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Contemporary Issues in Toxicology

Proceedings of a workshop on DNA adducts: Biological significance and applications to risk assessment Washington, DC, April 13–14, 2004

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Abstract

In April 2004, the Health and Environmental Sciences Institute, a branch of the International Life Sciences Institute, with support from the National Institute of Environmental Health Sciences, organized a workshop to discuss the biological significance of DNA adducts. Workshop speakers and attendees included leading international experts from government, academia, and industry in the field of adduct detection and interpretation. The workshop initially examined the relationship between measured adduct levels in the context of exposure and dose. This was followed by a discussion on the complex response of cells to deal with genotoxic insult in complex, interconnected, and interdependent repair pathways. One of the major objectives of the workshop was to address the recurring question about the mechanistic and toxicological relevance of low-concentration measured adducts and the presentations in the session entitled "Can low levels of DNA adducts predict adverse outcomes?" served as catalysts for further discussions on this subject during the course of the workshop. Speakers representing the regulatory community and industry reviewed the value, current practices, and limitations of utilizing DNA adduct data in risk assessment and addressed a number of practical questions pertaining to these issues. While no consensus statement emerged on the biological significance of low levels of DNA adducts, the workshop concluded by identifying the need for more experimental data to address this important question. One of the recommendations stemming from this workshop was the need to develop an interim "decision-logic" or framework to guide the integration of DNA adduct data in the risk assessment process. HESI has recently formed a subcommittee consisting of experts in the field and other key stakeholders to address this recommendation as well as to identify specific research projects that could help advance the understanding of the biological significance of low levels of DNA adducts.

Keywords: Risk assessment; DNA; DNA adducts; DNA damage; Workshop report

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Introductory remarks

Syril Pettit (HESI)

The Health and Environmental Sciences Institute (HESI) was established in 1989 as a global branch of the International Life Sciences Institute (ILSI). HESI is a non-profit international organization whose mission is to understand and resolve scientific issues related to human health, toxicology, risk assessment, and the environment. HESI members include representatives from the chemical, agrochemical, petrochemical, pharmaceutical, biotechnology, and consumer products industries in the United States, Europe, and Japan, and representatives from government and academic sectors. HESI programs provide a unique forum for dialogue between government, academic, and industrial scientists, bringing together different perspectives and complementary expertise.

In the fall of 2003, the HESI Emerging Issues Committee identified the biological significance of DNA adducts as a key area for future study. The subcommittee on the Biological Significance of DNA Adducts was formed and first met in November 2003 to begin planning this workshop. The National Institute of Environmental Health Sciences co-sponsored the workshop, whose program and

format were designed to promote discussion, interaction, and consensus development relating to the application of DNA adduct data to risk assessment.

Introductory Address

Aflatoxin story: a case study in genotoxicity evaluation

John Groopman (Johns Hopkins University)

John Groopman presented an overview of approximately 40 years of scientific research related to aflatoxin toxicity and the biological significance of aflatoxin-DNA adducts. Groopman began working on aflatoxin in collaboration with Thomas Kensler at Johns Hopkins University (Groopman et al., 1987; Kensler et al., 1986). When the risk of aflatoxin exposure to humans from dietary sources was discovered in 1960, the hepatoxicity of aflatoxin had been known for a long time due to animal research. A potential link between aflatoxin and human cancer was first noted in the mid-1960s, but aflatoxin was not listed as a confirmed human carcinogen until 1994.

Research on aflatoxin genotoxicity constitutes a paradigm for developing and validating a DNA adduct as a molecular biomarker. This process requires initial carcinogenesis studies in animals and development of sensitive and specific methods to quantify the DNA adduct in samples from exposed humans. Subsequent studies in animal systems define the relationship of the DNA adduct to exposure and to disease in the presence and absence of preventive interventions. In parallel with animal studies, human epidemiological methods are then applied using cross-sectional, longitudinal, and prospective study designs. Optimally, this approach can establish a DNA adduct or another molecular event as a biomarker of exposure and/or disease in humans (Fig. 1). In the case of aflatoxin, aflatoxin-DNA adducts are considered biomarkers of exposure and of risk for aflatoxin-induced liver cancer in humans.

Liver cancer is a leading cause of cancer deaths in some parts of Asia and Africa, where incidence of hepatocellular carcinoma (HCC) has been correlated with dietary intake of aflatoxin (reviewed in Kensler et al., 2003). China has especially strong regional variation in HCC incidence and includes large populations at high risk for HCC; this regional variation was exploited in early epidemiological

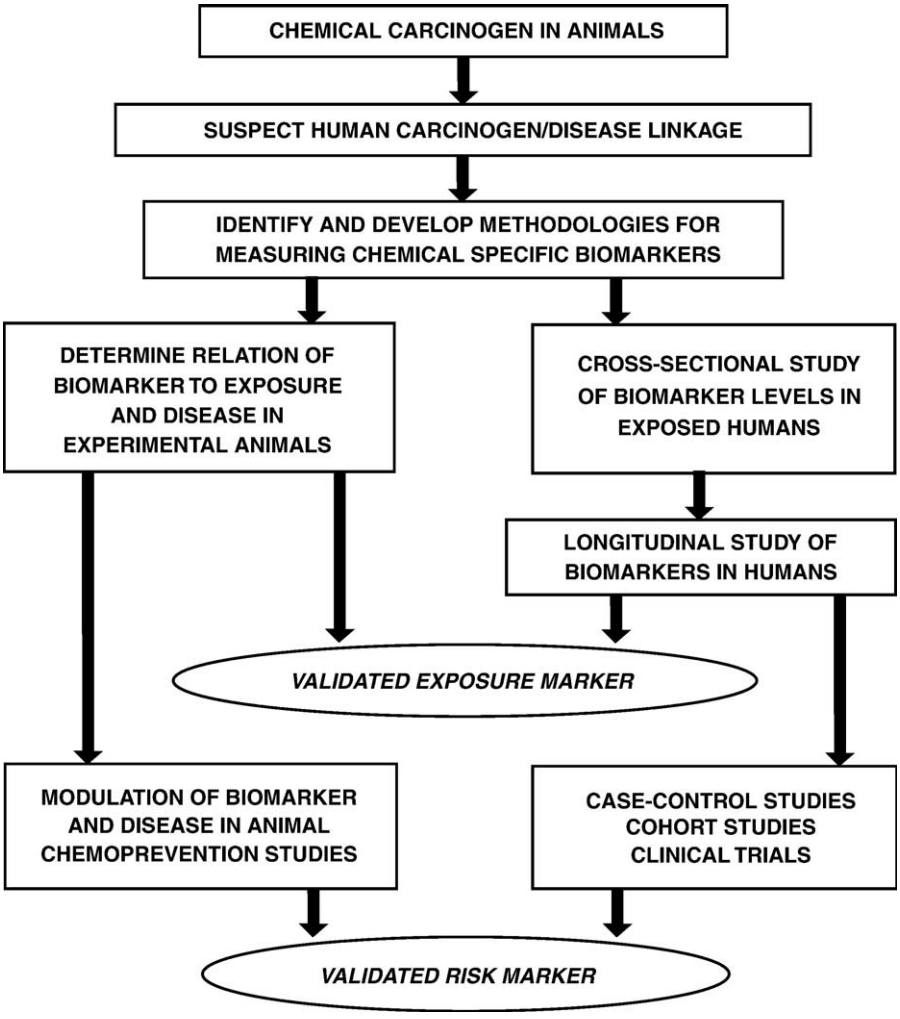

Fig. 1. Model for validating chemical-specific biomarkers (Groopman and Kensler, 1999).

studies of aflatoxin-induced DNA adducts and aflatoxin-related HCC. Using sensitive methods to detect N7-guanine-aflatoxin DNA adducts (AFX-N7G) in urine, a major cohort study with 18,000 residents of Shanghai, China, showed that individuals who tested positive for urinary AFX-N7G have a 3.4-fold higher risk of HCC than controls. A 7.3-fold higher risk was observed for individuals who were positive for hepatitis B virus (HBV). However, the risk for HCC was even higher for individuals who were positive for HBV and AFX-N7G; these individuals had a 60-fold higher risk of developing HCC than control individuals lacking both biomarkers. These and other results of cohort studies which monitor AFX-N7G and/or HBV in relation to HCC have established a causal relationship between aflatoxin exposure and HCC. They also demonstrate a strong interaction between exposure to aflatoxin and hepatitis B.

Several preventive interventions that alter aflatoxin metabolism in exposed individuals have been developed to reduce HCC incidence in high-risk populations in Asia. One such intervention is oltipraz, an agent that inhibits activation and stimulates detoxification of aflatoxin. In rats, oltipraz reduced incidence of HCC from 80% to 50% with corollary reduction in urinary AFX-N7G. Oltipraz was also tested in humans in a randomized placebo-controlled phase IIa clinical trial in 236 individuals in Qidong, China (Wang et al., 1999). Individuals receiving differing doses of oltipraz either daily or once a week showed a 50% reduction in urinary aflatoxin adducts, increased glutathione S-transferase activity, and decreased cytochrome *P*4501A2. These results are consistent with high efficacy of oltipraz as a chemopreventive agent in humans exposed to high levels of aflatoxin. However, because of the relatively high cost of oltipraz, it is not feasible to use this agent in developing countries where there is a large population at risk for aflatoxin-related HCC. An inexpensive alternative to oltipraz is chlorophyllin, a chemical that is approved for use by the U.S. Food and Drug Administration. When administered three times per day, chlorophyllin reduced urinary aflatoxin-DNA adducts by 55%. In rats, 55% reduction in aflatoxin DNA adducts correlated with more than 90% reduction in incidence of liver tumors (Egner et al., 2001). Current investigations are exploring the use of a broccoli sprout tea, which may also enhance patient compliance and increase efficacy of the intervention in high-risk populations exposed to aflatoxin in Asia.

