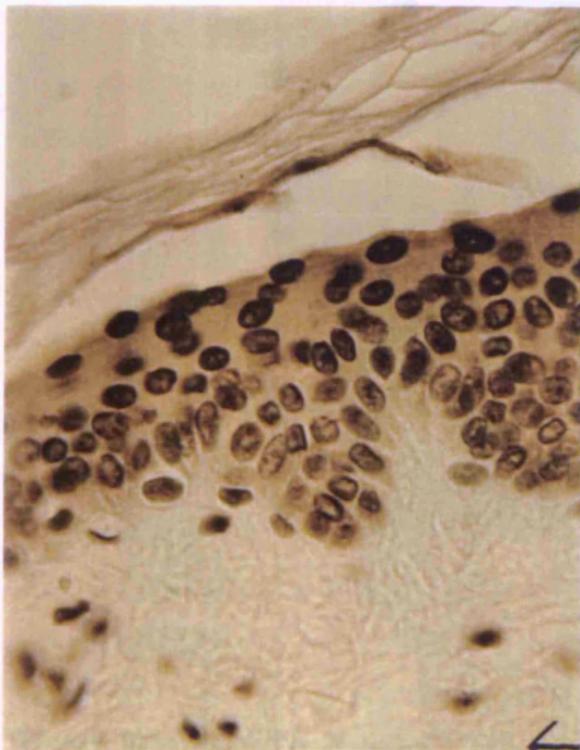


Figure 6.6.3. Section of UVB irradiated (2 MED), normal human skin, from a biopsy taken two hours post-irradiation, probed with Ab529, using a peroxidase-labelled secondary antibody. Intense staining of the nuclei in the outer layers of epidermis is very apparent. (x400 magnification).

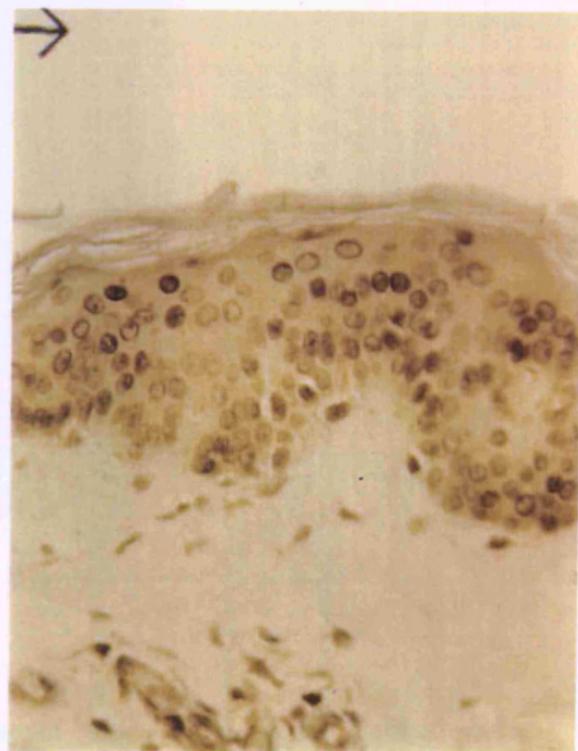


Figure 6.6.4. Section of UVB irradiated (2 MED), normal human skin, from a biopsy taken four hours post-irradiation, probed with Ab529, using a peroxidase-labelled secondary antibody. An overall reduction in staining is seen throughout the section when compared with figure 7.5.2. (x200 magnification).



Figure 6.6.5. Section of unirradiated, normal human skin, from a biopsy, probed with Ab529, using a peroxidase-labelled secondary antibody. Non-specific staining, which is not restricted to nuclei, is seen within the section. (x200 magnification).

DISCUSSION

Discussion of the high background seen in the negative control must first be undertaken before useful interpretation of the other results can begin. It is important to note that whilst the pattern of staining in the irradiated sections are representative of those seen in sections taken from a number of individuals, the control slides available were only derived from one individual. It is therefore vital that sections from others are obtained and analysed, as this anomaly may only be seen in the one person, suggesting artefactual exposure of these sections to UV (e.g. sunlight). Comparison of staining seen in the control with that in the other sections suggests that the results seen in the irradiated sections are real and the staining in the control appears to simply be a non-specific "wash", not apparent in the irradiated sections due to intense nuclear staining. Eggset *et al.* (1983) also noted non-specific staining of their polyclonal antiserum, which had been raised to UVC-irradiated DNA, in cryostat section of UV irradiated mouse skin. This problem was overcome by purification of the antibodies specific for UV irradiated DNA by the use of an affinity column. A limited amount of biopsy material precluded this approach, although this would represent essential future work.

It is proposed that greater dilutions of the antiserum will be investigated to remove the staining seen in the control, in addition to obtaining other controls from collaborators in Cardiff, UK. The affinity column approach of Eggset *et al.* (1983) may be used, although a reduction in positive antibodies by such an enrichment technique is possible. The staining protocol described here represents a number of advantages over that of Eggset *et al.* (1983); the antiserum (Ab529) possessed a sensitivity amenable to the use of a peroxidase label, allowing formalin-fixed, rather than frozen, sections to be used. Also denaturation of the nuclear DNA in sections was not required.

The gradation of nuclear staining seen throughout the sections from irradiated individuals would suggest that a depth penetration effect of the broad band UVB is occurring. A similar feature was noted by Chadwick *et al.* (1995) using narrow band 260, 280, 300 and 320nm UV sources. Such a phenomenon is not surprising as penetration would be expected to increase as wavelength increases. Positive staining of dermal nuclei, a feature rarely reported, although seen by Eggset *et al.* (1983), would suggest that UV wavelengths, effective at inducing thymine CPD, are penetrating deep into the skin. Reports that far UV penetration into the skin is limited due to absorption (Bruls *et al.*, 1984) would suggest that

longer wavelengths, possibly UVA, are responsible for CPD formation within the dermis, particularly as UVA does possess the ability to penetrate well into the dermis (Anderson, 1983).

From this work the benefits of immunochemical techniques are clear. Whereas a technique relying on endonuclease sensitive sites would require the destruction of the tissue (Freeman *et al.*, 1989), immunohistochemistry can provide location of damage within the section. Location of such damage is clearly important as tumours frequently arise from the cells associated with the basal layer of the epidermis (see Chapter 1. Introduction). On this basis, damage to dermal cells appears to be of little pathological relevance. It would, however, be valuable to judge as to whether cells at different locations within the epidermis possess varying repair efficiencies.

Both *in vivo* and *in vitro* keratinocyte repair studies have been performed by a number of groups, although Muramatsu *et al.* (1992) suggest caution when extrapolating repair data derived from cells *in vitro* to skin cells *in vivo*. It has been reported that approximately 50% of the UV-induced CPD are excised within one hour of irradiation with less than 10% remaining 24 hours post-irradiation (D'Ambrosio *et al.*, 1981a). However, such a study did not provide data as to the location of repair or persistence of dimers and most immunochemical repair studies have focused on the repair kinetics of CPD and (6-4)PP (Muramatsu *et al.*, 1992; Qin *et al.*, 1994). Vink *et al.* (1993) compared the induction of dimers in the basal and suprabasal cells of mouse epidermis. Discrimination between the two cell types was achieved by irradiating the skin *in situ*, taking a biopsy and producing a cell suspension in which the suprabasal cells were stained using an anti-cytokeratin 10 antibody. Detection of CPD was performed by fluorescent immunocytochemistry. Similar patterns of CPD induction and removal were noted in both cell types. These results are open to conjecture as there would appear to be some controversy as to the repair efficiencies of rodent cells to repair CPD (Ruven *et al.*, 1993). However the distinction appears to be whether the cells are *in vitro*, in which case there is little CPD repair (Mitchell *et al.*, 1990), or *in vivo*, where Vink *et al.* (1993) claim efficient repair to occur. Examination of repair efficiencies within a section of tissue may be possible for the biopsy sections described earlier. Comparison of Figures 6.6.2. and 6.6.4. would suggest that the repair which appears to have occurred was greatest amongst the outermost epidermal layers, with notable levels of damage remaining in the cells of the basal layer. However, the most likely explanation,

with consideration of the data of Vink *et al.* (1993), is that the outermost cells have died and sloughed off resulting in, what appears to be, greater repair, nevertheless some repair would appear to have occurred. These results illustrate that Ab529 to be eminently suitable for immunohistochemically studying the induction and repair of UV-induced DNA damage in *ex vivo* samples. Clearly, it remains as future work for the other damage probes to be used likewise, in particular, results with the anti-8-oxodG antibody may prove most interesting.

6.7 CHAPTER SIX SUMMARY

This chapter represented the application of the immunochemical probes, used in previous chapters, to the detection of UV-induced DNA damage formed as a consequence of the irradiation of cells *in vitro* and *in vivo*.

Antiserum 529, having established its greater sensitivity over X18 in Chapter 5, was used to successfully detect thymine CPD in cells irradiated with biologically relevant doses of UVB. No discernible binding was seen in cells irradiated with UVA. However, the experimental probe of oxidative DNA damage, avidin, unequivocally showed the formation of lesions following either UVA or hydrogen peroxide treatment. Furthermore it was shown that pre-treatment of cells with α -tocopherol ameliorated generation of such products, implicating a free radical mechanism of damage induction. The contrast in the applicability of avidin to the detection of lesions in cells and naked DNA, as evidenced in Chapter 4, was noted and will be more fully discussed in Chapter 7.

