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Journal of Photochemistry and Photobiology A: Chemistry 93 (1996) 185–192

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY A: CHEMISTRY

A sensitive immunoassay technique for thymine dimer quantitation in UV-irradiated DNA

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Received 15 June 1994; accepted 17 August 1995

Abstract

This paper describes an immunological technique for thymine dimer quantitation which is more sensitive than the high performance liquid chromatography (HPLC) method used previously in our laboratory. Calf thymus DNA was irradiated in the presence of the photosensitizer acetophenone to induce the formation of *cis–syn* thymine dimers exclusively. These DNA solutions were then denatured and injected into New Zealand white rabbits to raise antibodies against the thymine dimer. Blood was drawn from the rabbits at regular intervals and the crude serum was used in an immunoblotting protocol which immobilized the antigen–antibody complex on a membrane system. Subsequent detection and quantitation of the thymine dimer antigen was performed by enhanced chemiluminescence (ECL). Dilutions of the antibody used in the above protocol could be quantitatively related to the UV-irradiated DNA antigen, i.e. the thymine dimer. It is shown that this technique is 4000–8000 times more sensitive than the HPLC technique used previously. The above immunoblotting/ECL protocol was then used to test the validity of a proposed mechanism for acetophenone-photosensitized dimerization of thymine in DNA at concentrations more relevant to cellular systems, but previously undetectable by HPLC.

Keywords: Immunoassay technique; Thymine dimer; UV-irradiated DNA

1. Introduction

Exposure of the skin to UV irradiation induces both physiological and pathological events. Several lines of evidence implicate DNA as the target for most of the biological effects of UV, such as lethality, mutation and transformation [1,2]. Some of these effects have been attributed to the formation of the cyclobutane pyrimidine dimer in DNA [3], although the pyrimidinones have also been implicated in this process [4]. The most prevalent lesion, however, is the *cis–syn* cyclobutane thymine dimer generated by a cycloaddition reaction between two contiguous thymine moieties. The work performed by Clemmett [5], Rutherford et al. [6] and Thomas [7] has focused on the understanding of the kinetic and mechanistic processes underlying the dimerization process in DNA in vitro and in vivo, and for this the identification and quantitation of the photoproduct is crucial. The analytical techniques typically used in the literature include ion-exchange chromatography [8], gas–liquid chromatography and high performance liquid chromatography (HPLC). This laboratory has developed an HPLC assay which satisfactorily detects and quantitates UV-induced cyclobutane photoproducts and, especially, the thymine dimer generated from a number of thymine-containing systems [5–7]. However, this technique is destructive as it necessitates the degradation of DNA to its constituent parts prior to analysis. More importantly, the detection limit of the method does not allow the quantitation of the thymine dimer at the lower levels present in cellular systems.

In this work, we present an alternative, more sensitive means of quantitating pyrimidine dimer formation at these lower concentrations. The investigative tools currently in use are non-destructive in nature and include enzymatic [9], immunological and radiolabelling [10] assays. The sensitivity of these in situ techniques is inherently greater than that obtained chromatographically. Both immunoassays and radiolabelling assays have allowed the quantitation of photodamage at low (less than 10 J m^{-2}) UV fluences [10]. Although the sensitivity afforded by radiolabelling is comparable with that of immunological detection, the former technique requires sophisticated instrumentation and carries with it all the hazards of working with radioactive species. Included in the latter is the observation of base damage induced by the radioactive label itself [11]. Environmental

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SSDI 1010-6030(95)04196-6

considerations are also beginning to militate against radioactive material usage, chiefly because of the expense and difficulties associated with waste disposal.

However, immunoassays offer a safer, cheaper and sensitive alternative. Here antibodies are generated against UV-irradiated and denatured DNA [12] in an experimental animal. The detection and quantitation of these antibodies is then necessary. One technique which has been developed to accomplish this is the enzyme-linked immunosorbent assay (ELISA) [13]. Here, the antigen, antibody and a secondary antibody conjugated to an enzyme are immobilized on a support medium (a 96-well ELISA plate). When the enzyme substrate is added to the complex, the chromogenic reaction product allows the quantitation of the bound antigen. However, reproducibility is often a problem with ELISA, partly due to variations in the adsorption of the antigen onto the well of the plate. In the light of this, Nehls et al. [14] described an immunoblotting technique which immobilizes the complexes of interest on a synthetic membrane system instead. This technique has been used successfully to demonstrate the repair kinetics of UV DNA damage [15] and to detect pyrimidine dimers in calf thymus DNA irradiated at sublethal UV fluences [10].

