PROCESSING OF UV-INDUCED DNA DAMAGE IN HUMAN SKIN CELLS

VERWERKING VAN DOOR UV VEROORZAAKTE DNA BESCHADIGINGEN IN MENSELIJKE HUIDCELLEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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De foto op de omslag toont een gefuseerde menselijke cel met twee kernen en drie andere cellen met een enkele kern. Met een fluorescerend antilichaam zijn cyclobutaan thymine dimeren in het DNA, geïnduceerd door bestraling met ultraviolet licht, zichtbaar gemaakt. De gefuseerde cel vertoont een geringe fluorescentie omdat de thymine dimeren waren verwijderd via fotoreaktivering.

Aan mijn ouders

Voor Elly, Kim, Martijn en Eline

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I GENERAL INTRODUCTION

I.1 INTRODUCTION

In Western societies, changes in life style such as the increased leisure time, the popularity of "sun holidays" and the use of clothing that leaves larger parts of the body uncovered, have resulted in a substantial increase in exposure of the human skin to ultraviolet radiation (UV) from the sun and from artificial sources. Also, the degradation of the ozone layer in the stratosphere probably due to atmospheric pollution may result in an increase in the amount of shorter-wavelength UV that reaches earth. UV is known to induce damage in DNA, which may lead to alterations (mutations) in the genetic information of the cell. When these mutations affect the expression or function of genes controlling cell growth and differentiation, this can result in the initiation of cancer. Therefore, an increased exposure to UV has to be considered as a health hazard. In man, skin tumors are among the commonest cancers and it is one of the few human tumor types for which the predominant carcinogen is known, *viz.* sunlight (O'Rourke and Emmett, 1982; Fitzpatrick and Sober, 1985). This has resulted in a stimulation of research on the deleterious effects of UV on DNA, which topic is dealt with in this thesis.

The precise cellular damage(s) responsible for cell-death or mutation following UV-irradiation have not been identified with certainty. A wide variety of DNA lesions can be induced by UV at wavelengths <310 nm. However, there is massive evidence that the two most abundant lesions, the cyclobutane pyrimidine dimer and the (pyrimidine-pyrimidone) (6-4)photoproduct are closely associated with the above mentioned biological effects of UV. The number of lesions induced varies with the wavelength, in a way which differs for each lesion; however, for UV-induced DNA damages other than cyclobutane dimers accurate action spectra (*i.e.* the relation between a certain effect and the wavelength) for the total UV-region are not available.

Living cells possess efficiently working enzyme systems to restore the integrity of their DNA once damage has been induced (Friedberg, 1985 for a review on DNA repair). This applies also to UV-induced damages, for the removal of which different mechanisms of DNA repair are operative in the cell. Not all lesions will be removed equally fast; rapid repair kinetics of a certain lesion may be an indication of its deleterious effect for the cell. In one type of DNA repair, photoreactivation, specific restoration of cyclobutane dimers to the original pyrimidines is brought about under the influence of visible light (Sancar and Sancar, 1987). The predominant

mechanism, however, according to which UV lesions appear to be removed from cellular DNA is the so-called excision repair. In this repair process a number of enzymes and other proteins cooperate to excise a stretch of the damaged DNA strand and to replace it by newly synthesized DNA (Sancar and Sancar, 1988).

One of the best characterized examples of the relationship between exposure to agents that damage DNA and carcinogenesis, and of the importance of cellular repair in this respect, is directly associated with the effects of UV. This concerns the human heriditary disease xeroderma pigmentosum (XP). Patients suffering from this autosomal recessive disorder are marked by an extreme sensitivity of the skin to (the UV part of) sunlight. There is a high tendency to develop neoplasia (basal and squamous cell carcinomas and melanomas) in particular on sun-exposed areas of the skin. The basis of the elevated tumor-incidence, which is about 1000-fold enhanced, is thought to be a defect in DNA repair. In cells from most XP patients, UV-induced DNA damage is not removed efficiently (Zelle and Lohman, 1979), resulting from a deficiency in the excision repair pathway (Cleaver, 1968).

I.2 OUTLINE OF THIS THESIS

It was the aim of the experimental work presented in this thesis to identify -in mammalian cells- the lesions responsible for UV-induced biological effects, such as cell-killing, to study the relation between the removal of lesions and the occurrence of repair synthesis of DNA ("unscheduled DNA synthesis") and to investigate the possible role and practical significance of photoreactivation in humans.

The chapters II to IV summarize the literature on the effects of UV on mammalian cells, with regard to the DNA lesions induced, the protective mechanisms to cope with cellular damages, and the consequences of DNA damage. The experimental work presented in this thesis is described in appendix papers I to V. In summary, survival of cells was determined after irradiation with various wavelengths of UV, which alters the ratios of DNA damages induced. Also, UV-induced specific DNA damages were detected and photoreactivation was used as a tool to eliminate cyclobutane dimers and -thus- to investigate the noxiousness of other DNA damages. For the detection of cyclobutane thymine dimers, the most abundant UV-induced DNA lesion, a specific immunochemical method was developed and used in combination with photoreactivation to investigate which lesions are involved in excision repair. In addition, the presumed occurrence of photoreactivation in human skin, a possible acquired mechanism for humans to reduce the deleterious effects of UV light, was studied with this new method.

II EFFECTS OF UV ON MAMMALIAN CELLS IN PARTICULAR ON HUMAN SKIN

II.1 THE SPECTRUM OF ELECTROMAGNETIC RADIATION

From the sun a wide spectrum of electromagnetic energy radiates through space in all directions. Electromagnetic radiation exhibits both wave-like and particle-like properties. The radiant energy is present in discrete quantities (quanta), the unit being the photon, with an energy content proportional to the frequency of the radiation. The spectrum of electromagnetic radiation can be subdivided -in order of increasing wavelength and decreasing energy- into cosmic rays, gamma rays, X-rays, ultraviolet radiation, visible light, infrared radiation, micro waves and radio waves. Reactions of organic chemicals which follow upon the absorption of quanta of visible or ultraviolet radiation are classified as photochemical reactions. The ratio of the number of molecules reacting and the number of quanta absorbed is defined as the quantum efficiency. Visible light (wavelengths of approximately 400-700 nm) consists of radiation that is able to pass through the lens of the human eye, and is absorbed in the retina. Visible light also penetrates through the upper layer of the skin and is absorbed in the underlying tissue, but usually the photons do not carry enough energy to cause tissue alterations or damage. UV with its shorter wavelength compared to visible light, however, is absorbed mainly in the upper layer of the skin and it consists of photons that contain sufficient energy to damage essential macromolecules in the cells. The spectrum is usually subdivided into three parts, *viz.* UV-C (from 200-280 nm), UV-B (from 280-315 nm) and UV-A (from 315-400 nm) based on a combination of physical properties and biological effects of the radiation in these regions.

II.2 GENERAL EFFECTS OF UV ON HUMAN SKIN: PROTECTIVE MECHANISMS AND HEALTH EFFECTS

An important function of human skin is protection of underlying tissue and organs against exogenous damaging agents. As a result, skin itself is exposed to these influences. An example is the effect of solar UV, which -due to its limited penetration- does not reach tissues underneath the skin. In human skin several mechanisms exist to minimize (light- or UV-induced) damage. The stratum corneum -the outer, dead layer of the skin- is composed principally of a fibrous protein,

keratin, which not only absorbs significantly in UV-B and UV-C regions of the spectrum, but also, because of its structural characteristics, scatters most visible radiation. The presence of melanin in the skin protects this organ further from damage by light. This pigment, which is synthesized in the melanocytes and packaged in organelles called melanosomes, is "injected" into the keratinocytes (Fitzpatrick *et al.*, 1979). The dark pigment may serve to absorb and scatter UV radiation physically. Also, it may play a role in trapping free radicals generated as a result of UV exposure (Harber and Bickers, 1981).

Increases in epidermal melanin pigmentation (tanning) occur upon exposure to UV. Two different types of tanning can be distinguished: immediate tanning and delayed tanning. UV-A radiation is very effective in immediate tanning, which begins very shortly after onset of radiation and peaks within 1-2 h (Epstein, 1977). It does not involve melanin synthesis, but arises from alterations in melanin already present (Pathak and Stratton, 1968; Kaidbey and Kligman, 1978). The ability to show immediate tanning is a function of both the natural coloration of the unexposed individual and the extent of tanning previously acquired. Delayed tanning becomes noticeable much later after exposure than does the immediate process. It first becomes apparent between 2 and 3 days after UV exposure, peaks at 13 to 21 days, and subsides over a period of several months. In contrast to immediate tanning, which is caused by UV-A radiation but apparently not by UV-B, the major peak in the action spectrum for delayed tanning resides in the UV-B region. However UV-C, UV-A, and visible light in the blue region are also capable to some extent of inducing delayed tanning. As with immediate tanning, the relative dose of radiation required to produce delayed tanning depends on both the constitutive nature of the exposed skin and its history of previous exposure. Delayed tanning is a result of new melanin production in the epidermal skin layer serving to decrease transmission of UV in the deeper layers. This protective effect is further increased due to thickening of the stratum corneum and the epidermal cell layer (Cripps, 1981; Gange and Parrish, 1983; Kaidbey and Kligman, 1978). This response results from a sustained increase in epidermal mitosis, associated with increases in DNA, RNA and protein synthesis that follow already a single exposure to UV (Epstein *et al.*, 1970). Additional protection of the cells is offered by intracellular glutathione, through scavenging of active oxygen radicals that are produced by irradiation with long-wavelength UV (Tyrrell and Pidoux, 1986; Tyrrell and Pidoux, 1988).

UV-induced erythema or sunburn is the most obvious of the photobiological responses evidenced by skin. Virtually all humans are susceptible to this inflammatory reaction to a greater or lesser degree, depending on the extent of

skin pigmentation. Erythema is perhaps one of the most extensively studied photobiological responses in humans. A faint, transient redness may be seen within minutes upon exposure to UV, reaches its optimum at 12-24 h, and then fades over several days (Farr *et al.*, 1988). Erythema is probably induced in the epidermal cell layers, where cellular substances are released which, after diffusion, cause a dilatation of blood vessels in the dermis (Van der Leun, 1965).

The only well-documented and proved beneficial effect of UV for humans is the conversion of 7-dehydrocholesterol into pre-vitamin D_3 in skin. Pre-vitamin D_3 is further metabolized to vitamin D_3, which then is transformed in the liver and kidneys into 1,25-dehydroxyvitamin D_3. Vitamin D_3 is required for calcium absorption and calcification of the bone; insufficient UV exposure is reported to cause rickets in children due to a lack of (pre-)vitamin D3 (Loomis, 1970).

When UV damage to DNA is induced, DNA-repair systems come into action which, in most cases, operate without making errors. Apparently, normal human skin is very well adapted to cope with UV-induced DNA lesions, since exposure to low UV-doses that do not cause sunburn or any other detectable effect on the skin, nevertheless can result in the induction of up to one million of DNA damages per epidermal cell (Gange *et al.*, 1985; Roza *et al.*, 1988, Appendix paper III), and this may be related to the induction of skin cancer. Unquestionably, skin cancers are among the commonest tumors in man. Basal cell carcinoma and squamous cell carcinoma are the predominantly detected types, but fortunately their tendency to metastasize is small. In contrast, the rapidly growing melanomas metastasize easily and these cause a high mortality (Urbach, 1984).

Another skin-related effect induced by UV exposure is premature aging, often seen with outdoor workers. It is the result of prolonged exposure over many years to solar UV. Characteristic features are dryness and cracking of the skin, keratosis, and a loss of elasticity (Gilchrest, 1979; Kligman, 1979).

II.3 CELLULAR DAMAGE INDUCED BY UV-EXPOSURE

Research in recent decades has shown that the genetic material of the cell, DNA, is the most important target of UV exposure. It is essential for the cell that the information in DNA stays intact to assure its continued existence and functioning. Although it has been reported that UV can cause damage to proteins (Smith, 1976) or cellular membranes (Moss and Smith, 1981), it is generally assumed that the adverse biological effects of solar UV are primarily due to its absorption by nucleic acids, as at wavelengths <310 nm the degree of this absorption correlates to the

biological effects, including cell-killing, mutation induction and carcinogenesis. Nucleic acids absorb UV strongly, owing to aromaticity of the bases, with a maximum around 260 nm. Although the absorption decreases sharply towards higher wavelengths, it is still significant at 300 nm. Proteins absorb in the UV region from 220-300 nm due to the aromatic amino acids and the peptide bond, but the maximum absorption coefficient is about one order of magnitude lower than that of nucleic acids. Biological substances with unsaturated bonds, such as flavins, porphyrins, steroids, quinones and carotenoids may be involved in the effects of UV too, especially when high fluences are involved in those UV regions where nucleic acids do not absorb strongly.

II.4 UV-INDUCED DNA PHOTOPRODUCTS

It is generally accepted that an important breakthrough in DNA photochemistry was made by Beukers and Berends (1960) and by Wang (1960), who, independently, isolated and identified the cyclobutane thymine dimer from a UV-C irradiated frozen solution of thymine. Evidence for the occurrence of thymine dimers in irradiated DNA was initially obtained by Beukers *et al.* (1960) and substantiated by Wacker *et al.* (1961). Since then, this structure has received much attention in prokaryotic and eukaryotic photobiology. Cyclobutane dimerization was shown by Setlow and Carrier (1966) to extend to pyrimidines in general. In DNA a cyclobutane pyrimidine dimer is formed by linkage of two adjacent pyrimidines at the carbon atoms in the positions 5 and 6 (figure 1). Only the cis-syn dimer is formed in native DNA (Patrick and Rahn, 1976); here cis, refers to the orientation of the CH_3 residues, and syn to the orientation of the pyrimidines. The presence of a cyclobutane dimer leads to an distortion of the DNA helix by 19.7° and introduces a kink of 27.0° that protrudes into the major groove: at the site of the dimer the helical displacement is 2.66 Å (Pearlman *et al.*, 1985). Other important photoproducts induced in DNA by UV-C are the pyrimidine-pyrimidone (6-4)photoproducts(Lippke *et al.*, 1981; Rycyna and Alderfer, 1985).

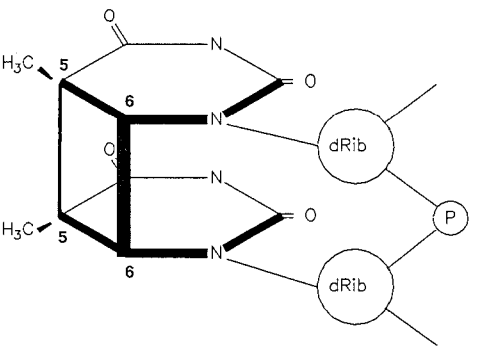

Figure 1. Structure of the cis-syn cyclobutyl thymine dimer.

Analyses of the structure of the (6-4)photoproduct in a TC dinucleotide revealed that the plane of the 3' cytosine base was shifted 90° relative to the 5' thymine base, besides a transfer of the NH_2-group from C to T (Franklin *et al.*, 1985). In principle, 4 different products of, both, cyclobutane dimers and (6-4)photoproducts can be formed containing thymidine and cytidine. At low UV-C fluences about 50% of all cyclobutane dimers are formed at TT sequences, 40% at TC/CT, and only 10% at CC sequences (Ellison and Childs, 1981). In contrast, (6-4)photoproducts are observed in DNA only at TC, CC, and occasionally, at TT sequences (Lippke *et al.*, 1981; Sancar and Rupp, 1983). The (6-4)photoproduct is subject to photolysis upon irradiation with 320-nm UV: a "dewar" isomer is produced (Taylor and Cohrs, 1987). In figure 2 the structure formulas of the TC(6-4)photoproduct and its "dewar" isomer are shown.

Saturation of pyrimidines at the C5-C6 bond can lead to cytosine hydrate and thymine glycol (figure 3, Boorstein *et al.*, 1989; Patrick and Rahn, 1976). Recently, a new photoproduct was suggested to be formed at ACA sequences;

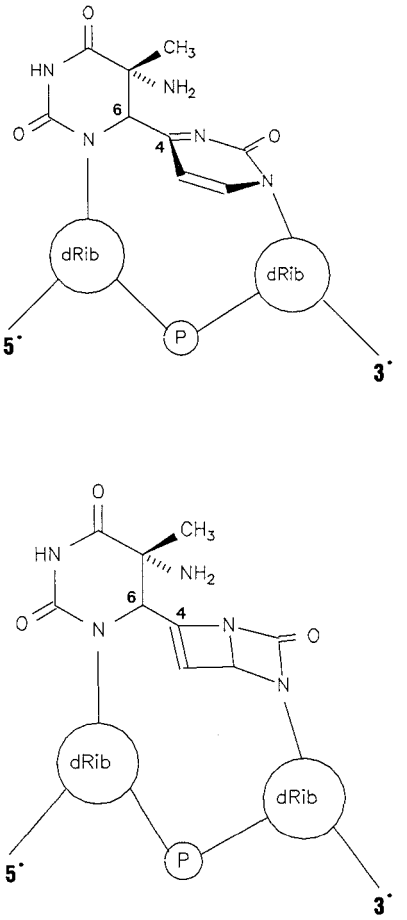

Figure 2. Structures of the TC (6-4)-photoproduct and its "dewar" isomer.

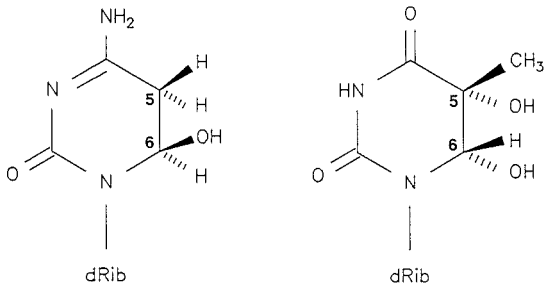

Figure 3. Stuctures of cytidine hydrate and thymidine glycol.

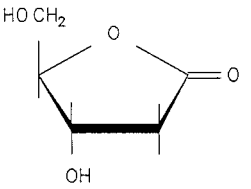

Figure 4. Structure of 2-deoxyribonolactone photoproduct formed from cytidine at ACA sequences.

the cytosine is removed and the a-basic site is converted into 2-deoxyribonolactone (figure 4, Urata *et al.*, 1989). Purines are involved less frequently in UV-induced photoproducts; the lesions found comprise two different dimeric adenine photoproducts (Gasparro and Fresco, 1986; Kumar *et al.*, 1987), a not yet identified purine photoproduct (Gallagher and Duker, 1986), and a photoproduct at TA sequences (Bose and Davies, 1984). The structure formulas of these purine-containing lesions are presented in figure 5. In addition, miscellaneous other DNA damages are induced by UV, including single-strand breaks and DNA-protein crosslinks (Peak *et al.*, 1985; Peak *et al.*, 1987; Rosenstein and Ducore, 1983; Shetlar, 1980).

Not so much is known about the induction of DNA photoproducts at UV-wavelengths >310 nm. Thymine glycols could be induced in cells at 313 nm (Hariharan and Cerutti, 1977). The other photoproducts known to be produced at these wavelengths comprise cyclobutane dimers (Enninga *et al.*, 1986), single-strand breaks (Peak *et al.*, 1987; Roza *et al.*, 1985a, Appendix paper I) and DNA-protein crosslinks (Han *et al.*, 1984; Peak *et al.*, 1985). Nothing is known about the induction of purine-photoproducts by long-wavelength UV; when DNA was irradiated with broad-wavelength UV (250-400 nm), some incision at purines mediated by a UV-specific endonuclease was found (Gallagher and Duker, 1986), but the incision sites were not consistent with the photoproducts known so far.

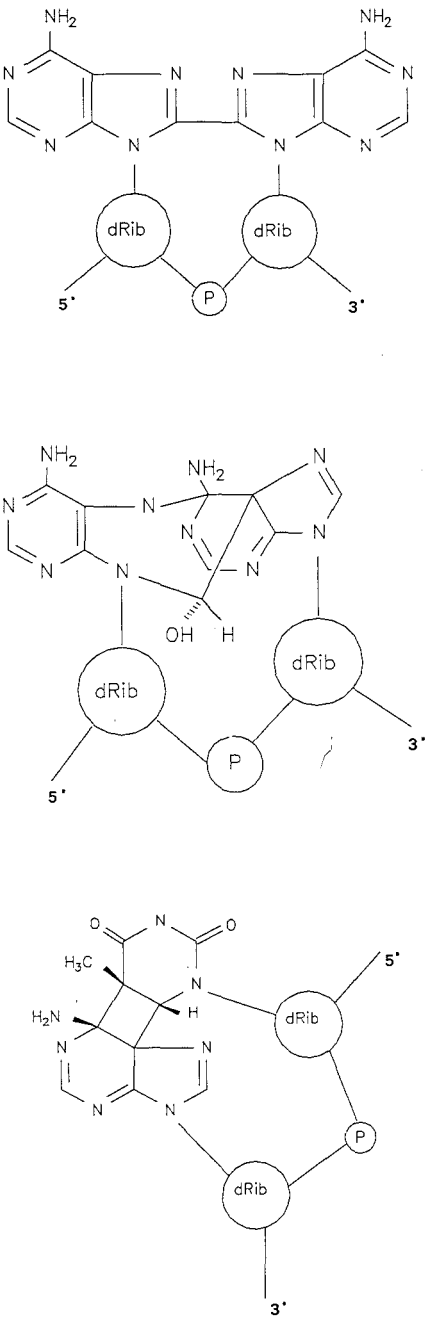

Figure 5. Structures of the dimeric purine-containing photoproducts.

II.5 DETECTION OF PHOTOPRODUCTS IN DNA

In general the nature and the amount of each photoproduct present in irradiated DNA can be determined in two ways: (1) enzymatic or acid hydrolysis of the nucleic acid followed by separation of the individual components (including photoproducts) by some type of chromatographical procedure, and detection of their presence by absorbance or radioactivity measurements and (2) detection of a certain photoproduct *in situ* on the basis of a specific property of the product (*e.g.,* absorbance, fluorescence, affinity to specific antibodies).

The cyclobutane dimer is the DNA damage for the detection of which by far the most methods have been described. It is acid-stable and after hydrolysis of DNA it can be isolated chromatographically (Reynolds *et al.*, 1981); either paper chromatography, thin layer chromatography or high performance liquid chromatography have been used. Recently, a new powerful variant on chromatographical analysis was described, based on enzymatic DNA hydrolysis, (post)labelling of the (oligo)nucleotides and gel electrophoresis (Weinfeld *et al.*, 1989). Biochemical quantification of the dimer in DNA is possible with specific endonucleases (Paterson *et al.*, 1973; Wilkins, 1973): an endonuclease present in an extract of *Micrococcus luteus* or the enzym purified from phage T4 infected cells (Friedberg and Clayton, 1972), which both make single-strand breaks at the dimer site in UV-irradiated DNA. Sedimentation of (nicked) DNA in an alkaline sucrose gradient can be used to detect the breaks, down to one break per 10^8 daltons, the sensitivity of the method depending on the molecular weight of the DNA. A much improved sensitivity is reached by studying the molecular weight distribution of the DNA fragments with alkaline elution (Fornace, 1982). Several methods have been described to circumvent the need of radioactively labelled DNA in these UV-endonuclease assays. Fluorescent staining of DNA with ethidium bromide (Brash and Hart, 1983), but also immunochemical detection of DNA in alkaline sucrose gradient fractions have been described (Vijg *et al.*, 1986). Another modification on the UV-endonuclease assay involves alkaline gel electrophoresis, membrane-transfer of DNA and hybridization with a specific DNA-probe, which offers the possibility to detect dimers at the level of the gene (Bohr *et al.*, 1985). The UV-endonuclease assay, making use of the *M. luteus* extract or the T4 enzyme, is specific for cyclobutane dimers and does not detect other lesions, in contrast to the chromatographic assays. However, when the *E. coli* UvrABC excinuclease is used, the method is non-specific and a wide variety of lesions can be studied (Thomas *et al.*, 1988).

A different approach for analysing lesions is the immunoassay. This assay is

based on the binding of antibodies to specific lesions in DNA. The first antibodies with specificity for thymine dimers have been prepared by Levine *et al.* (1966). As an immunogen UV-irradiated DNA complexed to methylated bovine serum albumin was used. This has the disadvantage that antibodies against all kinds of UV-induced photoproducts are to be expected in the serum, which can give rise to misleading results (Eggset *et al.*, 1983; Eggset *et al.*, 1987; Clarkson *et al.*, 1983; Mitchell *et al.*, 1982; Mitchell *et al.*, 1985a; Mitchell and Clarkson, 1984b). Radioimmunoassays with specific tracer-DNA molecules containing for instance only cyclobutane thymine dimers can circumvent this drawback (Mitchell *et al.*, 1985a). On the other hand, immunization of rabbits with a specific hapten, *i.e.* a thymine dimer, coupled to a carrier protein yielded highly specific polyclonal antibodies (Klocker *et al.*, 1984). Also various monoclonal antibodies against UV-induced photoproducts have been isolated, which have the advantage of unlimited supply and single specificity. Monoclonal antibodies directed against the thymine dimer in DNA (Strickland and Boyle, 1981; Roza *et al.*, 1988, Appendix paper III) as well as against (6-4)photoproducts (Mori *et al.*, 1988) have been reported.

In literature, a wide variety of sensitive immunoassays for detection of UV-induced DNA lesions has been described, including radioimmunoassay (Klocker *et al.*, 1982; Mitchell and Clarkson, 1981), enzyme-linked immunosorbent assay (Wani *et al.*, 1984; Roza *et al.*, 1988, Appendix paper III), immuno-slot blot assay (Wani *et al.*, 1987), flow cytometry (Wani *et al.*, 1984) and (quantitative) immunofluorescence microscopy (Lesko *et al.*, 1989; Mori *et al.*, 1989; Roza *et al.*, 1988; Roza *et al.*, 1990, Appendix papers III, IV and V). The latter method offers the possibility to quantitate DNA damage at the level of the single cell and it can be used on skin biopsies obtained after *in vivo* irradiation (Roza *et al.*, Appendix paper V).

II.6 RELATIVE AMOUNTS OF UV-INDUCED DNA DAMAGE

The induction of DNA lesions by UV is wavelength dependent. Formation follows photon absorption. Most photoproducts are induced efficiently at the absorption maximum of DNA, *i.e.* 260 nm, but higher yields can be reached at somewhat longer wavelengths (Ellison and Childs, 1981). Cyclobutane dimers are the most prevalent lesions upon irradiation with UV-C, although at certain dipyrimidine sites (6-4)photoproducts occur at a greater frequency (Brash *et al.*, 1987; Brash and Haseltine, 1982). The relative rates of induction of the dipyrimidine photoproducts are highly dependent on the AT/GC ratio of the DNA (Patrick and Rahn, 1976). In human cells, the total amount of (6-4)photoproducts, detected as

sites labile to hot alkali, is 15-35% of that of cyclobutane dimers detected as sites sensitive to a UV-endonuclease (Brash *et al.*, 1987). This relative level was found to be 25-50% when both lesions were detected immunochemically (Mitchell, 1988). It has been suggested that in some assays for detection of UV-induced non-cyclobutane dimers, *i.e.* (6-4)photoproducts and "dewar" isomers, the relative amount of these lesions may have been underestimated to a significant degree due to their lability towards alkali (Mitchell *et al.*, 1990). In fact, in an approach in which ABC excinuclease was used to incise DNA at lesion-sites in combination with the use of photolyase to remove cyclobutane dimers, it was estimated that (6-4)photoproducts are formed at about two-thirds the rate of cyclobutane dimers, thus comprising about 40% of total photoproducts (Thomas *et al.*, 1989). However, in making these calculations one has to take into account that ABC excinuclease makes incisions only at about 50% of the lesions (Myles *et al.*, 1987). (6-4)Photoproducts are most prevalent at sequences in DNA in which cytidine is located 3' to a pyrimidine (Lippke *et al.*, 1981; Sancar and Rupp, 1983). Single-strand breaks are major lesions at longer wavelengths, but they do not seem to make an important contribution to lethality (Roza *et al.*, 1985a, Appendix paper I); the latter was found to be the case also for cyclobutane dimers at these wavelengths (Enninga *et al.*, 1986; Smith and Paterson, 1982; Roza *et al.*, 1985a, Appendix paper I). The relative number of DNA-protein crosslinks induced per lethal hit seems to **increase** with increasing wavelength and to reach a maximum at 405 nm (Peak *et al.*, 1985), in contrast to cyclobutane dimers (Enninga *et al.*, 1986; Roza *et al.*, 1985a, Appendix paper I). Lesions in which purines are involved are much less frequent because the quantum efficiency for photoproduct formation is 10-100 times lower.

III DNA REPAIR

III.1 DNA REPAIR AND REPAIR-ASSOCIATED HUMAN SYNDROMES.

