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GENETIC TOXICOLOGY OF COMPLEX MIXTURES

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FOREWORD

Contained in this volume are the proceedings of the international conference on the "Genetic Toxicology of Complex Mixtures," held from July 4-7, 1989, in Washington, DC. This meeting was a satellite of the "Fifth International Conference on Environmental Mutagens" and the seventh in a biennial series of conferences on "Short-term Bioassays in the Analysis of Complex Environmental Mixtures."

Our central objective in calling together key researchers from around the world was to extend our knowledge of the application of the methods of genetic toxicology and analytical chemistry in the evaluation of chemical mixtures as they exist in the environment. This conference emphasized the study of genotoxicants in air and water, and the assessment of human exposure and cancer risk. The latest strategies and methodologies for biomonitoring of genotoxicants (including transformation products) were described in the context of the ambient environment. Source characterization and source apportionment were discussed as an aid to understanding the origin and relative contribution of various kinds of complex mixtures to the ambient environment. Similarly, investigations of genotoxicants found in the indoor environment (sidestream cigarette smoke) and in drinking water (chlorohydroxyfuranones) were given special attention in terms of their potential health impacts. New molecular techniques were described to enable more precise quantitation of internal dose and dose-to-target tissues. The emphasis of presentations on exposures/effects assessment was on integrated quantitative evaluation of human exposure and potential health effects. It is clear that the sophistication of complex mixture research technologies has increased dramatically since the first conference in 1978 with the application of state-of-the-art genetic and molecular methods. It is now apparent that interdisciplinary approaches are essential in order to assess the contribution of mixtures of genotoxic agents in the environment to total human exposure and potential cancer risk.

We are indebted to the speakers and chairpersons who presented their data at the meeting and in the excellent chapters that follow.

A conference of this type requires the cooperative efforts of many individuals. The organizing committee is grateful for the generous support of Dr. Don Hughes of Procter & Gamble, Cincinnati, Ohio, and Dr. Steve Haworth of Hazelton Laboratories, Kensington, Maryland, who contributed to these proceedings. I would like to acknowledge Dr. Bruce Casto of the Environmental Health Research and Testing, Inc., who

helped to organize the meeting, and his associates, Dale Churchill and Kathy Rous, who helped on-site in Washington, DC. Special thanks to Claire Wilson & Associates, Washington, DC, for conference management and technical editing of these proceedings.

Michael D. Waters, Ph.D.
Senior Editor

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Exposure/Effects Assessment

DEVELOPMENT AND APPLICATION OF NEW METHODOLOGIES APPLICABLE TO RESEARCH ON COMPLEX ENVIRONMENTAL MIXTURES

P.H.M. Lohman, E.W. Vogel, B. Morolli, A.A. v. Zeeland, and H. Vrieling

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Although the induction of cancer in man and in experimental animals by exposure to ionizing radiation or chemicals has been known for a long time, major insights into the mechanisms that underlie naturally occurring and induced cancers began to emerge only since the early 1970s. There is now persuasive evidence which documents that (i) many carcinogens are mutagens; (ii) most forms of cancer are due, at least in part, to changes (mutations) in the DNA (genetic material) contained in cells; and (iii) such genetic changes play a pivotal role in the initiation of cancer at the cellular level. A wide variety of test systems developed during the last 20 years--ranging from bacteria and mammalian cells including human cells in culture to whole mammals--is now available to examine the "mutagenic potential" of different chemicals, but they are only suitable for a qualitative determination of the level of cancer risk resulting from exposure of man to such agents. Agents that are capable of damaging the DNA are called "genotoxic" and a general characteristic of these is their electrophilic reactivity towards DNA and other cellular macromolecules. Interaction of chemicals with DNA has been considered as the initial step in the formation of cancer and hereditary effects in mammals, in spite of the (often spectacularly efficient) DNA repair processes in the individual cells of the organism (Fig. 1). The assumption is made that DNA lesions may escape correction by DNA repair processes, others may be erroneously repaired or not repaired at all. Moreover, the spectrum of lesions in the DNA of exposed cells is often complex and strongly dependent on the agent involved. Furthermore, DNA repair processes are found to be dependent on organ and cell type, chromosomal structure, and whether genes are active or inactive.

