The Photocarcinogenicity of Anthracene: Photochemical Binding to Deoxyribonucleic Acid in Tissue Culture

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Anthracene becomes covalently bound to high-molecular-weight DNA in mammalian tissue culture as a result of irradiation at 365 nm after the incubation of cells with the hydrocarbon. At high radiation doses, the extent of binding exceeds one hydrocarbon molecule per 10^3 bases, and is lethal. At low radiation doses, much decreased binding is observed, but a majority of cells remain viable and can be recultured.

The carcinogenic properties of certain polycyclic aromatic hydrocarbons are well known, and have been correlated to some extent with their ability to bind DNA *in vivo* (Brookes & Lawley, 1964). Whereas the metabolic mechanism of this process remains undefined, certain essential structural requirements relating to size and planarity of the hydrocarbon molecule have been identified by Craig & Isenberg (1970). These criteria are well satisfied by anthracene, which readily forms a physical complex with DNA *in vitro* (Ts'o & Lu, 1964). For anthracene, however, the extent of such complex-formation, which is probably intercalative in nature, relative to that for other polycyclic aromatic hydrocarbons, contrasts with its apparent non-carcinogenicity. It seems possible therefore that the latter results from the high chemical reactivity of anthracene, which renders it susceptible to ready detoxification *in vivo*.

This situation does not hold if topical application of anthracene is augmented with subsequent long-wavelength u.v. irradiation, a treatment which Heller (1950) has shown to be efficient in the formation of carcinomas in mice. He attributed this phenomenon to a photosensitizing effect of anthracene, since the long-wavelength u.v. irradiation alone proved to be inactive. Our observation that anthracene can be bound covalently to DNA *in vitro* by 300–400 nm irradiation (Blackburn *et al.*, 1973) indicates a possible alternative origin for this photocarcinogenicity, namely that this behaviour is associated with the covalent interaction of anthracene with DNA, as appears to be the case for the majority of chemical carcinogens (Miller, 1970).

Tissue culture is now a widely used method for the study of the interaction of carcinogens with cellular macromolecules and, in particular, for the metabolic binding of certain aromatic hydrocarbons to DNA (Diamond *et al.*, 1967). We therefore chose to examine the influence of long-wavelength u.v. light on the interaction of anthracene with high-molecular-weight DNA in mammalian tissue cultures.

Materials and methods

Monkey kidney epithelial cells (Vero cells, obtained from the Ministry of Defence Establishment, Porton Down) and human skin epithelial cells (HD7, supplied by the Department of Human Genetics, University of Sheffield) were cultured on Eagle's medium supplemented with 10% foetal bovine calf serum albumin (Flow Laboratories, Irvine, Ayrshire, U.K.). [*methyl*- ^3H]Thymidine (18.6 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the culture medium to a final radioactivity of 5 mCi/litre. After the cells had grown to half-confluence in 10 cm Pyrex Carrel flasks, carrier-free [^{14}C]anthracene (31 mCi/mmol; The Radiochemical Centre), dissolved in 0.15 ml of dimethyl sulphoxide, was added to give a final concentration of $30\text{ }\mu\text{M}$ and a radioactivity of 1 mCi/litre in 15 ml of culture medium.

Cells were irradiated from below, either immediately or after a dark-incubation period, by using light from a Phillips 125W Blacklight source filtered through a 5 mm polyacrylate sheet and providing approx. 100 nW/m^2 to the air-cooled base of the Carrel flasks, principally at 365 nm. The cells were harvested immediately after irradiation, and their DNA was isolated by the procedure of Hopkins *et al.* (1972) and then either hydrolysed or examined by gel-filtration chromatography on Sephadex G-200 (Blackburn *et al.*, 1974a). Dual-isotope scintillation counting was performed on a Packard 3358 machine by using automatic external standardization. In addition, the DNA–hydrocarbon photoproduct was characterized by alkaline-sucrose-density-gradient centrifugation by the procedure of McGrath & Williams (1966) modified as described by Peterson *et al.* (1973).

