Annu. Rev. Pharmacol. Toxicol. 1994. 34:41–67

MACROMOLECULAR ADDUCTS: Biomarkers for Toxicity and Carcinogenesis[1]

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KEY WORDS: biomonitoring and molecular epedimology, prediction of toxicity and carcinogenesis, exposure index of chemicals, DNA adducts

INTRODUCTION

Researchers have recently explored new approaches to recognizing and predicting toxic exposures and their potential consequences. Such efforts require the development of indicators enabling these identifications and predictions to be made with some certainty. Biological markers (biomarkers) allow reactions, responses, or adverse changes in a biological system or component to be recognized early and with certainty. Biomarker research seeks to give molecular biologic findings an immediate application to human health care.

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Macromolecular adducts can provide reliable indicators of exposure to certain chemicals and can help to predict toxicological consequences, including carcinogenesis. Comprehensive reviews of recent advances in biomarker research for various toxicological endpoints are available (1). Here we treat the use of macromolecular adducts as biomarkers, focusing on (*a*) methods for studying macromolecular adducts, (*b*) the molecular dosimetry of macromolecular adducts and carcinogenesis, and (*c*) the application of findings from macromolecular adduct research to human carcinogenesis.

METHODS FOR STUDYING MACROMOLECULAR ADDUCTS

New biomarkers are constantly being discovered and developed. Sensitive and sophisticated analytical methods have been developed during the past decade that make it possible to detect and quantify various macromolecular adducts generated by reactive chemicals or their metabolites. The prospect of studying such adducts in human subjects exposed to toxicants is particularly exciting. Several reviews published during the last five years provide an overview of the various methodological approaches to the study of macromolecular adducts (1–6).

DNA Adducts

Owing to their mechanistic significance in chemical carcinogenesis, DNA adducts have been the focus of most efforts to develop new biomarkers. At present, three distinct analytical methods are commonly used for the detection and quantitation of DNA adducts: immunological methods, fluorometric methods, and ^{32}P-postlabeling. These methods have extended the detection limits for DNA adducts to 1 per 10^6–10^{10} normal nucleotides. All three approaches have advantages and disadvantages and thus play different and often complementary roles in biomarker research and human biomonitoring.

Immunological Methods

The availability of antibodies recognizing specific DNA adducts has enabled us to understand the toxic, mutagenic, and carcinogenic properties of these adducts (for a review, see 7). At present, the most commonly used immunological methods for DNA adduct quantitation include competitive radioimmunoassay (RIA), solid-phase, competitive or noncompetitive enzyme-linked immunosorbent assay (ELISA), and ultrasensitive enzymatic radioimmunoassay (USERIA). In addition, antibodies have been used in the development of various immunopurification techniques.

In RIA the concentration of antigen in a sample is determined by measuring its ability to compete with a fixed amount of radiolabeled antigen

for a limiting quantity of antibody. ELISA employs a solid phase–bound antigen and a second antibody conjugated to an enzyme, which increases assay sensitivity because one molecule of enzyme can hydrolyze many substrate molecules. USERIA is similar to ELISA except that in this experimental approach the substrate for the second antibody is radiolabeled. USERIA is at least as sensitive as, if not more sensitive than, ELISA. For example, using a standard competitive procedure with 1 μg DNA in the antigen-antibody reaction mixture, USERIA has been shown to be approximately 500 times as sensitive as RIA and 5 times as sensitive as ELISA for the detection of benzo(a)pyrene-DNA adducts (8). At present, many sensitive immunoassays can detect specific DNA adducts (see below). Detection limits in the range of 1 adduct per 10^6-10^8 normal nucleotides are routinely attained when milligram quantities of DNA are available for analysis (6). However, with high-affinity antibodies, DNA requirement can be drastically reduced. For example, with an ELISA using monoclonal antibody 6A10 specific for the imidazole ring-opened aflatoxin B_1 (AFB_1)-DNA adduct, a detection limit of 5 adducts per 10^7 normal nucleotides in various biological samples was achieved with 50 μg of DNA (9).

It is important to stress that the sensitivity of the various immunoassays (or of any other analytical methods for the detection of DNA adducts) should be considered in two different ways—i.e. as absolute and relative sensitivities. Absolute sensitivity involves the amount of a given DNA adduct required for precise quantitation, whereas relative sensitivity denotes the lowest relative modification level to be detected in DNA (10). At a given absolute sensitivity, the relative sensitivity of a method depends on the amount of DNA required by the system. The distinction between absolute and relative sensitivities was illustrated in a comparison of RIA results in two studies for the quantitation of O^6-methyldeoxyguanosine (O^6-MedG) in adducted DNA (7). A dramatic difference in relative sensitivities (three orders of magnitude in the DNA modification level detected) was observed between a direct RIA using 5 μg of DNA for a single analysis and another RIA using 10 mg of DNA plus an additional high-performance liquid chromatography (HPLC) purification step.

Overall, for the quantitation of DNA adducts, immunoassays offer the advantage of ease and low cost in analyzing large numbers of samples, a critical consideration in conducting molecular-epidemiological studies in human populations. However, the production of antibodies, whether they be polyclonal or monoclonal, requires extensive development efforts. Prior knowledge of the structure of the DNA adduct under investigation is essential, as is the ability to synthesize the appropriate antigen. The use of antibodies has also proven extremely valuable in immunopurification. Monoclonal antibodies have been used in various affinity chromatography proce-

dures to eliminate interfering substances prior to analysis by either immunoassays or non-immunological assays. For example, an immunoaffinity purification step based on a monoclonal antibody specific for O^6-MedG and immobilized on a periodate-activated Sepharose gel has been successfully used in combination with ^{32}P-postlabeling for the detection of O^6-MedG adducts in DNA from human tissues (11). Recovery of bound material from the gel exceeds 95%; the detection limit is 1 adduct per 10^8 normal MedG when 100 μg of DNA are used.