Although different doses of UV had been used in the work described above, no dose/responsive increases in fluorescent intensity could be noted, highlighting a need for a method by which quantitative assessment can be made. Data was presented to suggest that UVB irradiation of cells induces the formation of both oxidative and dimer damage and that these events may occur at very low doses of UV. Although not possible to state with certainty, it appeared that oxidative damage, as represented by antiserum 532 binding, made an appreciable contribution to the total UV damage to DNA. Assuming the formation of 8-oxodeoxyguanosine in UV-irradiated cells to be a sensitive marker of oxidative DNA damage, measurement of this lesion by established methods such as HPLC-ECD or GC-MS, would confirm the findings with Ab532. However, these techniques require considerable amounts of DNA (60 μ g per sample for GC-MS compared to 2.5 μ g for ELISA), limiting their applicability to biological samples.

Upon irradiation with UVA, it was concluded that no contribution was made by dimeric lesions, as an increase in antiserum 529 binding was not seen. In contrast a significant increase in 532 was noted, illustrating oxidative DNA damage was occurring with increasing doses of UVA. A similar increase in binding was also seen with X18. As it was likely that the formation of thymine CPD was not occurring, it was concluded that binding

was due to the fraction of antiserum which recognised UV irradiated guanines, as described in Chapter 5.

With no evidence for the immunochemical detection of UVA-induced thymine CPD by ELISA, the sensitive technique of flow cytometry was applied. Ab529, in conjunction with a monoclonal antibody to p53 protein, was used assess UVA-induced, formation of CPD, the induction of DNA damage and sensitivity of stage of the cell cycle to irradiation. Whilst allowing quantitative assessments of cellular DNA damage to be made *in situ* no significant increase was seen with either antibody. However, some evidence was presented to support existing theories that the effectiveness of UV to induce DNA damage varies throughout the cell cycle.

The ability for Ab529 to successfully detect damage in the nuclei of sections from biopsies taken following the UVB irradiation of subjects, was shown. Examination of the pattern of staining appeared to show a depth penetration effect, with some nuclei in the dermis staining. Also the reduction in staining in sections from biopsies taken four hours post-irradiation suggested the repair of these lesions.

CHAPTER SEVEN

GENERAL DISCUSSION

“Science is spectrum analysis: art is photosynthesis.”

Karl Kraus - Half truths One and a half truths

GENERAL DISCUSSION

The interaction of UV light with cells can lead to DNA damage. Such damage may arise, either directly, via the absorption of UV by DNA (Mitchell *et al.*, 1989), or indirectly via free radicals, a consequence of UV absorption by other cellular chromophores (Rahn, 1979). The *cis,syn* cyclobutane thymine dimer is an example of a lesion produced by the former route and the oxidative lesion, 8-oxodeoxyguanosine, is an example of a lesion derived via the latter. The overall objective of this thesis was, principally using immunochemical methods, to assess the relative induction of direct and indirect DNA damage in naked DNA and ultimately in cultured human cells, following irradiation with either UVA or UVC. From this, the relative importance of each damage type with respect to the consequences of cellular irradiation, may be implied. The *cis,syn* cyclobutane thymine dimer (cyclobutadithymine) was used as a marker of UV-induced direct damage. However, due to difficulty in producing an immunochemical probe which reliably detected oxidative DNA changes, a number of markers of indirect damage were considered, although principally 8-oxodeoxyguanosine was chosen.

It was anticipated that the development of antibodies to oxidatively damaged DNA would prove to be most difficult and a number of approaches were undertaken. These approaches were broadly classified into: raising polyclonal antisera using ROS-modified DNA, containing numerous different lesions or raising monoclonal antisera to a specific, isolated lesion. In the first instance the ROS generating systems included the use of iron (II) sulphate (Alam, 1993), ascorbate (Blount, 1990) or UVC (Ara, 1993), all in conjunction with hydrogen peroxide. These immunogens, despite being oxidatively modified, failed to produce a successful antiserum to ROS-damaged DNA. However, they did provide an opportunity for the effect of modification on the immunogenicity and antigenicity of DNA to be examined, along with its possible implications for autoimmune disease (Cooke *et al.*, 1997; Appendix III).

Whilst a number of research groups have raised monoclonal or polyclonal antibodies to 8-oxo-2'-deoxyguanosine, using the modified ribonucleoside conjugated to a carrier protein as an immunogen (Degan *et al.* 1991; Park *et al.*, 1992; Yin *et al.*, 1995; Osawa *et al.*, 1995; Bespalov *et al.*, 1996) these have, with the exception of Osawa *et al.* (1995), largely been unable to detect 8-oxodG *in situ* within the DNA polymer. Described within this thesis is

the development of a monoclonal antibody which, although not recognising 8-oxodG in double-stranded DNA, showed a greater than two-fold recognition of oxidatively modified DNA over native when rendered single-stranded. From this evidence, a hypothesis was generated in which glycosidic conformation of the modified guanine, in addition to hydroxylation at the C-8 position, produces recognition of 8-oxodG over dG in single-stranded DNA (Cooke *et al.* *In press*. Appendix III). However, the clone producing this antibody was concluded to be unstable when it ceased secreting immunoglobulin.

A serendipitous finding, consequential of attempting to produce antisera to ROS damaged DNA (Cooke *et al.*, 1997), resulted in antiserum which appeared to recognise UVC irradiated DNA. This antiserum was produced using UVC/H₂O₂ damaged DNA and designated "X18". This antiserum, along with one previously produced (Ab529; Herbert *et al.*, 1994), was characterised by competitive ELISA (Chapter 5.). Both antisera were demonstrated to detect cyclobutane thymine dimers, but within the context of specific flanking sequences; to our knowledge, a facet of an antiserum not previously reported outside this group. The major epitope recognised by X18 was a trinucleotide sequence containing the dimer and a 5' cytosine residue. Ab529 was reported, by Herbert *et al.* (1994), to recognise dimers within a less specific sequence, that is to say, a dimer with either a 5' or 3' pyrimidine. The demonstration that the dimer, recognised by both antisera, is of a cyclobutane type, was reported in this thesis.

In general, the limits of detection of antisera have been previously established by calculating the number of lesions present, based on the theoretical numbers induced at the lowest dose of UV at which antiserum binding is still detected (Mori *et al.*, 1991). However, use of a DNA standard, containing a known amount of lesion would allow for a more accurate determination. Whilst gas chromatography-mass spectrometry methods for the measurement of oxidative DNA lesions are well established, no methods exist for cyclobutane dimers. In order to begin the development of such an assay, the isotopically labelled internal standard was synthesised, according to the method of Wang (1961), but using deuterated thymine. The identity and purity of both the ²H₈- and ²H₀- *cis,syn* cyclobutane thymine dimer were confirmed by HPLC-UV and GC-MS. The derivatisation of both the non-labelled analyte and internal standard was shown, by GC-MS, to have occurred and calibration curves were produced. However, successful derivatisation was only achieved based on a modified high temperature method of Dizdaroglu and Bergtold

(1986). Room temperature derivatisation, adopted by the Division of Chemical Pathology, University of Leicester to minimise artefactual oxidative damage, appears not to derivatise pyrimidines and this is equally true for cyclobutadithymine. The method was shown to be applicable for the quantitation of lesion in DNA, with increasing dose of UVC (this method was published during the course of this thesis, Podmore *et al.*, 1996; Appendix III). Doetsch *et al.* (1995), reported increases in the levels of monomeric lesions, such as 8-oxoG and FapyGua, by GC-MS, subsequent to the UVB or UVC irradiation of DNA. However, levels of dimeric damage were not examined and a dose-response for 8-oxodG was not shown. Although aware of the problems with artefactual damage described above, the levels of 8-oxoG and cyclobutadithymine were examined in UVC DNA using GC-MS. As expected the background levels of 8-oxoG were high, but despite this an appreciable increase was seen post-irradiation. Comparison between the data of Doetsch *et al.* (1995) and that presented here revealed differences in the percentage increases for a given dose of UV. Doetsch *et al.* (1995) produced a 68% increase in 8-oxoG over the control value, compared to 31% for the above experiment. Although a reason for this discrepancy may have been the source DNA used, plasmid versus calf thymus, a more reasonable explanation may have related to possible inaccuracies in the dose given at which comparisons were made. In contrast to Doetsch *et al.* (1995), who quantified the 8-oxoG levels following a specific dose, the value of 31% from the above work was calculated from the corresponding point on a dose-yield plot.

Examination of the same damaged DNA by three different “affinity probes” was also performed, using ELISA-based technology. Clark *et al.* (1996) reported an affinity technique involving avidin to be applicable for the detection of 8-oxodG in DNA. This was utilised as well as a monoclonal antibody to single-stranded DNA and a polyclonal antiserum to ROS-damaged DNA. Ab529 acted as a positive control for the UVC treated DNA - the model damage system of choice. The anti-ROS DNA antiserum showed a dose responsive increase, however the exact nature of the changes this antiserum recognises is not, at present, clear but is under investigation within this group.