For the reasons given above, immunoblotting was chosen in this work for the detection of anti-UV DNA antibodies. Quantitation of these antibodies was achieved by enhanced chemiluminescence (ECL) because immunofluorescence [16] is not sufficiently sensitive to reveal lesions induced by low UV fluences, and a radioimmunoassay [17] was discounted for the reasons cited previously. Support for this choice comes from the observation that luminescent assays are finding varied applications in the field of clinical biochemistry, where increasingly lower concentrations of analyte are being detected. This assay is based on the production of light when a substrate is added to an immobilized antigen/antibody/secondary antibody–enzyme complex. It is the intensity of this emitted light which is quantitated, thus allowing quantitation of the bound antigen. The assay is quick to perform, requires no sophisticated instrumentation and, most importantly, is particularly sensitive. It is this sensitivity which has allowed adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) to be detected at 10^{-10} and 10^{-16} mol respectively [18].

This paper describes an experimental procedure for generating anti-UV DNA antibodies, immobilizing them during the course of an immunoblotting protocol and, finally, allowing their detection and quantitation by ECL. A comparison is also made between the use of crude serum and serum purified by affinity chromatography in this procedure. The enhanced sensitivity of this technique over HPLC is described, and its application in testing the validity of a proposed mechanism for the acetophenone photodimerization of thymine in DNA [19] at concentrations more characteristic of cellular events is discussed.

2. Experimental details

2.1. Irradiation of calf thymus DNA

Irradiation of aqueous solutions of calf thymus DNA (Sigma) and 2×10^{-2} M acetophenone was performed using an Osram HBO 500 W/2 high pressure mercury lamp in conjunction with a 10 mm optically flat Pyrex filter. The rate of light absorption was approximately 6.3×10^{16} photons s^{-1} under these conditions, but varied with the age of the UV source. Samples for irradiation were contained within a 400 μl quartz cuvette of 1 mm path length. This system allowed light of wavelengths greater than 290 nm to induce photosensitized dimerization of adjacent thymine moieties. DNA was used at concentrations ranging between 1.0 and 0.025 mg ml^{-1} and was dissolved in 0.15 M phosphate-buffered saline (PBS). Hydrolysis of the irradiated DNA solutions (9.2 M perchloric acid at 100 °C, followed by neutralization with 10 M KOH [7]) was performed prior to *cis–syn* thymine dimer quantitation. HPLC was employed for separation and quantitation of the thymine dimers. The HPLC system consisted of a Waters 600 multisolvent delivery system, a U6K injector and a Waters 990 photodiode array detector connected to an NEC APCII personal computer containing the Waters 1988 software package. Optimum resolution of the *cis–syn* thymine dimer was achieved using a Spherisorb 5ODS2 column (0.46 cm × 25.0 cm) and a mobile phase of Milli-Q water (pH 6) at a flow rate of 0.7 ml min^{-1}. Quantitation of the *cis–syn* thymine dimer was performed by correlating the observed peak area to a *cis–syn* thymine dimer concentration with the use of the relevant calibration graph.

2.2. Production of UV-irradiated DNA antigen

It is uncommon for nucleic acids to be immunogenic when used as pure materials. Yet, if they are denatured, the exposed purine and pyrimidine bases are capable of inducing an immune response. Included here is the *cis–syn* thymine dimer, which has been shown to be the antigenic determinant in UV-irradiated DNA [12,17,20]. In this context, 1 mg ml^{-1} DNA solutions were irradiated for 10 h in the presence of acetophenone, and subsequently denatured by heating at 100 °C for 10 min and then placing the denatured solution on crushed ice for a further 10 min. The carrier protein, methylated bovine serum albumin (MBSA, Sigma), was added as a 1:1 mass ratio as suggested by Plescia et al. [21]. Freunds incomplete adjuvant (Sigma) was used to enhance antibody production, and this was employed at a 1:1 volume ratio with the DNA–MBSA conjugate. Eight individual UV-irradiated DNA immunogens were administered over a 4 week period to each of two New Zealand white rabbits. Subcutaneous and intramuscular injection routes were employed. Bleeds were performed prior to each injection and 10 and 20 days after completion of the injections. The blood was centrifuged and the serum frozen until required. In order to keep antibody titres high, a further series of injections was performed 2

months after the initial series using a 0.5 mg ml^{-1} DNA antigen.

