BIOMARKERS, 2000, VOL. 5, NO. 5, 323–340

DNA adducts in relation to lung tumour outcome are not markers of susceptibility following a single dose treatment of SWR, BALB/c and C57BL/6J mice with *N*-nitrosodiethylamine

V. OREFFO[1], R. SINGH[1], K. J. RICH[2], D. E. G. SHUKER[1], P. CARTHEW[1], R. CORDERO[3], J. H. M. VAN DELFT[4] and P. B. FARMER[1,*]

[1] Medical Research Council, Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK

[2] Proctor & Gamble (HABC) Ltd, Lovett House, Lovett Road, Staines, Middlesex, TW18 3AZ, UK

[3] Toxicology Unit, Imperial College London, St Dunstan Road, London, W6 8RP, UK

[4] Department of Molecular Toxicology, TNO Food and Nutrition Research Institute, PO Box 360, 3700 AJ Zeist, The Netherlands

Received 14 December 1999, revised form accepted 11 June 2000

Keywords: DNA adducts, susceptibility, *N*-nitrosodiethylamine, mouse strains, lung tumours, immunoslot-blot, alkyltransferase.

Abbreviations: ATase, alkyltransferase; GC–MS, Gas chromatography–mass spectrometry; NDEA, *N*-nitrosodiethylamine; *N*-3EtA, *N*-3 ethyladenine; *N*-7EtG, *N*-7 ethylguanine; O^4EtT, O^4 ethylthymidine; O^6EtG, O^6 ethylguanine.

The formation of DNA adducts by the covalent binding of genotoxic chemicals to DNA represents a valuable marker for assessing exposure to carcinogens but as yet the role of DNA adducts as a biomarker of carcinogenic susceptibility still needs to be clearly ascertained. To address this question an animal study was instigated using mice (SWR (high), BALB/c (intermediate) and C57BL/6J (low)) varying in their susceptibility to lung carcinogenesis. Groups of animals from each strain were dosed with a single intraperitoneal injection of saline or *N*-nitrosodiethylamine (NDEA) at 15 or 90 mg kg^{-1} body weight. Lung and liver tissues were removed at different time points following dosing. Further groups of mice dosed with the same regime had urine samples collected 24 h post dosing and were then left up to 18 months to allow for the development of tumours. Immunoslot-blot analysis was used for the determination of *N*-7 ethylguanine (*N*-7EtG) and O^6 ethylguanine (O^6EtG) adduct levels in the DNA from the tissues and gas chromatography–mass spectrometry (GC–MS) was used to determine *N*-3 ethyladenine (*N*-3EtA) adduct levels in the urine samples. Levels of alkyltransferase (ATase) were also determined in the tissues. The results showed that the DNA adduct levels and persistence were similar across the three strains of mice following dosing with 15 and 90 mg kg^{-1} NDEA. High levels of adducts were observed in the urine of the BALB/c strain, implying an increased metabolic or repair capacity in this strain. However there were no differences in the levels of ATase in the lung and liver of the three strains of mice following dosing with 15 mg kg^{-1} NDEA. The incidence of tumours in C57BL/6J mice was lower compared with the other two strains and showed a dose dependent increase. The results from this study show that the differences in susceptibility to lung carcinogenesis between the three strains of mice do not appear to be linked to the formation of the two adducts detected. These results imply that dosing with NDEA resulted in toxicity which may have led to cell death and induction of tumours by compensatory cell proliferation. Although these results do not allow decisive conclusions

* Corresponding author: P. B. Farmer, Medical Research Council, Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK. e-mail: pbf1@le.ac.uk

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2000 Taylo
http://www.tandf.co.uk/journals

to be drawn concerning the relationship between total levels of DNA adducts and differences in carcinogenic susceptibility for the three strains of mice it is clear that the increased presence of a DNA adduct in the target tissue increases the likelihood of tumour development.

Introduction

DNA adducts can be used as simple markers of internal dose but they have the potential to be used to investigate different host factors in response to carcinogen exposure and to gain insight into the susceptibility of certain individuals to cancer. DNA adducts have an important role to play in the multistage process of chemically-induced carcinogenesis as DNA adduct formation appears to be the first critical event involved in its initiation (Wright 1991, Gaylor *et al*. 1992, Dipple 1995, La and Swenberg 1996). Experimental evidence in the literature suggests that the levels of DNA adducts produced are dependent upon the dose of chemical carcinogen, and the general assumption is that there is a positive correlation between the extent of DNA adduct formation and tumour induction in a specific tissue, with tumours rarely being formed in tissues containing no adducts (Yuspa and Poirier 1988).

A number of animal models have been used to assess the relationship between DNA adducts and carcinogenesis by measuring levels of DNA adducts in target tissues from animals that were continuously exposed to carcinogenic compounds (Beland and Poirier 1993). The advantage of using an animal model over short-term genotoxicity tests such as the Ames test is that the latter do not provide any information regarding involvement of chemical carcinogens in the stages of promotion or progression of carcinogenesis. Furthermore, short-term tests do not account for the detoxification of carcinogens, DNA repair and replication, all of which can be assessed using DNA adduct determinations in an animal model. The importance of DNA adducts for evaluating exposure to chemical carcinogens has been clearly established, however their role as markers for carcinogenic susceptibility still remains to be determined.

Mouse lung carcinogenesis, which was first observed in mice 100 years ago, provides a valuable biological model for studying the different stages of tumour development and the mechanism by which carcinogenesis is influenced by genetic and environmental factors. Furthermore, mouse lung tumours have analogous morphological, histogenic and biochemical features to those found in human lung adenocarcinoma and represent a good model for studying human lung carcinogenesis (Malkinson 1992). Inbred strains of mice have differing susceptibilities to spontaneous and induced tumour formation. A strain which develops spontaneous tumours within approximately a year after birth will also develop many tumours within a few months following carcinogen administration. The opposite is true for a strain that does not develop spontaneous tumours, which will be more resistant to the carcinogen (Malkinson 1989). Inbred mice which have been brother–sister mated for at least 20 generations have a theoretical genetic similarity of greater than 95%, thus eliminating any genetic variability that may arise in experiments (Dragani *et al*. 1995, Festing 1997).

NDEA was chosen as the model compound for this study since extensive background literature has been published describing its metabolism and carcinogenic properties in detail (Preussmann and Wiessler 1987, Gray *et al*. 1991, Peto *et al*. 1991, Swenberg *et al*. 1991). NDEA is a procarcinogen requiring

metabolic activation by cytochrome P450 2E1 and 2A6 whose activities vary among individual mice and humans, to form the ultimate genotoxic species, a diazonium ion, which acts as an ethylating agent (Yamazaki *et al*. 1992, Camus *et al*. 1993). A number of DNA adducts can be formed which include those ethylated at the O^6, N^2, N-7 and N-3 positions of guanine, the N-7 and N-3 positions of adenine, the O^2 position of cytosine and the O^2 and O^4 position of thymine as well as the phosphodiester backbone (Singer 1985, Beranek 1990). The lesions thought to be important in initiating carcinogenesis result from alkylation of the O^6 position of guanine and O^4 position of thymine, which can lead to GC to AT and AT to GC base pair transitions, respectively (Singer and Essigmann 1991). These lesions can be repaired by ATase which catalyses the direct stoichiometric transfer of the ethyl group from the DNA onto itself resulting in irreversible inactivation. Basal ATase levels have been shown to vary among species, tissues and cells and an inverse relationship between ATase levels and tumour formation has been observed in rodents (Myrnes *et al*. 1983, Rudiger *et al*. 1989, Pegg 1990).

Single doses of NDEA can be used to generate mouse lung tumours, making this a highly appropriate model for studying the relationship between adducts and carcinogenesis. In the study presented here we administered tumorigenic doses of NDEA to different strains of mice varying in their susceptibility to lung carcinogenesis, SWR (high), BALB/c (intermediate) and C57BL/6J (low) (Malkinson 1989). Levels of N-7EtG and O^6EtG were measured in lung and liver as well as the levels of ATase and excretion of N-3EtA in urine, for comparison with lung tumour incidence and multiplicity in the three strains of mice.

Materials and methods

Materials

Ribonuclease A (RNase A), proteinase K and NDEA were purchased from Sigma Chemical Co. Ltd, Dorset, UK.

Animals

Three strains of specific pathogen free (SPF) female mice supplied by Harlan UK Ltd (Blackthorn, Bicester, UK) were used, differing in their susceptibility to lung carcinogenesis, SWR (high), BALB/c (intermediate) and C57BL/6J (low).

All the animals were electronically tagged using a Trovan Tag^{TM} and randomized prior to cage assignment. When the animals were obtained from the suppliers, they were left to acclimatize for 2 weeks prior to dosing, so that they were approximately 8 weeks old at the time of dosing.

