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Methods of DNA Adduct Determination and Their Application to Testing Compounds for Genotoxicity

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At the International Workshop on Genotoxicity Test Procedures (IWGTP) held in Washington, DC (March 25–26, 1999), a working group considered the uses of DNA adduct determination methods for testing compounds for genotoxicity. When a drug or chemical displays an unusual or inconsistent combination of positive and negative results in in vitro and in vivo genotoxicity assays and/or in carcinogenicity experiments, investigations into whether or not DNA adducts are formed may be helpful in assessing whether or not the test compound is a genotoxin. DNA adduct determinations can be carried out using radiolabeled compounds and measuring radioactive decay (scintillation counting) or isotope ratios (accelerator mass spectrometry) in the isolated DNA. With unlabeled compounds adducts may be measured by ^{32}P-postlabeling analysis of the DNA, or by physicochemical methods including mass spectrometry, fluorescence spectroscopy, or electrochemical detection, or by immunochemical methods. Each of these approaches has different strengths and limitations, influenced by sensitivity, cost, time, and interpretation of results. The design of DNA binding studies needs to be on a case-by-case basis, depending on the compound's profile of activity. DNA purity becomes increasingly important the more sensitive, and less chemically specific, the assay. While there may be adduct levels at which there is no observable biological effect, there are at present insufficient data on which to set a threshold level for biological significance. Environ. Mol. Mutagen. 35:222–233, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

Circumstances In Which DNA Binding Studies May Be Appropriate or Informative

Investigation of DNA adduct formation can be viewed as a supplementary test that may, in some instances, provide information clarifying the assessment of a compound with an unusual or puzzling profile of activity in statutory genotoxicity assays, animal bioassays, or both.

There are prominent examples of carcinogenic compounds that are uniformly negative in short-term tests for genotoxicity but for which there is nevertheless good evidence, including the formation of DNA adducts, to suggest that they are in fact genotoxic carcinogens; one such example is the antiestrogenic drug tamoxifen [Han and Liehr, 1992; White et al., 1992]. In other cases, a compound may give positive results in some in vitro tests but be negative in short-term in vivo tests. In the absence of animal bioassay data, this could raise concerns about the possible tissue specificity of metabolic activation and genotoxic effects. If the compound showed activity in in vivo tests but not in vitro, this might indicate a failure to effect efficient metabolic activation in the latter tests. How such DNA binding studies should be designed and conducted is ultimately dependent on the profile of the compound and the concerns that this raises; each case must be considered on its own merits.

The toxicological significance of DNA adducts has been the subject of a previous workshop [Nestmann et al., 1996]. Recommended procedures for some methods of detection

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have been published [Martin et al., 1993]. It is the purpose of the present article, which arose from the International Workshop on Genotoxicity Test Procedures (IGWTP) held in Washington DC, March 25–26, 1999, to identify strengths and limitations of the various DNA adduct analytical methods in use, and to consider what factors and parameters should be considered in the design of experiments to determine the DNA binding potential of a new compound.

RADIOLABELED COMPOUNDS

Strengths

The use of radiolabeled compounds permits the most straightforward determination as to whether or not DNA adduct formation has occurred. These experiments are typically conducted by administering a single dose of the radiolabeled test substance to laboratory animals. After a suitable period of time (hours to days), the animals are killed, DNA is isolated from the organs of interest, and the amount of radioactivity associated with the DNA is determined by liquid scintillation counting (the use of accelerator mass spectrometry will be considered in a subsequent section). An increase in radioactivity compared to DNA isolated from control animals can be taken as presumptive evidence for the formation of DNA adducts. Protocols for conducting experiments with radiolabeled material have been given in detail (e.g., by Lutz [1982, 1986] and by Martin et al. [1993]).

Most radiolabeled experiments are performed with either ^{3}H or ^{14}C. ^{3}H-Labeled compounds are typically less expensive to prepare and can be obtained in higher specific activities than ^{14}C-labeled chemicals. Compounds labeled with ^{14}C are less likely to lose the radiolabel through exchange processes but may present a greater radiohazard disposal problem. With both ^{3}H and ^{14}C, it may be difficult to prepare the material in the requisite amount and with sufficiently high specific activity and stability to be useful. For example, a compound with a specific activity of 1.4 Ci/mmol that binds to DNA at a level of 1 adduct per 10^{8} nucleotides will only give 100 dpm/mg DNA. This specific activity precludes the use of ^{14}C, which is only available at activities of less than 100 mCi/mmol. If less DNA is available or if it is necessary to detect lower adduct levels (e.g., in some instances a level of 1 adduct per 10^{10} nucleotides has been considered necessary [Nestmann et al., 1996]), even higher specific activities will be required.

Limitations

When preparing radiolabeled test substances, it is essential that the label be located in a position that is resistant to loss during either metabolism or adduct formation. For instance, with arylamides the amide moiety is generally removed before or during adduct formation [Beland and Kadlubar, 1990], and therefore is not a suitable location for radiolabeling. In addition, a significant proportion of arylamine adducts result from covalent linkage to the carbon ortho to the amine nitrogen [Beland and Kadlubar, 1990]; thus, this position is not suitable for tritiation.