An appreciable increase was also seen with the anti-single stranded DNA antibody in conjunction with a 96-well membrane plate - no such increase was seen with a standard 96-well ELISA plate, the two solid phase supports utilised. This discrepancy was postulated to be due to ability of the membrane plate to retain small, highly fragmented pieces of DNA,

allowing recognition by the antibody, whereas such fragments are washed from the standard plate. It is established that hydroxyl radicals may produce strand breaks (Chiu *et al.*, 1995) and Blount *et al.* (1992) implied these lead to regions of single-strandedness. However, the mechanism of UVC-induced damage to DNA is postulated to be hydroxyl radical independent (Doestch *et al.*, 1995) and therefore an alternative mechanism, perhaps involving base radicals, must exist. Such a mechanism was described by Von Sonntag (1984) for radiation induced strand breaks. In the case of γ radiation, base radicals formed by water radicals, attack the sugar moiety producing sugar radicals; these in turn give rise to not only, strand breakage, but also base release. UVC can give rise to base radicals (Gräslund *et al.*, 1979) and may likewise lead to strand breakage, along with base loss. This latter process may be responsible for revealing the antigenic determinant of the anti-single strand antibody, on the complimentary DNA strand, along with some relaxation of the DNA structure. Whilst this appears to represent a sound hypothesis, it should be noted that work by Cullis *et al.* (1996) illustrated that, whilst guanine radical cations are precursors of 8-oxodG in 248nm UV-irradiated DNA, they do not lead to immediate strand breaks, but instead alkali labile sites.

The results using avidin were less clear. Although increases in binding were determined to have occurred in conjunction with both solid phase supports, the extent of such increases were difficult to judge due to the large error bars. The data of Clark *et al.* (manuscript in preparation), which shows that avidin can be used as a probe of oxidative damage, appears convincing and is supported by some of the data here, in particular the cellular work with UVA irradiated keratinocytes (Chapter 6.). However, at present, without full and exhaustive competitive studies of the sort utilised by Degan *et al.* (1991) and Park, *et al.* (1992), for the characterisation of their poly- and monoclonal antisera to 8-oxodG, the specific binding to 8-oxodG can only be implied.

The GC-MS method for cyclobutadithymine allowed a DNA standard to be produced. Utilising this standard, a method for determining an antiserum's limit of detection was established, allowing comparison with other methods of analysis. The antisera studied included Ab529 (Herbert *et al.*, 1994) that raised in Chapter 3. (X18) and represented part of their overall characterisation (Cooke *et al. In preparation*). The limit of detection of the GC-MS assay was found to be 20-50fmol, compared to 0.9-1.9fmol for Ab529 and X18 respectively. This rather surprising result may be explained, in part at least, by a number of

factors. Firstly, the most intense ion generated by GC-MS analysis of cyclobutadithymine is at m/z 255, however analysis of DNA samples displayed interfering ion currents and therefore the second most intense (43% of the most intense) was selected. Clearly, this will result in a loss of sensitivity and is probably a primary cause. Secondly, the ELISA method, used for the antiserum limit of detection determination, involves an enzyme-substrate system, likely to amplify the signal from the bound antibodies. The values for limits of detection of the two antisera are very similar to those reported by Mori *et al.* (1991) and Wani *et al.* (1987) who both used the calculation involving the theoretical number of dimers, suggesting some agreement between this and the GC-MS method.

Having established a method for producing a dimer-containing DNA standard, it may be possible to produce a calibration curve using an ELISA format, whereby a given absorbance, proportional to antiserum binding, relates to a known amount of damage. Such a curve may then be applied to DNA extracted from UV irradiated cells (as described in Chapter 6.). The result of which would provide a method, standardised by a "benchmark" technique (GC-MS), which could directly quantitate absolute levels of dimer in DNA, without need for the time-consuming sample workup of GC-MS.

Antisera to DNA lesions are of most use if they can detect damage within the polymer of DNA and particularly in cells, precluding the need for sample manipulation, such as protease treatment or DNA extraction. Demonstration of the applicability of both Ab529 and X18 to naked DNA was shown by the convincing dose-response curves in Chapter 5. The data also revealed information regarding wavelength-dependent lesion induction. UVC was shown to induce high levels of lesion at relatively low doses, indicated by a steep gradient, with a plateau at approximately 1kJm^{-2} . UVB, however, displayed a slower induction, for an equivalent dose. Most surprisingly, UVB lead to a greater total number of lesions at the maximal dose, compared to UVC. The favoured explanation for this concerns the equilibrium that exist between dimerisation and monomerisation of thymine on UV irradiation (Johns *et al.*, 1962). The data suggests that the position of the equilibrium for UVB is such that it favours the dimerisation reaction more, compared to UVC. The significance of this finding, considering the biological relevance of UVB, is yet to be determined. Thymine dimers have been detected in the DNA of cells irradiated with UVA (Tyrrell, 1973). However in this thesis, no binding to UVA irradiated calf thymus was immunochemically detectable with either Ab529 or X18, concurring with other groups

(Peak *et al.* 1973; Blanc *et al.* 1976). It was concluded from this, in conjunction with the absorption spectrum of DNA, that thymine dimers were unlikely to be formed by the direct absorption of UVA by DNA. However, the induction of thymine dimers by UVA in keratinocytes warrants further investigation.

With the exception of a primary cell line, transformed human keratinocytes represent the best *in vitro* model in which to examine the effects of UV radiation. In Chapter 6, the induction of both CPD and oxidative damage were examined qualitatively and quantitatively following UV. Unequivocal immunocytochemical staining was seen with Ab529 following 108mJcm^{-2} UVB, less than three MED. Although other doses were also used, quantitative comparisons between doses proved to be unreliable, illustrating the need for an assay in which such measurements are possible. Again, no discernible binding was seen to UVA irradiated cells.

It would appear from the data presented in this thesis that avidin can detect nuclear damage to cells, induced either by UVA irradiation, or hydrogen peroxide and that such damage can be prevented by pre-incubation with the antioxidant, α -tocopherol. UVA irradiation of cells has been shown to induce oxidative stress (Tyrrell, 1991), DNA-protein crosslinks (Peak *et al.*, 1985), pyrimidine dimers (Tyrrell, 1973; Freeman *et al.*, 1987) and single-strand breaks (Gange and Rosen, 1986). An apparent paradox of avidin binding has been highlighted by the work performed here. In naked DNA, greater binding to damage does not appear to be conclusive and certainly does not occur dose-responsively over the doses used. Yet, a clear difference in avidin binding was demonstrated between treated and untreated cells in Chapter 6. Again, as was speculated in Chapter 6, with regard to the lack of Ab529 staining in the basal cells of skin sections, the "thin blue line", conformation of the DNA may be a factor. In this way, native, double-stranded calf thymus DNA, may possess a more closed conformation, impeding access of the probe to the lesion. Whereas mitotically and metabolically active cells are likely to have a more open DNA structure, allowing transcription and translation and consequentially access of the probe. Such an explanation may offer the basis of an explanation for avidin binding, described above. This represents the first documented use of avidin, as a means of detecting oxidative damage, in UV irradiated keratinocytes. As such avidin would appear to be a useful probe for cells in culture, although not for extracted, naked DNA.

The immunocytochemical work described above, although providing data regarding qualitative information, such as localisation, appeared to be a system unsuitable for quantitative measurements. This problem was addressed by the extraction of DNA from irradiated cells and assessment by ELISA as described previously (Chapter 6.). Such a protocol appeared perfectly amenable to providing quantitative data. Although some groups use the extracted DNA in a competitive ELISA format (Roza *et al.*, 1988), assessment by direct ELISA is more straightforward (Mizuno *et al.* 1991). In both cases the extracted DNA was denatured before being assayed, a procedure unnecessary for the method described in this thesis.

In each experiment, three probes were used, two antisera to thymine CPD (Ab529 and X18) and the anti-ROS DNA (Ab532) antiserum, thus non-oxidative and oxidative damage assessments could be made simultaneously. As such, this represents the first time quantitative, immunochemical assessments of both direct (dimer products) and indirect (oxidative changes) have been made simultaneously in cellular DNA. Such information may allow the relative contribution of each damage type to the total DNA damage to be determined, or at least inferred. Until the procedure for producing calibration curves of binding, described in Chapter Five, has been developed for each antiserum, comparison of levels of damage by ELISA cannot be wholly relied upon. Despite this, it would appear that damage due to ROS, as measured by Ab532, makes some contribution towards the sum of cellular DNA damage induced by UVB *in vitro*. Thymine CPD induction, as measured by Ab529 binding, does increase with dose, although apparently not as dramatically as the ROS damage. In Chapter 4., the induction of oxidative damage in naked DNA, as measured by the anti-ROS DNA antiserum, supported by GC-MS data, was shown to occur on irradiation with UVC. However, no increases in binding could be detected immunochemically following UVB irradiation. Levels of thymine CPD increase dramatically in the same samples of DNA following UVC irradiation (Chapter 5.). Mitchell *et al.* (1991) used T4 endonuclease V and endonuclease III (endo III), the latter of which is reported to remove such pyrimidine lesions as; thymine glycol, dihydrothymine and cytosine photohydrates (Ramotar and Demple, 1993; Dizdaroglu *et al.*, 1993), to examine the relative induction of dimer and monobasic damage following UV radiation *in vitro* and *in vivo*. It was shown that in both experimental systems, the endo III sensitive sites represented only 1-2% of the T4 endo V sites. The data of Mitchell *et al.* (1991) would appear to contrast with the *in vitro* work in this thesis relating levels of oxidative damage to

dimer damage. This can perhaps be explained by considering the specificity of the antibody and the mechanisms of damage induction in the two systems. Initial characterisation of the anti-ROS DNA antiserum, within the Division of Chemical Pathology, has shown the major antigenic site to be a glyoxal-DNA adduct. More specifically, this may involve glyoxal-guanine which has been shown to be derived mainly from free radical attack to DNA and reported to be present in up to 17 times greater levels than 8-oxodG (Murata-Kamiya *et al.*, 1995). As a purine product this would be likely not to represent a substrate for endo III, therefore differences may be seen in levels of lesion detection. Furthermore, the lesions themselves may be formed from very different mechanisms. Preliminary results, in this thesis and as part of its characterisation, would suggest that the glyoxal-guanine adduct is formed in greater amounts in cellular systems than in naked DNA (Chapter 4.). Explanation, in part, for this may be due to the adduct being more easily formed by ROS in association with metal ions (Murata-Kamiya *et al.*, 1995), than via base and sugar radicals. In contrast, whilst some substrate products of endo III are formed by all ROS-generating systems, some are exclusive to γ radiation.