To ensure existential continuity, normal cells have at their disposal efficient mechanisms to repair damage to DNA. DNA repair may be defined as "those cellular responses associated with the restoration of the normal nucleotide sequence and stereochemistry of DNA following damage" (Friedberg, 1985). Several enzymatic pathways are involved in the repair of DNA damage, varying with the type of damage introduced. When something is wrong with the DNA repair system, it will have direct consequences for the cell or the organism to which it belongs. A direct correlation between unrepaired DNA damage and carcinogenesis in humans was established with the cancer-prone hereditary disease xeroderma pigmentosum (XP) which orginates from a defect in the repair of DNA lesions such as those produced by UV (Cleaver, 1968; Cleaver and Bootsma, 1975). Other human hereditary diseases with predisposition to malignancy have also been shown to be related to an anomaly in the processing of damaged DNA. These include the chromosome breakage syndromes Bloom's syndrome (BS), Fanconi's anemia (FA) and ataxia telangiectasia (AT) (Hanawalt and Sarasin, 1986; Kraemer, 1983). Cockayne's syndrome (CS), like XP, is characterized by an elevated UV sensitivity, but induction of neoplasia at an early age is not enhanced. Also here, the hypersensitivity to DNA-damaging agents appears to be a consequence of defective DNA repair, as was substantiated recently (Mayne *et al.*, 1989): repair of lesions in actively transcribed genes is impaired (see section III.3). For BS cells there is evidence that the primary defect is a DNA ligase I defiency in accordance with the high level of sister chromatid exchanges found in these cells (Chan *et al.*, 1987; Willis and Lindahl, 1987). Cells from FA patients are sensitive to DNA-crosslinking agents and it has been assumed that these cells are (partly) deficient in short-patch excision repair (Fujiwara *et al.*, 1977; Ishida and Buchwald, 1982). AT cells are sensitive to ionizing radiation and other clastogenic agents. After exposure to X-rays, inhibition of DNA synthesis is less compared to normal cells, suggesting that the primary defect in AT is at the level of cell cycle control in relation to the presence of DNA damage (Jaspers, 1985).

Apart from BS, the above mentioned human disorders share a considerable genetic heterogeneity. For XP, CS, FA and AT, respectively 8, 3, 2 and 4 genetically distinct groups have been found (XP: De Weerd-Kastelein *et al.*, 1972; Bootsma *et al.*, 1989; CS: Lehmann, 1982; FA: Duckworth-Rysiecki *et al.*, 1985; AT: Jaspers *et*

al., 1988). The excistence of so many classes of mutants in humans suggests that mammalian DNA repair is a highly complex process in which many genes are involved. Further evidence for this stems from the identification -to date- of 8 complementation groups of UV-sensitive rodent mutant cells isolated in the laboratory (see Section III.2), which are believed to represent defects in different repair genes. These cell-strains are used to clone the complementary human genes (Van Duin, 1988).

III.2 MECHANISM OF DNA EXCISION REPAIR

One of the major repair routes in the cell to overcome DNA damage is excision repair, which appears responsible for the removal of many types of lesions, including cyclobutane dimers and (6-4)photoproducts. This type of repair is easily detected by visualization of incorporated ^{3}H-thymidine (*e.g.,* with autoradiography) or thymidine analogs in UV-irradiated cells. Excision repair is brought about by the cooperative action of several enzymes or enzym-complexes, which includes: (1) recognition of the DNA damage; (2) incision in the phosphodiester backbone at or near the site of the defect; (3) excision of the damaged segment in the affected DNA strand; (4) repair replication to replace the excised nucleotides (which can be measured as unscheduled DNA synthesis, UDS); and (5) ligation to close the gap in the DNA strand at the 3' end of the repaired patch. The first two steps, recognition and incision, may be carried out by the same enzyme. The enzyme T4 endoV, encoded by the *den*V gene of the bacteriophage T4, and the UV-endonuclease of *Micrococcus luteus* act in this way. They are cyclobutane dimer-specific and cleave first the glycosylic bond at the 5'-pyrimidine of the dimer (glycosylase activity), followed by incision at the apyrimidinic (AP) site (AP endonuclease activity), cleaving the phosphodiester bond between the two pyrimidines (Gordon and Haseltine, 1980; Haseltine *et al.*, 1980; Nakabeppu *et al.*, 1982). Both enzymes have been used frequently in sensitive assays for detection of cyclobutane dimers (Paterson *et al.*, 1973; Van Zeeland *et al.*, 1981). In *E. coli*, which to date is the only organism for which nucleotide excision repair has been characterized at the molecular level, incision of DNA with cyclobutane dimers or other lesions occurs on both sides of the damage. The UvrA, UvrB, and UvrC proteins, referred to collectively as "ABC excinuclease", initiate nucleotide excision repair by incising the DNA at the 8th phosphodiester bond counted from the 5' side and at the 4th phosphodiester bond from the 3' side of the damage (Sancar and Rupp, 1983). The Uvr proteins have each been purified and characterized in some

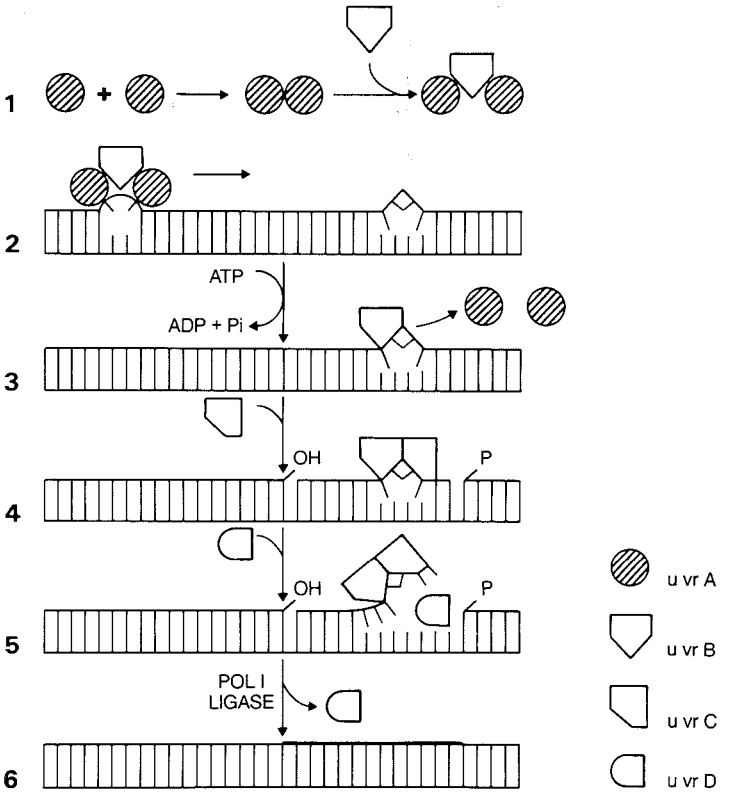

Figure 6. Mechanism of the *E. coli* uvrABC excinuclease (Orren and Sancar, 1989). Cartoon by courtesy of Dr NGJ Jaspers.

detail (Sancar and Sancar, 1988). UvrA is an ATPase and a DNA-binding protein. UvrA has higher affinity to damaged DNA than to non-damaged DNA; it delivers the UvrB subunit to the damaged site, and dissociates from DNA. The UvrC subunit then binds to the complex of UvrB and damaged DNA and the incisions are made on both sides of the DNA damage (Orren and Sancar, 1989). The oligonucleotide containing the lesion is hydrolysed to nucleotides and the resulting gap is filled in by DNA polymerase I and sealed by ligase (Sancar and Sancar, 1988). In figure 6 the sequential actions of the ABC excinuclease are illustrated. Mutations in any of the three *uvr* genes in *E. coli* make cells extremely sensitive to UV as well as other genotoxic agents. This wide range of substrates is one of the most striking features

of the ABC excinuclease. Attempts to correct the repair defect of XP cells *in vivo* with the ABC excinuclease either by nuclear microinjection (Zwetsloot *et al.*, 1986) or by DNA transfection (Dickstein *et al.*, 1988) have failed sofar. However, correction of the XP defect with ABC excinuclease could be demonstrated in a cell-free system (Hansson *et al.*, 1990). Introduction of T4 endoV or *M. luteus* UV-endonuclease into human XP cells by permeabilization or microinjection restored UDS after UV exposure and increased cell survival (De Jonge *et al.*, 1985; Tanaka *et al.*, 1975; Tanaka *et al.*, 1977; Yamaizumi *et al.*, 1989). Also introduction of the T4 *den*V gene into XP cells and into UV-sensitive Chinese hamster mutant cells partially restored UV-resistance (Arrand *et al.*, 1987; Valerie *et al.*, 1985; Valerie *et al.*, 1987). Notwithstanding the phenotypic correction by T4 endoV and *M. luteus* UV-endonuclease, in human cells separate endogenous glycosylase and AP endonuclease activities associated with cyclobutane dimer sites were not detected (La Belle and Linn, 1982; Weinfeld *et al.*, 1986). A mechanism proposed for the initial reaction in excision repair acting on cyclobutane dimers in human cells is cleavage of the intradimer phosphodiester linkage (Weinfeld *et al.*, 1986; Paterson *et al.*, 1987).

After the excision of the defective DNA segment, a new patch of DNA is synthesized; it is unclear whether this is brought about by polymerase α, and/or δ (Nishida *et al.*, 1988). A report on *in vitro* SV40 DNA replication implicates polymerase α and polymerase δ as the lagging and leading strand polymerase, respectively (Prelich and Stillman, 1988). Dependent on the type of lesion, long-patch or short-patch repair has been identified (Hanawalt *et al.*, 1979; Regan and Setlow, 1974). In mammalian cells UV-induced DNA damage and other bulky lesions, which block replication, are subject to the long-patch type of repair. A stretch of 30-100 nucleotides is renewed (Walker and Th'ng, 1982; Th'ng and Walker, 1983). Short-patch repair in mammalian cells comprises renewal of only a few nucleotides upon induction of DNA damage by, *e.g.*, alkylating agents.

Cells from XP patients are impaired in the removal of UV-induced cyclobutane dimers (Zelle and Lohman, 1979). This deficiency is held responsible for the high incidence of skin cancer in these patients (Kraemer *et al.*, 1987). The (at least) 8 different XP-complementation groups reported to date (Kraemer *et al.*, 1987; Bootsma *et al.*, 1989) are believed to represent defects in as many different repair genes. From mainly Chinese hamster cells, UV-sensitive mutant cells have been derived (Thompson, 1988) which belong to 8 complementation groups (Zdzienicka *et al.*, 1988a; Thompson, 1988; Thompson *et al.*, 1988). Recently, human genes controlling DNA repair have been cloned on the basis of their ability to correct repair

deficiency when transfected into certain of these UV-sensitive Chinese hamster cells (Westerveld *et al.*, 1984; Van Duin *et al.*, 1986; Weber *et al.*, 1988; Hoeijmakers *et al.*, 1988; Weber *et al.*, 1990; Weeda *et al.*, 1990). The first of these genes was the *ERCC*-1 gene correcting the repair defiency in complementation group 1 hamster mutant cells. Surprisingly, this gene was found not to complement cells from any of the XP complementation groups, nor Cockayne's Syndrome cells (Van Duin *et al.*, 1989), but cyclobutane-dimer excision in Chinese hamster mutant cells was stimulated by transfection with this gene (Zdzienicka *et al.*, 1987); resulting in restoration of the normal pattern of preferential DNA repair (Bohr *et al.*, 1988). The first indication of an overlap between the 8 XP complementation groups and the 8 rodent mutant groups was found with the isolation of the human *ERCC*-3 gene, which corrects the defiency of complementation group 3 hamster mutant cells (Weeda *et al.*, 1990). Microinjection of the cDNA of this gene restores the XP-B phenotype (Weeda *et al.*, in preparation). At present several other human repair genes are being identified and characterized in rodent cells (Van Duin, 1988).

III.3 PREFERENTIAL DNA REPAIR IN THE MAMMALIAN GENOME

DNA repair in cultured mammalian cells appears to occur in a non-random fashion. This has been shown for several DNA damages induced by chemical and physical agents, *e.g.*, the cyclobutane dimer (Bohr *et al.*, 1985; Bohr *et al.*, 1986; Madhani *et al.*, 1986; Mellon *et al.*, 1986), the (6-4)photoproduct (Thomas *et al.*, 1989), aflatoxin B1-induced damage (Leadon, 1986) and O^6-methylguanine (Ryan *et al.*, 1986). In Chinese hamster cells cyclobutane dimers are removed more efficiently from the coding region of the (continuously expressed) dihydrofolate reductase (*DHFR*) gene than from its 5' flanking non-coding sequences or from the overall genome (Bohr *et al.*, 1985; Bohr *et al.*, 1986). Similar results were obtained in experiments with the human *DHFR* gene (Mellon *et al.*, 1986). Moreover, this repair appears to occur selectively in the transcribed strand (Mellon *et al.*, 1987). Furthermore, it was shown that the efficacy of dimer removal correlates with the activity of transcription; in 24 hours 85% of dimers were removed from the active mouse *c-abl* gene and only 20% from the silent mouse *c-mos* gene (Madhani *et al.*, 1986). Also, DNA repair in the Chinese hamster metallothionein gene is enhanced upon induction of the expression of this gene (Okumoto and Bohr, 1987). Preferential repair of active genes is not a phenomenon observed for all kinds of DNA lesions. It was demonstrated that repair of N-methylpurines (Sciccitano and Hanawalt, 1989) and N-deoxyguanosin-8-yl-2-aminofluorene lesions (Tang *et al.*,

1989) in the CHO *DHFR* gene is comparable with repair in a non-transcribed, downstream region of the DNA.

Xeroderma pigmentosum cells of complementation group C exhibit residual repair activity at levels of 10-20% of normal cells. It was found that this repair was restricted to a limited portion of the genome (Mansbridge and Hanawalt, 1983), namely to those parts which are associated with the nuclear matrix (Mullenders *et al.*, 1984). In such regions transcriptionally active genes are located (Reeves, 1984). In addition it was found that removal of cyclobutane dimers from the *ADA* (adenosine deaminase) and the *DHFR* genes was much more efficient than from an inactive locus (Venema *et al.*, 1990). There is evidence that repair in active genes is disturbed in cells of patients with the heriditary disease Cockayne's Syndrome (Mayne *et al.*, 1989; Mullenders *et al.*, 1988). Apparently, DNA repair processes in active and inactive chromatin are at least partially independent.

Preferential DNA repair of active genes might be an important mechanism for cells to firstly restore the vital process of transcription, which is blocked by DNA injury. Preferential DNA repair might be an explanation also for the phenomenon that human and rodent cells differ greatly in removal of dimers from the overall genome, whereas these cells exhibit similar survival after UV exposure (Ganesan *et al.*, 1983; Vijg, 1987). This may implicate that primarily removal of DNA damage from (the transcribed strand of) active genes is essential for cell survival.

III.4 ENZYMATIC PHOTOREACTIVATION OF CYCLOBUTANE DIMERS

Cyclobutane dimers in DNA can be repaired by the action of a single "photoreactivating" enzyme (photolyase) in combination with light in the range of 350-500 nm (Rupert, 1975). This enzyme contains chromophores that utilize the energy of light to break the cyclobutane ring joining the two pyrimidines of the dimer. All photolyases analysed to date appear to contain two chromophores bound to their apoenzymes via non-covalent interactions (Sancar and Sancar, 1987). $FADH_2$ (reduced flavin adeninedinucleotide) is present in all of these enzymes, which points at a central role for this chromophore. The second chromophore (pterin or a deazaflavin derivative) is the major light-absorbing cofactor and may function by transfer of energy to $FADH_2$. The mechanism suggested for dimer repair by a photolyase is donation of an electron from a photo-excited reduced flavin to the dimer. A cyclobutane dimer anion is formed which collapses to yield two monomeric pyrimidines (Jorns *et al.*, 1987; Sancar *et al.*, 1987); the flavin then is regenerated. The photolyase interacts with a 6-7 basepair region around the dimer, but only on

the damaged DNA strand (Husain *et al.*, 1987), in contrast to the *E. coli* ABC excinuclease complex. Interestingly, it has been found that photolyase does not hamper, but actually stimulates the incision of UV-irradiated DNA by ABC excinuclease (Sancar *et al.*, 1984).

Photoreactivating activity is clearly detectable in bacteria, protozoa and cells of yeast, insects, crustacea, echinodermata, fish, amphibians, reptiles and marsupials (Eker, 1983; Rupert, 1975; Sutherland, 1981). In birds, only cells from the embryo and the adult brain show photoreactivation (Cook and McGrath, 1967). Studies on the occurrence of photoreactivation in mammalian cells have yielded conflicting results (Harm, 1980; Ananthaswamy and Fisher, 1981). Although photoreactivation has been reported to occur in human leukocytes (Sutherland, 1974; Sutherland and Castellani, 1982), fibroblasts (Sutherland *et al.*, 1976) and human skin (D'Ambrosio *et al.*, 1981b; Sutherland *et al.*, 1980), data are controversial. The activity in cultured cells seems to depend on the culture medium (Mortelmans *et al.*, 1977; Sutherland and Oliver, 1976). Endogenous photoreactivation could not be detected in cultured human fibroblasts, whereas microinjection of purified yeast photolyase into such cells followed by exposure to photoreactivating light resulted in efficient dimer repair (Roza *et al.*, 1990, Appendix paper IV). Efficient photorepair of cyclobutane dimers in human skin has been reported (Sutherland *et al.*, 1980; D'Ambrosio *et al.*, 1981b; Eggset *et al.*, 1983), but in the immunochemical study by Eggset *et al.* it turned out that instead of cyclobutane dimers, (6-4)photoproducts had been subject of detection (Eggset *et al.*, 1987), which upon 320-nm UV-exposure are converted into "dewar" isomers (Taylor and Cohrs, 1987). In another immunochemical study, too, photorepair in human skin was found, but only after repeated UV-irradiation and illumination (Roza *et al.*, Appendix paper V). Photoreactivating activity has been detected in different human fetal tissues (Ogut *et al.*, 1989). Considerable variation in specific activity between different tissues was found, with the highest activity in skin. Two separate activities were present with a molecular weight below as well as above 10,000 dalton, which both are sensitive to proteolytic degradation. The existence of a real photoreactivating enzyme (photolyase) in human cells still remains to be proved, although it appears that cyclobutane dimers can be removed at a higher rate in the presence of light. The differences in the efficiency of this photorepair as found by different groups of investigators may reflect interindividual variation as only small numbers of volunteers have been subject of study. Also with respect to repair of dimers in human skin without illumination considerable interindividual variations appear to exist, among data for different individuals in the same study as well as among data from different authors (D'Ambrosio *et al.*, 1981a;

Freeman, 1988; Sutherland *et al.*, 1980).

The formation of cyclobutane dimers is a reversible process. The photochemical monomerization reaction is favoured at wavelengths in the 230-nm region. However, this wavelength is filtered out completely by the stratospheric ozone layer and consequently this process has no biological importance, unless artificial UV-sources are used. Also reversal of cyclobutane dimers can be mediated by light-excited tryptophan residues present as the free amino acid or as a constituent of peptides (Charlier and Helene, 1975). This requires UV <310 nm where tryptophan absorbs (Sutherland and Griffin, 1980). It has been proposed that such peptides might be responsible for photoreactivating activities detected in mammalian cells (Mortelmans *et al.*, 1977). Supposedly, photochemical monomerization of cyclobutane dimers can be mediated also by flavin derivatives, which are not part of a regenerating enzyme.

UV-induced lethality in *E. coli* can be reduced by exposure to 313-nm UV (Husain *et al.*, 1988; Ikenaga *et al.*, 1970; Patrick, 1970). In isolated DNA or in oligonucleotides photochemical monomerization of cyclobutane dimers at 313-nm UV was not observed (Mitchell and Clarkson, 1984a; Patrick, 1970). Whereas the (6-4)photoproduct is subject to photolysis at this wavelength, resulting in the "dewar"-isomer (Taylor and Cohrs, 1987). The effect in *E. coli* makes it conceivable that these isomers are less disruptive to the DNA structure and allow DNA replication to proceed.

IV CONSEQUENCES OF DNA REPAIR

IV.1 RELATIVE BIOLOGICAL IMPORTANCE OF CYCLOBUTANE DIMERS AND (6-4)PHOTOPRODUCTS IN MAMMALIAN CELLS.

The DNA lesions induced by UV with wavelengths <320 nm, comprise mainly the cyclobutane dimers and (6-4)photoproducts. With UV-C (254 nm) about 2-3 times as many dimers are induced as (6-4)photoproducts (see above). Action spectra for the induction of either lesion are very similar between 265-302 nm (Rosenstein and Mitchell, 1987), but at wavelengths between 310-330 nm the relative induction of (6-4)photoproducts is 2- to 3-fold lower than that observed for dimers, probably owing to photolysis of the (6-4)photoproduct at these wavelengths. The kinetics of cyclobutane dimer and (6-4)photoproduct removal are different. After moderate UV-irradiation of cultured normal human cells 80% of the latter lesions were shown to be removed within the first 3 h after irradiation by means of immunochemical detection (Mitchell *et al.*, 1985b; Mitchell, 1988; Mori *et al.*, 1988). This repair proceeds considerably faster than the dark repair observed for cyclobutane dimers, *i.e.* 70-80% removed in 24 h (Vijg *et al.*, 1984; Wani *et al.*, 1987; Zelle and Lohman, 1979; Roza *et al.*, 1988; Roza *et al.*, 1990, Appendix papers III and IV). The kinetics of (6-4)photoproduct removal closely resemble those for UDS in human fibroblasts (Vijg *et al.*, 1984) and for removal of blocks to DNA synthesis (Painter, 1985).

Under proper experimental conditions the contribution of cyclobutane dimers and other lesions to biological consequences such as the occurrence of repair replication, the formation of mutations or cell killing can be assessed separately. An elegant approach to this end is to analyse these effects with and without removal of dimers by means of enzymatic photoreactivation. However, in human cells biological effects attributable to endogenous photoreactivation have not been reported. From experiments on the kinetics of reduction of UDS after photoreactivation of cyclobutane dimers by microinjected photoreactivating enzyme in human fibroblasts, it appears that early UDS originates from repair of (6-4)photoproducts, whereas the repair synthesis occurring after 2-3 h of repair is sensitive to photoreactivation for about 80% and, consequently due to mainly repair of cyclobutane dimers (Roza *et al.*, 1990, Appendix paper IV). Also, repair replication in chicken embryo fibroblasts is decreased upon photoreactivation, but distinctly only at later time points after UV irradiation (Roza *et al.*, 1985b, Appendix paper II). In general, photoreactivation of dimers in cells proficient in this type of repair reduces

UV-induced cytotoxicity and mutation induction (Bronk *et al.*, 1984; Freed *et al.*, 1979; Shima and Setlow, 1984; Van Zeeland *et al.*, 1980; Wade and Lohman, 1980; Wade and Trosko, 1983), indicating that cyclobutane dimers are important genotoxic lesions.

Another approach to investigate the noxiousness of the different UV-induced DNA lesions is the use of different cell types, *i.e.* from different species or mutant cells with a deficiency in a certain DNA repair process. Rodent cells survive UV irradiation to the same extent as do human cells, but have considerably lower capacity to remove dimers from their genome than human cells (Meyn *et al.*, 1974; Vijg *et al.*, 1984; Yagi, 1982). On the other hand, removal of (6-4)photoproducts in rodent cells occurs at almost the same rate as in human cells (Mitchell *et al.*, 1985b; Mitchell, 1988). From studies with Chinese hamster mutant cells it appears that both dimers and (6-4)photoproducts have comparable cytotoxicity (Mitchell, 1988; Mitchell and Nairn, 1989). The main evidence for this is based on studies with UV5 and UV61 cells, the former of which are deficient in removal of both types of lesions (Zdzienicka *et al.*, 1988b) whereas the latter cells are able to remove (6-4)photoproducts normally, but are at least in part deficient in removal of cyclobutane dimers (Thompson *et al.*, 1989). Experiments with a partial revertant of an SV40-transformed XP-A cell line, which removes (6-4)photoproducts from DNA but is largely deficient in removal of dimers, show that this revertant survives UV- to about the same extent as normal cells do (Cleaver *et al.*, 1987; Cleaver *et al.*, 1988). This suggests that the cyclobutane dimer is of minor importance for lethality and, consequently, that the (6-4)photoproduct is the predominant lethal damage induced by UV. Recent observations have shown, however, that the revertant is able to repair cyclobutane dimers in active regions of DNA (L. Lommel, as cited in Broughton *et al.*, 1990).

Trichothiodystrophy (TTD) is a genetic disease which in the majority of cases studied is associated with a deficiency in the ability to repair UV-induced damage in cellular DNA. A cell line from a TTD patient was described, which has reduced levels of repair incision, removal of (6-4)photoproducts and UDS, but a normal level of cyclobutane dimer removal (Lehmann *et al.*, 1988). The UV cytotoxicity for this cell line is normal, suggesting that (6-4)photoproducts are of minor importance in this case. Supposedly, preferential repair of cyclobutane dimers in addition to repair of (6-4)photoproducts is responsible for survival of cells after UV irradiation.

IV.2 MUTAGENICITY OF UV PHOTOPRODUCTS IN HUMAN CELLS.

In general, UV of all wavelengths is mutagenic if a sufficient fluence is available (Enninga *et al.*, 1986). Recently, UV-induced mutagenesis was studied in human cells by transfection with shuttle vectors. These vectors carry a cloned target sequence and are derived from viral genomes such as SV40, which ensures that they form nucleosomes and replicate by use of host enzymes. The vector may be irradiated prior to transfection or after it has entered the cell. After replication the target sequence is recovered from the cell, screened for mutations and sequenced (Lebkowski *et al.*, 1985). In this system, large deletions and rearrangements are not amenable to investigation because these occur already as a consequence of the transfection. In most studies, the UV-induced point mutations found were predominantly G:C -> A:T transitions, besides a few transversions. This predominance has been explained to occur through preferential insertion of adenine opposite noncoding lesions by DNA polymerases during replication (Tessman, 1985). Most transitions occurred at pyrimidine-pyrimidine sequences, suggesting the involvement of a dipyrimidine photoproduct.

Data about the *E. coli sup*F gene that contained UV-type lesions when it was introduced into normal human and xeroderma pigmentosum cells, indicate that the major (pro)mutagenic photoproducts are the TC, CT and CC cyclobutane dimers, the C of which undergoes a C -> T transition. About 80% of the UV-induced mutations in the *sup*F gene could be eliminated by photoreactivation of the UV-irradiated plasmid. This was found for both cell types, although the frequency of mutation induction in the XP cells was much higher (Protic-Sabljic *et al.*, 1986). About 10% of the mutations were due to a dipyrimidine photoproduct which was not a cyclobutane dimer but which still gave a G:C -> A:T transition. However, the relative proportion of transversions was increased after photoreactivation of the shuttle vector, indicating that these were largely due to a non-photoreactivable photoproduct (Protic-Sabljic *et al.*, 1986).

In studies on the effect of UV on the endogenous *APRT* gene of Chinese hamster cells, 50% of the mutations were G:C -> A:T transitions at TC and CC; no deletions, rearrangements, or multiple mutations were observed (Drobetsky *et al.*, 1987). A study with normal (V79) and repair deficient (V-H1) Chinese hamster cells revealed that 65% of base-pair changes in the *HPRT* gene of V79 consisted of transversions. G:C -> A:T transitions comprised only 18% of the base changes that had occurred. In the V-H1 cells, which have a 7-fold higher UV-induced mutation frequency compared to V79 cells (Zdzienicka *et al.*, 1988b), all base-pair changes

involved G:C -> A:T transitions, which seem to be prefentially induced by lesions in the transcribed strand (Vrieling *et al.*, 1989). The V-H1 cells were found to remove (6-4)photoproducts almost at the same rate as normal cells do, but they fail to remove cyclobutane dimers from the *HPRT* gene (Mitchell *et al.*, 1989). Apparently, in this cell line cyclobutane dimers are the primary cause of the induction of mutations.

In summary, UV-induced mutations in mammalian cells occur most frequently at (the C of) dipyrimidine sites; probably the primary lesions responsible for the effects are cyclobutane dimers and, less frequently, (6-4)photoproducts.

IV.3 UV-CARCINOGENESIS

Skin cancer is among the most prevalent human cancers. The evidence that sunlight is responsible for the introduction of skin tumors is that (1) tumors appear most frequently on sun-exposed parts of the body, such as the face and neck; (2) tumor incidence increases with occupational exposure, *e.g.*, among farmers and sailors; (3) tumor incidence increases with decreasing latitude; (4) tumor incidence is highest in light-skinned individuals; (5) genetic diseases associated with sensitivity to sunlight give rise to high tumor incidence (albinism, xeroderma pigmentosum); (6) UV induces tumors in laboratory animals.

Recently, an action spectrum for the induction of skin tumors in hairless mice has become available (Slaper, 1987; Sterenborg and Van der Leun, 1987). The spectrum peaks at 300 nm and declines steeply at higher wavelength, but tends to level off in the UV-A range (>330 nm), where the effect of the radiation is smaller than that at the peak value by 3 to 4 orders of magnitude. Nevertheless, it was possible to induce tumors in these mice with no other than UV-A radiation (Van Weelden *et al.*, 1986; Sterenborg and Van der Leun, 1990). The action spectrum for carcinogenesis is very similar to the action spectrum for human skin-tanning (Parrish *et al.*, 1982; Roza *et al.*, 1989; Slaper, 1987).

In humans, types of skin cancer are subdivided in melanomas and non-melanomas. The non-melanomas are the most prevalent, accounting for 90% of total skin-tumor incidence. Mortality due to these tumors is low, about 1%. The estimated incidence in the Netherlands is about 1 tumor per 1000 inhabitants per year. Non-melanomas can be subdivided in basal and squamous cell carcinomas, of which the former are estimated to occur at 75-90% of the total incidence of non-melanomas. The incidence of melanoma appears to increase rapidly. In the Netherlands the increase is 7% per year over the last 35 years (Health Council of the Netherlands,

1986). Mortality is high, 30-40%, because of the high frequency of metastasis. The correlation between UV exposure of skin and the incidence of melanoma is not as clear as it is with non-melanoma. Melanoma sites are associated with precursor lesions, the dysplastic (melanocytic) nevi, the etiology of which can have a familial component (Fitzpatrick and Sober, 1985).

