Biomonitoring human exposure to environmental carcinogenic chemicals*

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A coordinated study was carried out on the development, evaluation and application of biomonitoring procedures for populations exposed to environmental genotoxic pollutants. The procedures used involved both direct measurement of DNA or protein damage (adducts) and assessment of secondary biological effects (mutation and cytogenetic damage). Adduct detection at the level of DNA or protein (haemoglobin) was carried out by \textsuperscript{32}P-postlabelling, immunochemical, HPLC or mass spectrometric methods. Urinary excretion products resulting from DNA damage were also estimated (immunochemical assay, mass spectrometry). The measurement of adducts was focused on those from genotoxicants that result from petrochemical combustion or processing, e.g. low-molecular-weight alkylating agents, PAHs and compounds that cause oxidative DNA damage. Cytogenetic analysis of lymphocytes was undertaken (micronuclei, chromosome aberrations and sister chromatid exchanges and mutation frequency was estimated at a number of loci including the \textit{hppt} gene and genes involved in cancer development. Blood and urine samples from individuals exposed to urban pollution were collected. Populations exposed through occupational or medical sources to larger amounts of some of the genotoxic compounds present in the environmental samples were used as positive controls for the environmentally exposed populations. Samples from rural areas were used as negative controls. The project has led to new, more sensitive and more selective approaches for detecting carcinogen-induced damage to DNA and proteins, and subsequent biological effects. These methods were validated with the occupational exposures, which showed evidence of DNA and/or protein damage and/or chromosome damage in workers in a coke oven plant, garage workers exposed to diesel exhaust and workers exposed to ethylene oxide in a sterilization plant. Dose response and adduct repair were studied for methylated adducts in patients treated with methylating cytostatic drugs. The biomonitoring methods have also demonstrated their potential for detecting environmental exposure to genotoxic compounds in nine groups of non-smoking individuals, \textsuperscript{32}P-postlabelling of DNA adducts being shown to have the greatest sensitivity.

Introduction

Thirteen laboratories from eight European countries participated in this coordinated study on the development, evaluation and application of biomonitoring procedures for populations exposed to environmental genotoxic pollutants. It is well known that environmentally polluted atmospheres contain a range of genotoxic substances, including (i) polycyclic aromatic hydrocarbons (PAHs) and lower-molecular-weight alkenes (e.g. ethylene, butadiene) which are metabolized to reactive epoxides; (ii) aromatic amines and nitroaromatics; (iii) low-molecular-weight alkylating (e.g. methylating) agents; and (iv) oxides of nitrogen (NO\textsubscript{x}) which may generate genotoxic nitrosamines by reaction with amines. Additionally both metabolism of the above to generate DNA-reactive species and other compounds present in the atmosphere stimulate the production of active oxygen radicals (e.g. \textit{\textsuperscript{1}O}_2). These can also damage DNA. The monitoring of human exposure to this complex mixture of toxic chemicals is clearly a desirable goal; knowledge gained from such studies would enable an assessment to be made of the relative significance and hazard associated with the 'environmental' exposure to a particular compound in comparison with other possible modes of exposure.

Exposure assessment for carcinogenic environmental pollutants may be carried out either by environmental monitoring of the inhaled air for genotoxic compounds or by biological monitoring at different stages in the process that can lead to the development of tumours (Figure 1) (Farmer, 1995). For example, body fluids (blood, urine) may be analysed for the
Fig. 1. Biomonitoring exposure to genotoxic carcinogens.

chemicals and/or their metabolites in order to gain a clearer insight into the amount of chemical absorbed. Measurements of the interaction products of these chemicals with cellular macromolecules (e.g. DNA, protein) may be made, which gives the biologically effective dose of the genotoxic compound that has reached the tissue under study. The biological effects caused by those chemical interactions (e.g. mutations, production of micronuclei) may also be studied. The ability of these approaches to predict risk would be expected to increase as one goes further down the process from exposure to tumour. Correspondingly, the predictive value for identifying the chemicals involved in the exposure decreases as one goes from exposure monitoring to mutation determination. Therefore, a multifactorial approach seems the most appropriate for studying complex exposures where information on both the chemicals involved and the risk from the exposure is required.

Many of the above-mentioned biomonitoring methods are at an early stage of development. Others were widely used only for exposures to much larger amounts of genotoxic substances (or their analogues) than are found in environmental pollution, e.g. in occupational surroundings. For this reason this collaborative project paid particular attention to development of methods suitable for biomonitoring low levels of genotoxicants present in complex mixtures.

Regarding the populations studied, attention was paid to those exposed to urban pollution and in particular to the genotoxicants present in such samples that result from petrochemical combustion or processing. Blood and urine samples from individuals in urban locations were collected and distributed amongst the programme's participants. Air samples were collected and analysed. Populations exposed through occupational or medical sources to larger amounts of some of the genotoxic compounds, or their analogues, present in the environmental samples were used as positive controls for the environmentally exposed population. Samples from rural areas were used as negative controls.

Adduct detection at the level of DNA or protein (haemoglobin) was by $^{32}$P-postlabelling, immunochemical, HPLC or mass spectrometric methods. Urinary excretion products resulting from DNA damage were also estimated (immunochemical assay, mass spectrometry). The measurement of adducts was focused on those from (i) low-molecular-weight alkylating agents; (ii) compounds that cause oxidative DNA damage; and (iii) some more complex constituents of urban pollution (e.g. aromatic hydrocarbons and nitroaromatic hydrocarbons). Although established methods were selected for use, considerable method development took place in all assays.

Cytogenetic analysis of lymphocytes was undertaken (micronuclei, chromosome aberrations and sister chromatid exchanges) and mutation was estimated at a number of loci, including the hprt gene and genes involved in cancer development.

It was intended that the project should allow (i) a thorough interlaboratory comparison of different biomonitoring procedures for genotoxic exposure; (ii) assessment of the relationship between the internal dose of the genotoxic agent (determined from adduct levels) and the external dose; (iii) assessment of the relationship between the internal dose of the genotoxic agent and the subsequent genetic damage associated with the exposure (measured by mutation and cytogenetic effects); (iv) study of interindividual variation amongst the subjects in a population; (v) development of novel, more sensitive, specific and practical methods for biomonitoring such exposures; (vi) comparison of the relative sensitivity of different techniques within individual laboratories; and (vii) evaluation of interlaboratory variability of established techniques.

Materials and methods

Within this collaborative project, the main focus of attention was on the application of methods to assess environmental exposure of defined populations in Europe to genotoxic, potentially carcinogenic chemicals. However, parts of the contributions of various participants have dealt with further development and refinement of methodology, in order to achieve higher sensitivity, better reproducibility or more reliable interlaboratory calibration. This is particularly the case for $^{32}$P-postlabelling, where extensive developmental work, which is described in some detail below, was carried out.

Methodological developments and improvements that were achieved during the course of this project pertained to biochemical, immunochemical and physicochemical methods to determine genotoxic damage. In addition to $^{32}$P-postlabelling of DNA lesions, these also included mass spectrometric analysis of protein adducts and urinary DNA adducts, immunoaffinity purification and immunochemical detection of DNA damage. In addition, improved methods to detect mutations in blood lymphocytes were presented. Furthermore, developments and improvements were reported with respect to automation, statistical approaches in population studies and biologically-based mathematical modelling.