Dosing of animals with NDEA

Part 1—Early biomarkers: Each mouse (three mice per group) received either a single intraperitoneal dose of saline or NDEA in saline at 15 mg or 90 mg kg^{-1} body weight. Lung, liver and kidney tissues were removed 5 h, 10 h, 24 h, 4 d, 7 d, 28 d and 56 d after dosing and immediately frozen in liquid nitrogen for subsequent DNA adduct analysis. In a further experiment the same three strains of mice were dosed with saline or NDEA in saline at 15 mg kg^{-1} with lung and liver tissues being removed at 2 h, 5 h, 10 h, 24 h, 72 h and 7 d after dosing. The tissues were immediately frozen in liquid nitrogen for subsequent DNA adduct analysis and measurement of ATase levels.

Part 2—Tumour development: A separate group of animals treated with the same dosing regime of NDEA were left up to 18 months to develop tumours (figure 1). Prior to dosing with NDEA the animals were placed in individual metabolism cages and 24 h pre-dosing urine samples were collected and stored at –20 °C. The animals (25 per strain) were dosed with NDEA or 0.9% saline, replaced into the metabolism cages and 24 h post-dosing urine samples collected and again stored at –20 °C. These urine samples were subsequently used for the determination of N-3EtA adduct levels by GC–MS analysis. All the mice were sacrificed by cervical dislocation, and the major organs were examined and fixed in 10% neutral buffered formalin. The multiplicity of lung tumours was determined at autopsy by counting the

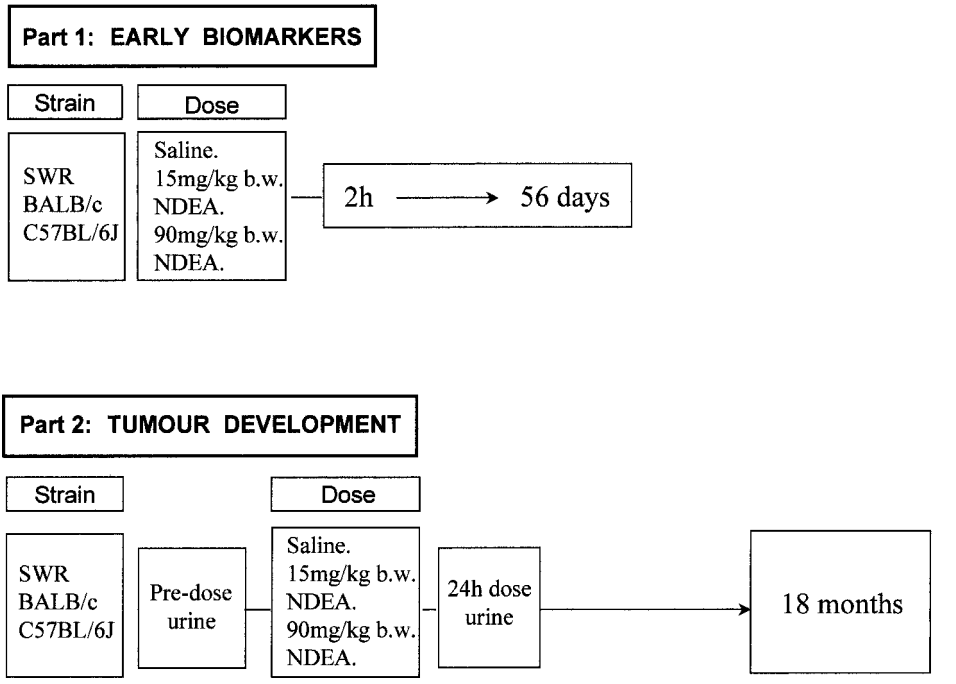

Figure 1. Scheme showing the design of the study.

number of tumours visible per tumour-bearing mouse. Sections (5 μm) of paraffin wax embedded tissues were prepared and examined histologically.

DNA isolation

DNA was isolated from animal tissues, which had first been crushed in liquid nitrogen to a fine powder and dissolved in 50 mM Tris–HCl, 100 mM EDTA, pH 8.0, using a standard phenol/chloroform–isoamylalcohol method following pre-treatment with RNase A (100 μg ml^{-1}), SDS (0.5 %) at room temperature and finally with proteinase K (500 μg ml^{-1}) at 37 °C overnight. An equal volume of the phenol extraction buffer (10 mM Tris-equilibrated phenol: chloroform: *m*-cresol: isoamyl alcohol (47.2:47.2:4.7:0.94) and 8-hydroxyquinoline (0.1 % by weight)) was added to the tissue solution. The two phases were separated by centrifugation at 3000 rpm for 10min at 4 °C (GPR Beckman centrifuge, Beckmann Instruments Inc., Palo Alto, CA, USA) and the top layer was removed, re-extracted with an equal volume of the phenol extraction buffer and centrifuged again. An equal volume of chloroform:isoamyl alcohol, (24:1, v/v) was added to the top layer and centrifuged as before. The DNA was precipitated by the addition of 3 M sodium acetate, pH 6.5 (0.1 volume) and 0.8 volume of ice-cold isopropanol (assuming the total volume was equal to 1.0) to the top aqueous layer and washed with absolute alcohol followed by 70 % ethanol in water before resuspending in SCC buffer (1.5 mM NaCl, 150 μM trisodium citrate, 1.0 μM EDTA, pH 7.3). The purity of the DNA was checked by the determining the ratio of the absorbance at 260 and 280 nm (Kontron UVikon 860 spectrophotometer) and the concentration determined by assuming that $A_{260nm} = 1$ is equivalent to 50 μg ml^{-1} for double-stranded DNA.

Determination of N-7EtG *in calf thymus DNA standards used for the immunoslot-blot by HPLC-electrochemical detection*

The *N*-7EtG level in the calf thymus DNA treated with *N*-ethyl-*N*-nitrosourea *in vitro* was determined by HPLC with electrochemical detection. The first step in the analysis involved the release of the adducted base from the DNA backbone, which was achieved by hydrolysing the DNA sample in 0.1 M formic acid, pH 2.3 at 70 °C for 1 h. An enrichment step was performed on the sample by using reverse phase HPLC to separate *N*-7EtG from guanine and adenine, which were also generated by the

hydrolysis conditions utilised. The HPLC fraction containing *N*-7EtG was lyophilized, redissolved in water and analysed by electrochemical detection (Singh *et al*. 1997). This calf thymus DNA was used as a standard to construct the calibration line for the immunoslot-blot.

Determination of N-7EtG and O⁶EtG levels in DNA from tissues obtained from the 90 mg kg⁻¹ NDEA dose group by immunoslot-blot analysis

Preparation of DNA samples: The method has been described previously by Mientjes *et al*. (1996). Briefly, DNA samples to be analysed were diluted with 10 mM di-potassium hydrogen orthophosphate (K_2HPO_4), pH 7.0, to a concentration of 100 μg ml^{-1} in a final volume of 200 μl and then sonicated for 5 s (Branson sonifier 250, Dawe Ultrasonics, Middlesex, UK). The DNA samples were incubated with 4.7 μl of 3 M NaOH at 37 °C for 30 min followed by the addition of 6.0 μl of 1 M potassium dihydrogen orthophosphate (KH_2PO_4) and then 15–16 μl of 1 M HCl to maintain the pH at approximately 7.5. The DNA samples were heated to 100 °C for 5 min following the addition of 75 μl of phosphate buffered saline (PBS) and then cooled to 0 °C for 10 min. The UV absorbance was determined at 260 nm using 2 M ammonium acetate:PBS (1:1, v/v) as the blank, following the addition of 2 M ammonium acetate (500 μl) and the concentration of DNA in each sample was calculated by assuming that $A_{260nm} = 1$ is equivalent to 40 μg ml^{-1} for single stranded DNA.

Immunoslot-blot analysis: The DNA samples were applied onto the nitrocellulose filter (0.1 μm, Schleicher and Schuell, Dassel, Germany) by pipetting a volume that contained 1 μg of DNA into each well of the immunoslot-blot apparatus (Minifold S, SRC 60D, Schleicher and Schuell) followed by 1 M ammonium acetate (200 μl). The filter was heated at 80 °C for 1 h and then bathed in 50 ml of PBS-T (PBS plus 0.1 % Tween-20 (polyoxyethylene-sorbitan monolaurate, Sigma)) plus 5 % non-fat milk powder (Marvel, Nestle Ltd) at room temperature for 1 h. The filter was then washed twice for 5 min with PBS-T and incubated with the primary antibody for *N*-7EtG (obtained from Dr J. H. M. Van Delft, AJ Zeist, The Netherlands) or *O*⁶EtG (obtained from Prof. M. F. Rajewsky, Essen, Germany) at 4 °C overnight. Both antibodies were diluted in 20 ml of PBS-T plus 0.5 % milk powder (0.1 g) 1000× and 60×, respectively. The filter was washed with PBS-T (30 ml) for 1 min followed by a further two 5 min washes and incubated at room temperature for 1 h with either the secondary antibody for *N*-7EtG (goat anti-mouse immunoglobulin horseradish peroxidase conjugated, Dako A/S, Denmark) or *O*⁶EtG (rabbit anti-rat immunoglobulin horseradish peroxidase conjugated, Dako A/S, Denmark) and then washed with PBS-T (30 ml) for 15 min followed by a further two 5 min washes. Each antibody was diluted 2000× in 16 ml of PBS-T plus 0.5 % milk powder. Adduct levels in each sample were determined by comparison with a series of standards derived from the dilution of calf thymus DNA incubated with 5 mM *N*-ethyl-*N*-nitrosourea dissolved in 0.1 M Tris–HCl, pH 7.5 at 37 °C for 1.5 h, which were blotted onto the same nitrocellulose filter. The chemiluminescence generated following bathing of the nitrocellulose filters with ECL reagents (Amersham International plc, Buckinghamshire, UK) was determined by densitometry (Molecular Dynamics instrument and ImageQuant software (v. 3.3) Sunnyvale, CA, USA) following exposure of Hyperfilm-ECL (Amersham).

