Assessment of the potential in vivo genotoxicity of fluoranthene

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Fluoranthene is a ubiquitous environmental pollutant. Although fluoranthene is mutagenic in bacterial and mammalian in vitro cell systems following metabolic activation by rat liver fraction, information on in vivo mutagenicity is lacking and studies on tumour initiating activity in mice are equivocal. In the present study, the potential genetic hazard to man was assessed using the mouse bone marrow micronucleus and rat liver unscheduled DNA synthesis test systems. Fluoranthene did not show any evidence of genotoxicity in either of the in vivo assays following acute oral administration at levels of up to 2000 mg/kg b.w.

Introduction

Fluoranthene is frequently found as a major component in environmental mixtures of polyaromatic hydrocarbons (PAHs) and occurs as a product of incomplete combustion and in fossil fuels. Fluoranthene and methylated derivatives are found in coal tar and coal tar products, engine oil and exhaust, cigarette smoke, smoke from open fires, smoked and grilled foods, waste water and in water supplies (International Agency for Research on Cancer/IARC, 1983; Busby et al., 1984; LaVoie et al., 1994). Concern in the UK has recently been centred on the elevated levels of PAHs found in some drinking water supplies. High levels of PAH in drinking water result from leaching of coal tar which is used to protect the internal surfaces of some mains water pipes (Drinking Water Inspectorate, 1994). In nearly all cases, contraventions of the standard limit of PAH in drinking water (0.2 μg/l as specified by the Water Supply Regulations, 1989) can be attributed to the presence of fluoranthene.

Fluoranthene has shown clear evidence of mutagenic potential in various short-term in vitro tests after metabolic activation by exogenous rat and mouse liver fractions (Kaden et al., 1979; Thilly et al., 1980; Barfknecht et al., 1982; LaVoie et al., 1982; Palliti et al., 1986, Vaca et al., 1992). A study presented by Gorelick et al. (1989) indicated that [3H]fluoranthene was systematically distributed in the rat following i.p. administration and was cleared primarily by hepatobiliary excretion; although 95% of the administered dose was excreted within 24 h, some radiolabel required 55 days for complete removal. Using 32P-postlabelling, these workers also demonstrated the appearance of a particular DNA-adduct in most tissues examined from rats following sub-chronic dietary administration of fluoranthene. The same adduct was identified in various tissues of mice following three i.p. administrations of fluoranthene (Wang et al., 1995) although no adducts had been detected in rats following a single i.p. administration (Gorelick et al., 1989). In contrast, Palitti et al. (1986) failed to find any evidence of sister chromatid exchange in the bone marrow of male mice following either i.p. or oral dosing at levels of ≤1000 mg/kg even though the compound was shown to be absorbed and systemically distributed.

Although a conventional dietary carcinogenicity assay has yet to be performed, based on the results of mouse skin-painting and subcutaneous carcinogenicity studies, it has previously been concluded that fluoranthene does not show any evidence of tumour-initiating activity in experimental animals (IARC, 1983). Bos (1987) postulated that the outcome of the skin-painting studies could have been influenced by the volatility of the test substance. In comparison, new-born mice treated with three i.p. doses of fluoranthene showed an increased incidence of lung tumours; some evidence of liver tumorigenicity was also obtained (Busby et al., 1984; Wang and Busby, 1993), although the compound was considered to be a weak carcinogen compared with other PAHs such as cyclopenta[cd]pyrene and benzo[a]pyrene which, on a molar basis, are ~15 and 60 times more potent respectively in the same bioassay (Wang and Busby, 1993).