Fluorometric Methods

An alternative methodology for DNA adduct analysis utilizes the inherent fluorescent properties of many polycyclic aromatic hydrocarbons (PAHs) and compounds with extended heterocyclic structures. One commonly used approach is synchronous scanning fluorescence spectrophotometry (SFS). In this technique, simplified spectra are generated by scanning of both excitation and emission simultaneously with a fixed wavelength difference. The limit of SFS detection of AFB_1-DNA adducts was 1 AFB_1 residue per 1.5×10^7 normal nucleotides (12), which is comparable to that of USERIA. While SFS provides a sensitive means for the detection of selected DNA adducts, its ability to distinguish between closely related compounds is limited. That is, the resolving power of SFS is not high enough to determine a single adduct in a complex mixture. In addition, like other fluorometric assays, SFS is vulnerable to interference by other fluorescence-emitting substances. Therefore, an additional sample cleanup step prior to SFS analysis, such as the use of immunoaffinity chromatography, is often necessary. An alternative approach is based on HPLC coupled with fluorescence detection. For example, a two-step fluorometric HPLC assay has been developed for the detection of benzo(a)pyrene diol-epoxide-DNA adducts in human lung samples (13). Neither immunochromatography nor solvent extraction is used, and the technique achieves a >90% recovery of the adducted DNA. The detection limit of this assay using 100–500 μg of DNA is 1 adduct per 10^8 normal nucleotides.

^{32}P-Postlabeling Assay

The third and increasingly popular approach to DNA adduct analysis is the ^{32}P-postlabeling assay (for a review, see 14). The version of this assay currently in use involves the following sequence of biochemical steps: 1. enzymatic DNA digestion to the deoxyribonucleoside-3′-monophosphates (dNps); 2. labeling the adducted nucleotides with [^{32}P]ATP and T_4 polynucleotide kinase (T_4 PNK) to produce the deoxyribonucleoside-3′,5′-biphosphates (dpNps); 3. enrichment of adducted nucleotides by the elimination of normal nucleotides; and 4. separation and detection by high-res-

olution thin-layer chromatography (TLC) and autoradiography, respectively. A number of advantages make ^{32}P-postlabeling assay the most widely used method in DNA adduct studies. First, the assay is noted for its sensitivity. Detection limits of 1 adduct in 10^6-10^8 normal nucleotides are readily attained with bulky and hydrophobic adducts using the original procedure published by Randerath and his colleagues (15). More recently, new procedures have been developed to enhance the sensitivities to a reported 1 adduct per 10^{10} normal nucleotides. Second, minimum DNA requirements for the assay have been reported to be as low as 1–10 μg, an important consideration in view of the limited quantities of human DNA generally available to researchers. Third, it is a versatile assay, as prior knowledge of adduct composition is not necessary. Thus, it is capable of detecting multiple adducts resulting from exposures to complex mixtures. On the other hand, with an unknown adduct, absolute quantitation may not be possible with the ^{32}P-postlabeling assay. This is mainly because the efficiency of the T_4 PNK reaction differs with the various DNA adducts. In addition, only those adducts that can be chromatographically separated will be quantified reliably.

The most crucial but often overlooked phase of the ^{32}P-postlabeling assay is adduct enrichment. The standard and ATP-deficient versions of the assay lack an enrichment step. At present, the most commonly used techniques are butanol extraction and nuclease P_1-mediated enrichment. Both were developed after publication of the standard assay. The butanol procedure (16) involves an organic extraction in which adducted dNps are partitioned from undamaged nucleotides in the presence of a phase transfer agent (tetrabutyl ammonium chloride) at acid pH. In the nuclease P_1 procedure, normal dNps are preferentially hydrolyzed to deoxyribonucleosides which are not substrates for T_4 PNK (17). Nuclease P_1 enrichment has the advantage of being much less time consuming than butanol extraction. However, certain aromatic amine-adducted nucleotides, e.g. the deoxyguanosine-C8 adduct of 4-aminobiphenyl (18, 19), appear to be highly susceptible to nuclease P_1 dephosphorylation, a susceptibility resulting in little or no recovery.

The ^{32}P-postlabeling assay is best suited for detecting bulky and hydrophobic adducts, e.g. those produced by the PAHs or aromatic heterocyclics. In contrast, its success with the smaller adducts (such as those generated by the alkylating agents) has been questionable. Several laboratories have recently reported new procedures that significantly improve the efficiency and utility of the ^{32}P-postlabeling assay in analyzing these small ("unbulky") adducts. For example, a ^{32}P-postlabeling assay incorporating both ion-pair reverse-phase HPLC and weak anion exchange HPLC has been found to be sensitive and highly specific for the detection of 7-methyl-2′-deoxyguanosine-3′-monophosphate (7-MedGp) and 7-ethyl-2′-deoxyguanosine-3′-mono-

phosphate (7-EtdGp) in human lung samples (19). The detection limit using 100 μg of DNA was 1 adduct per 10^8 normal nucleotides. The overall recovery with this method was 58% for 7-MedGp and 98% for 7-EtdGp. A similar detection limit has been attained for the detection of O^6-MedG with a procedure combining immunoaffinity chromatography with ^{32}P-postlabeling (11, 20).