Determination of N-7EtG and O⁶EtG levels in DNA from tissues obtained from the 15 mg kg⁻¹ NDEA dose group by immunoslot-blot analysis

A modified procedure with improved sensitivity of the immunoslot-blot method described above was used to detect adduct levels in the low dose group. DNA samples were prepared as described above and applied onto the nitrocellulose filter by pipetting a volume that contained 1 μg of DNA into each well of the immunoslot-blot apparatus, Minifold II microfiltration apparatus (Schleicher and Schuell). The filter was heated at 80 °C for 1.5 h under vacuum and then bathed in 50 ml of PBS-T plus 2 % non-fat milk powder at room temperature for 1 h. The filter was then incubated with the primary antibody for *N*-7EtG or *O*⁶EtG in 20 ml of PBS-T plus 1 % non-fat milk powder at 4 °C overnight using a Hybaid (Teddington, Middlesex, UK) roller hybridization chamber. Following two washes with PBS-T the filter was incubated with the secondary antibody diluted 2000× in 20 ml of PBS-T plus 1 % non-fat milk powder for 2 h at room temperature. The filter was immersed in Supersignal® ULTRA chemiluminescent substrate (Pierce, Rockford, IL, USA) and the signal detected as described above.

Determination of DNA binding to the nitrocellulose filters: The nitrocellulose filters were checked for equivalency of DNA binding using Southern hybridization with sonicated single-stranded genomic mouse DNA. The filters were hybridized overnight in 5× SSC buffer (0.75 M sodium chloride, 0.075 M sodium citrate), Denhardt's solution (2 % (w/v) bovine serum albumin, 2 % (w/v) Ficoll™, 2 % (w/v) polyvinylpyrrolidone), 100 μg ml^{-1} denatured salmon sperm DNA and 10 % dextran sulphate at 65 °C using 10^6 cpm ml^{-1} of [α-^{32}P] dCTP labelled probe prepared by random primer synthesis. Non-specifically bound probe was three times washed in 0.5× SSC and 0.1 % SDS for 20 min at 65 °C followed by 0.1 % SSC and 0.1 % SDS. Quantitation of band intensity was performed by storage phosphoimage detection (Molecular Dynamics instrument and ImageQuant software (v. 3.3) Sunnyvale, CA, USA).

Determination of N-3EtA in urine by GC–MS

The method has been described previously by Prevost *et al*. (1993). Briefly *N*-3EtA was isolated from urine (5 ml) by immunoaffinity chromatography. The purified extract was treated with derivatizing agent and analysed by gas chromatography–mass spectrometry. *N*-3EtA was quantitated by reference to a deuterium labelled internal standard.

Determination of ATase levels in lung and liver tissues obtained from mice dosed with the 15 mg kg^{-1} NDEA

Following the addition of phenylmethylsulphonyl fluoride (final concentration 87 μg ml^{-1}), frozen lung and liver tissues were homogenized and sonicated in 50 mM Tris pH 8.3, 1 mM EDTA, 3 mM dithiothreitol, centrifuged to remove cell debris and then extracts were assayed for activity. Assay mixtures (300 μl) contained [^{3}H] methylated DNA (~2000 dpm, excess substrate conditions) and various amounts of tissue extracts. Alkylated DNA substrate was prepared by treatment of calf thymus DNA with [^{3}H]-*N*-methyl-*N*-nitrosourea (18.7 Ci mmol^{-1}, Amersham International). The assay was based on the transfer of the O^6 methyl group from methylated guanine in DNA to the alkyltransferase protein present in the homogenate. Protein and DNA were precipitated by the addition of 10 mg ml^{-1} BSA (100 μl), 4 M perchloric acid (100 μl) and 1 M perchloric acid (2 ml) following incubation for 1 h at 37 °C. The precipitate was incubated at 75 °C for 50 min to ensure hydrolysis of DNA substrate and centrifuged. The pellet was washed with 1 M perchloric acid (4 ml) and centrifuged. The supernatant was discarded and the pellet neutralized with 10 mM NaOH (300 μl). The radioactivity present in the protein precipitate was measured by scintillation counting. Each determination for ATase activity for the different strains is the mean value from three tissues from individual animals (unless indicated). Each tissue was assayed such that at least three points were obtained for the linear portion of a protein dependence curve. Bicinchoninic acid protein assay was used for protein determinations (Sigma).

Data analysis

Statistical evaluation of adduct levels with respect to sacrifice time and strain was carried out by a general linear model two-way analysis of variance (ANOVA).

Results

Immunoslot-blot analysis was performed on the DNA samples to ascertain if there was any differences in the levels of adducts between the strains. The immunoslot-blot method is a rapid and sensitive way to measure DNA adduct levels, offering the advantage of not requiring radiolabelled standards which can be difficult to synthesise or costly to purchase. Previous studies have mostly relied upon ^{32}P-postlabelling methodologies which though highly sensitive have certain disadvantages when used to determine small alkyl DNA adducts (Randerath and Randerath 1994). The requirement of a 3′-monophosphate-2′-deoxynucleoside standard of the adduct to determine labelling efficiency and the normally long period of time required for the assay to be performed make ^{32}P-postlabelling unsuitable for this type of study in which large numbers of samples need to be analysed. Furthermore, the sensitivity of the immunoslot-blot method is greatly enhanced since only 1 μg of DNA is utilized per adduct determination, with the added advantage that each sample is analysed in triplicate. Since quantitation of adduct levels is based on a calibration line derived from standards with known amounts of modification blotted onto the same filter as samples, the results can be standardized for different analyses.

Initial experiments showed that the levels of adducts were below or close to the limits of detection in the 15 mg kg^{-1} NDEA dose groups, with results only being obtained from the livers of SWR and BALB/c mice. Attempts to improve the sensitivity of the immunoslot-blot method were made by increasing the amount of DNA loaded onto the nitrocellulose filters; Nehls *et al*. (1984) have shown that the nitrocellulose filters can bind up to 3–4 μg of DNA. However we found that increasing the amount DNA resulted in a corresponding increase in the

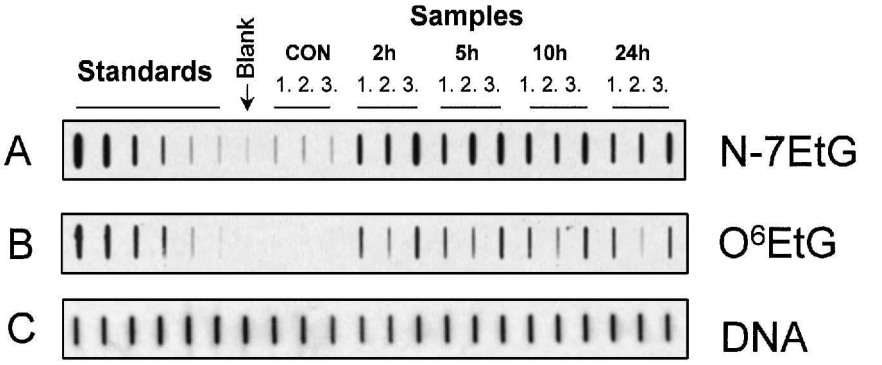

Figure 2. Typical image obtained of the immunoslot-blot filter. DNA (1 μg per slot) was blotted onto the nitrocellulose filter and then incubated with primary monoclonal antibody recognizing *N*-7EtG (lane A) O^6EtG (lane B) adducts followed by secondary horseradish peroxidase-linked antibody. The bands were detected by bathing the filters with chemiluminescent substrate. Results obtained for the standard curve (ranging from 50 to 200 adducts per 10^8 nucleotides) and lung samples (1, SWR; 2, BALB/c; 3, C57BL/6J) at different times following dosing with 15 mg kg^{-1} NDEA are shown. Each sample was blotted in triplicate and adduct levels determined from a calibration line generated by densitometric evaluation of the standards. Lane C shows filters hybridized with single-stranded mouse DNA to show equivalency of binding.

background signal from the control DNA due to non-specific binding of the antibody, and hence an improvement in sensitivity was not obtained. An improvement in sensitivity of the method was, however, obtained by using Supersignal® ULTRA chemiluminescent substrate, which allowed for detection of adducts in the 15 mg kg^{-1} dose group (Mattson and Bellehumeur 1996). Figure 2 represents a typical image obtained of the nitrocellulose filter using this reagent. Detection of *N*-7EtG and O^6EtG is shown in lane A and B respectively. Lane C shows the same filter probed for mouse genomic DNA to confirm equal loading in each band (1 μg per slot). The immunoslot-blot method used for detection of adducts in the 90 mg kg^{-1} dose group had a detection limit of 100 adducts per 10^8 nucleotides compared with the improved sensitivity of the method used for the detection of adducts in the 15 mg kg^{-1} NDEA dose group which had a limit of detection of 5–10 adducts per 10^8 nucleotides.