Results

The gel-filtration elution pattern is shown in Fig. 1 for a washed sample of DNA isolated from a single Carrel flask which had been exposed to u.v. light for

Fig. 1. Gel-filtration pattern for the anthracene-DNA complex

The complex was produced by u.v. irradiation of Vero cells for 24h. Gel-filtration was carried out by using Sephadex G-200, eluted with 10mM-potassium phosphate buffer, pH6.8, at a flow rate of 1ml/10min; 1.3 ml fractions were collected. —, [^3H]Thymidine; ----, [^{14}C]anthracene; ····, [^{14}C]anthracene (for control) incubated for 24h in the dark.

24h immediately after the addition of anthracene to the tissue-culture medium. The specific radioactivity for the ^{14}C label indicates an association in excess of 1 hydrocarbon molecule per 10^3 bases for an irradiation dose of 10mJ/m^2 . This amount of binding is raised over twofold if the same irradiation dose is applied in 4h pulses interspersed by 4h dark periods. Control flasks, maintained for 24h in the dark, show a low, somewhat variable, association of hydrocarbon with the DNA peak of about 1 anthracene molecule per 10^5 base pairs (Fig. 1).

Alkaline-sucrose-gradient centrifugation of the DNA of cells lysed after 4.5h incubation in the dark with hydrocarbon and 2h irradiation shows a sedimentation of the ^3H label which corresponds to DNA of mol.wt. 10^9 (Fig. 2). The anthracene ^{14}C label sediments in coincidence with ^3H except at the top of the gradient. The DNA from cells incubated in the dark for 6h with anthracene and lysed without irradiation show approx. 5% of this amount of ^{14}C in association with the DNA peak. Similar gradient-centrifugation patterns are obtained after irradiation exposures of 15min or 24h. At an exposure of 1min, which corresponds to $70\mu\text{J/m}^2$, the ^{14}C label associated with the DNA band is some three times that of the dark controls.

Fig. 2. Sedimentation of the anthracene-DNA complex in alkaline sucrose gradient

A saline-washed suspension of Vero cells, irradiated for 2h in the presence of $30\mu\text{M}$ -anthracene, was layered in 0.3M -NaOH on a preformed sucrose gradient (5–20% in 0.3M -NaOH containing 1 mM-EDTA, 0.06M -*p*-aminosalicylic acid, 0.7M -NaCl and 0.01M -Tris) and incubated for 1h before centrifugation at $100000g$ for 1.5h in an SW50L swing-out rotor. Fractions (20 drops) were collected and diluted with carrier DNA ($200\mu\text{g}$ in 1ml) and the DNA was precipitated with cold 12.5% trichloroacetic acid (1ml). The precipitates were collected on Whatman GF/C discs and washed repeatedly with cold 10% trichloroacetic acid, ethanol and ether before dual-isotope scintillation counting in a Packard 3385 spectrometer; —, [^3H]thymidine; ----, [^{14}C]anthracene.

The residual viability of cells after irradiation in the presence of anthracene diminishes with increasing u.v. exposure. After 1min of irradiation, cells show unimpaired growth and can be passaged. After 15min exposure, cultures exhibit a retardation in growth, but remain attached to the glass of the flasks, and contain sufficient survivors to seed new cultures. After 2h or more irradiation, no cells survive, although they retain adhesion to the surface of the Carrel flasks. Throughout all of these experiments, no difference was detected between the responses of the two types of tissue under investigation.

Discussion

The above results show that long-wavelength u.v. irradiation of mammalian epithelial-cell cultures after treatment with anthracene produces an association (of that hydrocarbon with DNA) whose characteristics are those of a covalent attachment. Three principal features emerge from sedimentation studies. First, the coincidence of distribution of anthracene and thymidine labels (Fig. 2) indicates a

homogeneous hydrocarbon-DNA complex. Secondly, the DNA is still of high molecular weight even at the high u.v. doses which result in the binding of 1 anthracene molecule per 10^3 bases and in cell death. Thirdly, provided that cells are preincubated with anthracene to allow permeation by the hydrocarbon, a u.v. dose as low as $10 \mu\text{J}/\text{m}^2$ gives detectable photochemical binding of hydrocarbon to DNA, of the order of 3 anthracene molecules per 10^5 base pairs. Since this does not appear to impair cell growth, it must be presumed that the major part of this binding is either non-mutagenic in character or is subject to repair processes.

