DETECTION OF DNA DAMAGE IN STUDIES ON CANCER ETIOLOGY AND PREVENTION

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This international conference on methods for detecting DNA damage and their application in studies on cancer etiology and prevention concerns a very timely and important subject. The process of risk assessment is being used increasingly by regulatory decision-making bodies in formulating policies intended to minimize health risks resulting from exposure to hazardous substances. The process of risk assessment requires the use of factual data to define the health effects of exposure of individuals or of populations to such substances. In one current definition (National Academy of Sciences/National Research Council, 1983), the process of risk assessment includes three elements: hazard identification, dose-response assessment and risk characterization. Exposure assessment and epidemiology data are key components of the dose-response assessment, the objective of which is to define dose-incidence relationships for adverse health effects (such as cancer) in human populations. These components, together with animal bioassay data, appropriate extrapolations of information concerning dose-effects in animals and interspecies differences in response effectively determine the quantitative features of the risk estimation, and are therefore of critical importance.

Epidemiological studies designed to evaluate the health significance of environmental chemicals, including carcinogens, are seriously compromised by a lack of quantitative data on individuals in exposed populations. Data on levels of compounds in environmental media often represent the only information available, and average population exposure is therefore the only quantitative parameter that can be calculated. Biological monitoring, i.e., measurements that can be made on cells, tissues or body fluids of exposed people, has the objective of defining 'internal dose', or 'biologically effective dose', on an individual basis. Information gained from such measurements can be utilized to detect potentially hazardous exposures before adverse health effects appear, and also to establish exposure limits that minimize the likelihood of significant health risks. Because measurements are made on individual bases, they provide for that person an indication not only of external exposure to a given substance, but also of the amount absorbed, metabolically transformed to inactive and activated derivatives, and the fraction bound to functionally important cellular sites. Thus, biological monitoring data are complementary to information derived from analysis of environmental media, inasmuch as they can be interpreted in the context of known mechanisms of action, and are therefore more directly relevant to assessment of health risks. Because the monitoring strategy can be designed to take into account exposure through multiple routes and to integrate the consequences of intermittent as well as continuous exposures, it can also provide evidence of total risk from multiple sources.

Certain attributes are required of a method adequate to provide accurate,

quantitative measurements of exposure to environmental carcinogens and to fulfil the objective of providing early indication of long-term risk of cancer. Some of those indispensable to attainment of these objectives include the following: (1) the analytical methodology should be adequate to detect and quantify exposure to carcinogens and mutagens at ambient levels in the environment; (2) the methods should be applicable to cells or body fluids that are readily accessible; (3) measured values should be related quantitatively to exposure levels over a wide range; and (4) the methods should integrate consequences of intermittent or continuous exposures to multiple agents. All of these attributes are applicable to accurate dosimetry of *exposure* alone. In order to be applicable to the assessment of *health risk,* the method should also make it possible to detect early biological effects predictive of long-term adverse health risks (e.g., cancer). All of the methods discussed in this volume are potentially applicable to detection of exposure; at present, none can be considered adequately validated to provide direct evidence of cancer risk. Strategies for using exposure information in the prospective assessment of genetic risk represent important future applications of these methodological developments, the validation of which will require a great deal of additional research.

Biological monitoring and its applications in the field that has come to be known as 'molecular epidemiology' have been the subjects of several recent, comprehensive reviews. This volume, and therefore this discussion, deals specifically with detection methods. Broader perspectives can be found in the reviews of Berlin *et al.* (1979, 1984), Bridges (1980), Sorsa and Norppa (1986), Wogan and Gorelick (1985), Garner (1985), Perera (1987) and Farmer *et al.* (1987).

Indicators of genotoxic exposures

Currently available methods for detecting DNA damaging agents fall into two categories on the basis of the character of the experimental systems used and the endpoints detected: measurements of levels of genotoxic chemicals, their metabolites and derivatives in cells, tissues, body fluids and excreta; and measurements of biological responses such as cytogenetic changes in exposed individuals. This discussion emphasizes recent developments in the former category, and only a brief summary of biological indicators of genotoxic exposure is included, consistent with the emphasis reflected in this volume.

Biological indicators

Cytogenetic changes in chromosomes are among the earliest manifestations of genetic injury in persons exposed to ionizing radiation. The linear interdependence of dose of ionizing radiation with the number of structural chromosomal aberrations in circulating lymphocytes has made it possible for the method to be used for biological dosimetry, even at low radiation levels (Evans *et al.*, 1979). Cytogenetic monitoring based on the detection of chromosomal damage in somatic cells is finding increasing application, especially in the occupational setting (Vainio *et al.*, 1983).

Damage to chromosomes can take various forms: structural aberrations, sister chromatid exchanges (SCE) and numerical abnormalities (Bloom, 1981; Evans, 1983). The type of alteration produced depends on the lesions induced in the chromosomes and, therefore, on the nature of the genotoxic injury in question. Structural chromosomal aberrations result from the breakage and rearrangement of whole chromosomes and are most efficiently induced by those substances that directly break the backbone of DNA (e.g., ionizing radiation and radiomimetic chemicals) or significantly distort the DNA helix (such as intercalating agents). SCE result from the

breakage and rejoining of DNA strands, without observable morphological distortion of chromosomal structure. They are efficiently induced by genotoxic agents that form covalent adducts with DNA or interfere with DNA synthesis or repair. Numerical alterations (aneuploidy) represent gains or losses of whole chromosomes or parts thereof. It is believed that aneuploidy arises following exposure of the cell to substances that interfere with the apparatus of cell division. Because the three types of cytogenetic endpoints respond to different cellular lesions, information about them is complementary, and all should be taken into account in assessing the possible genotoxicity of environmental chemicals.