2.3. Purification of the crude serum

Antibodies (immunoglobulins) were purified from contaminating serum proteins using an affinity chromatography technique. Here, protein A molecules which bind specifically to the major class of antibodies, IgG, were housed in a Memsep chromatography 1000 cartridge (Millipore). The cartridge was used in conjunction with a Waters Delta Prep 4000 HPLC solvent delivery system to which a Waters 486 tunable absorbance UV detector and a Waters 745 data module were attached. Serum samples were diluted and filtered through Millex 0.45 μm filtration units (Millipore) prior to loading. A 0.05 M Tris/HCl, 0.1 M NaCl, pH 8 solution was used as the loading buffer, while 0.1 M glycine/HCl, pH 2.5 brought about efficient elution of the bound IgG component, thus separating this desired fraction from the contamination proteins. A detection wavelength of 280 nm and a flow rate of 1.5 ml min^{-1} were employed. The relevant fractions were collected (by comparing their retention times with those of commercial rabbit IgG samples) and used in the subsequent immunoblotting protocol.

2.4. Immunoblotting

Details of the protocol developed are given. Immobilon N membranes (Millipore) were cut into 4 cm × 1 cm strips and, after pre-wetting separately in methanol, Milli-Q water and PBS, were transferred to an ELISA plate modified by the removal of the base of each well. Membranes were blotted with 4 × 5 μl aliquots of UV-irradiated denatured DNA, allowed to dry, and thereafter all unreacted sites on the membrane surface were blocked using 2% dried milk dissolved in 0.1% Tween 20 (Riedel-de-Haën) in PBS. The membranes were allowed to sit in the blocking solution for 1 h. Blocking and all subsequent washing steps were performed in small plastic boxes (20 cm × 10 cm × 10 cm) positioned on a shaking water bath (Protea). Membranes were washed three times using 0.1% Tween 20 in PBS, and thereafter incubated overnight on the shaking bath in dilutions of the crude serum. Usually dilutions of 1:50 to 1:10 000 were employed. These were prepared in 5 ml glass vials into which the membranes were inserted for incubation. After a second washing protocol, the membranes (now immobilized with the antigen–antibody complex) were incubated for 6 h in a 1:500 dilution of a secondary antibody (from donkey) conjugated to the enzyme horseradish peroxidase (Amersham). A final washing protocol then followed.

2.5. Detection and quantitation of antigen using ECL

Detection and quantitation of the immobilized antigen–antibody complexes was performed using ECL (the sustained emission of light). In an enzymatic reaction, luminol (a substrate for horseradish peroxidase) is converted to the aminophthalate dianion and emits light of wavelengths between 425 and 430 nm. The intensity of the light emitted is proportional to the amount of antigen bound to the membrane.

An ECL Western blotting detection system (Amersham) was used, as it is more sensitive for detection than standard colorimetric assays. The system consisted of detection solutions containing luminol and unspecified enhancers. Hyperfilm ECL (Amersham), a double-sided high performance luminescence film, was used to capture the light emitted by the enzymatic reaction, and exposure of the film to emitted light took place in an 18 cm × 24 cm Hypercassette (Amersham).

A working protocol involved incubating the membranes with the detection reagent for 1 min and then wrapping the membranes in clingfilm. It was imperative to work as quickly as possible. With the light off, pre-cut luminescent film was placed over the light-emitting membranes and exposed for varying lengths of time. Thereafter, standard photographic techniques were used to develop the luminescent film. The film was then viewed. Dark spots on the film confirmed the presence of the antigen with a known antibody dilution. A cut-off dilution was established at which point the antigen–antibody complex was no longer detectable by the naked eye.

2.6. Application of immunoblotting/ECL protocol for the validation of a previously proposed mechanism

Solutions of 0.025 mg ml^{-1} DNA in the presence of 2×10^{-2} M acetophenone were irradiated for varying lengths of time. The solutions were hydrolysed and the thymine dimer quantitated by means of immunoblotting with ECL detection. The calibration graph was used to convert cut-off antibody dilutions to thymine dimer concentrations. The previously reported mechanism and associated rate constants [19] for photosensitized thymine dimerization in DNA in the presence of acetophenone were used to calculate the expected thymine dimer yields. In order to do this, the computer program, COMPUTER ANALYSIS OF KINETIC EQUATIONS (CAKE) [22,23], was used. Here, rate equations, rate constants, the initial concentrations of the species used and the reaction times were specified. The calculated dimer yields (from CAKE) were compared with those obtained experimentally from the immunoblotting protocol.