In contrast, cells incubated in the dark under otherwise identical conditions show only a fraction of such anthracene-DNA association. With the specific radioactivity of anthracene used throughout this work it was not possible to determine accurately the significance of these controls, since their radioactivity was close to background counting values. The apparent value of 1 anthracene molecule per 10^5 base pairs could either result from a metabolic uptake of anthracene into DNA in the dark *in vivo* or indicate the limit of experimental efficiency of desorption of hydrocarbon which is only physically bound to DNA.

In general, the quantitative uptake of anthracene has not proved sufficiently reproducible to sustain accurate analysis of a dose-binding relationship. Nonetheless, this is clearly non-linear over the range of exposures studied, and falls off markedly with progressive increase in the duration of irradiation.

The mechanism underlying this photochemical interaction *in vivo* is probably closely related to that which obtains *in vitro*. In the latter case, the photochemical binding of aromatic hydrocarbons to DNA is attended by a loss of ^3H from the hydrocarbon (Blackburn *et al.*, 1973, 1974b). Moreover, the structures established for the photochemical binding of benzo[a]pyrene to 1-methylcytosine (Cavalieri & Calvin, 1971) and to thymine (Blackburn *et al.*, 1972) show an oxidative coupling of hydrocarbon to these bases. In the present work, preliminary studies have revealed that a majority of the anthracene molecules associated with the DNA can be detached by depurination (Peterson & Burton, 1964) and that there is a marked loss of ^3H from doubly labelled anthra-

cene on binding to DNA in tissue culture. These clues all suggest that the phenomenon under investigation here is not a photoaddition process, but occurs via an oxidative linkage of anthracene to DNA bases which may well involve photoionization of the anthracene to a cation radical.

Mechanistic considerations apart, this manifestation of light-induced binding of anthracene to DNA in tissue culture may be regarded as a potentially mutagenic lesion, and one which might well be responsible for the photocarcinogenicity of this hydrocarbon, which has been demonstrated by Heller (1950). In that case, the combined topical exposure of skin cells to anthracene and atmospherically transmitted u.v. radiation could constitute an environmental hazard.

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- Blackburn, G. M., Fenwick, R. G. & Thompson, M. H. (1972) *Tetrahedron Lett.* 589-592
Blackburn, G. M., Buckingham, J., Fenwick, R. G., Taussig, P. & Thompson, M. H. (1973) *J. Chem. Soc. Perkin Trans. 1*, 2809-2813
Blackburn, G. M., Flavell, A. J. & Thompson, M. H. (1974a) *Cancer Res.* 34, 2015-2019
Blackburn, G. M., Taussig, P. E. & Will, J. P. (1974b) *J. Chem. Soc. Chem. Commun.* 907-908
Brookes, P. & Lawley, P. D. (1964) *Nature (London)* 202, 781-784
Cavalieri, E. & Calvin, M. (1971) *Photochem. Photobiol.* 14, 641-653
Craig, M. & Isenberg, I. (1970) *Biopolymers* 9, 689-696
Diamond, L., Defendi, V. & Brookes, P. (1967) *Cancer Res.* 27, 890-902
Heller, W. (1950) *Strahlentherapie* 81, 529-548
Hopkins, H. A., Flora, J. B. & Schmidt, R. R. (1972) *Arch. Biochem. Biophys.* 153, 845-849
McGrath, R. A. & Williams, R. W. (1966) *Nature (London)* 212, 534-535
Miller, J. A. (1970) *Cancer Res.* 30, 559-576
Peterson, A. R., Fox, B. W. & Fox, M. (1973) *Biochim. Biophys. Acta* 299, 385-396
Peterson, G. B. & Burton, K. (1964) *Biochem. J.* 92, 666-672
Ts'o, P. O. P. & Lu, P. (1964) *Proc. Natl. Acad. Sci. U.S.A.* 51, 272-280