To better assess the potential genotoxic hazard to man and because of the paucity of information on in vivo mutagenic effects of fluoranthene, a micronucleus test was commissioned to determine whether the mutagenic activity shown in vitro is expressed in vivo. Although the micronucleus test is a rapid, reliable and sensitive technique for detection of genotoxic carcinogens with systemically distributed ultimate metabolites, it is insensitive to some tissue-specific carcinogens; the bone marrow assay was therefore complemented by the rat liver unscheduled DNA synthesis (UDS) assay which is sensitive to short-lived reactive metabolites. In particular the liver is an appropriate organ for examination because it has a high metabolic capability and it generally receives relatively high exposure to agents which are absorbed from the gut, especially those which are efficiently excreted without achieving significant systemic levels (Ashby, 1986; Kennelly et al., 1993). Both assays were performed according to the current recommendations of the United Kingdom Environmental Mutagen Society (UKEMS) (Richold et al., 1990; Kennelly et al., 1993) and most recent draft guidelines of the Organisation for Economic Co-Operation and Development (OECD, 1995a,b).

The oral route of administration was chosen for both assays as being most relevant to human exposure from drinking water. Although low levels of DNA adducts have been detected in mice after multiple i.p. injections of fluoranthene, the use of the i.p. route for these assays was rejected since this is not relevant to the expected major route of human exposure and single oral exposure is the generally recommended exposure method for the UDS assay.

Materials and methods

Chemicals

Fluoranthene (CAS No. 206-44-0, 98% pure) was obtained from Aldrich Chemical Co, Gillingham, UK. 2-Acetylaminofluorene (CAS No. 53-96-3,
and continuing incubation for a further 4 h. The medium was replaced by carbon dioxide for 90 min to allow cells to attach, then cultures were used containing a circular glass coverslip. Cultures were incubated at 37°C in 5% Amersham International, Little Chalfont, UK) at a final activity of 10 ^Ci/ml with WEI (Williams' medium E without serum) before replacing the medium into the 35 mm diameter wells of multi-well tissue culture plates, each well suspended in WEC at 0.2 x 10^6 hepatocytes/ml then dispensed in 2 ml aliquots and mean cell yield was 408 x 10^6 viable hepatocytes per liver. Cells were at a rate of 10 ml/min and was allowed to run to waste via a puncture in the sub-

addition, hepatocytes were obtained from two animals treated with dimethyl-2-acetylaminofluorene at 50 mg/kg (14 h expression time). Animals were in each group at two expression times after treatment: 2 and 14 h. In groups of male rats were treated orally by gastric intubation with a single dose of the vehicle control (aqueous 1% methylcellulose), fluoranthene at 500, 1000 and 2000 mg/kg b.w. using a standard dose volume of 20 ml/kg b.w. A preliminary toxicity test was performed and showed that dose levels of ≤2000 mg fluoranthene/kg b.w. (the standard limit for in vivo genotoxicity assays) would be expected to be tolerated in mice and rats without minor clinical signs. Mouse micronucleus test

Groups of mice were treated orally by intragastric gavage with a single dose of the vehicle control (aqueous 1% methylcellulose), fluoranthene at 500, 1000 and 2000 mg/kg b.w. using a standard dose volume of 20 ml/kg b.w. Five males and five females from the vehicle control and from each of the three fluoranthene treated groups were killed by cervical dislocation 24 and 48 h after dosing. The positive control group was killed 24 h after dosing. Both femurs were dissected out from each animal and a direct bone marrow smear was made after dilution of the marrow with foetal calf serum. The smears were fixed in methanol, air-dried and stained for 10 min in aqueous 10% Giemsa. Slides were rinsed in distilled water, differentiated in buffered distilled water (pH 6.8). air-dried and mounted.