Recent studies demonstrate that tumor DNA is sufficiently abundant in human blood and saliva to be used for biomarker studies. One method useful for analyzing this tumor DNA is short oligonucleotide mass analysis (SOMA). SOMA was used to study the correlation between specific mutations in p53 or HBV and liver tumors in aflatoxin-exposed individuals (Jackson et al., 2003). In particular, a G:T transversion at p53 codon 249 and a double mutation in the X gene and E antigen of HBV were tested as pre- or post-diagnostic biomarkers for HCC in aflatoxin-exposed individuals. The data suggest that p53 mutation is a late event in aflatoxin-induced liver carcinogenesis, thus it is a valuable post-diagnostic marker for cancer etiology. In contrast, presence of the mutant HBV virus may occur very early in aflatoxin-induced liver carcinogenesis, and this DNA biomarker may have predictive diagnostic value in aflatoxin-exposed individuals (Kuang et al., 2004).

In closing, Groopman pointed out that cancer is a multifactorial disease; therefore, multiple biomarkers may be needed to accurately describe the relationship between causal factors and disease. If the etiology of liver cancer is well enough understood, it will be possible to devise interventions that shift the age of onset for clinical disease (i.e., average age of onset will increase). Biomarkers of exposure or of early disease are needed to help reach this goal.

Session one—relationship between measured adduct levels and exposure and dose

Discussion Leader **Lawrence Marnett** (Vanderbilt University) introduced the first workshop session, in which presenters were asked to discuss the relationship between measured DNA adduct level and exposure and dose. Marnett indicated that numerous approaches and technologies are needed to develop an understanding of DNA adduct dosimetry and the biological significance of specific DNA adducts. Scientists can address these issues using biochemistry, chemistry, epidemiology, genetics, and molecular genetics. DNA adducts are one of the many types of DNA damage that accumulate in the genome due to ongoing exposure to endogenous and exogenous compounds and chemicals. The DNA damage generated by endogenous and exogenous sources has multiple fates and multiple potential impacts on the cell. Damaged DNA bases can be excreted in the urine after spontaneous or enzymatically-catalyzed release from nucleic acids. Alternatively, when unrepaired, different DNA adducts differentially generate mutations in replicating cells. DNA adducts can lead to altered cell signaling and altered cell fate, including apoptosis (reviewed in Poirer, 2004). The process of inflammation, which is associated with numerous noxious compounds including oxidizing, nitrosating, and chlorinating agents, is particularly likely to have an effect on cell–cell signaling and the general metabolic status of the cell.

The talks in this session focus on the relationship between DNA adducts and prior exposure. Marnett pointed out that their experiments tend to be carried out at very high doses, and there is much left to be learned about low dose effects for many genotoxic compounds (Waddell et al., 2004). The sensitivity of methods that measure DNA adducts and biological endpoints induced by DNA damage will need to be improved for this purpose.

Marnett identified several important questions and issues relevant to the relationship between DNA adduct dosimetry and exposure. What is the most appropriate molecular

marker for a particular exposure? What are the limits of sensitivity for each method of detection? How specific is the assay? What is the relationship between dose and DNA adduct level in target vs. non-target tissues? What is an appropriate control DNA sample? How should the level of artifactual DNA adducts be assessed? Are endogenous and exogenous adducts with the same structure biologically equivalent? How can we account for differential rates of DNA repair and different degrees of persistence for different adducts?

Oxidative DNA damage

James Swenberg (University of North Carolina, Chapel Hill)

Normal cellular metabolism and exogenous radiation generate oxygen free radicals, which have the potential to damage biological macromolecules. Cells express several homeostatic mechanisms to manage and minimize the deleterious effects of oxidative compounds; nevertheless, oxidative damage is thought to contribute to several human diseases including neurodegenerative diseases, cancer, and aging. Oxidative DNA damage is the most common type of DNA damage and 8-hydroxy-deoxyguanosine (8OHdG) is the most commonly used marker of oxidative DNA damage. The European Standards Committee on Oxidative DNA damage (ESCODD) recently conducted a large study of variability in measurement of oxidative DNA damage. They noted large differences (almost three orders of magnitude) in measurements of background levels of 8OHdG in different laboratories and using different methods (ESCODD, 2002, 2003). James Swenberg emphasized that in general, minimizing artifactual oxidative DNA damage can be achieved by use of free radical scavenging agents and metal chelating agents during DNA isolation and in vitro DNA manipulations (Ham et al., 2004).

Swenberg presented studies of induced oxidative lesions in DNA exposed to several different agents, emphasizing results at low doses and the relationship of endogenous and exogenous damage. A complex non-linear dose response was observed when 8OHdG or aldehydic DNA lesions were measured as a function of concentration of H_2O_2. The shape of the dose-response curve for H_2O_2 was unusual with a moderate asymmetric peak at low dose range (0–1 mM) followed by a decline to a broad minimum (2–5 mM) and then a low slope increase to the higher dose range (5–20 mM) (Nakamura et al., 2003). Similar dose-response curves were observed for H_2O_2-induced 8OHdG and H_2O_2-induced aldehydic DNA lesions (ADL) (Fig. 2). Swenberg calculated that the DNA adduct-forming efficiency of H_2O_2 is at least 40-fold higher at 0.1 mM than at 2 mM or higher.

The source of endogenous ethenodeoxyguanosine (εG) was examined using immunoaffinity/GC/HRMS (high resolution mass spectrometry) analysis to quantify εG adducts and ^{13}C-labeled ethyl linoleate to differentially label adducts induced by lipid peroxidation. The results

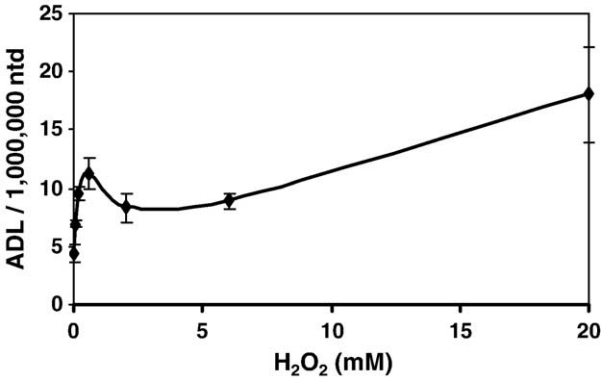

Fig. 2. Aldehydic DNA lesions in HeLa cells exposed to H_2O_2 (0.06–20 mM) for 15 min (Nakamura et al., 2003).

show that lipid peroxides are likely to be the predominant source of εG adducts, since ^{13}C-labeled adducts were detected in excess of unlabeled adducts under most conditions tested. In untreated rat brain and hepatocytes, εG adducts increase from 28 days to 2 years of age. Adult human tissues such as liver and colon may also accumulate a significant number of εG adducts due to endogenous oxidative damage (Barbin et al., 2003a, 2003b).

Malondialdehyde is another reactive species that can generate an endogenous exocyclic guanine adduct, pyrimido[1,2-alpha]purin-10(3H)-one (M_1G), in human cells. M_1G quantification is significantly improved if the adduct is stabilized with an aldehyde-reactive probe. Swenberg quantified M_1G in genomic DNA and showed a baseline level of between 1×10^{-8} and 2×10^{-8}, significantly lower than previous estimates. This method also showed a non-linear dose-response for bleomycin-induced M_1G adducts. M_1G was also formed efficiently in cells exposed to H_2O_2, TCBQ, and estrogen metabolites.

In closing, Swenberg pointed out that the rate of repair of oxidative DNA lesions varies not only with the specific lesion and cell type, but also with the state of the cell in which lesions are measured. For example, peroxisome proliferators induce many but not all base excision repair enzymes in a PPARα-dependent manner, and the rate of repair will influence steady-state adduct level.

High performance liquid chromatography electrospray (HPLC-ES) ionization tandem mass spectrometry (MS/MS) for the detection and quantitation of DNA adducts: application to DNA adducts derived from tamoxifen and acrylamide

Frederick A. Beland (National Center for Toxicological Research)

Frederick Beland presented case studies of tamoxifen and acrylamide to demonstrate use of HPLC-ES-MS/MS in the study of DNA adducts. Tamoxifen is an anti-estrogenic chemotherapeutic agent which reduces the rate of recurrence in breast cancer patients. Recently, tamoxifen has also been used for the chemoprevention of breast cancer. However,

use of tamoxifen as a chemoprevention agent is controversial because it increases the risk of endometrial cancer up to 4-fold in patients receiving tamoxifen therapy for 5 years. To optimize and perhaps improve tamoxifen therapy, it has therefore become critical to determine if tamoxifen induces endometrial cancer by a genotoxic mechanism or promotes endometrial cancer by another mechanism perhaps involving hormonal signaling.

Two metabolites of tamoxifen, α-hydroxytamoxifen and α-hydroxy-*N*-desmethyltamoxifen, are known to form two specific DNA adducts with guanosine nucleotides: (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen (dG-Tam) and (*E*)-α-(deoxyguanosin-N^2-yl)desmethyltamoxifen (dG-DesMeTam). Beland showed that, following treatment with tamoxifen, significant amounts of both DNA adducts are detected in rat liver but not uterine tissue and that dG-Tam is formed in monkey liver and uterine tissue. Using a limited number of human samples, Beland also showed the absence of detectable dG-Tam or dG-DesMeTam adducts in human endometrial or breast tissue samples from tamoxifen-treated subjects. Beland concluded that tamoxifen does not act as a tumor initiator in human tissues via a genotoxic mechanism involving dG-Tam or dG-DesMeTam. However, in discussion, Beland acknowledged the possibility that tamoxifen generates a low level of another DNA adduct that was not measured in this analysis (Beland et al., 2004; Gamboa da Costa et al., 2003a).