$^{32}$P-postlabeling

Established postlabeling procedures were used for the detection of bulky adducts of aromatic carcinogens with DNA (Gupta, 1985; Reddy and Randerath, 1986), although several improvements to these assays were made. These procedures were calibrated between the participating laboratories. For postlabeling of low-molecular-weight adducts, standard adducted nucleotides were synthesized to act as standards. Additionally, a novel approach was developed for postlabeling of DNA adducts due to metabolites of 1,3-butadiene.

Improvement of the $^{32}$P-postlabeling procedure to quantify aromatic DNA adducts. As reliable adduct quantification via $^{32}$P-postlabeling is a matter of ongoing debate, various efforts were made to improve the methodology of the assay (Steenwinkel et al., 1993; Baan et al., 1994). These improvements include:

(i) The use of standard DNA samples carrying known amounts of benzo[a]pyrene (BP) adducts, determined by use of an independent analytical technique
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Fig. 2. FPLC analysis of a DNA digest prior to $^{32}$P-postlabelling of DNA adducts. A 1 μg aliquot of DNA, digested with micrococcal endonuclease and spleen phosphodiesterase, is loaded onto an anion-exchange column (Mono Q, Pharmacia) and eluted with a NaCl-gradient. The effluent is monitored at 260 nm. The numbers 1-4 (panels A and B) indicate the normal deoxynucleotides 3’dCMP, 3’dTMP, 3’dAMP and 3’dGMP respectively. In panel B, a and b indicate nucleotides 3’UMP and 3’GMP, derived from contaminating RNA. The efficiency of digestion can be assessed from the relative ratios of the peaks in panel A. The amount of DNA used in each $^{32}$P-postlabelling sample can be accurately quantified on the basis of the area of the 3’dGMP peak, eluting at 9.2-9.3 min (detection limit 15 ng DNA). RNA contamination can be detected to a lower level of 0.1% (wt/wt). This FPLC analysis thus contributes to a more accurate quantification of DNA adducts by $^{32}$P-postlabelling.

Fig. 3. Negative electrospray mass spectrum of N-7-methyl-2’-deoxyguanosine-3’-monophosphate.
Fig. 4. HPLC enrichment of the DEB-dAMP adduct in calf thymus DNA hydrolysate. The location of the adduct is indicated.
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UK) provided with a radioactivity detector. A direct ratio of DEB-dAMP to normal dAMP was then determined. By using $^{14}$C-radiolabelled DEB to measure the level of DEB–adenine adducts, it was shown that when the adducted nucleotide was labelled with $^{32}$P it was at an efficiency comparable to undamaged nucleotides. The presence of this DEB–adenine adduct in DEB-exposed mammalian cells was also verified (Leuratti et al., 1994). Hence, taking into account that the N6-position is involved in hydrogen bonding, it is likely to be a biologically relevant lesion.

Future studies of the metabolic capabilities in man, e.g. on the extent of formation of DEB in humans, should reveal the applicability of this procedure to assess human exposure to butadiene.

Other approaches for detecting DNA adducts

In addition to postlabelling, the other analytical approaches used for DNA adducts included immunochemical methods, mass spectrometry, HPLC with electrochemical detection, mobility-shift gel electrophoresis and a competitive repair assay (see below). An established technique was applied for the immunoaffinity GC–MS quantitation of N3-alkyladenines that were excreted in urine following repair of alkylated DNA (Shuker and Bartsch, 1994).

Development of immunochemical methods to detect various alky–DNA adducts

Immunochemical methods were developed (participant 4) for the detection of guanine-$N_7$-alkylations. Antibodies were raised in mice against various alkyldeoxyguanosines in the chemically stable, ring-opened form (ROM7-AlkOGuo) (Van Delft et al., 1991a, 1993, 1994). Monoclonal antibodies have become available for the analysis of $N_7$-ethyl-$N_7$-ethyl- or $N_7$-(2-hydroxyethyl)-guanine ($N_7$-McGu, $N_7$-EtGuo or $N_7$-EtOHGuo) in DNA. As the antibodies only recognize the ring-opened form of these lesions, the DNA is treated with alkali prior to analysis in the competitive ELISA (detection limit 1–2 adducts/$10^8$ nucleotides). In addition, an immunoslot-blot assay (ISB) was developed with the monoclonal antibody against ROM7-EtOHGuo (detection limit 3 adducts/$10^7$ nucleotides). In this assay, this detection limit can be reached with <1 μg of DNA (Figure 6).

Development of an HPLC method with electrochemical detection (HPLC-EC), to determine various types of alkylguanines. A method was developed (Van Delft et al., 1991b, 1993) to determine $N_7$-EtOHGuo (the major base adduct formed in DNA upon exposure to ethylene oxide) via HPLC-EC. The adduct is released from the alkylated DNA by neutral thermal hydrolysis, separated from unmodified nucleobases by HPLC and detected electrochemically. The detection limit of this HPLC-EC analysis is 1 $N_7$-EtOHGuo/$6 \times 10^6$ nucleotides.

Development of a mobility-shift gel electrophoresis assay for examining DNA adducts. A mobility-shift gel electrophoresis inhibition assay was developed (McConnell et al., 1993a, b; Dingley et al., 1994) to assay aflatoxin $B_1$.
Development of clonal assays for the measurement of hprt mutant frequencies in humans, rats and mice. Substantial efforts were made (participants 6 and 11) to improve and validate the clonal assay for the determination of hprt mutations in human lymphocytes (Jansen et al., 1992, 1994, 1995; Cole et al., 1994; Cole and Arlett, 1993; Cole and Skopek, 1994; Gies et al., 1994; Robinson et al., 1994). In broad outline, the clonal assay was already operational at the beginning of the project, but its performance and reproducibility has gradually and substantially been improved through numerous smaller or larger modifications in (i) composition of culture media; (ii) choice of suitable T-cell growth factors; (iii) procedures for collection, isolation, freezing and thawing of lymphocytes; and (iv) choice of feeder cells. The influence of parameters such as gender, age and smoking on the mutant frequency was also carefully studied. The clonal assay was validated by extensive comparison of data sets from laboratories in France, England, The Netherlands and the USA. This has resulted in various recommendations for use of the clonal assay in biomonitoring studies.

The clonal assay was used to monitor mutations in (i) normal populations in cities or rural areas (participants 6 and 11; Tates et al., 1991a, b); (ii) people exposed to radon in their homes (participant 11); (iii) cancer patients exposed to cytostatic drugs (Tates et al., 1994a); (iv) workers occupationally exposed to ethylene oxide, epichlorohydrin or styrene/dichloromethane (Tates et al., 1994a); and (v) people exposed to airborne crude petroleum (participant 11).

Participants 6 and 11 were also actively involved in the development of methodology for molecular analysis of hprt mutations (Rossi et al., 1992; Vrielings et al., 1992a, b; Steinhrisnott et al., 1993). This has resulted in the analysis of mutations in the hprt gene of lymphocytes from (i) normal smoking and non-smoking donors; (ii) DNA-repair-deficient donors; and (iii) workers exposed to ethylene oxide. It could be established that there is a hot spot for mutation induction by ethylene oxide.

Apart from a reliable clonal assay for hprt mutations in lymphocytes from humans exposed to physical or chemical genotoxic agents, there is a need for clonal assays that can be used to perform model studies with laboratory mammals like rats and mice. Therefore, participant 6 was actively engaged in adapting the clonal assay for human lymphocytes for use in rats and mice (Van Dam et al., 1992; Tates et al., 1994b). In principle, the assays for the three species are similar. Nevertheless, it rapidly became apparent that quite a number of subtle species specific modifications in culture techniques had to be taken into consideration for optimal functioning of the assay in a particular species. It has also become clear that it is rather difficult to accomplish a stable output of the clonal assay in mice and rats. Apparently, a number of still unknown factors can influence the performance of the clonal assay in an unpredictable fashion. Furthermore, it has become apparent that mutant frequencies are influenced by the age of the animals at the time of treatment. It was also found that the expression time for mutations is species-dependent.