The system used most frequently in monitoring exposure to clastogenic agents involves the study of mitogen-stimulated lymphocytes in short-term cultures of blood samples. Because of the long life span of lymphocytes, detection of aberrations in mitogen-stimulated cells offers some possibility for detecting both short-term and accumulated damage, although the kinetics of damage of this type are poorly understood. Structural aberrations can be classified as either unstable or stable, depending on whether they persist in dividing cell populations. Unstable aberrations (dicentrics, rings, deletions and other asymmetrical rearrangements) lead to cell death. Stable alterations consist of balanced translocations, inversions and other symmetrical rearrangements, and are transmitted to progeny at cell division. The biological relevance of these stable rearrangements in somatic cells is not well defined, but recent evidence suggests that certain of them, such as stable translocations, can persist and may confer a growth advantage in dividing cell populations. For example, translocation of an oncogene, the *c-myc* gene, to a site at which its transcription is placed under the control of regulatory elements of the immunoglobulin genes has been observed in patients with Burkitt's lymphoma, and the abnormal expression of these genes in such patients may be causally related to the rearrangement. An oncogene has also been mapped to the site of the rearrangement in chronic myelogenous leukaemia, and other rearrangements in malignancy may be related to the translocation of other oncogenes.

Recent developments in chromatin staining methods have made possible the detection of intrachromosomal SCE. Although the molecular mechanisms underlying these changes have not been fully characterized, it is clear that measurements of SCE offer a sensitive indicator of DNA damaging agents. While the biological consequences of SCE formation are poorly understood, formation of these lesions in a chromosome represents, at the very least, the breakage and rejoining of four DNA strands. It has been amply demonstrated that the frequency of SCE formation is dramatically increased when cells, animals or people are exposed to known carcinogens (Latt, 1981).

The assay methods are quantitative and sensitive, and the occurrence of SCE has been linearly and positively correlated with specific locus mutations *in vitro* and with lung tumour induction in mice. Experiments in rats, mice and rabbits have demonstrated that it is possible to measure an increase in SCE in peripheral blood lymphocytes for several days following exposure *in vivo.* Increased SCE frequencies have been observed in the lymphocytes of cigarette smokers, workers exposed occupationally to ethylene oxide, individuals undergoing cancer chemotherapy and those exposed to certain drugs. As a result of these findings, SCE techniques are considered a valuable adjunct to measurement of chromosomal aberrations in cytogenetic monitoring. The significance of cytogenetic endpoints in the assessment of genotoxic effects of ethylene oxide and their relevance to human cancer has been reviewed recently by Kolman *et al* (1986).

Recent advances in recombinant DNA procedures are being applied successfully to the identification of molecular defects in man that account for certain heritable

diseases, and also to somatic mutations associated with neoplasia. Recombinant DNA methods are highly sensitive and discriminating in the detection of DNA mutations and sequence alterations. As noted above, oncogenes can be activated by point mutations or chromosomal rearrangements, as in the case of Burkitt's lymphoma. Recently, T-cell leukaemias have been found to be associated with translocations that involve the *c-myc* gene and the *a* chain of the T-cell receptor. The precise site of joining for the translocation has been identified and sequenced. Each junction was initially identified by DNA hybridization analysis with *c-myc* and T-cell receptor probes. If the translocation occurs reproducibly, through aberrant homologous recombination involving specific nucleotide sequences, precise and simple methods for detecting the translocation should be possible. This might in turn make possible the detection of early-stage effects of environmental chemicals capable of inducing leukaemias through this mechanism. Other leukaemias associated with chromosomal translocations include chronic myelogenous leukaemia and acute promyelocytic leukaemia, indicating the possibility of applying a similar approach to their early detection. Restriction fragment length polymorphism (RFLP) analysis is based on the combined use of restriction endonucleases which cleave DNA at specific recognition sites together with oligonucleotide probes to identify changes in fragment length induced by point mutations or insertion or deletion alterations in DNA sequences. The use of RFLP analysis to identify changes in DNA sequence associated with environmental exposures and their possible relationships to cancer risk are discussed elsewhere in this volume.

Biochemical markers

The majority of chemical carcinogens and mutagens exert their effects only after metabolic conversion to chemically reactive forms, which bind covalently to cellular macromolecules, including nucleic acids and proteins, to form addition products (adducts). Certain low-molecular-weight chemicals important in cellular functions, such as water and glutathione, are also readily attacked. In some cases, formation of these adducts may be catalysed by enzymes such as glutathione-5-transferase. The overall process whereby electrophiles react with nucleophiles forms a central theorem of carcinogenesis, and particular emphasis has been placed on DNA adducts, since these are thought to represent the initiating events leading ultimately to mutation or malignant transformation. It has been established empirically that the carcinogenic potency of a large number of chemicals is proportional to their ability to bind to DNA — the so-called covalent binding index — when reacted *in vivo* with DNA (Lutz, 1979). Covalent adducts formed in RNA and proteins have no putative mechanistic role in carcinogenesis but are expected to be related quantitatively to total exposure and activation and therefore to represent dosimeters for both exposure and activating capability. The proportionality of response between different target molecules in different tissues and cells forms the basis of an approach to biological monitoring in which the goal is to determine the accumulated dose of the ultimate carcinogenic form of a chemical at the critical target which leads to the unwanted biological response.

A practical approach to the problem of monitoring the critical target dose must be based on the complex relations among the stages of exposure, metabolic and physiological processing, and the ultimate biological effects of genotoxic chemicals. Any measure of exposure or target dose should reflect the capacity of the individual for absorption, metabolism and excretion of the particular carcinogen to give a more accurate and relevant index than simple measurement of the concentration of the

compound in air, water or food. Several types of chemical and biochemical methods have been developed for detecting DNA damage in human populations exposed to genotoxic substances. These include measurement of chemicals and their metabolites in blood and urine by chemical analysis and immunoassay, detection of mutagens in urine and measurement of covalent adducts of haemoglobin and DNA. Several analytical methods have been devised to detect covalent adducts, including gas chromatography-mass spectrometry (GC-MS) for determining haemoglobin adducts and physicochemical, immunological and postlabelling for detection and quantification of DNA adducts. For the purposes of this discussion, it is necessary only to summarize briefly the analytical strategies, inasmuch as the reviews cited earlier provide detailed discussion of the various methods involved.