Autoradiography is most commonly used to locate radioactive spots on a chromatogram. These areas are then excised for quantitation by liquid scintillation or Cerenkov counting. However, on a chromatogram with multiple adducts, it can be difficult to quantify partially resolved adducts and determine background radioactivity levels. Recently, a technique known as storage phosphor imaging, which has been used for medical diagnostic radiography, was adapted for mapping and quantitation of DNA adducts on chromatograms generated by the ^{32}P-postlabeling assay (21). Storage phosphor imaging yields a substantial improvement (about 10-fold) in sensitivity compared to screen-enhanced autoradiography for the detection of ^{32}P. In addition, it exhibits a greater linear range of response, has a resolution that compares favorably to film, and has a lower background radioactivity than does liquid scintillation counting. More recently, a method of analyzing ^{32}P-postlabeled DNA adducts on reverse-phase HPLC with on-line ^{32}P detection has been developed (22). This method permits direct injection of the ^{32}P-postlabeling mixture into the analytical system without prior purification. The background radioactivity is kept at a moderate level by the use of high salt concentration (2 M ammonium formulate buffer) in the HPLC eluent. Another alternative approach to TLC analysis has been reported in which the ^{32}P-postlabeling mixture is separated using HPLC and then isolated by immunoprecipitation with monoclonal antibody (10).

Protein Adducts

Protein adducts have been found to be invaluable biomarkers of exposure (for a review, see 23). To date, the proteins most studied in this connection are those found in circulating blood, namely, hemoglobin (Hb) and albumin (Ab). From the quantitative viewpoint, these protein adducts are better dosage indicators than are DNA adducts. While most DNA adducts in human cells are efficiently repaired via the excision repair enzyme pathway, Hb adducts tend to persist for the lifetime of circulating erythrocytes. In addition, both serum Ab and Hb are abundant in blood and can be isolated easily. The overall reactivity of tissue proteins to common monofunctional alkylating agents such as ethylene oxide is 1–5 times higher than that of DNA. Thus, the use of protein adducts as biomarkers of exposure means a gain of at least three orders of magnitude in resolving power (24). Hb or Ab adducts each offer distinct advantages as biomarkers. Hb is longer-

lived than other blood proteins. The turnover rate of Hb is related to the life span of the erythrocytes (approximately 120 days in humans). Thus, Hb adducts may be detected several months after the last exposure period. In contrast, Ab adducts can only be used for assessing recent exposure because of Ab's faster turnover in humans (it has a half-life of 20–25 days). However, Ab adducts are generally more abundant than those of Hb—perhaps because (*a*) Ab exists in serum and thus is not isolated by the erythrocyte membrane, and (*b*) Ab is synthesized in the liver, a major site of xenobiotic activation.

Both Hb and Ab adducts are commonly analyzed using chemical methods, though immunoassays are also applicable. Three general strategies have been employed in the chemical analysis of protein adducts. One strategy involves the isolation of the adducted protein of interest, which is then subjected to mild acid- or base-catalyzed hydrolysis to release the adducting species from the protein. The free molecule is then isolated and quantified by conventional analytical methods such as HPLC or gas chromatography-mass spectrometry (GC-MS). For example, mild hydrolysis has been used in the analysis of Hb adducts generated by tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (25) and 4-aminobiphenyl (26). A second strategy starts with a harsh and complete acid hydrolysis of the protein adduct to individual amino acids. The adducted amino acids are then isolated and quantified by conventional means. For example, a S-phenylcysteine adduct resulting from occupational exposure to benzene has been detected after subjecting serum Ab to a complete hydrolysis in 6 N HCl at 110° C for 24 hr (27). A third strategy was recently developed specifically for the release of adducted N-terminal valines in Hb using a modified Edman degradation method with pentafluorophenyl isothiocyanate (28). The pentafluorophenylthiohydantoin derivative of N-alkylvaline was analyzed by GC-MS. This approach offers high sensitivity as well as mild degradation conditions. For example, with ethylene oxide, a detection limit of 1 adduct per 5×10^6 N-terminal valines or globin chains has been achieved using this approach.

Several experimental observations from biomonitoring studies of protein adducts are worthy of mention. In general, the method of choice for isolating Ab from plasma has been affinity chromatography, but in special cases other means may be preferred. For example, a significant loss of the major AFB_1-Ab adduct in humans occurred (29) owing to the instabililty of this lysine adduct during the cibacron blue affinity chromatography step. Thus, in this case, a rapid ammonium sulfate precipitation step is preferable to the lengthy chromatography purification procedure.

Species specificity of the proteins is another area of interest. The cysteine residue at position 34 in human serum Ab is found across several species

and represents the only reactive sulfhydryl group in this protein (27). On the other hand, the major Hb adducts generated by the PAH fluoranthene in rats have been found to result from binding to the sulfhydryl group of the β-125 cysteine residue (30). Because this β-125 cysteine residue is peculiar to rats, guinea pig, and some but not all mouse Hbs (23), adduct formation in the rat at this site is not relevant for extrapolation to humans.

Excised Macromolecular Adducts

Both nucleic acids and proteins are broken down in the body by various catabolic processes under normal physiological conditions. Thus, the adducted portion of the degraded macromolecules is eliminated from the body via urination. Even though concentrations of the breakdown products in urine are extremely low, analysis of these excised macromolecular adducts is aided by the relatively large volumes of urine that can easily be obtained from exposed individuals. Even though these chemical species only reflect the most recent exposures to xenobiotics, valuable molecular epidemiology information has been derived by using these biomarkers in human populations. For the quantitation of excised DNA and protein adducts, both conventional analytical techniques (e.g. HPLC, GC, MS, or GC-MS) and immunoassays have been used. For example, increased excretion of both N-nitrososarcosine and N-nitrosothiazolidine 4-carboxylic acid has been established by GC-thermal energy analysis of human urine samples from a population in northern China with a high incidence of esophageal cancer (31). On the other hand, a monoclonal antibody–based ELISA in combination with an immunoaffinity chromatography clean-up procedure has been developed for the quantitation of urinary 3-methyladenine in human biomonitoring studies (32).

MOLECULAR DOSIMETRY OF MACROMOLECULAR ADDUCTS AND CARCINOGENESIS

For dosimetry of carcinogenesis, the actual amount of xenobiotic absorbed (internal dose) and the amount needed to induce a biological consequence (biologically effective dose) must be considered.