The presence of radioactivity in purified DNA does not prove that DNA adduct formation has occurred. Depending on the type of carcinogen and the location of the radiolabel, substantial metabolic incorporation can occur into normal nucleotides (e.g., urethane [Dahl et al., 1978; Ribovich et al., 1982]). Providing that sufficient radioactivity is available, it is possible to distinguish between the metabolic incorporation and adduct formation by hydrolysis of the DNA to nucleosides or purine/pyrimidine bases and chromatographic separation of the hydrolysate. ^{3}H-Labeled polycyclic aromatic hydrocarbons present an additional problem because on hydrolysis and chromatography of the DNA, substantial radioactivity is typically found to elute in the void volume and not with normal nucleosides [Martin et al., 1993]. The identity of the radioactivity is unknown.

Additional limitations of using radiolabeled material include the fact that it may not be possible to detect unstable adducts, such as N7-deoxyguanosine adducts that may be lost through depurination. The administration of radiolabeled material will not permit the detection of changes in endogenous DNA adducts, such as those arising from oxidative damage (e.g., 8-oxoguanine) or lipid peroxidation. Finally, due to the costs associated with the preparation of radiolabeled material, plus the hazards associated with its use, multidose experiments are generally difficult, if not impossible, to perform.

Sensitivity

Ideally, the amount of radiolabeled material administered should mimic as closely as possible that used in experiments to elicit a biological response (e.g., tumors). In practice, this may not be possible because unacceptably large amounts of radioactivity would have to be given. Nonetheless, a sufficient amount of compound (and thus, radiolabel) must be administered to allow detection of chemicals that may be weak genotoxic carcinogens. This quantity can be estimated through the use of covalent binding indices, as developed by Lutz [1982, 1986], and will typically involve the use of millicurie amounts of radiolabel per animal. DNA adduct levels as low as approximately 1 adduct in 10^{9} nucleotides have been detected using radiolabeled material [Buss et al., 1990].

Criteria for Establishing Positive or Negative Results

The criteria for determining positive and negative responses have been considered by Lutz [1979, 1982] and Martin et al. [1993]. When no increase in radioactivity is

detected compared to a DNA sample from control animals that has been isolated in an identical manner, the result can be considered negative. When low levels of radioactivity are detected (i.e., less than twice the level in the control samples), the situation is less clear. In these instances, further information can be obtained by counting greater quantities of DNA or by conducting additional dosing experiments at higher dose levels or with greater specific activity.

In summary, radioactivity binding to DNA is an indicator of potential genotoxicity, and where possible further studies (e.g., digestion followed by high-performance liquid chromatography (HPLC)) may be necessary to determine the nature of the binding or incorporation of the label in DNA.

ACCELERATOR MASS SPECTROMETRY

Accelerator mass spectrometry (AMS) is a technique for measuring isotope ratios with high selectivity, sensitivity, and precision [Elmore, 1987; Vogel et al., 1995]. In general, AMS counts (radio) isotopes using a variety of nuclear physics techniques. This involves selection of ions on the basis of charge-changing, momentum, charge, energy, and energy loss before each ion is uniquely identified and counted in the detector.

Strengths

The principal advantage of AMS for DNA adduct analysis is sensitivity. For radioisotopes, decay measurements (scintillation counting) are efficient if performed for a large fraction of the isotopic mean life, or on large samples that can provide significant numbers of decay events. For isotopes with long mean lives ($t_{1/2} > 10$ years) these conditions are seldom met. For ^{14}C, a primary isotope used in DNA adduct analysis, less than 0.01% ($\tau = 8{,}340$ years) decays in 1 year and the detection of DNA adducts below levels of a few DNA adducts per 10^9 nucleotides is not possible. Furthermore, sample size is often limited by the availability of DNA. In practice, large isotope concentrations are used to overcome the efficiency problem for measuring DNA adducts by decay counting. This strategy can require either high-specific-activity compounds (often Ci/mmol), high isotope loads in the test system (>mCi amounts/subject), or, where possible, large DNA samples (> 200 μg DNA).

AMS efficiently detects low concentrations (10^{-8} to 10^{-15} rare nuclei per total ions) of isotopes with high precision (0.3% to 5%). AMS measurements are independent of decay rate and measure directly the number of isotope nuclei. AMS measures between 1% and 10% of the isotope in the sample relative to the 0.01% for decay counting of radiocarbon [Beukens, 1992]. The actual sensitivity improvement relative to decay counting is a function of τ. For ^{14}C, AMS offers a 10^5-fold to 10^6-fold improvement relative to decay counting, based on a comparison of the time it takes to count a sample to 1% precision. For ^3H, AMS offers approximately a 10^3-fold improvement in sensitivity. In radiocarbon dating of organic substances, for example, sample size reduction by a factor of 200,000 relative to dating by decay counting is possible, and reduction of sample size by a factor of 1,000 has been reported in biological studies [Polach, 1984; Gove, 1992; Turteltaub et al., 1992]. DNA adduct analysis by AMS at these low isotope ratios can be done with lower specific activities and smaller quantities of isotope than has been practical with decay counting [Vogel et al., 1990; Vogel and Turteltaub, 1992; Turteltaub et al., 1995]. It is particularly useful for analysis of DNA digests, when separation and analysis of specific DNA adducts is necessary, or when there is a need to be able to detect DNA adducts at levels below 1 adduct per 10^9 nucleotides [Creek et al., 1994]. This may be necessary to show that a compound does not bind DNA. In addition to ^3H and ^{14}C, AMS allows for the utilization of other isotopes [Freeman et al., 1995], including ^{79}Se, ^{59}Fe, ^{129}I, ^{63}Ni, and ^{59}Ni.