Mutation induction is only considered a first step (initiation) in the long chain of events leading to malignant transformation of cells. Still many other, mostly unknown, steps are involved in the complicated pathway leading from an initiated cell to an established tumor. Especially noticeable among unknown factors are those that influence the progression

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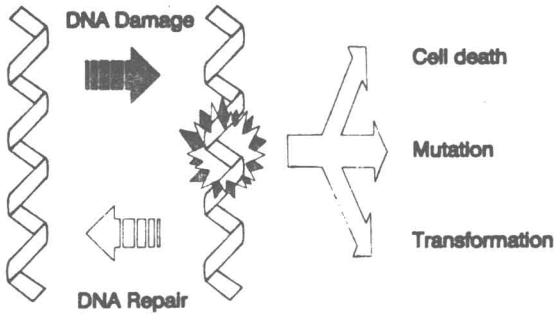

Fig. 1. DNA repair processes.

of transformed cells and those that determine the often strong strain, organ, tissue, or cell dependence of tumor formation as a result of exposure of a mammal to a particular genotoxic agent and the role of immunological defense mechanisms. Therefore, it is more surprising than logical to find that primary genotoxic damage to DNA, especially after low acute or low chronic exposures, can stochiometrically lead to mutation and transformation of cells, ultimately leading to cancer.

The complexity of the effects of environmental mixtures containing genotoxic components is not only due to the fact that exposure may occur due to a variety--both in amount and nature--of chemicals. The lack of knowledge of the number, kind, and nature of steps involved in tumor formation does not allow any prediction of the possible additivity or synergism of the biological effects of identified components of such mixtures. The situation is so uncertain that one even has to consider seriously whether the addition of any piece of knowledge on the genotoxicity of mixtures will necessarily lead to the resolution of scientific conflicts and problems in the interpretation of what is safe or unsafe; paradoxically increasing knowledge in this respect may make the world seem to be a more hazardous place than it actually may be.

One possible way to overcome part of the paradox may be a better appreciation of general toxicological principles in the evaluation of the results of genotoxic tests; the field has been too dominated by the theory that in carcinogenesis no safe dose exists. As an example, in Fig. 2 an experiment is shown where gene mutation [at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus] is measured in cultured Chinese hamster cells exposed to ethyl methane sulphonate (ENU), 254 nm ultraviolet (UV) light, X-rays, and methyl methane sulphonate (MMS), respectively. It will be obvious from the results of this experiment that X-rays, for instance, are much less mutagenic per surviving fraction than ultraviolet light and also that relatively small changes in the structure of otherwise similar alkylating agents can lead to a dramatic difference in biological response. Simply, one may conclude from this experiment that sometimes genotoxic agents are much more lethal than mutagenic (e.g., X-rays versus ENU or UV). Obviously, there seem to be "key" lesions causing lethality and "key" lesions being less lethal but more mutagenic. The actual number of such kinds of "key" lesions can differ dramatically from agent to agent (10). After irradiation with X-rays, for instance, mammalian cells are killed when more than 3,000 DNA lesions per cell are

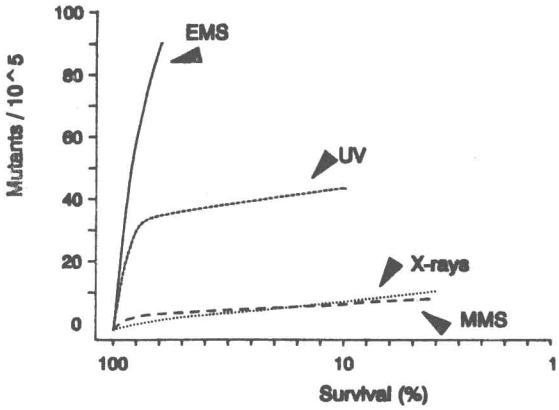

Fig. 2. Gene mutation in Chinese hamster cells.

introduced. The same kind of cells will still survive after the initiation of more than 300,000 DNA lesions are induced in their genome by UV.

From a toxicological standpoint experiments like the one shown in Fig. 2 suggest that relative hazard assessment (quantitative ranking of test results from various chemicals or mixtures of chemicals) may be much more relevant for practical situations than absolute risk assessment of a single chemical or situation. Several attempts have been undertaken to design approaches for relative hazard estimation of genotoxic agents such as the comparison of genetic activity profiles (11,23) and the relation between chemical structure and test responses (13,14). Such analysis can be strengthened by adding information on the actual mechanism by which chemicals react with cellular macromolecules. An example of such an approach is given in Fig. 3. In this experiment, Vogel and Zijlstra (21,24) compared the response of a large number of bifunctional agents (chemicals that can cause intra- and inter-crosslinks in DNA) in two tests with the fruitfly Drosophila melanogaster. Each test measures a different independent biological endpoint, i.e., chromosomal aberrations (ring-X loss) and gene mutation (recessive lethals), and the tests are done at doses which are not toxic for the animal. Although all chemicals depicted in Fig. 3 have a similar reaction mechanism towards interaction with DNA, it will be obvious that there are large differences in the test results. Moreover, a significant ranking of the chemicals is observed as a function of the response in the two independent Drosophila tests. Interestingly, the ranking of these cross-linking agents corresponds with the carcinogenic potential in rodents and/or humans of a significant number of the indicated chemicals.