The poor absorption of UVA by DNA largely invalidates any *in vitro*, naked DNA experiments, clearly thymine dimers are not formed over the doses used (Ellison and Childs, 1981; Peak *et al.*, 1984). The quantitative determination of oxidative and non-oxidative DNA damage induced by UVA showed that the emphasis of damage type differed from that following UVB, with significant increases in oxidative damage and no detectable levels of thymine CPD. This, in agreement with others (Peak *et al.*, 1985; Hattori-Nakakuki *et al.*, 1994), suggests that ROS and other free radical mechanisms to be important in UVA-induced damage and that oxidative DNA lesions may play an important role in the cellular consequences of UVA radiation (Valavanidis, 1994; Nataraj *et al.*, 1995). In this experiment, avidin and a monoclonal anti-8-oxodG antibody were utilised, in addition to antisera 529, X18 and 532. However, these probes failed to detect any changes, suggesting the assay format to be unsuitable, as discussed previously for avidin. Following consideration of the hypothesis in Chapter 3., relating glycosidic conformation of 8-oxodG and antibody recognition, it was subsequently shown that an increase in binding with the commercial antibody could be achieved on denaturation of the DNA. The results with this antibody strongly support those obtained with the anti-ROS DNA polyclonal, suggesting that oxidative processes are the primary insult to DNA following UVA irradiation of cells, at least over the doses used in this thesis.

Important advantages possessed by immunochemical techniques for detecting DNA damage include, economical in cost of reagents and equipment, versatility in application (extracted DNA, affinity columns, direct detection in cells and tissues) and the potential for *in situ* demonstration of damage in cells and tissues, avoiding possible artefactual damage during extraction. The quantitative data obtained above required sample manipulations, such as DNA extraction, prior to immunochemical probing. Whilst artefactual contribution to the dimer damage by such manipulations is unlikely, it is a more realistic risk when considering oxidative damage. Therefore *in situ* immunocytochemical quantitative analysis is highly desirable. Flow cytometry allows the sensitive detection of antibody binding in large numbers of cells individually. Application of flow cytometry to the detection of thymine dimers and p53 protein in UVA irradiated cells suggested there to be a relationship between stage of the cell cycle and sensitivity to UV radiation. Such a finding may, again have relevance for the variations in probe binding seen in earlier Chapters, particularly where viable, mitotically active cells were involved. A key element in the aim of this work was to establish immunochemically, whether UVA radiation was capable of inducing thymine dimers in cells. However, it was concluded that insufficient doses of UVA were used and any increase in binding remained equivocal, making the use of greater doses an important future experiment.

Whilst *in vitro*, cell culture systems are useful for elucidating mechanisms of UV-induced damage, *in vivo* investigations are also required to illustrate such questions as the location of damage within the tissue, the time scale for *in vivo* repair and whether differential repair occurs according to location. These are questions to which immunochemical-based techniques are perhaps most readily applicable. The ability for an antiserum to detect damage *in situ* within the DNA of cells would allow localisation of damage in the cell and distribution across a tissue section indicating, in the case of UV irradiation, penetrance into the tissue. This also circumvents any possible artefact formation during, for example, DNA extraction. Frozen, irradiated, sections of skin were investigated first, to minimise the number of parameters affecting the section, such as formalin fixation. The tissue samples were snap frozen before being sectioned on a chilled microtome. The resultant sections were fixed in cold acetone prior to irradiation. Strict nuclear localisation of staining was seen in the irradiated sections, in contrast with the unirradiated, where no staining was seen. It was noted that within the epidermal basal layer, some cells were not staining, despite

exposure to high doses of UVC. Penetrance effects of the UV through the section were ruled out as an explanation because cells deep in the section and in other areas were staining. The possible conclusions from this phenomenon were either, some cells of the basal layer were not being damaged by the UV, which seems unlikely, or the sites of damage are inaccessible to the antiserum, perhaps due to chromatin organisation. This suggested a role for nucleoproteins such as histones, particularly as the DNA used in the ELISA assays was relatively free of protein. Formalin fixation of the section prior to irradiation did not alter the antiserum's ability to detect damage. Protease treatment, as an antigen revealing technique, was investigated and did lead to a greater intensity of staining within nuclei. Irradiation of sections with UVA did not produce any staining, suggesting that, for thymine dimers to be induced by UVA, not only did the DNA have to be within a cellular context, but also the cell very likely had to be viable on irradiation, or at least possess some attribute more associated with viability.

In order to gauge the antiserum's binding ability in sections irradiated with biologically relevant doses of UVB* a dose-response was established. Although assessment was only possible by eye, a definite increase in binding was noted from 2mJcm^{-2} to 120mJcm^{-2} (where the MED for skin type I is $10\text{-}30\text{mJcm}^{-2}$) with binding still apparent at the lowest dose. This result suggested the antiserum to be eminently suitable for the detection of dimers within biopsies from UVB-irradiated subjects. Using sections from biopsies of subjects irradiated with 2 MED UVB, positive staining was shown throughout the cells of the epidermis, with staining extending into those in the dermis, suggesting the penetrance of wavelengths effective at inducing thymine dimerisation. It is important to note that such wavelengths may be acting via a photosensitiser, not necessarily by direct action. Furthermore a gradation of staining throughout the section was observed, with heaviest staining in the stratum granulosum of the epidermis. Some reduction in staining was seen on comparison of sections from biopsies at the two and four hour time periods. This was, in part, attributed to rapid early repair of these lesions, although some loss of staining may be due to the sloughing of damage-containing cells as part of the cytomorphosis process. This work clearly illustrates how amenable immunohistochemistry is to providing information regarding, not only localisation, but also repair of DNA damage induced *in vivo*.

CONCLUSIONS

The following aims, essential to the achievement of the objective (page 48, Chapter One, Introduction), have been accomplished:

1. Polyclonal antibodies were developed which recognise thymine-containing cyclobutane dimers within a specific sequence context and were determined, by ELISA-based methodology, to possess good specificity for such.
2. A novel and robust GC-MS assay for the detection and quantitation of cyclobutane thymine dimers in DNA was established.
3. The GC-MS assay for cyclobutane thymine dimers was subsequently used to produce DNA standards containing a known amount of dimer, allowing a secondary assessment of antibody binding and the determination of limits of detection.
4. Polyclonal antibodies characterised here (Ab529 and X18), along with other probes currently under investigation within the Division of Chemical Pathology (Ab532 and avidin) and commercially available (anti-8-oxodG monoclonal antibody), were used to successfully detect damage in naked DNA, fixed tissues, cultured human keratinocytes and human skin biopsies, following irradiation with either UVC or UVA.

From the above work and in the context of relevant, current research, it was concluded that in naked DNA, direct mechanisms of damage induction predominate. This results in large levels of cyclobutane thymine dimers following UVB and in particular UVC irradiation. Consistent with this mechanism of damage, free radical-induced base lesions *i.e.* 8-oxoguanine and glyoxal-DNA adducts, also occur in significant numbers with UVC, as evidenced by GC-MS and Ab532. No binding was seen with Ab529, following the UVA irradiation of DNA, due to relatively poor absorption of DNA at these wavelengths.

Formalin-fixed, paraffin-embedded tissue was shown to be a good model system for the establishment of an antiserum's applicability to detect and localise damage in tissues, along

with determining pertinent dose ranges for later work. However, it was established as not representing a more *in vivo* model than naked DNA, for the study of UVA radiation effects.

With UVA, however, no contribution from dimers could be detected immunochemically over the dose ranges used. In contrast, free radical damage increased with dose as shown by Ab532 and the anti-8-oxodG antibody in ELISA. Avidin proved to be a useful immunocytochemical probe for free radical DNA damage, detecting damage either by UVA or hydrogen peroxide. Furthermore, vitamin E amelioration of the damage caused by hydrogen peroxide was also unequivocally shown. Taken together, these results suggest the indirect mechanisms are the primary, if not sole processes leading to cellular DNA damage following UVA irradiation.

The principle advantage of an immunohistochemical technique for the demonstration of damage is its ability to locate damage *in situ* within a section, with the potential for quantitation and thereby removing the risks of artefact formation by DNA extraction and processing. However, not all antibodies are amenable to such techniques. This chapter reports the successful detection and localisation of lesions in the nuclei of UV-irradiated, formalin fixed and paraffin embedded sections of human skin. Differential staining was noted between cell types within a section and in all nuclei following protease digestion. From this, it was concluded that DNA conformation is important in either lesion formation or antibody interaction with the lesion. Application of this technique to various UV-related pathologies failed to show any positive staining. This is understood to be due to the rapid repair of these potentially mutagenic lesions. The semi-quantitative demonstration of a dose-response of antiserum binding in UVB irradiated sections of skin, down to doses as low as 2mJcm^{-2} suggested the technique to be suitable for the *in situ* detection of lesions in sections of biopsies from the UV irradiated skin of subjects. Availability of biopsy material limited the investigation of *in vivo* induced damage in *ex vivo* samples to UVB radiation and Ab529. Nonetheless, binding was shown to occur throughout the epidermis and into the dermis, suggesting that the UVB source emitted wavelengths effective at penetrating deep into the skin and inducing cyclobutane thymine dimers. Furthermore, decreases in staining intensity at two and four hours post-irradiation, suggested rapid repair of these lesions was occurring.