Limitations

AMS is a technique that measures isotope ratios only. It provides no information on the nature or chemical form of the isotope and thus has the potential to provide false positives in DNA binding studies where genomic DNA isolates are measured. Isotope in a DNA isolate can arise from metabolic incorporation if the test compound degrades. Additionally, contamination with RNA adducts, protein adducts, or unbound metabolites of the test chemical can be misinterpreted to represent DNA binding when measuring low levels of adducts, particularly if the compound binds to protein or RNA with greater affinity than to DNA. Isotopes added during processing could cause similar false positives. Thus, additional studies are often necessary to show that isotopes in the DNA are actually due to DNA adducts.

Current AMS instrumentation has been designed primarily for very high-precision and low count rates, and for the ability to analyze isotopes requiring energies much greater than that necessary for ^{14}C and ^3H [Bennett, 1979; Elmore, 1987; Beukens, 1992; Gove, 1992; Duggan, 1994]. This existing instrumentation is large and expensive due to the need to use high potentials for analysis of medium to heavy radioisotopes. Presently, few investigators have direct access to the equipment or expertise needed to utilize AMS for DNA adduct analyses. Studies using this methodology will require working with a facility offering AMS analysis services. This situation is likely to exist for the next 2–5 years until the smaller and less expensive biomedical instruments now under development become more widely available [Mous et al., 1996; Chen et al., 1997].

Limits of Detection

The limit for detection of DNA adducts by AMS is dependent on the isotopic natural abundance of the isotope in the experiment (for ^{14}C this is 6 fCi ^{14}C/g carbon, or 1 part ^{14}C per 10^{12} parts total carbon) and on contamination introduced into the sample during the experiment or sample processing. The absolute detection limit for AMS is approximately 6 aCi ^{14}C/g carbon; 1 part ^{14}C per 10^{15} parts total carbon. In practice, DNA adducts have been detected to levels of 6 adducts per 10^{12} nucleotides using AMS [Frantz et al., 1995; Creek et al., 1997; Kautiainen et al., 1997]; however, further work is needed to understand dynamic ranges, limits of detection, and procedures to limit contamination in laboratories routinely using isotopes. Based on preliminary studies, similar limits of detection are expected with the use of ^{3}H.

Criteria for Establishing Positive or Negative Results

For a positive result, isotope levels in DNA isolated from animals dosed with a potential DNA binding agent should be at least twice the level of the isotope present in a set of controls (undosed). The levels of isotope in the controls should be consistent with the natural abundance of the isotope being analyzed. Because of the potential for false positives when analyzing genomic DNA isolates, DNA should be digested to deoxyribonucleosides and separated using a suitable method such as HPLC. No isotope above the natural abundance should be detectable by AMS in the normal nucleotide fractions and no isotope should be detectable at points where ribonucleic acids migrate (based on R_t of standards such as uracil). If an adduct standard is available, it should be shown to comigrate with the isotope. If no standard is available, it is useful, although not definitive, to show that an isotope peak can be detected that is well separated from the normal nucleotides. Finally, for a positive result, the isotope in the DNA should be stable to dialysis.

For a negative result, no dose response should be present and the isotope levels in the DNA should be consistent with the abundance of the isotope in DNA of undosed controls.

^{32}P-POSTLABELING ASSAY

The assay generally comprises four steps: (1) enzymatic digestion of DNA; (2) enrichment of the adduct fraction of the digest by solvent or solid-phase extraction, immunoaffinity chromatography, HPLC, or further enzymatic digestion; (3) labeling of the 5′-position of the adducts by polynucleotide kinase-mediated transfer of ^{32}P-orthophosphate from [γ-^{32}P]ATP; (4) chromatographic separation, by thin-layer chromatography (TLC) or HPLC, followed by detection and quantitation by measuring ^{32}P-decay.

Strengths

^{32}P-Postlabeling is a highly sensitive assay, requiring only small quantities (1–10 μg) of DNA [Randerath et al., 1981; Gupta, 1985; Reddy and Randerath, 1986]. The assay allows the detection of adducts from different chemicals with diverse chemical structures, such as polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines, alkenylbenzene derivatives, benzene and its metabolites, styrene, mycotoxins, simple alkylating agents, unsaturated aldehydes from lipid peroxidation, pharmaceuticals, reactive oxygen species, and UV radiation [Reddy et al., 1984; Randerath and Randerath, 1994; Phillips, 1997; Izzotti, 1998; Stiborova et al., 1998]. One unique strength of the assay is that it has been useful for the detection of adducts from complex mixtures such as cigarette smoke and environmental pollutants [Randerath and Randerath, 1994; Phillips, 1997; Izzotti, 1998; Stiborova et al., 1998]. The assay can be applied to measure adducts after multiple dosing in any tissue or to examine the removal of adducts after cessation of exposure.