It would be interesting to test whether the genotoxic properties of chemicals with known reaction mechanisms with DNA as a function of the response in two or more independent biological responses in experimental animals would, just as in Drosophila, also quantitatively mimic carcinogenic potential. There are still only a limited number of techniques available that allow testing in vivo, especially at low nontoxic dose ranges, although recently a number of promising tests have been developed. For studying gene mutation, the molecular analysis of point mutations has become available after the introduction of the polymerase chain

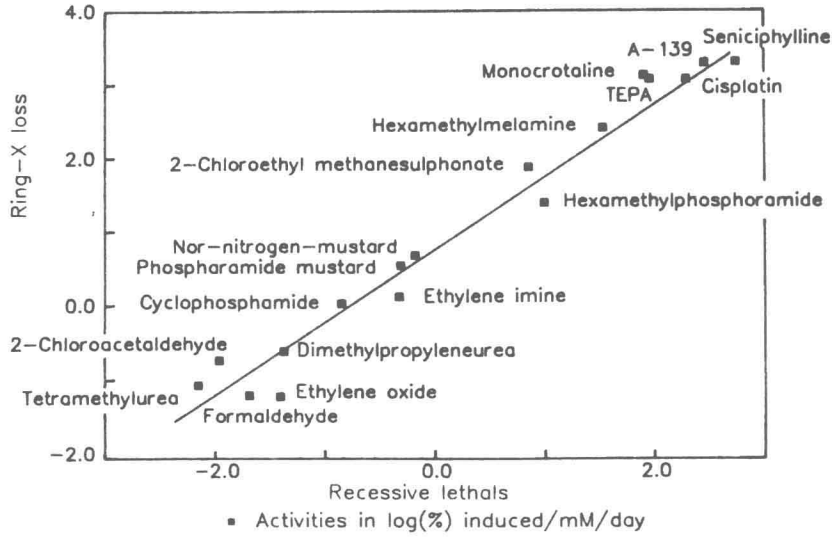

Fig. 3. Comparison of bifunctional agents.

reaction (PCR) by Saiki et al. (16). Vrieling et al. (22) have adapted the PCR technique for the use of cDNA derived from mRNA of an expressed gene instead of genomic DNA; the gene used in these studies was HPRT which is located on the X-chromosome of rodent and human cells. By comparison of the known DNA sequence of the undamaged HPRT gene with the DNA sequence in the mutants, it is possible to determine exactly which mutations (deletions, point mutations = single base changes) have been formed in the isolated mutants. An example of the results obtained with rodent cells treated with two different agents is given in Fig. 4. It can be seen that the kind of DNA sequence alterations are totally different for different agents: X-rays induce many more deletions than single base changes; ultraviolet light, more point mutations than deletions. Further evidence obtained by using alkylating lesions has recently revealed that the spectrum of mutations as scored in the HPRT gene of mammalian cells is unique for the genotoxic compound used (data not shown here), suggesting that the PCR technique for measuring mutations in endogenous genes may be a promising new biological endpoint for in vivo studies on the effect of low concentrations of genotoxic agents and mixtures of agents.

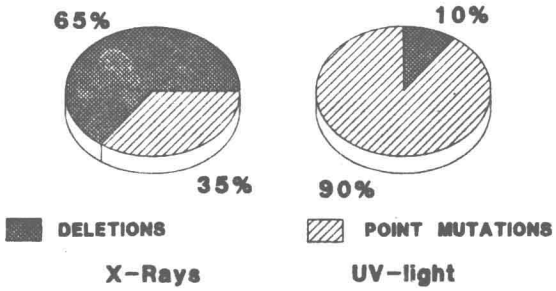

Fig. 4. Induced mutations in the HPRT gene in irradiated rodent cells.