The use of DNA adducts to determine the critical tissue dose may be approached in two ways. First, measurements can be made of the levels of DNA adducts derived from a chemical of interest in cells of an accessible tissue, such as white blood cells or biopsy or autopsy material. If the chemical nature and stability of the DNA adducts for the compound of interest have been fully characterized, qualitative as well as quantitative identification of adduct levels can, in principle, provide an indication of exposure history as well as individual ability to activate the carcinogen to DNA-binding forms. A second approach takes advantage of the fact that some adducts are known to be removed from cellular DNA (and also from RNA) and excreted in urine. Detection and measurement of their excretion rates can provide information on recent exposure of the subject, and possibly also indications of that individual's capability for DNA repair. Thus, studies of urinary excretion of adducts may provide data complementary to measurement of adduct levels in cellular DNA in the same individual.

Although DNA adducts offer the most direct biological monitor for a carcinogen in which DNA is clearly the ultimate target, interpretation of data derived from DNA adduct measurements is in fact highly complex. It is well established that carcinogens — varying in structural complexity from simple alkylating agents to more complex molecules that require multiple steps of activation — react to form covalent bonds at a variety of nucleophilic sites on all four DNA bases as well as on the phosphate backbone of DNA. Thus, from a qualitative viewpoint, detection of all DNA adducts derived from even a single carcinogen is a very complex analytical problem. From a quantitative point of view, the problem is complicated even further by the fact that adducts are removed from DNA by chemical and enzymatic processes at different rates. The rates may vary for each type of adduct, from one tissue to another, or even for the same adduct in different types of cells in the same tissue. Singer and Grunberger (1983) gave a comprehensive review of the types of DNA adducts formed by different carcinogens and mutagens, as well as their repair characterises.

In the past few years, analytical methods of several types have been devised for the detection and quantitative analysis of carcinogen-DNA adducts at levels reflecting exposure to ambient levels of the agents. Most of the available information concerning DNA adducts in experimental systems has been obtained through the use of physicochemical or radiochemical detection. The usefulness of these methods of detection in human monitoring is limited by their relative insensitivity and inapplicability, respectively. In a few instances (e.g., benzo[*a*]pyrene and aflatoxins), ultrasensitive physicochemical methods based on the inherent property of fluorescence have been successfully applied to the detection of carcinogen-DNA adducts in human material. A postlabelling method, in which modified DNA bases are detected by P-labelling of mononucleotides produced by enzymatic hydrolysis of DNA, has been devised and shown to be effective in detecting adducts of a large number of carcinogens

of differing chemical structures (Randerath *et al.*, 1985). Immunoassays have been developed with sensitivities that approach the levels required for the detection of modified DNA in exposed populations (Poirier, 1981; IARC/IPCS Working Group, 1982). These methods are currently being evaluated in studies of workers and others known to be exposed to polycyclic aromatic hydrocarbons (PAH) and other environmental carcinogens, and representative results are summarized in the following section.

The chemical modification of proteins is a well-established phenomenon. Indeed, clinical application of a haemoglobin dosimeter has already been used successfully in monitoring excessive exposure of diabetics to blood glucose. Circulating glucose reacts *via* a carbonylamine condensation to form a Schiff's base, predominantly with the TV-terminal amino groups of the globin chains, to yield a glycosylated haemoglobin named haemoglobin A_{ic}. Levels of this modified haemoglobin are significantly elevated in diabetics, and its level in blood in a given patient reflects the degree of diabetic control. This approach has been regarded as more useful than measurement of glucose levels in blood or urine because it gives an integrated picture of exposure. The projected use of haemoglobin alkylation products formed as a result of exposure to environmental carcinogens is based on the same principles.

The main nucleophilic centres in proteins are the sulphur atoms of cysteine and methionine, nitrogens of amino groups, guanido groups, ring systems and oxygen atoms. The chemical reactivity of the nucleophilic sites of protein is determined by several factors, such as polarizability, protonation state and pK_a, among others. Experience has shown that the simple concept of electrophile reacting with nucleophile is not sufficient to predict the nature of the site participating in protein-carcinogen adduct formation. The type of adduct formed is frequently unpredictable and may depend on the affinity of the carcinogen for induced binding sites on the protein which steer the proximal reactive form of the carcinogen to a particular nucleophiiic site on the protein.

Ehrenberg and his colleagues conducted the pioneering work on the use of protein adducts as dosimeters and have made many contributions to the field, only the main points of which can be mentioned here. Ehrenberg and Osterman-Golkar (1980) reviewed the rationale and technical requirements for the use of protein alkylation for detecting mutagenic agents. Important among the requirements is that exposure must result in the formation of stable covalent derivatives of amino acids for which assay methods of adequate sensitivity and specificity can be devised. Further, the target protein should be found in easily accessible fluids (e.g., blood) and should be present in concentrations adequate to provide sufficient material for analysis. Although any protein could, in principle, be used for monitoring alkylated derivatives, haemoglobin was suggested by Osterman-Golkar *et al.* (1976) as a suitable dose-monitoring protein, and the majority of the relevant literature concerns studies of haemoglobin alkylation. More recently, albumin has been recognized as a potentially useful dose monitor, since it is abundant and reactive, has a long half-life and is synthesized in the hepatocyte — a site where many carcinogens are metabolized to their most reactive forms. Also, since albumin is a component of the interstitial fluid which bathes all cells, it may capture carcinogen metabolites from any tissue.

In an extensive series of studies, Ehrenberg and his colleagues characterized the essential attributes of haemoglobin as a dosimeter, which can be summarized as follows. The stability of alkylated residues in haemoglobin modified by ethylene oxide or N-nitrosodimethylamine was established, and the half-life of alkylation levels produced by a single dose of either agent was found to be equivalent to the life span of erythrocytes in mice. The validity of the steady-state level of alkyl residues in haemoglobin as a measure of chronic, repeated exposure was subsequently established

in mice dosed repeatedly with methylmethane sulphonate. Osterman-Golkar *et al.* (1983) reviewed studies of haemoglobin alkylation in people occupationally exposed to ethylene oxide. Blood samples were obtained from workers exposed to known levels of ethylene oxide (established by analysis of air samples), and haemoglobin was analysed for the presence of A^r-3-(2-hydroxyethyI)histidine by mass spectrometry and by ion-exchange amino acid analysis. The authors concluded that the haemoglobin alkylation values accurately reflected exposure and were in good agreement with earlier data derived for ethylene oxide in mice.