Limitations

The ^{32}P-postlabeling assay does not provide structural information of adducts. The assay could give false-negative results due to loss of adducts. In addition, the false-negative results are likely to occur more often with nonaromatic adducts (i.e., adducts with substitutions such as methyl and ethyl groups) than aromatic adducts (i.e., adducts with one or more aromatic ring substitutions) because of the lower sensitivity of the assay for nonaromatic adducts. Polynucleotide kinase can label nonnucleic acid components, such as some hydroxylated metabolites, leading to false-positive results [Masento et al., 1989; Vulimuri et al., 1994; Scates et al., 1995]. It is, therefore, important to obtain good quality DNA free from a test compound or its metabolites after an in vitro or in vivo exposure. A DNA isolation procedure using organic solvent extractions has been shown to be adequate for this purpose [Masento et al., 1989]; however, controls such as labeling DNA without micrococcal nuclease and spleen phosphodiesterase treatments may be used to identify potential nonnucleic acid components in DNA producing adduct-like spots. Endogenous DNA adducts called I-compounds [Randerath et al., 1993] can interfere with the detection of adducts formed from a test compound exposure. Some of these I-compounds increase with age but decrease during carcinogenesis, whereas others are increased by carcinogen treatment and have the characteristics of oxidative DNA lesions [Nath et al., 1996b; Randerath et al., 1999]. Some I-compounds are present at levels of 1 adduct in 10^7 or more DNA nucleotides and migrate on TLC plates similar to adducts derived from aromatic carcinogens [Randerath et al., 1993]. Treatment-related adducts may go undetected if they are very weak and

comigrate with I-compounds. Also, adduct scoring can be subjective if I-compounds are too many or too strong, while treatment-related adducts are very weak. Different TLC solvents or different versions of the postlabeling assay may be explored, as needed, to resolve adducts of interest from I-compounds. For example, a putative major benzoquinone adduct was resolved from a background spot in bone marrow DNA when analyzed as a monophosphate adduct, rather than as a bisphosphate adduct [Reddy et al., 1990]. Low levels of DNA adducts have been reported with classic nongenotoxic carcinogens, such as estrogens [Liehr et al., 1993] and peroxisome proliferators [Randerath et al., 1993], causing some concerns that the assay is too sensitive and is not selective enough to distinguish genotoxic carcinogens from nongenotoxic carcinogens.

Sensitivity

The ^{32}P-postlabeling method is more sensitive for the detection of aromatic DNA adducts than non-aromatic adducts because of analytical difficulties in the separation of non-aromatic adducts from unmodified (normal) nucleotides. The limit of detection for aromatic adducts varies from 1 to 100 adducts in 10^9 nucleotides depending on the separation of adducts relative to I-compounds [Gupta, 1985; Reddy and Randerath, 1986; Randerath et al., 1993]. The limit of detection for nonaromatic adducts that are labeled and mapped together with unmodified nucleotides is 1 adduct in 10^5 to 10^6 nucleotides [Reddy et al., 1984]. An exception to nonaromatic adducts is N7-alkylguanines and other positively charged adducts, for which an enrichment method has been reported that improves their limit of detection to one that is similar to that achievable with aromatic adducts [Kumar et al., 1995]. Note that the recovery of adducts is assumed to be quantitative for these calculations.

Quantitation

The ^{32}P-postlabeling assay may underestimate adduct levels because of incomplete DNA digestion, inefficiency of adduct labeling by polynucleotide kinase, and/or loss of adducts during enrichment and TLC stages. In an interlaboratory study, different adduct measurement methods gave variable results on adduct levels for DNA samples modified with benzo[a]pyrene and 4-aminobiphenyl, making it difficult to ascertain true adduct levels and evaluate the recovery of adducts by ^{32}P-postlabeling relative to other methods [Phillips et al., 1999]. The recovery of DNA adducts from polycyclic aromatic hydrocarbons is similar with the butanol and nuclease P1 enrichment methods [Gupta and Earley, 1988; Gallagher et al., 1989]. The nuclease P1 method gives a lower recovery of adducts derived from some aromatic amines [Reddy and Randerath, 1986; Gupta and Earley, 1988; Gallagher et al., 1989], while butanol enrichment gives a lower recovery of adducts from benzoquinone, mitomycin C [M. V. Reddy, unpublished observations], and reactive oxygen species [Carmichael et al., 1992]. For an unknown compound, the use of both enrichment methods is recommended. [γ-^{32}P]ATP at 1–2 μM is sufficient for optimal labeling of some adducts [Randerath et al., 1989; Reddy et al., 1990; Hemminki et al., 1993], while [γ-^{32}P]ATP as high as 60 μM is necessary for optimal labeling of thymine glycol adducts [Reddy et al., 1991] and even higher concentrations may be required for efficient labeling of aromatic amine-DNA adducts [Mourato et al., 2000]. Contamination of DNA with RNA would give an overestimation of adduct levels, because the standard TLC conditions described in the literature are generally not adequate to resolve DNA adducts from RNA adducts [Godschalk et al., 1998].