Laboratory animals exposed to a single identified carcinogen develop tumors with the same activated oncogene in each animal. The particular oncogene may depend on the carcinogen used (Guerrero *et al.*, 1984; Perantoni *et al.*, 1987; Zarbl *et al.*, 1985). For the mouse Ha-*ras* oncogene, there is a correlation between the type of initiating chemical used and the mutation observed in the oncogene, which supports the hypothesis that the mutation is caused by direct interaction of the carcinogen with DNA. However, in other model systems the mutational event leading to oncogene activation appeared to be independent of the mutagenic activity of the initiating carcinogen (Doniger *et al.*, 1987). Recent observations showed a 5- to 10-fold increase of c-Ha-*ras* gene expression in mouse epidermal tumors induced with single-dose UV-B irradiation (Husain *et al.*, 1990).

In general, human tumors display one or more of a wide range of activated oncogenes (Barbacid, 1987; Bos, 1989), probably because humans are exposed to a wide variety of carcinogens, which usually are complex mixtures of substances. Also, human skin cancer may show preferential oncogene activation. In 7 of 37 patients with cutaneous melanoma, mutations in the N-*ras* gene were found. The primary tumors of these 7 patients were exclusively localized on parts of the body continuously exposed to sunlight. Moreover, the *ras* mutations were all at or near dipyrimidine sites (TT or CC), suggesting the involvement of a cyclobutane dimer or a (6-4)photoproduct (Van 't Veer *et al.*, 1989). The same N-*ras* mutation has also been found in melanoma cells of an XP patient (Keijzer *et al.*, 1989). Furthermore, introduction of an *in vitro* UV irradiated N-*ras* gene in rodent cells resulted in transformed cells with the same mutations in this gene as found *in vivo* (Van der Lubbe *et al.*, 1988). In a study on human basal and squamous cell carcinomas mutational activation of *ras* genes was found too, but the rate at which these mutations occur appeared to be relatively low (Van der Schroef *et al.*, 1990).

Tumor progression may be due to death of normal cells. After the induction of cell killing by UV, surviving cells -proliferating to replace the dead cells- may include mutated cells which thus undergo clonal expansion. Death of normal tissue would change the mutagenic effect of sunlight for the worse, favouring growth of mutated cells.

Recent research also indicates an important role of the immune-system in UV-

initiated carcinogenesis, as reviewed by Morison (1984; 1989). Experiments with UV-B-induced tumors in mice, which tumors were transplanted to genetically identical mice, were pioneering. Transplanted tumors were rejected by unirradiated mice and only grew in mice that had been pre-irradiated with a (low) UV-dose, or in mice treated with agents that suppress the immune-system. Rejection of tumors did not happen when skin tumors induced by chemical agents were transplanted (Kripke, 1974; Kripke and Fisher, 1976). Mice irradiated with (large) UV-A doses also failed to reject UV-induced tumors transplanted from syngeneic animals (Morison, 1985). Latency periods for tumor induction in certain areas were shortened by previous exposures of other skin areas, strongly suggesting selective immuno-suppression (De Gruijl and Van der Leun, 1982; De Gruijl and Van der Leun, 1983).

A likely UV-induced step in carcinogenesis is thus mutation of a gene such as a *ras* proto-oncogene to an activated form, *e.g.,* by a point mutation resulting from a (dipyrimidine) photoproduct. The consequence may be the initiation of tumor cells. On the other hand UV exposure may lead to suppression of the immune system which results in the absence of adequate removal of the tumor cells. Also cell killing by UV irradiation may facilitate growth of mutated cells which have escaped cell-death.

V CONCLUDING REMARKS AND INTRODUCTION TO THE PAPERS

Skin cancer is the most common neoplasm in caucasian populations and the primary etiologic factor appears to be solar UV (Scotto *et al.*, 1981; O'Rourke and Emmett, 1982). The incidence of skin cancer today is increasing at an alarming rate, which might be the direct consequence of increased human exposure to UV. The current aesthetic value placed upon a good suntan results in individuals subjecting themselves to prolonged exposures to natural sunlight and -more recently- to sunlamps. In addition, the amount of UV-B radiation reaching the earth's surface from the sun may be increasing because of reduction of the ozone layer probably due to atmospheric pollution. Therefore, investigation of the effects of exposure of human cells to UV is very important. The biological effects of UV, including cell killing, mutagenicity, and carcinogenicity at shorter wavelength (<310 nm), correlate to the absorption of light by DNA (Harm, 1980). This has led to the suggestion that DNA is the target that mediates most of the biological effects. However, the contribution of the different UV photoproducts to cell death, mutation induction and carcinogenesis is not well understood and thus needs further study.

In the work presented in this thesis the processing of UV-induced DNA damage has been investigated by studying (1) the removal of lesions, (2) the effect of different lesions on cytotoxicity, and (3) the kinetics of repair synthesis. The induction of single-strand DNA breaks and cyclobutane dimers at different UV wavelengths, as well as the contribution of these lesions to cell-death is investigated in paper I. At shorter wavelength (UV-C) cyclobutane dimers (Roza *et al.*, 1985a, Appendix paper I) and (6-4)photoproducts (Mitchell, 1988) appear responsible for the UV-induced biological effects. It has been demonstrated that the distribution of damage in cellular DNA induced at longer wavelengths (UV-A) are clearly different from that of lesions induced by UV-B and UV-C radiation (Smith and Paterson, 1982; Roza *et al.*, 1985a, Appendix paper I). UV-A-induced DNA lesions that have been studied in cultured mammalian cells comprise cyclobutane dimers, single-strand breaks and DNA-protein crosslinks (Enninga *et al.*, 1986; Peak *et al.*, 1985; Peak *et al.*, 1987; Rosenstein and Ducore, 1983; Smith and Paterson, 1982; Roza *et al.*, 1985a, Appendix paper I). The cyclobutane dimers and the single-strand breaks could be ruled out as the lethal lesions induced by the longer wavelength UV (Roza *et al.*, 1985a, Appendix paper I). The relative importance of DNA-protein crosslinks is not clear: per lethal event substantially less of these lesions were formed at 365 nm than

at 334 nm and 405 nm (Peak, 1988), indicating that, possibly besides DNA-protein crosslinks, other lesions must be involved in cell killing by 365 nm UV. Repair of cyclobutane dimers and other UV-induced photoproducts is described in paper II. The cells used in this particular study were chicken embryo fibroblasts, which cells perform photoreactivation of dimers, thus enabling us to study effects of other lesions than cyclobutane dimers. After removal of cyclobutane dimers by photoreactivation immediately after UV-C exposure, it was found that this repair had only a small effect on the initial UV-induced UDS but greatly reduced UDS occurring at 2 h after the UV irradiation. Apparently, after this period the majority of UDS is caused by repair of cyclobutane dimers (Roza *et al.*, 1985b, Appendix paper II).

Alteration of DNA by the induction of DNA damage is considered to be an important step in gene mutation and the initiation of cancer. For this reason, molecular dosimetry of DNA damage is of importance, in particular with regard to the target organ, to estimate the extent of the biologically effective genotoxic exposure. However, before these data can be used for the assessment of risk involved in the exposure, a correlation should be established between the presence and persistence of well defined DNA lesions and their biological consequences, such as cell killing or mutation induction. In this respect, the clear role of solar UV in the etiology of non-melanoma skin cancer is advantageous. The carcinogen is known and it is one single agent rather than a complex mixture of chemicals such as found in, *e.g.*, tobacco smoke. Still, up to now, no studies have been performed to detect sunlight-induced DNA lesions in human skin during chronic exposure. For this purpose an immunochemical detection method appears most suitable (Roza *et al.*, 1987; Baan *et al.*, 1989), which could provide the molecular dosimetry of dimers in UV-exposed human skin needed for the collection of essential data for risk-assessment of sun-exposure. In paper III the isolation and characterization of a monoclonal antibody against the cyclobutane thymine dimer in DNA is described. This antibody has proved to be suitable for studying dimers in isolated DNA as well as in cells *in situ* (Roza *et al.*, 1987; Roza *et al.*, 1988, Appendix paper III). In paper IV the antibody is used to investigate repair of dimers in cultured human fibroblasts. The effect of photoreactivation of dimers by microinjected photoreactivating enzyme on excision repair was studied in normal and in xeroderma pigmentosum fibroblasts. It was found that the initial UDS to a large extent was due to repair of non cyclobutane dimer photoproducts, probably (6-4)photoproducts, whereas the majority of the UDS at later time points (>2 h) after exposure was due primarily to cyclobutane dimers (Roza *et al.*, 1990, Appendix paper IV). Evidently, differences exist in the processing of cyclobutane dimers and (6-4)photoproducts. Probably, in

mammalian cells different pathways are involved in the repair of these two types of lesions, which is also supported by the existence of mutant cells in which repair capacity is evident for one photoproduct and not for the other (Broughton *et al.*, 1990; Mitchell and Nairn, 1989).

The antibody against thymine dimers enabled us to investigate the appearance of photoreactivation in human skin irradiated *in vivo*, which is described in paper V. Photorepair in human skin *in vivo* could be detected, but in contrast to the literature not after single UV exposure and subsequent illumination. After repeated UV exposure and illumination some reduction of dimers was found compared to skin exposed to UV but shielded from (photoreactivating) light (Roza *et al.*, Appendix paper V). Still, photorepair of cyclobutane dimers appears to be a repair process of some importance in human skin. Measurements of the kinetics of cyclobutane dimer removal in UV-irradiated human skin in the dark revealed that this can be faster than throughout found for cultured human fibroblasts (D'Ambrosio *et al.*, 1981a; Reusch *et al.*, 1988; Sutherland *et al.*, 1980). This difference between *in vivo* and *in vitro* is found still more strikingly for rodent cells (Mullaart *et al.*, 1988; Vink, pers. comm.). In a comprehensive study with 17 volunteers by Freeman (1988) on removal of dimers in human skin an average half-life of 11.0 (± 4.3) h was found. His data indicated a significant inter-individual variation in the ability of human skin to repair cyclobutane dimers. The few data available on removal of (6-4)photoproducts *in vivo* indicate that its rate may be comparable to that of cyclobutane dimers *in vivo* (Mitchell and Nairn, 1989). It appears possible that in mammalian cells *in vitro* a factor is missing, which is involved in untangling the DNA structure and/or recognizing certain DNA lesions. This might result in slower repair in those parts of the genome, which are not actively transcribed (heterochromatin) and therefore are less accessible to proteins.

The mechanisms of DNA repair in mammalian cells still remain to be solved. The process is studied by use of mutant cells, variation of amounts and types of lesions, use of (specific) drugs that act on certain (repair) enzymes, and cloning of human repair genes. Apparently, mammalian DNA repair is a highly complex process in which many genes are involved, in contrast to the process of photoreactivation. Strikingly, it was found that in *E. coli* as well as in *S. cerevisiae* excision repair of cyclobutane dimers is enhanced by photolyase even without addition of light (Sancar *et al.*, 1984; Sancar and Smith, 1989; Yamamoto *et al.*, 1983; Yamamoto *et al.*, 1984). Recently, it was proposed that this enhancement is a general phenomenon and that photolyase should be considered as an accessory protein in the excision repair pathway (Patterson and Chu, 1989; Sancar and Smith, 1989). Whether this

role of photolyase extends to repair in mammalian cells remains to be answered. Unequivocal demonstration of enzymatic photoreactivation in these organisms has been problematic and any homology in nucleotide sequence with either the bacterial or yeast photolyase gene has not been observed in hybridisation studies (Meechan *et al.*, 1986; Sancar, 1985; Yasui and Langeveld, 1985). This absence of homology suggests that the nucleotide sequences of photolyase genes are not highly conserved in evolution. On the other hand, at the protein level differences might be smaller. Considerable homology exists in amino acid sequences between the human repair protein ERCC-1 and the yeast repair protein RAD10 as well as between (the C-terminal part of) ERCC-1 and the *E. coli* repair proteins UvrA and UvrC (Doolittle *et al.*, 1986; Van Duin *et al.*, 1986; Van Duin *et al.*, 1988). The hypothesis that sequences of repair proteins might be strongly conserved in evolution is strengthened by the recent finding of Weber *et al.* (1990) that the human *ERCC-2* gene is very similar to the yeast repair gene *RAD3*.

One may speculate that also in mammalian cells photolyases are enzymes with a dual function: firstly as a photoreactivator involved in cleavage of cyclobutane dimers, and secondly as a recognition factor accessory in the excision repair pathway. The first function appears to be lacking in cultured cells. Although photorepair is a phenomenon observed in human skin, the existence of a true photoreactivating enzyme in human cells remains to be established. Probably only cloning of a mammalian photolyase gene can resolve the inconsistencies about photoreactivation in mammalian cells. Still, photorepair of cyclobutane dimers in human cells can be brought about by photochemical instead of photoenzymatic reactions, which does not neccessarily exclude a flavin derative as the donor of electrons required for dimer-splitting, analogous to the mechanism of real photolyases.

VI SUMMARY

Human skin is continuously exposed to a wide variety of potentially harmful environmental agents. One of these is ultraviolet radiation (UV). In western countries, changes in life style have resulted in a substantial increase in exposure of the human skin to UV from the sun or from artificial sources. The most important detrimental effect of UV on the living cell, known so far, is the induction of damage in the DNA. Various UV-induced lesions have been identified. For each type of lesion, the amount induced varies with the wavelength of UV in a different way. The major photoproducts induced in DNA by UV-C (wavelengths between 200 and 280 nm) and UV-B (280-315 nm) are the cyclobutane-type pyrimidine dimers, which are involved in UV-induced cell-killing, mutagenesis and carcinogenesis. Other important photoproducts in DNA induced by UV-C comprise the (6-4)photoproducts, which as well can be lethal or mutagenic. Fortunately, cells have efficient mechanisms at their disposal to remove these lesions from their DNA. For UV-induced DNA lesions at least two enzymatic pathways are known. Cyclobutane dimers can be monomerized in a reaction that requires visible light (photoreactivation, PHR) and which is catalyzed by a DNA photolyase (photoreactivating enzyme, PRE). In the other mechanism, excision repair, a stretch of the DNA strand containing the damage is taken out, followed by insertion of new nucleotides and sealing of the gap. The mechanism of this process has been characterized at the molecular level for *Escherichia coli*. In the cells of higher eukaryotes similar mechanisms appear to be operative.

In this thesis the results of a study on the processing of UV-induced DNA damages in cultured human skin fibroblasts, chicken embryo fibroblasts and human skin *in vivo* have been described. The aim of the study was to gain insight into the damaging potentials of UV. In addition to cells from normal individuals, skin fibroblasts derived from persons with the rare recessive disease xeroderma pigmentosum (XP) were also investigated. Cultured cells of XP patients show a markedly increased sensitivity to UV; they are deficient in the repair of UV-damaged DNA. This deficiency appears to be closely correlated with the early events in the development of neoplasia in these patients.

In *Appendix paper I* the effects of UV-C (mainly 254 nm) on cultured human skin fibroblasts have been compared with the effects of UV-A (> 330 nm) on these cells. Both UV-C and UV-A induced DNA damage and cell-killing. After UV-A irradiation, single-strand breaks could be detected in DNA; these lesions were rapidly repaired. The induction of these breaks was almost absent when irradiation was

performed in the presence of catalase, an enzyme that catalyses the lysis of hydrogenperoxide. However, the presence of catalase did not reduce cell-killing by UV-A. Only a very small amount of cyclobutane dimers was induced by this type of UV, which is consistent with the virtual absence of excision repair in cultured human fibroblasts after UV-A exposure. XP fibroblasts of complementation group A, which are extremely sensitive to UV-C irradiation, showed the same sensitivity to UV-A (with regard to cell-killing) as did normal fibroblasts. The results confirm the biological importance of cyclobutane dimers in the case of UV-C irradiation, particularly in the repair-deficient XP fibroblasts, but also indicate that non-cyclobutane dimer damage is responsible for UV-A induced cell death. Single-strand breaks, however, do not seem to be important in this respect.

Cultured chicken-embryo fibroblasts possess the capacity to photoreactivate cyclobutane dimers in their DNA. To determine the effect of dimer removal by PHR on the occurrence of excision repair, in *Appendix paper II* the time course of excision repair, monitored as unscheduled DNA synthesis (UDS), following UV irradiation has been examined. PHR had little effect on the initial UDS, but a distinct difference in rate and cumulative amount of UDS was seen several hours after irradiation. The results of the experiments described in *Appendix paper II* suggest that excision repair of cyclobutane dimers is mainly responsible for the UDS seen in the period between 2 and 24 h after UV exposure but not for early UDS.

In order to study the induction and repair of cyclobutane dimers at the single-cell level in UV-irradiated cultured cells as well as in human skin irradiated *in vivo*, monoclonal antibodies directed against thymine dimers were isolated. The thymine dimer is one of the four possible cyclobutane dimers found in UV-irradiated DNA. The isolation and characterization of the antibody is described in *Appendix paper III*. The antibody was found to be highly specific for thymine dimers in DNA; binding of the antibody was decreased upon *in vitro* repair of cyclobutane dimers by treatment of UV-irradiated DNA with PRE under illumination. Methods were developed to determine the amount of thymine dimers in isolated DNA from UV-irradiated cells or directly in cells *in situ*. The removal of dimers detected immunochemically was comparable to that observed with biochemical methods. Advantages of the immunochemical methods are that radioactive labelling of DNA is not required and that detection can be performed with small numbers of cells.

In *Appendix paper IV* the kinetics of excision repair and dimer removal in UV-irradiated cultured normal human and XP fibroblasts, which had been microinjected with purified yeast photoreactivating enzyme (PRE), have been investigated. Thymine dimers were determined at the single-cell level by quantitative immunofluorescence

microscopy based on the application of the thymine dimer-specific antibody. Injection with PRE and subsequent illumination resulted in rapid disappearance of dimers: within 15 min virtually all dimers had been removed in normal as well as in XP fibroblasts, whereas non-injected normal cells still retained 25% of their dimers after 27 h. In general, PHR of dimers strongly reduces UV-induced UDS. However, when PHR was applied immediately after UV irradiation, UDS remained unchanged initially and started to decrease after about 30 min. When PHR was performed 2 h after UV exposure, UDS dropped without delay. This difference can be explained by the preferential removal of some type of non-cyclobutane dimer lesions, *e.g.*, (6-4)photoproducts, which is responsible for the PHR-resistant UDS immediately following UV irradiation. After the rapid removal of these photoproducts, the bulk of UDS is due to dimer repair. Also in XP fibroblasts the effect of PHR was investigated. Although dimers were removed by PHR as rapidly in these cells as in normal cells, the decrease of the (residual) UDS due to PHR was much more delayed than in normal cells, or even absent. This supports the idea that repair of non-cyclobutane dimer lesions does occur in certain XP cell-strains, but at a much lower rate.

To investigate the possible occurrence of PHR in human skin, the effect of visible light on the level of UV-induced thymine dimers in human epidermal cells *in vivo* was examined (*Appendix paper V*). Volunteers were exposed to a moderate dose of only UV-B, or to a serial combination of UV-B and visible light. Dimers were assayed in skin sections by quantitative immunofluorescence microscopy. After a single UV-exposure a significant amount of dimers was measured, but no difference could be detected between skin kept in the dark after UV-irradiation and that exposed to visible light. In other experiments the UV-dose was split into 3 parts, given at 2.5 h intervals. Following each dose fraction, half of the skin area was exposed to light. After the second and third dose fraction, illuminated skin areas clearly showed lower levels of dimers than did skin kept in the dark. The results provide evidence that photorepair (possibly PHR) does occur in human skin, but not immediately after a first UV-exposure.

Upon exposure of cells to UV, various DNA damages are induced. Primarily, excision repair appears responsible for the removal of the lesions induced by short-wavelength UV. After irradiation, most of the initial repair synthesis is due to repair of non-cyclobutane dimer lesions, but at later time points (> 2 h) the bulk of excision repair is due to dimer removal. PHR, when occurring, can significantly contribute to removal of UV-induced DNA lesions, thus decreasing the amount of excision repair. Whereas PHR does not occur in cultured human cells, evidence has

been found for the occurrence of a light-enhanced removal of cyclobutane dimers in human skin *in vivo*.

SAMENVATTING

De menselijke huid wordt voortdurend blootgesteld aan allerlei schadelijke invloeden van buitenaf. Eén hiervan is ultraviolette straling (UV). Veranderingen in de leefgewoonten in westerse landen hebben geleid tot een toename van de blootstelling van de menselijke huid aan UV afkomstig van de zon of van kunstmatige UV-bronnen. Het belangrijkste schadelijke effekt van UV voor de levende cel, voorzover nu bekend, is beschadiging van het DNA. Een aantal verschillende beschadigingen is geïdentificeerd. De mate waarin deze door UV in het DNA worden geïnduceerd blijkt op een voor elk type verschillende wijze te variëren met de golflengte van het UV. De belangrijkste fotoprodukten die door UV-C (golflengten tussen 200 en 280 nm) en door UV-B (280-315 nm) in DNA worden gevormd, zijn de cyclobutaan-pyrimidine dimeren, welke betrokken zijn bij de door UV veroorzaakte celdood, mutagenese en tumorigenese. Andere belangrijke fotoprodukten die door inwerking van UV-C in DNA ontstaan zijn de (6-4)fotoprodukten, welke ook de dood van de cel of de vorming van mutaties kunnen veroorzaken. Gelukkig hebben cellen efficiënte mechanismen tot hun beschikking, waarmee de DNA-beschadigingen uit het DNA kunnen worden verwijderd. Voor DNA-beschadigingen die door UV zijn veroorzaakt, zijn tenminste twee verschillende enzymatische herstel-processen bekend. Cyclobutaan dimeren kunnen worden gemonomerizeerd in een enzymatische reaktie (fotoreaktivering), die wordt verzorgd door DNA-fotolyase (fotoreaktiverend enzym, PRE) onder absorptie van zichtbaar licht. Bij het andere herstelmechanisme, excisieherstel, wordt een deel van de DNA streng waarin de beschadiging zich bevindt, verwijderd. Dit wordt gevolgd door de aanmaak van een nieuw stuk DNA en sluiting van de ontstane breuk in de DNA keten. Echter, het mechanisme van dit proces op moleculair niveau is tot nu toe slechts gekarakteriseerd voor de bacterie *Escherichia coli*. In cellen van hogere eukaryoten lijken vergelijkbare mechanismen een rol te spelen.

In dit proefschrift zijn de resultaten beschreven van onderzoek naar de wijze waarop UV-geïnduceerde DNA-beschadigingen worden verwerkt door gekweekte fibroblasten uit de menselijke huid en uit het kippeëmbryo, en door cellen in menselijke huid *in vivo*. Het doel van het onderzoek was inzicht te krijgen in de DNA-beschadigende werking van UV. Behalve onderzoek met cellen van normale personen, is ook onderzoek verricht met huidfibroblasten afkomstig van personen

die lijden aan de zeldzame, recessieve ziekte xeroderma pigmentosum (XP). Gekweekte cellen van XP-patiënten vertonen een uitgesproken gevoeligheid voor UV en zijn slecht in staat de door UV geïnduceerde beschadigingen uit het DNA te verwijderen. Dit hersteldefekt lijkt een rol te spelen bij de vroege verschijnselen in het ontstaan van neoplasieën bij deze patiënten.

In *Appendix paper I* zijn de effekten van UV-C (voornamelijk 254 nm) op gekweekte menselijke huidfibroblasten vergeleken met de effekten van UV-A (> 330 nm) op deze cellen. Zowel UV-C als UV-A veroorzaken beschadigingen in het DNA en kunnen de dood van de cel teweegbrengen, maar er zijn opvallende verschillen. Na bestraling met UV-A kunnen enkelstreng DNA-breuken worden gedetekteerd, welke zeer snel worden gerepareerd. De vorming van deze breuken kan bijna geheel worden voorkomen indien de bestraling wordt uitgevoerd in de aanwezigheid van katalase, een enzym dat de splitsing van waterstofperoxide katalyseert. Echter, de aanwezigheid van katalase blijkt niet te leiden tot een verhoging van de celoverleving. UV-A bestraling geeft aanleiding tot vorming van slechts geringe hoeveelheden cyclobutaan dimeren, hetgeen in overeenstemming is met de afwezigheid van het optreden van excisieherstel in gekweekte menselijke fibroblasten na bestraling met UV-A. Dit is in tegenstelling met hetgeen dat na bestraling met UV-C wordt gevonden. XP-fibroblasten van de complementatiegroep A, welke bijzonder gevoelig zijn voor UV-C-bestraling, vertoonden in celoverleving eenzelfde gevoeligheid voor UV-A als normale fibroblasten. De resultaten in *Appendix paper I* bevestigen het biologische belang van cyclobutaan dimeren in het geval van bestraling met UV-C, in het bijzonder bij de herstel-deficiënte XP-fibroblasten, maar duiden er ook op dat andere beschadigingen dan dimeren verantwoordelijk zijn voor de door UV-A geïnduceerde celdood. Enkelstrengbreuken in DNA lijken in dit verband echter niet van belang.

Gekweekte fibroblasten uit het kippeëmbryo zijn in staat cyclobutaan dimeren in het DNA te herstellen via fotoreaktivering. Om het effekt van de verwijdering van dimeren via fotoreaktivering op het optreden van excisieherstel te bepalen, is de kinetiek van excisieherstel die het gevolg is van UV-bestraling onderzocht. De resultaten zijn beschreven in *Appendix paper II.* De DNA-synthese (UDS) die optreedt bij het herstel is hierbij gemeten. Fotoreaktivering had slechts een gering effekt op de initiële UDS, maar enkele uren na de bestraling werd een duidelijk verschil waargenomen zowel wat betreft de snelheid van UDS als de cumulatieve hoeveelheid. De resultaten van de experimenten beschreven in *Appendix paper II* rechtvaardigen de veronderstelling dat vooral herstel van dimeren verantwoordelijk is voor het gemeten excisieherstel in de periode tussen 2 en 24 uur na de bestraling, maar niet

voor het vroege excisieherstel.

Om de vorming en het herstel van cyclobutaan dimeren op het niveau van de enkele cel te kunnen bestuderen, zowel in UV-bestraalde gekweekte cellen als in menselijke huid die *in vivo* is bestraald, werden monoclonale antilichamen tegen thymine dimeren geïsoleerd. Het thymine dimeer is één van de vier mogelijke cyclobutaan dimeren die kunnen worden gevormd in UV-bestraald DNA. De isolatie en karakterisering van het antilichaam is beschreven in *Appendix paper III*. Het antilichaam bleek bijzonder specifiek voor thymine dimeren in DNA te zijn; binding van het antilichaam bleek niet meer op te treden na *in vitro* herstel van de dimeren door behandeling van UV-bestraald DNA met PRE in aanwezigheid van licht. Er werden methoden ontwikkeld om de hoeveelheid thymine dimeren te bepalen, zowel in DNA geïsoleerd uit UV-bestraalde cellen als direkt, in cellen *in situ*. De gegevens omtrent immunochemisch bepaalde verwijdering van dimeren waren vergelijkbaar met resultaten verkregen via biochemische methoden. De immunochemische bepalingsmethoden hebben het voordeel dat het niet nodig is het DNA radioaktief te merken en dat de bepaling kan worden uitgevoerd met geringe aantallen cellen.

In *Appendix paper IV* is de kinetiek van het excisieherstel en de verwijdering van dimeren onderzocht in UV-bestraalde normale menselijke en XP-fibroblasten, die waren geïnjekteerd met PRE. De thymine dimeren werden bepaald op het niveau van de enkele cel met kwantitatieve immunofluorescentiemikroskopie waarbij gebruik gemaakt werd van het thymine dimeer-specifieke antilichaam. Injektie van PRE in cellen gevolgd door belichting resulteerde in een snelle verdwijning van de dimeren: binnen 15 min bleken vrijwel alle dimeren verwijderd te zijn, zowel in normale als in XP-fibroblasten, terwijl cellen die niet geïnjekteerd waren 27 uur na de bestraling nog 25% van de oorspronkelijke hoeveelheid dimeren bevatten. In het algemeen blijkt fotoreaktivering de UV-geïnduceerde UDS sterk te verminderen. Echter, indien fotoreaktivering onmiddelijk na de UV bestraling plaatsvindt blijft de UDS aanvankelijk onveranderd: pas na ongeveer 30 min begint de daling van de UDS op te treden. Wanneer daarentegen fotoreaktivering plaatsvindt 2 uur na de bestraling is er een onmiddelijke afname van de UDS. Dit verschil kan worden verklaard door de preferentiële verwijdering van andere typen DNA-beschadigingen dan cyclobutaan dimeren, bijvoorbeeld (6-4)fotoprodukten. In deze verklaring is dit verschijnsel verantwoordelijk voor de "fotoreaktivering-resistente" UDS die plaatsvindt onmiddelijk na de bestraling. Na de snelle verwijdering van dit type fotoprodukten is het overgrote deel van de UDS het resultaat van herstel van dimeren. Het effekt van fotoreaktivering is ook in XP-fibroblasten onderzocht. Hoewel bij fotoreaktivering de dimeren in deze cellen net zo snel werden verwijderd als in normale cellen, was de

verlaging van de (residuele) UDS tengevolge van fotoreaktivering erg vertraagd vergeleken met normale cellen of zelfs afwezig. Dit ondersteunt de gedachte dat herstel van andere beschadigingen dan cyclobutaan dimeren wel optreedt in bepaalde XP cellijnen, maar met een lagere snelheid.