Pereira and Chang (1981) surveyed the ability of carcinogens and mutagens representing a broad spectrum of chemical classes to bind covalently to haemoglobin in rats. Animals were dosed with ^{14}C-labeIled carcinogens and blood was collected 24 h later. Covalent binding was determined by analysis of purified haemoglobin for ^{14}C bound to the protein. All the carcinogens studied were found to form covalent haemoglobin adducts in a dose-related manner, but the absolute binding level was *not* related to known carcinogenic potency.

In order for protein alkylation to be useful as a monitoring procedure, reliable dose-response relationships between exposure dose and production of alkylated amino acids must be established. This requirement has been satisfied for the exposures studied to date, all of the observations having been made in experimental animals dosed with various known carcinogens. Thus, GC-MS determination of the levels of 5-methylcysteine in haemoglobin of rats following injection of methylmethane sulphonate showed that the level of alkylated amino acid was linearly related to dose. For ethylene oxide, a virtually linear relationship was observed between dose and production of vV-3-(2-hydroxyethyl)histidine in haemoglobin, in experiments in which the alkylating agent was administered by inhalation at doses of 0-100 ppm in air, 30 h per week for two years. Other agents for which dose-response relationships have been established include franj-4-dimethylaminostilbene. chloroform, jV-nitrosodimethyl-amine and 4-aminobiphenyi, which are discussed in more detail below.

On this basis, it can be concluded that the amount of alkylated haemoglobin is directly related to erythrocyte dose, and that erythrocyte dose and exposure dose are almost always linearly related to each other. Thus, many of the requirements for validating haemoglobin adducts as exposure dosimeters have been met. It is important to consider whether this parameter could also be used to estimate carcinogenic risk. The observation of haemoglobin alkylation *per se* can be taken as an indicator of genotoxic risk only when it has been shown that such alkylation correlates with reactions at the target DNA site, i.e., that the erythrocyte dose is directly related to the target dose. This relationship has been studied in only a few experimental systems, in which the amounts of DNA and haemoglobin binding products have been compared following dosing with carcinogens. Observed levels of alkylation of guanine in DNA of liver and testis induced by ethylene oxide deviated by no more than two-fold from the amount expected on the basis of haemoglobin alkylation. Thus, the degree of alkylation of DNA could be estimated approximately from the dose of this compound in erythrocytes. Similar relationships have been shown for /ra/w-4-dimethyl-aminostilbene and 2-acetylaminofluorene. Thus, in at least *some* instances, it seems possible to predict DNA binding by measuring protein binding, and therefore the latter may in some cases be taken as an indication of genotoxic risk.

Detection of DNA damaging agents in man

The development of these methods has made possible their evaluation for detecting DNA damaging agents in man. Results of several such studies have been published,

Table 1. Indicators of genotoxic exposure: urinary excretion of markers

Compound analysed	Exposure source	Method of analysis[a]	Principal findings[b]		Reference
Nitrosamino acids	Unknown	GC-TEA	High-risk area (44) Low-risk area (40)	21.2 μg/day 5.6 μg/day	Lu *et al.* (1986)
N-Nitrosoproline	Cigarette smoke	GC-TEA	Smokers (13) Nonsmokers (13)	5.9 μg/day 3.6 μg/day	Hoffman *et al.* (1986)
N-Nitrosoproline	Unexposed	GC-MS	Nonsmokers (24)	3.3 μg/day	Garland *et al.* (1986)
Aflatoxin B_1, aflatoxin B_1-7-guanine	Diet	IA-HPLC	Exposed subjects (20)	0.1–10 μg/day aflatoxin B_1 equivalent	Groopman *et al.* (1985)
Aflatoxin B_1-7-guanine	Diet	HPLC-SSFS	Low/high-risk areas (983)	12% positive	Autrup *et al.* (1987)
3-Methyladenine	Unexposed	GC-MS (SIM)	Excretion rate (9)	4.5–16.1 μg/day	Shuker *et al.* (1987)
Thymine glycol, thymidine glycol	Unexposed	HPLC	Excretion rate (9)	0.39 nmol/kg/day 0.10 nmol/kg/day	Cathcart *et al.* (1984)

[a] GC, gas chromatography; TEA, thermal energy analysis; MS, mass spectrometry; IA, infra-red analysis; HPLC, high-performance liquid chromatography; SSFS, synchronous scanning fluorescence spectrophotometry; SIM, single-ion monitoring
[b] In parentheses, no. of subjects

and it is useful to summarize the nature of the findings in the context of the objectives of the present volume. Tables 1-5 contain data extracted from published reports dealing with various aspects of biomonitoring of exposure of humans to genotoxic agents of a variety of types and sources. In preparing these tables, an attempt has been made to select data that are representative of the results obtained, with particular emphasis on those aspects of the study that are pertinent to method validation.

Urinary excretion of markers

Table 1 summarizes findings from a series of studies in which urinary excretion of markers of the interactions of carcinogens with proteins or nucleic acids were measured as indices of carcinogenic exposures. Lu *et al.* (1986) determined the daily excretion of nitrosamino acids in population groups residing in areas of high and low risk with respect to development of cancer of the oesophagus. This study was designed to gain evidence about the possible etiological role of iV-nitroso compounds, some of which are effective oesophageal carcinogens in animals, as risk factors in populations at differing levels of risk. The substantially higher values for persons in the high-risk area indicate a higher level of exposure to these carcinogens during the study period. A similar approach was used by Hoffmann and Brunnemann (1983) to assay the potential of inhaled cigarette smoke for endogenous iV-nitrosation of amines. The data revealed not only the existence of a substantial background of endogenous nitrosation in nonsmoking controls, but also a significant increase in smokers. Garland *et al.* (1986) conducted an extensive investigation of individual and interindividual differences in the excretion of N-nitrosoproline in healthy volunteer subjects, all but two of whom were nonsmokers. As indicated in Table 1, the average value for these subjects is very close to that for the nonsmoking controls and for residents of the low-risk areas in the earlier studies. In each of these studies, intake of ascorbic acid was shown to lower the excretion rates, as expected. The general interlaboratory agreement with respect to the values reported indicates the potential value of this measurement in detecting exposure to nitrosating agents in the environment.