The slides were examined (under code) by light microscopy using oil immersion optics. The incidence of micronucleated cells per 2000 polychromatic erythrocytes for each animal was assessed by examination of at least 1000 erythrocytes. A record of the number of micronucleated normochromatic erythrocytes was also kept. Results were analysed using appropriate non-parametric statistical analysis based on rank

Groups of mice were treated orally by gastric intubation with a single dose of the vehicle control (aqueous 1% methylcellulose) or fluoranthene at 600 and 2000 mg/kg b.w. Hepatocytes were isolated and cultured from four animals in each group at two expression times after treatment: 2 and 14 h. In addition, hepatocytes were obtained from two animals treated with dimethyl-N-nitrosamine at 4 mg/kg (2 h expression time) and two animals treated with 2-acetylaminofluorene at 50 mg/kg (14 h expression time). Animals were killed by exposure to a gradually increasing atmospheric concentration of carbon dioxide. Hepatocytes were isolated using the two-stage enzymatic dissociation method developed by Seglen (1976). The hepatic portal vein was cannulated and the liver perfused with EGTA solution in buffer (Butterworth et al., 1987) for 5 min, then with collagenase solution (Williams' medium E containing 10 mM HEPES, 3 mM CaCl₂, 4.9 mM NaOH and 100 U heparinized collagenase/ml) for 10 min. Perfusate (at 37°C) was passed via a bubble trap at a rate of 10 ml/min and was allowed to run to waste via a puncture in the sub-hepatic vena cava. Hepatocytes were comb in suspended collagenase medium then filtered through a 200 µm mesh. The hepatocytes were partially purified by differential centrifugation then resuspended in Williams' medium E supplemented with 10% fetal calf serum (WEC), and a viable cell count made using Trypan Blue exclusion. Viabilities ranged between 69 and 89% and mean cell yield was 408 x 10^6 viable hepatocytes per liver. Cells were suspended in WEC at 0.2 x 10^6 hepatocytes/ml then dispensed in 2 ml aliquots into the 35 mm diameter wells of multi-well tissue culture plates, each well containing a circular glass coverslip. Cultures were incubated at 37°C in 5% carbon dioxide for 90 min to allow cells to attach, then cultures were rinsed with WEI (Williams' medium E without serum) before replacing the medium with WEI containing [methyl-3H]thymidine (specific activity 83 Ci/mmol), Amersham International, Little Chalfont, UK) at a final activity of 10 µCi/ml and continuing incubation for a further 4 h. The medium was replaced by WEI containing 250 µM unlabelled thymidine and incubation continued for 24 h. Coverslips were removed from medium, given three 5 min washes in Hank's balanced salts solution then fixed in 2.5% v/v acetic acid (higher concentrations have been found to cause cytoplasmic lysis) in ethanol, air-dried and mounted with the cell layer uppermost.

Results

Mouse micronucleus test

Fluoranthene did not cause any significant increase in the incidence of micronucleated polychromatic erythrocytes or any significant decrease in the ratio of polychromatic to normochromatic erythrocytes at either sampling time. Mice treated with mitomycin C showed the expected large increase in the incidence of micronucleated polychromatic erythrocytes together with slight decreases in the ratio of polychromatic to normochromatic erythrocytes (see Table I).

Rat liver unscheduled DNA synthesis test

Fluoranthene did not cause any significant increase in the gross or net nuclear grain counts at either sampling time. The positive control agents both caused large highly significant increases in both gross and net nuclear grain counts (see Table II).

Discussion

Palitti et al. (1986) have previously argued that fluoranthene is probably inactive in bone marrow because only very low levels of mutagenic metabolites are distributed systemically; a situation which contrasts markedly with the in vitro situation where relatively high levels of genotoxins can build up when metabolism is simulated by induced rat liver fraction. The results of the present study confirm the absence of genotoxic effects in the mouse bone marrow following acute oral administration and, in addition, indicate absence of significant genotoxic activity in the rat liver.