Om het mogelijke optreden van fotoreaktivering in de menselijke huid te onderzoeken, werd het effekt van zichtbaar licht op het aantal door UV geïnduceerde thymine dimeren in menselijke epidermale cellen *in vivo* bekeken (*Appendix paper V*). Vrijwilligers werden blootgesteld aan UV-B, of aan een opeenvolgende combinatie van UV-B en zichtbaar licht. De dimeren werden bepaald in coupes van huidbiopten, met kwantitatieve immunofluorescentiemikroskopie. Na een enkelvoudige blootstelling aan UV werden signifikante hoeveelheden dimeren waargenomen, maar er werd geen verschil waargenomen tussen huid die na de bestraling in het donker was gehouden en die welke was blootgesteld aan licht. In andere experimenten werd de UV dosis in drieën gesplitst met intervallen van 2½ uur. De helft van het bestraalde huidoppervlak werd na elke bestraling aan licht blootgesteld. Na de tweede en de derde dosering vertoonden de belichte huidgedeelten duidelijk minder dimeren dan de huid die in het donker was gehouden. De resultaten duiden dat in mensenhuid fotoherstel (mogelijk fotoreaktivering) optreedt, echter niet na een enkele UV-bestraling.

Bij blootstelling van cellen aan UV worden er verschillende beschadigingen in het DNA gevormd. In eerste instantie lijkt het excisieherstel verantwoordelijk voor de verwijdering van de beschadigingen die door kortgolvig UV zijn geïnduceerd. De UDS die optreedt na de bestraling is aanvankelijk grotendeels het resultaat van herstel van andere beschadigingen dan cyclobutaan dimeren, echter op latere tijdstippen ligt vooral de verwijdering van dimeren eraan ten grondslag. Wanneer fotoreaktivering optreedt, kan dit een belangrijke bijdrage leveren aan het herstel van DNA-beschadigingen die door UV zijn geïnduceerd, met als gevolg dat in mindere mate excisieherstel optreedt. Hoewel gekweekte menselijke cellen niet in staat zijn tot fotoreaktivering van UV schade in DNA, zijn er aanwijzingen gevonden voor het optreden van versneld herstel van dimeren in menselijke huid *in vivo* indien deze wordt belicht na herhaalde bestraling.

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CURRICULUM VITAE

Op 25 april 1956 werd de schrijver van dit proefschrift te Vlaardingen geboren. Na het behalen van het diploma Gymnasium B aan de Christelijke Scholengemeenschap Groen van Prinsterer te Vlaardingen in 1974, begon hij in hetzelfde jaar een studie aan de subfaculteit scheikunde van de Rijks Universiteit te Leiden. Het kandidaatsexamen S2 werd behaald in 1977. Voor het doctoraalexamen werd onderzoek verricht op de afdelingen Eiwitbiosynthese van het Biochemisch Laboratorium van de R.U. Leiden en Celbiologie en Genetica van de Medische Faculteit van de Erasmus Universiteit Rotterdam. In 1980 was hij tevens als student-assistent aan laatstgenoemde afdeling verbonden. In april 1981 werd het doctoraalexamen afgelegd. Vanaf november 1981 was hij als wetenschappelijk assistent verbonden aan de afdeling Celbiologie en Genetica van de Erasmus Universiteit Rotterdam. Het onderzoek was mogelijk door financiële steun van het Ministerie van Volksgezondheid en Milieubeheer (later Welzijn, Volksgezondheid en Cultuur) en werd uitgevoerd binnen de sektie Genetische Toxikologie van het Medisch Biologisch Laboratorium TNO te Rijswijk, eerst onder leiding van Dr. Ir. P.H.M. Lohman en later onder leiding van Dr. R.A. Baan. Sinds september 1987 is hij als wetenschappelijk medewerker in dienst van het Medisch Biologisch Laboratorium TNO.

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APPENDIX PAPER I

Mutation Research, 146, 89-98 (1985)

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DNA Repair Reports
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The induction and repair of DNA damage and its influence on cell death in primary human fibroblasts exposed to UV-A or UV-C irradiation

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Summary

Irradiation with UV-A of normal human fibroblasts in phosphate-buffered saline induced cell death, measured as lack of colony-forming ability. A specially filtered sunlamp, emitting wavelengths > 330 nm, was used as UV-A source. After UV-A irradiation, single-strand breaks (alkali-labile bonds) could be detected in DNA; these lesions were rapidly repaired. The induction of these single-strand breaks was almost eliminated when irradiation was performed in the presence of catalase. However, catalase, when present during UV-A irradiation, did not reduce cell death of the fibroblasts. Excision repair, monitored as unscheduled DNA synthesis, was induced strongly by irradiation with UV-C (predominantly 254 nm), but could not be detected after UV-A irradiation. Moreover, very little accumulation of incision breaks during post-irradiation incubation with hydroxyurea and 1-β-D-arabinofuranosylcytosine (araC) was detected after UV-A. This is consistent with the low amount of pyrimidine dimers (measured as UV-endonuclease susceptible sites) induced by UV-A. Xeroderma pigmentosum fibroblasts of complementation group A, which are extremely sensitive to UV-C irradiation, showed the same sensitivity to UV-A as normal fibroblasts. The results indicate that lethality by UV-A wavelengths > 330 nm is caused by lesions other than single-strand breaks (alkali-labile bonds) and pyrimidine dimers.

The most important detrimental effect of ultraviolet radiation on the living cell, so far known, is the induction of damage in the DNA. In addition to studies with cells from normal individuals, studies with skin fibroblasts derived from persons with the rare autosomal recessive disease xeroderma pigmentosum (XP; for a review, see Kraemer, 1980) have given us insight into the damaging potentials of UV. Cultured cells of XP patients show a marked sensitivity to UV and have defective repair of UV-damaged DNA, which appears to be intimately involved in the early events in neoplasia in the patients (Cleaver and Bootsma, 1975; Hart et al., 1977).

Many of the investigations with cultured human cells have employed UV-C (< 280 nm) radiation as the damaging agent; usually 254-nm UV was used. The major UV-C products in DNA are the cyclobutane-type pyrimidine dimers and these have been implicated in UV-induced lethality, mutagen-

Abbreviations: ESS, UV-endonuclease susceptible sites; PBS, phosphate-buffered saline; UDS, unscheduled DNA synthesis; FCS, fetal calf serum; araC, 1-β-D-arabinofuranosylcytosine; SSB, single-strand DNA breaks; ^{3}HdT, [^{3}H]thymidine; D37, the fluence that reduces survival to 37%.

icity and tumorigenicity (Hart et al., 1977; Rothman and Setlow, 1979; Suzuki et al., 1981). However, only longer-wavelength UV (UV-A: 315–380 nm and part of UV-B: 280–315 nm) reaches the earth's surface, since the ozone layer in the upper atmosphere effectively filters out UV wavelengths from sunlight < 290 nm. It has been demonstrated that the types of damage produced by irradiation at UV wavelengths > 310 nm differ from those induced by UV-C radiation (Tyrrell et al., 1974; Hariharan and Cerutti, 1977; Ellison and Childs, 1981; Smith and Paterson, 1982). Moreover, it has been suggested that UV-irradiation, in particular at the longer wavelengths, has deleterious effects on cellular repair systems for damaged DNA (Tyrrell and Webb, 1973; Smith and Paterson, 1982; Holmberg, 1983). Despite these differences in effects of UV-C and longer wavelength UV, most photobiological studies, in particular those with cultured human cells, have been performed with UV-C radiation, which is environmentally not relevant.

In this paper we report on a comparative study of the effects of UV-C and UV-A on cultured normal human skin fibroblasts. The induction of single-strand DNA breaks (SSB, including alkali-labile bonds) was investigated, as well as the formation of lesions that are converted into SSB under the influence of site-specific endonucleases present in extracts from *Micrococcus luteus* bacteria (endonuclease-susceptible sites, ESS, probably identical to pyrimidine dimers). The repair of SSB during a post-irradiation incubation was followed. The occurrence of long-patch excision repair after UV-C or UV-A irradiation was monitored by measuring unscheduled DNA synthesis (UDS). Repair was also studied by determination of the accumulation of incision breaks when irradiated cells were treated with HU (hydroxyurea) and araC (1-β-D-arabinofuranosylcytosine); this treatment blocks repair-DNA synthesis, and breaks introduced by cellular endonucleases, which would otherwise be resealed after repair synthesis, now accumulate within minutes of irradiation. Finally, normal human cells were compared with XP-cells with regard to sensitivity to UV-C and UV-A. The results confirm the biological importance of pyrimidine dimers in the case of UV-C irradiation, but also indicate that non-dimer damage is responsible for UV-A induced lethality. UV-A induced SSB, however, do not seem to be important in this respect.

Materials and methods

Cell strains and culture conditions

Monolayer cultures of cells were grown in Ham's F10 medium (Flow Laboratories, Irvine, U.K.) supplemented with 15% fetal calf serum (FCS; Flow), 1 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The absence of mycoplasma infections was periodically checked by the method of Chen (1977). The normal human skin fibroblast strain 82MB2 was derived from foreskin in our laboratory. The xeroderma pigmentosum fibroblast strain XP2CA has been assigned to complementation group A (Hashem et al., 1980).

UV irradiation

The UV-C source was a single low pressure mercury vapour lamp (Philips, 15-W TUV). The incident fluence rate was 0.38 $W \cdot m^{-2}$ at 254 nm as determined with a calibrated thermopile (Hewlett Packard 8334A/8330A). A correction was made for the contribution of radiation with longer wavelengths, the intensities of which were given by the manufacturer. In some experiments a 6-W TUV lamp (Philips) was used, with a fluence rate of 0.02 $W \cdot m^{-2}$. Cells were irradiated, while attached to 3- or 6-cm plastic culture dishes (Greiner, Nürtingen, F.R.G.), after they had been washed free of medium with ice-cold phosphate-buffered saline (PBS, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.6 mM KCl). Irradiation was from above, at room temperature, directly after draining off the cold PBS.

The UV-A source was a 1200 W type Sellas UV-A sunlamp. The radiation was filtered with a selected piece of 8-mm plate glass; less than 10^{-6} of the emitted energy was of wavelengths below 320 nm. A relative spectral energy distribution curve is presented in Fig. 1. The fluence rate at a distance of about 55 cm was 165 $W \cdot m^{-2}$ as determined with a Waldmann PUVA meter calibrated with a Kipp E11 thermopile. Before irradiation cells were rinsed as above; they were irradiated from beneath, through the bottom of

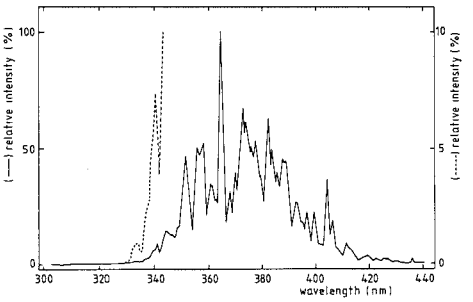

Fig. 1. The emission spectrum of the Sellas UV-A sunlamp type Sellasol 1200, filtered by an 8-mm thick plate of glass to eliminate light of wavelengths below 330 nm. The relative spectral energy distribution was measured through a Jarrel Ash monochromator operating at a bandwidth of 1.2 nm. Calibration was done by comparison with the radiant output of a HPK 125 Watt of Philips. In addition, the spectrum between 305 and 345 nm, recorded at a 10-fold higher sensitivity, has been plotted (- - - - -).

the dishes which contained 1 or 3 ml of ice-cold PBS per 3-cm or 6-cm dish, respectively. The temperature of the PBS was checked to remain below 14°C during and after irradiation with the highest UV-A dose. In some experiments (Figs. 2–4) catalase was added to the PBS (Sigma Chemical Co., St. Louis, MO; 1600 units/ml).

γ-Irradiation

Air-equilibrated cells were irradiated in monolayer in complete medium at 0°C in a ^{60}Co-γ-source (Gammacell 100, Atomic Energy of Canada Ltd.) at a dose rate of 13 Gy · min^{-1}.

Cell survival

Cell survival was determined as described by Arlett et al. (1975), with some modifications. Briefly, on the day prior to irradiation, cells of the strain under test were harvested and an appropriate dilution in Ham's F10 medium was made to allow plating of 2×10^5 cells per 6-cm culture dish (Greiner). The remainder of the harvested cells was irradiated with 40 Gy of ^{60}Co-γ-radiation and diluted to a final concentration of 4×10^3 cells/ml in Eagles MEM (Flow) + 15% FCS. 10-ml aliquots of this suspension were then dispensed onto the appropriate number of 9-cm culture dishes (Greiner) to serve as feeder layer. All dishes were incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. The next day the cells on the 6-cm culture dishes were UV-irradiated and trypsinized. Control and UV-irradiated cell samples were seeded at appropriate densities on feeder-layer plates; 5 plates per fluence point were used. The cells were spread evenly; the plates were incubated for 17–20 days to allow colony formation. The medium was changed once during this period. At the end of the growth period, 2 ml of 0.5% methylene blue in 50% methanol was added to each plate. After a 60-min staining period, this solution was drained off and the plates were air-dried. Then the plates were rinsed with distilled water and dried again. Colonies estimated to contain more than 50 cells were considered as survivors. The mean of 5 replicate plate counts was used to calculate the surviving fraction at each fluence.

Unscheduled DNA synthesis

Unscheduled DNA synthesis (UDS) after UV exposure was measured autoradiographically by the incorporation of [^{3}H]thymidine (^{3}HdT, spec. act. 24 Ci · mmole^{-1}, Amersham, U.K.) in cells in the G1 or G2 phase of the cell cycle. Cultures (5×10^4 cells per 3-cm dish) grown for 2 days after seeding, were exposed to different UV doses. Then, they were given fresh medium containing 10 μCi ^{3}HdT per ml and were incubated at 37°C for 3 h. Subsequently, they were fixed with Bouin's fixative, mounted onto glass slides and dipped in liquid photographic emulsion (Kodak NTB2). After 4 days at 4°C, the slides were developed and stained with toluidine blue. The level of UDS was assessed by measuring the area occupied by the grains overlaying the nucleus of 40 lightly labelled cells (heavily labelled cells were regarded as being in S phase), with an Artek Model 880 automatic grain counter (Lonati-Galligani et al., 1983). The data obtained were processed by computer analysis and displayed as histograms and average area percentages per nucleus (the mean area occupied by the grains relative to the total surface of the nuclei). Background values have been subtracted.

Determination of UV-endonuclease susceptible sites (ESS)

The technique used to determine the amount of

sites susceptible to strand-breaking by endonuclease(s) present in a *M. luteus* extract, was essentially that originally reported by Paterson et al. (1973), but modified to give higher control molecular weights and more reproducible results (Zelle and Lohman, 1979; Wade and Lohman, 1980). The breaks were enumerated by velocity sedimentation in alkaline sucrose gradients and computer analysis of the resulting distribution profiles. The determinations were done in duplicate and the results are expressed as the number of ESS per 10^9 dalton of DNA. Values of controls incubated without *M. luteus* extract, and those of the non-irradiated controls, have been subtracted.

The values for the ESS induction per unit dose of UV-A and UV-C given in the text and in Table 1 were obtained by linear regression analysis of the results of several experiments at various dosages. UV-A: 7 determinations at 3 different doses in the range 45–335 kJ · m^{-2}; the accuracy is limited due to the relatively low number of ESS induced (approx. 11 per 10^9 dalton at the highest dose) but the cells will not support a further increase of dose (they become detached). UV-C: 14 determinations at 5 doses in the range 1.9–7.6 J · m^{-2}.

Detection of single-strand breaks (SSB) by alkaline elution

The method applied was mainly that of Kohn et al. (1976) and Fornace et al. (1976) with some modifications as described by Shiloh et al. (1983). In each experiment, a γ-irradiated sample, which had been prelabelled with [^{14}C]thymidine (specific activity 56.5 mCi · $mmole^{-1}$, Amersham, U.K.), served as a standard. Under the conditions mentioned above it has been found earlier (Van der Schans et al., 1983) that 1 Gy induces 2.5 SSB per 10^{10} dalton of DNA.

Detection of incision breaks after hydroxyurea-araC treatment

^{14}C-labeled cells, prepared as for SSB-detection by alkaline elution, were drained and fresh medium containing 4 mM hydroxyurea (HU) was added. After an incubation at 37°C for 1 h, the cells were irradiated with UV and incubated for 1 h in medium containing 4 mM HU and 50 μM 1-β-D-arabinofuranosylcytosine (araC). DNA breaks were detected by the alkaline elution method.

Results

UV-induced lesions

The effects measured when normal human fibroblasts were irradiated with UV-C (254 nm) or UV-A (> 330 nm; see spectrum in Fig. 1) are summarized in Table 1. In addition to the effect on cell survival (D37, the fluence that reduces survival to 37%), the yields of ESS and SSB are given. The D37's show the expected large difference (a factor of about 4×10^4). Irradiation with various dosages of UV-C induced — on the average — 50 ESS per J · m^{-2}, per 10^9 dalton of DNA, in contrast to only 3.2×10^{-5} found for UV-A, a difference of a factor of 1.6×10^6 (see methods section for details). The value determined for the induction of ESS by 254-nm UV is somewhat higher than values published earlier for hu-

TABLE 1

COMPARISON OF LETHALITY AND INDUCTION OF ESS AND SSB AFTER UV-C AND UV-A IRRADIATION OF NORMAL HUMAN FIBROBLASTS (82MB2)

	UV-C	UV-A
D37 (J · m^{-2})	4.1 ± 0.2	(1.76 ± 0.16) × 10^5
ESS per J · m^{-2}, per 10^9 dalton	50 ± 2	(3.2 ± 0.4) × 10^{-5}
SSB per J · m^{-2}, per 10^9 dalton	n.d.	(1.10 ± 0.08) × 10^{-5}
idem, with catalase	n.d.	(0.11 ± 0.01) × 10^{-5}
ESS/cell/lethal hit (D37) [a]	(72 ± 5) × 10^4	(2.0 ± 0.3) × 10^4
SSB/cell/lethal hit (D37) [a]	n.d.	(0.68 ± 0.08) × 10^4
idem, with catalase	n.d.	(0.068 ± 0.009) × 10^4

D37, UV dose which reduces survival of the cells to 37%. The data with the standard errors indicated for ESS and SSB were obtained by linear regression analysis of the lesion induction curves.

n.d., not determined.

[a] An approximate DNA content of 3.5×10^{12} dalton per cell was assumed.

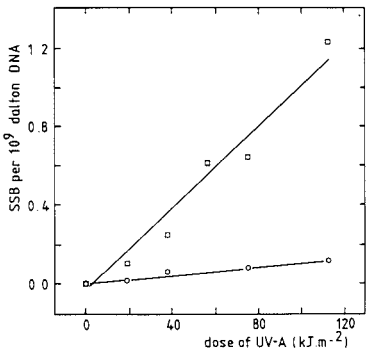

Fig. 2. Induction of single-strand breaks and alkali-labile bonds (SSB) by UV-A irradiation of normal human fibroblasts (82MB2). SSB were detected with alkaline elution. The results of a single, representative experiment are presented, which was performed with duplicate dishes of irradiated cells for each data point. □, irradiation in PBS; ○, irradiation in PBS with catalase.

man fibroblasts: 34 (Zelle and Lohman, 1979), 37 (Smith and Paterson, 1982) and 22 (Ritter and Williams, 1981) ESS per $J \cdot m^{-2}$ per 10^9 dalton DNA, whereas the value found for UV-A is some-

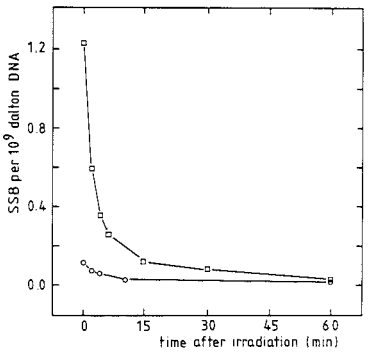

Fig. 3. Removal of SSB induced in normal human fibroblasts by irradiation with 110 $kJ \cdot m^{-2}$ UV-A during a subsequent incubation at 37°C in a 5% CO_2 incubator (except the samples incubated for 2 and 4 min, which were placed on a wet brass plate with a temperature of 37°C). After the periods indicated in the figure, samples were used for the SSB determination. A single representative experiment is shown, performed in duplicate as in Fig. 2. □, irradiation of cells in PBS; ○, irradiation of cells in PBS with catalase.

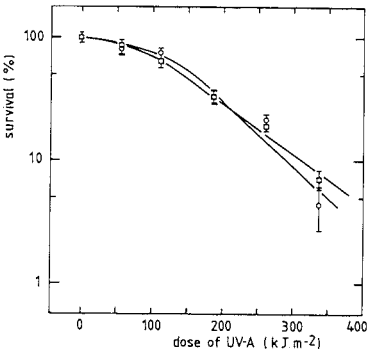

Fig. 4. Survival of normal human fibroblasts after UV-A irradiation. □, UV-A irradiation of cells in PBS; ○, UV-A irradiation of cells in PBS with catalase. Mean control plating efficiency was: 46.0 ± 3.7% (with and without catalase). Data points (± SEM) represent mean values of two experiments with 5 replicate plates per fluence point.

what lower than the 5.6×10^{-5} mentioned by Smith and Paterson (1982) for monochromatic 365-nm UV.

Only after UV-A irradiation were significant levels of strand breakage observed; these were induced at about a third of the frequency at which ESS are formed. In Fig. 2 the dose dependence of the induction of SSB by UV-A is presented.

To study whether this formation of SSB is a direct effect of the irradiation, or possibly is mediated by peroxides formed at the high dose of UV-A needed to obtain effects, irradiation was performed in the presence of catalase, an enzyme that inactivates hydrogen peroxide. Under these conditions far less SSB were induced; according to the dose–response graph, the difference amounted to a factor of 9–10. This result suggests that SSB induction by UV-A is due to the formation of hydrogen peroxide, which in turn is converted into highly reactive OH-radicals which damage the DNA in the cells.

Fig. 3 shows the repair of SSB induced by UV-A irradiation (110 $kJ \cdot m^{-2}$) during a post-irradiation incubation of the fibroblasts at 37°C. After 15 min, 90% of the breaks have disappeared. A similar rapid repair was observed after irradiation in the presence of catalase (Fig. 3).

Although the presence of catalase greatly reduces the amount of SSB induced by UV-A, no

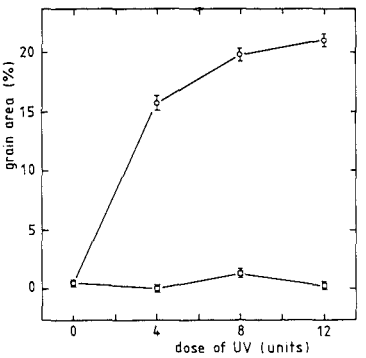

Fig. 5. Induction of unscheduled DNA synthesis (UDS) in normal human fibroblasts (82MB2) by UV-C and UV-A irradiation. Data points represent the amount of repair replication of DNA, derived from the autoradiographically determined incorporation of [^{3}H]thymidine in nuclear DNA. The area occupied by silver grains in the photographic emulsion covering the cell nucleus was measured rather than the number of grains. Per data point the results of 40 non-S phase nuclei were averaged, and plotted with SEM (background has been subtracted). UDS was measured over the first 3 h after irradiation. UV-C fluence rate used was 0.38 $W \cdot m^{-2}$. ○, UDS after UV-C irradiation (1 unit = 1 $J \cdot m^{-2}$); □, UDS after UV-A irradiation (1 unit = 10^4 $J \cdot m^{-2}$).

effect of this enzyme on the lethality of UV-A irradiation was observed (Fig. 4).

DNA-excision repair

UV-C was found to induce unscheduled DNA synthesis (UDS), which was measured by autoradiography, in a dose-dependent manner (Fig. 5), but no such repair was detected in UV-A irradiated cells, not even after a dose of 120 $kJ \cdot m^{-2}$. With respect to cytotoxicity, this dose is comparable with about 3 $J \cdot m^{-2}$ of UV-C which does result in a high level of UDS (see Fig. 5). However, this highest dose of UV-A used induces only 3.8 ESS per 10^9 dalton DNA, the repair of which is not expected to cause a measurable amount of UDS. On the other hand, this result indicates that other, probably more numerous DNA damages that are possibly induced by UV-A, do not lead to a significant amount of repair replication.

A more sensitive way to detect excision repair than by means of UDS is the determination of incision breaks which accumulate when the cells, after UV irradiation, are incubated in the presence of HU and araC (Fig. 6). After 60 min of incubation with these repair inhibitors, in cells exposed to UV-C, 8.3 SSB per $J \cdot m^{-2}$ per 10^9 dalton DNA

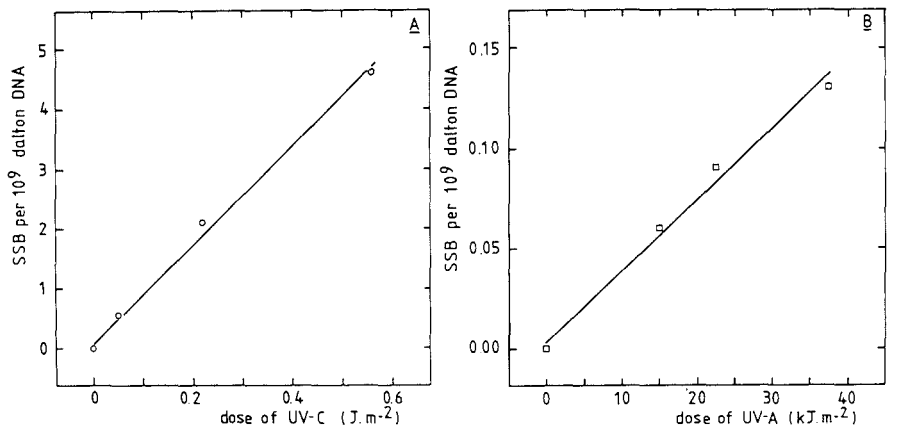

Fig. 6. Accumulation of incision breaks as a function of UV dose in normal human fibroblasts (82MB2). Panel A: incision breaks after UV-C irradiation; panel B: incision breaks after UV-A irradiation. The breaks were measured via alkaline elution. Accumulation was achieved by incubating the cells with hydroxyurea (HU) and araC. Cells were incubated 1 h before irradiation in medium containing 4 mM HU, irradiated as described in Materials and methods and then incubated for 1 h in medium containing HU + araC. The number of breaks in DNA of unirradiated cells, incubated in medium without HU + araC, has been subtracted. A single representative experiment is shown, performed in duplicate as in Fig. 2. UV-C fluence rate used was 0.02 $W \cdot m^{-2}$.

were detected, which corresponds to about 17% of the total amount of ESS induced. The same experiment with UV-A resulted in 3.5×10^{-6} SSB per $J \cdot m^{-2}$ per 10^9 dalton DNA, which corresponds to 11% of the amount of ESS induced. Taking into account the limited accuracy of these numbers (in particular for UV-A), this means that the number of sites undergoing excision repair after irradiation with UV-A or UV-C is not significantly different when equal numbers of ESS are compared. The results are not inconsistent, however, with a somewhat lower yield of araC-detectable sites after UV-A exposure, caused by a selective inhibition of dimer repair as demonstrated by Smith and Paterson (1982).

Comparison of survival of normal and XP cells

The survival responses of normal and XP-A fibroblasts to UV-C and UV-A are shown in Fig. 7. In comparison with normal cells, the XP strain shows enhanced sensitivity only to UV-C irradiation. After UV-A irradiation the curves of the two cell strains are comparable. The ratio of D37 values for normal and XP cells is 23 for UV-C and 1.0 for UV-A.