The molecular analysis of hprt mutations in rats proved to be more difficult than anticipated because, unlike the situation in human lymphocytes, it proved to be difficult to expand mutant clones. In the case of mice, an expansion of clones proved to be impossible. Consequently, new procedures had to be developed for molecular analysis of mutations. The laboratory of participant 6 was successful in developing procedures for the molecular characterization of mutations in the complete coding sequence of the hprt gene. Using this technology, mutants were sequenced from rats that had been exposed to vivo to ethylenimethanesulfonate, N(2-hydroxyethyl)-N-nitrosourea and N-ethyl-N-nitrosourea. Marked chemical-specific differences were found in the role of OP-alkylguanine in hprt mutagenesis in T lymphocytes from spleen of rats (Jansen et al., 1992, 1994, 1995).

Cytogenetics

Automation of the micronucleus test on human lymphocytes. Cytogenetic studies can be considerably improved by the use of automated acquisition and processing of data (Castleman et al., 1993). Image analysis was applied as a useful tool to achieve this. With respect to analysis of micronuclei (MN), various aspects should be taken into account, e.g. cell classification on the basis of the number of nuclei per cell, the number of micronuclei in the different cell-classes and the statistical analysis. A flexible programme for the automation of micronucleus detection was worked out (participant 13) which allowed for the regular variation between slides and experiments. Comparison of the manual and the automated procedures revealed that nearly the same MN frequencies were scored with these methods. Further improvements as to algorithms and the use of more powerful statistical analyses were implemented.

Miscellaneous methods

Development of methods for data collection and statistical analysis during biomonitoring studies.

(i) Questionnaire. A questionnaire that was specifically designed to assess possible confounding factors during biomonitoring of environmental exposures.
was developed (participant 11) and distributed. This questionnaire was a simplified version of that published by ICPEMC (Carrano and Natarajan, 1988). It was designed to be self-administered, with a reasonable chance of obtaining correct information, in particular about the smoking habits and smoking history of subjects. The questionnaire was used during studies of hprt mutant frequencies in human T-lymphocytes.

(ii) Database analysis. A serious problem encountered during analysis of hprt mutant frequencies among humans is the large variation in background frequencies in allegedly non-exposed individuals. Within a given age group, the variation may be >10-fold. In order to determine the sources of such variation, large databases obtained during the last decade were combined (participant 11) and suitable statistical procedures were worked out to analyse the data. Some experimental procedures and subject-related factors that contribute to the experimentally determined mutant frequency were identified. Most importantly, on the basis of this analysis, recommendations could be proposed with respect to sample size requirements to detect a given increase in hprt mutant frequency: 50–50 subjects per group (control or exposed) are needed to detect a 1.5-fold increase with 90% probability (Cole and Skopek, 1994; Green et al., 1994; Robinson et al., 1994).

Results

Biomonitoring of occupational exposure

Studies of populations undergoing deliberate, controlled exposure to genotoxic chemicals can provide important information relevant to environmental risk assessment. In the current project, in addition to the investigation of genetic damage in members of the general population exposed to ambient environmental agents, a number of other related activities were included to examine populations exposed to specific agents. Thus populations exposed through occupational sources to large amounts of genotoxic PAH compounds were used as positive controls for the environmentally exposed population.

PAH exposure in a coke oven plant (Øvrebo et al., 1992, 1995).

PAH–DNA adduct levels were determined by an ultrasensitive radioimmunoassay (USERIA) and 32P-postlabelling in white blood cell DNA samples from coke oven workers and a reference group (participants 2, 4 and 7). Thirty per cent of the samples had a detectable adduct level by USERIA (polyclonal rabbit antibody against BPDE-modified calf thymus DNA), with a mean of 12.9 adducts/10^8 nucleotides for the exposed group and 10.4 adducts/10^8 nucleotides for the reference group. The mean adduct level with 32P-postlabelling was 1.67/10^8 nucleotides for the exposed group and 1.54 adducts/10^8 nucleotides for the reference group. (This indicates an unusual higher selectivity of the postlabelling technique compared with the immunochemical method.)

Based on job description, the workers were then divided into four groups: reference, and low, medium and high exposed groups. Both assays produced a positive correlation coefficient between estimated exposure and PAH–DNA adduct levels. Adduct levels determined by the postlabelling method showed a correlation with tobacco smoking in the reference group. No significant correlation between BPDE–DNA adduct levels measured by USERIA and PAH–DNA adduct levels by postlabelling was found.

N-(2-Hydroxyethyl)valine (HOEtVal) concentrations in globin were analysed (participant 1). This adduct has previously been shown to be a biomonitor of exposure to ethylene oxide and other hydroxyethylating agents. A significant difference was found between smokers (256.8 ± 128.9 pmol adduct/g globin) and non-smokers (49.1 ± 25.0 pmol adduct/g globin). Higher levels of HOEtVal were present in the non-smoking workers (57.3 ± 32.8 pmol adduct/g globin) versus the non-smoking controls (42.2 ± 16.2 pmol adduct/g globin), indicating that coke oven workers were exposed to hydroxyethylating agents. However, additional samples have to be analysed to show if there is a significant difference.

Urinary levels of 1-hydroxypyrene correlated with exposure to PAH, but considerable variation in the level of 1-hydroxypyrene was detected. Mean level varied from 1.11 to 4.26 μmol 1-hydroxypyrene/mol creatinine in the exposed group, while the mean value for the reference group was 0.14 μmol/mol creatinine. A significant correlation between estimated exposure levels for PAH and urinary levels of PAH was observed. Furthermore, a correlation was observed between smoking and levels of urinary PAH.

Diesel exhaust-exposed garage workers. Postlabelling techniques, determination of haemoglobin adducts and 1-hydroxy-pyrene analysis were employed to investigate exposure to PAH and the level of PAH–DNA adducts in lymphocytes from garage workers (Nielsen and Autrup, 1994; Nielsen et al., 1994). Garage workers and bus mechanics were found to have been shown to be a biomonitor of exposure to ethylene oxide and other hydroxyethylating agents. A significant difference was found between smokers (256.8 ± 128.9 pmol adduct/g globin) and non-smokers (49.1 ± 25.0 pmol adduct/g globin). Higher levels of HOEtVal were present in the non-smoking workers (57.3 ± 32.8 pmol adduct/g globin) versus the non-smoking controls (42.2 ± 16.2 pmol adduct/g globin), indicating that coke oven workers were exposed to hydroxyethylating agents. However, additional samples have to be analysed to show if there is a significant difference.

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HOEtVal in haemoglobin was determined. The median adduct level for HOEtVal in the exposed workers had a significantly higher value (34.4 pmol/g globin, range 25.4–58.8) than the control group (23.2 pmol/g globin, range 8.0–37.0; \( P < 0.01 \)). Biological monitoring of exposure to PAH using a method for the detection of 1-hydroxypyrene in urine showed that the excretion of 1-hydroxypyrene in the postshift samples was significantly higher in the exposed workers (median 0.11 \( \mu \text{mol/mol creatinine, range 0.05–0.16} \)) compared with the controls (median 0.05 \( \mu \text{mol/mol creatinine, range 0–0.11} \); \( P < 0.0065 \)). The 1-hydroxypyrene results did not correlate with adduct levels in lymphocyte DNA. No association between exposure and SCE, HFC or MN was observed.