3. Results and discussion

3.1. Purification of the crude serum

Antibodies directed against UV-irradiated DNA antigens were generated in experimental animals and then used in an immunoblotting protocol to quantitate antigen–antibody complexes. It is important that the crude serum containing the antibodies is purified from extraneous proteinaceous material. Here, affinity chromatography was successfully

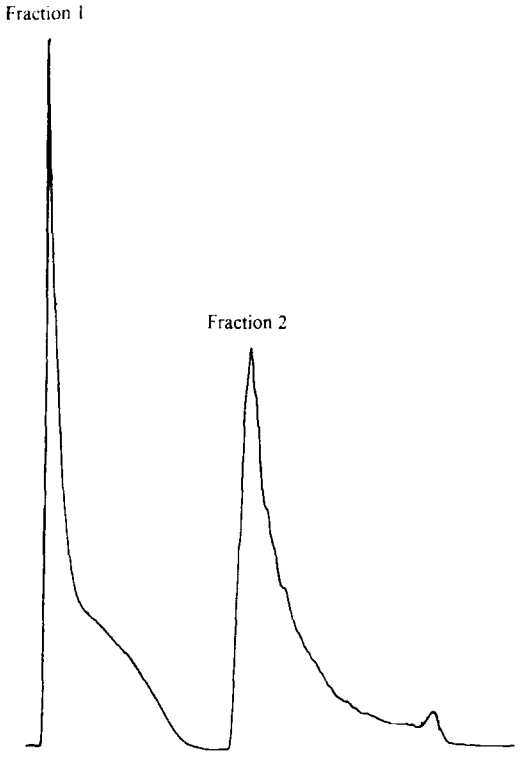

Fig. 1. High performance liquid chromatogram illustrating separation of IgG antibodies from serum proteins and other immunoglobulins using a Memsep chromatography 1000 cartridge. Samples were loaded using 0.05 M Tris/HCl, 0.1 M NaCl, pH 8 buffer over 0–2 min. Samples were eluted using 0.1 M glycine/HCl, pH 2.5 buffer over 2–20 min. A flow rate of 1.5 ml min^{-1} was employed. Fraction 1: serum proteins and the immunoglobulins IgM, IgA and IgE. Fraction 2: the immunoglobulins IgG produced in response to the UV DNA antigen.

employed to separate the IgG component from the unwanted fractions. A typical chromatogram is illustrated in Fig. 1. The percentage recovery of commercial IgG was estimated to be 94%. This calculation was based on an extinction coefficient of 1.43 $(mg/ml)^{-1}$ cm^{-1} [24] at the detection wavelength of 280 nm for IgG. However, despite the high recoveries achieved and the many successful applications of affinity chromatography, this technique is unable to resolve antibody classes from one another. If the latter is required, anion-exchange chromatography has been found to separate IgG, IgM and IgA from one another [25]. Purified and crude serum were subjected to the same immunoblotting/ECL protocol (see later). However, no difference in cut-off dilution was observed between the two serum types. It thus appeared satisfactory to employ crude serum in all subsequent immunoblotting runs. In this work, it was not necessary to quantitate the absolute antibody production in the various serum samples obtained. As will be shown later, cut-off antibody dilutions could be related to thymine dimer concentrations without knowing or calculating the specific thymine dimer concentrations in any serum samples.

3.2. Immunoblotting with ECL detection

Immunoblotting with ECL detection has been used successfully to detect thymine dimers in UV-irradiated DNA. A protocol was developed and reagent concentrations and incubation times optimized to provide the strongest signal of the immune complex against the clearest background. Since the major aim of this work was the development of a technique more sensitive than HPLC for the quantitation of thymine dimers in DNA, it was found that, in practical terms, immunoblotting has a number of advantages over HPLC. Prior to immunoblotting analysis, the UV-irradiated DNA antigen does not require pre-treatment. This is in contrast with the acid hydrolysis which is required prior to HPLC analysis. Furthermore, a single 400 μl aliquot of irradiated DNA allows more analyses to be performed, since only 5 μl is required per immunoassay determination in contrast with the 30 μl of hydrolysate required for HPLC analysis.

Crude and purified serum were subjected to the same immunoblotting protocol. As the cut-off dilution obtained was not altered by the serum type (data not shown), crude serum was employed in all subsequent runs. In order to detect low concentrations of thymine dimer, it is necessary to establish a calibration graph of cut-off antibody dilutions against thymine dimer concentrations. This involved irradiating a 0.5 mg ml^{-1} DNA solution in the presence of 2×10^{-2} M acetophenone, diluting the irradiated sample, and performing an HPLC analysis on half of each successive dilution after the hydrolysis step. The HPLC analysis served to determine the thymine dimer concentration in each dilution and also to establish that the lowest concentrations of thymine dimer which can be quantified with this technique are in DNA samples of between 0.05 and 0.025 mg ml^{-1}. At the former DNA concentration, the thymine dimer was present at a concentration of 2×10^{-6} M. For those DNA concentrations at which the dimer was not detectable by HPLC, the dimer concentration was calculated by a simple dilution calculation from the quantity of dimer in the 0.5 mg ml^{-1} DNA solution. The remaining half of each dilution of irradiated DNA was subjected to immunoblotting with ECL detection. Each DNA concentration was incubated in a number of crude serum dilutions in order to find the antibody dilution which could just detect the antigen–antibody complex (i.e. the cut-off dilution). Those luminescent films containing a "possibly visible" (with the naked eye) antigen–antibody complex were further examined using scanning densitometry in order to obtain a reliable cut-off dilution. Table 1 shows the antibody cut-off dilutions for the entire range of thymine dimers quantitated by immunoassay with ECL detection. Since the detection limit of HPLC was determined to lie at 2×10^{-6} M thymine dimer, it can be seen that the immunoassay technique described in this paper allows the thymine dimer to be detected at concentrations previously undetectable by HPLC. Table 1 also illustrates that the detection limit achieved with the immunoblotting/ECL technique is approximately 2.5×10^{-10} M. The calibration graph for the entire range of DNA concentrations investigated (6.25×10^{-7} mg ml^{-1} DNA to 0.5 mg ml^{-1} DNA) is shown in Fig. 2. In order to use the calibration data to convert antibody cut-off dilutions to thymine dimer concentrations, simple linear regression was