Formation and persistence of N-7EtG adducts in SWR, BALB/c and C57BL/6J mice

Lungs: Figure 3(A) represents the levels of *N*-7EtG adducts in DNA from the lungs of SWR, BALB/c and C57BL/6J mice dosed with 90 mg kg^{-1} NDEA. Levels of *N*-7EtG ranged from 170 to 660 adducts per 10^8 nucleotides. DNA from C57BL/6J lung contained the highest level of *N*-7EtG at 5 and 10 h, which decreased to levels similar to those found in the other two strains at 24 h. The level of the adduct in the C57BL/6J strain was significantly different ($p < 0.0001$) from the other strains. The C57BL/6J strain also exhibited a difference in the time course of adduct build up and elimination ($p < 0.05$). Figure 3(B) represents the equivalent results obtained following dosing with 15 mg kg^{-1} NDEA with the lung DNA containing approximately 4–10-fold lower adduct levels than observed with

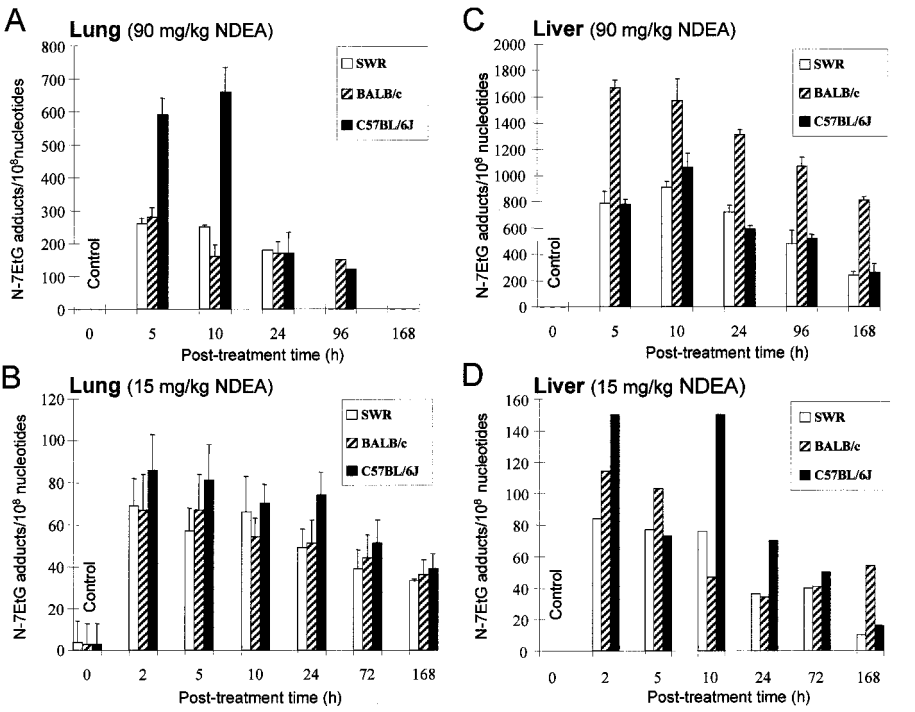

Figure 3. Formation and removal of *N*-7EtG adducts in DNA extracted from lungs (A and B) and livers (C and D) of SWR, BALB/c and C57BL/6J mice treated with a single intraperitoneal dose of 15 and 90 mg kg^{-1} NDEA. Adduct levels were determined by immunoslot-blot analysis using a specific antibody recognizing the ring-opened form of *N*-7EtG. The values shown are the means obtained from three animals per time point (except for the SWR strain and liver at the low dose where the values represent the mean of two animals) and the error bars indicate the standard deviation.

the 90 mg kg^{-1} dose. Adduct levels could be detected until 168 h with peak adduct levels being observed at the earliest time point measured after dosing (2 h). Removal of the adduct was relatively slow, with 50 % of the 2 h levels still present after 168 h for all three strains. Again significantly higher levels of adducts were found in the lungs of C57BL/6J strain compared with the SWR and BALB/c strains at 5 h ($p < 0.03$) but no statistical difference in the build up or removal of *N*-7EtG was found between the three strains.

Liver: Figure 3(C and D) shows that *N*-7EtG was detectable in the liver until 168 h following dosing with 15 and 90 mg kg^{-1} doses of NDEA. The liver contained 590–1670 *N*-7EtG adducts per 10^8 nucleotides over 24 h (Figure 3(C)) following dosing with 90 mg kg^{-1} NDEA. Adduct levels in the BALB/c strain peaked between 5 and 10 h and this strain exhibited a significant 1.5–2-fold higher adduct level than the other two strains ($p < 0.01$). Levels of *N*-7EtG in C57BL/6J mice ranged from 70 to 150 adducts per 10^8 nucleotides between 2 and 24 h following dosing with 15mg kg^{-1} NDEA (figure 3(D)). These levels were approximately eight-fold lower than those observed following dosing with 90 mg kg^{-1} NDEA. The liver contained 3–5-fold higher *N*-7EtG adduct levels than the lung following dosing with 90 mg kg^{-1} NDEA. In summary no marked difference in adduct levels was found between the DNA from the liver tissues between the three strains of mice following dosing with 15 mg kg^{-1} NDEA.

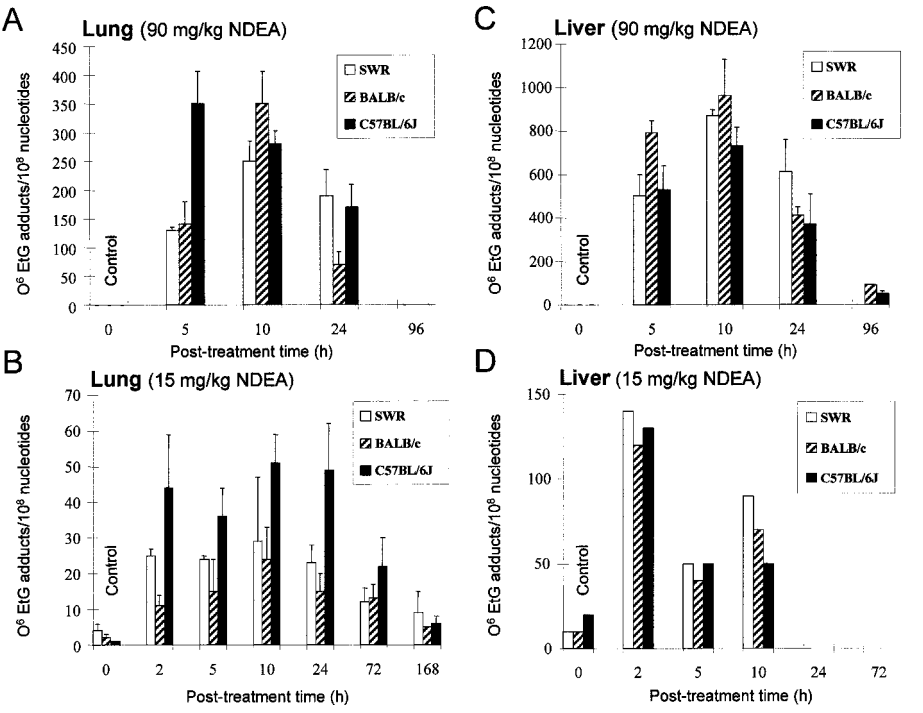

Figure 4. Formation and removal of O^6EtG adducts in DNA extracted from lungs (A and B) and livers (C and D) of SWR, BALB/c and C57BL/6J mice treated with a single intraperitoneal dose of 15 and 90 mg kg^{-1} NDEA. Adduct levels were determined by immunoslot-blot analysis using a specific antibody recognizing O^6EtG. The values shown are the means obtained from three animals per time point (except for the SWR strain and liver at the low dose where the values represent the mean of two animals) and the error bars indicate the standard deviation.

Formation and persistence of O⁶EtG adducts in SWR, BALB/c and C57BL/6J mice

Lung: Figure 4(A) shows the levels of O^6EtG ranging from 70 to 350 adducts per 10^8 nucleotides in the lung DNA from the three strains of mice following dosing with 90 mg kg^{-1} NDEA. Significantly higher levels of adducts were found in the lungs of the C57BL/6J strain compared with the SWR and BALB/c strains at 5 h ($p < 0.02$). A time and strain effect was also observed with adduct levels peaking at 5 h in C57BL/6J mice before declining whereas levels peaked at 10 h in SWR and BALB/c mice. Levels of O^6EtG were detectable up to 168 h in the lungs of mice dosed with 15 mg kg^{-1} NDEA (figure 4(B)). However no statistical differences in the level of the adduct determined at the different time points were found between the three strains.