CHAPTER EIGHT

FURTHER WORK

“Let us not go over old ground, let us rather prepare for what is to come.”

- Marcus Tullius Cicero (106-43 BC.)

9.0 FURTHER WORK

Much of the future work directly pertinent to this thesis has been suggested in the discussion sections at the end of each chapter, although is outlined here. However, application of the techniques and information drawn together in this thesis makes the following proposed work not only possible, but also important.

From the work with the monoclonal which appears to recognise 8-oxodG in single-stranded DNA (3/8/1) described in Chapter 3, it is clear that further characterisation is required. This antibody would appear to be most promising and when characterised, may have the potential to detect 8-oxodG in applications, such as immunohistochemistry, a property elusive to many of the previously reported antibodies to 8-oxodG. The ability to produce monoclonal antibodies is now well established within the Division of Chemical Pathology, making the raising of monoclonal antibodies to detect, UV-induced DNA damage, such as cyclobutane thymine dimers, not only possible, but an important goal. Potential uses, in addition to those demonstrated in this thesis, are outlined in Future Work, below.

It may be possible for the GC-MS thymine dimer assay to be fully incorporated into the existing assay for oxidised purines (8-oxoG and 8-oxoA). This would rely on high temperature derivatisation, making removal of native purine bases essential to avoid artefact formation. Such a process may be achieved by HPLC pre-purification, or enrichment with monoclonal antibody columns, as described in Chapter 1, Introduction, page 43. This would allow the simultaneous quantitation of both oxidised purines and cyclobutane thymine dimers with the potential for the assay to be extended to include other lesions. With the development of other antibodies to oxidative and non-oxidative DNA lesions, the capacity for relevant, lesion-containing DNA standards, calibrated by GC-MS to be used for accurate quantitative ELISA assessments is clear. Indeed, this is an approach proposed for antisera 529 and X18 in Chapter 5, allowing quantitation of damage in extracted cellular DNA and requiring small amounts of DNA, compared to GC-MS or HPLC.

The possibility of immunohistochemically detecting and localising both oxidative and non-oxidative DNA damage in biopsies from subjects exposed to UVB, UVA and ultimately solar UV represents an important continuation of the work in this thesis. Results of such an

investigation may allow comparative studies of *in vivo* lesion repair and correlation with skin type, to be made.

9.1 FUTURE WORK

General introduction

The rates of incidence of malignant melanoma, basal cell carcinoma and squamous cell carcinoma, are all increasing amongst the general population (Cascinelli *et al.*, 1989; MacKie, 1993; Arlett *et al.*, 1993). Furthermore, both epidemiological and clinical studies suggest solar UV radiation to be the primary aetiological agent in each of these tumour types. Furthermore, molecular epidemiology suggests a link between UV-induced DNA lesions, mutagenesis (Tornaletti *et al.*, 1993; Tornaletti and Pfeiffer, 1994) and skin carcinogenesis (Dumaz *et al.*, 1994). It is acknowledged that DNA, a major cellular chromophore for UV, can absorb UV directly, giving rise to damage products ranging from base lesions and strand breaks, to DNA-protein crosslinks. A common photoproduct formed subsequent to the UV irradiation of DNA is the *cis, syn* cyclobutane thymine-thymine dimer (cyclobutadithymine) This lesion, produced uniquely by UV, is proposed to be important in cell cytotoxicity (Tornaletti *et al.* 1993), mutation (Tornaletti and Pfeiffer, 1994) and carcinogenesis (Alcalay *et al.*, 1990). Increasingly, interest is focusing on a possible role for oxidative DNA damage, which is also known to occur subsequent to UV irradiation.

On this basis, the future work proposed here will investigate the events which affect the cyclobutane dimer, post-excision from DNA, and (ii) the investigation of oxidative stress in UV-associated cutaneous tumours.

9.1 Investigation of the cellular processing of the excised oligomer containing cyclobutane thymine dimers.

The appearance of 8-oxo-2'-deoxyguanosine, a possible repair product of 8-oxoguanine from DNA, in human urine lead to the proposal that levels of this lesion in the urine may be used to non-invasively monitor *in vivo* oxidative stress (Ames, 1989; Simic, 1992). Likewise, it is here proposed that an assay to monitor urinary levels of cyclobutadithymine may possess the potential to:

- (i) monitor an individual's exposure to UV.
- (ii) determine an individual's ability to process this form of damage.

A number of prerequisites exist before such an assay can be developed:

- (a) it must be determined whether the lesion appears in the urine.
- (b) a suitable means of detection must be available - this must be specific and sensitive.

It is established within the literature that cyclobutadithymine lesions are a substrate for nucleotide excision repair (NER) and that the DNA repair product consists of a 24-29mer containing the lesion (Huang *et al.*, 1992). However, the post-excision processing of this oligomer has not been examined. Other lesions, such as 3-methyladenine, have been isolated from urine with polyclonal antisera prior to their quantitation (Friesen *et al.*, 1991), indicating another precedent for the detection of DNA damage products in urine. Experiments reported in the literature regarding the stability and pharmacokinetic characteristics of oligonucleotides suggest that, although they are degraded, urinary excretion represents the major elimination route for the degradative products (Agrawal *et al.*, 1995). This suggests that the possibility for a urinary assay is very real.

Clearly, it is important to elucidate the events pertaining to this oligo, following its excision. Experiments are proposed in which cultured cells are irradiated with non-lethal doses of UVB, known to induce the formation of cyclobutadithymine. The cells are subsequently allowed to recover and the loss of lesion from the DNA, along with its appearance in the culture medium, compared to controls, would be assessed by a suitable chromatographic technique.

It is known, from studies with antisense oligos, that only the degradative products of oligomer digestion appear in the urine. There must, therefore, exist intra- or extracellular processes, probably enzymic, which act on oligos, reducing their size. It is expected that the undamaged nucleotides within the dimer-containing oligo would be recycled by a cell salvage process, utilising an exonuclease. There exists some evidence to suggest that the excised oligos rapidly become subject to 5'→3' exonucleolytic attack (Galloway *et al.*, 1994) which the authors speculate may continue until the dimer is encountered, resulting in a 6- or 7-

mer. Whether this results in the deoxynucleotide form of free dimer has not been reported, and this would be established by the study.

Whilst the dimer is within the oligomer, it is believed that the existing polyclonal antisera (Ab529 and X18) would be applicable for the detection and cleanup of samples. Isolation of the dimer, once free from the oligomer, would require the generation of separate antisera, raised specifically to the free lesion. Studies utilising competitive ELISA have shown that Ab529 and X18 are unsuitable for the detection of free dimer (see Chapter 5).

On the basis of the successful establishment of dimer in the cell culture medium, attention will then be focused on determining whether this lesion then appears in urine of individuals exposed to UV. Enrichment of the urine for the dimer would be performed by an immunoaffinity column (possibly in conjunction with another form of solid phase extraction). The enriched sample could then be analysed by GC-MS, shown to be a specific technique for the *cis-syn* cyclobutane thymine dimer (Podmore *et al.*, 1996; see Chapter 4). For the analysis of UV-irradiated DNA by GC-MS, interfering peaks were noted, preventing monitoring of the strongest ion; as the urine sample would contain free dimer it is expected that the strongest ion may be used, increasing the sensitivity of the technique.

The final stage would involve modification of the existing GC-MS assay for the examination of dimers in urine, largely involve optimising solid-phase extraction procedure and rigorous testing. Once established and following ethical approval, a clinical analysis would be undertaken to assess levels of DNA damage and repair in healthy volunteers. The sample group would comprise of individuals using sunbeds, sunbathers and outdoor workers compared against a control group of individuals in which UV exposure is minimal, e.g. office workers. In order to investigate the effect of therapeutic UV exposure, patients undergoing photodynamic therapy and PUVA treatment will also be screened. Samples of urine would be obtained on recruitment, immediately prior and post UV exposure. The measurement of urinary 8-oxodeoxyguanosine, a recognised marker of oxidative stress, could also be carried out simultaneously by GC-MS according to the method of Teixeira *et al.* (1996). This would provide two indices of DNA damage and repair and their comparison and correlation to UV exposure would be examined.

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“I suppose every old scholar has had the experience of reading something in a book which was significant to him, but which he could never find again. Sure he is that he read it there, but no one else ever read it, nor can he find it again, though he buy the book and ransack every page.”