Internal Dose

The internal dose may be defined as the amount of the xenobiotic absorbed into the organism. It can be determined by measuring the xenobiotic or its metabolite in the body fluids (blood, milk, amniotic fluid, saliva, bile), tissues (hair, nails, fat, teeth), or excretory products (feces, urine, sweat). Owing to their accessibility and quantity, blood and urine are most commonly analyzed. For example, plasma cotinine levels serve as an indicator of

tobacco smoke exposure, p-nitrophenol in urine as an indicator of exposure to parathion, aflatoxin B_1 in urine as an indicator of exposure to dietary aflatoxin, and chlorinated pesticides and polychlorinated biphenyls in adipose tissues as markers of exposure to these lipophilic chemicals. Pharmacokinetic data such as half-life, circulating peak, and cumulative dose may also be generated. For reproductive toxicants, concentration of the compound in follicular fluids, amniotic fluids and cells, and/or semen may be determined.

Measuring internal dose avoids the uncertainties of determining the concentration of the toxicant in the environment, the route of exposure, the duration of exposure, the frequency of exposure, and the toxicant absorption rate; but the technique also has disadvantages. It overestimates the biological effect of chemicals that require activation, because the metabolic rate is not taken into consideration, and it underestimates the biological effect of chemicals that act directly, because these chemicals act on the tissue of first contact.

Biologically Effective Dose

The biologically effective dose may be viewed as the amount of chemical needed to induce a biological response or consequence. The interaction of electrophiles with nucleophiles is recognized as the primary step in carcinogenesis. DNA-adduct formation was first demonstrated by the Millers (33) in the studies of activation of carcinogenic compounds such as aminoazo dyes, acetylaminofluorene, safrole, and aflatoxin B_1. The adducts represent the amount of material interacting with the critical subcellular, cellular, and tissue targets and are considered to be the biologically effective dose of xenobiotics. Researchers have found the nucleophilic sites on DNA at which electrophiles from direct acting alkylating chemicals or from metabolically activated intermediates bind covalently to form adducts (34). Binding depends on the nucleophilicity of the reaction site and the electrophilic strength of the reactive chemical. The 7-methyldeoxy-guanosine adducts are not directly mutagenic (35). Adducts formed at O^6-guanine, O^2- and O^4-thymidine, or O^2-cytosine are considered promutagenic. Thus, in DNA-adduct quantitation it is essential that the position of the adduct be identified (36). However, N^7-methylguanines are more abundant and, because they are repaired more slowly than O^6-methylguanines, persist longer. They are therefore more readily available for assay (37) and may serve as a surrogate marker for the O^6-methylguanines.

Unrepaired promutagenic adducts can cause cell death; they can also cause the initiation of neoplastic development by inducing DNA damage and gene mutation that lead to oncogene activation, perturbation of the regulation of cell proliferation, triggering of uncontrolled cellular growth. Even if repair occurs, repair errors may produce a similar outcome. Thus, measurement of DNA

adducts is a relevant indicator of the biologically effective dose (molecular dose) of a carcinogen and is more mechanistically relevant to carcinogenesis than are internal dose determinations. Because adduct levels also compensate individual or species differences in absorption, distribution, metabolism, pharmacokinetics, and DNA repair, DNA-adduct determinations have been employed to evaluate occupational and environmental exposures in humans, which are usually intermittent and involve low doses.

Because target tissue DNA in humans is inaccessible, DNA adducts in white blood cells, sperm, placenta, or skin; protein adducts in hemoglobin and albumin; and urinary (DNA base) adducts have been used as surrogates for exposure estimates. Hcmoglobin and albumin adducts are especially useful because they are accessible, plentiful, persistent, and stable (not subject to repair). Their quantity is also dose related and correlates well with DNA-adducts. The quantitative relationships between adducts in the DNA of target and surrogate tissues must, of course, be ascertained; such relationships vary for different chemicals and species.

Various assay methods have been developed to determine DNA and protein adducts. These methods (see above) are sensitive, specific, and quantitative, and they can be applied to body fluids or small samples of cells.

ANIMAL MODELS FOR DOSIMETRY OF MACROMOLECULAR ADDUCTS AND CARCINOGENESIS

N-Nitrosamines

Nitrosamines form adducts with guanine at the N^7 and O^6 sites and phosphotriesters with the phosphate of nucleic acids. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a specific carcinogen in tobacco smoke, formed by nitrosation of nicotine. In rat carcinogenesis studies, NNK in drinking water induced tumors in the lung, liver, nasal cavity, and exocrine pancreas (38). α-Hydroxylation of NNK produces unstable intermediates which upon spontaneous decomposition produce methyldiazo-hydroxide, keto aldehyde, 4-(3-pyridyl)-4-oxobutanediazohydroxide, and formaldehyde. Methyldiazohydroxide methylates hemoglobin, forming unknown adducts. It also methylates DNA to form N^7-methylguanine, O^6-methylguanine, and O^4-methylthymidine. The oxobutanediazohydroxide reacts with hemoglobin, producing a carboxylic ester adduct which upon hydrolysis yields 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). Reaction of the oxobutanediazohydroxide

with DNA gives rise to an adduct which upon acid hydrolysis also yields HBP. The derivatized HPB-pentafluorobenzoate is quantifiable by negative ion chemical ionization mass spectrometry after HPLC separation (38).

In groups of rats treated with 4 daily interperitoneal injections of [^{3}H]NNK ranging from 3 to 10,000 μg/kg/day and sacrificed 4 hr after the last injection, binding of NNK to hemoglobin, as measured by release of HPB, increased linearly with dose. However, linear relationships between dose and methylation of lung and liver DNA were not observed (38). In a similar study in which rats were treated subcutaneously with NNK at 0.1–100 mg/kg/day, 3 times a week for 4 weeks, the O^6-methylguanine levels in Clara cells, determined by fluorescence-linked HPLC, reached a steady state in the lung after 3 weeks. The adduct levels in Clara cells were nonlinear in relation to dose. This nonlinearity was attributed to the high affinity of the O^6-methylguanine for NNK activation at low concentrations (39, 40). Other factors, such as P-450 isozyme activity, repair, and cell proliferation, may also contribute to the nonlinearity observed. Lung tumor incidences correlated well with lung O^6-methylguanine levels (40).