Assessment of human exposure to aflatoxins through measurement of urinary excretion of the major DNA adduct has been reported in two studies. Groopman *et al.* (1985) used the technique of immunoaffinity purification coupled with high-performance liquid chromatography (HPLC) detection to identify the guanine adduct of aflatoxin B, in the urine of persons residing in a commune in Guangxi Province, China, where the dietary content of the carcinogen was known to be high. The analytical method was adequately sensitive to detect the presence of the adduct in persons exposed to high levels and also to quantify the excretion of aflatoxin M_t, a metabolite, as well as of unmetabolized carcinogen. Aflatoxin exposure was also measured by urinary excretion of the guanine adduct in populations living in areas with different liver cancer incidence in Kenya (Autrup *et al,* 1987). The adduct, detected by HPLC used in combination with synchronous scanning fluorescence spectrophotometry (SSFS) was found in 12% of a large series (983) of samples collected over a period of years in different areas of the country. Both analytical methods require sophisticated analytical instrumentation and are not yet suitable for routine monitoring.

Two additional approaches based on analysis of urinary components are being developed as potential monitors for exposure to genotoxic exposures. Shuker *et al.* (1987) have explored the measurement of 3-methyladenine in urine as an indicator of exposure to methylating agents. As indicated in Table 1, they have developed a GC-MS method using single-ion monitoring that is capable of detecting the methylated base in nominally unexposed individuals. Further validation of the method will be required to determine its usefulness for monitoring exposure. Cathcart *et al.* (1984) have developed an HPLC assay for free thymine glycol and for thymidine glycol in urine, in order to provide a noninvasive assay for oxidative DNA damage, since these compounds are products of DNA damage caused by ionizing radiation and other oxidative mutagens. While they were able to determine excretion rates for both compounds in healthy individuals, the method in its present form is not applicable for routine application but will require further development.

Haemoglobin adducts

A summary of representative data on measurement of haemoglobin adducts derived from exposure to carcinogens is presented in Table 2. Most of the work done to date has concerned measurement of vV-3-(2-hydroxyethyl)histidine and jV-(2-hydroxyethyl)-valine as monitors of exposure to ethylene oxide. This methodology, in which the alkylated derivative was measured by GC-MS, was developed by Professor Ehrenberg and his colleagues and was used in a study of occupationally exposed workers (Calleman *et al,* 1978). Average data shown in Table 2 for exposed subjects and unexposed controls indicate the sensitivity of the method for detecting exposure. Other findings in this study indicate the general agreement between data obtained in man and those predicted from earlier studies in mice and also demonstrate the superiority of the method for providing a cumulative measure of exposure as compared to point monitoring of air levels. The same method was subsequently applied by Van Sittert *et al* (1985) in a study of workers in an ethylene oxide manufacturing plant, in which cytogenetic and immunological endpoints were measured simultaneously in the same individuals. These investigators reported a substantially higher background of *N-3-(2*-hydroxyethyl)histidine in their control subjects and reported no significant difference between exposed and unexposed persons. Farmer *et al* (1986) compared levels of this compound as determined by GC-MS on protein hydrolysates in which the adduct was concentrated by ion-exchange chromatography, with levels of 7V-(2-hydroxyethyl)valine determined by GC-MS analysis of haemoglobin subjected to Edman degradation

Table 2. Indicators of genotoxic exposure: haemoglobin adducts

Compound analysed	Exposure source	Method of analysis[a]	Principal findings[b]		Reference
N-3-(2-Hydroxyethyl)histidine	Ethylene oxide (occupational)	GC-MS	Exposed subjects (5) Control subjects (2)	0.5–13.5 nmol/g Hb 0.05 nmol/g Hb	Calleman *et al.*. (1978)
N-3-(2-Hydroxyethyl)histidine	Ethylene oxide (occupational)	GC-MS	Exposed subjects (32) Control subjects (31)	2.08 nmol/g Hb 1.59 nmol/g Hb	Van Sittert *et al.* (1985)
N-3-(2-Hydroxyethyl)histidine	Ethylene oxide (occupational)	Ion exchange + GC-MS	Exposed subjects (7) Control subjects (3)	0.68–8.0 nmol/g Hb 0.53–1.6 nmol/g Hb	Farmer *et al.* (1986)
N-(2-Hydroxyethyl)valine	Ethylene oxide (occupational)	Edman degradation + GC-MS	Exposed subjects (7) Control subjects (3)	0.02–7.7 nmol/g Hb 0.03–0.93 nmol/g Hb	Farmer *et al.* (1986)
N-(2-Hydroxyethyl)valine	Cigarette smoke	Edman degradation + GC-MS	Smokers (11) Nonsmokers (14)	389 pmol/g Hb 58 pmol/g Hb	Törnqvist *et al.* (1986)
4-Aminobiphenyl	Cigarette smoke	GC-MS (NCI)	Smokers (15) Nonsmokers (26)	154 pg/g Hb 28 pg/g Hb	Bryant *et al.* (1987)

[a] GC-MS, gas chromatography-mass spectrometry; NCI, negative-ion chemical ionization
[b] In parentheses, no. of subjects

before analysis. They found that the two methods of analysis gave consistent results, especially at high levels, and also that higher levels of background alkylation (of unknown origin) were obtained in measurements of iV-3-(2-hydroxyethyl)histidine than with A^r-(2-hydroxyethyl)valine, suggesting that the latter assay would show greater sensitivity in monitoring exposure to ethylene oxide. This method was subsequently applied in a study of cigarette smokers and nonsmokers (Tornavist *et al.*, 1986). The results demonstrated an elevation of N-(2-hydroxyethyl)valine levels in smokers that was quantitatively compatible with measured levels of ethene in the smoke to which they were exposed.