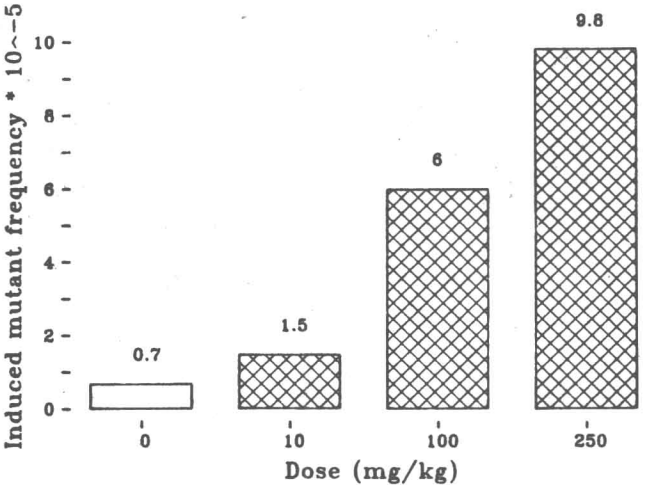

Fig. 5. ENU mutations in the brain of transgenic mice.

A limiting factor in using the PCR technique for the in vivo detection of the spectrum of DNA alterations induced by genotoxic agents is caused by the fact that the technique can so far only be applied to the HPRT gene as present in special cells of the body, primarily the T-cells isolated from the blood. Gossen et al. (5), however, developed a new method which, at least for experimental animals, can be applied to measure mutation spectra in all cells of the body. This method uses so-called "marker genes" which are being incorporated in fertilized eggs of mice. Subsequently, these eggs are reimplanted in pseudopregnant foster mice. The offspring of these mice, called transgenic animals, contain in all cells of the body the foreign genes that were transferred to the fertilized eggs. The authors used in their experiment the bacterial marker gene LacZ located on a bacteriophage lambda shuttle vector. This vector can be rescued from total genomic DNA from all cells of the body with high efficiency. If the marker gene is mutated in transgenic animals treated with a genotoxic agent, also the mutated genes can be analyzed in Escherichia coli and the spectrum of DNA alterations can be determined. In Fig. 5, the frequency of mutations induced in marker genes isolated from brain cells of transgenic mice one week after treatment with the genotoxic agent ENU are depicted; a dose-dependent increase of LacZ mutations is found with the peculiar spectrum of DNA lesions (predominantly G.C $\longrightarrow$ A.T transitions). In the same animals it was found that the induction of mutations in organs other than the brain was much less. This emphasizes the applicability of this system to detect organ/tissue-specific mutation induction.

Methods for measuring mutation induction in cells of humans exposed to genotoxic agents are so far restricted to the above-mentioned PCR technique of measuring DNA alterations in a single gene (the HPRT gene of T-cells). Uitterlinden et al. (20) have developed a new method that allows the detection of "hotspots" of mutations in a large part of the genome of mammalian cells. The technique these investigators used is called "DNA fingerprinting" and is based on the fractionation of genomic

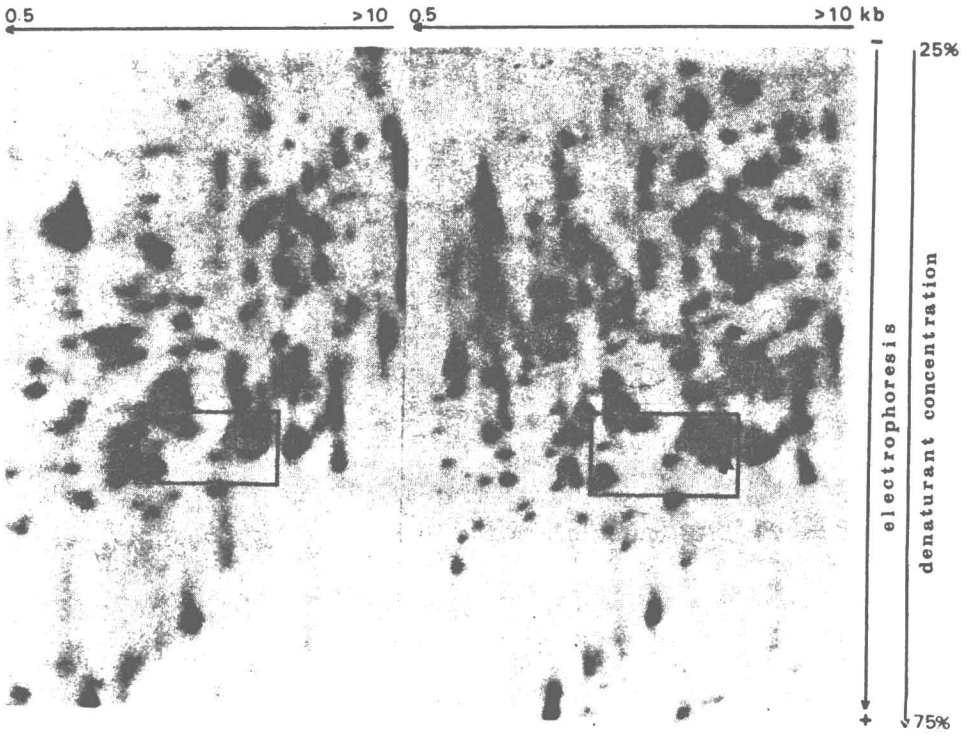

Fig. 6. (Left panel) "DNA fingerprint" of nonirradiated T-cells; (right panel) "DNA fingerpring" of a T-cell clone of the same individual irradiated with 0.4 Gy X-rays.