Acrylamide is a carcinogen, mutagen, and neurotoxin that was recently found to be present at low levels in fatty and starchy foods such as French fried potatoes, potato chips, and bread (0.3–0.8 μg acrylamide/kg bw/day estimated average adult human exposure). The mechanism of action of acrylamide in humans is controversial and may involve interaction with DNA. Beland measured DNA adducts in mice or rats treated with acrylamide or with its epoxide metabolite glycidamide. When glycidamide is incubated with DNA in vitro, the major adduct is formed via interaction with guanine N7 (N7-(2-carbamoyl-2-hydroxyethyl) guanine), with minor species interacting with adenine N3 or N1. In rats injected with a single intraperitoneal dose of acrylamide (50 mg/kg), glycidamide (50 mg/kg), or water, HPLC-ES-MS/MS analysis showed very high levels of the N7-guanine adduct in all tissues of glycidamide-treated animals. Highest levels were observed in testes (10,000 adducts/10^8 nucleotides (nt)), mammary gland, and leukocytes, and acrylamide-treated animals had fewer adducts than glycidamide-treated animals in all tissues. Similar results were observed in mice, except that the difference in the adduct levels between acrylamide and glycidamide was not as great. N7-guanine adducts were detected at 4- to 7-fold above the background level of 1×10^{-8} adducts in the liver of mice dosed with 100 μg/kg acrylamide or glycidamide. Because a high level of DNA adducts is detected in many tissues in exposed rats and mice, and because glycidamide forms adducts more efficiently than acrylamide in treated animals, Beland concluded that acrylamide induces tumors through a genotoxic mechanism involving metabolism to glycidamide and subsequent DNA adduct formation (Gamboa da Costa et al., 2003b).

Radiation-induced damage to cellular DNA: formation and measurement

Jean Cadet (French Atomic Energy Commission-Grenoble)

Jean Cadet is interested in characterizing the effects of ionizing radiation on DNA and identifying and quantifying radiation-induced DNA lesions. Ionizing radiation acts on DNA directly by one-electron oxidation, or indirectly via water radiolysis radicals, producing modified bases, abasic sites, single- or double-strand breaks, and DNA-protein cross-links (Fig. 3). Many radiation-induced modifications of thymine, cytosine, adenine, and guanine have been identified from model studies involving nucleosides or DNA fragments. It may be noted that the adenine moiety within DNA appears to be less susceptible to $^{\bullet}$OH radical and one-electron oxidation than the other pyrimidine and purine bases.

Cadet and others isolated and characterized as many as 70 radiation-induced oxidative base lesions in model studies (Cadet et al., 2003). Cadet showed it is now possible in a single HPLC run to measure several oxidized nucleosides including 5,6-dihydroxy-5,6-dihydrothymidine, 5-(hydroxymethyl)-2′-deoxyuridine, 5-hydroxy-2′-deoxyuridine, 5-formyl-2′-deoxyuridine, 8-oxo-7,8-dihydro-2′-deoxyadenosine, and 8-oxo-7,8-dihydro-2′-deoxyguanosine. In addition, the two formamidopyrimidine derivatives of guanine and adenine are also measured by the HPLC-MS/MS method. When DNA was analyzed from cultured human monocytes exposed to γ-irradiation (Pouget et al., 2002), 11 base lesions were quantified with the following abundances (lesions/10^9 bases/Gy): 5,6-dihydroxy-5,6-dihydrothymidine (4 diastereoisomers) (97), 5-(hydroxymethyl)-2′-deoxyuridine (29), 5-hydroxy-2′-deoxyuridine (<0.2), 5-formyl-2′-deoxyuridine (22), 8-oxo-7,8-dihydro-2′-deoxyadenosine (3), adenine formamidopyrimidine (5), 8-oxo-7,8-dihydro-2′-deoxyguanosine (20), guanine formamidopyrimidine (39).

Another useful method for analyzing radiation-induced DNA damage is the comet assay and particularly its modified version which involves the use of bacterial DNA repair enzymes to reveal classes of oxidized purine and pyrimidine lesions. The assay is particularly appropriate for low dose exposures and when only small amounts of biological material are available (<10,000 cells). Thus base modifications were detected in γ-irradiated monocytes for doses as low as 0.2 Gy. These measurements clearly show the modified comet assay is more sensitive than chromatographic methods (Collins et al., 2004).

Cadet summarized his main conclusions concerning radiation-induced DNA damage in cellular DNA as follows: abundance of modifications recognized to date decreases in

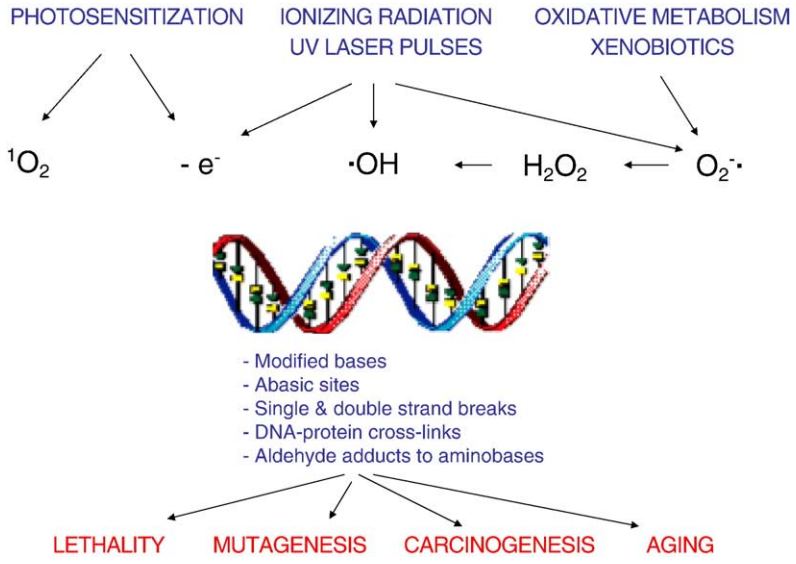

Fig. 3. Oxidative damage to DNA.

the following order: thymine > guanine ≫ adenine lesions; it may be added that purine base damage appears more frequently as formamido derivatives rather than 8-oxoderivatives. Another major observation deals with the low radiation-induced formation yields of single DNA damage; this underlines the major biological role played by clustered lesions that constitute the molecular signature of ionizing radiation. A final remark concerns the steady-state level of oxidized bases that is in the range $1/10^6$ to $1/10^7$ normal bases and that is, at least, 10-fold lower than that estimated a few years ago. In that respect, one of the major recommendations of European Standards Committee on Oxidative DNA Damage (ESCODD) was that values of 8-oxodGuo higher than 1 lesion per 10^6 normal bases in the DNA of untreated cells should be considered as overestimated (Collins et al., 2004).

Session two—relationship between DNA adducts and biological response

Discussion Leader **Bennett Van Houten** (National Institute of Environmental Health Sciences) introduced the second workshop session in which presenters were asked to define the relationship between DNA adducts and biological response. Van Houten emphasized the complexity of the biological response to DNA damage. It has recently become clear that multiple DNA damage response and DNA repair pathways are intricately interconnected and interdependent. For example, some nucleotide excision repair (NER)-deficient mice have a phenotype of premature aging, which suggests that NER and base excision repair (BER) may coordinately repair oxidative DNA damage; in contrast, many earlier studies suggest that BER repairs most oxidative DNA damage.

Citing the recent discovery of several lesion bypass polymerases in eukaryotic cells, Van Houten also drew attention to the fact that DNA lesion-induced mutagenesis can be enhanced when an error-prone DNA lesion bypass polymerase is recruited to sites of DNA damage. However, DNA lesion-induced mutagenesis can also be minimized in damaged cells when DNA repair enzymes that remove and repair DNA lesions are induced due to DNA damage-stimulated signaling. On a cellular level, DNA damage can also induce a cell cycle checkpoint, apoptosis, or a transcriptional response. Thus, different aspects of the biological response to DNA adducts and other types of DNA damage should be considered in attempting to correlate distinct cellular events with each other.

Tissue specificities in genomic instability and DNA repair pathways in aging

Jan Vijg (University of Texas Health Science Center)

The number of accumulated mutations in genomic DNA appears to increase as organisms increase in age (Fig. 4), and this may be a reflection of decreased DNA repair efficiency as a function of age (Vijg, 2000). Jan Vijg explored the relationship between mutation rates, genomic instability, and aging using a mouse transgenic model (Boerrigter et al., 1995) to assess mutagenesis in wild-type and mutant backgrounds. Vijg observed that mutant frequency increases in mice with age in a tissue-specific manner; mutant frequency increased moderately in liver and heart and significantly in small intestine from birth through 32 months of age, but mutant frequency reached a flat plateau at ≈5 months in testis and brain and a nearly flat plateau in spleen (Dollé et al., 1997, 2000; Vijg and Dollé, 2002). In general, animal and cell culture experiments using mouse embryo fibroblasts are consistent with the hypothesis that oxidative lesions generate a major fraction of age-dependent mutations.