Liver: Figure 4(C) shows the levels of O^6EtG ranging from 370 to 960 adducts per 10^8 nucleotides in the liver DNA from the three strains of mice following dosing with 90 mg kg^{-1} NDEA over a 24 h period. The levels of the adduct were maximal at approximately 10 h in the liver in all three strains. Again no statistical differences in the level of the adduct determined at the different time points were found between the three strains. Approximately two orders of magnitude lower adduct levels were detected in the mice dosed with 15 mg kg^{-1} NDEA, with levels ranging from 40 to 140 adducts per 10^8 nucleotides detectable up to 10 h (figure 4(D)).

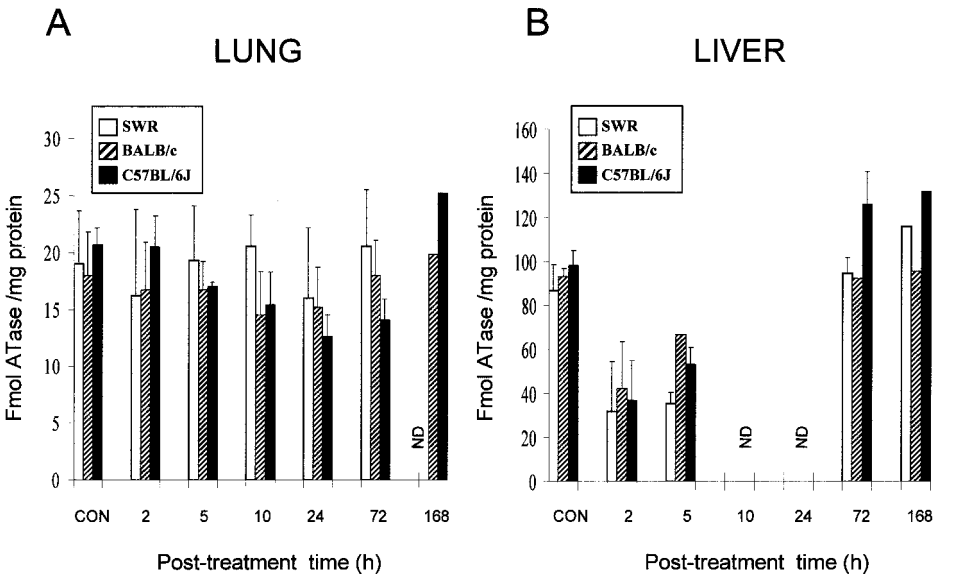

Figure 5. ATase levels (fmol mg^{-1} protein) in lung and liver from SWR, BALB/c and C57BL/6J at different time points following a single dose of 15 mg kg^{-1} NDEA. ATase activity was determined in lung (A) at 0, 2, 5, 10, 24, 72 ($n = 3$) and 168 h ($n = 1$) and in liver (B) at 0, 2, 5, and 72 h ($n = 2$). For BALB/c 5 and 168 h $n = 1$, ND = not determined.

Levels of ATase in lung and liver of SWR, BALB/c and C57BL/6J mice dosed with 15 mg kg^{-1} NDEA

ATase enzyme levels were measured in the lung and liver of the three strains of mice dosed with 15 mg kg^{-1} NDEA to determine whether differences in tumourigenicity correlated with the level of the repair enzyme. Approximately 19 fmol mg^{-1} protein of ATase were present in untreated lung tissues (figure 5(A)) whilst higher levels, 88–98 fmol mg^{-1} protein were present in untreated liver tissues (figure 5(B)). In the lung ATase levels were similar to control levels amongst the three mouse strains at different time points following dosing with 15 mg kg^{-1} NDEA. In the liver dosing with NDEA resulted in the rapid depletion of ATase levels to 30–35% of control levels after 2 h and these remained depleted until at least 5 h in all three mouse strains (figure 5(B)). The levels of ATase were the same or exceeded control levels when measured at 72 h and 168 h with the rate of recovery of ATase activity being similar between the three strains of mice.

Levels of N-3EtA excreted in the urine of SWR, BALB/c and C57BL/6J mice dosed with 15 and 90 mg kg^{-1} NDEA

Analysis of *N*-3EtA levels by GC–MS in the urine 24 h following dosing with 15 and 90 mg kg^{-1} NDEA showed that there was no significant difference in the level of adducts between the three strains. A considerable inter-individual variation of the levels of *N*-3EtA was noted for each of the strains. However there was no correlation between levels of *N*-3EtA and tumour incidence for individual animals. Following dosing with 15 mg kg^{-1} NDEA the average levels of *N*-3EtA excreted in 24 h were 4.2 ± 4.0. 14.1 ± 11.2 and 5.5 ± 5.9 pmol for SWR, BALB/c and

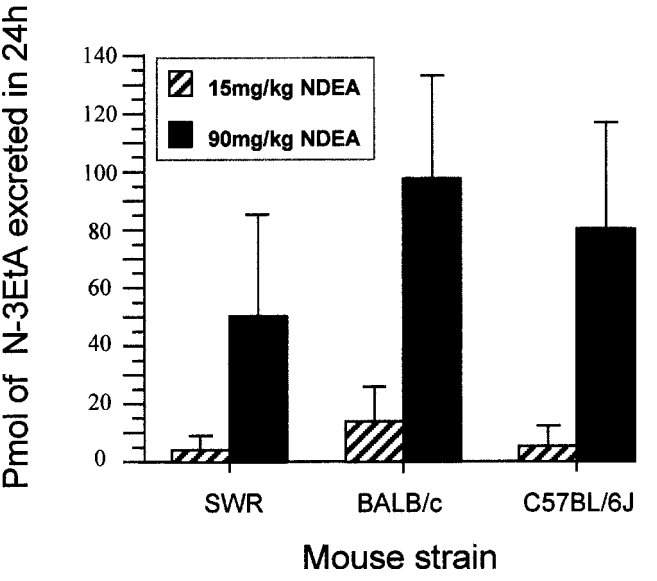

Figure 6. The excretion of *N*-3EtA into the urine at 24 h from SWR, BALB/c and C57BL/6J mice following dosing with a single intraperitoneal dose of saline, 15 or 90 mg kg^{-1} NDEA.

C57BL/6J mice, respectively. Dosing with 90 mg kg^{-1} NDEA resulted in average levels of *N*-3EtA excreted in 24 h that were 50.4 ± 34.1, 97.6 ± 53.3 and 80.3 ± 43.2 pmol for SWR, BALB/c and C57BL/6J mice, respectively (figure 6). In both dose groups the highest levels of *N*-3EtA were obtained in the BALB/c mice.

Tumour incidence in SWR, BALB/c and C57BL/6J mice dosed with 15 and 90 mg kg^{-1} NDEA

Histological and ultrastructural examination of mouse tissues obtained from the Part 2 tumour development study was consistent with previous reports in the literature (Rehm *et al*. 1994) indicating lung tumours as originating from either alveolar type II or possibly Clara cells. Interestingly, polyps which are very rare tumours in mice were detected in the small intestine. However these tumours were found not only in NDEA dosed BALB/c mice but also in control mice. Deaths occurring before the 18 month autopsy were mainly restricted to the 90 mg kg^{-1} NDEA dose group with the C57BL/6J strain having the greatest number of animals that survived. Tumours were found in the majority of the animals that did not survive to 18 months. The lung tumour incidence and multiplicity in SWR, BALB/c and C57BL/6J mice is shown in figure 7. The tumour multiplicity increased with the administered dose of NDEA for both SWR and BALB/c mice but only a slight difference was observed for the C57BL/6J mice. At the 90 mg kg^{-1} NDEA dose a difference in lung tumour multiplicity could be observed. SWR mice had the highest tumour multiplicity (7.5±3.6, n = 16) followed by BALB/c mice (6.1 ± 3.8, n = 21) with C57BL/6J mice having the lowest multiplicity (2.6 ± 1.7, n = 24). A high tumour incidence in susceptible and intermediate BALB/c mouse strains was observed at both doses while in the C57BL/6J strain a dose-dependent