Criteria for Establishing Positive or Negative Results

A positive response in the ^{32}P-postlabeling method is indicated by the presence of adducts as additional spots on the maps of treated DNA samples and the absence of such spots on the maps of DNA from control animals or cells. Control DNA maps generally show weak or no background spots when analyzing adducts at levels of 1 adduct in 10^7 DNA nucleotides. At lower adduct levels, background adducts (I-compounds) may be seen; this can make adduct scoring subjective and interpretation of results difficult. An increase in the intensity of one or more background spots (I-compounds) after treatment could indicate comigration of adducts with background spots, which should be interpreted as a positive response, if the increase in mean adduct levels for a group of treated animals relative to the mean of controls is reproducible and significant. Alternatively, the increase in the intensity of background spots could be due to intensification of I-compounds by the treatment [Randerath et al., 1993]; however, this result should also be conservatively interpreted as a positive response, since neither the structure nor the function of I-compounds is not well understood. A negative response is indicated by the absence of additional spots or the lack of significant increase in I-compounds or background spots in the treated DNA maps compared to the control DNA maps. Demonstration of a dose response is desirable but the detection of adducts at a single high dose would be adequate for identifying a positive response.

IMMUNOASSAYS

Antisera elicited against carcinogen-modified DNA samples have been used to establish immunoassays able to quantify DNA adducts in biological samples, immunohistochemical staining methods able to localize and semiquantify DNA adducts in nuclei of cells and tissues, and immunoaffinity chromatography techniques able to separate DNA containing adducts of a specific class, which can be quantified by other methods [Poirier, 1991, 1993; Poirier

and Weston, 1991; Weston and Poirier, 1994]. The immunoassays [Poirier, 1991, 1993] are most commonly performed in a competitive mode on microtiter plates, and inhibition of binding of the specific modified DNA antiserum to a coated immunogen DNA is observed with either biological sample DNAs or a standard curve composed of DNA modified in the same range as the biological samples (approximately 1 adduct in 10^6 nucleotides). Samples are typically assayed twice on different days using triplicate microtiter wells for each assay. The amount of adduct in the unknown sample is obtained by comparison with the known standard.

Strengths

Immunoassays are highly sensitive and have good relative specificity because the antisera usually recognize multiple adducts from the same chemical as well as adducts of other carcinogens in the same chemical class. Immunoassays are inexpensive and relatively easy to perform. One person can assay about 25 samples per day and the daily reproducibility of the standard curve provides internal quality control. The major unique advantage of this approach, again in a testing situation, is the potential ability to perform cost-effective long-term dosing and determine DNA adduct processing over time in animal and cell culture models.

Limitations

Disadvantages of this approach include the requirement to immunize rabbits (for polyclonal antibodies) and the necessity to characterize the antiserum and validate the assays. In addition, the data obtained generally give values for relative and not absolute quantitation (unless the standard used and the unknown sample are both modified by the same single compound). For the application under consideration here, prior knowledge that the test compound is bound to DNA must be obtained, probably by using radiolabeled compounds. It must be possible to obtain a DNA modified in the range of 1% to use as immunogen. The assay requires relatively large amounts of biological sample DNA for maximal sensitivity. It is not uncommon to use more than 200 μg of DNA in total if approximately 25 μg DNA are assayed per microtiter well. In addition, this approach does not work with unstable adducts.

Sensitivity

The last assay step (colorimetric, fluorescent, chemiluminescent) for detecting antiserum bound to a microtiter plate can vary, usually giving sensitivity in the range of 1 adduct in 10^8 nucleotides [Poirier, 1993] but a recent dioxetane chemiluminescent end point has allowed detection of 2.6 tamoxifen adducts per 10^9 nucleotides [Divi et al., 1999].

Quantitation

The amount of DNA adduct in an unknown sample is obtained by comparing the extent of inhibition of antibody binding to the microtiter well, using a known quantity of standard in a standard curve, with that produced by the unknown sample. The standard curve must be included in each assay, and constitutes an internal standard for assay variability. Quantitation of adduct in the standard material (either specific adduct or adducted DNA) must be determined by an alternate method, such as ultraviolet spectroscopy, atomic absorbance spectrometry, radiolabeling, or gas chromatography-mass spectrometry (GC-MS). Typically, samples are assayed twice and each assay includes three experimental wells (coated with immunogen) and one well not coated with adduct. If the standard is a modified DNA, the amount of DNA in the standard curve wells must match the amount of DNA in the sample wells.

Criteria for Establishing Positive or Negative Results

Immunoassays can be direct or competitive, but the competitive assays are generally more sensitive. Competitive immunoassays are inverse in that high antibody binding indicates availability of a low level of adduct competing in the sample. The results can be expressed in different ways but are commonly designated "% inhibition," which is inhibition of antibody binding to the well due to the presence of the competitor. The wells with no sample and no standard are therefore very important because they form the basis for all of the calculations. The lower limit of detection is in the range of 15% inhibition but should be determined empirically for each assay based on the slope of the standard curve. A positive sample should read more than 15% inhibition in two separate assays on different microtiter plates, preferably performed on different days. Competitive immunoassays can also take the form of radioimmunoassays, in which tracer replaces the microtiter plate and the reaction is in a tube. In the case of radioimmunoassay the same principles apply.

Thus, in the context of testing compounds for genotoxicity, immunoassays might be appropriate for their extended evaluation, in conjunction with radiolabeling studies, where long-term administration of compound is required.