**Ethylene oxide.** Workers exposed to ethylene oxide in a sterilization plant were analysed for \( N_7 \)-EtOHGua levels in WBC DNA by the HPLC-EC method (participant 4). No evidence of this adduct was found among exposed and non-exposed individuals (detection limit for this assay is 40 fmol). The immunoslot-blot assay was also used to detect the ring-opened \( N_7 \)-EtOHGua. The median adduct levels of the exposed individuals was 50% higher than that of controls. The differences between exposed and non-exposed individuals are not significant, due to large inter-individual variation.

After the publication of results from an investigation into induction of haemoglobin adducts and several types of genetic damage in workers exposed to ethylene oxide in the former GDR (Tates et al., 1991a, b), two more occupationally or incidentally exposed populations were analysed. These populations were again investigated for hprt mutants, chromosome aberrations, micronuclei and SCEs in lymphocytes and haemoglobin adducts in erythrocytes (participant 6). The first population consisted of 22 controls and 75 workers employed in an industry in Brazil (Ribeiro et al., 1994). During the 3 month sampling period, the time-weighted average for an 8 h working day was 2–5 p.p.m. Results indicate that exposure to ethylene oxide led to a statistically significant enhancement of chromosomal aberrations (\( P = 0.01 \)) and of micronuclei in binucleated lymphocytes (\( P < 0.001 \)). For the frequencies of micronucleated cells in buccal mucosa, there was no statistically significant difference between exposed and control groups. Due to the fact that blood samples were of poor quality upon arrival in The Netherlands, it was impossible to measure hprt mutations. The mean values for haemoglobin adducts (HOEtVal) in a selected group of exposed and unexposed donors were statistically different.

The second study involved biological monitoring in industrial workers following incidental exposure to high concentrations of ethylene oxide (Tates et al., 1995). The study consisted of four groups of seven workers from a chemical manufacturing plant in The Netherlands. Group I workers were incidentally exposed to acute high doses of ethylene oxide ranging from 52 to 785 mg/m\(^3\). Group II and III workers were chronically exposed to low doses of ethylene oxide for <5 years or >15 years respectively. Group IV were controls from the Occupational Health Department at the plant. Haemoglobin adduct levels in group I workers were very high and ranged from 1461 to 19913 pmol HOEtVal/g Hb ~1 month after the accident. HOEtVal values for group II and III workers fluctuated between 0 and 190 pmol/g Hb, corresponding with average ethylene oxide exposure levels in the range of <0.01 and 0.06 mg/m\(^3\) ethylene oxide. The statistical analysis of genetic data for hprt mutations, micronuclei in binucleated lymphocytes and SCEs did not reveal any statistically significant differences between any combination of worker groups. This finding implies that the incidental exposure to high concentrations of ethylene oxide did not cause any measurable permanent mutational/cytogenetic damage in exposed lymphocytes. The data accumulated in our studies on genetic effects of ethylene oxide were used for an exploratory assessment of genetic risk made during an EC/US workshop on human genetic risks from exposure to chemicals, focusing on the feasibility of the parallelogram approach (Natarajan et al., 1995).

The molecular nature of hprt mutants was analysed for 51 mutants collected from ethylene oxide-exposed workers and 27 mutants from a parallel control group. Among other things, a hot spot was noted for base pair changes at position 617 of the coding sequence of the hprt gene (five times a GC → AT transition in different donors). Such hot spot mutants were not recovered from the present control group or any other control group investigated in our laboratory. It thus seems that the hot spot mentioned is indicative of EO exposure (Vrieling et al., 1992a, b).

**Styrene, dichloromethane and epichlorohydrin.** Frequencies of hprt mutants, chromosomal aberrations with or without gaps, aberrant cells, micronuclei, SCEs and cells with high frequencies of SCEs (HFCs) were measured in lymphocytes collected from 46 workers occupationally exposed to styrene and dichloromethane (DCM) in the former GDR (Tates et al., 1994a) (participant 6). These parameters were also determined in 23 controls. Samples were collected and partly analysed by Dr Grummt from the Bundesgesundheitsamt Forschungsstelle, Bad Elster, Germany. Time-weighted average values for styrene and DCM exposure during an 8 h working day were respectively 70 and 108 mg/m\(^3\). These values correspond to TWA values of 17 p.p.m. styrene and 31 p.p.m. DCM. In exposed
workers all cytogenetic parameters were significantly enhanced ($P < 0.0001$), but, due to lack of appropriate control data, no definite conclusions could be drawn concerning the mutagenicity of styrene/DCM exposure. Duration of exposure was not correlated with genetic effects analysed. The TWA value for styrene was not correlated with the extent of genetic damage detected, but that for DCM was positively correlated with the frequencies of chromosome aberrations and aberrant cells. These observations make it difficult to decide whether styrene or DCM or both chemicals induced the genetic effects observed in exposed workers.

Frequencies of hprt mutants, chromosomal aberrations, micronuclei, SCEs and HFCs were measured in 15 workers occupationally exposed (16 ± 9 years) in the former GDR to epichlorohydrin and in 14 unexposed controls (participant 6). Samples were received and partly analysed by Dr Grummt. For the major part of the working day, the external exposure was 0.11–0.23 p.p.m. (1 p.p.m. = 3.78 mg/m$^3$) and during 3×15 min/day it was 0.19–2.57 p.p.m. Dr Hindso Landin from the Department of Radiobiology in Stockholm analysed dihydroxypropyl valine adducts in haemoglobin and found no significant difference between exposed and non-exposed subjects. Mean values ± SD for the two groups were respectively 15.6 ± 14.8 and 11.4 ± 10.9 pmol/g globin. These data are now being compared with data from Swedish controls. Final conclusions cannot yet be drawn. With respect to genetic effects, the most significant effect was found for HFCs ($P < 0.0001$), SCEs ($P < 0.0001$) and chromosomal aberrations (minus gaps) ($P = 0.0008$). Effects on micronuclei were on the borderline of significance, whereas there was no effect for hprt mutants.

**Medicinal and other exposures**

*Patients treated with methylating cytostatic drugs (participants 4, 5, 6 and 10).* This activity was included in the project for two reasons: (i) such individuals could serve as positive controls for a better understanding of the factors affecting the formation, fate and consequences of DNA adducts in man; and (ii) methylation damage similar to that induced by the cytostatic drugs examined here can be induced by genotoxins of environmental significance. It was expected, therefore, that by studying the consequences of methylated DNA adducts induced by cytostatic drugs, it should be possible to obtain information useful for the assessment of the risks associated with the exposure of the general population to environmental methylating agents.