N-nitrosodimethylamine (NDMA) is a rat lung and liver carcinogen; it forms O^6-methylguanine adducts in these tissues. The O^6-methylguanine levels reached a steady state in the lung after 3 weeks of NDMA administration. At equimolar doses, NNK was more tumorigenic than NDMA (39, 41, 42). However, Hecht et al (42) reported the O^6-methylguanine levels in the lung were significantly higher following administration of a single dose of NDMA than following an equimolar dose of NNK. On the other hand, repeated subcutaneous injections of NNK induced a higher O^6-methylguanine level in the lung than did NDMA at equimolor doses (39). Adduct levels induced by a single dose differed from those induced by multiple doses, and adduct levels obtained after single doses alone cannot predict the degree of carcinogenicity. The affinities of activation and repair enzymes and cell proliferation also play a role so that steady-state adduct levels correlate well with tumor incidence.

Treating rats with NDMA or with a combination of aminopyrine and nitrite resulted in dose-related urinary excretion of N^7-methylguanine. Using chromatographic separation and radioimmunoassay, Degan et al (37) showed that the levels of 7-methyldeoxyguanosine and O^6-methyldeoxyguanosine in rat peripheral blood cells were similar to those in liver DNA up to 24 hr after oral NDMA administration. The data indicated that adducts in urine and in peripheral blood lymphocyte DNA may serve as surrogates for adducts in liver, the target organ.

In rats treated with varying doses of diethylnitrosamine (DEN) for up to 70 days, the concentration of O^4-ethyldeoxythymidine (O^4-EdT) adducts in liver

determined by RIA was dose related at up to 40 ppm. Liver tumor incidence in the DEN-treated rats correlated well with adduct levels (36, 43).

Polycyclic Aromatic Hydrocarbon

Polycyclic aromatic hydrocarbons such as benz(a)pyrene (BP) are activated by cytochrome P-450 and epoxide hydrolase to BPDEs which bind covalently to the exocylic amino group of guanosine (N^2 position) or react with phosphate to produce phosphotriesters.

Ashurst et al studied the relationship among dose of BP, quantity of DNA adduct, and tumor incidence (44). Hydrolyzed epidermal DNA isolated from mice treated topically with varying doses of [^{3}H]BP were separated by HPLC and their radioactivity was determined by scintillation counting. The results showed linear dose responses in adduct levels and incidences of skin papilloma. In separate studies, Pereira et al (45) and Albert et al (46) also reported that BP-induced skin tumor incidence was linearly correlated with dose.

Adriaenssens et al (47) and Stowers & Anderson (48) demonstrated that the BPDE-DNA adduct levels in the stomach, lung, liver, brain, colon, muscle, and blood of mice and rabbit appear to be similar in the same animal regardless of whether the tissue is the target tissue or not. Adriaenssens et al (47) further demonstrated that the adduct levels in the stomach, the target tissue for oral administration, were linear to dose; the levels in the lung and liver, the nontarget tissues, were linear at low doses of BP but nonlinear at high doses. The nonlinear dose-response relationship may be due to P-450 isozyme activity, repair, and cell proliferation at high doses (38, 39).

Aromatic Amines

Aromatic amines form major adducts at C8 of guanine and minor adducts at the exocyclic nitrogens and oxygens of guanine and adenine. Aromatic amines are known to induce tumors in liver, urinary bladder, mammary gland, and intestine in experimental animals (50).

The relationship between the adduct-dose, N-(deoxyguanosin-8-yl)-2-aminofluorene (C8-dG-DNA), and the liver and urinary tumor incidences in female BALB/c mice exposed to 2-acetylaminofluorene (2-AAF) was investigated by Poirier et al (51). The C8-dG-DNA adducts were identified by HPLC-radioimmunoassay or ^{32}P-postlabeling. The adduct levels in liver and bladder were found to be linear in relationship to 2-AAF dose; the levels were higher in bladder than in liver. The relationship between liver tumor incidence and adduct levels was linear; however, the relationship between bladder tumor incidence and DNA adduct level was nonlinear (52). The data suggested that in liver carcinogenesis a single adduct-related step

is involved, whereas in bladder carcinogenesis multiple adduct-related events are involved. These steps may be related to the affinity of the aromatic amines to metabolic enzyme, repair enzyme, cytotoxicity, and/or cell proliferation. Similar observations were obtained in rats (50–52).

4-Aminobiphenyl induced high liver and low bladder tumor incidences in female BALB/c mice and low liver and high bladder tumor incidence in male BALB/c mice (53). In a study using female BALB/c exposed to 4-aminobiphenyl in their drinking water, the liver N-(deoxyguanosin-8-yl)-4-aminobiphenyl (C8-dG-ABP) levels and the dose had a linear relationship up to 75 ppm. The relationship between liver adduct levels and liver tumor incidence was also linear (54). However, the adduct levels in bladder were nonlinear to the dose. In male BALB/c mice, the adduct levels in bladder were linear with respect to dose, but the relationship of adduct levels and bladder tumor incidence was nonlinear (54). Liver C8-dG-AABP levels were also dose related in male BALB/c mice, but owing to low tumor incidence their relation to liver tumors was not evaluated.

The 2-AAF and 4-iminobiphenyl studies illustrate that the relationships among dose, adduct level, and tumor incidence are complex. The relationship varies depending on sex, biology of specific target organs, and properties of the adducts. At the same dose level, the 2-AAA-DNA adducts appear to be more efficient than are 4-aminobiphenyl-DNA adducts in inducing liver tumors (54).