- Ralph Waldo Emerson

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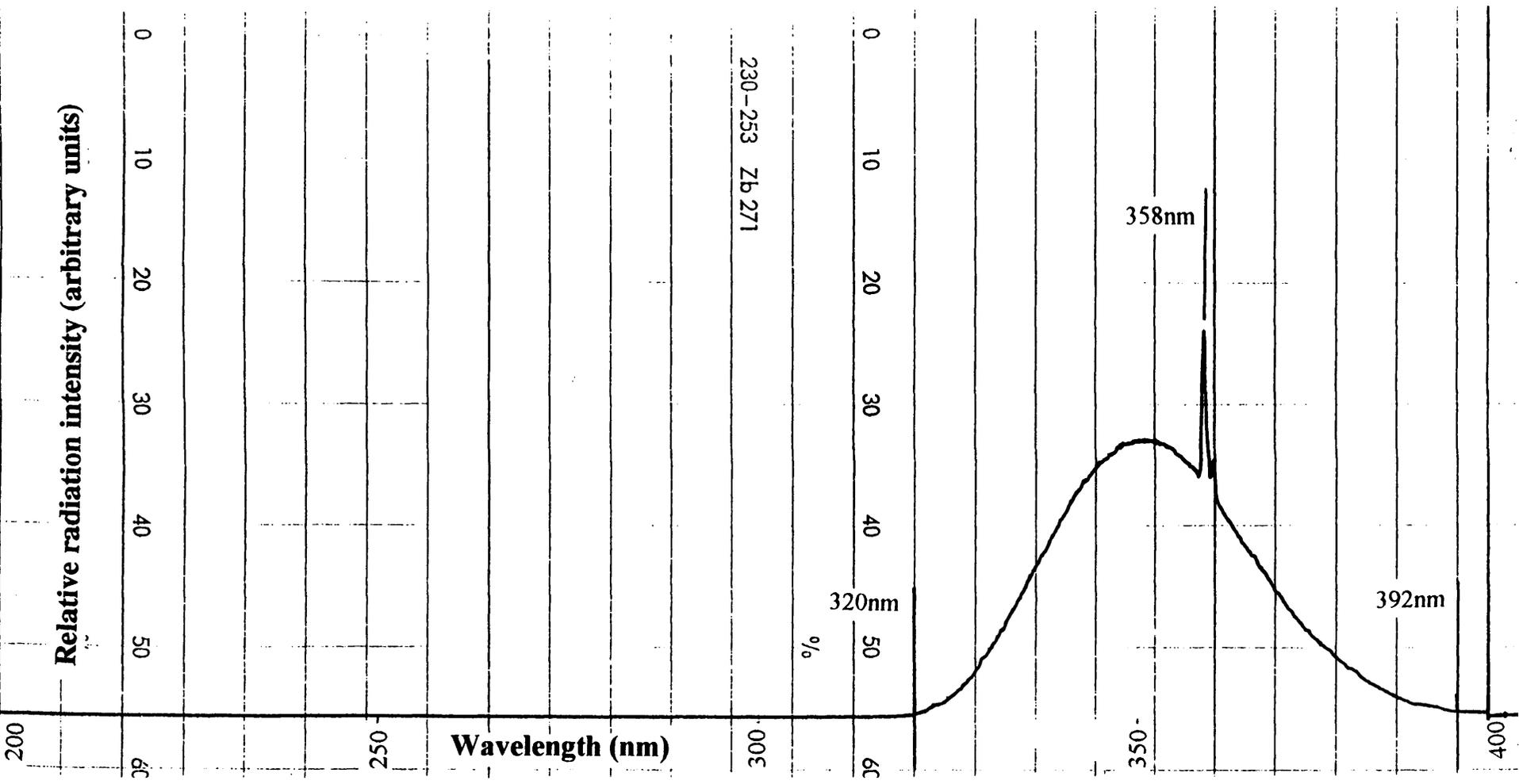
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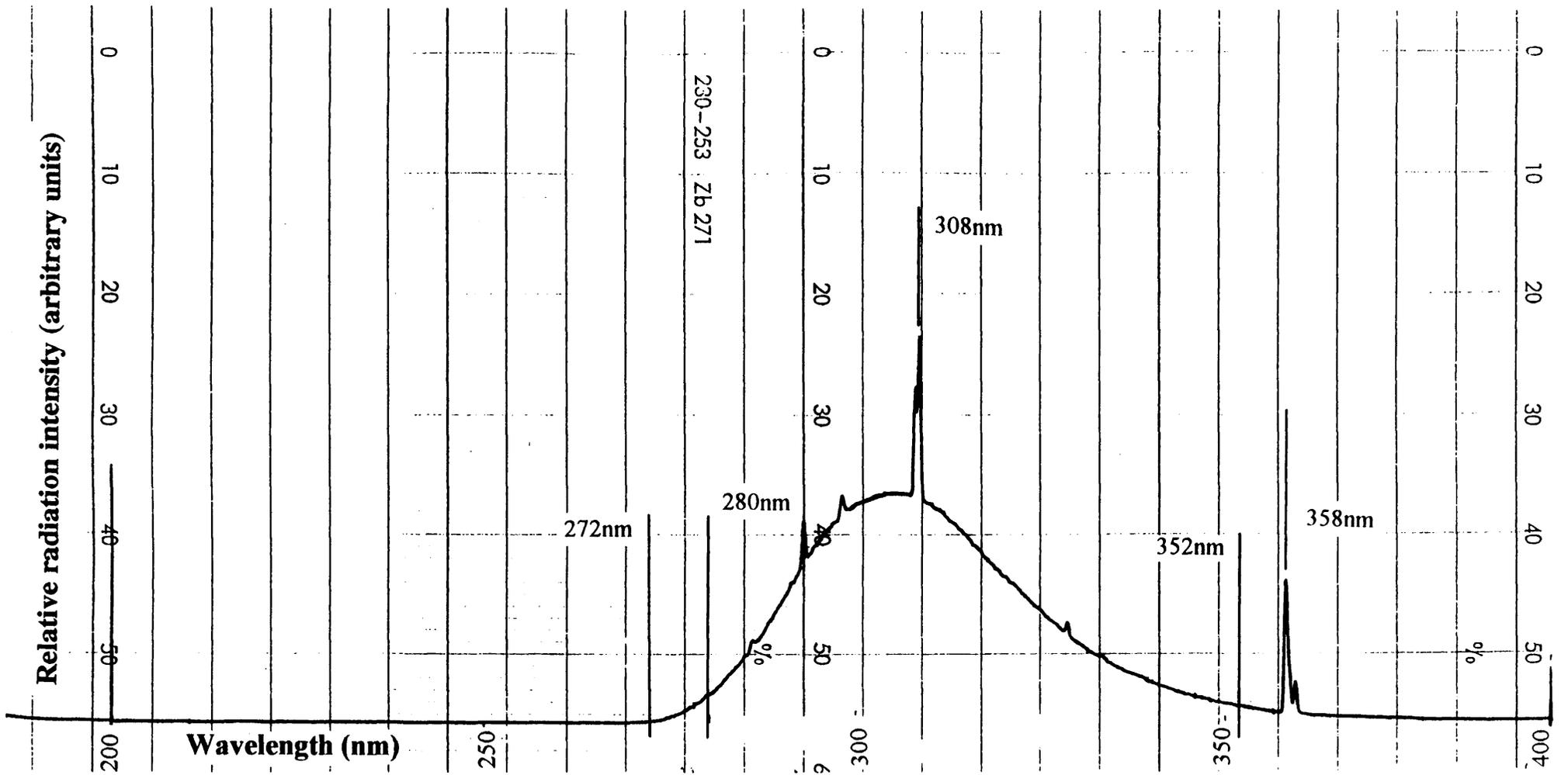
APPENDIX I**COMPOSITION OF MITOGENS IN RM+ MEDIUM**

| | |
|-------------------------|------------------------|
| Hydrocortisone | 0.41µg/ml |
| Choleratoxin | 1×10^{-10} M |
| Transferrin | 51µg/ml |
| Lyothyronine | 2×10^{-11} M |
| Adenine | 1.8×10^{-4} M |
| Insulin | 51µg/ml |
| Epidermal growth factor | 10ng/ml |