MASS SPECTROMETRY

Strengths

Of the physicochemical methods used to detect DNA binding, mass spectrometry has the greatest potential because of its high chemical specificity that allows for unequivocal characterization of the DNA binding products [Sweetman et al., 1998]. Thus, genuine DNA adducts derived from the chemical being tested may be distinguished

from unrelated adducts and from products of endogenous DNA damage, which is not possible, for example, by the use of ^{32}P-postlabeling. Where necessary, detailed structural information on DNA adducts may also be obtained (especially by the use of tandem mass spectrometry), which may be valuable in the study of mechanisms, metabolic polymorphism, and structure-activity relationships. A further advantage of mass spectrometric techniques is that very low analytical backgrounds are achievable by the use of high-resolution chromatography coupled with selected ion recording or, when using a tandem mass spectrometer, with multiple reaction monitoring. High mass spectrometric mass resolution has also been used to enhance signal-to-noise ratios for some adducts [Ranasinghe et al., 1998]. Elimination of background noise from the analysis is less easily achievable with the use of most other nonmass spectrometric adduct measuring techniques.

Limitations

The increased chemical selectivity of mass spectrometry is offset by decreased sensitivity, in comparison with ^{32}P-postabeling or AMS. It is hoped that current improvements in mass spectral interface technology (e.g., nanospray) and ion detection will lead to mass spectrometry achieving the sensitivity of ^{32}P-postlabeling. A further drawback of mass spectrometry, when compared to ^{32}P-postlabeling and AMS, is its inability to screen unknown mixtures of adducts, as the high sensitivity for mass spectrometric detection is only achievable when one knows the structure of the analyte.

It is possible, however, that tandem mass spectrometry with the use of, for example, constant neutral loss or precursor ion scanning could be used to screen for adducts within a certain chemical class, e.g., N-7 guanine adducts or adducts derived from polycyclic aromatic hydrocarbons.

To date, mass spectrometry has been extensively used for the detection of protein adducts but considerably less so for DNA adducts [Sweetman et al., 1998]. The reasons for this are twofold. Firstly, adducted proteins are available in greater quantity (several hundred milligrams) from in vivo studies, in contrast to adducted DNA where only microgram quantities are normally available. Secondly, the earlier mass spectrometry technology was based on compound separation by gas chromatography using electron impact or chemical ionization, which was more applicable to modified amino acids than to modified nucleotides. The rapid recent development of liquid chromatography-mass spectrometry (LC-MS) techniques means that this is certainly the methodology of the future for DNA adduct detection by mass spectrometry.

Detection and Quantitation

Current literature references to the use of mass spectrometry for DNA adduct detection refer mostly to GC-MS, and include detection of adducts with 4-aminobiphenyl [Lin et al., 1994], benzo[a]pyrene [Weston et al., 1989], tobacco-specific nitrosamines [Foiles et al., 1991], 4,4′-methylene dianiline [Schutze et al., 1996], and 2-amino-l-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [Friesen et al., 1994]. For all of those compounds, the adducted DNA is hydrolyzed to release a carcinogen-derived molecule that is derivatized and subjected to GC-MS. Highly sensitive analyses result, although because of the hydrolysis procedure all information on the chemical nature of the adduct and its position in the sequence is lost. Levels of detection down to 1 adduct per 10^9 nucleotides have been achieved [Foiles et al., 1991]. High purity of the DNA is required because RNA and protein adducts would also yield similar products upon hydrolysis.

Other GC-MS analytical methods have been developed for modified DNA bases, such as the malondialdehyde-guanine adduct [Chaudhary et al., 1994; Rouzer et al., 1997], N-7-(2-hydroxyethyl)guanine [Ranasinghe et al., 1998], and oxidative damage products such as 8-hydroxy-guanine [Halliwell and Dizdaroglu, 1992; Dizdaroglu, 1993; Ravanat et al., 1995], thymine glycol [Farooq et al., 1997], and 5-hydroxymethyluracil [Djuric et al., 1991]. These modified bases are relatively abundant in DNA compared to most adducts produced by exogenous agents. The use of LC-MS to date has included analysis of methylguanines [Arimoto-Kobayashi et al., 1997], N^2,3-ethenoguanine [Yen et al., 1996], malondialdehyde adducts [Chaudhary et al., 1995], 8-hydroxy-2′-deoxyguanosine [Ravanat et al., 1998], diepoxybutane adducts [Tretyakova et al., 1997, 1998], and 7-(2-oxopropyl)-1-N^2-etheno-2′-deoxyguanosine [Liu et al., 1996].

Quantitation is best achieved by the use of a stable-isotope–labeled internal standard, coupled with selected ion recording or multiple reaction monitoring detection. Close structural analogues may also be used as internal standards. Calibration lines are established using mixtures of the internal standard and varying amounts of the adduct. The latter should preferably be in the form of adducted DNA in order that the calibration line takes into account any losses of adduct during the DNA work-up procedure. Unfortunately, the availability of such adducted DNA standards is limited at present.

Criteria for Establishing Positive or Negative Results

A positive mass spectral detection of a compound by selected ion recording or multiple reaction monitoring is generally thought to be the presence of a signal at the correct chromatographic retention time with an intensity greater than a specified signal-to-noise (S:N) ratio. The accepted value of this S:N ratio differs according to local laboratory practice and the requirements of the assay but could be in the range of 3:1 for detection and 10:1 for quantitation.