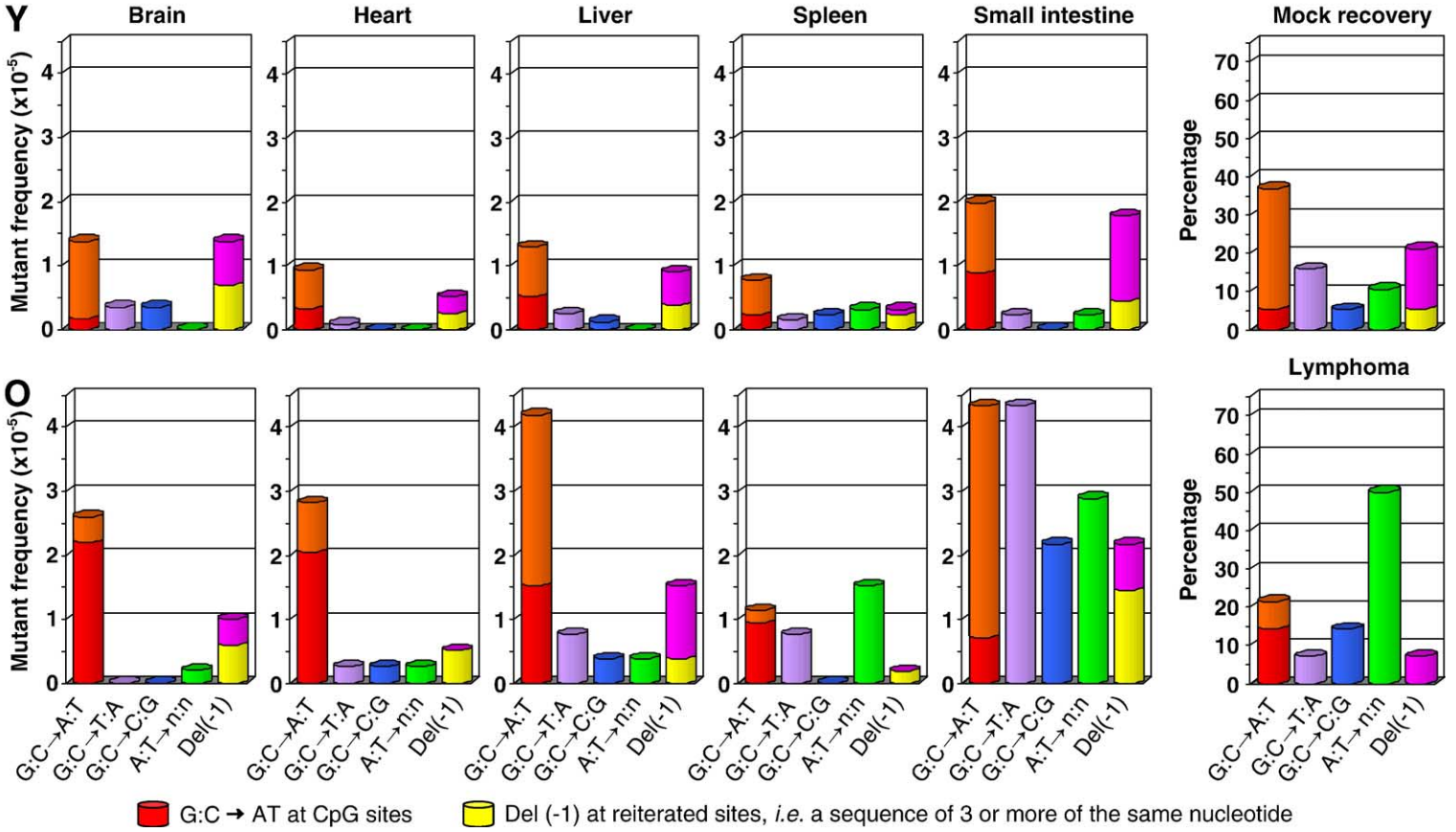

Fig. 4. Point mutational spectra in organs from young (Y; 3 month) and old (O; 32 month) mice (based on Dollé and Vijg, 2002).

Vijg examined life span and age-dependent increases in mutation frequency in DNA repair-deficient mouse strains. The life span of the Xpa (*Xeroderma pigmentosum* complementation group a) or the Xpd^{TTD} (Trichothiodystrophy) mouse is slightly shorter than wild-type or Csb (Cockayne's syndrome group b) mutant mice (median survival 118 or 110 weeks vs. 125 or 123 weeks), but not as short as the life span of Ercc1 (excision repair cross-complementing group 1) mutant mice which are deficient in repair of DNA cross-links (23 weeks). The relative reduction in life span roughly correlates with increase in number of age-dependent mutations in these strains. Thus, mutant frequency in liver DNA increases fastest with age in the Ercc1-deficient mice and is significantly higher in Xpa knockout mice. In Xpa mutant mice, the mutations that increase with age are primarily −1 frameshifts. The Xpd and Csb mutant mice did not show an increase in mutation frequency, possibly because the defects in these models primarily reflect a transcription problem rather than DNA repair defects.

Overall cellular response to MMS

Leona Samson (Massachusetts Institute of Technology)

Leona Samson and her colleagues utilized genomic phenotyping to analyze the system-wide response of *Saccharomyces cerevisiae* and *Escherichia coli* to MMS, UV, 4-nitroquinoline 1-oxide (4NQO), or t-butyl hydroperoxide (t-BuOOH).

Using genomic phenotyping, Samson's group identified a distinct group of sensitivity and resistance genes for different DNA damaging agents including 1441, 447, 819, and 288 genes that confer sensitivity to MMS, t-BuOOH, 4NQO, and UV, respectively. The majority of these genes are uncharacterized and have unknown function; the previously characterized genes have highly diverse functions including DNA repair, protein synthesis, cell cycle, DNA replication, cellular signaling, protein degradation, amino acid metabolism, transcription, and others. The broad representation of cellular functions involved in the response to MMS and other types of DNA damage indicates that these agents may have deleterious effects on diverse cellular components including nucleic acids, proteins, and membranes. Several subnetworks, in addition to the DNA damage response itself, were identified as playing a role in the response to MMS; these were RNA processing, protein degradation, protein synthesis, chromatin remodeling, cytoskeleton remodeling, and chromatin segregation (Begley et al., 2002).

Samson also analyzed the subcellular localization of proteins involved in DNA damage response pathways. Referring to the results of a recent global analysis of yeast protein localization (Huh et al., 2003), Samson observed that proteins involved in the MMS response network are enriched in the nucleus, endosome, microtubules, and vacuolar membranes. A distinct pattern of subcellular enrichment locations was noted for proteins involved in the response to t-BuOOH, 4NQO, and UV, but each response pathway (except t-BuOOH) localized components to the nucleus; this result emphasizes the overlapping but distinct nature of different DNA damage response pathways. These studies also underscore the overall complexity and interconnectedness of the multifaceted response to DNA damage.

Functional genomics approaches in the study of the cellular responses to genotoxic agents

Albert Fornace (National Cancer Institute/NIH)

Albert Fornace used ionizing radiation as a model agent to study the response to DNA damage at the level of gene expression. The goal of these studies was to identify characteristic gene expression signatures in different cell types and to use these data for risk assessment and/or to improve therapeutic treatments for cancer and other disease states.

Fornace studied the response to low dose radiation (50 cGy or lower) in human p53 wild-type myeloid ML-1 cells and in human peripheral blood lymphocytes (PBLs), which are non-growing cells. Specific genes, including WAF1 (*CDKN1A*) and GADD45 (*GADD45A*), responded in a dose-dependent manner in ML-1 cells (Amundson et al., 2000a, 2003). In general, apoptosis response appeared to be dose-rate dependent while checkpoint response appeared to be dose-rate independent. Acute phase response genes were not induced in ML-1 cells. In PBLs irradiated ex vivo, cyclin G1, XPC, DDB2, PCNA, and WAF1 showed dose-dependent effects and XPC, DDB2, and WAF1 showed a linear response from 0.2 to 2 Gy. These results suggest that PBLs may be a useful surrogate tissue for the study of radiation exposure in humans. This possibility was confirmed using PBLs from patients undergoing whole body irradiation in preparation for bone marrow transplant. Fornace identified dose-specific gene expression patterns in PBLs from these patients.

Agent-specific DNA damage responses were also studied in two isogenic cell lines: TK6, a non-tumor spontaneously-immortalized human lymphoblastoid cell line, and NH32, a p53-null derivative of TK6 (Amundson et al., 2000b). Cells were treated with a non-cytotoxic dose of 12 genotoxic and nongenotoxic agents (UVB, 43 MeV neutron, γ-ray, hydrogen peroxide, sodium arsenite, MMS, adriamycin, camptothecin, cisplatin, TPA, osmotic shock, and heat shock). Gene expression profiles were analyzed by microarray with a human cDNA array library; 1338 unselected genes, which were stress responsive in at least 5 cell lines, were studied. The expression profiles fell into recognizable patterns which correlated with type of stress exposure (i.e., oxidative stress, infrared radiation-like, non-IR-like genotoxic, nongenotoxic). These agent classes were further defined and could be differentiated using a specific subset of discriminator genes. Examining only genotoxic agents, discriminator genes were identified that distinguish a p53-dependent stress response from a p53-independent stress response.

Session three—low levels of DNA adducts as potential predictors of adverse outcomes

Discussion leader **Fred Kadlubar** (National Center for Toxicological Research) presented data collected several years ago which demonstrated that low levels of DNA adducts correlate well with tumorigenesis as assayed by either a skin tumor promotion assay or a mouse neonatal assay. He also presented data on DNA adduct levels in human epithelial cells from breast milk which suggest that DNA adducts in these cells may have potential as biomarkers of exposure to hair dye or cigarette smoke (second or first hand; Gorlewska et al., 2002a, 2002b; Turesky et al., 2003). Kadlubar then challenged the speakers of the session to provide further evidence for whether low levels of DNA adducts may be useful for predicting adverse outcomes.