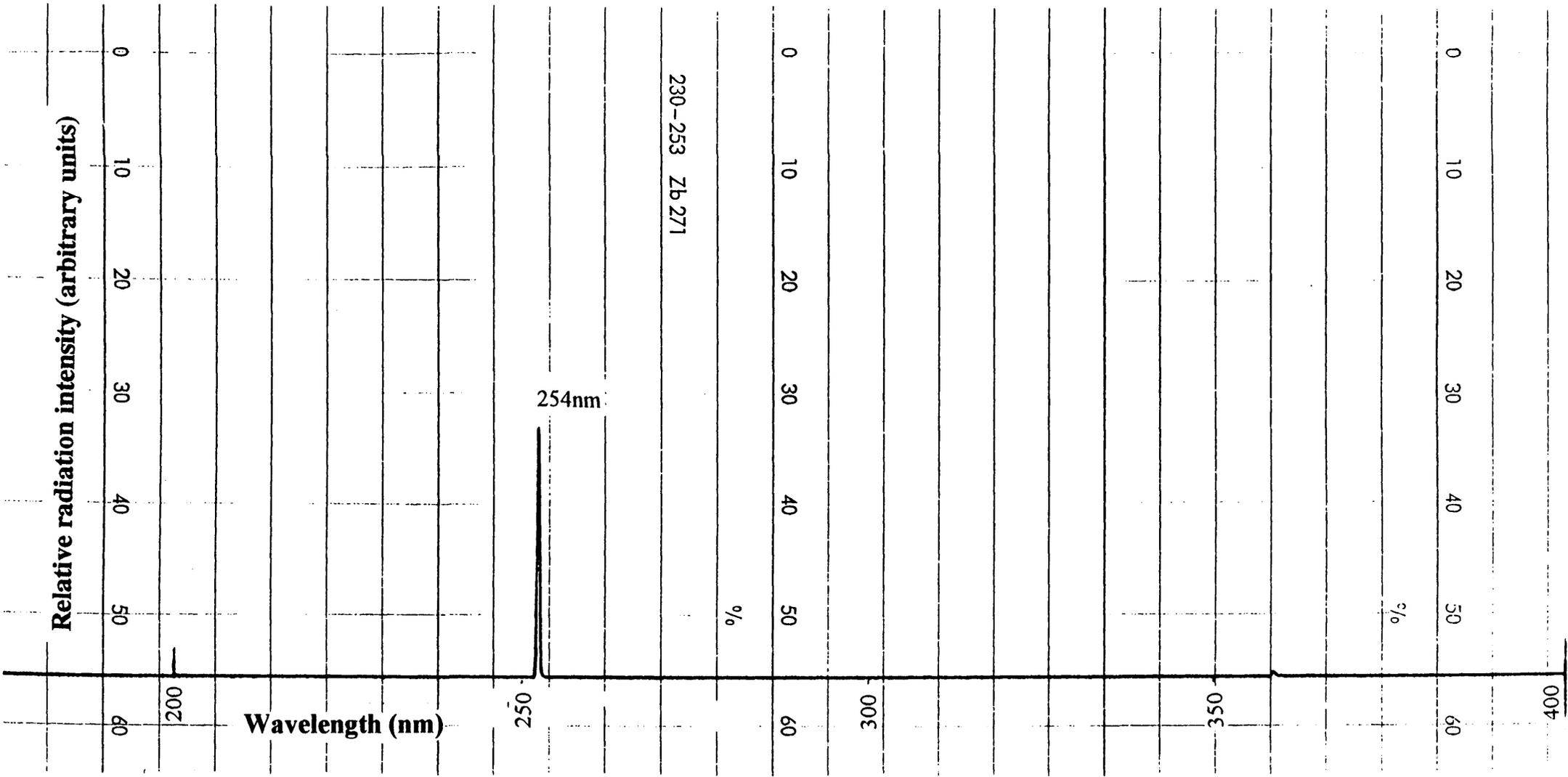
SPECTRAL CHARACTERISTICS OF UV LAMPS



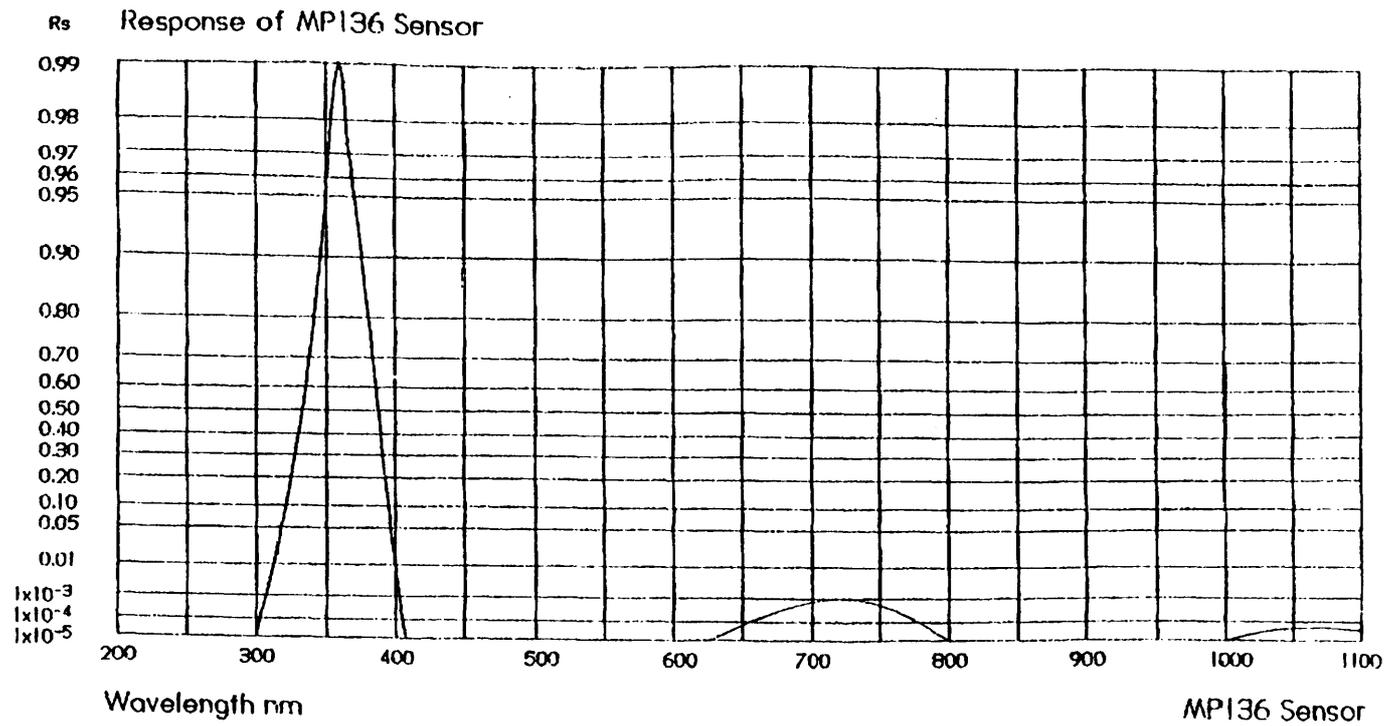
Spectral characteristics of the Chromatovue, model-UVL-56 lamp, described as UVA (320-392nm) and used for the experiments described in this thesis.



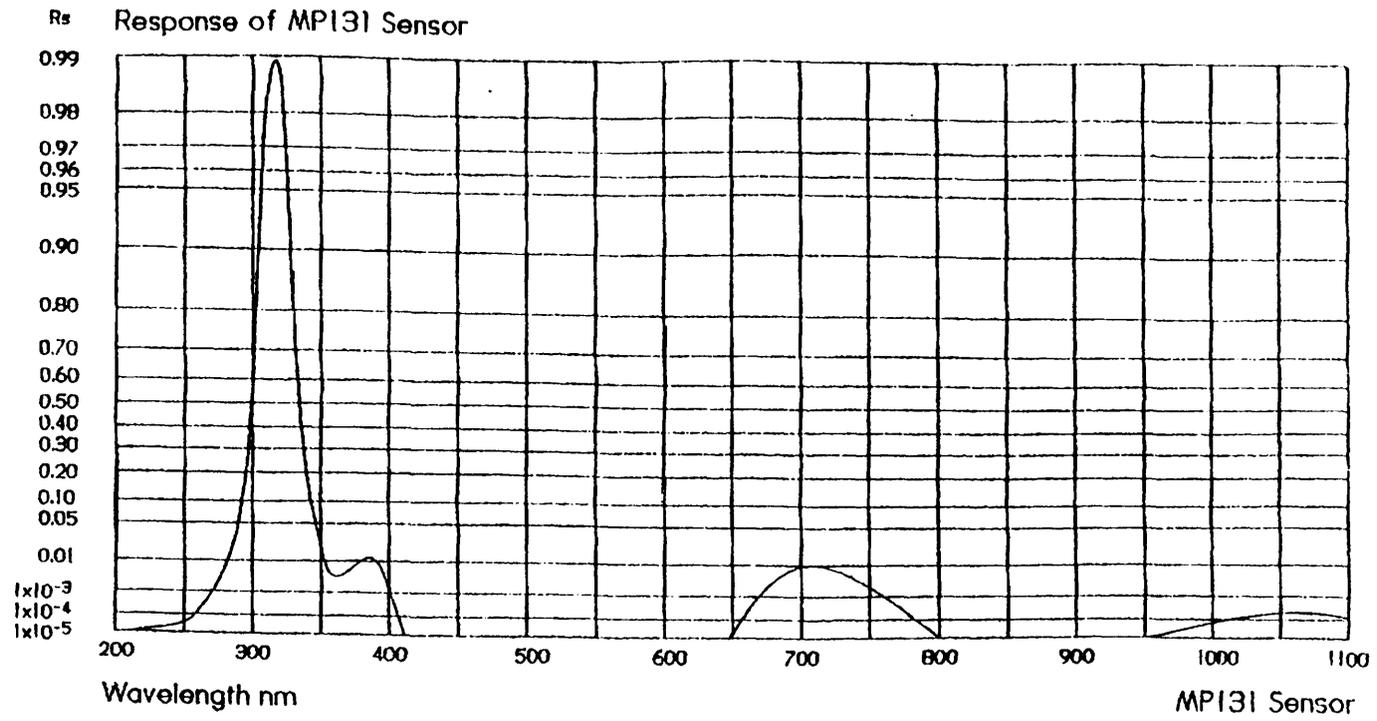
Spectral characteristics of the Chromatovue, model-UVM lamp, described as UVB (primary output between 272-352nm) and used for the experiments described in this thesis.