Bryant *et al.* (1987) have developed a method for the analysis of 4-aminobiphenyl covalently bound as the sulphinic acid amide to the 93-/3 cysteine of human haemoglobin. The method involves hydrolysis of the haemoglobin followed by GC-MS determination of the parent amine after derivatization. Application of the method to smokers and nonsmokers revealed consistently elevated levels in smokers and a detectable background of undetermined origin of the adduct in nonsmokers. Collectively, these results indicate the applicability of analysis of haemoglobin adducts as monitors of exposure to carcinogens of different structural types and modes of action.

Detection of benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) adducts in DNA by immunoassay and chemical analysis

Several studies have been conducted that were designed to determine exposure to the ubiquitous PAH benzo[#]pyrene by the detection of derivatives covalently bound to DNA (Table 3). Immunoassays and physicochemical methods have been applied to detect derivatives formed through the major intermediate in the activation pathway, BPDE. Immunoassays have been applied in two modes, enzyme-linked immunosorbent assay (ELISA) and ultrasensitive enzyme radioimmunoassay (USERIA), both employing polyclonal antisera that recognize BPDE-DNA adducts, with various levels of cross-reactivity with structually related congeners. In an early pilot study in humans,

Table 3. Detection of BPDE adducts in DNA by immunoassay or chemical analysis[a]

Type of assay	Principal findings			Reference
ELISA (PC)	Lung DNA (tumorous/non-tumorous)	(5/27)	0.14–0.18 fmol/μg DNA	Perera *et al.* (1982)
ELISA (PC)	WBC Roofers	(7/28)	2–120 fmol/50 μg DNA	Shamsuddin *et al.* (1985)
	WBC Foundry workers	(7/20)		
	WBC Controls (smokers)	(2/9)	37–47 fmol/50 μg DNA	
SSFS	WBC Aluminium workers	(1/30)	Positive	Vähäkangas *et al.* (1985)
	WBC Controls	(0/10)		
USERIA (PC)	WBC Coke-oven workers	(18/27)	0.4–34.3 fmol/μg DNA	Harris *et al.*. (1985)
SSFS	WBC Coke-oven workers	(31/41)	Positive	
		(11/41)	Positive for serum Ab	
USERIA (PC)	WBC Coke-oven workers	(13/38)	0.1–13.7 fmol/μg DNA	Haugen *et al.* (1986)
SSFS	WBC Coke-oven workers	(4/38)	0.38–2.2 fmol/μg DNA	
		(13/38)	Positive for serum Ab	
ELISA (PC)	WBC Foundry workers (heavy, medium, light exposure)	(22)	1.2 fmol/μg DNA 0.53 fmol/μg DNA 0.32 fmol/μg DNA	Perera *et al.* (1987)
	Controls	(10)	0.06 fmol—μg DNA	

[a] PC, polyclonal; WBC, white blood cells

Perera *et al.* (1982) demonstrated that the ELISA is sufficiently sensitive to detect adducts in DNA extracted from lung tumours as well as from nontumour tissue of lung cancer patients; but the small number of subjects studied precluded conclusions concerning exposure history. Shamsuddin *et al.* (1985) employed ELISA and USERIA to investigate the levels of BPDE-DNA adducts in the white blood cells of roofers and foundry workers, in view of their exposure to high levels of benzo[a]pyrene. Adducts were detected in a significant proportion of exposed individuals and also in two of nine control subjects, both of whom were cigarette smokers. The SSFS technique was applied in the analysis of DNA in white blood cells collected from workers in an aluminium plant for the presence of BPDE-DNA adducts (Vahakangas *et al.,* 1985). The limit of detection of this method as applied is about one adduct in 10^7 nucleotides. One sample of white blood cell DNA from the series of 30 exposed subjects showed the presence of a detectable level of BPDE. Harris *et aL* (1985) analysed the DNA of white blood cells from coke-oven workers by USERIA and by SSFS to determine the frequency and levels of BPDE adducts as markers of exposure, since these workers are known to be exposed to high levels of benzo[a]pyrene and are also at an elevated risk of lung cancer. Approximately two-thirds of the workers had detectable levels of BPDE-DNA adducts as determined by immunoassay, and an even larger proportion showed evidence of the presence of BPDE by the SSFS assay. Antibodies to the DNA adducts were also detected in the serum of 27% of the workers. Coke-oven workers were also the subjects of a study by Haugen *et al.* (1986), who sought to evaluate the genotoxic effects of their occupational exposure through a study of BPDE-DNA adducts, with simultaneous measurements of urinary excretion of PAH metabolites and air monitoring. As in the earlier study, DNA adducts were measured by USERIA and SSFS, and the sera were examined for the presence of anti-DNA adduct antibodies. The results of the study were in close agreement with those obtained earlier by Harris *et al.* (1985) in all respects. Perera *et al.* (1987) have recently applied the ELISA in the study of PAH adducts in the DNA of white blood cells collected from Finnish foundry workers classified as having high, medium or low exposure, as well as from unexposed control subjects. Mean levels of DNA adducts increased with exposure, and there was a highly significant difference between the control and pooled exposure group means.