Discussion

This report describes the DNA-damaging and lethal effects of UV-C and UV-A irradiation on cultured human fibroblasts. Both UV-C and UV-A induce lethality, but there is a marked difference in the cause of lethality. XP-A fibroblasts, which are not able to repair pyrimidine dimers, are extremely sensitive to UV-C irradiation, but show the same sensitivity to the UV-A irradiation used as normal fibroblasts. This indicates that pyrimidine dimers, which are considered the main lethal lesions in case of UV-C irradiation, are of minor importance for exposures to UV-A with wavelengths beyond 330 nm. This could also be demonstrated by the amount of ESS per lethal hit for normal fibroblasts: per lethal hit, UV-C induced 35 times more ESS than UV-A. Clearly, other lesions than pyrimidine dimers are involved in cell killing by UV-A. However, this conclusion is justified only when two assumptions are correct: (i) only pyrimidine dimers are measured as ESS and (ii) pyrimidine dimers induced by UV-C are as lethal as those induced by UV-A. The former assumption has already been verified by Zelle et al. (1980), who used the same determination proce-

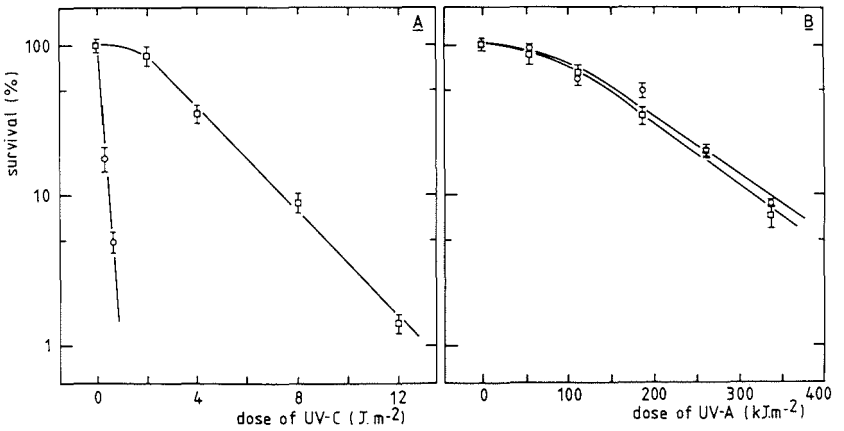

Fig. 7. Survival of normal human fibroblasts (82MB2) and xeroderma pigmentosum group A fibroblasts (XP2CA) as a function of UV-C dose (panel A) and UV-A dose (panel B). □, 82MB2; ○, XP2CA. Mean control plating efficiencies were: 28.2% (82MB2) and 10.8% (XP2CA) for the experiment with UV-C irradiation, and 33.0% (82MB2) and 5.1% (XP2CA) for the experiment with UV-A irradiation. Data points (± SEM) represent mean values of two experiments with 5 replicate plate counts per fluence point, except survival of XP2CA cells after UV-C irradiation which data are from a single experiment. UV-C fluence rate used was 0.38 $W \cdot m^{-2}$ and 0.02 $W \cdot m^{-2}$ for 82MB2 and XP2CA inactivation, respectively.

dures. The second has not been checked yet, but it does not appear to be unreasonable. Similar conclusions have been reported by other authors about monochromatic 365-nm irradiation of HeLa and rodent cells (Danpure and Tyrrell, 1976), of human fibroblasts (Smith and Paterson, 1982) or following polychromatic irradiation with fluorescent sunlamps of rodent cells (Elkind et al., 1978; Zelle et al., 1980) or of human fibroblasts (Ritter and Williams, 1981).

The lethal lesion induced by long wavelength UV has not been characterized; it may differ with the genetic constitution of the cell strain (Webb, 1978). Some evidence of this has been presented by Smith and Paterson (1982) who showed XP group D cells to be more sensitive in their responses to monochromatic 365-nm irradiation than normal cells. This suggests that XP group D cells have difficulties, not only in the repair of dimers, but also in handling non-dimer lesions. In contrast to this, cells of XP group A appear to have the same ability to repair UV-A induced nondimer lesions as normal cells.

Single-strand breaks or alkali-labile bonds (SSB) in DNA are known to be induced by UV of all wavelengths (Rosenstein and Ducore, 1983), but the ratio of SSB to ESS induced becomes higher for longer wavelengths (Smith and Paterson, 1982). An interesting contribution to the insight into the reaction(s) leading to these SSB comes from the use of catalase during exposure to UV-A. The strong protection against the induction of SSB indicates that hydrogen peroxide is involved in this induction. A possibility is that hydrogen peroxide is generated in the buffer during irradiation and is converted into the active OH radical by means of the Haber–Weiss reaction (Mello Filho and Meneghini, 1984). The hydroxyl radical is the molecular species that ultimately damages DNA.

The repair of SSB in cultured fibroblasts after UV-A irradiation appears to be a very rapid process, since 90% are removed within 15 min. About the same has been reported for repair of SSB induced by γ-irradiation, which are also supposed to be the consequence of radical formation (Van der Schans et al., 1982). These results differ from those of Hoffmann and Meneghini (1979a), who reported a substantially slower repair of SSB after exposure of mammalian cells to 'near UV' in medium containing riboflavin and L-tryptophan; elimination of 90% of the breaks took about 5 h. This difference might be caused by the different experimental conditions, such as the larger contribution of short-wavelength UV in the radiation used by these authors, which hampers a comparison on the basis of energy, and the fact that we irradiated the cells in the absence of riboflavine and tryptophan, which results in a very much lower induction of SSB (Hoffmann and Meneghini, 1979a).

As the catalase experiments show, the SSB induced by UV-A irradiation have no detectable lethal effect on normal human fibroblasts (Fig. 4). They also demonstrate that the amounts of hydrogen peroxide generated by the UV-A irradiations were too small to cause lethality through other lesions, although hydrogen peroxide is known to be toxic for human fibroblasts (Hoffmann and Meneghini, 1979b).

The theoretical possibility remains that a residual fraction of SSB, still present after a prolonged repair period, is responsible for cell death (both after irradiation in the presence and absence of catalase). At present, this hypothesis escapes experimental verification, since the fraction of SSB remaining after a 60-min repair period is already down to the detection limit and too small, therefore, to allow the detection of any significant difference after irradiation in the absence or presence of catalase.

Very little excision repair occurs after UV-A irradiation (detected as accumulated incision breaks), but it corresponds to the amount of accumulated incision breaks after UV-C irradiation when compared on the basis of an equal number of ESS originally induced.

In conclusion, relative to cytotoxicity many more SSB are induced by UV-A irradiation than by UV-C, but these lesions appear not to be lethal. Pyrimidine dimers are induced by UV-A with very low efficiency. They are not important for lethality, as is shown by the survival characteristics of XP group A fibroblasts, which are the same as those of normal fibroblasts. These arguments strongly suggest that other DNA lesions, such as thymine glycols (Hariharan and Cerutti, 1977) or DNA–protein crosslinks (Eisenstark et al., 1982; Han et al., 1984), are responsible for cell death

after UV-A irradiation. However, an alternative is that cell death is caused by non-DNA effects, for instance membrane damage.

Acknowledgements

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APPENDIX PAPER II

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Kinetics of unscheduled DNA synthesis in UV-irradiated chicken embryo fibroblasts

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Summary

Unscheduled DNA synthesis induced by 254-nm UV radiation in chicken embryo fibroblasts was examined for 24 h following irradiation, while cells were kept in the dark. The effect on this repair process of a 2–4-h exposure to photoreactivating light immediately after UV was studied. Initial [^{3}H]thymidine incorporation in the light-treated cells was only slightly different from that in cells not exposed to light, but a distinct difference in rate and cumulative amount of unscheduled DNA synthesis was seen several hours after irradiation. By varying the UV dose and the time allowed for photoreactivation, the amount of dimers (determined as sites sensitive to a *M. luteus* UV-endonuclease) and non-dimers could be changed. The results of these experiments suggest that excision repair of dimers, rather than non-dimer products, is responsible for the unscheduled DNA synthesis seen after UV irradiation.

Chicken embryo fibroblasts have been shown to possess the light-dependent ability to photoreactivate UV-damaged DNA (Paterson et al., 1974; Wade and Lohman, 1980; Van de Merwe and Bronk, 1981; Bronk and Van de Merwe, 1983). Only certain aspects of the occurrence of a light-dependent repair process in UV-irradiated chicken embryo fibroblasts have been studied extensively. Photorepair of pyrimidine dimers was found to increase in efficiency when cells were incubated in the dark for several hours at 37°C following UV irradiation, but prior to exposure to photoreactivating black light (Van de Merwe and Bronk, 1981; Bronk and Van de Merwe, 1983). The incubation in the dark between UV exposure and photoreactivation also led to an increase in survival (Bronk et al., 1984).

In the dark, too, a significant amount of repair of UV-induced lesions occurs. This has been demonstrated by measuring the disappearance of *Micrococcus luteus* UV-endonuclease-sensitive sites (ESS) from the DNA. Although the excision is slow, about 80% of the ESS (pyrimidine dimers) are removed in 24 h (Wade and Lohman, 1980). Occurrence of excision repair also has been studied, by measurement of unscheduled DNA synthesis (UDS) with autoradiography. Cells that were kept in the dark for 2.5–3 h after UV irradiation were compared with cells exposed to photoreactivating light during that period; a similar incorporation of [^{3}H]thymidine ([^{3}H]dT) was observed (Paterson et al., 1974; Wade and Lohman, 1980). It was not

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Abbreviations: ESS, UV-endonuclease-sensitive sites; [^{3}H]dT, [^{3}H]thymidine; PBS, phosphate-buffered saline; UDS, unscheduled DNA synthesis; UV, ultraviolet light.

established whether this repair synthesis resulted from the excision of a small number of dimers, or from the removal of non-dimer photoproducts (or from a combination of both).

To discriminate between the different possibilities, in particular to determine the relative importance of dimer removal for the occurrence of repair replication, and to further characterize the excision repair process in chicken embryo fibroblasts, we designed several experiments to examine the time course of UDS following UV irradiation, using different UV doses and different photoreactivation periods.

Materials and methods

Cell cultures

The isolation of chicken embryo fibroblasts, the composition of standard and special media and the photoreactivation conditions have been previously described (Wade and Lohman, 1980). All in vivo operations were carried out at 41°C, which is the body temperature of the chicken and the temperature at which optimal cell growth is achieved (Wade and Lohman, 1980).

Unscheduled DNA synthesis (UDS)

For measurement of UDS, 1.5×10^5 cells were seeded into 60-mm plastic dishes (Greiner, Nürtingen, F.R.G.) containing glass coverslips and grown in standard Ham's F-10 medium (Flow Laboratories, Irvine, Great Britain) with 15% fetal calf serum (Flow Labs.) for 48 h at 37°C in a humidified atmosphere with 5% CO_2. UV irradiation of the cells and subsequent photoreactivation were carried out as described by Wade and Lohman (1980). When UDS during the photoreactivation period had to be measured, the special medium (Wade and Lohman, 1980) was supplemented with [^{3}H]dT (10 μCi/ml, 17 or 25 Ci/mmole, Amersham, Great Britain). After the photoreactivation period (2 or 4 h) the medium was removed, standard F-10 medium added and all dishes were placed in the dark. At consecutive intervals during the 18–22 h after photoreactivation, [^{3}H]dT (as above) was added to the various dishes. At the end of each interval the cells incubated with [^{3}H]dT were rinsed twice with PBS, fixed in Bouin, mounted onto glass slides, and dipped in liquid emulsion (Kodak NTB 2). After 4 days at 4°C, the slides were developed and stained with toluidine blue. 25 lightly labeled nuclei were scored (cells undergoing normal DNA replication were easily distinguished by their densily labeled nuclei) and the accumulated number of grains minus the background was calculated for each time point. The standard error of the mean was less than 2 grains at each slide and is not plotted. The unirradiated controls showed no increase in grain number above the background. In the experiment the data of which are shown in Fig. 3, the level of UDS was assessed with an automatic grain counter that became operative in the course of this study. With this apparatus, an Artek model 880 connected to a Leitz microscope, the amount of silver grains in the photographic emulsion overlying the nucleus of a cell was quantitated by measuring the total surface of the grains rather than their number, and by expressing the surface occupied by the grains relative to the total nuclear area measured (grain area percentage; Lonati-Galligani et al., 1983). This method is more accurate than the counting of grain numbers when clusters are present. The results obtained are proportional to grain numbers up to at least 28% of the surface of the nucleus occupied by grains (all measurements shown were below 10%). Each determination was based on measurements on 35 lightly labeled cells, the results of which were subjected to computer analysis (a.o. for distribution anomalies) yielding the average grain area % per nucleus. The background-counts of the film never exceeded values higher than 1% and have been subtracted.

Determination of UV-endonuclease-sensitive sites (ESS)

Simultaneously with the UDS measurements, the number of ESS was determined. The technique used was essentially that originally reported by Paterson et al. (1973) but modified to give higher control molecular weights and more reproducible results (Zelle and Lohman, 1979; Wade and Lohman, 1980). These determinations were done in duplicate; the results are expressed as the number of ESS/10^9 molecular weight of DNA. Values of controls incubated without *M. luteus* extract and of unirradiated controls have already been subtracted.

Results

The effect of exposure of UV-irradiated chicken embryo fibroblasts to photoreactivating light was studied after 2 dosages of 254-nm UV, 5 and 10 J/m^2. The results are summarized in Table 1. Irradiation at 5 J/m^2 resulted in the induction of 190 ESS/10^9 dalton of DNA, while 10 J/m^2 induced about twice this number of ESS. When the cells were kept in the dark after the UV irradiation, a gradual removal of these sites occurred which reduced the number of ESS, over a period of 24 h, to about 16 and 31%, respectively, of the original value. During the first 2 h, only some 10% of the ESS were removed by dark repair. Exposure to photoreactivating light during that same period, however, resulted in the disappearance of almost 90% of the sites (166) after 5 J/m^2, and of about 60% (221) after 10 J/m^2. In these cells, virtually no ESS remained after a subsequent incubation in the dark for 22 h. When the photoreactivation period was extended to 4 h after an initial UV dose of 5 J/m^2, the number of ESS remaining directly after photoreactivation was reduced to less than 5% (Table 1).

UDS was measured in cells irradiated with 254-nm UV at 5 J/m^2. Following irradiation, the cells were either subjected to a 2-h photoreactivation treatment or kept in the dark; subsequently, cells were incubated in the dark for a maximum period of 22 h. UDS was determined over the initial 2-h period and over the next 11 consecutive 2-h periods. The accumulated results are given in Fig. 1.

There is only a small difference between light- and dark-treated cells in the number of grains measured 2 h after UV irradiation, but in the later periods the amount of excision repair in the dark-treated cells clearly surpasses that of the photoreactivated cells. Over the total post-UV-irradiation period of 24 h, photoreactivated cells incorporate about half (53%) the amount of [^{3}H]dT as compared to cells kept in the dark. In cells exposed to light, virtually no further UDS occurs after about

TABLE 1

THE REPAIR OF *M. luteus* UV-ENDONUCLEASE-SENSITIVE SITES IN UV-IRRADIATED CHICKEN EMBRYO FIBROBLASTS WITH AND WITHOUT PHOTOREACTIVATION

	UV dose (J/m^2)			
	5		10	
Photoreactivation:	−	+	−	+
Sites present after				
0 h	190±10		380±60	
2 h	167± 7	24±5 [a]	348±55	159±8 [a]
4 h	176±14	9±1 [b]	n.d.	n.d.
24 h	30± 4	1±4 [a]	117±10	14±4 [a]

[a] Photoreactivating light between 0 and 2 h after UV irradiation.

[b] Photoreactivating light between 0 and 4 h after UV irradiation.

n.d., not determined.

The irradiated cells were incubated in the dark, unless otherwise indicated, at 41°C. After the periods indicated in the table, cells were sampled, DNA was isolated and assayed for the presence of sites sensitive to UV-endonuclease. The number of sites per 10^9 dalton of DNA is given at 4 moments after UV irradiation. Control values of DNA from unirradiated cells and of DNA incubated without the *M. luteus* extract have been subtracted. The errors given are estimated errors of 4 Expts. (4 and 24 h) or standard errors of the mean of 10 determinations (0 and 2 h).

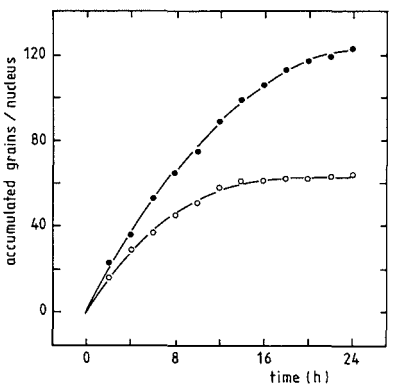

Fig. 1. Unscheduled DNA synthesis in chicken embryo fibroblasts irradiated with 5 J/m^2 UV followed by 2-h treatment in the dark (closed circles) or with photoreactivating light (open circles). UDS was measured via autoradiography, over consecutive 2-h periods. For each period, the silver grains in the photographic emulsion above 25 nuclei were counted, corrected for background and averaged. Each point represents the accumulated number of grains over all preceding 2-h periods. S.E.M. was less than 2 grains for each slide. For both curves the 12–14-h samples were missing; for these, estimated values were taken by averaging the preceding and subsequent values.

14 h, but in the dark-treated cells, incorporation still continues at 24 h.

In a second experiment, the same UV dose was used (5 J/m^2), but now the photoreactivation period was extended to 4 h. UDS was measured during 9 consecutive 2-h periods after the initial 4-h light or dark treatment. The accumulated results are given in Fig. 2. The UDS measured over the first 4 h after UV irradiation is slightly lower for photoreactivated cells than it is for dark-treated cells. After this initial 4-h period the photoreactivated cells show almost no further [^{3}H]dT incorporation, whereas without photoreactivation UDS continues throughout the whole incubation period, in agreement with the experiment shown in Fig. 1.

In a third experiment, the UV dose was varied. Fig. 3 shows the accumulated results of the UDS measurement after 5 and 10 J/m^2 with and without a 2-h exposure to photoreactivating light. During and after this period, measurements were performed over the intervals indicated. Over each of the intervals, cells that received 10 J/m^2 of UV show more UDS than those irradiated at 5 J/m^2. For cells subjected to photoreactivation lower values were found than for corresponding cells kept in the dark. After 5 J/m^2, UDS over the total

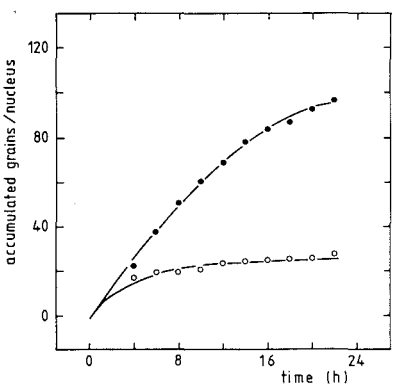

Fig. 2. Unscheduled DNA synthesis in chicken embryo fibroblasts irradiated with 5 J/m^2 UV followed by 4-h treatment in the dark (closed circles) or with photoreactivating light (open circles). For both curves the 14–16-h samples were missing; for these, estimated values were taken. Other conditions as in Fig. 1.

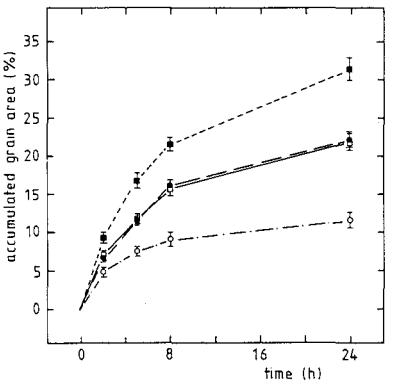

Fig. 3. Unscheduled DNA synthesis in chicken embryo fibroblasts irradiated with 5 or 10 J/m^2 UV followed by 2-h incubation in the dark or with photoreactivating light. The level of repair synthesis was assessed by measuring the grain surface area per nucleus (grain area %) with an automatic grain counter. Points represent the accumulated grain areas of the means of 35 nuclei, with the standard error of the mean (background is subtracted). ●, cells irradiated with 5 J/m^2 UV; ○, cells irradiated with 5 J/m^2 UV + photoreactivation; ■, cells irradiated with 10 J/m^2; □, cells irradiated with 10 J/m^2 + photoreactivation.

period of 24 h in the cells subjected to 2 h photoreactivation is 53% of that in the cells kept in the dark, in full agreement with the ratio found for the experiment presented in Fig. 1. For cells irradiated with 10 J/m^2, this percentage amounts to 70%.

Discussion

Irradiation with 254-nm UV induces pyrimidine dimers in DNA of cells as the predominant lesion, next to a number of less studied damages. The fact that chicken embryo cells can photoreactivate pyrimidine dimers, enabled us to reduce the amount of dimers and the ratio to non-dimer lesions. In this way a discrimination could be made between the repair processes involved in the repair of dimers and non-dimers, respectively. For this purpose we studied the time course of UDS following 254-nm UV irradiation at 2 different doses with and without photoreactivation, and compared the results with the number of ESS (= dimers) removed.

A 2-h photoreactivation period reduces the rate of UDS (Fig. 1), probably because much less dimers remain (Table 1) and therefore less excision repair occurs. Prolongation of the photoreactivation period from 2 h to 4 h results in cells that show hardly any further UDS (Fig. 2), in agreement with the observation that this procedure decreases the number of ESS to a very low level (Table 1). As photoreactivation is supposed to be specific for pyrimidine dimers and not to affect non-dimer photoproducts, these results suggest strongly that the UDS seen is caused by excision repair of dimers.

A doubling of the UV dose to 10 J/m^2 doubles the number of ESS induced, but UDS over 24 h in the dark (Fig. 3) is increased with only 42%; the number of ESS removed over this period increases from 160 to 263 (65% increase, which is not significantly different from 42%). After 10 J/m^2, a 2-h photoreactivation period reduces the subsequent UDS to the level measured after 5 J/m^2 without photoreactivation. The same is observed for the removal of ESS: in light-treated cells exposed to 10 J/m^2, 145 ± 9 ESS are removed between 2 and 24 h after irradiation, while over the same period after 5 J/m^2 in dark-treated cells 137 ± 8 ESS are removed. This, too, suggests a direct relationship between the removal of ESS and the occurrence of UDS.

During the initial 2-h or 4-h photoreactivation period less UDS was observed in light-treated cells than in cells kept in the dark. The difference is only small, however, in particular when it is compared to the large fraction of ESS that is removed by photoreactivation during this period. This small difference was found consistently, in all experiments we performed, which is not in agreement with earlier observations (Paterson et al., 1974; Wade and Lohman, 1980).

Table 2 gives a survey of the number of ESS removed between 2 and 24 h after UV exposure, together with the amount of UDS measured over that period, both in cells kept in the dark and in photoreactivated cells. The ratios in this table show that not in all cases the same relation between the two phenomena exists. Whereas, in general, the results seem to be compatible with a ratio of about 0.1 (arbitrary units), the cells irradiated at 5 J/m^2 and subsequently photoreactivated for 2 h appear

TABLE 2

COMPARISON OF UNSCHEDULED DNA SYNTHESIS (UDS) AND REMOVAL OF ESS BETWEEN 2 AND 24 h AFTER UV IRRADIATION

UV dose (J/m^2)	Photoreactivation [a]	UDS (% area)	ESS removed per 10^9 dalton of DNA	Ratio UDS/ESS removed
5	–	15.5 ± 1.1	137 ± 8	0.11 ± 0.01
5	+	6.8 ± 1.1	23 ± 6	0.30 ± 0.09
10	–	22.0 ± 1.7	231 ± 56	0.10 ± 0.03
10	+	14.8 ± 1.2	145 ± 9	0.10 ± 0.01

[a] Photoreactivation was between 0 and 2 h after UV irradiation.

Cells were treated as described in the legends to Table 1 and Fig. 3. UDS data have been taken from Fig. 3, ESS data are from Table 1. Data are given ± S.E.M.

to perform a higher amount of UDS per removed ESS (0.30 ± 0.09). We cannot offer a documented explanation for this deviating value. It might be speculated, though, that the high level of DNA repair relative to ESS removal results from repair synthesis involved in the excision of non-dimer lesions, the contribution of which to total [^{3}H]dT incorporation becomes significant when very few dimers remain after photoreactivation, as is the case in these cells. Alternatively, the high ratio might be understood if (1) the size of the reinsertion patches depends on the location of the dimer sites in the genome and (2) the last dimers to be removed give rise to longer patches.

The results of our study agree with earlier conclusions that chicken embryo fibroblasts do have dark excision capabilities if given extended time for repair (Wade and Lohman, 1980). In this respect these cells appear somewhat less capable than human cells; after 5 J/m^2 without photoreactivation it takes 5–8 h to complete 50% of the UDS measured over 24 h, which is somewhat longer than the values reported in literature for human cells: 4.5 h (Ehmann et al., 1978); about 5 h (Zelle et al., 1980; Vijg et al., 1984). The removal of ESS in chicken embryo fibroblasts in the dark measured over 24 h (70–80%) is equal to that found in human fibroblasts (Zelle et al., 1980; Vijg et al., 1984), but chicken cells appear to have a lag in removal of ESS between the first hour and 9 h

after irradiation (Wade and Lohman, 1980), which is not seen in human cells.

According to our results, the majority of the UDS after UV irradiation is caused by the repair of photoreactivatable lesions, presumably the pyrimidine dimers. If non-dimer lesions give rise to repair replication, this can only be responsible for a minor fraction of the UDS observed, in particular in cells kept in the dark. A non-dimer product, about 10 times less abundant than the pyrimidine dimers, is the (6–4)-photoproduct which may be formed between combinations of dipyrimidine sequences (Franklin et al., 1982; Haseltine, 1983). Small amounts of other photoproducts, too, may be formed, such as thymine glycols (Hariharan and Cerutti, 1977).

Although a full interpretation of our results cannot be given, the original goal has been achieved by establishing that dimer removal is primarily responsible for UDS after UV irradiation in chicken embryo fibroblasts. This result supports the earlier findings suggesting that the dimer is responsible for cell killing and inhibition of DNA synthesis (Wade and Lohman, 1980). Other investigators have also demonstrated, by means of photoreversal, the deleterious effect of pyrimidine dimers in paramecia (Smith-Sonneborn, 1979), in frog cells (Freed et al., 1979), in rat kangaroo cells (Wade and Trosko, 1983), in fish cells (Shima and Setlow, 1984) and in *Xenopus laevis* cells (Van Zeeland et al., 1980).

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APPENDIX PAPER III

Photochemistry and Photobiology, 48, 627-633 (1988)

DETECTION OF CYCLOBUTANE THYMINE DIMERS IN DNA OF HUMAN CELLS WITH MONOCLONAL ANTIBODIES RAISED AGAINST A THYMINE DIMER-CONTAINING TETRANUCLEOTIDE

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Abstract—A hybrid cell line (hybridoma) has been isolated after fusion between mouse-plasmacytoma cells and spleen cells from mice immunized with a thymine dimer-containing tetranucleotide coupled to a carrier protein. Monoclonal antibodies produced by this hybridoma were characterized by testing the effect of various inhibitors in a competitive enzyme-linked immunosorbent assay (ELISA). The antibodies have a high specificity for thymine dimers in single-stranded DNA or poly(dT), but do not bind UV-irradiated $d(TpC)_5$. Less binding is observed with short thymine dimer-containing sequences. *In vitro* treatment of UV-irradiated DNA with photoreactivating enzyme in the presence of light, or with *Micrococcus luteus* UV-endonuclease results in disappearance of antigenicity. Antibody-binding to DNA isolated from UV-irradiated human fibroblasts (at 254 nm) is linear with dose. Removal of thymine dimers in these cells during a post-irradiation incubation, as detected with the antibodies, is fast initially but the rate rapidly decreases (about 50% residual dimers at 20 h after 10 J/m^2). The induction of thymine dimers in human skin irradiated with low doses of UV-B, too, was demonstrated immunochemically, by ELISA as well as by quantitative immunofluorescence microscopy.

INTRODUCTION

Exposure of living matter to ultraviolet radiation (UV)† induces damages in cellular DNA. This may result in gene mutations, cell death or cellular transformation (Hanawalt *et al.*, 1979; Suzuki *et al.*, 1981). For human skin, UV-exposure may result in cell death or in precancerous lesions (Urbach, 1984). The major photoproducts induced in DNA by UV are the cyclobutane pyrimidine dimers, which are among the most extensively studied DNA lesions; several methods have been described for their detection based on chromatographical analysis (Reynolds *et al.*, 1981), and on biochemical analysis with endonucleases specific for UV-irradiated DNA (Paterson *et al.*, 1973). Also methods utilizing antibodies specific for pyrimidine dimers and other UV-induced DNA lesions have evolved, which permit the study of the induction and repair of these lesions without the requirement of *in vivo* radiolabelling of DNA. This facilitates investigations on the role of UV-induced pyrimidine dimers in the process of photocarcinogenesis in human skin.

Radioimmunoassays have been described in which dimers induced by UV-irradiation (at 254 nm) of cells with doses as low as 2.5 J/m^2 were detected with rabbit antisera (Klocker *et al.*, 1982a; Mitchell and Clarkson, 1981). This demonstrated the usefulness of antibodies. With an enzyme-linked immunosorbent assay (ELISA), pyrimidine dimers could be detected in DNA irradiated at 254 nm with 1 J/m^2 (Wani *et al.*, 1984). Recently, these authors were able to detect dimers induced by even lower UV-doses using immuno-slot blot analysis (Wani *et al.*, 1987). With a dimer-containing synthetic hapten, polyclonal antibodies with high specificity for thymine dimers were obtained (Klocker *et al.*, 1984). Also monoclonal antibodies against pyrimidine dimers have been isolated (Strickland and Boyle, 1981), which have the advantage of almost unlimited supply of material of constant quality and uniform specificity.

Here we report on a study aiming at the isolation of monoclonal antibodies specific for cyclobutane thymine dimers and their application in the detection of such dimers in DNA of UV-irradiated human skin. The monoclonal antibodies were obtained through immunization of mice with a specific hapten. Application in an ELISA allowed the detection of thymine dimers in single-stranded DNA isolated from human fibroblasts, which had been

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†*Abbreviations*: CgG: chicken-gamma-globulin; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA: enzyme-linked immunosorbent assay; FCS: foetal calf serum; FPLC: fast protein liquid chromatography; GTTG: dGpdTpdTpdG; HPLC: high performance liquid chromatography; SDS: sodium dodecyl sulphate; T<>T: *cis-syn* thymine dimer; TWEEN-20: polyoxyethylene sorbitan monolaurate; UV: ultraviolet radiation.

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irradiated with a UV-dose (254 nm) as low as 1.5 J/m^2. With the same method, also in DNA isolated from UV-irradiated human skin, dimers could be detected. In addition, the kinetics of removal of thymine dimers in cultured human fibroblasts were examined. In a different approach, the antibodies were used for studying dimers in single cells by means of indirect immunofluorescence microscopy. On this basis a method for the quantitative detection of thymine dimers in epidermal cells isolated from UV-irradiated human skin was developed.