Table 1
DNA and dimer concentrations used for immunoblotting, together with the antibody cut-off dilutions observed using scanning densitometry of the luminescent films

[DNA] (mg ml^{-1})	[Dimer] (M)	Cut-off dilution (densitometry)
5.0×10^{-1}	1.926×10^{-5}	1:45000
1.0×10^{-1}	4.621×10^{-6}	1:19000
5.0×10^{-2}	2.013×10^{-6}	1:10000
2.5×10^{-2}	1.006×10^{-6}	1:8100
1.25×10^{-2}	5.030×10^{-7}	1:5400
6.25×10^{-3}	2.516×10^{-7}	1:10000
3.125×10^{-3}	1.258×10^{-7}	1:5400
7.812×10^{-4}	3.145×10^{-8}	1:3000
1.953×10^{-4}	7.863×10^{-9}	1:400
3.125×10^{-5}	1.258×10^{-9}	1:50
6.250×10^{-7}	2.516×10^{-10}	1:1

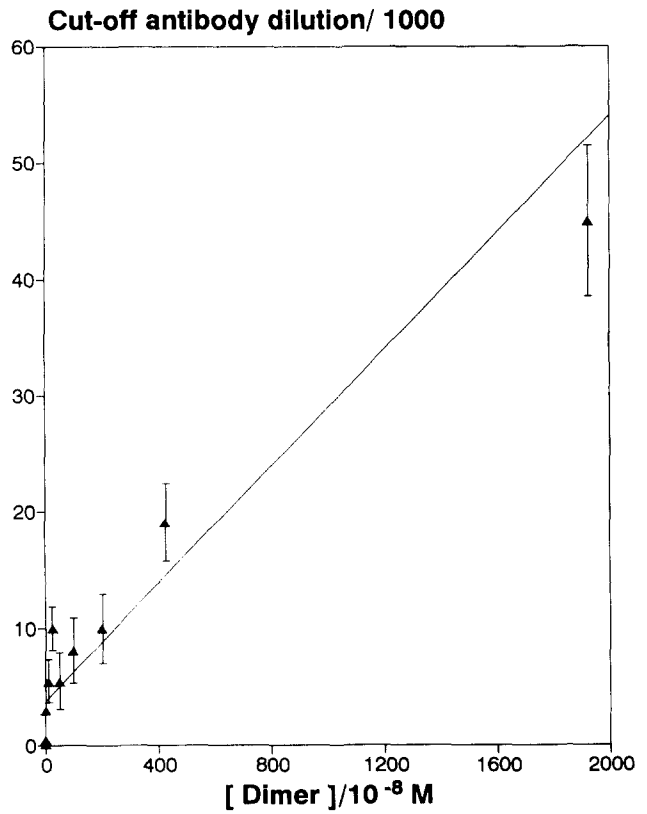

Fig. 2. Calibration graph of *cis–syn* thymine dimer concentrations vs. cut-off antibody dilutions for DNA concentrations of 5×10^{-1} to 6.25×10^{-7} mg ml^{-1}. The full line was obtained by performing linear regression on all the data points.

performed on the entire range of calibration data points and the resulting relationship was used for the conversion. It can be seen that the resulting line does not pass through the origin. This non-zero intercept is proposed to reflect antibody production against non-thymine dimer moieties (see next section).