Etheno DNA adducts: their formation and biological consequences in mammals under conditions of acute or chronic exposure

Alain Barbin (International Agency for Research on Cancer)

Alain Barbin presented studies of the biological consequences of etheno DNA adducts using rodent model systems. Etheno DNA adducts are formed by environmental carcinogens such as vinyl chloride and urethane or by endogenous compounds such as lipid peroxides. The most common etheno-nucleoside adducts observed in vivo are 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine, and 1,N^2-ethenoguanine. These adducts are mutagenic in cells, both *E. coli* and mammalian cells. Exposure to vinyl chloride induces hepatocarcinomas and angiosarcomas in humans and rodents. These tumors often carry characteristic mutations in *ras* or *p53* that are consistent with formation of etheno-adenine adducts (Barbin, 2000). Similar observations have been made regarding tumors associated with exposure to vinyl carbamate or urethane.

Barbin showed that εA and εC accumulate with a linear dose-response in liver DNA of rats exposed subchronically to vinyl chloride (Guichard et al., 1996). εA is repaired more efficiently in lung and kidney of exposed rats, and εC accumulates differentially in these tissues. In humans, alkylpurine DNA glycosylase (ANPG) is the primary repair enzyme that acts on εA and εG. In knockout mice deficient in the mouse ANPG homolog, higher levels of εA accumulated after exposure to vinyl carbamate, and the half-life of εA adducts was 2- or 3-fold longer in liver or lung from knockout animals than in wild-type mice, suggesting that εA is repaired by ANPG in vivo in mice (Barbin et al., 2003a, 2003b). In contrast, repair of εC did not appear to be altered in ANPG knockout mice. Barbin also looked at tumor induction in wild-type and ANPG mutant mice exposed to vinyl carbamate. Surprisingly, the ANPG mutant mice did not demonstrate an exposure or adduct-associated increase in latent tumors 1 year after treatment was terminated. Barbin also observed that the number of proliferating and apoptotic hepatocytes was similar in ANPG-deficient and wild-type mice exposed to vinyl carbamate. This result suggests that ANPG-catalyzed repair of εA is not essential to prevent vinyl carbamate-induced liver tumors; nevertheless, Barbin calculated that DNA repair, cell proliferation, and apoptosis are mechanisms that contribute to removing εA adducts that form after exposure to environmental carcinogens such as vinyl carbamate (Fig. 5). Numerous factors can lead to persistent etheno DNA adducts in vivo, including inhibition of DNA repair enzymes, elevated endogenous lipid peroxides, or

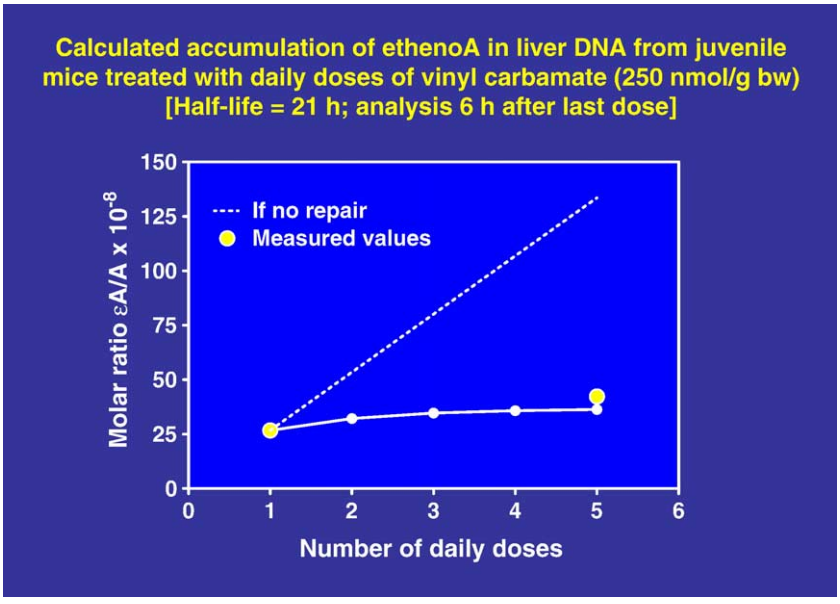

Fig. 5. Calculated accumulation of ethenoA in liver DNA from juvenile mice treated with daily doses of vinyl carbamate (250 nmol/g bw).

accumulation of poorly repaired intermediates, and these adducts may play a role in carcinogenesis in humans. This possibility is consistent with the observation that the carcinogenic potency of etheno-adduct forming compounds correlates well with the covalent DNA binding index of these compounds. Barbin also suggested that levels of εA and εC adducts may be useful biomarkers of chronic oxidative stress and its associated risk of disease in humans.

DNA adducts: biomarkers for tamoxifen-induced endometrial cancer

Arthur Grollman (State University of New York, Stony Brook)

Tamoxifen is used in the treatment of breast cancer and decreases the risk of developing breast cancer by 50% in women at high risk of developing this disease. However, tamoxifen therapy is also associated with a significantly increased risk of developing endometrial cancer. Several mechanisms have been offered to explain this carcinogenic effect. For example, it has been proposed that tamoxifen may act as a partial estrogen agonist. Arthur Grollman presented evidence supporting an alternative mechanistic hypothesis; namely, that tamoxifen acts through genotoxic effects. Strong support for this view is provided by studies that demonstrate the presence of tamoxifen-DNA (Tam-DNA) adducts in human and primate endometrial tissues (Shibutani et al., 2000, 2003).

Tamoxifen and its desmethyl derivative are α-hydroxylated by CYP3A4, which is then O-sulfonated by hydroxysteroid sulfotransferase, generating a metabolite that reacts with DNA. Site-specific mutagenesis studies in Simian kidney cells were used to demonstrate that tamoxifen DNA adducts are mutagenic and can be repaired by the nucleotide excision repair pathway. Terashima et al. (2002) identified four possible isomers of the tamoxifen guanine adduct (dG-N^2-Tam) in human tissues using a ^{32}P-post-labeling/HPLC analytical method (Terashima et al., 2002). High-resolution acrylamide gel electrophoresis has also been used to resolve dG-N^2-Tam isomers. These methods were used to analyze endometrial DNA samples from 16 women treated with tamoxifen for treatment periods of 4–72 months. dG-N^2-Tam was detected in eight of the 16 samples (Shibutani et al., 2000). The level of tamoxifen-DNA adducts did not correlate with the length of tamoxifen therapy and may reflect inter-individual variability in activation of tamoxifen and/or repair of tamoxifen adducts. dG-N^2-Tam was also detected at low levels in uterine DNA obtained from one of three Cynomolgous monkeys, in ovarian DNA from two of three monkeys, in liver DNA from three of three monkeys, and in brain cortex from three of three monkeys (Shibutani et al., 2003).

Based on these data, Grollman argued that tamoxifen-DNA adducts play a central role in initiating endometrial cancer by a genotoxic mechanism. Importantly, clinically-effective anti-estrogens, such as toremifene and raloxifene, have little or no potential to form DNA adducts (Shibutani et al., 2001) and may be safer yet equally efficacious alternatives to tamoxifen in the treatment and prophylaxis of breast cancer.

DNA alkylation and cancer risk at low levels of exposure in animal models and humans

David Shuker (Open University, United Kingdom)

David Shuker analyzed the biological consequences of low-level exposure to alkylating agents in humans and animal model systems. Exogenous environmental compounds and endogenous sources generate DNA alkylation adducts in DNA from animals and humans. The primary DNA adducts observed in vivo include 3-methyladenine, O^6-methylguanine, and N7-methylguanine. However, there is large interindividual variability in the level of DNA adducts detected in human DNA samples, which may reflect differential rates of metabolic activation of parent compounds or repair of DNA adducts.

Low-dose responses are generally poorly understood for many carcinogenic agents, and when DNA adducts form, the correlation between adduct level and tumorigenicity is unpredictable. Nevertheless, in animal studies, a few carcinogens such as aflatoxin and the alkylating agent dimethylnitrosamine show linear correlation between dose, adduct level, and tumor incidence. The dose range relevant to human exposure is however a difficult one to analyze in animal models due to high background levels of adducts, which leads to insufficient sensitivity.

A sensitive immuno-slotblot assay was developed to quantify O^6-carboxymethylguanine (O^6-CMdG) adducts induced by low-dose exposure in DNA from experimental animals or human clinical samples (Cupid et al., 2004). These experiments showed that stomach DNA accumulates a significant level of O^6-CMdG in experimental animals exposed to exogenous diazoacetate. Immunohistochemical analysis also indicated that DNA of the small intestine contains O^6-CMdG, which may reflect endogenous exposure to alkylating compounds. In human clinical DNA samples, gastric biopsy samples contained DNA with a very low level of O^6-CMdG, which was also confirmed by immunohistochemical staining.

In the last part of his talk, Shuker emphasized that, especially at low levels of adducts, the specific location of an adduct within a gene or non-coding sequence and the precise nature of the adduct are important determinants of the biological consequences of the adduct. Thus, methods that analyze adduct location may be needed to assess the biological impact of a particular DNA adduct. One such method is atomic force microscopy, with which individual DNA molecules are visualized and adducts can be precisely mapped. Shuker also suggested that there may be a critical minimal number of adducts required per genome before there is strong potential for the adducts to

have significant biological consequences. This level may be at or somewhat below one adduct per gene. For mammalian genomes, this is somewhere between 1 adduct in 10^8 nucleotides and 1 adduct in 10^9 nucleotides. This may be equivalent to a threshold for carcinogenesis for genotoxic agents.