Immunoassay techniques have also been used to detect adducts of DNA damaging agents other than BPDE (Table 4). Radioimmunoassay of O^6-methyldeoxyguanine was performed by Wild *et al.* (1986) on human oesophageal and cardiac stomach mucosal DNA from tissue samples obtained during surgery in Linxian County, China, an area of high risk for both oesophageal and stomach cancer. Similar analyses were conducted on samples collected from hospitals in Europe. Using this methodology, O^6-methyldeoxyguanine was detected in about two-thirds of samples from the high-risk area, as compared to five out of 12 from the control area. Adduct levels were also higher in samples from people living in the high-risk areas. DNA adducts induced by the anticancer drug cisplatin were detected in the white blood cells of cancer patients treated with the drug through application of ELISA methodology (Fichtinger-Schepman *et al.,* 1987). The immunoassay procedure involved four antisera capable of detecting different adducts, including: intrastrand cross-links on pGpG sequences (which proved to be the major adduct); intrastrand cross-links on pApG sequences; inter- or intrastrand cross-links on two guanines separated by one or more bases; and a monofunctional adduct bound to guanine. Results indicated that the susceptibility of white blood cells to adduct formation can show strong individually determined differences. Reed *et al.* (1987) also used ELISA methodology to study adducts derived from cisplatin in white blood cells of ovarian cancer patients being treated with the drug. Values for median adduct levels were grouped by complete response, partial

Table 4. Detection of O^6-methylguanine and DNA adducts of cisplatin by immunoassay[a]

Compound analysed	Type of assay	Principal findings			Reference
O^6-Methyldeoxy-guanine	RIA	Oesophagus/Stomach (tumorous/nontumorous)	(27/37)	25–160 fmol/mg DNA	Wild *et al.* (1986)
Cisplatin-DNA	ELISA (4 PC)	European controls WBC Infused patient	(5/12)	25–45 fmol/mg DNA pGpG 5.6 fmol/μg DNA pApG 1.9 fmol/μg DNA $(GMP)_2$ 1.1 fmol/μg DNA GMP 0.06 fmol/μg DNA	Fichtinger-Schepman *et al.* (1987)
Cisplatin-DNA	ELISA (PC)	WBC Ovarian cancer patients	(55) (peak)	Complete response 212 amol/μg DNA Partial response 193 amol/μg DNA No response 62 amol/μg DNA	Reed *et al.* (1987)

[a] RIA, radioimmunoassay; PC, polyclonal; WBC, white blood cells.

response and no response, and statistical analysis of the data showed that higher level of adduct formation correlated with clinical responsiveness to the drug.

Taken together, these data show that adducts formed in DNA of accessible cells of people exposed to DNA damaging agents of a variety of types can be detected, and in many instances quantified, by currently available immunological and chemical methods. Application of these methods in well-designed longitudinal studies in man will permit further evalutions of their validity and of their limitations in actual use.

Detection of aromatic DNA adducts by ^{32}P-postlabelling

The postlabelling procedure developed by Randerath and his collaborators has been extensively applied to studies of DNA adduct formation in a variety of experimental systems, and the capacity of the method to detect adducts of a large number of carcinogens (about 50) has been reported. The procedure has recently been extended to studies in humans (Table 5). Everson *et al.* (1986) investigated the presence of DNA adducts in human term placentas by both ELISA and the ^{2}P-postlabelling assay. The immunoassay revealed a small but insignificant increase in BPDE adduct levels in placentas from smokers compared to nonsmokers; however, the postlabelling assay detected a number of adduct types, the major one of which was strongly related to maternal smoking during pregnancy. Subsequently, Randerath *et al.* (1986) compared adduct levels in bronchus and larynx from smokers with those produced in mouse skin treated with cigarette tar. The human tissues were found to contain detectable levels of adducts, one of which (designated adduct 1) was identical to a major and persistent adduct formed in mouse skin. Dunn and Stich (1986) used the postlabelling assay to investigate DNA adducts in exfoliated mucosal cells collected from the oral cavity of three groups at high risk for oral cancer. Five chromatographically distinct adducts were found in both the high-risk groups and nonsmoking controls. Individual adducts were detected in 30-90% of samples, and no adduct was found in high-risk groups that did not also appear in control groups. Thus, although the method was useful for detecting adducts derived from unknown sources, it did not differentiate between exposed and unexposed populations. Using the same approach, Chacko and Gupta (1987) analysed the DNA from oral mucosal cells of cigarette smokers and nonsmokers to determine whether smoking-related adducts could be identified. Two chromatographically distinct adducts of unknown identity were found in smokers but not in nonsmokers. In addition, the levels of these major and several minor adducts were substantially higher in smokers. Phillips *et al.* (1986) analysed DNA from normal human bone-marrow mononuclear and non-mononuclear cells for the presence of aromatic adducts. Ten out of ten individuals showed the presence of adducts that were not present in fetal bone marrow but were present at lower levels in the DNA of peripheral white blood cells. Their data suggest that the adducts result from environmental exposure to unidentified genotoxic agents. Phillips *et al.* (1987) also employed the postlabelling assay to analyse DNA from white blood cells of foundry workers for the presence of adducts that might reflect differing levels of exposure to benzo[a]pyrene. Adducts were found in ten of ten workers at levels detectable by the analytical method, but none of these had the chromatographic properties characteristic of adducts derived from benzo[a]pyrene. Adducts were also identified in unexposed control subjects. The results indicated significant interindividual differences in DNA binding among people exposed to similar levels. Reddy *et al.* (1987) investigated white blood cell and placental DNA for the possible presence of adducts derived from exposure of pregnant women to residential wood combustion smoke. Detectable levels of unidentified adducts were found in all placentas of exposed and

Table 5. Detection of aromatic DNA adducts by ^{32}P-postlabelling[a]