DNA, cut by restriction enzymes, by two-dimensional gel electrophoresis: in the first-dimension, the DNA fragments are fractionated on size and, subsequently, in the second-dimension, the DNA fragments are fractionated on a denaturing gradient gel on base-composition. The DNA fragments are visualized after blotting with a probe consisting of minisatellite core sequences (6). The minisatellite core sequences are spread out over a large part of the human genome and, therefore, if restricted genomic DNA of human cells is analyzed on the two-dimensional gels, information is obtained on a large number of different DNA fragments in which the minisatellite core sequences are present. We have applied this technique to human T-cell clones from various persons before and after irradiation with X-rays. In Fig. 6 (left panel), the "DNA fingerprint" is shown of a T-cell clone of the same individual irradiated with 0.4 Gy X-rays. In one out of six T-cell clones irradiated with 0.4 Gy X-rays, we found an additional spot to be present in the "DNA fingerprint" (arrow in the box indicated in the righthand panel of Fig. 6). This finding may be an indication that the two-dimensional gel electrophoresis system is sensitive enough to measure mutations in a large fraction (about 10-15%) of the genomic DNA of human T-cells after treatment with genotoxic agents. However, at this moment there is not yet sufficient evidence available that demonstrates that indeed such mutations can reproducibly be observed through "DNA fingerprinting."

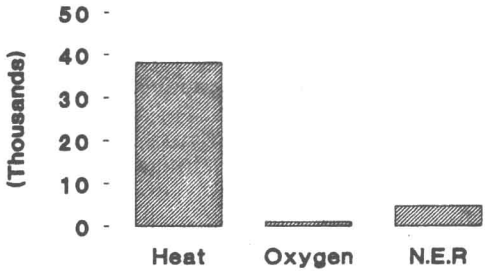

Fig. 7. Endogenous sources of DNA damage.

The new developments described in this chapter suggest that it will be possible in the near future to quantitatively measure the induction of mutations in experimental animals and humans exposed to low levels of genotoxic agents. Together with already existing sensitive techniques to measure chromosomal damage, these techniques may then allow, as in Drosophila (see Fig. 3), a comparison of the response of independent biological endpoints in mammals exposed to genotoxic agents with a common mechanism of interaction with DNA. However, one has to realize that induction of DNA damage by exogenous sources is on a daily basis often minor in comparison to DNA lesions induced in the human body due to endogenous sources. Perhaps the most ubiquitous natural cause of DNA damage is heat. Due to the thermodynamic instability of DNA, certain alterations in its structure can occur at the normal body temperature of 37°C (4,7,8,9,17). In Fig. 7 the estimated extent of possible DNA damage/cell/day in humans due to body heat is depicted. Another important class of endogenous DNA damage is free radicals, in particular the active oxygen species (2,12,18,19), although the average number of DNA lesions formed in cells of the human body per day is estimated to be lower than what would be expected due to body heat (Fig. 7). There are other important endogenous sources that cause spontaneous DNA damage, for instance due to the nonenzymatic reaction [(NER) Fig. 7] of glucose and other reducing sugars with the amino groups of DNA bases (3) or spontaneous alkylation of DNA by S-adenosyl-L-methionine, the normal methyl-group donor in cells (15).

In summary it can be stated that, surprisingly, during the lifetime of humans, the induction of DNA damage and DNA alterations may be rather more abundant than rare. A major task still remains to identify the "key" DNA lesions and/or DNA alterations caused by exogenously applied genotoxic agents or mixtures of genotoxic agents that lead to induction of cancer and/or heritable disease in addition to those induced by endogenous causes or unavoidable exposure to "natural" agents. Prioritizing chemicals or situations to be studied seems at this moment more relevant than rigorous attempts at ad hoc and absolute risk assessment. Ames (1) has suggested that exposure levels, the toxicological profile, and the use of chemicals are a more important criteria than a simplified interpretation of hazards on the basis of available short-term and long-term tests. However, as indicated in this paper, new technologies are under development that may allow a more definitive ranking of the genotoxic properties of chemicals and chemical mixtures and, therefore, will provide a significant improvement in setting priorities for the study of specific chemicals or groups of chemicals.

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