Aflatoxin B_1

Aflatoxin B_1 (AFB_1) is converted by cytochrome P-450 enzymes to the reactive electrophilic 8,9-epoxide. The 8,9-epoxide reacts with liver DNA and serum albumin, forming aflatoxin-N^7-guanine (AFB-N^7-gua) and lysine adducts, respectively. Experimental animals fed AFB_1 developed liver, kidney, and colon tumors. Binding of the reactive AFB_1 metabolite to lymphocyte DNA or to hemoglobin is poor.

In rat studies, many investigators have shown a linear relationship between dietary aflatoxin dose and binding to serum albumin and liver DNA (55–57). Dietary AFB_1 dose also correlated well with urinary and liver AFB-N^7-gua levels (58). AFB_1-hepatic DNA adduct levels in rats and rainbow trout correlate well with incidence of liver tumors. These results demonstrated that AFB_1-albumin and urinary AFB-N^7-gua levels can serve as surrogates for the target liver DNA adduct level.

Risk Extrapolation

In experimental settings where it is possible to establish the dose levels of the carcinogen, exposure frequency, experimental conditions, rate constant for adduct formation, and half-life of the adduct, the linear relationship

among carcinogen exposure, adduct formation, and tumor incidence can be demonstrated. However, it is still difficult to predict cancer risk in experimental animals based on adduct levels because of the multistage nature of carcinogenesis (affinities of metabolic enzyme, DNA repair, cell proliferation, promotion activity) and because little is known about the ultimate lesion induced by a specific type of adduct at a specific tissue dose. The mouse lung O^6-methylguanine levels induced by NDMA and NNK (discussed above) exemplify this difficulty. Even less is known about how to monitor exposure to nongenotoxic chemicals because the preneoplastic events that occur during the process of carcinogenesis caused by these chemicals are poorly understood. Nevertheless, Gaylor et al (49) have reported the DNA adduct levels of several carcinogens that corresponded to a 50% tumor incidence in animal studies. Attempts to correlate adduct levels in surrogate tissues to tumor incidence in target tissue have not yet been reported. More data relating adduct levels with tumor incidence in different species are needed before DNA adducts in target or surrogate tissues can be used to assess tumor risk.

The relationship between molecular dose and tumor incidence in humans is difficult to establish because of uncertainties about exposure history (chemical identity, exposure to mixtures, variations in dose, exposure frequency, interval since exposure); low disease specificity of the causative agent; and interindividual variation in age, sex, genetic factors, life-style habits (diet, alcohol, and drug use), immune status, and environmental conditions. Thus, in developing potential biomarkers for monitoring exposure in humans, a wide dose range of the toxicant must first be studied in at least two species of experimental animals to provide coefficients relating dose and response and to demonstrate that the coefficients do not vary between species (59). Dosimetry data and dose-response relationship in different tissues should be evaluated under chronic exposure to obtain steady-state kinetics of adduct formation (43, 60). The kinetics of adduct removal in different tissues after cessation of exposure should also be evaluated to determine tissue and species differences in repair. Furthermore, the pattern of accumulation and removal of adducts, as well as dose-response curves during intermittent exposures, should be explored. Data from studies with human tissues and cells in vitro and in vivo can be incorporated into a dose-response computer model for extrapolation between species. A computer model for the formation and removal of hemoglobin adducts has recently been described (61). These experimental procedures are especially necessary when surrogate biomarkers are used. At present, adduct levels are useful only for identifying high-risk population groups. The goals of using adduct levels to monitor human exposure and to predict cancer risk of individuals are still distant.

MACROMOLECULAR ADDUCTS AND HUMAN EXPOSURE TO TOXICANTS

Cancer is among the most feared diseases in the modern industrialized world. Often a progressive fatal condition, cancer is one of the three leading causes of death in most countries. Estimates have linked 80–90% of human cancer to "extrinsic" or "environmental" factors (62). Thus the incidence of human cancer could be minimized if the causative extrinsic factors could be identified and if humans could be protected from them either by elimination of exposure or by reduction of host susceptibility.

The onset of cancer is often delayed 10 or more years after exposure to a chemical carcinogen. Such a latency period makes retrospective epidemiological studies difficult and confounds attempts to measure exposure to environmental agents or to trace the chronology of tumor progression. However, sensitive methods are now available to quantitate an individual low-level environmental exposure to a particular carcinogen. The data produced by these methods may prove critical to establishing a causal relationship between an agent and cancer.

Significant progress has been made in understanding the complex causes of the major cancers. With many chemical carcinogens, the critical initial event in tumor induction appears to be covalent binding of the ultimate carcinogen to DNA, which leads subsequently to mutation (63). Recent advances in analytical biochemistry make it possible to measure levels of DNA adducts in humans. However, practical considerations limit the types of target tissue available for analysis of DNA adducts in epidemiologic studies. Because the reactive derivatives of carcinogens are strong electrophilic reagents, many will react with the nucleophilic residues present in cellular macromolecules (DNA, RNA and proteins). Many epidemiologic studies have used surrogate tissues for indirect measurement of DNA adducts in target tissue. Because differences in metabolic activation, deactivation, and cell proliferation exist among tissues, questions of whether these surrogates actually provide reliable estimates of DNA adduct levels in target cells still remain.

Molecular Dosimetry of Human Aflatoxin Exposure

Primary liver cancer is one of the leading causes of cancer mortality in Asia and Africa. Several epidemiological studies have been conducted to relate the incidence of primary liver cancer to hepatitis B virus (HBV) infection, estimated dietary intake of aflatoxin, alcohol intake, and other possible risk factors in different parts of the world (64–66). However, several questions remain unanswered. Does a synergy occur between aflatoxin and hepatitis B virus? What is the public health impact of aflatoxin exposure

in the absence of chronic HBV infection? These questions can be addressed by measuring individual human exposure to aflatoxin.