Source of exposure	DNA analysed	Principal findings		References
Cigarette smoke	Placenta	Smokers (16/17)	Adduct 1: 1.4 per 10^8 nucleotides (postlabelling) 2.0 per 10^6 nucleotides (ELISA)	Everson *et al.* (1986)
		Nonsmokers (3/14)	Adduct 1	
Cigarette smoke	Bronchus, larynx	Smokers (2)	Total adducts: 1 per ($1.7–2.9 \times 10^7$ nucleotides) [0.10–0.18 fmol/μg DNA] Adduct 1: 8–14% of total	Dunn & Stich (1986)
Betel chewing, tobacco chewing, inverted smoking	Oral mucosa	Exposed (59)	Adducts found in 30–95% [1 per 10^9 nucleotides to 1 per 10^7 nucleotides]	Chacko & Gupta (1987)
Cigarette smoke	Oral mucosa	Smokers (11/14)	Total adducts: 0.1–210 amol/μg DNA	Phillips *et al.* (1986)
		Nonsmokers (2/8)	Total adducts: 0.4–1.7 amol/μg DNA	
Foundry workers	WBC	Exposed (10/10)	Total adducts: 0.2–11.6 per 10^8 nucleotides	Phillips *et al.* (1987)
		Controls (5/10)	0.4 per 10^8 nucleotides	
Wood smoke	Placenta, WBC	Exposed: Placenta (4)	Total adducts: 12 per 10^9 nucleotides	Reddy *et al.* (1987)
		WBC (8)	ND	
		Control: Placenta (5)	12 per 10^9 nucleotides	
		WBC (8)	ND	

[a] WBC, white blood cells; ND, not determined

unexposed women; none of the nine adducts found was present in DNA of white blood cells from any of the subjects. The results suggest that residential wood smoke does not elicit aromatic DNA adducts at detectable levels, but that placental DNA contains detectable levels of adducts of unknown identity and origin. Collectively, these results demonstrate the capacity of the postlabelling method to identify DNA damage arising from a variety of environmental exposures. However, additional experience with its applications in human studies will be required to establish fully the important analytical characteristics of the method.

DNA adducts and haemoglobin adducts as measures of exposure and susceptibility

The information summarized above provides valuable evidence concerning some features of currently available methods for detecting and quantifying covalent adducts to DNA and haemoglobin in human material. Most of the studies constitute feasibility trials designed to test the use of the methods for certain specific purposes. Taken together, the available data show that detection of exposure to DNA damaging agents of a variety of chemical types will be feasible using the methods currently in hand or being developed. Substantial additional technological validation will be required for routine application of any of the current methods, but it seems likely that many of them will eventually come into broader application in epidemiological surveys. In interpreting the data generated by their application, it is important to take into account all of the available information concerning the parameters being measured. Some of the important points in this context can be summarized as follows.

Concerning the use of DNA adduct levels as measures of exposure and susceptibility, virtually all of the available evidence comes from experimental systems, especially experiments in which adduct formation in carcinogen-treated animals was studied in relation to tumour formation in response to the treatment. Most carcinogens have been shown to form complex spectra of DNA adducts, and qualitatively similar adduct profiles can be formed in sensitive and resistant species, strains and tissues. Adduct persistence may or may not be related to susceptibility and target tissue specificity. In target tissues, maximal total adduct levels usually reflect carcinogen potency and dose, and maximal total adduct levels are linearly related to dose over a wide range. Many factors have been identified that can affect the levels of DNA adducts in cells of treated animals at any given time. The exposure-sampling interval can markedly affect observed levels, as determined by the pharmacokinetic properties of carcinogens of specific chemical types. An important component of this process is the capacity for metabolic activation, which can be affected by a variety of genetic and environmental factors (e.g., inducers and inhibitors). Exposure to protective agents such as antioxidants and other dietary ingredients can also exert marked effects. DNA repair capacity, as determined by genetic factors, kinetics (e.g., saturability) and modulating factors, is also an important determinant of adduct stability. Collectively, these factors indicate the complexity of the problem of accurately interpreting DNA adduct levels as quantitative measures of exposure.

The use of DNA adduct levels as indicators of long-term risk is complicated by additional factors. Generally, the target tissue at risk to a given genotoxic exposure is unknown, and reliance must be placed upon measurements of adduct levels in surrogate cells such as peripheral white blood cells. The validity of analysing DNA in surrogate cells for target cell DNA is difficult to assess. Extrapolation of DNA adduct levels as indicators of cancer risk necessarily entails simplifying assumptions, which cannot be evaluated on the basis of current information, about the multistage nature of

the cancer process. In addition, point measurements of DNA adduct levels may or may not provide evidence of multiple and variable exposures.

As discussed earlier, protein adducts have no putative mechanistic role in carcinogenesis and are primarily regarded as measures of exposure alone. Evidence from experimental animals and from humans supports the interpretation that protein adduct levels are exposure monitors. Measurement of haemoglobin adducts has certain obvious technical advantages, inasmuch as the protein can readily be obtained in abundant quantities. Sensitive and specific methods have been developed for two classes of carcinogens, and additional ones are in the process of development. Carcinogens of diverse chemical structures have been shown to bind haemoglobin *in vivo*. Adducts are stable over the erythrocyte life span, thereby giving an integrated measure of multiple exposures over a substantial time period. Levels of haemoglobin adducts have been shown to be linearly related to dose for at least seven carcinogens of different types. Importantly, haemoglobin adduct levels have been shown to be related, within a factor of two, to DNA adduct levels in target tissues for three agents, ethylene oxide, *trans*-4-dimethylaminostilbene and 2-acetylaminofluorene. Haemoglobin adducts therefore provide very useful complementary data to DNA adduct levels as dosimeters of carcinogen exposure.

The information summarized above concerning the application of current methods for detecting and quantifying levels of DNA and haemoglobin adducts in human samples indicates that adequate levels of sensitivity have been attained for several classes of DNA damaging agents to make possible the detection of adducts resulting from ambient levels of exposure. Specificity for the detection of compounds of known structure is also a common feature of most of the methods, and some are suitable for detecting total DNA damage from multiple sources. With respect to practicability, certain of the methods, e.g., immunoassays, are applicable to large numbers of samples, whereas others, e.g., postlabelling and thymine glycol analysis, are technically more complex and time-consuming. Validation will be required to avoid systematic errors in measurement before the methods are applicable to large-scale epidemiological studies. Methodological attributes such as accuracy, reproducibility (intra- and interlaboratory) and applicability *to* stored samples must be determined by appropriately designed collaborative studies among qualified laboratories. Additional factors such as variations in observed values due to age, sex and race, as well as effects of possible interfering factors (e.g., diet, smoking, alcohol) must be defined in properly designed human studies of limited scope in order to validate the approach for epidemiological surveys.

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