Session four—DNA adducts in risk assessment: current practice

In opening the fourth conference session, discussion leader **Sheila Galloway** (Merck) emphasized the different use of DNA adduct data ranging from use in mechanistic studies when the only data available on a new compound are in vitro genotoxicity data, through the formal process of quantitative risk assessment, with a need to take into account such factors as DNA replication and repair, toxicity, metabolism, detoxification, tissue specificity, and species differences.

DNA adduct data may be useful early in drug development as an indicator of safety issues that might arise later in the drug evaluation process. We have to be concerned not only about potential human safety, but also about potential impacts of tumorigenicity at high doses in rodents, where protective mechanisms may be overwhelmed and homeostasis is lost. Galloway indicated that in most cases it is not practical or feasible to analyze adduct structure early in drug development; thus adducts are not used alone in screening, since the mutagenic potential of uncharacterized adducts is not known. When a compound is positive in a genotoxicity assay and then is found to form adducts, structurally similar compounds may be screened with a DNA adduct assay to identify alternative compounds with lower or no DNA adduct-forming potential. DNA adduct data are also useful in mechanistic studies when a compound is positive in a genotoxicity assay that does not discriminate between indirect and direct interaction with DNA.

For predictive value in risk assessment, there are key areas of uncertainty in how to interpret DNA adduct data. These include how to extrapolate animal data to humans, the importance of target tissue data vs. surrogate tissue data, and whether there is an acceptable level of risk for low levels of adducts, especially in light of the known endogenous and/or background levels of DNA adducts.

Adducts. . .but no measurable effects: examples from industrial chemicals

Lynn Pottenger (The Dow Chemical Company)

Lynn Pottenger summarized studies on ethylene and ethylene oxide, describing the differences in potential hazards to exposed humans and the status of these compounds as regulated environmental chemicals. Ethylene is ubiquitous in the environment, arising from natural sources including plants and animals, and from human activities including combustion (automotive) and industrial applications. Ethylene is used in many industrial manufacturing processes and is a high production volume chemical (>235 billion pounds per year). Ethylene has a large toxicology database and is negative in genotoxicity assays; it is recognized as an asphyxiant, with narcotic effects at high doses, and is a significant explosive and flammability risk (IARC, 1994).

Ethylene is poorly absorbed and poorly metabolized by animals and humans, with the majority inhaled being exhaled unchanged. The absorbed fraction can be metabolized to ethylene oxide (EO) by Cytochromes P450. This is a saturable process, producing a limited steady-state amount of ethylene oxide in vivo. The fraction of total ethylene intake converted to EO is estimated at 2% in humans and 4% in rats (Csanády et al., 2000; Ehrenberg et al., 1977; Filser et al., 1993). EO, unlike ethylene, is a mutagen and carcinogen in animals and is classified as a known or probable human carcinogen in many countries. EO is a reactive molecule, which forms DNA adducts including N7-hydroxyethylguanine (N7HEG), N^2HEG, and O^6HEG (Tornqvist, 1996; Walker et al., 2000).

N7HEG adducts, which represent the major EO-related adduct, are present at a significant level (3-270 HEG/10^8 nt) in control human tissues (Walker et al., 2000). In rats and mice exposed to ethylene, N7HEG adducts can be detected above background levels in all tissues examined, including liver, spleen, brain, and lung, with the highest level of adducts found in liver. However, the presence of these N7HEG adducts following exposure to ethylene does not correlate with the lack of increases in mutations or tumor incidence following ethylene exposure, based on the extensive toxicology database.

In 1999, ethylene was classified as a Category 3 Mutagen by German regulatory authorities, the first time any classification was based on DNA adduct evidence alone. This decision is controversial because the presence of ethylene-induced DNA adducts per se was considered sufficient to assign mutagenic potential to ethylene, even though mutagenicity and carcinogenicity tests for ethylene have been uniformly negative. Subsequent to the German decision, the European Union reviewed the data for ethylene and decided not to classify ethylene for mutagenicity.

These observations underscore the sensitivity gap that exists between the detection limits of DNA adducts and the detection limits of mutations. Pottenger estimates that this gap is as large as five orders of magnitude. Nevertheless, Pottenger suggests that it is most appropriate to use DNA adduct data for risk assessment in the context of a total weight-of-evidence approach. Thus, if the total toxicology database for a compound strongly supports negative mutagenicity, that result should have greater weight than DNA adduct-forming potential per se. Mechanistic information that explains the lack of biological effects of a compound should, in some cases, dictate acceptance of an NOAEL level for that compound (i.e., practical threshold).

DNA adduct study with raloxifene (Evista®)
Michael Garriott (Eli Lilly and Company)

Raloxifene is a non-steroidal, tissue-specific, selective, estrogen receptor modulator developed and approved for use to prevent osteoporosis in post-menopausal women. Michael Garriott presented studies on the potential genotoxic and carcinogenic activities of raloxifene which he contrasted with tamoxifen. In vitro and in vivo genotoxicity tests with raloxifene were uniformly negative in dose ranges that cause significant cellular toxicity. However, raloxifene was tumorigenic in 2-year bioassays in rats and mice, causing ovarian tumors in a dose-dependent manner. The tumorigenic effects of raloxifene in rodents have been attributed to raloxifene-dependent interference with feedback inhibition of pituitary hormone signaling. This leads to higher than normal levels of gonadotropin releasing hormone, luteinizing and follicle stimulating hormones (Risma et al., 1995; Willemson et al., 1993). A DNA adduct analysis with a ^{32}P-postlabeling assay to test this hypothesis was conducted, assuming that lack of DNA adduct detection supports the proposed nongenotoxic mechanism for raloxifene-induced carcinogenesis. Rats were exposed to three doses of raloxifene for 7 days and ovary DNA was analyzed for DNA adducts using two DNA extraction procedures. The results indicated that raloxifene exposure stimulated formation of an endogenous adduct in rats, but it did not generate new adduct species. These data were considered indicative of a nongenotoxic basis for the tumorigenicity of raloxifene. Preliminary data from human clinical studies are consistent with this hypothesis (Neven et al., 2002).

DNA adducts in hazard evaluation: IARC's perspective
Robert Baan (International Agency for Research on Cancer)

Robert Baan summarized the methods and principles used by the International Agency for Research on Cancer (IARC) in generating the IARC monographs on human carcinogens, which are critical reviews of information relevant to cancer hazard assessment in humans.

IARC classifies compounds into one of five cancer hazard classes as follows: Group 1, human carcinogen; Group 2A, probable human carcinogen; Group 2B, possible human carcinogen; Group 3, inadequate data to determine carcinogenic potential in humans; Group 4, probable non-carcinogen in humans. When limited human data are available, the compound can be classified as 2A or 2B. When human data are inadequate, but animal data are available, the compound can be classified as 2B or 3.

Baan summarized the rationale behind IARC classification of four agents. Two of these agents, *d*-limonene (Group 3) and *p*-dichlorobenzene (Group 2B) (IARC, 1999), are associated with renal cell carcinoma and two other agents, amitrole (Group 3) and thiouracil (Group 2B) (IARC, 2001), are associated with thyroid follicular cell carcinoma.

Three mechanisms have been proposed for renal carcinogenesis in rodents: direct genotoxic DNA damage, oxidative stress-induced indirect DNA damage, or inappropriate stimulation of cell proliferation. The latter mechanism can occur when the male-specific, species-specific protein α-2u-globulin binds exogenous compounds and accumulates in the proximal tubules of the kidney; the overabundance of α-2u-globulin in the tubules stimulates sustained cell proliferation leading to kidney tumors (Capen et al., 1999). Baan reviewed data establishing that kidney carcinogenesis in rats exposed to *d*-limonene is α-2u-globulin-dependent and male-specific. Kidney carcinogenesis was not observed in female rats or in mice, and *d*-limonene showed no evidence of genotoxicity. These and other supporting data were sufficient to establish that the mechanism by which *d*-limonene increases the incidence of kidney cancer in male rats is not relevant to humans. Therefore, IARC identified *d*-limonene as Group 3: not classifiable as to its carcinogenicity to humans. In contrast, *p*-dichlorobenzene is classified as 2B by IARC. Although *p*-dichlorobenzene is also a renal carcinogen in male rats (not in female rats or mice) via an α-2u-globulin-dependent mechanism, *p*-dichlorobenzene binds to DNA, causes weak cytogenetic effects, and induces adenomas and carcinomas in mouse liver. Baan explained that IARC could not confidently exclude a DNA-reactive mechanism for *p*-dichlorobenzene-induced carcinogenesis in the mouse, which justifies the 2B vs. 3 classification.

In rodents but not in humans, thyroid tumors can be induced by hormonal imbalances (i.e., overproduction of thyroid stimulating hormone). In general, rodents are more sensitive to thyroid carcinogenesis than humans (see Capen et al., 1999). Baan summarized data for amitrole and thiouracil, both of which appear to cause thyroid cancer in rodents by a hormonal mechanism. There was inadequate human epidemiological data for either agent. However, amitrole was determined to be a Group 3 agent and thiouracil was determined to be a Group 2B agent, because genotoxicity assays were adequate to rule out significant genotoxicity for amitrole but inadequate to rule out genotoxicity for thiouracil.

U.S. EPA perspective on research needs to support application of adduct data in risk assessment: framework based on mode of action
Annie Jarabek (U.S. Environmental Protection Agency)

Annie Jarabek proposed a framework in which regulatory risk assessment guidelines might begin to integrate molecular biomarker data and molecular mechanistic data based on genomic-based technology (i.e., global transcriptome or proteome analysis). It is imperative to develop a new framework now because of several emerging trends in biomedical science: these trends include exponentially increasing amount of biomolecular data in data- and knowledgebases, increasing sophistication of biological

data, increased detail in molecular analysis, appreciation and understanding of genetic susceptibility, and enhanced quantitative and qualitative computational capacity.