MATERIALS AND METHODS

Preparation of antigen. Two μmol of d(GpTpTpG) (GTTG, a kind gift of Dr. van Boom, State University of Leiden, The Netherlands) dissolved in 2.5 mℓ buffer A (6 m*M* KH_2PO_4, 4 m*M* K_2HPO_4, pH 7.0) was irradiated in a quartz cuvette with monochromatic 280-nm UV for the preparation of the hapten GT<>TG (GTTG containing cyclobutane thymine dimer). The light source was a 1000-W HgXe arc (Conrad Hanovia Inc., Newark, NJ, USA). The light was collimated on the entrance slit of a monochromator (Oriel Corp., Stamford, CT, USA) after passage through a cooled water-filter to absorb infrared radiation. The entrance and exit slits had a width of 2 mm which resulted in a half-maximum bandwidth of 12 nm. The dose rate of the light that reaches the cuvette was 28 W/m^2 as determined with a calibrated thermopile (Hewlett Packard 8334A/8330A, Hewpak, Geneva, Switzerland). Total (incident) dose was 1000 kJ/m^2. During irradiation, the solution was stirred continuously, at room temperature.

The products generated by UV-irradiation of GTTG were separated by high performance liquid chromatography (HPLC) as described by Demidov and Potaman (1984), on a reversed phase column (Ultrasphere ODS 4.6 × 150 mm, Beckman, Berkeley, CA, USA), with two Beckman (112) pumps and a Beckman 412 Controller, with a Beckman Model 165 detector set at 260 nm. Elution (1 mℓ/min) was carried out with a linear (1%/min) gradient of methanol in 20 m*M* ammonium acetate pH 7.0. GT<>TG was eluted at 15–16 min; it was pooled from several runs and lyophilized. Subsequently a 5′-phosphate group was added by incubating 400 nmol GT<>TG with 25 units of the enzyme T4 polynucleotide kinase (Boehringer, Mannheim, FRG) and 2 μmol ATP in 300 μℓ buffer (70 m*M* Tris–HCl, 10 m*M* $MgCl_2$, 10 m*M* dithioerythritol, pH 7.6) for 8 h at 37°C. Efficiency of phosphorylation was about 80%. Phosphorylated tetranucleotide (pGT<>TG) was isolated by chromatography on a MonoQ anion-exchange column (Pharmacia, Uppsala, Sweden), incorporated in a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). Upon elution (1 mℓ/min) with a linear gradient (0–500 m*M* NaCl in 30 min) in 5 m*M* Tris–HCl, pH 7.2, pGT<>TG was eluted after ATP. Fractions containing pGT<>TG were pooled, concentrated by flash evaporation and desalted by gel filtration. Subsequently, the hapten was coupled to carrier-protein: 125 nmol pGT<>TG was reacted with 0.1 *M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma Chemical Co., St. Louis, MO, USA) in 250 μℓ 0.1 *M* N-methylimidazole (Chu *et al.*, 1983), pH 6.0, for 1 h at room temperature; thereafter 250 μℓ chicken-gamma-globulin (CgG; 1 mg/mℓ; Sigma) was added. The mixture was further incubated for 16 h and then dialysed against 0.1 *M* sodium phosphate buffer pH 7.0. The amount of hapten coupled to CgG was determined from the change in the absorbance spectrum.

Immunization of mice and isolation of hybridoma cells. Mice (female BALB/c, 12 weeks old) were injected intraperitoneally with 50 μg of alum-precipitated CgG-GT<>TG together with 2 × 10^9 killed *Bordetella pertussis* bacteria (Department of Public Health, Bilthoven, The Netherlands). After 5 weeks an intravenous booster injection was given with again 50 μg CgG-GT<>TG. Three days later spleen cells (10^8) were fused with 2 × 10^7 SP2/0 plasmacytoma cells (Shulman *et al.*, 1978) in 50% poly(ethylene glycol) (PEG 4000, Merck, Darmstadt, FRG). The cells were incubated for 24 h in complete medium [RPMI 1640, Gibco, Grand Island, NY, USA, supplemented with 15% foetal calf serum (FCS), Flow Labs, Irvine, UK] and then divided over 10 25-cm^2 culture flasks (Costar, Cambridge, MA, USA) to which 5 × 10^5 mouse peritoneal macrophages were added in HAT medium (RPMI 1640, supplemented with 15% FCS, 0.1 m*M* hypoxanthine, 0.4 μ*M* aminopterin and 16 μ*M* thymidine). Eight days after the fusion, cells from the flasks were seeded in 96-well plates (Costar) at 100 cells per well. When cell growth was apparent, supernatants were tested with ELISA (see below). Cells from positive wells were cloned by limiting dilution in 96-well plates and tested again after an appropriate time of cultivation. Antibodies from monoclonal hybridoma cells were purified from culture supernatant by affinity chromatography with protein A-Sepharose (Pharmacia), according to procedures given by the manufacturer.

Enzyme-linked immunosorbent assay (ELISA). The assay was as described by Fichtinger-Schepman *et al.* (1985), except that only 50 μℓ of the various solutions were added per well. In short, 50 ng DNA (control or UV-irradiated, see next section) were adsorbed to each well of poly-L-lysine precoated plates by incubation for 16 h at 37°C. After washing of the plates with 0.05% TWEEN 20 (polyoxyethylene sorbitan monolaurate; Sigma), hybridoma culture supernatant diluted in PT buffer (8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 0.14 *M* NaCl, 2.6 m*M* KCl, 0.05% TWEEN 20) + 1% heat-inactivated FCS was added. Second antibody was goat anti-mouse IgG alkaline phosphatase conjugate (1/500 in PT buffer + 1% FCS; Sigma). Incubations were at 37°C for 45 min. Unbound antibody was washed away with PT buffer. Finally, the wells were rinsed with 100 m*M* diethanolamine (Merck) pH 9.8, and incubated with substrate [0.4 m*M* methylumbelliferyl phosphate (Sigma) in 10 m*M* diethanolamine pH 9.8, 1 m*M* $MgCl_2$] for 3 h at 37°C or 16 h at 25°C. The fluorescence was recorded with a Fluoroskan (Flow Labs) detector. For the competitive ELISA the plates were incubated with competition mixtures instead of supernatant dilutions. These mixtures contained fixed amounts of antibody (IgG fraction, comparable to 1/5000 diluted culture supernatant) and various amounts of inhibitor, both diluted in PT buffer + 1% FCS; they were incubated for 20 min at room temperature before being added to the wells.

UV-irradiation. DNA used for coating of ELISA plates was from calf thymus (Sigma), purified after RNase digestion by repeated phenol extraction and alcohol precipitation. It was irradiated in buffer A (0.2 mg/mℓ) with 5 kJ/m^2 monochromatic 280-nm UV (see above), then denatured by heating at 100°C for 5 min followed by rapid cooling (0°C). Competitors (calf thymus DNA, poly(dT), oligo dT_{12-18}, d(TpT), $d(TpC)_5$, poly U; all from Sigma) were irradiated in a glass Petri dish (0.2 mg/mℓ in buffer A; layer thickness 0.5 mm) with UV-C from a low-pressure mercury vapour lamp (Philips, 15-W TUV). The incident fluence rate was 0.4 W/m^2 at 254 nm as determined with a calibrated thermopile and corrected for the emission spectrum given by the manufacturer. Cultured human fibroblasts were irradiated with UV-C while attached to plastic culture dishes (Greiner, Nürtingen, FRG). UV-B irradiation of skin samples was carried out with a Philips TL12/20W sunlamp with a fluence rate of 9 W/m^2 as

determined with a Waldmann UV-meter (Waldmann, Schwenningen, FRG).

Modifications of UV-irradiated DNA. Enzymatic incision of UV-irradiated calf thymus DNA was brought about by incubation with a UV-endonuclease preparation from *Micrococcus luteus*, corresponding with fraction II in the purification method of Carrier and Setlow (1970), in 10 m*M* Tris–HCl, pH 7.6, 100 m*M* NaCl, 1 m*M* EDTA for 30 min at 37°C. Enzymatic photoreactivation of DNA was performed by treatment with photoreactivating enzyme from *Streptomyces griseus* (a kind gift of Dr. Eker, Rotterdam, The Netherlands) plus white fluorescent light filtered through 8 mm plate glass, for 30 min at 37°C in 40 m*M* NaCl, 0.1% (wt/vol) bovine serum albumin (Sigma), 5 m*M* 2-mercaptoethanol, 10 m*M* potassium phosphate pH 7.0. DNA was digested with DNase I (100 μg/mℓ, Sigma) for 60 min at 37°C in buffer A.

DNA isolation from cells and skin samples. Cells were scraped off the culture plates with a rubber policeman and lysed in 50 m*M* Tris–HCl pH 7.6, 10 m*M* EDTA, 150 m*M* NaCl, 0.5% SDS, 0.1 mg/mℓ proteinase K (Boehringer) for 1 h at 37°C. DNA was purified by phenol extraction, RNase digestion, chloroform/isoamylalcohol (24:1) extraction and ethanol precipitation (Fichtinger-Schepman *et al.*, 1987), and denatured by heating (5 min at 100°C) and rapid cooling. Epidermal cells were isolated from human skin as described by Vijg *et al.* (1986). DNA was isolated as above.

Quantitative immunofluorescence microscopy. Epoxy-coated microprint slides (8 wells, Cel-line Associates, Newfield, USA) were coated with poly-L-lysine (0.1 g/ℓ) for 1 h. Isolated epidermal cells were suspended in serum-free medium (RPMI 1640) and brought onto the slides. After 10 min the cells were fixed with 0.1 *M* sodium phosphate, 45% ethanol for 5 min and subsequently with 70% ethanol for 10 min. Slides were stored dry at −20°C. For immunostaining they were processed by a modification of the method described by Muysken-Schoen *et al.* (1985). Slides were thawed and treated for 30 min in 50 m*M* Tris–HCl, pH 7.2, 1 *M* KCl, 0.3% triton X-100 and washed twice with TBS (20 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl). To denature DNA *in situ*, slides were incubated in 0.5 *N* HCl, 0.05% pepsine (Sigma) at 37°C for 30 min and washed with TBS. Anti thymine dimer antibody solution (1/25 diluted culture supernatant) in TBS containing 5% FCS and 0.05% TWEEN 20 was added; incubation was for 60 min at 37°C. After washing with TBS, the cells were incubated for 60 min at 20°C with fluorescein-labelled goat anti-mouse IgG (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) diluted 1/150 in TBS containing 5% FCS and 0.05% TWEEN 20. Unbound antibodies were washed away with TBS, nuclear DNA was stained with propidium iodide (Sigma; 5 min, 40 ng/mℓ in TBS) and the slides were mounted in 5 m*M* Tris–HCl pH 8.1, 10 m*M* NaCl, 75% glycerol, coverslipped and sealed with paraffin. The slides were examined with a laser-scan microscope LSM-41 (Carl Zeiss, Oberkochen, FRG) connected to a DEC VAX8250 computer. The procedure for the acquisition of the fluorescence images will be described in detail elsewhere (Van der Wulp *et al.*, in preparation). It involves the recording of twin images, both after excitation with a 488-nm Ar^+ laser, which are digitized in a format of 512 × 512 pixels. The first image, obtained with a BP 515-560 nm emission filter, represents the fluorescence from the indirectly fluorescein-labelled antibodies. The second image of the same group of nuclei, recorded as propidium fluorescence with a LP 590 nm filter, serves to determine the exact position of the nuclei within the image, which is then used in the computation of the total fluorescein-fluorescence within each nucleus. The image-processing software package (TCL-Image) has been developed by TNO–TPD Delft in collaboration with the Technical University of Delft, The Netherlands.

Determination of thymine dimers by means of HPLC. Forty 9-cm culture dishes (Greiner) with human fibroblasts (16×10^7) were UV-irradiated (254 nm, 18 J/m^2) and DNA was isolated as above. Before being irradiated, the cells in 2 dishes were labelled for 3 days with [^{3}H]thymidine (Amersham, Buckinghamshire, UK). DNA of these cells was isolated separately. The number of thymine dimers in the radioactive DNA was determined with HPLC according to the procedure of Niggli and Cerutti (1983). DNA of the unlabelled cells served as calibration DNA in the competitive ELISA.

RESULTS

Preparation of thymine dimer-containing hapten and immunogen

Thymine dimers were induced in the tetranucleotide d(GpTpTpG) (GTTG) by irradiation with 280-nm UV. The photoproduct with the cyclobutane thymine dimer (GT<>TG) was purified by reversed-phase HPLC. Two major UV-absorbing peaks were eluted, and two smaller ones. The peak with the shortest retention time was attributed to GT<>TG, since it contained a product that was stable to heating at 90°C in 0.1 *M* KOH (unlike (6–4)photoproducts; Franklin *et al.*, 1982), it had an absorbance spectrum closely resembling that of guanosine and it yielded unmodified GTTG molecules upon irradiation with 230-nm UV. The peak with the longest retention time corresponded to unmodified GTTG; one of the 2 intermediate minor peaks was characterized as the (6–4)photoproduct.

The tetranucleotide with the cyclobutane thymine dimer was isolated, phosphorylated at the 5′-position and coupled to chicken-gamma-globulin (CgG). The coupling was based on the method described by Halloran and Parker (1966), with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The procedure was modified to increase the coupling efficiency: pGT<>TG was first dissolved in 0.1 *M* N-methylimidazole and allowed to react with EDC (0.1 *M*; Chu *et al.*, 1983). This leads to good yields of the 5′-phosphorimidazolide, which is very reactive towards amino-groups. Thereafter, CgG was added to the mixture. From the absorbance spectrum of the resulting product it was deduced that the molar ratio CgG: GT<>TG was about 1:12. A coupling efficiency (ratio hapten coupled/total hapten × 100%) of about 16% was reached, much higher than in the absence of N-methylimidazole (about 5%; recent experiments show that the efficiency can be increased further by raising the EDC concentration to 1 *M* (manuscript in preparation)).

Isolation of a monoclonal antibody specific for thymine dimers

Spleen cells from mice immunized with the hapten-coupled CgG were pooled and fused with plas-

macytoma cells to yield proliferating hybrids. Culture supernatants of the hybrids were tested for the presence of antibodies with specificity for UV-irradiated DNA relative to untreated DNA. Twelve positive hybrids were cloned.

Antibody characterization

The culture supernatants of the 12 clones were retested in an ELISA with control DNA and UV-irradiated DNA, respectively, as the coating antigens. With the antibodies of the most promising clone (H3), exclusive binding to UV-irradiated DNA was observed (Fig. 1). This clone was selected for further study. The H3 antibodies were found to be of the IgG1 (lambda) subclass.

Their specificity was analysed in detail by competitive ELISA. Different UV-irradiated DNA samples and poly- and oligonucleotides were used as competitors. Results are summarized in Table 1: the amount of competitor needed to reduce antibody-binding to the coating antigen, i.e. UV-irradiated single-stranded DNA, by 50% is given. The antibodies had affinity only for single-stranded T<>T-containing sequences. Inhibition of antibody-binding was no longer found after monomerization of the dimers in competitor DNA, which was accomplished by treatment with photoreactivating enzyme from *Streptomyces griseus* under illumination. Inhibition also disappeared after treatment of the competitor with a UV-endonuclease preparation, which specifically introduces strand breaks at each dimer site. More competitor appeared to be needed when shorter sequences with dimers were used, as is illustrated by the loss of antigenicity of UV-irradiated DNA after digestion with DNase I

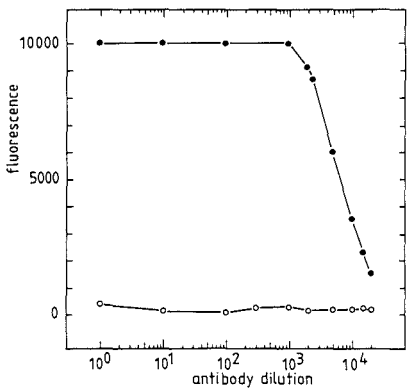

Figure 1. Binding of monoclonal antibodies to immobilized UV-irradiated (280 nm, 5 kJ/m²) and unirradiated single-stranded calf thymus DNA. Per well of a microtiter plate 50 ng DNA was adsorbed, and different dilutions of supernatant of a culture of H3 cells were tested. The ELISA was performed as described; mean values of duplicate samples are shown. O–O, unirradiated DNA; ●–●, UV-irradiated DNA. Fluorescence is in arbitrary units.

Table 1. Inhibition of antibody-binding to immobilized single-stranded UV-irradiated DNA (280 nm, 5 kJ/m²) by non-irradiated single-stranded DNA and various UV-irradiated oligo- and polynucleotides

Inhibitor	UV-dose (J/m²)	Amount required for 50% inhibition (ng)
DNA	0	>1250
DNA	20	82
DNA + UV-endonuclease	20	>1250
DNA + PRE	20	>1250
DNA + DNase I	20	>1250
DNA	100	27
DNA (non-denatured)	100	>1250
poly(dT)	100	14
oligodT_{12-18}	100	88
poly U	100	>1250
d(TpT)	100	>1250
d$(TpC)_5$	100	>1250
GT<>TG	*	27

Nucleic acids were irradiated with 254-nm UV. Subsequently, they were denatured by heating or treated with UV-endonuclease (*Micrococcus luteus*), photoreactivating enzyme (PRE; *Streptomyces griseus*) or DNase I and then denatured. Triplicate DNA samples were tested at 5 concentrations in the competitive ELISA.

*100% T<>T.

and by comparison of the amounts of poly(dT), oligo dT_{12-18} and d(TpT) needed to give 50% inhibition, which were 14, 88 and >1250 ng, respectively. Also the relatively high amount of GT<>TG required for 50% inhibition (27 ng) underlines this observation, as this material contains at least 500–1500 times as many dimers per ng as UV-irradiated (100 J/m²) DNA and poly(dT) (Patrick and Rahn, 1976).

Application of the antibody on UV-irradiated cultured human cells

The H3 monoclonal antibodies were used to study UV-induced dimers in DNA of cultured human fibroblasts and of human skin. First, a dose-response study was performed on the induction of dimers in the cultured cells. To this aim DNA was isolated from human fibroblasts after irradiation with various UV-C doses, and used as competitor in the ELISA, which yielded a set of inhibition curves. Calibration was done with a reference DNA sample from UV-irradiated human fibroblasts which—according to a T<>T determination with HPLC—contained 80 T<>T per 10^6 nucleotides (results not shown). A linear dose-response relationship for the induction of T<>T was observed (Fig. 2).

Removal of dimers in cultured human fibroblasts was determined by isolating DNA at different times after irradiation (10 J/m²), followed by dimer detection with the competitive ELISA. An initially fast removal was observed, with a gradually diminishing

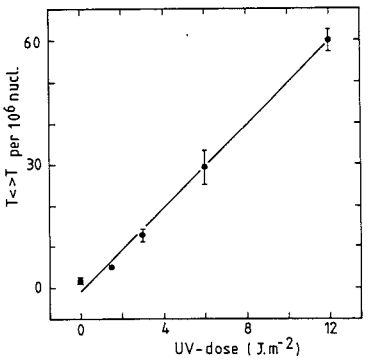

Figure 2. Induction of thymine dimers by UV-irradiation (254 nm) of cultured human fibroblasts in dependence on UV-dose. Dimers were detected as antibody-binding sites with the competitive ELISA: a fixed amount of H3-antibody was incubated with various amounts of DNA isolated from the cells and then allowed to bind to immobilized single-stranded UV-irradiated DNA. The assay was performed in triplicate at four different DNA concentrations. The 50% inhibition values were determined and compared with a calibration curve obtained with a DNA sample isolated from human fibroblasts, which contained 80 T<>T per 10^6 nucleotides. Data points (± SEM) represent mean values of three ELISA experiments.

rate; after about 6 h hardly any further repair appeared to occur (Fig. 3), with the result that after 20 h post-incubation 46% of the initially induced thymine dimers were still present.

Application of the antibody on UV-irradiated human skin

UV-induced thymine dimers were also assayed in the DNA of human skin. Following exposure of excised mammary skin to 2 kJ/m² UV-B, the epidermal cells were isolated. The DNA of these cells was isolated and tested in the competitive ELISA (Fig. 4). A clear difference was observed between DNA from untreated and UV-irradiated skin.

In our laboratory, for several years special attention has been devoted to the use of specific antibodies for the detection of DNA damages in individual cells, by means of immunofluorescence microscopy (Muysken-Schoen *et al.*, 1985; Baan *et al.*, 1986, 1988). To this end, methods have been developed for the labelling of these damages in the nuclei of cells attached to glass-slides, followed by computer-mediated processing of the microscope images and quantification of the nuclear fluorescence. The H3 antibodies were applied in these methods to study the induction of thymine dimers in samples of excised human skin, which had been irradiated with various dosages of UV-B. From these samples epidermal cells were isolated and brought onto glass-slides. After fixation, the slides were processed for the immunofluorescence microscopy. The nuclear fluorescence showed a linear dependence on the dose of UV-B (Fig. 5).

DISCUSSION

A monoclonal antibody specific for cyclobutane thymine dimers has been isolated, after immunization of mice with a specific hapten, *viz.* a tetranucleotide containing a thymine dimer, attached to a protein. A substantial improvement was obtained in the efficiency of the reaction used for coupling this hapten to an immunogenic carrier protein, by the introduction of N-methylimidazole as an intermediary reactant in the EDC coupling according to Halloran and Parker (1966). After immunization,

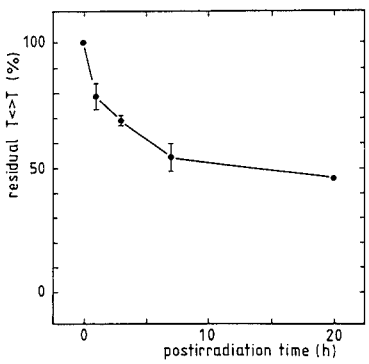

Figure 3. Removal of thymine dimers in DNA, in cultured human fibroblasts irradiated with UV-C (254 nm, 10 J/m²). Dimer detection was as described in Fig. 2. The percentage residual dimers was obtained by relating the 50% inhibition value at X h postirradiation to the value at 0 h. Data points (± SEM) represent mean values of three experiments.

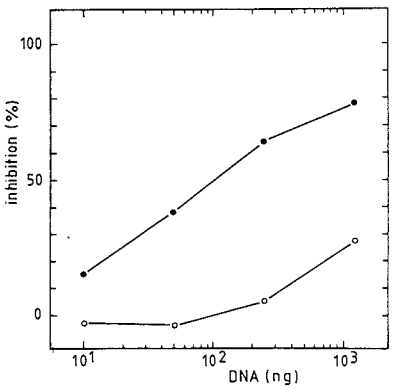

Figure 4. Competitive inhibition of antibody-binding to UV-irradiated DNA by DNA isolated from UV-irradiated human skin. The competitive ELISA was performed as described in Fig. 2, with the amounts of DNA indicated on the abscissa. Excised skin was irradiated with UV-B (●–●; 2 kJ/m²) or held in the dark (○–○). Thereafter, epidermal cells were isolated from which DNA was purified.

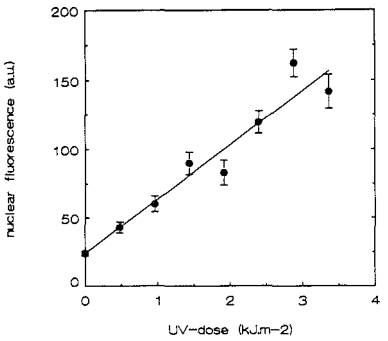

Figure 5. Detection of thymine dimers in isolated epidermal cells from UV-irradiated human skin, in dependence on UV-B dose. After trypsinisation, cells were brought onto glass-slides and prepared for immunostaining. Subsequently, they were incubated with H3 antibodies followed by fluorescein-labelled anti-mouse IgG antibodies. Nuclear immunofluorescence was quantified by computer-mediated digitization and image-processing. Data points represent mean nuclear fluorescence ± SEM. About 60 nuclei divided over 10 images per dose were measured.

cell fusion and selection, monoclonal hybridoma cells were obtained that produce antibodies highly specific for thymine dimers in single-stranded DNA.

The antibodies appeared to have a lower affinity for the dimer in short oligonucleotides. Others also have reported that specific binding of antibodies to dimer-containing nucleotides increased with chain length (Strickland and Boyle, 1981; Seaman *et al.*, 1972), which likely is due to an increased stability of the antibody–antigen complex. There is no binding of the antibodies to thymine dimers in double-stranded DNA or to dimers in DNA that has been nicked by *Micrococcus luteus* UV-endonuclease. There seems to be no cross-reaction with TC or CT dimers or with (6–4)photoproducts in DNA, as is shown by the absence of inhibition by UV-irradiated $d(TC)_5$ and by UV-irradiated DNA after photoreactivation, respectively. The lack of effect found for $d(TC)_5$ might be attributed to its short length; however, as it is comparable in size to dT_{10}, the difference in effect with oligo dT_{12-18} appears too large for such an explanation. Although binding of the antibodies to CC dimers or CC (6–4)photoproducts has not been tested, the specificity for T<>T seems very high, as also indicated by the absence of binding to UV-irradiated poly U.

Application of the antibodies in the competitive ELISA resulted in a sensitive assay, allowing the detection of about 5 dimers per 10^6 unmodified nucleotides. Data from Table 1 and extrapolation of Fig. 2 indicate that 50% inhibition of antibody-binding is obtained at 20 fmol T<>T, with the UV-irradiated DNA used.

The induction of dimers in irradiated human fibroblasts was found to be linear with the UV-dose; inhibition of antibody-binding by DNA from cells irradiated with 1.5 J/m^2 UV-C still could be clearly distinguished. The kinetics of dimer removal observed are comparable to other data obtained by immunochemical methods (Mitchell *et al.*, 1985; Klocker *et al.*, 1982b).

The use of these monoclonal antibodies in the ELISA permits the detection of thymine dimers in DNA isolated from UV-B-irradiated human skin (Fig. 4). The dose of UV-B applied for this irradiation (2 kJ/m^2) was about 1.5 times the minimal erythema dose (Schothorst *et al.*, 1987); evidently, also relatively low doses of UV-B can well be detected in our system. The results obtained with human skin when the antibody was applied in quantitative immunofluorescence microscopy (Fig. 5) underline the high sensitivity that can be reached. Moreover, in this approach only a very limited number of cells is required.

The advantage of the immunochemical detection is that there is no need for radioactivity. Together with the high sensitivity reached with our antibodies, it will allow studies on the induction and repair of thymine dimers, and on the further effects of dimers, in human skin UV-irradiated *in vivo*. Currently, the development of methods for the quantitative detection of dimers in cells *in situ*, in small biopsies, is in progress.

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APPENDIX PAPER IV

Cancer Research, 50, 1905-1910 (1990)

Effects of Microinjected Photoreactivating Enzyme on Thymine Dimer Removal and DNA Repair Synthesis in Normal Human and Xeroderma Pigmentosum Fibroblasts[1].

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ABSTRACT

UV-induced thymine dimers (10 J/m^2 of UV-C) were assayed in normal human and xeroderma pigmentosum (XP) fibroblasts with a monoclonal antibody against these dimers and quantitative fluorescence microscopy. In repair-proficient cells dimer-specific immunofluorescence gradually decreased with time, reaching about 25% of the initial fluorescence after 27 h. Rapid disappearance of dimers was observed in cells which had been microinjected with yeast photoreactivating enzyme prior to UV-irradiation. This photoreactivation (PHR[3]) was light-dependent and (virtually) complete within 15 min of PHR illumination. In general, PHR of dimers strongly reduces UV-induced unscheduled DNA synthesis (UDS). However, when PHR was applied immediately after UV irradiation, UDS remained unchanged initially; the decrease set in only after 30 min. When PHR was performed 2 h after UV exposure, UDS dropped without delay. An explanation for this difference is preferential removal of some type(s) of nondimer lesions, *e.g.,* (6-4)photoproducts, which is responsible for the PHR-resistant UDS immediately following UV irradiation. After the rapid removal of these photoproducts, the bulk of UDS is due to dimer repair. From the rapid effect of dimer removal by PHR on UDS it can be deduced that the excision of dimers up to the repair synthesis step takes considerably less than 30 min.

Also in XP fibroblasts of various complementation groups the effect of PHR was

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[3]The abbreviations used are: FCS, fetal calf serum; PHR, photoreactivation; PRE: photoreactivating enzyme; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum; TBS, 20 mM Tris-HCl-150 mM NaCl, pH 7.4.

investigated. The immunochemical dimer assay showed rapid PHR-dependent removal comparable to that in normal cells. However, the decrease of (residual) UDS due to PHR was absent (in XP-D) or much delayed (in XP-A and -E) compared to normal cells. This supports the idea that in these XP cells preferential repair of nondimer lesions does occur, but at a much lower rate.

Introduction

Cyclobutane-type pyrimidine dimers are the major photoproducts induced in DNA by UV. Evidence has been collected that these lesions initiate the process of UV-induced mutagenesis and carcinogenesis (1,2). Other photoproducts in DNA induced by UV comprise the (6-4)photoproducts, which are mutagenic as well (3,4). In living cells, UV-induced DNA lesions may be repaired by a multienzyme process (excision repair), or via a light-dependent enzymatic reaction known as photoreactivation (PHR), which is specific for pyrimidine dimers (see Ref. 5, for a review). PHR has been found to occur in a wide range of organisms (6,7); studies on the occurrence of PHR in mammalian cells, however, have yielded conflicting results (8,9). A correlation between unrepaired DNA damage and carcinogenesis in humans was established when it was shown that the cancer-prone hereditary disease xeroderma pigmentosum (XP) involves a defect in the excision-repair mechanisms acting on UV-induced DNA lesions (10).