It can also be seen that the antibody cut-off dilutions in Table 1 do not vary monotonically as expected. This can possibly be explained in terms of a "masking effect" on the Immobilon N membrane. At the higher DNA concentrations, application of the UV DNA antigen could oversaturate all the available sites. Hence, a layering of antigen occurs, thereby limiting the access of antibody and secondary antibody to the thymine dimer. During the ECL detection procedure, access of luminol is even further restricted. However, at lower DNA concentrations, this "site saturation" effect does not occur and there is easy access of all components during the immunoblotting protocol. Thus each antigen molecule comes into contact with primary and secondary antibodies, resulting in a darker spot for the antigen–antibody complex on the luminescent film than obtained with a higher antigen concentration, and thereby extending the range of antibody dilutions which must be tested in order to obtain the cut-off dilution. This problem could possibly be averted by reducing the volume of antigen delivered to the membrane.

3.3. Sensitivity and specificity of immunoblotting analysis for thymine dimer quantitation

Immunoassay sensitivity is conventionally monitored by assessing the lowest UV fluence required to bring about detectable dimer quantitation [13,17]. In this work, however, the restraints imposed by a number of graduate students using the same UV lamp for a variety of applications necessitated that an alternative approach was adopted for determining the immunoassay sensitivity. A UV source of fixed intensity was employed for the irradiation of calf thymus DNA, the DNA solution was diluted and the lowest dimer concentration detectable by immunoblotting with ECL detection was determined. As mentioned above, HPLC analysis set the lower limit for detection at approximately 0.05 mg ml^{-1} DNA (2.1×10^{-6} M thymine dimer), whilst immunoblotting with ECL detection extended this range to 6.25×10^{-7} mg ml^{-1} DNA (2.5×10^{-10} M thymine dimer). Importantly then, immunoblotting has been proved to be about 4000–8000 times more sensitive than HPLC, and this result will facilitate the planned extension of this work to the quantitation of thymine dimer production in in vivo systems.

The question of antibody specificity was approached by comparing the cut-off antibody dilution in an immunoblotting protocol for an irradiated denatured DNA antigen with that of a denatured (and not irradiated) DNA antigen. Such an assay allowed antibody production against non-thymine dimer constituents to be set at approximately 60%. Although this value could be considerably lowered by using monoclonal antibodies [26,27], the use of crude, polyclonal serum has satisfactorily allowed an immunoblotting protocol to be developed for thymine dimer quantitation. It should be noted that the data presented here remain uncorrected for antibody production against non-dimer constituents.

3.4. Application of immunoblotting/ECL protocol for the validation of a previously proposed mechanism

Kinetic mechanisms for thymine dimerization in the presence of various photosensitizers have been proposed [5–7].

However, these mechanisms have only been tested at those thymine dimer concentrations detectable by HPLC. The immunoblotting/ECL protocol described here has now made it possible to detect much lower thymine dimer concentrations, and was therefore used to test whether the mechanism proposed by Salter and Thomas [19] for the acetophenone-photosensitized dimerization of thymine in DNA still held at lower DNA concentrations. In Ref. [19], the range of DNA concentrations examined was 0.1–10 mg ml^{-1}.

The mechanism proposed is shown below

$gap \longrightarrow sap \quad k_1 = 1.6 \times 10^{-3}\ s^{-1}$, singlet excitation

$sap \longrightarrow tap \quad k_2 = 6.0 \times 10^{10}\ s^{-1}$, intersystem crossing

$tap + tt \longrightarrow gap + tt$

$k_3 = 1.3 \times 10^{8}\ dm^3\ mol^{-1}\ s^{-1}$, quenching

$tap + tt \longrightarrow gap + ttt$

$k_4 = 4.0 \times 10^{9}\ dm^3\ mol^{-1}\ s^{-1}$, triplet transfer

$tap + gap \longrightarrow gap + gap$

$k_5 = 1.8 \times 10^{8}\ dm^3\ mol^{-1}\ s^{-1}$, self-quenching

$tap \longrightarrow gap \quad k_6 = 5.0 \times 10^{2}\ s^{-1}$, non-radiative decay

$tap + x \longrightarrow x + gap$

$k_7 = 1.0 \times 10^{8}\ dm^3\ mol^{-1}\ s^{-1}$, quenching

$tap + O_2 \longrightarrow gap + O_2 \quad k_8 = 2 \times 10^{9}\ dm^3\ mol^{-1}\ s^{-1}$,

oxygen quenching

$dim + tap \longrightarrow ga + dim$

$k_9 = 1.3 \times 10^{8}\ dm^3\ mol^{-1}\ s^{-1}$, quenching

$ttt \longrightarrow tt \quad k_{10} = 2.0 \times 10^{9}\ s^{-1}$, decay

$ttt \longrightarrow dim \quad k_{11} = 1.1 \times 10^{7}\ s^{-1}$, dimerization

where gap is ground state acetophenone, sap is singlet state acetophenone, tap is triplet state acetophenone, x is any non-contiguous thymine pair, tt is a contiguous thymine pair, ttt is a triplet state thymine pair and dim represents the thymine dimer. This proposed mechanism assumes the reacting species to be the contiguous thymine pair within the DNA matrix and collision of triplet acetophenone with the other bases in DNA results in quenching [19].