OTHER PHYSICOCHEMICAL METHODS

Other physicochemical methods for adduct detection include HPLC with fluorescence detection (e.g., for aflatoxin B_1-guanine [Autrup et al., 1985] or for benzo[a]pyrene-adducted DNA [Weston et al., 1989]), and HPLC with electrochemical detection (e.g., for 8-hydroxy-2′-deoxyguanosine [Halliwell and Dizdaroglu, 1992]). The use of these techniques, although very valuable, is clearly limited to those compounds possessing a fluorophore or an electrochemically active group.

For DNA adducts that are formed at the N-7 position of guanine or the N-3 position of adenine, depurination occurs and the adducted purine may be excreted in urine [Shuker and Farmer, 1992]. This phenomenon has been used to great advantage in the analysis of urinary aflatoxin B_1-guanine adducts as an indicator of DNA damage [Qian et al., 1994]. The urinary excretion of such an adduct, however, does not indicate the source of the damaged macromolecule (i.e., DNA or RNA) or in which tissue the adducts had been formed.

ENDOGENOUS DNA ADDUCTS

Recent research has shown that in addition to exposure to exogenous electrophiles, the mammalian genome is also under attack from endogenous DNA reactive substances. Normal cellular function is known to release electrophiles. Many of these agents are highly reactive, and thus, do not need further metabolic activation. Various types of endogenous DNA damage include those from DNA instability, errors in replication and repair, oxidatively damaged bases and adducts derived from reaction of bases with aldehydic lipid peroxidation products. There are several recent publications describing the chemistry of endogenous DNA adducts, methods for their detection, and the biological significance of their formation [Nath and Chung, 1994; Ames et al., 1995; Chung et al., 1996; Burcham, 1998; Chung et al., 1999; Gupta and Lutz, 1999; Marnett, 1999; Nair et al., 1999]. Oxidation of membrane lipids leads to the generation of a large number of aldehydes. The three most studied groups of aldehyde-derived adducts are malondialdehyde-derived adducts, the propano adducts from acrolein and crotonaldehyde, and the etheno adducts formed from the epoxides of aldehydes [Chung et al., 1996; Marnett, 1999; Nair et al., 1999]. The major malondialdehyde-derived adduct is a pyrimidopurinone called M1G, which has been detected in human liver, white blood cells, pancreas, and breast. Methods used to detect and quantify this adduct include mass spectrometry, ^{32}P-postlabeling, and immunochemical techniques. The estimated levels of M1G are 1–120 adducts per 10^8 nucleotides [Marnett, 1999].

Acrolein- and crotonaldehyde-derived 1,N^2-propanodeoxyguanosine adducts (Acr-dG and Cro-dG, respectively) have been detected in various untreated rodent and human tissues using ^{32}P-postlabeling-HPLC methodology. The human tissues analyzed included liver, breast, and white blood cells. The levels of these adducts are in the range of 1 adduct in 10^6–10^7 nucleotides [Nath and Chung, 1994; Nath et al., 1996a). Depletion of the endogenous antioxidant glutathione, stimulation of lipid peroxidation by carbon tetrachloride treatment, and increase in age lead to increases in the levels of Acr-dG and Cro-dG in rat liver DNA, thereby suggesting an endogenous origin [Nath et al., 1997; Chung et al., 1999]. Higher levels of these adducts are also found in Long Evans Agouti coat rats with elevated hepatic lipid peroxidation due to abnormal copper metabolism [Chung et al., 1999]. Because acrolein and crotonaldehyde are also environmental pollutants originating from cigarette smoke and automobile exhaust, it is possible that the Acr-dG and Cro-dG detected in humans may be formed from exogenous as well as endogenous sources.

Endogenous aldehydes like *trans*-4-hydroxy-2-nonenal, which are released during lipid peroxidation, can be epoxidized. The epoxides of aldehydes are a probable source of another class of adducts, the etheno adducts. Examples are 1,N^6-ethenodeoxyadenosine (etheno-dA) and 3,N^4-ethenodeoxycytidine (etheno-dC). These adducts are detected in untreated rodents and humans using ^{32}P-postlabeling after immunoaffinity enrichment [Nair et al., 1999]. The levels of these adducts are approximately two orders of magnitude lower than the propano adducts. It is interesting to note that, like the propano adducts, the conditions in which lipid peroxidation is stimulated lead to increases in etheno-dA and etheno-dC as well [Nair et al., 1999].

In summary, endogenous DNA damage is present at relatively high levels in mammalian tissues. Certain treatments may alter endogenous DNA damage levels, but the biological significance of this is unknown. The presence of higher levels of endogenous adducts does not negate the importance of lower levels of exogenous adducts.

IMPORTANCE OF ADDUCT IDENTIFICATION

Generally, the measurement of total adducts in DNA is considered an adequate indicator of genotoxicity. Such total adduct measurements will clearly include all the modified DNA bases from the compound and from each of its metabolites. Despite the fact that only some of these adducts may be of particular mutagenic significance (e.g., O^6-adducts on guanine) the measurement of their sum should correlate with the amount of the significant ones, and is therefore a valid biomonitor of genotoxicity.

There may be circumstances where a particular individual adduct needs to be measured. These situations might include, for example, when a structure-activity relationship study was being performed, when there is a suspicion of contaminants, where mechanistic studies on species differences in metabolism are needed, or when genetic polymorphisms need to be investigated.