The effects of two methylating drugs (procarbazine and dacarbazine) (Kolar, 1984; IARC, 1986; Prough and Tweedie, 1987; Bonnadonna and Santoro, 1992) were studied in different patient groups treated with protocols involving their use alone or in combination with other drugs (Van Delft et al., 1992; Kyropoulos et al., 1993; Souliotis et al., 1994). The combination of selected patient groups (Table II) permitted the examination of the effects of different doses for a given agent, as well as interactions between different agents administered in the same protocol. The end-points examined included hprt and

**Table II. Patient groups examined**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Patient group</th>
<th>Treatment protocol</th>
<th>No. of individuals</th>
<th>End-points examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procarbazine</td>
<td>Hodgkin's lymphoma</td>
<td>MOPP (3 × 50 mg/day pcz p.o., 10 days/cycle, in combination with mechlorethamine, vincristine and prednisone)</td>
<td>21</td>
<td>O'-meGua</td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>Hodgkin’s and non-Hodgkin’s lymphoma melanoma</td>
<td>ABVD (1 × 80 mg/kg dcz i.v. once per cycle, in combination with adriamycin, bleomycin and vincristine)</td>
<td>20</td>
<td>O'-meGua</td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>melanoma</td>
<td>1 g/m$^2$ dcz i.v., once per cycle, alone or in combination with hydroxyureas</td>
<td>35</td>
<td>O'-meGua, AGT</td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>melanoma</td>
<td>225 mg/m$^2$ i.v. on 3 successive days for 1 or 2 cycles, in combination with BCNU and cis-Pl</td>
<td>3</td>
<td>O'-meGua, N7-meGua</td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>melanoma</td>
<td>800 mg/m$^2$ i.v.</td>
<td>5</td>
<td>O'-meGua, N7-meGua</td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>melanoma</td>
<td>800 mg/m$^2$ i.v., once per cycle, for 8 cycles at 21 days' interval</td>
<td>8</td>
<td>Micronuclei (with CREST staining)</td>
</tr>
</tbody>
</table>

![Fig. 10. Dose–response relationships for the formation of methylated adducts in human leukocyte DNA after treatment with procarbazine or dacarbazine. Left, O'-meGua in Hodgkin's lymphoma patients during MOPP treatment 4 h after the last dose of procarbazine (left axis), or in Hodgkin's disease or melanoma patients 2–4 h after treatment with dacarbazine (right axis). Right, N7-meGua in Hodgkin's disease or melanoma patients 2–4 h after treatment with dacarbazine.](https://academic.oup.com/mutage/article-abstract/11/4/363/995295)
Table III. Age and gender of donors

<table>
<thead>
<tr>
<th>Cohort (participant)</th>
<th>Mean age ± SD</th>
<th>% Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aarhus (2)</td>
<td>23 ± 2</td>
<td>100</td>
</tr>
<tr>
<td>Copenhagen (2)</td>
<td>42 ± 8</td>
<td>100</td>
</tr>
<tr>
<td>Rural (2)</td>
<td>19 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>Athens (5)</td>
<td>34 ± 11</td>
<td>90</td>
</tr>
<tr>
<td>Crete (5)</td>
<td>67 ± 15</td>
<td>74</td>
</tr>
<tr>
<td>Wales (3)</td>
<td>50 ± 10</td>
<td>40</td>
</tr>
<tr>
<td>York (9)</td>
<td>43 ± 11</td>
<td>60</td>
</tr>
<tr>
<td>Genoa (10)</td>
<td>35 ± 5</td>
<td>82</td>
</tr>
</tbody>
</table>

Table IV. HOEtVal levels in Hb in the general population (pmol/g globin)

<table>
<thead>
<tr>
<th>Place (participant)</th>
<th>Number</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copenhagen, DK (2)</td>
<td>12</td>
<td>20.9 ± 9.1</td>
</tr>
<tr>
<td>York, UK (9)</td>
<td>10</td>
<td>35.3 ± 7.6</td>
</tr>
<tr>
<td>Swansea, UK (3)</td>
<td>13</td>
<td>27.1 ± 6.3</td>
</tr>
<tr>
<td>Mo, N (7)</td>
<td>11</td>
<td>49.1 ± 25.0</td>
</tr>
</tbody>
</table>

Fig. 11. (a) Levels of N-(2-hydroxyethyl)valine in the globin from four non-smoking European populations. (b) Variation with time in the levels of N-(2-hydroxyethyl)valine in globin from a non-smoking population in Swansea. Three sampling times were used (1/6/92, 3/8/92 and 23/1/93).

$O^\alpha$-MeGua, the repair enzyme $O^\alpha$-alkylguanine-DNA alkyltransferase (AGT), hprt mutations and micronuclei in blood lymphocytes.

The main results and conclusions of these studies are as follows.

(i) Dose-response relationships. Repeated daily exposure to procarbazine resulted in the accumulation in blood leukocyte DNA of $O^\alpha$-MeGua in a manner linearly related to the cumulative dose up to a total of ~20 mg/kg (800 mg/m$^2$) taken over 10 days (Figure 10). The levels of $O^\alpha$-MeGua induced were of the order of 0.4 pmol/mol Gua ($n = 21$) on day 10 and did not result in significant depletion of AGT.

Two hours after i.v. treatment of Hodgkin's lymphoma patients with a low dose of dacarbazine (mean dose 178 ± 13 mg/m$^2$ (4.9 ± 0.6 mg/kg)), the mean level of $O^\alpha$-MeGua was 0.38 ± 0.19 pmol/mol Gua ($n = 20$), and again, no depletion of AGT was observed. Four hours after a dose of 225 mg/m$^2$ dacarbazine (in combination with BCNU/cis-Pt), the corresponding adduct level was 1.85 ± 0.50 pmol/mol Gua ($n = 5$). Finally, 2 h following i.v. treatment with dacarbazine alone at a dose of 1 g/m$^2$ (~28 mg/kg), the average $O^\alpha$-MeGua level was 3.3 ± 0.9 pmol/mol Gua ($n = 35$). It should be noted that, in this case, significant depletion of AGT occurred,
the enzyme reaching 67% of pre-treatment levels 2 h after
treatment. It is likely that this decrease accounts for the
supralinear increase in adduct formation observed.

Four hours after i.v. treatment with dacarbazine, the mean
\(N^7\)-MeGua levels were 42 ± 13 µmol/mol Gua for 225
mg/m\(^2\) (\(n = 5\)), 87 ± 6 µmol/mol Gua for 800 mg/m\(^2\)
(\(n = 3\)) and 80 ± 37 µmol/mol Gua for 1000 mg/m\(^2\) (\(n =
26\), without hydroxyurea). These data suggest a dose–response
relationship which is sublinear in this dose range, possibly due
to saturation of the metabolic capacity at high doses of
dacarbazine.

Repeated, daily exposure to dacarbazine (225 mg/m\(^2\)/day)
for 3 days gave rise to continuous accumulation of \(N^7\)-MeGua
in total blood DNA (40 ± 14 µmol/mol Gua 24 h after the
first dose and 97 ± 46 µmol/mol Gua 24 h after the third
dose). \(O^6\)-MeGua did not show analogous accumulation
under the same conditions, its levels being 1.20 ± 0.45 µmol/mol
Gua and 0.60 ± 0.20 µmol/mol Gua respectively. While this
behaviour of \(O^6\)-MeGua is in contrast to that observed after
procarbazine, it is in line with experimental observations that
this adduct rapidly reaches a steady state during repeated
exposure to methylating agents under conditions which do not
affect its repair (Souliotis et al., 1995).

(ii) Adduct repair. By comparing adduct levels at different
times after exposure, it was possible to obtain an indication
of the stability of methylated DNA adducts in human leukocytes
in vivo. In the case of patients treated with procarbazine or
low doses of dacarbazine, the \(t_{1/2}\) of \(O^6\)-MeGua could be
estimated to be in the range 25 ± 5 h. In patients treated with
a high dose of dacarbazine a longer \(t_{1/2}\) (40–58 h) was observed,
probably as a result of the depletion of AGT occurring under
these conditions.