Essentially, the rate constants in this mechanism were assigned in the following manner. Rate constant k_1 was determined from Hatchard and Parker actinometry [28], while k_2 is the value obtained by Hochstrasser et al. [29] for benzophenone intersystem crossing from the singlet to the triplet state in ethanol. Rate constants k_3, k_4, k_7 and k_9 were originally assumed to be diffusion controlled, although the work of Charlier and Helene [30] on free bases, the dimer and acetophenone indicated that the quenching of triplet acetophenone by the thymine dimer would be of the order of 1×10^{8} $dm^3\ mol^{-1}\ s^{-1}$. Thus k_3, k_7 and k_9 were optimized in this range. However, k_4 was calculated to be 4.0×10^{9} $dm^3\ mol^{-1}\ s^{-1}$ with DNA modelled as a rigid rod of known length. The Stokes equation for a prolate ellipsoid was used in conjunction with the Debye–Smoluchowski equation to yield this value. k_5 was assigned a value of 1.8×10^{8} $dm^3\ mol^{-1}\ s^{-1}$ on the basis of the reported self-quenching rate constant for benzophenone triplet in water [31], while the value of k_6 was based on the measured acetophenone triplet lifetime of 125 μs [30] in conjunction with the self-quenching rate constant k_5. k_8 was optimized from the value of 3.7×10^{9} $dm^3\ mol^{-1}\ s^{-1}$ reported by Charlier and Helene [30], while k_{10} and k_{11} were estimated from the data of Meistrich and Lamola [32] who irradiated phage DNA under conditions in which it was completely coated by photosensitizer. A more detailed description of how the rate constants were obtained for each elementary step in the mechanism is given in Ref. [19].

In this work, solutions of 0.025 mg ml^{-1} DNA and 2×10^{-2} M acetophenone were irradiated for varying lengths of time and used as the antigen in the immunoblotting protocol. The DNA concentration chosen was such that dimer production was not detectable by HPLC. The cut-off antibody dilutions obtained were used to estimate the thymine dimer yields from the calibration graph (Fig. 2). The above mechanism was then used to calculate (with the aid of the computer program CAKE) the expected thymine dimer yields for the experimental conditions investigated. A comparison of the experimentally observed and calculated dimer yields is shown in Fig. 3. In both cases it can be seen that the dimer yields level off with increasing irradiation time. It is thought that this levelling off reflects a limitation in the substrate (i.e. contiguous thymines along the DNA strand), rather than a limitation in the acetophenone available for the photosensitized reaction to proceed [7]. Another possibility is the occurrence of DNA–acetophenone association, thereby reducing the amount of acetophenone available for photosensitized dimerization. This saturation of the thymine dimer yields at longer irradiation times was also observed by Alliwell et al. [33,34] on irradiation of 0.1 mg ml^{-1} pUC19 plasmid DNA in the presence of 1×10^{-2} M *para*-aminobenzoic acid (PABA) as photosensitizer. In this case, the effect could be partially explained in terms of the formation of a number of PABA photoproducts, including a PABA–thymine adduct, which would again reduce the availability of the photosensitizer for dimerization.

From Fig. 3, it can be seen that, although the calculated thymine dimer yields agree fairly well with the experimentally observed values at longer irradiation times, the same is not true at shorter irradiation times. This is not surprising since, even for experiments performed with the higher DNA concentrations [7], the initial rate of dimer formation was found to be sensitive to the value set in the simulation for the number of dimerizable contiguous thymines [19]. This value can be estimated from the plateau value of the dimer formed at different irradiation times, or from statistical arguments, but in each case there is an associated uncertainty. It is believed that direct (non-photosensitized) dimerization may