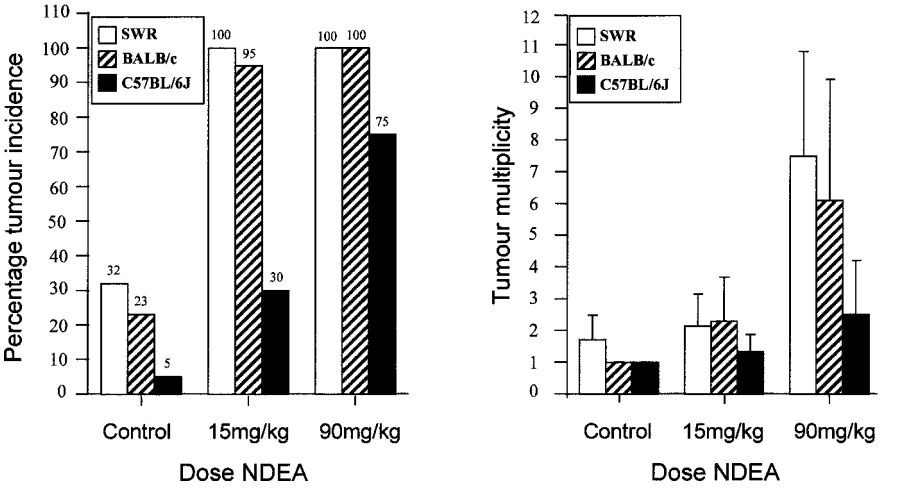

Figure 7. Representation of the lung tumour incidence (A) and multiplicity (B) in animals left up to 18 months following dosing with a single intraperitoneal dose of saline, 15 or 90 mg kg^{-1} NDEA.

increase in tumour incidence was observed (30% at 15 mg kg^{-1} and 75% at 90 mg kg^{-1}). In contrast no clear difference in tumour multiplicity is observed for the three mice strains dosed with 15 mg kg^{-1} NDEA. In the control dose groups the incidence of spontaneous tumours resulting from an epigenetic mechanism or endogenous DNA damage, parallels the expected susceptibility for each strain. Figure 8 shows that both *N*-7EtG and O^6EtG adduct levels measured in the lungs of C57BL/6J mice at 24 h following dosing with NDEA correlated with tumour incidence. Similar correlations were not observed for the SWR and BALB/c strains because virtually all the animals had tumours at the lower dose.

Discussion

Chemical carcinogenesis appears to be a multistep process that in many cases requires the formation of DNA adducts for its initiation. In an animal model we have addressed the question whether DNA adduct levels can explain reasons behind the differences in susceptibility to carcinogenesis. In this study we have examined the formation and persistence of *N*-7EtG and O^6EtG in the lung and liver following administration of a single dose of NDEA in three mouse strains of known differing susceptibility to spontaneous lung tumours. The highest level of adducts were initially present in the livers of all three mouse strains, but the lung remained the target tissue for tumour formation. We found lung adduct levels, tumour multiplicity and incidence increased with NDEA dose in all three strains. The results obtained for the SWR and BALB/c strains showed that tumour incidence was saturated at the 15 mg kg^{-1} NDEA dose but tumour multiplicity was dose-dependent. However the C57BL/6J strain exhibited a linear relationship of tumour incidence and multiplicity with NDEA dose. Urinary excretion of *N*-3EtA was similar between the three strains of mice with only significant differences in levels related to NDEA dose. Given the rapid rate of repair of *N*-3EtA following excision by DNA glycosylases and its lack of direct mutagenic effect these observations confirm the use of this adduct as predominantly a marker of exposure

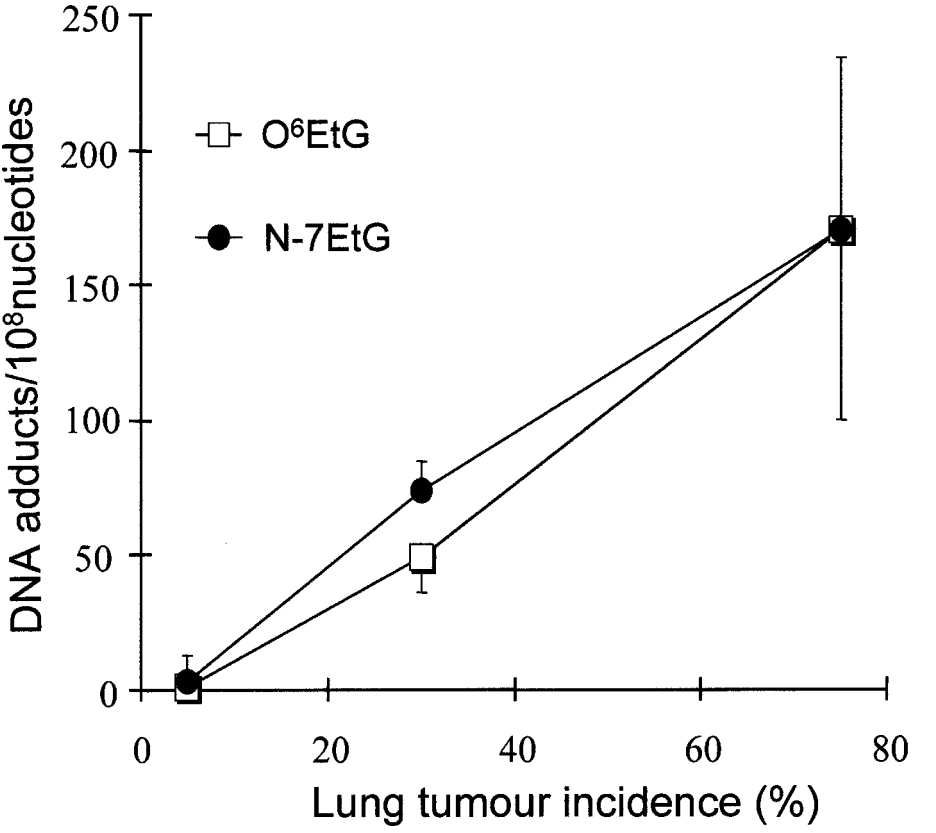

Figure 8. Correlation of O^6EtG and *N*-7EtG adduct levels determined at 24 h with lung tumour incidence for C57BL/6J mice.

to ethylating agents (Krokan *et al*. 1997). No significant differences in the levels of ATase were observed in the lung, implying that the differences in lung tumour susceptibility are not related to the capacity to repair DNA damage for the three strains of mice.

At early time points following administration of NDEA adduct formation and persistence was dose- and tissue-specific within a given mouse strain. However the resistant C57BL/6J strain had the highest DNA adduct levels in the lung at 15 and 90 mg kg^{-1} doses of NDEA. The results obtained indicated that levels of *N*-7EtG and O^6EtG determined in the lungs of the three mice strains did not explain the reason for the differences in susceptibility to lung carcinogenesis. Similar conclusions have been reached by Papanikolaou *et al*. (1998) and Yan *et al*. (1998) who have found that DNA adducts are not predictive of tumour susceptibility in different strains of mice and rats, respectively. In other model systems where polycyclic hydrocarbons have been used, there has been no association found between different strain susceptibility to carcinogenesis and DNA damage (Phillips *et al*. 1978).

Clearly to derive any conclusions from these results we must determine the mechanistic nature of NDEA induced carcinogenesis in this study. The formation of tumours resulting from the presence of DNA adducts may be as a result of either increased cell death due to toxicity induced by NDEA leading to increased

cell proliferation (Cohen and Ellwein 1990) or mutational events where a number of genes may be involved, leading to increased cell replication (Dragani *et al*. 1996). From these results it seems likely that differences in cell turnover may have more of an impact on the role of DNA adducts in tumour incidence. It has been shown that susceptible mouse strains have a higher turnover rate of alveolar type II and Clara cells than resistant strains (Malkinson 1991, La and Swenberg 1996). Increased cell turnover decreases the time available for DNA repair. Thus at high doses, cytotoxicity of NDEA may result in regenerative hyperplasia with increased rates of cell proliferation and enhanced tumour incidence. In particular the 90 mg kg^{-1} dose of NDEA may have produced toxicity in the cells of the lung, which may have resulted from the saturation of DNA repair pathways and detoxifying enzymes, and thus the levels of adducts may not be representative of the normal homeostasis, even though the LD_{50} of NDEA in mice following intraperitoneal injection is 200 mg kg^{-1} (Diwan and Meier 1976). Therefore tumour incidence in the three strains following exposure to NDEA may not be related directly to DNA adduct levels and could be a consequence of a non-specific mechanism, such as increased cell division to compensate for the cell death that has occurred following the toxic effects of NDEA. This would explain the high incidence of tumours observed in all three mice strains at the high dose.

The results obtained from the low dose group should provide more relevant information concerning the relationship of DNA adducts and tumour development, since one would not expect cell toxicity at this dose, the levels of adducts would be expected to be produced under conditions of non-saturated metabolism and DNA repair. Thus an explanation for the lower tumour incidence in the C57BL/6J mice may be because these mice have a greater capability of detoxifying or metabolizing NDEA in the liver compared with the other strains, so that less of the compound is available to cause DNA damage in the lungs. Evidence to support this hypothesis comes from results in the literature obtained following the partial hepatectomy of rats which have been dosed with low doses of *N*-nitrosodimethylamine (NDMA) leading to an increased incidence of kidney tumours. This was explained in terms of the partial removal of the liver resulting in an increased proportion of the dose of NDMA reaching the kidney due to decreased detoxification in the liver (Craddock 1975). The restriction of tumour development to lung following a single intraperitoneal administration of NDEA probably reflects the greater DNA repair capacity in the liver and also cell division (turnover) might be lower in the liver compared with the lung, thus allowing for the complete repair of the adducts before replication of the DNA ensues.