The loss of \(N^7\)-MeGua in DNA from total white blood cells
is due to active repair and chemical instability (\(t_{1/2}\) of ~150 h
in vitro). Data from patients treated with 800 mg/m\(^2\) and
monitored during three subsequent days suggest that
in vivo, the \(t_{1/2}\) of \(N^7\)-MeGua is ~75 h, suggesting that active
repair is more important than loss by spontaneous depurination.

Maximal levels of \(N^7\)-MeGua are reached at a later time
(4–24 h) after treatment with dacarbazine than \(O^6\)-MeGua
(~1 h), suggesting that adduct formation continues for 4–24
h, but that shortly (~1–2 h) after treatment, the repair of
\(O^6\)-MeGua is already faster than its rate of formation.

Comparison of the levels of \(O^6\)-MeGua and \(N^7\)-MeGua at
24 h post-treatment within patient groups suggests a correlation
between the levels of these two adducts for 225 mg/m\(^2\)
dacarbazine (\(n = 5\); \(r^2 = 0.98\); \(P = 0.001\)), but not for 1000
mg/m\(^2\) (\(n = 16\); \(r^2 = 0.01\); \(P = 0.71\)). Whether these
contradicting findings are due to differences in dose, other
drugs, etc. is unknown. The mean level of \(N^7\)-methylguanine
2 h post-dacarbazine treatment (1000 mg/m\(^2\)) was 57.7 ± 28.1
µmol/mol Gua, giving a \(O^6/N^7\)-MeGua ratio of 0.058.

(iii) Relationship between AGT levels and \(O^6\)-MeGua
accumulation. A non-statistically significant negative relation-
ship was observed between the pre-treatment AGT levels in
lymphocytes and the rate of \(O^6\)-MeGua accumulation in whole
blood DNA during treatment with procarbazine. On the other
hand, a statistically significant negative correlation (\(r^2 = 0.53\);
\(P = 0.013\)) was observed between pre-treatment levels of
AGT and \(O^6\)-MeGua levels 24 h after treatment of melanoma
patients with dacarbazine alone. It is of interest, in terms of
the variation of individual susceptibility to environmental
methylating agents, to note that, for individuals lying at the
extremes of AGT variation (in the range 5—11 fmol/g DNA),
the corresponding variation of \(O^6\)-MeGua accumulation
covered an ~2-fold range.

(iv) Intra- and inter-individual variations in adduct and AGT
levels. Since many patients were examined over a number of
treatment cycles, it was possible to examine the intra- and
inter-individual variation of response to exposure to methylat-
ing agents. For patients treated with procarbazine, 8.9% of the
total variance of \(O^6\)-MeGua levels observed after a given dose
could be accounted for by intra-individual variation, while
84.5% was due to inter-individual variation. The corresponding
figures for Hodgkin's lymphoma patients treated with low-
dose dacarbazine are 5.0 (intra-individual) and 69.8% (inter-
individual). Thus it is clear that most variation in adduct
formation is due to inter-individual differences in susceptibility.
However, despite this large difference between intra- and
inter-individual variation factors, the actual range of adduct levels
observed, either within the same or between different indi-
viduals exposed to a given dose of methylating agent was
surprisingly small, being only ~3-fold. This range is much
smaller than that reported for adducts found in individuals
exposed to supposedly equivalent levels of environmental
mutagens.

As no significant differences in the levels and distribution
of AGT were observed between the three groups of patients
examined, data from all groups were combined for statistical
analysis. Among the 43 melanoma and Hodgkin's disease
patients investigated, the mean value of AGT (pretreatment)
in blood lymphocytes was 9.04 ± 3.27 fmol/g DNA (range
4.8—18.6 fmol/g DNA). Statistical analysis indicated a uni-
modal distribution which did not deviate significantly from
normality (\(P = 0.016\)).

The inter-individual variation of \(N^7\)-MeGua levels for
patients receiving the same dose is maximally 7.7-fold at 24
h, being in most cases <3-fold. The observed variation may
be due to differences in kinetics of metabolic activation of
dacarbazine and/or inactivation of reactive intermediates.
The intra-individual variation of \(N^7\)-MeGua for patients followed
over two successive cycles was much less, generally covering
no more than a 1.3-fold range.

(v) \(hprt\) mutations and micronuclei. Attempts were made to
measure frequencies of \(hprt\) mutants and micronuclei in
lymphocytes in the blood samples collected (i) just prior to
the first course of chemotherapy; (ii) 24 h after the first course;
and (iii) just prior to the second and subsequent courses. Despite
problems associated with the transfer of viable lymphocyte
specimens between different countries, samples from 15
patients were examined, four patients for whom samples for
all of the above timepoints were available. No increase in
mutant frequency or micronucleus induction in cytochalasin
B-blocked binucleated lymphocytes which could be ascribed
to chemotherapy was found in these patients.

In another study, blood samples were collected just prior to
the first course of chemotherapy with dacarbazine alone
(800 mg/m\(^2\) and 2 h after the first and each of the seven
subsequent courses at 21 day intervals. A therapy-related,
progressive increase in the frequency of both CREST\(^+\) and
CREST\(^-\) micronuclei was observed, indicating induction of
structural and numerical chromosome damage.

(vi) Calculation of critical kinetic constants governing adduct
formation and repair in humans through mathematical model-
ing. Mathematical modelling of the kinetics of adduct and AGT
changes following treatment with the different methylating
Table V. Relative carcinogen-DNA adduct level in lymphocytes

<table>
<thead>
<tr>
<th>Location (participant)</th>
<th>No. of samples</th>
<th>Wales/Denmark (3/2)</th>
<th>Netherlands (4/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wales</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban (3)</td>
<td>55</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Rural (3)</td>
<td>7</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>DK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural (2)</td>
<td>29</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Aarhus (2)</td>
<td>74</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Copenhagen (2)</td>
<td>11</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Greece</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Athens (5)</td>
<td>21</td>
<td>9.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Crete (5)</td>
<td>20</td>
<td>5.6</td>
<td>1.9</td>
</tr>
<tr>
<td>York (9)</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of modified bases per 10^7 nucleotides.

• Zones 1 and 2 combined.

*Fifteen individuals, sampled up to six times at different periods, over 15 months.

cytostatic drugs has permitted the calculation of apparent rate constants for drug metabolism, O^-MeGua repair and AGT turnover. Comparison of these with independently observable pharmacokinetic parameters, as well as in vitro measurable parameters (e.g. repair rates), may allow extrapolation to the effects of methylating agents of environmental significance.

Individuals exposed to nitrogen oxides (participants 2 and 8). Nitrogen oxides (NO_x) constitute a widespread and significant environmental burden, particularly in cities. They are known to be efficient precursors of N-nitroso compounds, and there is evidence that human exposure to atmospheric NO_x may contribute to the endogenous formation of dimethylnitrosamine, a methylating carcinogen (Garland et al., 1986). In view of this possibility, a controlled exposure study was carried out (Rasmussen et al., 1995) in which volunteers (n = 15) were exposed for 6 h to different concentrations (0, 4, 77 and 395 p.p.m.) of NO_x in exposure chambers, and urine samples

Fig. 12. Levels of DNA adducts, enriched by butanol extraction or nuclease P1, in lymphocytes from a Welsh urban population. For each pair of adduct levels, that on the left is the level detected by butanol enrichment, whereas that on the right is the level detected using P1 nuclease.

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Human exposure to environmental chemical carcinogens

Fig. 13. Levels of DNA adducts, enriched by butanol extraction or nuclease P1, in lymphocytes from a Welsh rural population.