The aflatoxins are metabolized primarily by the microsomal mixed-function oxygenase system. These enzymes catalyze the oxidative metabolism of aflatoxin B_1 (AFB_1), forming various hydroxylated derivatives as well as a highly reactive epoxide metabolite, which can covalently bind to DNA and proteins (67). The structures of the major AFB_1-DNA and AFB_1-albumin adducts have been elucidated (68, 69).

Antibodies produced to recognize specific AFB_1-DNA adducts have been utilized in sensitive enzyme-linked immunosorbent assays (ELISA) for adduct quantitation in surgically removed human livers, placentas, and cord bloods (9, 70–72). A quantitative indirect immunofluorescence method has also been developed that uses the same antibodies to measure AFB_1-DNA adducts in human liver tissues (71, 73–74). This method can detect adducts down to a level of 1 in 10^6 nucleotides in small numbers of cells. Future case-control studies will find it useful for determining whether adduct levels are higher in liver cancer patients than in controls.

Antibodies developed to recognize specific AFB_1-lysine adducts have been used to quantitate levels of the covalent adducts of AFB_1 in albumin in human subjects (29, 75–79). Because it is obviously not possible to obtain the target tissue from healthy individuals, these studies utilized protein adduct levels as a marker of internal dose of aflatoxin exposure. The half-life of albumin (18–21 days in humans) will allow accumulation of adducts and thus the AFB_1-lysine levels will indicate an integration of exposure over a period of weeks. This measurement may not reflect sufficient duration to be of value in epidemiological case-control studies but would be appropriate for correlation and cohort studies for human tumor risk assessment, especially if repeated blood samples were obtained.

A study by Bennett et al (80) has established that the excretion of AFB_1-N^7-gua adduct into the urine occurs in a dose-dependent manner following single-dose exposures of rats to AFB_1 exposures. With this approach, a strong interaction between serological markers of chronic HBV infection and aflatoxin exposure in liver-cancer risk was found in a prospective study in Guangxi (81) and in Shanghai (82). Because of the short half-life ($t_{1/2}$ = 10 hr) of the AFB_1-N^7-gua adduct, this excised adduct in urine probably reflects DNA damage mediated by recent AFB_1 exposure. That the most toxicologically relevant metabolite, AFB_1-N^7-gua, is a minor urinary metabolite further complicates the routine, large-scale analysis of urine in general epidemiological studies. However, measurement of the urinary levels of AFB_1-N^7-gua should still provide easy and rapid means to assess the efficacy of chemoprotective interventions in aflatoxin-exposed individuals.

Molecular Dosimetry of Polycyclic Aromatic Hydrocarbon (PAH) Exposures in Human

PAHs have long been considered potential human health hazards. In humans, PAHs have been linked to increased risk of lung cancer in smokers and in occupational groups such as coke-oven workers (62, 83). Human exposure to PAHs occurs principally through inhalation of tobacco smoke and polluted air. Because it may also occur through the ingestion of contaminated and processed food and water, and through dermal contact with soot, tar, and oil, however, total human exposure is difficult to assess by air measurement alone. PAHs exert their biological effects through covalent interaction with DNA, so the presence of bound PAH adducts is a more appropriate indictor of human exposure.

The ^{32}P-postlabeling method developed by Randerath (84) can be applied to detect complex mixtures of DNA adducts, such as those PAHs derived from cigarette smoke and occupational exposures, with very high sensitivity (1 adduct per 10^9-10^{10} nucleotides) (16, 17). This is the most widely used measure of DNA adducts in humans today. Many studies in humans have applied ^{32}P-postlabeling to quantitate PAH-DNA adducts in different tissues from occupational groups and smokers (85–95). Elevated levels of DNA adducts have been observed in workers with high occupational exposure to PAHs and in smokers.

The major advantages of this method are that it requires low microgram amounts of DNA for analysis and can detect structurally unknown adducts. The technique's major limitation is that accurate quantitative and qualitative measurements are achievable only when appropriate authentic adducts are available for use in recovery determinations and as chromatographic standards. Thus, using ^{32}P-postlabeling requires careful epidemiological studies to determine clear quantitative and qualitative relationships between exposure biomarkers and the tumor incidence.

Incomplete combustion of organic materials, including fossil fuels, is a major source of PAHs. As a constituent of combustion mixtures, benzo-(a)pryrene (BP) is generally used as an indicator of total PAH concentrations. BP-DNA adduct has, therefore, been used as an indicator of total PAH-DNA adducts. Antibodies against benzo(a)pyrene-diol-epoxide-I-DNA (BPDE-I-DNA) adducts have been developed (96–98) and applied to population exposure studies. These antibodies may also cross-react with other DNA adducts whose stereochemistry is similar to that of BPDE-I-DNA, such as chrysene and benz(a)anthracene (99). Positive reaction with human DNAs would indicate the presence of multiple PAH-DNA adducts in human samples. With sensitive immunoassays, elevated levels of PAH-DNA adducts in white blood cell (WBC) DNA have been detected in workers with high

occupational exposure to PAH and in residents of the highly industrialized region. However, no significant difference in PAH-DNA adducts is detected in WBC or placental DNA from smokers and nonsmokers. Unless prior separation of adducts is employed, the absolute values obtained for human samples may reflect measurement of multiple, chemically similar DNA adducts.

Both ^{32}P-postlabeling and immunoassay have been used successfully in studying human DNA-adduct formation in an occupational setting with or without the help of exposure monitors (87, 88, 90–93, 95, 100). Although the absolve values of the quantities of BPDE-I-DNA adducts obtained by these two assays were different, they were highly correlated. Thus, because these two assays are based on different principles, comparisons of their absolute values should be cautious. It is noteworthy that adduct levels in the lung DNA and in total WBC DNA do not correlate (44, 101, 102). These findings stress the limitations of using total WBC for DNA adduct determinations in future risk assessments in humans. Because the levels of DNA adducts are dependent on pharmacodynamic and pharmacokinetic parameters, DNA repair rates, cell turnover, and adduct stability, better correlations might have been obtained if a specific cell type (e.g. lymphocytes, monocytes) had been used. Recent studies support this suggestion (103–105).