We have investigated the kinetics of dimer removal in UV-irradiated cultured normal human and XP fibroblasts, which had been microinjected with purified yeast photoreactivating enzyme (PRE). Thymine dimers were determined at the single-cell level by quantitative immunofluorescence microscopy based on the application of a dimer-specific monoclonal antibody and computer-assisted image processing and analysis (11).

Injection with PRE and subsequent illumination resulted in a rapid disappearance of dimers: within 15 min virtually all dimers (induced by 10 J/m^2) were removed in normal as well as in XP fibroblasts, whereas noninjected repair proficient cells still retained 25% of their dimers after 27 h. A comparison was made between the effect of PHR by microinjected PRE on dimer removal, as measured with immunofluorescence microscopy, and the indirect effect on UV-induced unscheduled DNA synthesis (UDS). This was done in parallel experiments with normal human as well as XP fibroblasts. The time interval between UV-irradiation of the cells, PHR and UDS measurement was varied.

MATERIALS AND METHODS

Antibodies. The monoclonal antibody H3, specific for thymine dimers in single-stranded

DNA, has been described (11). Culture medium of hybridoma cells or ascites fluid was used diluted 1:20 and 1:500, respectively. Fluorescein-labeled goat anti-mouse IgG (FITC-GaM; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) was used in 1:150 dilution.

Cell culture. Normal human fibroblasts (C5RO) were cultured in Dulbecco's modified Eagle's medium (Flow laboratories, Irvine, United Kingdom) supplemented with 10% FCS (Flow), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells used in microinjection experiments were cultured in Ham's F-10 medium (Flow) containing FCS and antibiotics as above. H3 hybridoma cells were cultured in RPMI 1640 (Gibco Ltd., Paisly, United Kingdom) supplemented with 10% FCS and antibiotics.

Photoreactivating enzymes. Purified PRE preparations from *Saccharomyces cerevisiae* were prepared as described (12,13). Enzyme concentration was approximately 8 μM as deduced from protein determination (14) and the absorbance at 377 nm (M.W. 56,000 from SDS polyacrylamide gel electrophoresis, ϵ_{377} = 27,850; Eker *et al.*, in preparation). PRE from Anacystis nidulans and *Escherichia coli* was purified as described (12). The enzymes are highly active in the *in vitro Haemophilus influenzae* transformation assay (see Refs. 13 and 15 for experimental details).

Microinjection of PRE and photoreactivation. Cell fusion was accomplished with inactivated Sendai virus, generating polykaryons with 2-10 nuclei in addition to nonfused monokaryons. Only homopolykaryons were injected. The microinjection has been described earlier (12). After injection, usually taking about 20 min, the cells were UV irradiated (see below) and either illuminated with photoreactivating light [4 Blacklite/Blue lamps F20/T12BLB (Sylvania) at a distance of 10 cm through a 6-mm-thick glass plate] or kept in the dark for periods varying from 30-120 min as indicated in the legends to the figures. During the illumination the cells were kept in culture medium under constant 5% CO_2 flush, at 34-38°C. These conditions are the same as those used earlier (12,13).

UV irradiation. The UV-C source was a single low-pressure mercury vapour lamp (Philips, The Netherlands; 15-W TUV). The incident dose rate was 0.4 J/m^2/s at 254 nm as determined with an International Light IL1500 dosimeter equipped with a SEE400 detector (International Light, Newburyport, MA). The UV dose rate in microinjection experiments was 0.6 J/m^2/s. Cells were irradiated while attached to glass at room temperature in a darkened room, directly after rinsing with phosphate-buffered saline.

Immunostaining of the cells. The procedure as described by Muysken-Schoen *et al.* (16) was followed with minor modifications. Briefly, cells were fixed while attached to glass with 70% ethanol or with methanol and acetone (microinjection experiments). After fixation the slides were stored at -20°C. For immunostaining, slides were thawed and incubated for 0.5 h in 50 mM Tris-HCl (pH 7.2)-1 M KCl-0.3% Triton X-100, washed with TBS, and treated for 1 h with RNase A (100 μg/ml; Sigma Chemical Co., St. Louis, MO) in TBS at 37°C. To denature DNA *in situ*, slides were incubated for 2 min in freshly prepared 0.07 N NaOH in 70% ethanol, dehydrated in graded ethanol series, and air dried. Then they were treated with proteinase K (10 μg/ml; Merck, Darmstadt, Federal Republic of Germany) for 10 min in 20 mM Tris-HCl-2mM $CaCl_2$, pH 7.4. After washing, anti-thymine dimer antibody solution (40 μl per cm^2) in TBS containing 5% FCS and 0.05% Tween-20 (Sigma) was added; incubation was for 60 min at 37°C. After washing with TBS, the cells were incubated for 60 min at 37°C with FITC-GaM (40 μl per cm^2) in TBS containing 5% FCS and 0.05% Tween-20. Unbound antibodies were washed away with TBS, nuclear DNA was stained with

propidium iodide (5 min, 40 ng/ml in TBS) and the slides were mounted in 5 mM Tris-HCl-10 mM NaCl-75% glycerol (pH 8.1), coverslipped and sealed with paraffin. All treatments were done at room temperature unless stated otherwise. A Orthoplan microscope (Leitz, Wetzlar, Federal Republic of Germany) was used to examine the slides. The filter combinations (Zeiss, Oberkochen, Federal Republic of Germany) used were BP 485/20 and BP 515-560 for fluorescein excitation and emission, respectively, and BP 515-560/KP 555 and LP 590 for propidium iodide excitation and emission, respectively. Photographs were taken on 35-mm 400 ASA Kodak Ektachrome film (Kodak Eastman, Rochester, NY).

Quantitative immunofluorescence. The equipment used to analyse the fluorescence images from the microscope consists of an image intensifier (Philips XXTV1500) placed in front of a normal light level TV camera (Philips; 0.3 lux for maximum video signal), which records the images (1 frame = 1 image). The images are passed to a home-made A/D converter and disk subsystem (FLEX system), to be digitized in a format of 256 x 256 pixels, whose grey values range from 0 to 255 (8 bit). Prior to recording the images, a test procedure is carried out yielding a correction matrix (image) of 256 x 256 pixels, with which each recorded image is corrected for uneven illumination due to the HBO 100-W mercury arc lamp and other factors.

The overall procedure followed requires the recording of twin images. The first one, recorded with the propidium iodide filter combination, serves to localize the nuclei in the image. The second image of the same nuclei, recorded with the fluorescein filter combination, is used to determine the dimer content, as indicated by the signal from the fluorescein-labeled secondary antibodies. From the first image the exact position of the nuclei within the computer image is calculated, which then is used to calculate the amount of fluorescein fluorescence within these nuclei from the second image. To determine dimer-specific fluorescence, background fluorescence of cells incubated without anti-thymine dimer antibody, but with fluorescein-labeled goat anti-mouse IgG is subtracted. The image-processing software package (TCL image) has been developed by TNO-TPD, Delft, The Netherlands, in collaboration with the Technical University of Delft, The Netherlands.

Unscheduled DNA synthesis. UDS was performed as described (12,13,17) by pulse-labeling cells in Ham's F-10 medium (without thymidine), supplemented with 10% dialysed FCS, 1 μM 5-fluoro-2'-deoxyuridine (Sigma), and 20 μCi/ml [^{3}H]-thymidine (Amersham, Buckinghamshire, United Kingdom; 117 Ci/mmol), for 15 or 30 min. Dialysis of the serum and the addition of 5-fluoro-2'-deoxyuridine increase the sensitivity of the assay by lowering exogenous and endogenous thymidine concentrations.

RESULTS

Quantitative Detection of Thymine Dimers and Dimer-Removal by Immunofluorescence. Monoclonal antibodies specific for thymine dimers were used to detect and quantitate dimers at the single-cell level with an immunofluorescence assay. To test whether the intensity of immunofluorescence is a reliable reflection of the amount of dimers present, a dose-response curve was determined. As shown in Fig. 1*A*, linear increase of dimer-specific fluorescence with UV dose was observed in normal human fibroblasts, over a dose range from 0 to 16 J/m^2 (254 nm UV). Increase of UV exposure up to 40 J/m^2 resulted in still increasing fluorescence

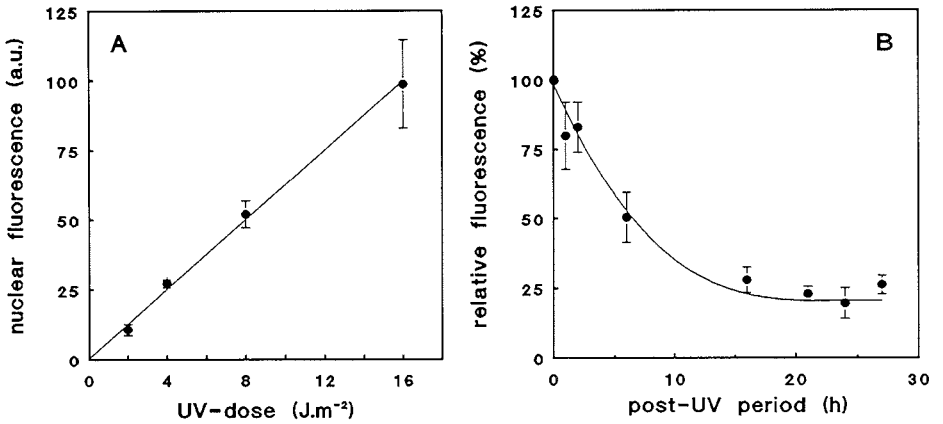

Fig. 1. Detection of thymine dimers in UV-irradiated human fibroblasts with monoclonal antibodies by means of immunofluorescence microscopy. Cells were either exposed to different dosages of UV (*A*), or UV irradiated at 10 J/m^2 followed by repair incubation for various time intervals (*B*). Thereafter cells were fixed and prepared for immunostaining. Subsequently, they were incubated with thymine dimer-specific antibodies, followed by fluorescein-labeled anti-mouse antibodies. Nuclear fluorescence was quantitated by applying computer-assisted image processing and analysis. Points, mean nuclear fluorescence (*A*) and relative mean nuclear fluorescence (*B*) ± SEM (*bars*) of 4 slides. Per slide, about 50 nuclei divided over 8-10 images were studied.

intensity (not shown).

To determine whether repair of dimers can be measured at the single-cell level, normal human fibroblasts were exposed to 10 J/m^2 UV and incubated for different periods of time prior to fixation and immunostaining. A gradual decrease of fluorescence was measured (Fig. 1*B*). In view of the specificity of the antibodies, this decrease can be attributed to thymine dimer removal. The results are in agreement with data published earlier about kinetics of dimer removal assessed with immunochemical methods (18,19) or with the UV-endonuclease assay (20).

On the basis of these results we conclude that the immunofluorescence assay used is suitable for quantitative detection of dimers at the single-cell level.

Kinetics of Dimer Removal by Microinjected Photoreactivating Enzymes. Photoremoval of thymine dimers was studied in normal human fibroblasts, microinjected with purified yeast PRE. To facilitate reidentification of the injected cells, a subpopulation of multinuclear fibroblasts was generated by cell fusion, and only those were injected. Then, cells were UV irradiated (10 J/m^2) and either exposed to PHR-light or kept in the dark, for 1 h at 37°C, in culture medium. Microinjected cells illuminated with PHR-light showed strongly reduced nuclear fluorescence in comparison to the highly fluorescent noninjected cells on the same slide (Fig. 2). Quantitative immunofluorescence data are summarized in Table 1. In microinjected

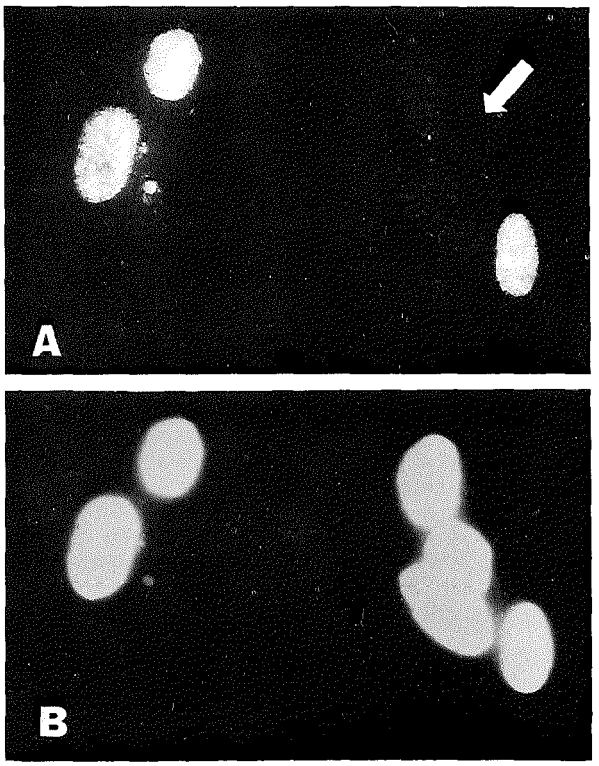

Fig. 2. The effect of photoreactivation, mediated by microinjected PRE, on thymine dimers in UV-irradiated (10 J/m^2) human fibroblasts, as detected by means of immunofluorescence microscopy. Cells were subjected to the immunostaining procedure as in Fig. 1 (*A*). Counterstaining of cell nuclei was with propidium iodide (*B*). Only multinuclear cells had been injected with PRE; after UV, all cells were illuminated with PHR-light for 1 h. The nuclei in the trinuclear injected cell are well stained with propidium iodide but are negative with regard to immunostaining of dimers (*arrow*), X 1000.

cells incubated in the dark, the level of fluorescence was not significantly different from that in cells not injected. Also in microinjected fibroblasts with larger amounts of pyrimidine dimers, induced by UV doses of 20 or 40 J/m^2, 1 h of PHR-light reduced the fluorescence to background values (Table 1). Microinjection with purified PRE from *A. nidulans* or *E. coli*, too, resulted in a PHR-light-dependent reduction of UV-induced immunofluorescence, to 4 and 56% respectively.

To determine the kinetics of PHR of dimers by PRE in injected UV-irradiated fibroblasts (10 J/m^2), the duration of illumination with PHR-light was varied. As shown in Fig. 3, already after a 5-min treatment with black light a pronounced reduction (about 75%) of immunofluorescence was observed, followed by a gradual further reduction after longer periods of illumination. In other experiments we have found that, when a UV dose of 40 J/m^2 was given, the bulk of dimers were removed within 15 min of exposure to PHR-light. These results show that injected PRE is present in sufficient amount and is able to efficiently reach and rapidly monomerize dimers in virtually all parts of the genome.

Table 1. *Effect of photoreactivation in UV-irradiated human fibroblasts on immunofluorescence associated with thymine dimers, and on UDS.*

Immediately after UV-irradiation, cells were illuminated or incubated in the dark for 1 h. Thymine dimer-specific immunofluorescence was measured. Data are given as nuclear fluorescence ± SEM of cells injected with PRE relative to that of noninjected cells. In each group about 30 nuclei were assayed.

UV dose (J/m^2)	PHR-light	Immunofluorescence (%) injected/noninjected	UDS (%)[a] injected/noninjected
10	-	111 ± 9	86 ± 6
10	+	8 ± 5	18 ± 2; 20 ± 2[b]
20	+	0 ± 6	41 ± 3
40	+	0 ± 6	105 ± 9[c]

a) The effect of photoreactivation on UV-induced UDS (1-3 h after UV irradiation; data from Ref. 12).
b) Result from recent repetition of the experiment performed to test reproducibility.
c) UDS induced by 35 J/m^2 instead of 40 J/m^2 UV (12).

Kinetics of Reduction of UDS after PHR of Dimers by Microinjected PRE. As has been shown by Zwetsloot *et al.* (12), UV-induced UDS in cultured human fibroblasts can be reduced by PHR occurring after injection of PRE. This reduction of UDS, measured over the period of 2 h immediately following the 1 h illumination with PHR-light, has been interpreted as the consequence of the sudden disappearance of dimers, which then no longer contribute to the repair synthesis by the endogenous excision repair system. Results on the effect of PHR of dimers on UDS obtained under conditions essentially identical to those of our immunochemical dimer detection experiments have been included in Table 1. Obviously, the degree of reduction in UDS shows a UV dose dependency not found in the dimer removal: after a UV dose of 10 J/m^2, PHR reduced UDS to about 20% of the value in noninjected cells; at higher doses this percentage increased, to 100% after 35 J/m^2, *i.e.*, PHR virtually had no effect on UDS anymore. Microinjection of PRE without PHR-illumination did not affect UV-induced UDS in these cells (Table 1).

Apparently, removal of dimers by PHR does not necessarily result in a reduced UDS. To obtain more information on the relationship between the two phenomena, a kinetic experiment was performed. In injected, UV-irradiated and "photoreactivated" fibroblasts, UDS was measured over short time intervals (15 or 30 min), at various moments after the illumination with PHR-light (15 or 30 min). Fig. 4*A* shows that when PHR is performed immediately following UV-irradiation, UDS is not reduced instantaneously. Only after a period of 30 min does a rapid decrease set in, and

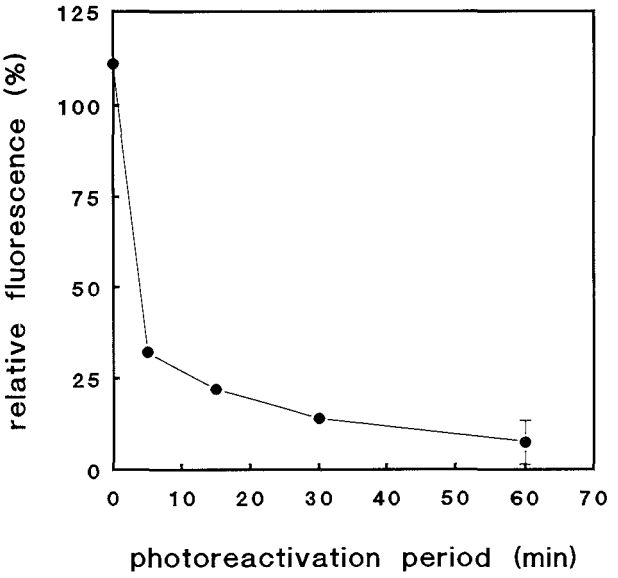

Fig. 3. Photoreactivation of thymine dimers in human fibroblasts by microinjected PRE. Cells were microinjected, exposed to UV (10 J/m^2) and subsequently incubated and illuminated with PHR-light for various time intervals just before fixation (1 h after UV irradiation). Thymine dimers were quantitated by means of immunofluorescence microscopy. Relative fluorescence was determined as the ratio of nuclear fluorescence of injected cells and noninjected cells on the same slide. *Bar*, SEM from 3 experiments. Other data are from single slides.

at 2 h after UV irradiation UDS is reduced to 17% of the level in cells not photoreactivated, in good agreement with our previous findings (12). These data show that there is a delay in the decrease of UDS after the disappearance of dimers (cf. Fig. 3). This delay was not detected in earlier experiments, because in these studies UDS was measured at later time points and over a 2-h time interval (12).

Several interpretations for the observed delay in drop of UDS are possible: (*a*) it can be due to a certain duration to complete the excision repair process from the initial recognition or removal of dimers up to the actual repair synthesis; (*b*) it may be caused by preferential excision repair of nondimer lesions occurring immediately after UV irradiation. In the former case, one expects that the delay is also observed when PHR is performed at later time points after UV irradiation. In the latter case, the delay is expected to be absent once the preferential repair of nondimer lesions is finished. To discriminate between these two main possibilities, the kinetics of UDS was also determined when PHR was performed at 2 h after UV (*i.e.*, well beyond the period of the delay observed immediately following UV irradiation). As shown in Fig. 4*B*, UDS was strongly reduced already over the first time interval studied (15 min) after PHR.

PHR of Dimers and Effect on Residual UDS in XP Fibroblasts. Previously we have found that PHR does not reduce the residual UDS of XP fibroblasts of complementation groups A, D, E and H, wheras it does lower the residual UDS of

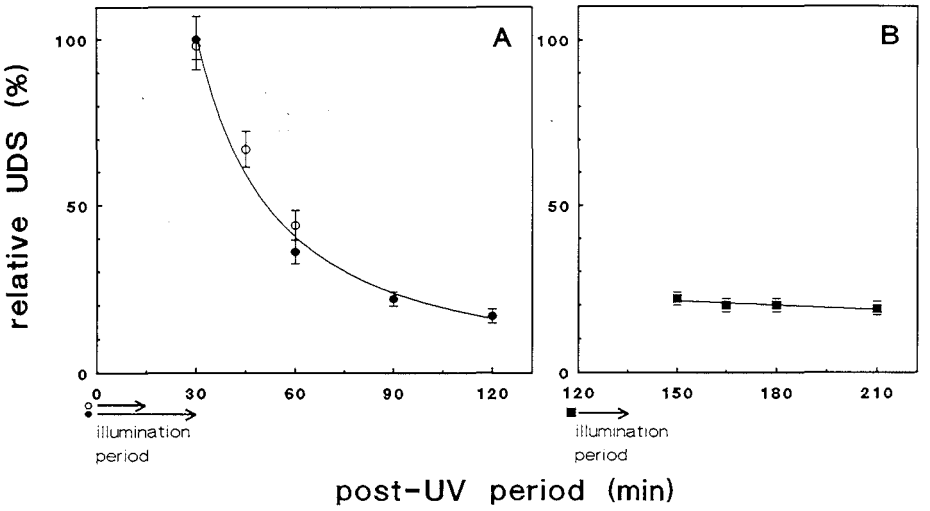

Fig. 4. Effect of PHR by microinjected PRE on kinetics of UV-induced UDS. *A*: Cells microinjected with PRE, exposed to UV (10 J/m^2) and to PHR-light for 15 min (o) or 30 min (o) and subsequently incubated in the dark. *B*: Cells treated as in *A* except after UV-irradiation they were incubated for 2 h in the dark, subsequently exposed to PHR-light for 15 min. UDS was assayed by pulse-labeling with [^{3}H]thymidine for 15 min (o) or 30 min (●,■). Cells were fixed immediately after pulse-labeling at the times indicated on the abcissa. Slides were processed for autoradiography and relative UDS was determined as the ratio of the grain number over nuclei of injected and noninjected cells. *Bars*, SEM.

all 4 XP-C strains tested, of XP-I (which now turns out to belong to XP-C as well), of XP-variant and (to a lesser extent) of XP-F (13). One of the explanations raised for the absence of PHR-induced reduction of (residual) UDS in XP cells of the complementation groups A, D, E and H is that dimers in these cells are inaccessible to the injected PRE. To directly test this hypothesis, dimer removal was determined in PRE-injected fibroblasts of XP-A, -C and -E using the immunochemical method. After UV irradiation (10 J/m^2) and 1 h illumination, microinjected cells showed background levels of thymine dimer-specific fluorescence (Table 2). Apparently, microinjected PRE is able to reach and monomerize dimers in the genome of XP fibroblasts of these complementation groups.

An alternative explanation for the absence of a PHR-induced decrease in (residual) UDS of XP-A, -D, -E and -H cells could be an extended lag phase between PHR and the onset of its effect on UDS, in these cells compared to normal fibroblasts (and XP-C, -variant and -F). In the earlier experiments UDS was measured over a 2-h time interval, 1-3 h post-UV irradiation. We therefore performed a kinetic experiment with UV-irradiated (10 J/m^2) XP-A, -D, and -E fibroblasts after PHR by

Table 2. *Effect of photoreactivation on UV-induced immunofluorescence associated with thymine dimers in normal and XP fibroblasts.*

After microinjection, cells were UV-irradiated (10 J/m^2) and illuminated for 1 h. Thymine-dimer specific immunofluorescence was measured in injected and noninjected cells on the same slide. Data are given as relative nuclear fluorescence ± SEM. Per group about 25 nuclei were assayed.

Cell strain[a]	Immunofluorescence (%) injected/noninjected	UDS (%)[b]
C5RO (normal)	4 ± 4	100 ± 5
XP25RO (XP-A)	2 ± 4	3 ± 3
XP8LO (XP-A)	2 ± 3	49 ± 4
XP21RO (XP-C)	8 ± 6	18 ± 1
XP2RO (XP-E)	10 ± 9	44 ± 4

a) Information in parenthesis, XP complementation group
b) Residual UDS relative to that in repair proficient fibroblasts accumulated over a 2 h period immediately following UV-irradiation (10 J/m^2). Data from Ref. 13 (XP25RO from Ref. 17).

microinjected PRE, in which UDS was studied over 30-min time intervals at later time points after UV irradiation (Fig. 5). Complementation group A cells normally have very low levels of a residual UDS (17); in this respect XP8LO fibroblasts are atypical because they have a residual UDS of about 50% compared to normal cells (13). This level of UDS offered the possibility to include XP-A cells in this kinetic study. In XP8LO (A) and XP2RO (E) a PHR-induced slow reduction of UDS was observed, which started 4-5 h after UV irradiation, in contrast to XP1BR (D) fibroblasts, in which at 9 h after UV irradiation in photoreactivated cells still the same level of UDS was measured as in noninjected cells (Fig. 5).

DISCUSSION

The results presented here illustrate the validity of the immunostaining technique for detection and quantitation of thymine dimers at a single-cell level. Immunostaining of UV-induced DNA damage *in situ* had already been described by Lucas (21). A monoclonal antibody directed towards thymine dimers has been used also to detect these lesions in UV irradiated mouse skin (22) although without quantitation. The specificity for thymine dimers of the monoclonal antibody we used was demonstrated in enzyme-linked immunosorbent assays (11). In agreement with this, the experiments with microinjection of PRE show that binding of the antibody is specific for lesions that can be photoreactivated, *i.e.*, pyrimidine dimers. In addition, these

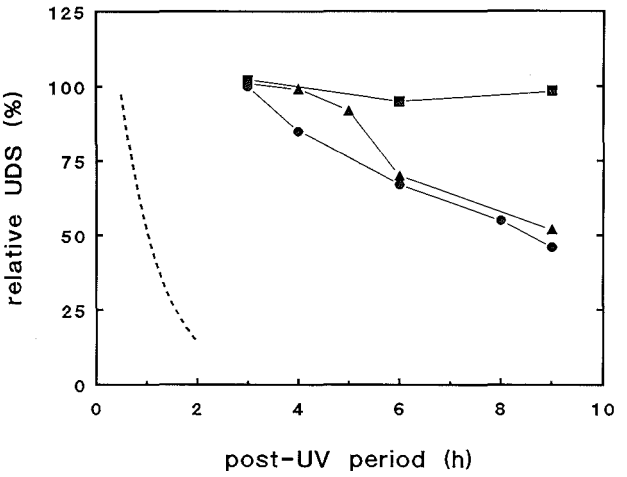

Fig. 5. Kinetics of UV-induced UDS after PHR by micro-injected PRE in xeroderma pigmentosum fibroblasts. Experimental procedure as in Fig. 4: PHR-light exposure time 30 min, applied immediately after UV-irradiation; [^{3}H]thymidine pulse-labeling 30 min. Dotted line represents data of normal fibroblasts from Fig. 4A. ●: XP8LO (XP-A); ■: XP1BR (XP-D); ▲: XP2RO (XP-E).

experiments show that the injected PRE efficiently monomerizes dimers in human chromatin, confirming conclusions reached earlier (12) on the basis of the indirect effect of PRE injection on UV-induced UDS.

The efficiency of PHR is indicated by the disappearance of more than 80% of the dimers upon illumination for periods as short as 5-10 min after a moderate dose of UV (10 J/m^2), and complete photoremoval within 1 h in fibroblasts irradiated with a UV dose as high as 40 J/m^2. The latter dose induces about 7.10^6 dimers per diploid nucleus (23). On the basis of an estimated injection volume of $1\text{-}5.10^{-14}$ l (24), we calculate that under these conditions each PRE molecule has to monomerize 10-100 dimers, assuming, rather optimistically, that all molecules injected are active and are transported into the nucleus.

In the foregoing it is assumed implicitly that the immunochemical assay detects all thymine dimer lesions in the nucleus. We cannot exclude the possibility that a fraction of dimers escapes detection by the antibody. This possibility appears not very likely, however, in view of the fact that the DNA is denatured (in alcohol and alkali) and subjected to treatment with high salt, non-ionic detergents and proteinase K prior to microscopy. These treatments remove a major fraction of the proteins attached to the DNA and make DNA readily accessible to antibody molecules. Moreover, the same procedures are employed in *in situ* hybridisation techniques in which probes are visualized by immunohistochemistry, and these procedures are known to be very efficient.

Our kinetic experiments show that dimer removal by PHR carried out immediately following UV irradiation has no immediate consequence for UDS; it results in a decrease but only after a delay. This finding can be explained in various ways. One

interpretation of this delay is that the repair synthesis, seen in the period directly following UV irradiation, is the result of repair of nondimer-type of lesions, such as (6-4)photoproducts which are known to be removed from DNA relatively rapidly. After moderate UV irradiation of normal human cells, 80% of the antibody-binding sites associated with (6-4)photoproducts were shown to be removed within the first 3 h after irradiation (25,26). This repair proceeds considerably faster than the dark repair observed for pyrimidine dimers (*cf.* Fig. 1). Other evidence for the preferential removal of (6-4)photoproducts stems from the characterization of an XP-A revertant cell strain that exclusively repairs (6-4)photolesions. The level of repair replication in cells of this strain resembles that of normal human cells over a period of 4 h after irradiation (27).