Fig. 14. Levels of DNA adducts in buccal mucosa from a Welsh urban population.

Human exposure to atmospheric pollution is poorly characterized. More than 3000 different chemical compounds were identified in urban air, some of which have genotoxic activity. Air pollution in large urban areas is dominated by traffic collected prior to, and at the end of, exposure, as well as 24 h after the end of exposure. Urinary 3-methyl-, 3-ethyl-, 3-(2-hydroxyethyl)- and 3-benzyladenine were measured by immunoaffinity purification followed by GC–MS spectrometry, to provide a possible measure of endogenous formation of alkylating N-nitroso compounds. No statistically significant association between exposure and urinary 3-alkyladenines was found, except for a marginally significant association for 3-ethyladenine ($P = 0.036$). Samples of serum were stored for future analysis of nitrotyrosine (to examine the value of this marker as a measure of exposure to NO$_2$) using immunoaffinity methodology developed in the context of the project.

Exposure to genotoxic compounds in the general population: a pilot study

Human exposure to atmospheric pollution is poorly characterized. More than 3000 different chemical compounds were identified in urban air, some of which have genotoxic activity. Air pollution in large urban areas is dominated by traffic...
emissions that consist of a gaseous phase and a particulate phase (IARC, 1989). The latter contains most of the known carcinogens present in urban air. An increased risk of lung cancer due to traffic-generated pollution was reported in a number of epidemiological studies, but the exposure is not well understood (Hemminki and Pershagen, 1994). Exposure monitoring has mostly been made by air quality measurement and biological analysis of collected material, i.e. metabolites in urine. In the present project, different biomarkers were used to assess exposure to genotoxic compounds present in ambient air (a biomarker being defined as the reaction product between the environmental toxin and cellular macromolecules) and potential consequences of these interactions, i.e. chromosomal changes or mutations in reporter genes.

**Environmental monitoring.** Information on ambient air pollution during the collection period of the biological samples was obtained by information obtained from the public monitoring system (Athens), personal samplers and PAH analysis (Genoa), and analysis of aromatic hydrocarbons containing 6-10 carbon atoms in samples collected by personal (diffusion) or pumped monitoring (Swansea). Historical data (1987–1990) were used to classify the air pollution level in Aarhus and Copenhagen, and the levels of particulate matter were 73 and 62 μg/m³ respectively. The level of particulate matter in Athens was in the range 35–88 μg/m³ on the day of collection of the blood samples. Exposure to BP, benzo[a]fluoranthene and benzo[b]fluoranthene was used as a marker of exposure to PAH in the Genoa study, the range being between 0.01 and 25.8, 0.1 and 6.7, and 0.1 and 16.5 ng/m³ respectively. Using personal monitors and benzene as a marker of exposure, in Aarhus and Copenhagen, and analysis of aromatic hydrocarbons containing 6-10 carbon atoms in samples collected by personal (diffusion) or pumped monitoring (Swansea), the combined results are shown in Table IV and Figure 11a.

The adduct levels were in the same range (background) as seen in previous studies. The levels were lower than seen in smokers and in occupationally exposed individuals. The high level in the Norwegian samples compared with the reference group in the other countries may be due to the high industrial activity close to the residential areas. Passive smoking, which may be a contributing factor, was not controlled for.

For most of the population from Swansea, blood sampling took place repetitively on two or three dates. The results of the HOEtVal analysis for each of these samples is shown in Figure 11b. The adduct levels for each individual in the population did not, in general, vary greatly with time of sampling, although one participant (no. 1) showed a >2-fold increase in HOEtVal level at the third sampling time. This is presumably due to an exogenous source of a hydroxyethylating agent.

The major reaction product of EtO with DNA is N7-EtOH Gua, and this adduct can be determined in DNA isolated from white blood cells by immunoslot-blot assay using a monoclonal antibody against imidazole ring-opened N7-EtOHGua. Only 4/29 (14%) of non-EtO occupationally exposed individuals had a detectable level of N7-EtOHGua with a median value of 0.065 adduct per 10⁶ nucleotides (range of 0–2.6).

**Exposure to polycyclic aromatic hydrocarbons (PAH).** BP is considered to be a marker of exposure to PAH, but the level of this adduct could not be determined in the biological samples. Figure 15 shows levels of DNA adducts in buccal mucosa from a Welsh rural population. The adduct levels in this population were lower than seen in smokers and in occupationally exposed individuals. The high level in the Norwegian samples compared with the reference group in the other countries may be due to the high industrial activity close to the residential areas. Passive smoking, which may be a contributing factor, was not controlled for.

**Fig. 15.** Levels of DNA adducts in buccal mucosa from a Welsh rural population.

**Fig. 16.** Mean DNA adduct levels in buccal mucosa and variation with sampling time.
of BP depends on the source of combustion products. The level of BP in urban air was estimated to be 4 pmol/m³ and in mainstream cigarette smoke, 20-40 ng/cigarette. The active form of BP reacts with serum proteins, i.e. albumin and cellular DNA. The BPDE-serum albumin level was determined by measuring BP-tetrols released by acid hydrolysis of isolated albumin by a competitive ELISA assay. The adduct level in the lungs of individuals exposed to ambient air pollution (Hemminki et al., 1993). In this study, the adduct levels in lymphocytes from non-smoking individuals living in rural and urban areas was analysed.

Two different enrichment procedures, P1-nuclease and butanol extraction (Beach and Gupta, 1992), can be used, but the latter was used in all the studies except in the Genoa traffic police officers’ cohort. Results obtained by the butanol procedure were consistently higher than the results from the nuclease P1 procedure. This is as expected, because the PI approach does not detect adduct formed between the C-8 position in guanine and, for example, aromatic amine or nitro-PAH, whereas the butanol method does. Both procedures are selective for large, bulky adducts and small alkylated nucleotides will not be detected. A large, interindividual variation was observed in addition to a seasonal variation (1.5-10-fold).

The results are expressed as RAL, and were corrected for day-to-day experimental variation in labelling efficiency by inclusion of a BPDE-modified DNA standard.

The results on the burden with genotoxic compounds in different geographic locations within the community are shown in Table V. DNA was isolated from lymphocytes isolated by lymphoprep centrifugation in the Danish and Welsh cohort, whereas total white blood cells were used in the samples from York and Athens.

Two different procedures were used for quantification of adduct levels. The values presented by the Netherlands group (participant 4) is based upon the number of total adducts in different zones of the chromatogram, zone 1 lower right and zone 2 upper left, whereas the method used by the Welsh and Danish groups (participants 3 and 2) is the sum of identifiable adducts.

The level of adducts was significantly higher in people living in the major cities, Copenhagen and Athens, compared with smaller cities and rural areas, suggesting that locally generated air pollution, e.g. traffic-generated combustion products, contributes to the burden with genotoxic compounds in the general population (Nielsen et al., 1996b). A positive association between environmental monitoring of particulate matter and adduct levels was observed in the samples collected in Athens. A comparison of the Athens and York data indicates a significant difference only in the adduct level in zone 2. This difference was only seen in samples collected during the winter, supporting the evidence of a large seasonal variation possibly due to different causes of air pollution or other meteorological differences. All donors in Wales were non-smokers with only a moderate to nil alcohol intake. The levels of adducts in the Welsh urban population are shown in Figure 12, and those for a control rural population in Figure 13.