The goal of this framework revision is to increase emphasis on toxicological mode of action and to facilitate incorporation of as much mechanistic information relevant to toxicological mechanism as possible. Mode of action describes the exposure-dose-response continuum and maps pathogenesis through the exposure-disease continuum using biomarkers of exposure and biomarkers of adverse effects. Observations should be considered in different species and should include studies at all critical life stages, tracking pathogenesis from pre-clinical to sub-clinical to early and late disease stages. Individual variation in animals and humans should also be addressed.

Mode of action provides a platform for the harmonization of noncancer and cancer endpoints, which are currently artificially associated with a dichotomy in analytical approach. This dichotomy separates effects as non-threshold vs. threshold, irreversible vs. reversible, and linear vs. non-linear, and focuses on either risk estimate or safety estimate. Instead, it may be appropriate to construct biologically-based or case-specific models based on a series of defined key events in pathogenesis.

It is critical to effectively establish causality, strength of association, consistency, specificity, and sensitivity in order to evaluate the reliability and predictive power of precursor lesions and key events. Understanding key events linked to mode of action that lie along the exposure-disease continuum is critical. This type of analysis will also help identify dose- and time-dependent components and differentiate adaptive from adverse effects.

Use of DNA adduct data in FDA's office of new drugs

David Jacobson-Kram (U.S. FDA)

Genotoxicity data play an important role in safety evaluation at the FDA, especially when carcinogenesis assays are not yet available. In these cases, genotoxicity data are considered to be a surrogate for the in vivo carcinogenesis tests, which are the definitive test for carcinogenicity at FDA. If a drug is nongenotoxic in a battery of three standard in vitro genotoxicity assays, clinical trials can proceed. However, if some evidence of genotoxicity is observed in these tests, additional tests are performed. If a drug shows evidence of genotoxicity, other factors are considered in determining whether the risk to clinical trial participants is outweighed by potential benefits. For example, FDA considers the severity of the condition for which the drug will be prescribed, the target population, the intended duration of use, and the availability of drugs that have equal benefit without genotoxicity.

Jacobson-Kram reviewed the genotoxicity of marketed drugs using information in the 1999 PDR and peer-reviewed literature (ICH S2A Technical Requirements for Registration of Pharmaceuticals for Human Use, 1996; ICH S2B Technical Requirements for Registration of Pharmaceuticals for Human Use, 1997). He identified 467 marketed drugs excluding anticancer drugs, nucleosides, steroids, biologicals, and peptide-based drugs. He summarized the results of a published review as follows: 115/467 marketed drugs have no genotoxicity data; 352 have one or more genotoxicity test result, of which 101 have 1 positive test; 201 have genotoxicity and rodent carcinogenicity test results; 124/201 are negative for carcinogenesis in rodents; 77/201 are positive or equivocal for carcinogenesis in rodents; and 100/124 noncarcinogens are nongenotoxic. Of the 24 noncarcinogens with positive genotoxicity studies, 19 are positive in cytogenics-based assays. Of the 77 rodent carcinogens, 26 are positive for genotoxicity.

In general, the FDA allows single-dose studies in volunteers with compounds giving positive in vitro genotoxicity results. Jacobson-Kram was not aware of requests by the FDA for DNA adduct data during regulatory review (Jacobs and Jacobson-Kram, 2004). Negative results in a DNA adduct assay would be given little weight, but positive DNA adduct data would contribute to evidence for genotoxicity. Jacobson-Kram indicated that DNA adduct data are useful to clarify carcinogenicity results, to elucidate the mechanism of carcinogenesis, or to evaluate low dose range effects.

Assessing the value of DNA adducts in a regulatory context: utility of a parallelogram approach

George Douglas (Health Canada)

George Douglas gave an overview of how DNA adduct data are used in regulatory decision-making at Health Canada. Douglas emphasized that Health Canada uses DNA adduct data as a qualitative, not quantitative, piece of evidence in assigning genotoxic potential to a specific compound. Alternatively, DNA adduct data can be viewed as a biomarker of exposure to a compound of interest.

Douglas questioned whether DNA adduct data were suitable as quantitative evidence for mutagenicity (Walker et al., 1992). If they were suitable as quantitative data, then he suggested it should be possible to define a four-way correlation between in vitro mutant frequency, in vitro adduct level, in vivo mutant frequency, and in vivo adduct level (Schut et al., 1997a,b). Similarly, the mutant frequency in two different tissues of the same exposed animal should correlate with the level of adducts in those two tissues (Arlt et al., 2004). In contrast, Douglas presented data showing that this "parallelogram" relationship breaks down for 3-nitrobenzanthrone (3-NBA) adducts in transgenic mice and FE1 lung epithelial cells (Arlt et al., 2003a, 2003b; Arlt et al., 2004). Douglas showed that the mutagenic potential of 3-NBA adducts is dramatically different for in vivo exposures compared with in vitro exposures, and for adducts in lung or bone marrow of 3-NBA exposed mice (Fig. 6). Douglas explained this result by suggesting that the mutant frequency

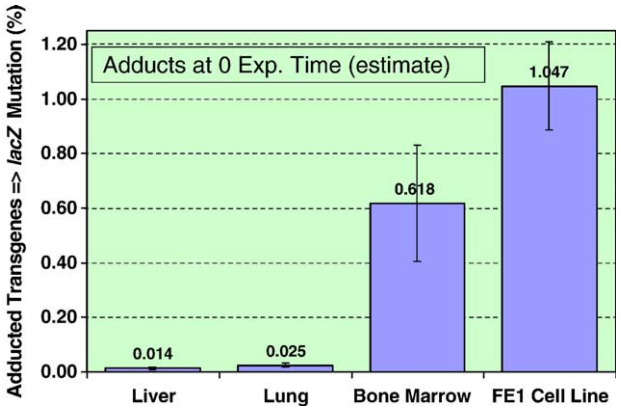

Fig. 6. Estimated likelihood of a mutation in an adducted *lacZ* transgene for liver, lung, bone marrow, and FE1 MutaMouse cells exposed to 3-nitrobenzanthrone. Relative adduct labeling (RAL) was determined using P1 nuclease enhanced ^{32}P postlabeling on selected tissues. Mutant frequency at the *lacZ* transgene in selected tissues was determined using the P-gal-positive selection system. Calculations assumed a mouse genome size of 5.22×10^9 bp, and transgene copy numbers of 33.6, 35.1, 45.3, and 17.5 per diploid cell for liver, lung, bone marrow, and the FE1 MutaMouse cell line, respectively.

of a DNA adduct is strongly influenced by tissue-specific variation in proliferative capacity. In this case, it may not be valid to extrapolate DNA adduct data from one tissue to another or from one experimental system to another without accurately accounting for differences in proliferative capacity.

Regulatory perspective on data gaps from Japan

Makoto Hayashi (National Institute of Health Sciences-Japan)

Makoto Hayashi summarized general regulatory guidelines for product safety in Japan. Hayashi indicated that a staged analysis procedure is used to evaluate genotoxic and carcinogenic potential of pharmaceuticals, food additives, agricultural chemicals, and industrial chemicals. Generally, the first stage of analysis requires a standard battery of mutagenesis tests (i.e., Ames, in vitro chromosomal aberration or mouse lymphoma assay, and rodent micronucleus assay). Depending on guidelines, genotoxicity of the chemicals has been evaluated and further test(s) required on a case-by-case basis. Generally, however, if all in vivo assays are negative, the agent is not considered an in vivo mutagen and its use is not regulated. If positive or equivocal results are obtained in the in vivo test(s), additional tests are chosen on a basis to confirm the result and characterize the carcinogenic potential of the agent. In vivo genotoxicity assays currently in use include liver UDS, comet assay, transgenic animal models, and ^{32}P-postlabeling assay.

Hayashi summarized data on genotoxicity and carcinogenicity of the heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). Humans are exposed to MeIQx in dietary sources such as baked fish at the level of 0.2–2.6 μg/day. MeIQx is positive in genotoxicity assays and causes hepatocarcinomas at high doses and adenomas at low doses in rodents. Low dose threshold effects were observed for several endpoints (8-OH-dG, GST-positive foci, in vitro and in vivo genotoxicity assays), but DNA adducts showed a linear dose-response curve at very low doses (Fukushima et al., 2002).

In contrast, Hayashi showed that exposure to Kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) causes thyroid adenomas in mice most likely by a nongenotoxic mechanism. Tests for Kojic acid-induced DNA adducts or oxidative DNA damage were negative (Hirose, M., personal communication).

For regulatory purposes in Japan, the minimal testing recommendations for genotoxicity are a three-test genotoxicity battery for pharmaceuticals (Ames test, chromosomal aberration or mouse lymphoma TK assay, and rodent micronucleus assay) and agricultural drugs and food additives (Ames test, chromosomal aberration assay, and rodent micronucleus assay), or the Ames test and chromosome aberration assay for industrial and occupational chemicals. The strategy for genotoxicity data evaluation and interpretation has been studied by the Japanese Environmental Mutagen Society ad hoc committee and in consultation with outside experts as needed, to arrive at appropriate risk assessment decisions.