DNA adducts have a role in the carcinogenic process, although the latter appears to be a multistep process which is not solely dependent on the presence of adducts. DNA adducts alone do not seem to explain the differences in carcinogenic susceptibility. Evidence from the literature suggests that lung carcinogenesis occurs following the accumulation of genetic damage resulting from the activation of more than one gene by point mutations, recombinations, amplifications and/or deletions (Sugimura *et al*. 1992). The multiplicities of lung tumour incidence may be governed by at least three genes according to Thaete *et al*. (1991), which are referred to as the pulmonary adenoma susceptibility (*Pas*) genes with *ras* oncogenes being one candidate for such genes. Genetic linkage analysis experiments for hepatocarcinogenesis, performed by crossing a highly susceptible mouse strain (C3H) with resistant strains (A/J, *M. spretus*), have shown that six different regions,

on chromosomes 2, 5, 7, 8, 12 and 19 were linked to tumour development. This leads to the conclusion that polygenic inheritance was probably responsible for the genetic basis of the strain variation in susceptibility to hepatocarcinogenesis (Dragani *et al*. 1995b).

The adducts that were investigated in this study were O^6EtG and *N*-7EtG which may or may not be the major adducts involved in the carcinogenicity attributable to NDEA in this animal model. Though Pegg (1984) has suggested that the promutagenic O^6 methylguanine is the most likely adduct present in DNA involved in the initiation of carcinogenesis by methylating agents, evidence from the literature is rather contradictory. A significant correlation was found between the pyridyloxobutyl adducts produced in alveolar type II cells following dosing of Fischer 344 rats with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and tumour incidence but no correlation was observed for the O^6 methylguanine adduct (Staretz *et al*. 1997). In contrast the *N*-7EtG adduct is not regarded to be promutagenic but may result in the formation of apyrimidic sites by spontaneous depurination thus altering the fidelity of DNA replication. Since the results show that the persistence of *N*-7EtG is greater than O^6EtG, the significance of this adduct may be more relevant. Results obtained by Jansen *et al*. (1994) have shown that O^6EtG was more rapidly lost than *N*-7EtG in both pouch skin fibroblasts and liver tissue from rats treated with *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea. There are also species differences in the formation and persistence of alkylguanine adducts following dosing with NDEA. The *N*-7EtG adduct in hamster liver DNA was found to be eliminated at a faster rate as compared with the rat liver. In contrast O^6EtG persisted longer in hamster than rat liver DNA (Becker and Shank 1985). Clearly the persistence of adducts is dependent on the dose used, as one would expect the adducts to be longer lived due to saturation of repair mechanisms.

An adduct which may have a more critical role in the initiation of carcinogenesis may be O^4 ethylthymidine (O^4EtT) (Travis *et al*. 1991). Evidence from the literature suggests that O^4EtT may be more promutagenic than the O^6EtG adduct. The O^4EtT adduct is longer lived than O^6EtG since it is less efficiently repaired by alkyltransferases. The O^4EtT adduct has been shown to accumulate in the liver DNA of 8-week-old Fischer 344 rats during continuous administration of NDEA as a consequence of less efficient repair compared to that of O^6EtG (Dyroff *et al*. 1986). Swenberg *et al*. (1982) have shown that continuous exposure of rats to NDEA results in a time dependent increase in the level of the O^4EtT adduct with steady state levels being reached at 28 d. Furthermore statistically significant higher levels of O^4EtT have been found in human liver autopsy samples of patients having liver and other cancers compared to those patients with non-cancerous diseases presumably as a result of exposure to ethylating agents *in vivo* (Huh *et al*. 1989). Clearly the levels of this adduct need to be determined in tissues from the three mouse strains since it may be a more relevant marker of carcinogenic susceptibility compared to *N*-7EtG and O^6EtG. An immunoslot-blot based method for determining O^4EtT has been described by Wani and D'Ambrosio (1987).

The results presented here clearly indicate that the presence of DNA adducts increases the likelihood of tumour development but the two adducts studied here (O^6EtG and *N*-7EtG) do not appear to give an insight into reasons behind the differences in susceptibility for the three mice strains. However it appears that the formation of adducts is just one event in the process of carcinogenesis and

subsequent events may modify the risk of developing tumours. Therefore the presence of DNA adducts implies that the risk is increased although its magnitude is not known. A more relevant marker for tumour susceptibility may be the O^4EtT adduct which persists for longer *in vivo* compared with the O^6EtG adduct since it is less well repaired. The relevance of phosphotriester and histone adducts in the carcinogenic process is not fully understood, and for example these could lead to alterations of the chromatin structure which may lead to aberrant gene expression.

In conclusion DNA adducts are relevant markers of carcinogenic risk since the likelihood of tumour development increases with presence of adducts, although carcinogenesis is not solely dependent on the presence of DNA adducts. The susceptibility of individuals or strains may be ultimately dependent on differences in bioactivation, detoxification pathways and DNA repair which is confined to different cell types within tissues, which ultimately leads to alteration of cellular proliferation.

Acknowledgements

Rajinder Singh acknowledges DOW Europe S.A. CH-8810 Hargen/Zurich for financial support. J. A. Baker for assistance with the immunoslot-blot, Dr M. Tucker for histological examination of the tissues, A. Poole for technical assistance, Prof. M. F. Rajewsky for providing the antibody for O^6EtG and Dr J. H. M. van Delft for providing the antibody for *N*-7EtG.

References

Becker, R. A. and Shank, R. C. 1985, Kinetics of formation and persistence of ethylguanines in DNA of rats and hamsters treated with diethylnitrosamine. *Cancer Research*, **45**, 2076–2084.

Beland, F. A. and Poirier, M. C. 1993, Significance of DNA adduct studies in animal models for cancer molecular dosimetry and risk assessment. *Environmental Health Perspectives*, **99**, 5–10.

Beranek, D. T. 1990, Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutation Research*, **231**, 11–30.

Camus, A., Geneste, O., Honkakoski, P., Bereziat, J., Henderson, C. J., Wolf, C. R., Barstch, H. and Lang, M. A. 1993, High variability of nitrosamine metabolism among individuals: role of cytochrome P450 2A6 and 2E1 in the dealkylation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in mice and humans. *Molecular Carcinogenesis*, **7**, 268–275.

Cohen, S. M. and Ellwein, L. B. 1990, Cell proliferation in carcinogenesis. *Science*, **249**, 1007–1011.

Craddock, V. M. 1975, Effect of a single treatment with the alkylating carcinogens dimethylnitrosamine, diethylnitrosamine and methyl methanesulphonate, on liver regenerating after partial hepatectomy. I Test for induction of liver carcinomas. *Chemico-Biological Interactions*, **10**, 313–321.

Dipple, A. 1995, DNA adducts of chemical carcinogens. *Carcinogenesis*, **16**, 437–441.

Diwan, B. A. and Meier, H. 1976, Carcinogenic effects of a single dose of diethylnitrosamine in three unrelated strains of mice: genetic dependence of the induced tumour types and incidence. *Cancer Letters*, **1**, 249–253.

Dragani, T. A., Manenti, G. and Pierotti, M. A. 1995a, Genetics of murine lung tumours. *Advances in Cancer Research*, **67**, 83–112.

Dragani, T. A., Manenti, G., Gariboldi, M., De Gregorio, L. and Pierotti, M. A. 1995b, Genetics of liver tumour susceptibility in mice. *Toxicology Letters*, **82/83**, 613–619.

Dragani, T. A., Canzian, F. and Pierotti, M. A. 1996, A polygenic model of inherited predisposition to cancer. *FASEB Journal*, **10**, 865–870.

Dyroff, M. C., Richardson, C., Popp, J. A., Bedell, M. A. and Swenberg, J. A. 1986, Correlation of O^4-ethyldeoxythymidine accumulation, hepatic initiation and hepatocellular carcinoma induction in rats continuously administered diethylnitrosamine. *Carcinogenesis*, **7**, 241–246.

Festing, M. F. W. 1997, Inbred strains of mice: a vital resource for biomedical research. *Mouse Genome*, **95**, 845–855.

Gaylor, D. W., Kadlubar, F. F. and Beland, F. A. 1992, Application of biomarkers to risk assessment. *Environmental Health Perspectives*, **98**, 139–141.

Gray, R., Peto, R., Brantom, P. and Grasso, P. 1991, Chronic nitrosamine ingestion in 1040 rodents:

the effect of the choice of nitrosamine, the species studied and the age of starting exposure. *Cancer Research*, **51**, 6470–6491.

HUH, N., SATOH, M.S., SHIGA, J., RAJEWSKY, M. F. and KUROKI, T. 1989, Immunoanalytical detection of O^4-ethylthymine in liver DNA of individuals with or without malignant tumors. *Cancer Research*, **49**, 93–97.