If the explanation given here for the lag phase in UDS decrease is correct, one would expect that the reduction of UDS due to PHR is UV dose dependent when studied over a fixed interval after irradiation. Data in Table 1 show this to be the case. The lack of PHR-induced reduction of UDS over the period 1-3 h after UV irradiation at 35 J/m^2, might be attributed to the fact that the capacity of the repair system responsible for UDS is limited; initial saturation is reached at UV doses exceeding 10-15 J/m^2 (23). At still higher UV doses the fraction of nondimer lesions induced apparently is large enough to saturate UDS for a number of hours even after PHR of dimers. Also the observation that PHR of dimers at 2 h after UV irradiation (10 J/m^2) results in a sharp decrease of UDS can be explained, if we accept that at that time most of the preferential repair of (6-4)photoproducts has been completed (which is in agreement with the 82% reduction in UDS over the period 1-3 h after UV; see Table 1). Furthermore, when most of the UDS occurring at 2 h after 10 J/m^2 UV is correctly attributed to removal of dimers, this fast decrease of UDS upon PHR at this time point (Fig. 4*B*) means that the time needed to repair a dimer lesion, *i.e.*, from the onset of dimer removal up to the repair synthesis, is (considerably) less than 30 min.

Residual UDS after PHR at 2-3 h after UV irradiation might be due to the repair of a few (6-4)photoproducts not repaired yet, or to removal of other types of non-dimer UV photoproducts. The fact that a low level of nonphotoreactivable UDS persists for at least 90 min (Fig. 4*A*) indicates that as a consequence of the disappearance of dimers the excision repair of the remaining nondimer lesions is not accelerated to such an extent that repair synthesis of these lesions fully compensates for the loss of UDS due to dimers.

Indications have been found that in *E. coli* and *Drosophila* photolyase stimulates dark (excision) repair (28-30). It appeared possible that also in human cells injection of PRE *per se* enhances excision repair of UV-induced lesions. In our control experiments in which PRE-injected fibroblasts were kept in the dark, thereby

preventing photoreactivation of dimers, we have not obtained evidence for this; the injected cells did not display significantly higher UDS levels in the time periods measured (*i.e.*, from 1-3 h after a UV dose of 10 J/m^2, which induces subsaturating levels of UDS; see Table 1) than noninjected neighbouring fibroblasts, nor was the immunofluorescence with the anti-thymine dimer antibody significantly lower (measured 1 h after UV irradiation; see Table 1).

Reduction of UDS upon PHR of dimers was observed earlier in chicken embryo fibroblasts (31). Also in these cells removal of dimers seemed to be more complete than was suggested by the reduction of UDS, especially at early times after UV irradiation. According to the interpretation given above, also in these cells preferential repair of nondimer lesions early after UV irradiation could explain this observation.

Xeroderma pigmentosum fibroblasts, which are (partly) deficient in excision repair, show reduced levels of UDS after UV irradiation (10,13). In some XP complementation groups, PHR of dimers by microinjected PRE results in hardly any or in a limited decrease of the residual UDS (13). One of the explanations proposed was an inaccessibility of dimers to the yeast PRE, *e.g.*, due to an altered chromatin structure or to a defective excision-repair protein that shields dimers. However, our experiments on immunofluorescence detection of thymine dimers clearly show complete dimer removal by microinjected PRE in UV-irradiated XP fibroblasts of various complementation groups.

Furthermore, reduction of UDS upon PHR of dimers was observed in XP8LO (A) and XP2RO (E) fibroblasts, albeit only as late as 4-5 h after UV irradiation. This suggests that the repair of pyrimidine dimers contributes to the late UDS, but that the majority of UDS early after UV irradiation should be attributed to repair of nondimer lesions. As no PHR-dependent reduction of UDS is observed in XP1BR (D) fibroblasts, in these cells UDS might be due to (impaired) repair of nondimer lesions exclusively. It has been shown that in XP-D fibroblasts removal of pyrimidine dimers is absent and that of (6-4)photoproducts is very slow, while in XP-E fibroblasts repair of both lesions is intermediate (26,32), which correlates with our data.

In conclusion, microinjected yeast photoreactivating enzyme is able to reach the active genome and to rapidly monomerize the dimers in normal human as well as in XP fibroblasts. Early after UV irradiation UDS appears to be due to repair of nondimer lesions, whereas in normal cells at 2 h post-UV the majority of UDS (80%) is photoreactivable and, therefore, due to repair of pyrimidine dimers. Certain nondimer lesions are repaired preferentially, which repair might proceed also in XP fibroblasts, although at a lower rate.

ACKNOWLEDGEMENTS

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APPENDIX PAPER V

Submitted for publication

DETECTION OF PHOTOREPAIR OF UV-INDUCED THYMINE DIMERS IN HUMAN EPIDERMIS BY IMMUNOFLUORESCENCE MICROSCOPY.

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ABSTRACT

To investigate the effect of visible light on the level of UV-induced thymine dimers in human epidermal cells *in vivo*, volunteers were exposed to UV-B alone, or to a serial combination of UV-B and visible light. Dimers were assayed in skin sections by immunofluorescence microscopy with a monoclonal antibody against the cyclobutane thymine dimer. The dimer-specific fluorescence from epidermal cell nuclei, identified by counterstaining with propidium iodide, was quantified through computer-mediated image processing and analysis. After a single UV-exposure significant dimer-specific fluorescence was measured, but no difference could be detected between skin kept in the dark after UV irradiation and that exposed to visible light. In three other experiments the UV-dose was split into 3 parts, given at 2.5-h intervals. Half of the skin area was exposed to visible light following each dose fraction. After the second and third dose fractions, skin areas treated with visible light clearly showed lower levels of dimers (*i.e.* about 40% reduced) than skin kept in the dark. The results provide evidence that photorepair of dimers does occur in human skin, but not immediately after a first UV-exposure of naive skin.

INTRODUCTION

The ultraviolet (UV*) radiation present in sunlight is thought to be the causative agent of the commonest human tumor type, *viz* skin cancer (1). Damage induced by UV in the chromosomal DNA of skin cells is considered to be at the origin of the carcinogenic process. There is ample evidence that the major DNA lesion caused by UV, the cyclobutane pyrimidine dimer, is responsible for UV-induced cytotoxicity

*Abbreviations:
FITC-GaM: Fluorescein-labeled goat anti-mouse Ig; MED: minimal erythema dose; PBS: phosphate-buffered saline; PHR: photoreactivation; UV: ultraviolet radiation.

and mutagenicity, although the pyrimidine-pyrimidone(6-4)photoproduct also plays an important role in the biological effects of UV-exposure (2,3). In living cells, UV-induced DNA lesions may be repaired by enzymatic processes, such as excision repair or the light-dependent reaction known as photoreactivation (PHR), the latter being specific for pyrimidine dimers (4). Removal of dimers by PHR in cells proficient in this type of DNA repair is rapid in comparison to excision repair of these lesions and is apparently error-free. PHR has often been used to study the biological effects of UV-induced DNA lesions other than pyrimidine dimers, by comparison of the effects with and without removal of the dimers by illumination (5,6). In this way it was possible to implicate pyrimidine dimers in UV-induced carcinogenesis (7,8), UV-erythema (9) and suppression by UV of immune reactions (10). It was demonstrated that carcinogenesis in hairless mice by UV-B radiation is decreased by combining it with moderate dosages of UV-A radiation (11). A similar decrease was found in the efficacy of UV-B to induce erythema in human skin, when the irradiation was followed by exposure to UV-A (12). PHR has been shown to occur in a wide range of organisms; however, studies on its presence in mammalian cells have yielded conflicting results (13,14,15), which has resulted in a general scepticism about the functioning of a photoreactivating enzyme in human cells. Light-enhanced repair (*i.e.* photorepair) of pyrimidine dimers in human skin has been reported by a few research groups (16,17,18). In the immunochemical study by Eggset *et al.* (18), however, (6-4)photoproducts (instead of dimers) had been the subject of study (19). These lesions are not a substrate for photoreactivating enzymes (20). Demonstration that in mammalian skin PHR indeed occurs to a substantial extent, has important implications, *e.g.*, in the study of the role of pyrimidine dimers and non-pyrimidine dimer lesions in UV-induced mutagenesis and carcinogenesis in skin.

Pyrimidine dimers in skin cells UV-irradiated *in vivo* have been detected either by the action of *Micrococcus luteus* UV-endonuclease on isolated DNA, via the detection of breaks at dimer sites caused by this enzyme (16,17,21,22,23), or immunochemically, with specific monoclonal antibodies (24,25). The latter method, although non-quantitative, provided information about the extent of damage induced per cell and its position in the skin.

In this paper we present an immunochemical method to detect and quantify thymine dimers in sections of UV-irradiated human skin by means of a monoclonal antibody that binds specifically to thymine dimers in DNA (26). The dimer-specific immunofluorescence of cell nuclei (localised by counterstaining the DNA with propidium iodide) is measured by computer-assisted image processing and analysis (6,26). This technique was developed and tested on UV-exposed human skin samples coming from cosmetic surgery. Subsequently, it was used on biopsies from the dorsal skin of volunteers that had been exposed to UV-B radiation to induce

thymine dimers, at dose levels by which erythema became manifest too. In these studies that were aimed at the demonstration whether or not photorepair of these lesions could be accomplished, part of the UV-treated skin was exposed to visible light. Various procedures were applied, including the use of repeated UV irradiation and illumination with photoreactivating light. The data suggest that photorepair does occur in human skin, but not immediately after a single dose of UV and visible light.

MATERIALS AND METHODS

Antibodies

The monoclonal antibody H3, specific for thymine dimers in DNA, has been described (26). Affinity-purified (Protein A-Sepharose; Pharmacia, Uppsala, Sweden) antibody (8 mg/ml) from culture supernatant was used at a dilution of 1:100. Fluorescein-labeled goat anti-mouse Ig (FITC-GaM; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) was used in a 1:150 dilution.

Irradiation and Illumination

UV-B radiation was provided by 4 fluorescent sunlamp tubes (TL 40W/12, Philips, The Netherlands; see Fig 1 for the emission spectrum). The incident dose rate was 26 J/m^2/sec as determined with a Waldmann UV-dosimeter (Waldmann, Schwenningen, Federal Republic of Germany) calibrated with a thermopile (Kipp E11). Illumination with visible light was carried out with a bank of 4 fluorescent daylight tubes (TL 40W/55, Philips; see Fig 1 for the emission spectrum). The incident dose rate was 48.3 J/m^2/sec (determined as above). The temperature of the skin increased less than 3 °C during a 20-min exposure (58 kJ/m^2).

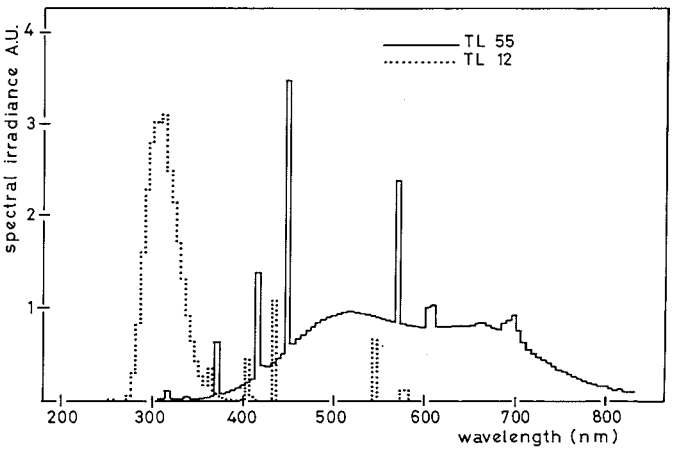

Figure 1. The emission spectra (given in arbitrary units) of the fluorescent UV-B tube, Philips TL 40W/12, and the fluorescent daylight tube, Philips TL 40W/55, measured in 5 nm intervals.

-Experiments *in vitro*

Human skin obtained upon cosmetic surgery was used with the informed consent of the patient. The skin was cut into small pieces (5 x 10 mm), rinsed in phosphate-buffered saline (PBS) and irradiated in 9-cm petri dishes (Greiner, Nürtingen, Federal Republic of Germany) at room temperature, with the stratum corneum facing the UV source. Immediately after irradiation each sample was cut into three equal pieces and fixed in methanol:acetic acid (3:1) for 1 h, rehydrated in 70% ethanol (1 h) and 5% sucrose (1 h), and subsequently frozen at -20 °C. Biopsies were embedded in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, Ind.). Sections (5 μm thickness) were cut with a cryostat at -25 °C and fixed on aminoalkylsilane-precoated slides (27). Slides were stored at -20 °C and thawed just before immunochemical staining.

- Experiments *in vivo*

For each volunteer, first the minimal erythema dose (MED) of UV-B was determined by irradiation of 9 skin areas (3 x 10 mm) to graduated UV doses with 41% increments. The lowest dose that induced a just perceptible pinkness at the exposure site after 24 h was identified as the MED. Then, untanned skin in the mid-dorsal area was exposed, once or repeatedly to a dose of UV-B related to the individual MED either or not followed by visible light. The total area of exposed skin was about 200 cm^2. Unexposed areas were covered with a non-transmitting black plastic sheet. Punch (4 mm) or shave (6 mm) biopsies were respectively frozen in liquid nitrogen or fixed in methanol:acetic acid (3:1), rehydrated and stored at -20 °C. Sections (8 μm thickness) were cut as described above.

Immunochemical Staining

Slides were thawed in freshly prepared 0.07 N NaOH in 70% ethanol for 4 min to denature DNA *in situ*. Then they were washed with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) and treated with proteinase K (1 μg/ml; Merck, Darmstadt, Federal Republic of Germany) for 10 min in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4. After washing, slides were incubated for 30 min in TBS containing 1% fetal calf serum (Flow laboratories, Irvine, United Kingdom) to prevent aspecific antibody-binding. Subsequently, slides were incubated with H3 antibodies followed by the fluorescent "second" antibody, *i.e.* FITC-GaM, counterstained with propidium iodide and mounted, as described in (6). Photographs were taken on 35-mm 400 ASA Kodak Tri-X pan film (Kodak Eastman, Rochester, NY).

Quantitative Immunofluorescence

The equipment used to examine the fluorescence of the preparations comprises a laser scanning microscope (LSM-41 Zeiss, Oberkochen, Federal Republic of Germany) interfaced to a Microdutch 100 workstation (Schreiner Electronics, Rijswijk, The Netherlands). Acquisition of the microscope images from the LSM was accomplished by software written in C; image processing and analysis were carried out with the software package TCL-image, developed at TNO, The Netherlands. The precise technical description of the software and the system will be published elsewhere (van der Wulp *et al.*, in preparation). A 40x objective (Plan-neofluar, N.A. 0.9; Zeiss) was used in combination with an electronic zoom factor of 18 to record the images, which were selected in such way that only epidermal cells were analysed. The overall procedure requires the recording of twin images, *viz* first the fluorescence of FITC-GaM, then the fluorescence from propidium iodide which serves to localize the nuclei in the image. This has been described earlier (6,26). Data are

presented as the mean dimer-specific fluorescence from various sections ± SEM processed simultaneously (Fig 3) or processed in independent experiments and averaged after normalization (Fig's 4 and 5).

RESULTS

Assay of Thymine Dimers in Skin Sections by Immunofluorescence.

Monoclonal antibodies specific for thymine dimers were used to detect and quantify dimers at the single-cell level with an immunofluorescence assay. In this assay, the fixed microscope preparations of UV-irradiated skin are stained immunochemically with the anti-dimer antibody, the binding of which is detected via attachment of a fluorescent "second" antibody. Nuclei are located through DNA-staining with propidium iodide. To test whether the intensity of the immuno-fluorescence signal is a reliable measure for the amount of thymine dimers present in the nuclear DNA, the fluorescence was recorded as a function of the UV dose. Data from the literature (21,23) indicate that the number of dimers shows a linear increase with dose. Human skin samples that became available from cosmetic surgery, were irradiated *in vitro* with various doses of UV-B. Images of a fluorescein- and propidium iodide-stained skin section are shown in Fig 2. Results of the measurements of nuclear fluorescence in the samples show the expected linear dose-response relationship (Fig 3). On the basis of these results we conclude that the immuno-fluorescence assay is suitable for quantitative detection of dimers in small samples of human skin.

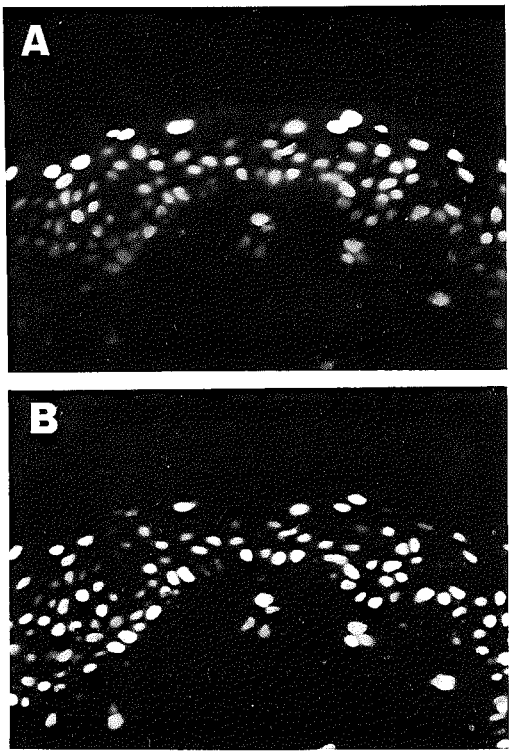

Figure 2. Detection of thymine dimers in human skin by immunofluorescence microscopy. Human skin resulting from cosmetic surgery was irradiated with UV-B (1000 J/m^2) and cryostat sections were subjected to the immunostaining procedure as described in Materials and Methods. A: FITC-fluorescence representing thymine dimers. B: Propidium iodide fluorescence representing DNA.

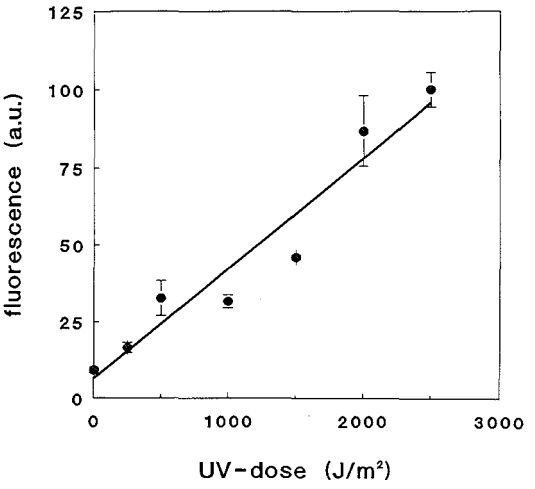

Figure 3. Thymine dimer induction in human epidermis in dependence on UV dose, as determined by immunofluorescence microscopy on skin sections. Skin biopsies were exposed to the doses of UV indicated on the abscissa. From each biopsy 4 cryostat sections were taken and processed simultaneously for immunofluorescence analysis with the anti-thymine dimer antibody. Of each cryostat section, 2 images were taken and the mean dimer-specific fluorescence in the epidermal cell nuclei was quantified by computer-mediated image analysis. The datapoints represent the average of the mean fluoresence per area of the 4 sections. The bars represent SEM. Per datapoint about 500 nuclei were sampled. (a.u.: arbitrary units).

Analysis of Photorepair in Human Skin.

To study the possible presence of photoreactivating activity in human skin, the effect of visible light on the removal of thymine dimers was investigated on human skin *in vivo*. A region of the dorsal skin of four volunteers was irradiated with UV-B (2x or 3x the individual "minimal erythema dose", MED) and illuminated with visible light for 20 min, just before or after irradiation. Another part of the skin received visible light only. All (punch) biopsies were taken at 20 min after UV-exposure, immediately after the illumination period. Cryostat sections were prepared and processed for immunofluorescence microscopy with the anti-dimer antibody. The mean dimer-specific fluorescence in the epidermal nuclei is given in Fig 4. Significant fluorescence was measured after these UV doses, but illumination of the skin for 20 min before or after UV-treatment did not significantly change the level of dimers. The data for the control, *i.e.* skin exposed to visible light only, were not significantly different from those of totally unexposed skin (not shown), for which all results have been corrected. These control data ranged from 15-36% of the maximal signal detected.

In other experiments (3 cases), the UV-dose was split into 3 parts (1 individual MED each), given at 2.5 h intervals. Following each dose fraction half of the skin area was exposed to visible light for 20 min, while the other half was kept in the dark. After the second and third illumination periods, (shave) biopsies were taken which were processed for immunofluorescence microscopy. The data on the mean dimer-specific fluorescence in the epidermal nuclei are given in Fig 5. Well measurable fluorescence levels were observed after UV exposure, which were

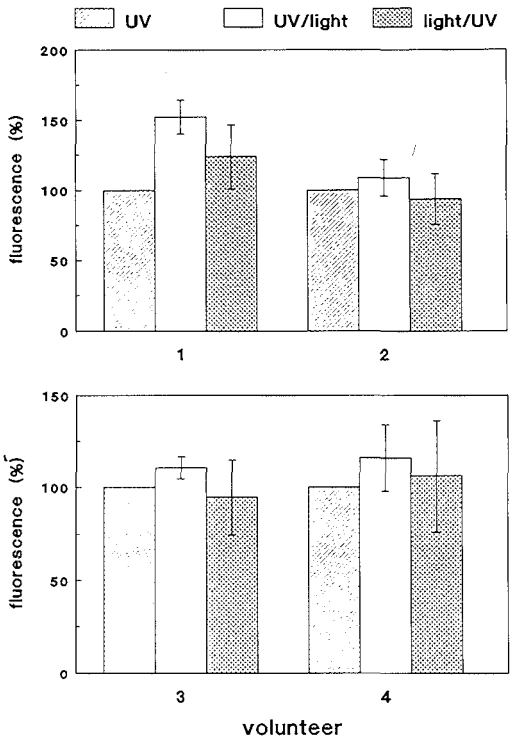

Figure 4. Effect of visible light on thymine dimers induced *in vivo* in human skin by a single dose of UV. Human volunteers received 2x MED (volunteers 1 and 2) or 3x MED (volunteers 3 and 4) of UV-B on the back. The MED values of the volunteers were 1: 360 J/m^2; 2: 370 J/m^2; 3: 470 J/m^2; 4: 480 J/m^2. Exposure to visible light (58 kJ/m^2 in 20 min) was just before or immediately after UV irradiation. One area was irradiated with UV only and another area was exposed to visible light only. Five biopsies were taken, one from each exposed area and an unexposed control. Cryostat sections from the biopsies were processed simultaneously for immunofluorescence analysis. Per section 6 images were taken and the nuclear fluorescence was averaged. Blanks, *i.e.* nuclear fluorescence in sections from unexposed epidermis were subtracted. The whole procedure of processing and analysis was repeated twice with new sections. To correct for the variation in the level of fluorescence from one set of data to another, data were normalized to the value obtained for epidermis exposed to UV only. The average of the normalized mean fluorescence of 3 separate analysis procedures is shown. The bars represent SEM. Per datapoint about 1000 nuclei were analysed.

increased after the third irradiation compared to the second; also epidermis illuminated with visible light showed a clearly lower fluorescence than epidermis kept in the dark after UV. The extent of this effect of illumination varied for the different experiments. In each of the six determinations a reduction was observed (Fig 5), which was significant in four cases ($p < 0.05$).

DISCUSSION

The results presented here illustrate the validity of the immunochemical staining technique to detect and quantify thymine dimers in sections of human skin, UV irradiated *in vitro* or *in vivo*. Sofar, *in vivo* studies on repair of UV-induced pyrimidine dimers in human epidermis concerned isolation of DNA from the biopsies taken, and biochemical determination of the number of pyrimidine dimers by means of UV-specific endonucleases (16,17,22,23). The sensitivities of the immunochemical and the biochemical methods are comparable *i.e.* the lowest UV dose that can be

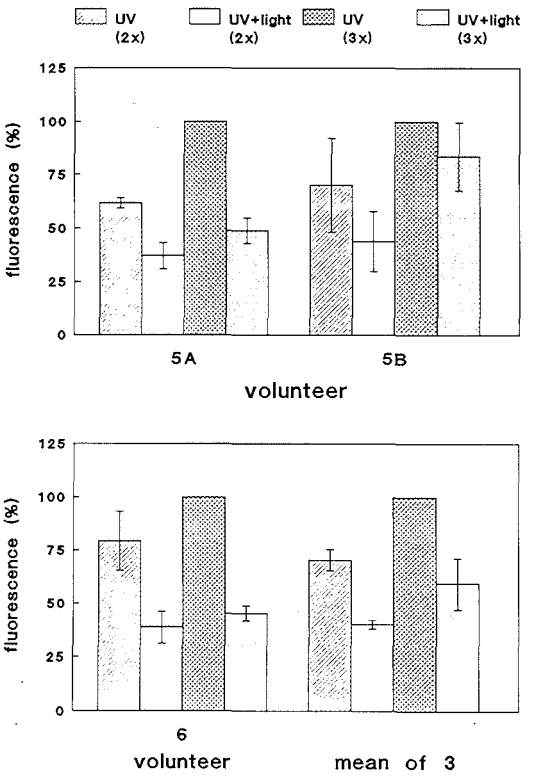

Figure 5. Effect of visible light on thymine dimers induced in human skin by several consecutive doses of UV-B. Volunteers were irradiated on the back with 3 x 1 MED of UV-B with 2.5 h intervals. Following each UV-exposure, part of the irradiated skin area was exposed to visible light (58 kJ/m^2 in 20 min), while the other part was kept in the dark. After the second and the third exposure periods biopsies were fixed and processed for quantitative immunofluorescence analysis. Data were derived as described in the legend of Fig 4, except that these were normalized to the values obtained for epidermis after the 3rd UV-exposure without illumination. Fluorescence in sections from an unexposed area was subtracted. The average of the normalized mean nuclear fluorescence of 3 separate analysis procedures is shown. The bars represent SEM. Per datapoint about 1000 nuclei were analysed. The upper panel shows data from the same volunteer (MED 600 J/m^2), irradiated and illuminated several months apart; the lower panel shows the data from another volunteer (MED 400 J/m^2) and the mean of the 3 experiments (5A, 5B and 6).

distinguished from background, but an advantage of the former is that morphology of the skin can be preserved, which allows examination of individual cells in specific regions of the skin. Another advantage is that the immunochemical method can be applied to small tissue samples. The monoclonal antibody used in the present study is specific for thymine dimers, as was demonstrated in enzyme-linked immunosorbent assays (26). In addition, earlier experiments with microinjection of photolyase into UV-irradiated human fibroblasts showed that binding of the antibody is specific for lesions that can be photoreactivated, *i.e.* pyrimidine dimers (6).

Photorepair of pyrimidine dimers in human skin irradiated *in vivo* has been reported by other investigators: in the study of Sutherland *et al.* (16) illumination for 20 min after UV irradiation reduced the amount of dimers by 33% in 3 out of 4 volunteers; in another study, by D'Ambrosio *et al.* (17), photorepair was very efficient, *i.e.* maximal 98% of dimers removed in a 10-min illumination period. The experiments reported here do not provide evidence for significant photorepair after a single UV-exposure and subsequent illumination (Fig 4), in contrast to the studies mentioned above. However, after 2 and 3 consecutive minimal erythemal doses of UV and subsequent illuminations with visible light, photorepair could be detected, on the

average the amount of dimers being reduced by about 40% relative to the amount present without illumination (Fig 5). The dependence of photorepair on repeated irradiation and/or illumination has not been reported before. An explanation might be the induction of the photoreactivating enzyme upon (repeated) UV-exposures; although a single UV-dose may also be effective in this respect: light-induced removal of dimers in cultured chicken-embryo fibroblasts increased when the UV-irradiated cells were first incubated in the dark for several hours before being exposed to the reactivating light (28). The latter effect and our data might also be explained by an increased susceptibility of the dimers to photorepair due to relaxation of chromatin caused by (incision and excision) repair enzymes. Such a relaxation has been observed in cultured human fibroblasts where it reached a peak at 3 h after irradiation (29).

The experimental evidence for the existence of a functional photoreactivating enzyme in adult birds and mammals is limited, and rather indirect. The capability to photoreactivate UV-lesions appears to diminish or disappear during development of the individual. It has been demonstrated in embryonal (chicken, 30) or neonatal cells (mice, 13), whereas in the adult animal the activity could no longer be demonstrated. Apart from the, not too well corresponding, data on photorepair in human skin, the presence of a photoreactivating activity was shown for crude preparations derived from different human embryonal organs (31). All results together suggest that at least the genetic information for a photoreactivating enzyme might be present, albeit rather poorly expressed. Whether the observed photorepair represents a true (human) photoreactivating enzyme, as found in *E. coli* and yeast (4,32), remains to be established. This uncertainty awaits the isolation and structural characterization of the enzyme or cloning of the gene involved.

In summary, our results show that thymine dimers can be detected in human skin after *in vivo* exposure to physiologically relevant UV doses. Part of these dimers can be removed by a light-induced reaction, possibly photoreactivation, but in our study visible light appeared not to be effective in this respect immediately following a single exposure to UV radiation. The experiments reported here show that 2 or 3 repeated irradiation and illumination doses do give rise to dimer removal. Further investigations with different irradiation/illumination regimens are in progress to establish the precise conditions under which photorepair occurs in human skin.

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