Panel discussion

Moderator: Julian Preston (U.S. Environmental Protection Agency)

Panelists:

George Douglas (Health Canada)

David Jacobson-Kram (U.S. Food and Drug Association)

Lynn Pottenger (The Dow Chemical Company)

M. Vijayaraj Reddy (Merck Research Laboratories)

Jeffrey Ross (U.S. Environmental Protection Agency (ORD))

Gary Williams (New York Medical College)

Julian Preston moderated the panel discussion, which was the last workshop session. In his opening remarks, Preston pointed out that use of DNA adduct data as a tool for hazard identification remains somewhat problematic, because of uncertainty concerning the biological significance of low levels of DNA adducts. Preston also questioned whether it is appropriate to use DNA adducts as a surrogate marker for tumors, or if another marker closer to the biological outcome might be more useful. Several recently published articles provide an informative discussion of the current situation (Baird and Mahadevan, 2004; Hemminki and Thilly, 2004; Preston, 2003; Waddell et al., 2004). These questions remained open for discussion during the remainder of the session.

Preston asked each of the panelists to succinctly present their thoughts on one of the discussion questions provided

by the workshop organizing committee. The relevant questions and the panelist who addressed them are listed below.

What kinds of data do we need to determine the biological relevance/risk of low-level DNA adducts?
Gary Williams

Williams presented two experimental results relevant to the panel discussion session. First, he showed that the nasal carcinogen 2,6-dimethylaniline causes adducts in its target tissue as well as in liver and testes, where no carcinogenic effects are observed (Jeffrey et al., 2002). Likewise, the bladder carcinogen 4,4′ methylene-bis(2-chloroaniline) produced liver and testes adducts. Thus, the presence of DNA adducts cannot per se be equated with tumorigenic potential. Secondly, Williams provided evidence for a No Observed Adverse Effect Level (NOAEL) for initiating activity of 2-acetylaminofluorene in rat liver. In particular, at the lowest doses tested, Williams argued that the rate of adduct repair greatly exceeded the rate of adduct formation, so that no adducts could be detected in rat liver for up to 12 weeks following continuous exposure (Williams et al., 2000, 2004).

When is it valuable to measure DNA adducts? With a new compound? To answer mechanistic questions?
M. Vijayaraj Reddy

Within the context of pharmaceutical drug development and review, Reddy indicated that DNA adduct data are useful when the test compound:

1) is positive in the chromosome aberration assay, but negative in the Ames test or DNA strand break assay;
2) positive in mammalian cell mutagenesis assay, but negative in all other genotoxicity assays;
3) includes a contaminant or degradation product that is positive in microbial genotoxicity assays;
4) is part of a screen for a nongenotoxic variant of a genotoxic compound;
5) belongs to a class of agents that has demonstrated genotoxicity; or
6) forms tumors in rodents but is negative in the standard battery of genotoxicity assays.

In general, Reddy recommended using additional tests (e.g., radiolabeling, PCR inhibition) when the postlabeling method is negative (Phillips et al., 2000). Radiolabeled methods allow for comparison of in vivo and in vitro results for estimation of cancer risk based on Lutz's in vivo covalent binding index (Otteneder and Lutz, 1999). The postlabeling assay is not sensitive for certain adducts. In scenarios 1 and 2, compounds that form adducts are generally not retained in the development pipeline unless their potential health benefit far outweighs health risk. He pointed out that compounds such as melatonin and serotonin cause adducts, as an example of the difficulty of interpreting potential risk from drug candidates that incorporate structures of, or are similar to, endogenous chemicals, and are found to induce adducts (Reddy et al., 2002). Reddy recommended that additional research be carried out to establish whether and in what circumstances low levels of adducts might constitute an acceptable risk relative to substantial levels of endogenous adducts.

When are adducts qualitatively useful?
When are adducts quantitatively useful?
David Jacobson-Kram

Jacobson-Kram suggested that DNA adduct data had the potential to be qualitatively and quantitatively useful when other genotoxicity tests are positive and when additional information on the mechanism of action of the compound is available. The primary concern within the FDA is safety, and the sponsor carries the burden of proof for safety of their product.

Are DNA adducts predictive metrics at low doses and/or a measure of exposure?
Jeffrey Ross

Ross addressed this question by presenting a quantitative kinetic analysis of DNA adducts and lung tumors in mice dosed with a series of polyaromatic hydrocarbon (PAH) carcinogens (Prahalad et al., 1997). Strain A/J mice were injected with a single intraperitoneal dose of five selected PAHs, and DNA adducts were quantified by ^{32}P-postlabeling 1, 3, 5, 7, 14, and 21 days after dosing. Each PAH was tested at 4 doses. Lung tumors were quantified 240 days post-dosing. Each compound displayed a distinct fingerprint of DNA adducts in lung DNA (Ross et al., 1995). The shape of the curve (DNA adducts vs. time) was qualitatively similar but quantitatively different for each compound. Initial analysis suggested that the tumorigenic potency of each compound as a function of administered dose differed by >100-fold; however, when Ross calculated effective dose for each administered dose using the area under the DNA adduct persistence curve, a different result was obtained. When the number of adenomas/mouse was plotted vs. time-integrated DNA adduct level, tumor potency was comparable for each compound. Thus, when DNA adduct measurements reflect total adduct persistence over a defined time period, PAH adduct level is predictive of tumor development in strain A/J mouse lung.

Future studies will focus on the low dose non-linear region of the dose-response curves which was not studied in detail in the experiments described above. Ross proposed that alternate endpoints must be tested for this analysis. The endpoints may include unstable adducts, oxidative DNA damage, mutations, or gene expression.

The role of PAH metabolizing enzymes may also be considered.

What is the relevance of DNA adduct measures in animals and humans?

Lynn Pottenger

Pottenger emphasized that DNA adduct data must be considered in the larger, biological context. For example, both endogenous and exogenous DNA adducts should be evaluated, target and non-target tissues should be compared, and the concordance of different types of animal data or human studies as well as the concordance across species and between animal and human studies should be considered. DNA adducts are potentially useful indicators of exposure, but the fate of the adduct must be considered (repair, mutation, persistence, etc.) as well as the fate of cells carrying DNA adducts (apoptosis vs. proliferation and DNA replication). In the absence of robust genotoxicity data from other standard test systems, compounds that form DNA adducts must be considered potentially hazardous. In such situations, additional data should be collected. In general, however, DNA adduct data should be considered in the context of the total dataset for any particular chemical using a weight-of-evidence approach.

Can adducts be used as a tool in quantitative risk assessment?

George Douglas

Douglas suggested that DNA adduct data were likely to be useful in hazard identification and to modulate interpretation of other data for risk assessment but not for risk assessment per se. It is important to consider the relative mutagenicity of different DNA adducts. Mutagenicity only recently began to be considered during risk assessment, and it must also be considered in the context of additional supporting data.

Open discussion

Preston opened the discussion to all meeting participants to voice specific concerns or address questions to panelists. He also asked meeting participants to consider the following questions:

What should we know and what are the future experimental needs?

Can we move hazard identification and/or risk assessment to a computational framework?

Adducts and mutagenesis

Discussants indicated that the relationship between DNA adducts and their mutagenic potential is not very clear, although some adducts are strongly linked to mutagenesis and the mechanism of this process is well characterized. This is an important area for future study. The example of oxidative DNA damage is important because it demonstrates that mutagenicity occurs via DNA damage in the absence or presence of DNA adducts. Induction of DNA repair activities, however, is not always protective and can lead to increased levels of mutagenesis.

Oxidative DNA damage

There was controversy among meeting participants over whether oxidative DNA damage should be considered in the same discussion with DNA adducts. Some discussants argued that carcinogens that form adducts and agents that generate oxidative DNA damage do so by distinct biological reactions that should be analyzed separately. Other discussants disagreed with this view, arguing for the high importance of oxidative damage for understanding the processes of mutagenesis and carcinogenesis on a broader level.

Sensitive biomarkers of effect

Several participants emphasized that most mutagenesis assays have a more limited dynamic range than assays for DNA adducts; similarly, the background rates of tumor development limit the dynamic range for carcinogenesis assays in vivo. Thus, there is a paucity of adequate markers of effect for exposures linked to DNA adducts. It is feasible to improve the sensitivity of mutagenesis assays, and this should be an area of future study.

Improved methods for detection of DNA adducts

Accelerated mass spectrometry (AMS) is a sensitive method for quantifying and characterizing DNA adducts. Wider use of this technology was encouraged by Paul Henderson (Lawrence Livermore National Labs). In particular, there is an NIH-funded research resource at LLNL which is willing to enter into collaborative projects on DNA adducts with interested scientists.

Summary remarks on panel discussion

Preston indicated that the strongest message from this discussion session was that additional research is needed to generate consensus on the biological significance of DNA adducts. Improved sensitivity in detecting mutations induced by very few DNA adducts/cell (if at all) could greatly facilitate progress in DNA adduct research.

Workshop concluding remarks

Jay Goodman (Michigan State University)

All DNA adducts are not equally mutagenic, and it cannot be assumed that the presence of a DNA adduct will

cause a mutation (Williams et al., 2000). Much more attention needs to be paid to the biological significance, if any, of low doses of possible mutagens. Furthermore, not all mutations are carcinogenic. It is important to understand that carcinogenesis is more than mutagenesis. It is a highly complex process, which is influenced by both genetic and epigenetic factors (Watson and Goodman, 2002). The latter are too often overlooked in experimental settings.

Research on DNA adducts should consider selection of rational doses, with emphasis on "real world" doses (accurate exposure assessment becomes important) plus definition of dose-response relationships, coupled with an appreciation for the fact that mutagenesis is dependent on a variety of parameters. These include dose of the mutagen, capacity for DNA repair, ability of the adduct(s) formed to cause mispairing, and rate of cell replication. Penetrance of particular mutations is an additional, important variable. Within this context, it may be possible to establish whether or not safe levels of DNA adducts can occur. For application to safety assessment, DNA adduct data need to be considered as one component of a larger body of information that is relevant to safety evaluation.

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