Fluoranthene has previously shown evidence of low level carcinogenic activity after three i.p. doses in the newborn mouse model (Busby et al., 1984; Wang and Busby, 1993) and of forming a specific DNA-adduct (a product of the reaction between a diol-epoxide metabolite with the N-2 of deoxyguanosine) following long-term dietary administration to rats (Gorelick et al., 1989) and multiple i.p. administration to mice (Wang et al., 1995). The relevance of the i.p. route in risk assessment is questionable; although it is recognized that the presence of the adduct may be useful in monitoring exposure, the biological consequences of persistence of low
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Table I. Summary of mouse micronucleus test results and statistical analysis

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Incidence mnp*</th>
<th>Total incidence mnp**</th>
<th>Mean c/d</th>
<th>Mean ratio p/n*</th>
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<tbody>
<tr>
<td>24 h</td>
<td>Control</td>
<td>-</td>
<td>1, 2, 1, 2, 0</td>
<td>3, 1, 1, 3, 0</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0, 1, 1, 1, 1</td>
<td>1, 0, 2, 3, 1</td>
<td>1.1 ns</td>
<td>1.2</td>
<td>0.715 ns</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>3, 2, 3, 1, 1</td>
<td>1, 1, 2, 1</td>
<td>1.6 ns</td>
<td>0.4</td>
<td>0.595 ns</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>2, 1, 2, 2, 1</td>
<td>0, 1, 2, 2, 0</td>
<td>1.2 ns</td>
<td>1.0</td>
<td>0.783 ns</td>
</tr>
<tr>
<td></td>
<td>Fluoranthene</td>
<td>12</td>
<td>52, 48, 69, 67</td>
<td>76, 49, 49, 58, 51</td>
<td>56.4 **</td>
<td>1.7</td>
</tr>
<tr>
<td>48 h</td>
<td>Control</td>
<td>-</td>
<td>1, 0, 1, 1, 1</td>
<td>2, 3, 3, 1, 1</td>
<td>1.4 ns</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1, 0, 2, 1</td>
<td>2, 3, 1, 2</td>
<td>1.4 ns</td>
<td>0.2</td>
<td>0.770</td>
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<tr>
<td></td>
<td>1000</td>
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<td>2, 2, 2, 0, 0</td>
<td>1.1 ns</td>
<td>1.0</td>
<td>0.800 ns</td>
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<tr>
<td></td>
<td>2000</td>
<td>1, 1, 1, 0</td>
<td>1, 2, 2, 4, 1</td>
<td>1.4 ns</td>
<td>0.8</td>
<td>0.795 ns</td>
</tr>
</tbody>
</table>

* mnp = Micronucleated cells observed per 2000 polychromatic erythrocytes.
** mnn = Micronucleated cells observed per 1000 normochromatic erythrocytes.

Table II. Summary of rat liver unscheduled DNA synthesis (UDS) test results and statistical analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Gross nuclear grain count</th>
<th>Net nuclear grain count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Individual animal mean</td>
<td>Group mean a</td>
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<tr>
<td>2 h expression time</td>
<td>Control</td>
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<td>9.8, 13.5, 10.5, 10.5</td>
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<tr>
<td></td>
<td>Fluoranthene</td>
<td>600</td>
<td>11.6, 9.3, 11.7, 10.7</td>
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<td></td>
<td></td>
<td>2000</td>
<td>12.5, 11.6, 10.4, 10.1</td>
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<td></td>
<td>Dimethylnitrosamine</td>
<td>4</td>
<td>29.7, 24.8</td>
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<tr>
<td>14 h expression time</td>
<td>Control</td>
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<td>15.2, 13.8, 13.3, 20.3</td>
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<tr>
<td></td>
<td>Fluoranthene</td>
<td>600</td>
<td>22.4, 26.6, 14.8, 23.5</td>
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<tr>
<td></td>
<td></td>
<td>2000</td>
<td>17.4, 16.4, 15.4, 11.9</td>
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<tr>
<td></td>
<td>2-Acetylaminofluorene</td>
<td>50</td>
<td>54.4, 56.4</td>
</tr>
</tbody>
</table>

*Results of statistical analysis (one-way analysis of variance followed by Student's t-test): ns = not significant, *P < 0.01; **P < 0.001 (one-sided probabilities).

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References