Molecular Dosimetry of Clinically Used DNA Damage Agents

Patients treated with genotoxic agents could provide a unique situation for the validation of different methods for DNA-adduct detection because such patients are exposed to high, well-defined doses, and unexposed control populations can easily be identified.

8-Methoxypsoralen (8-MOP) plus ultraviolet A light, termed PUVA, is used in the treatment of psoriasis and vitiligo (106); it is also used extracorporeally as a cytoreductive treatment in the leukemic phase of cutaneous T-cell lymphoma (107). PUVA is a known mutagen and animal carcinogen, producing squamous (108) and basal cell carcinomas in the skin (109). Several reports have suggested a higher risk of cutaneous carcinoma in treated than in untreated patients (110). A panel of antibodies that specifically recognize 8-MOP-DNA monoadducts and crosslinks has been developed (111, 112) and used to quantitate adduct levels in human samples (113). The 8-MOP-DNA antibodies have also been used for flow cytometric analysis of adducts formed in human keratinocytes during different stages of the cell cycle (114). This study indicated that adduct formation during replication may result in increased accessibility to the DNA. Thus, it is

possible to study basic mechanisms of chemical carcinogenesis with adduct-specific antibodies.

cis-Diamminedichloroplatinum (II), *cis*DDP, has been used successfully in the chemotherapy of several types of cancer, including testicular (115) and ovarian (116) varieties. Antineoplastic activity is thought to be related to the formation of *cis*DDP-DNA damage. A number of polyclonal and monoclonal antibodies recognizing *cis*DDP-DNA adducts have been developed (117–119) and used to monitor adduct formation in WBC DNA of testicular and ovarian cancer patients receiving *cis*DDP or carboplatin (120–125). These studies indicate that higher *cis*DDP-DNA adduct levels correlate with disease response, and the adduct can be removed slowly after final treatment with *cis*DDP. Thus, studies involving patients treated with genotoxic chemotherapeutic agents offer several benefits. They can help in the development and validation of immunologic methods for measuring DNA adducts, and they can shed light on the process of drug resistance.

Molecular Dosimetry of Alkylating Agents in Human Exposure

Exposure of humans to alkylating N-nitrosamines results from the presence of these carcinogens in the environment (e.g. through cigarette smoking, diet, and occupational exposure) and from their formation in vivo from various precursors (e.g. nitrates, nitrites, and secondary amines). These chemicals have been shown to be carcinogenic in a variety of animal species (126). However, data linking a particular type of human cancer with exposure to this group of carcinogens have been difficult to obtain. The low sensitivity of the epidemiological studies performed and the difficulty in defining an exposure quantitatively have impeded the causal inference. However, our recently acquired ability to measure specific alkylation products in DNA may increase the sensitivity of future epidemiological studies.

Antibodies to many alkylation DNA adducts have been developed and applied to human exposure studies. Umbenhauer et al (127) have observed higher levels of O^6-methyldeoxyguanosine (O^6-medGuo) in the DNA of esophageal or stomach mucosa of patients from Linxian, where high exposures to N-nitrosamines have been suspected. A study by Huh et al (128) indicates a higher level of O^4-ethylthymine (O^4-etdT) in the DNA of cancer patients, particularly liver cancer, than in that of patients with noncancerous diseases. This seems a highly promising approach to epidemiological inquiries into a causative role of nitrosamines in human cancer.

N^7-Methylguanine (7-MeGua) and N^3-methyladenine (3-MeAde), the products of efficient DNA repair after exposure to N-nitrosamines, have been found in urine (129, 130) and can be used as a biological marker for exposure to alkylating agents. For practical purposes, the detection of

carcinogen-derived 7-MeGua in urine is rendered impossible by the presence of large amounts of RNA-derived 7-MeGua. For 3-MeAde, no such background appears to exist, and its use as a marker is possible. Immunoaffinity chromatography followed by ELISA has been used in a series of human dietary intervention experiments (32). Because of its apparent natural occurrence, 3-MeAde itself may be of limited use as a marker in subjects eating uncontrolled diets, but it is extremely useful in model studies.

Because of its general availability and its long lifespan (which permits the determination of cumulative dose), hemoglobin (Hb) has been used to monitor tissue doses of ultimate carcinogens (131). Alkyl residues in Hb have been studied thoroughly by measuring alkylated cysteines, histidines, or alkylated N-terminal valine using GC/MS (132–136). N-hydroxyethylvaline (HOEtVal) in Hb has been demonstrated to be a good biological marker of ethylene oxide exposure associated with cigarette smoke (135, 136). Thus, alkyl protein adducts will also be a promising marker in future risk-assessment studies.

CONCLUDING REMARKS

Biological monitoring for human exposure to environmental and occupational carcinogens by means of macromolecular adducts can greatly strengthen traditional epidemiologic studies by providing more accurate assessment of exposure. The new analytical procedures for the determination of DNA and protein adducts, and an understanding of the dosimetry of the adducts, provide selective approaches for the detection and quantification of specific exposures. By virtue of their high resolving power and sensitivity, these methods can help to identify the agents that cause human cancers, to assess the efficacy of chemoprotective interventions, to elucidate the basic mechanisms of chemical carcinogenesis, and to treat cancer patients by enabling insight into the process of drug resistance.

ACKNOWLEDGEMENTS

This work is supported in part by the MITRE Corporation (MITRE-Sponsored Research Project No. 9587). The able assistance of Mrs. Evelyn Armstrong in the coordination of this manuscript's preparation is also appreciated.

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