A further possibility is that an exogenously supplied compound may affect the levels of endogenous adducts, i.e., the total adducts measured by for ^{32}P-postlabeling may not all be compound-related. In such circumstances individual adduct determinations may be required in order to distinguish between compound-related adducts and endogenous adducts.

In summary, a measurement of total DNA binding is generally an appropriate indicator of potential genotoxicity. Experiments to determine the structure of adducts may provide important mechanistic information but may not be necessary to assess potential genotoxicity.

QUANTITATIVE EVALUATION OF DNA BINDING

The relationship between DNA adduct levels and tumorigenesis in experimental animals has been studied for a number of classes of carcinogens including *N*-nitrosamines, aflatoxins, aromatic amines, heterocyclic aromatic amines, and polycyclic aromatic hydrocarbons (reviewed in Poirier and Beland [1992]; Beland and Poirier [1993]; Otteneder and Lutz [1999]). Typically, steady state adduct concentrations are obtained during continuous carcinogen administration; this usually requires approximately one month of dosing. Under these conditions, there is often a linear correlation between the administered dose levels and the steady state adduct concentrations in target tissues. Exceptions exist when the mechanism of activation changes (e.g., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in rat lung) or with the onset of significant toxicity (e.g., *N*-nitrosodiethylamine in rat liver). The steady state adduct levels are often linearly related to tumorigenic response, and because of this, attempts have been made to relate specific adduct levels with the tumor incidence [Gaylor et al., 1992; Otteneder and Lutz, 1999]. The typical metric is a 50% tumor incidence and this has been correlated with adduct levels of between 50 and 8,000 adducts per 10^8 nucleotides depending on the particular carcinogen and animal model investigated. From this relationship it is clear that an increase in tumorigenicity can be associated with low adduct levels.

It is accepted that there are uncertainties concerning the biological significance of low levels of DNA adduct formation. There are at present insufficient data on which to set an adduct level below which there is no measurable biological effect.

ASPECTS OF STUDY DESIGN

Before embarking on a study, a statistician should be consulted to ensure that the study has sufficient power to detect the required increase in adduct level. As stated earlier, the circumstances in which the DNA binding potential of a compound is to be determined will be dictated by the results of animal bioassays, in vitro or in vivo genotoxicity tests, and by the questions raised by those results.

For an in vivo test, the numbers of animals required per dose will depend on the variance of background levels of adducts in controls and the anticipated increase in adduct levels in treated animals above background for the particular assay used. There is a need for positive system controls, for example frozen tissue containing adducted DNA.

A two-stage approach may be appropriate. First, utilize the top dose used in the bioassay. Then, if a positive result is obtained, seek evidence for a dose-response by investigating one or more lower doses. Because adduct formation is usually linearly related to dose [Poirier and Beland, 1992], evidence of a dose response provides good confirmatory evidence of a positive response.

Single doses may result in adduct levels too low to be detectable. Substantial increases in adduct levels can result from multiple dosing.

Tissues from individual animals should be analyzed whenever possible. Tissues should be pooled only when this is necessary to obtain sufficient DNA for analysis. The tissues of interest will be determined to some extent by the reasons for conducting the experiment and should be related to the biological effects observed in animal experiments. Thus, recommendations on the choice of tissues can be less prescriptive than those made previously [Martin et al., 1993].

For *in vitro* binding studies, the conditions of the in vitro biological assay should be followed as closely as possible. Cells should be harvested for adduct determination at two time points (at least) within 24 hr, in order to guard against the possibility of a false negative due to slow rates of metabolic activation (leading to a negative result at an early time point) or due to rapid DNA repair (negative result at a late time point). Appropriate positive controls are necessary.

Highly sensitive assays require high chemical and/or radiochemical purity of administered compound.

ISSUES OF DNA PURITY

In general, it can be stated that the more sensitive a DNA binding assay, or the closer to the limit of detection of the assay the binding level of the compound becomes, the greater the requirement for the DNA to be of the highest purity attainable.

Where radioactivity associated with DNA appears to be lost readily on digestion, consideration should be given to the possibility that the apparent binding is in fact binding to contaminating material, such as peptides that have a strong affinity for DNA [Phillips et al., 1992; Adams et al., 1994].

In general, phenol-chloroform extraction is a suitable method for DNA preparation, provided precautions are taken against the artifactual generation of oxidative DNA damage if it is thought that the compound under investigation may cause DNA lesions of this type. Other methods (nonphenol) may also be preferable for samples intended for AMS analysis. Some workers prefer the use of commercial

kits for DNA isolation, but there is no consensus on which is the best procedure. In some instances, purer preparations can be obtained from nuclear preparations (as opposed to whole cell preparations). Glycogen can be removed from liver DNA preparations by ultracentrifugation of the DNA solution.

Guidelines for the preparation and storage of DNA samples for ^{32}P-postlabeling analysis have been described [Phillips et al., 1999]. These general principles may serve also for other methods of DNA adduct determination. It is generally accepted that protein and RNA contamination can cause problems in DNA adduct analysis, although no DNA sample is ever completely free of either. Contamination may also inhibit enzymatic digestion of DNA; thus, when using methods that require prior digestion, an estimate of digestion efficiency should be made [Phillips et al., 1999].

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