In a sub-cohort (Port Talbot, Wales) the level of aromatic-related DNA adducts in lymphocytes was compared with that in buccal mucosa DNA from the same individual. The adduct level in buccal mucosa was consistently higher than in WBC. The levels of adducts in mucosa for the urban and rural populations are shown respectively in Figures 14 and 15. The data are for a very limited population. Comparisons of adduct levels are shown in Figure 16. Here, the adducts in the buccal mucosa from the urban population were plotted on a temporal basis. Very high levels occurred in November 1993. Because internal standards of BP-adducted DNA were used between experiments, these differences are real. Although the small population size examined means that they are not statistically significantly different, it is clear that a more extensive study of donors from the region is warranted. Such a study should examine adduct levels, PA450 polymorphism, wind direction and PAH emissions from the coke oven plant. Using a competitive ELISA assay and a monoclonal antibody against BPDE-DNA, only 4 out of 30 samples gave a positive response, the range being 0.09-0.15 fmol BPDE-adduct/µg DNA. The difference in number of positive samples between the 32P-postlabelling and ELISA assay is due to the higher specificity of the latter procedure.

No association between carcinogen—serum protein levels and carcinogen—DNA levels in non-occupationally exposed individuals could be detected in the combined study.

Different methods were compared for the detection of PAH-DNA adducts in DNA from lung cancer patients (participants 2, 4 and 7). No correlation was found between SFS and a postlabelling technique when analysing for PAH—DNA adducts in normal lung tissue. An interlaboratory study on postlabelling is underway. A nearly significant correlation was found between the amount of cigarettes smoked and the BPDE-DNA adduct levels (t = 1.98, P = 0.06, n = 19, RSQ = 6%, linear regression). A higher level of PAH—DNA adducts was found in males than in females (t = 2.27; P = 0.069). No correlation between mutation in the p53 gene in lung tumours and PAH-DNA adduct levels in normal lung DNA was observed. However, a nearly significant correlation was seen between packyear and p53 mutations (t = 1.94; P = 0.07).

 Exposure to methylating agents. The O6-MeGua adduct, formed by small alkylating species, possibly generated by in vivo formation of methylating nitrosamines as a result of exposure to atmospheric nitrogen oxide, cannot be detected by either a competitive enzyme assay or by the 32P-postlabelling procedure. The limit of detection of the former procedure is 0.08 µmol/mol guanine, and no O6-MeGua could be detected in samples collected in an urban area (32 cases).

 Mutant frequency at the hprt locus. Other biomarkers include markers for early biological effects, e.g. hprt mutations in lymphocytes, MN. The mutation frequencies in the hprt loci in lymphocytes from people living in the centre of Athens and traffic police officers in Genoa were not significantly different from previously obtained results from the control group. The importance of the selection of sampling time was demonstrated.
by the observation of a seasonal variation in MN and SCE for the Welsh population (participant 13).

Mutant frequencies from 22 samples from the traffic-polluted exposed group from Genoa, and 10 samples from the York (non-polluted area) were also determined (participant 11). Mutant frequencies were in the normal range for age.

Exposure to environmental oil pollution. A further population became unexpectedly available for study during the course of this programme, when the oil tanker Braer ran ashore on the Shetland Islands during severe weather in December 1992. Samples were obtained from 26 non-smoking people exposed to airborne crude petroleum pollution following this incident (participant 11). Samples were obtained immediately (7–10 days) after the accident, 3 months later (when it might be expected that newly induced mutants would be expressed) and 1 year later. Samples from nine age-matched non-exposed Islanders were also obtained at the same time, and a series of experiments undertaken on each sample (with repeats wherever possible) to determine the mutant frequency at the hprt locus. Where results could be obtained at both first and second sampling, a slight tendency was seen for the mutant frequency to be higher at the later time; however, this effect was only small (11/20 higher versus 9/20 lower), and was also seen in the non-exposed group. In addition, all mutant frequencies were within the normal range of mutant frequencies seen in our extensive database. Studies such as this have proved invaluable in our assessment of the utility of assays aimed at monitoring the human population (of which hprt is an example).

It is clear that a wide variation in mutant frequency exists in the human population (as predicted on theoretical grounds, Green et al. (1994)), and where relatively small sample size is used, small effects, sometimes statistically significant, may be seen which may be ascribed to experimental artifacts or to chance, and which disappear when larger samples are analysed. 32P-postlabelling is currently being carried out on lymphocyte DNA from these Shetland Island populations (participant 1). There does not appear to be a significant difference in adduct levels between the exposed and control populations.

General conclusions

The participants in this multi-disciplinary programme have made substantial technical advances in the procedures used for biomonitoring human exposure to carcinogens, and have applied these techniques to over 400 individuals exposed to carcinogens through occupational, medicinal and environmental sources. During the course of this study, several reviews of this area of research have been published by participants in the programme (Marafante et al., 1991; Menichini and Abbondandolo, 1991, 1992; Shuker and Farner, 1992; Sorsa et al., 1992; Vrieling et al., 1992a, b; Farmer, 1993; Farmer et al., 1993), and other aspects of the analytical methods and their application, separate from those described above, have also been reported (Tates et al., 1991a, b; Cushman et al., 1992, 1993; Øvrebø et al., 1992; Törnvist et al., 1992; Herikstad et al., 1993; Sepai et al., 1993; Van Hummelen et al., 1993, 1994, 1995; Severi et al., 1994; Valavanis et al., 1994; Buchet et al., 1995).

Studies of occupationally exposed populations demonstrated the ability of the techniques employed to demonstrate exposure to genotoxic chemicals. For example, exposure to exhaust fumes was proved to result in both higher DNA adduct levels, as measured by postlabelling, and increased levels of the ethylene oxide adduct with N-terminal valine in haemoglobin. Exposure to methylating agents during cancer chemotherapy was also used to demonstrate the potential of the biomonitoring techniques for their application to monitor the exposure of the general population to environmental methylating agents. Human dose response and adduct repair data from these studies should assist in assessing the risk associated with exposures of this kind.

Studies with populations exposed to environmental genotoxic agents show that total carcinogen–DNA adduct using the postlabelling assay can be used to identify exposure to genotoxic compounds in non-occupational environments. 32P-postlabelling of DNA adducts was found to be the technique of highest sensitivity for such exposure monitoring. By using standards to calibrate the quantification of adducts between laboratories, the level of adducts in rural donors was extremely consistent. Thus, the differences in urban donors cannot be attributed to inter-assay or inter-laboratory variations. Additional and better designed studies are necessary to compare the exposure in different parts of the community member countries. Furthermore, the results should be stratified according to known genetic risk factors, i.e. polymorphisms in xenobiotic metabolizing enzymes. In any future studies, it is imperative that adequate numbers of individuals are analysed to provide a comprehensive view of particular populations.

The role of carcinogen–DNA adducts in the carcinogenesis process is still not fully established. However, increased adduct levels were reported in workers with increased risk of developing tumours, e.g. lung cancer. From animal experiments and short-term mutagenesis/transition assays, a positive association between adduct level and cancer incidence and mutation/transformation frequency has also been reported, suggesting that the carcinogen–DNA adducts may be a good marker of risk, although carefully designed prospective studies to establish clearly the association in man are still required. The confirmation that such an association exists in man would have a major positive impact on our efforts to minimize carcinogenesis in populations exposed to environmental genotoxic chemicals.

Acknowledgement

The authors acknowledge the financial support of the EU (STEP contract no. EV5V-CT91-0013).

References


human exposure to environmental chemical carcinogens
but no significant difference in mutation spectrum between a smoking and non-smoking human population. Carcinogenesis, 13, 1625–1631.

Received on December 11, 1995; accepted on March 12, 1996