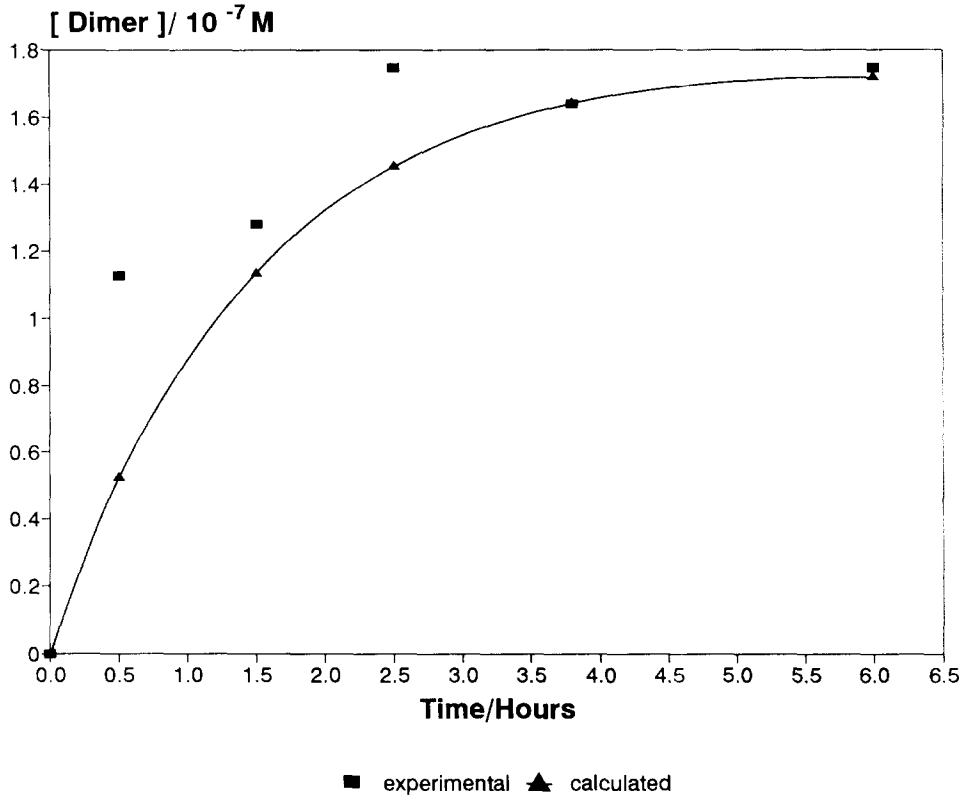

Fig. 3. Experimental (from immunoblotting) and calculated dimer yields for the analysis of 0.025 mg ml^{-1} DNA irradiated at a UV fluence of approximately 6.3×10^{16} photons s^{-1} in the presence of 2×10^{-2} M acetophenone.

contribute to the dimer yield at low acetophenone concentrations [19].

Taking cognizance of the above comments, there is reasonable agreement between the dimer yields calculated from the proposed mechanism and the experimental yields obtained for 0.025 mg ml^{-1} DNA. This suggests that the same, or a similar, mechanism is operating at a DNA concentration four times lower than that tested previously. This agreement is an important step towards elucidating the kinetics of photosensitized dimerization in vivo. No kinetic study of photosensitized dimerization in vivo has yet been carried out either in this or other laboratories. Thus it is not known how the kinetics would differ from the in vitro case. An in vivo mechanism would have to consider the various dimer repair processes occurring intracellularly as well as the scattering and absorption of radiation by the various cellular components. Patrick and Grey [35] note that any differences between the mechanisms would primarily be accounted for by the shielding from radiation in the in vivo case.

4. Conclusions

An immunoblotting/ECL protocol was developed for the detection and quantitation of thymine dimer at levels significantly lower than previously possible by HPLC. This procedure was then used to test whether a previously proposed mechanism for acetophenone-photosensitized thymine dimerization in aqueous solutions of DNA could reproduce the dimer yields at DNA concentrations closer to those of cellular systems. The fair agreement obtained implies that the same kinetic mechanism can explain the photosensitized process over a wide range of DNA concentrations (0.025–10.0 mg ml^{-1} DNA). It is postulated that this same mechanism may well apply at the DNA concentrations present in cellular systems. Support for this idea comes from the work of Alliwell et al. [33,34] who demonstrated that PABA (found in sunscreen preparations as an active UV absorber) photosensitized the dimerization of thymine in aqueous solutions of the following gradated systems: free thymine, thymidylyl-3′,5′-thymidine and pUC19 plasmid DNA. The kinetic mechanism proposed for the simplest system (the free base) was easily extended to be representative of the other two more complex systems.

As the immunoblotting procedure devised may well allow the quantitation of thymine dimer at cellular levels, and since the mechanism proposed by Salter and Thomas [19] may possibly be operational at this level, this work has set up the procedures which will allow neoplastic events within cells to be probed. The UV irradiation of an in vivo system has been attempted by Bolton [36] who, in order to be able to quantitate the yields of thymine dimer by HPLC, had to irradiate litres of cultured *E. coli* cells in the presence of acetophenone. A far simpler strategy is now available with the immunoblotting procedure described here. With the enhanced sensitivity afforded by immunoassay, far smaller quantities of cells

will be required for irradiation, and the DNA may be extracted and used in the immunoblotting protocol.

Acknowledgements

This work was supported by African Explosives and Chemical Industries Ltd., the Medical Research Council, the Foundation for Research Development and the University of Natal Research Fund.

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