JANSEN, J. G., DE GROOT, A. J. L., VAN TEIJLINGEN, C. M. M., LOHMAN, P. H. M., MOHN, G. R., VRIELING, H. and VAN ZEELAND, A. A. 1994, Formation and persistence of DNA adducts in pouch skin fibroblasts and liver tissue of rats exposed in vivo to the monofunctional alkylating agents *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*-nitrosourea. *Mutation Research*, **307**, 95–105.

KROKAN, H. E., STANDAL, R. and SLUPPHAUG, G. 1997, Review article: DNA glycosylases in the base excision repair of DNA. *Biochemical Journal*, **325**, 1–16.

LA, D. K. and SWENBERG, J. A. 1996, DNA adducts: biological markers of exposure and potential applications to risk assessment. *Mutation Research*, **365**, 129–146.

MALKINSON, A. M. 1989, The genetic basis of susceptibility to lung tumours in mice. *Toxicology*, **54**, 241–271.

MALKINSON, A. M. 1991, Genetic studies on lung tumour susceptibility and histogenesis in mice. *Environmental Health Perspectives*, **93**, 149–159.

MALKINSON, A. M. 1992, Primary lung tumours in mice: an experimentally manipulable model of human adenocarcinoma. *Cancer Research*, **52** (Suppl.), 2670s–2676s.

MATTSON, D. L. and BELLEHUMEUR, G. T. 1996, Comparison of three chemiluminescent horseradish peroxidase substrates for immunoblotting. *Analytical Biochemistry*, **240**, 306–308.

MIENTJES, M. J., HOCHLEITNER, K., LUITEN-SCHUITE, A., VAN DELFT, J. H. M., THOMALE, J., BERENDS, F., RAJEWSKY, M. F., LOHMAN, P. H. M. and BAAN, R. A. 1996, Formation and persistence of O^6-ethylguanine in genomic and transgene DNA in liver and brain of λ lacZ transgenic mice treated with *N*-ethyl-*N*-nitrosourea. *Carcinogenesis*, **17**, 2449–2454.

MYRNES, B., GIERCKSKY, K. and KROKAN, H. 1983, Interindividual variation in activity of O^6-methyl guanine–DNA methyltransferase and uracil–DNA methyl transferase and its application to human neoplastic tissues. *Carcinogenesis*, **4**, 1565–1568.

NEHLS, P., ADAMKIEWICZ, J. and RAJEWSKY, M. F. 1984, Immunoslot-blot: a highly sensitive immunoassay for the quantitation of carcinogen-modified nucleosides in DNA. *Journal of Cancer Research and Clinical Oncology*, **108**, 23–29.

PAPANIKOLAOU, A., SHANK, R. C., DELKER, D. A., POVEY, A., COOPER, D. P. and ROSENBERG, D. W. 1998, Initial levels of azoxymethane-induced DNA methyl adducts are not predicative of tumour susceptibility in inbred mice. *Toxicology and Applied Pharmacology*, **150**, 196–203.

PEGG, A. E. 1984, Methylation of the O^6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Investigation*, **2**, 223–231.

PEGG, A. E. 1990, Mammalian O^6alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Research*, **50**, 6119–6129.

PETO, R., GRAY, R., BRANTOM, P. and GRASSO, P. 1991, Dose and time relationships for tumor induction in the liver and oesophagus of 4080 inbred rats by chronic ingestion of *N*-nitrosodiethylamine or *N*-nitrosodimethylamine. *Cancer Research*, **51**, 6452–6469.

PHILLIPS, D. H., GROVER, P. L. and SIMS, P. 1978, The covalent binding of polycyclic hydrocarbons to DNA in skin of mice of different strains. *International Journal of Cancer*, **22**, 487–494.

PREUSSMANN, R., and WIESSLER, M. 1987, The enigma of the organ-specificity of carcinogenic Nitrosamines. *Trends in Pharmacological Sciences,* **8**, 185–189.

PREVOST, V., SHUKER, D. E. G, FRIESEN, M. D., EBERLE, G., RAJEWSKY, M. F. and BARTSCH, H. 1993, Immunoaffinity purification and gas chromatography-mass spectrometric quantification of 3-alkyladenines in urine: metabolism studies and basal excretion levels in man. *Carcinogenesis*, **14**, 199–204.

RANDERATH, K. and RANDERATH, E. 1994, ^{32}P-postlabeling methods for DNA adduct detection: overview and critical evaluation. *Drug Metabolism Reviews*, **26**, 67–85.

REHM, S., WARD, J. M. and SASS, B. 1994, Tumours of the lungs. In *Pathology of Tumours in Laboratory Animals, Volume II—Tumours of the Mouse*, V. Turusov and U. Mohr eds, IARC Scientific Publication No. 111 (Lyon: International Agency for Research on Cancer), pp. 325–355.

RUDIGER, H. W., SCHWARTZ, U., SERRAND, E., STIEF, M., KRAUSE, T., NOWAK, D., DOERJER, G. and LEHNERT, G. 1989, Reduced O^6-methylguanine repair in fibroblast cultures from patients with lung cancer. *Cancer Research*, **49**, 5623–5626.

SINGER, B. 1985, *In vivo* formation and persistence of modified nucleosides resulting from alkylating agents. *Environmental Health Perspectives*, **62**, 41–48.

SINGER, B. and ESSIGMANN, J. M. 1991, Site-specific mutagenesis: retrospective and prospective. *Carcinogenesis*, **12**, 949–955.

SINGH, R., SWEETMAN. G. M. A., FARMER, P. B., SHUKER, D. E. G. and RICH, K. J. 1997, Detection and characterisation of two major ethylated deoxyguanosine adducts by high performance liquid chromatography, electrospray mass spectrometry and ^{32}P-postlabeling. Development of an approach for the detection of phosphotriesters. *Chemical Research in Toxicology*, **10**, 70–77.

STARETZ, M. E., FOILES, P. G., MIGLIETTA, L. M. and HECHT, S. S. 1997, Evidence for an important role of DNA pyridyloxobutylation in rat lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: effects of dose and phenethyl isothiocyanate. *Cancer Research*, **57**, 259–266.

SUGIMURA, T., TERADA, M., YOKOTA, J., HIROHASHI, S. and WKABAYASHII, K. 1992, Multiple genetic alterations in human carcinogenesis. *Environmental Health Perspectives*, **98**, 5–12.

SWENBERG, J. A., BEDELL, M. A., BILLINGS, K. C., UMBENHAUER, D. R. and PEGG, A. E. 1982, Cell-specific differences in O^6-alkylguanine DNA repair activity during continuous exposure to carcinogen. *Proceedings of the National Academy of Sciences, USA*, **79**, 5499–5502.

SWENBERG, J. A., HOEL, D. G. and MAGEE, P. N. 1991, Mechanistic and statistical insight into the large carcinogenesis bioassays on *N*-nitrososdiethylamine and *N*-nitrosodimethylamine. *Cancer Research*, **51**, 6409–6414.

THAETE, L. G., NESBITT, M. N. and MALKINSON, A. M. 1991, Lung adenoma structure among inbred strains of mice: the pulmonary adenoma histologic (*Pah*) genes. *Cancer Letters*, **61**, 15–20.

TRAVIS, C. C., MCCLAIN, T. W. and BIRKNER, P. D. 1991, Diethylnitrosamine-induced hepatocarcinogenesis in rats: A theoretical study. *Toxicology and Applied Pharmacology*, **109**, 289–304.

WANI, A. A. and D'AMBROSIO, S. M. 1987, Immunological quantitation of O^4-ethylthymidine in alkylated DNA: repair of minor miscoding base in human cells. *Carcinogenesis*, **8**, 1137–1144.

WRIGHT, A. S. 1991, Emerging strategies for the determination of human carcinogens: detection, identification, exposure monitoring and risk evaluation. In *Human Carcinogen Exposure. Biomonitoring and Risk Assessment*, R. C. Garner, P. B. Farmer, G. T. Steel and A. S. Wright, eds, (Oxford: Oxford University Press), pp. 3–25.

YAMAZAKI, H., ODA, Y., FUNAE, Y., IMAOKA, S., INUI, Y., GUENGERICH, F. P. and SHIMADA, T. 1992, Participation of rat liver cytochrome P450 2E1 in the activation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine to products genotoxic in an acetyltransferase-overexpressing *Salmonella typhimurium* strain (NM2009). *Carcinogenesis*, **13**, 979–985.

YAN, Y., HIGASHI, K., YAMAMURA, K., FUKAMACHI, Y., ABE, T., GOTOH, S., SUGIURA, T., HIRANO, T., HIGASHI, T. and ICHIBA, M. 1998, Different responses other than the formation of DNA-adducts between the livers of carcinogen-resistant rats (DRH) and carcinogen-sensitive rats (Donryu) to 3′-methyl-4-dimethylaminoazobenzene administration. *Japanese Journal of Cancer Research*, **89**, 806–813.

YUSPA, S. H. and POIRIER, M. C. 1988, Chemical carcinogenesis: from animal models to molecular models in one decade. *Advances in Cancer Research*, **50**, 25–70.