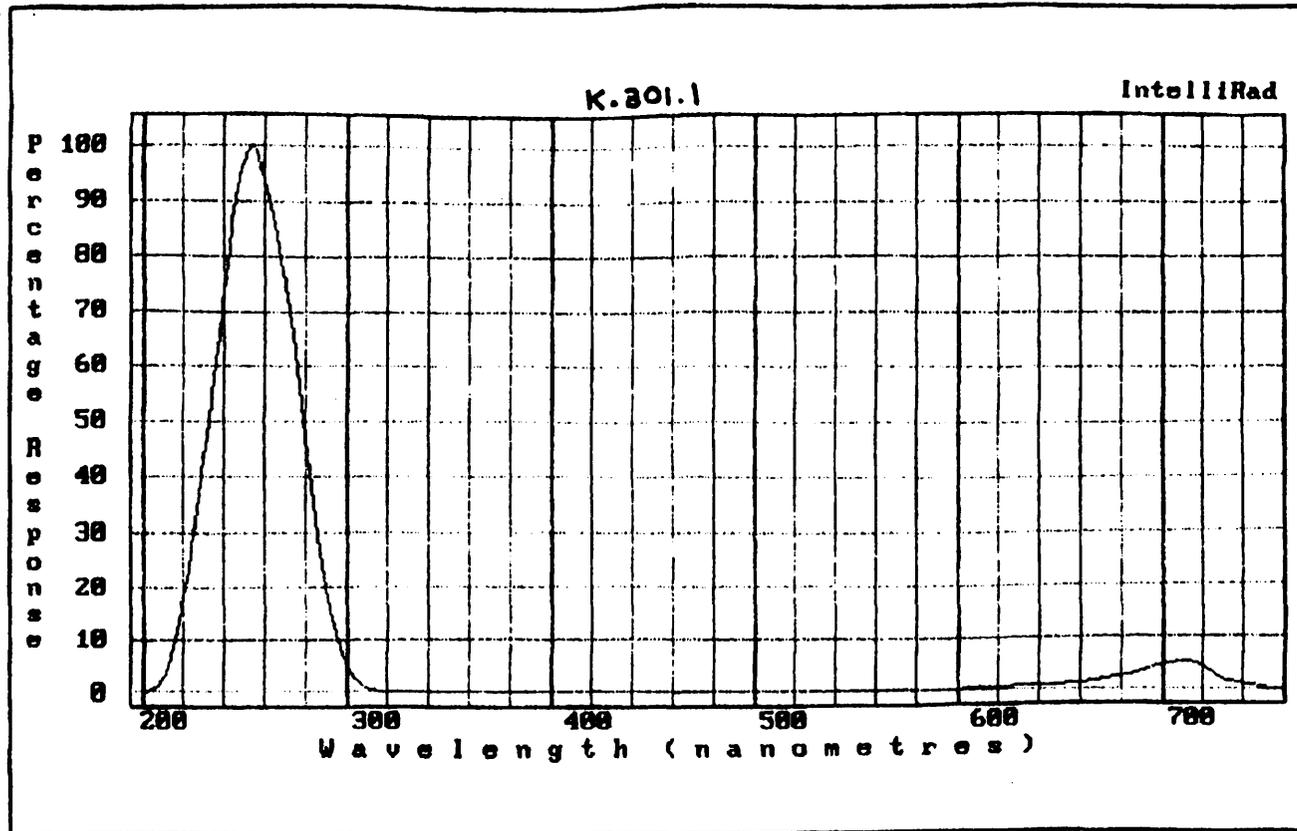
Spectral characteristics of the UVC (254nm) lamp, used for the experiments described in this thesis.



Spectral characteristic response of the MP136 sensor (UVA).



Spectral characteristic response of the MP131 sensor (UVB).



Spectral characteristics of the MC125 sensor (UVC).

APPENDIX IIa**CONVERSIONS****UV dose conversions**

$$1\text{Jcm}^{-2} = 10\text{kJm}^{-2}$$

$$1\text{Jm}^{-2} = 0.1\text{mJcm}^{-2} = 0.001\text{kJm}^{-2}$$

Units for biologically relevant doses of UVA and UVB are usually in Jcm^{-2} and mJcm^{-2} , respectively.

Conversions for DNA base damage

To obtain number modified DNA bases per 10^3 bases in DNA, from nmol/mg DNA (pmol/ μg), divide by 3.14 (or multiply by 0.318).

i.e. $1\text{nmol/mg} = 318$ modified bases per 10^6 DNA bases

APPENDIX III**PUBLICATIONS ARISING FROM THIS THESIS**

Cooke, MS., Finnegan, MT., Herbert, KE., Lunec, J. (1995) Cell cycle and dose-dependence of DNA damage and p53 expression following UVA irradiation. *Biochem. Soc. Trans.* **23**: 481.

Podmore, ID., **Cooke, MS.**, Herbert, KE., Lunec, J. (1996) Quantitative determination of cyclobutane thymine dimers in DNA by stable isotope-dilution mass spectrometry. *Photochem. Photobiol.* **64**, 2: 310-315.

Cooke, M., Podmore, I., Herbert, K., Lunec, J. and Thomas, S. (1996) UV-mediated DNA damage and its assessment. (Invited review) *Toxicology and Ecotoxicology News* **3**, 4: 101-109.

Cooke, MS., Mistry, N., Wood, C., Herbert, KE., Lunec, J. (1997) Immunogenicity of DNA damaged by reactive oxygen species - implications for anti-DNA antibodies in Lupus. *Free Radical Biology and Medicine.* **22**, 1/2: 151-157.

Cooke, MS., Herbert, KE., Butler, PC., Lunec, J. Role of conformation on the immunogenicity and antigenicity of the oxidative DNA lesion, 8-oxodeoxyguanosine. *Free Rad. Res.* (In press).

Cooke, MS., Podmore, ID., Mistry, N., Herbert, KE., Lunec, J. Investigation of UV-induced DNA damage by sequence-specific antisera and gas chromatography-mass spectrometry. (In preparation).

1. Quantitative Determination of Cyclobutane Thymine Dimers in DNA by Stable Isotope-dilution Mass Spectrometry.

ABSTRACT

In order to understand the role of UV[®]-induced DNA lesions in biological processes such as mutagenesis and carcinogenesis, it is essential to detect and quantify DNA damage in cells. In this paper we present a novel and both highly selective and sensitive assay using capillary gas chromatography combined with mass spectrometry for the detection and accurate quantitation of a major product of UV-induced DNA damage (cis-syn cyclobutadithymine). Quantitation of the cyclobutane thymine dimer was achieved by the use of an internal standard in the form of a stable ²H-labelled analogue. Both isotopically labelled and non-labelled dimer were prepared directly from their corresponding monomers. Each was identified as their trimethylsilyl ether derivative by GC-MS. Calibration plots were obtained for known quantities of both non-labelled analyte and internal standard. Quantitation of cis-syn cyclobutadithymine was demonstrated in DNA exposed to UVC radiation over a dose range of 0 to 3500Jm⁻². Under the conditions used the limit of detection was found to be 20-50fmol on column (equivalent to 0.02-0.05nmol dimer per mg DNA). The results of the present study indicate that capillary GC-MS is an ideally suited technique for selective and sensitive quantification of cis-syn cyclobutadithymine in DNA and hence UV-induced DNA damage.

2. UV-mediated DNA damage and its assessment. (Invited review)

ABSTRACT

The pathological consequences of ultraviolet irradiation is a topical subject of considerable concern both to the general public and to medical research. Photochemical events consequential to the absorption of ultraviolet radiation by cellular systems result in the formation of lesions to DNA via a variety of mechanisms, some of which involve free radical formation. The involvement of free radicals and reactive oxygen species has been implicated in a wide variety of toxicological and pathological processes, therefore understanding the mechanisms and pathological effects of UV-mediated DNA damage may provide a useful model system for understanding DNA damage in a wider context. The biological significance of UV-mediated DNA lesions is their suspected role in the mutagenic and carcinogenic potential of UV radiation. Considering the ubiquitous nature of this type of DNA damage, the development of methods not only for quantification but also for in situ demonstration of damage is essential. In this review we will primarily discuss UV-mediated

damage to DNA, however many of the technologies described will also have equal application to free radical- and oxidatively-mediated damage to DNA.

3. Immunogenicity of DNA damaged by reactive oxygen species - implications for anti-DNA antibodies in Lupus.

ABSTRACT

Reactive oxygen species (ROS) are implicated in the inflammatory, autoimmune, connective tissue disease, systemic lupus erythematosus (SLE), particularly in respect of processes leading to the formation of pathological anti-DNA antibodies. Exposure to ROS increases the antigenicity of DNA for SLE antibodies, but data on the immunogenicity of ROS-DNA are not conclusive. In this study, we have examined the immunogenicity in rabbits, of DNA modified by three hydroxyl radical generating systems. Additionally, we investigated the antigenicity of UVA, UVB and UVC irradiated DNA for lupus anti-DNA antibodies. Modification of DNA by both ROS and far UV dramatically increased its immunogenicity; the Fe^{2+} and H_2O_2 system resulted in antibodies which recognised both native and modified DNA. In our ELISA system, none of the UV antigens showed any antigenicity above native DNA for SLE sera. The data suggested that different profiles of antigenicity and immunogenicity arise dependent on the method of ROS production, but also that ROS-DNA may be a factor in antigen-driven immune complex formation in SLE.

4. Role of conformation on the immunogenicity and antigenicity of the oxidative DNA lesion, 8-oxodeoxyguanosine.

ABSTRACT

Damage to DNA by reactive oxygen species is acknowledged to be an important factor in a number of pathological conditions, including ageing and carcinogenesis. As a consequence, the development of methods for the sensitive detection and quantitation of oxidative DNA lesions has been of paramount importance. The oxidatively modified base product which has achieved most attention is 8-oxo-2'-deoxyguanosine (8-oxodG) and is a recognised marker of oxidative DNA damage.

Although both polyclonal and monoclonal antibodies have previously been raised to 8-oxodG these have, for the most part, failed to recognise this lesion within the DNA polymer. We have, through dilution cloning, produced a monoclonal antibody which appears to preferentially recognise 8-oxodG over deoxyguanosine (dG) in single-stranded

oxidatively modified DNA. Such discrimination was not apparent when the DNA was double-stranded. Previous work has shown that 8-oxodG favours the syn glycosidic conformation due to steric repulsion, whereas dG assumes the anti. We present initial data that appears to support the postulate that it is these differences in conformation, in addition to structural recognition of the lesion itself, which are responsible for the discrimination, by our antibody, of 8-oxodG over dG in single-stranded DNA.