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Early biochemical markers of effects: enzyme induction, oncogene activation and markers of oxidative damage

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Abstract

Experimental carcinogenicity studies focus on identification of single carcinogens. Humans, however, appear exposed to a variety of low doses of carcinogens. Furthermore, few chemical entities are carcinogenic or toxic per se, but require metabolic activation to form ultimate carcinogens or toxins. In contrast to experimental animals, humans show considerable difference in genetic properties. In that situation it is particularly important to estimate individual capability for metabolic activation. To an increasing extent, activation includes formation of toxic oxygen metabolites. Particular targets for activated species are DNA and lipids; in particular low-density lipoproteins (LDL). Modifications of DNA are important for initiating the multistep process of carcinogenesis, in particular if oncogenes are activated or if tumor supressor genes are inactivated. Such DNA modification can be identical regardless of the reactive specimens being a xenobiotic or an oxygen species. Modification of LDL can start the process of atherosclerosis by transforming macrophages into foam cells, deposited as fatty streaks in the arterial wall. Biomarkers for activation capacity of xenobiotics include the use of prototype substrates and molecular techniques to determine genetic polymorphisms. Oxidative DNA modification can be measured from urinary excretion of oxidatively modified deoxynucleosides, particularly guanosine. Future efforts have to include individual measurements in order to improve the 'resolution' of molecular epidemiological approaches.

Keywords: Early biochemical marker; Enzyme induction; Oncogene activation; Oxidative damage; Low-density lipoprotein

1. Introduction

Studies of the relationships between exposure to exogenous and endogenous compounds and disease are, to an increasing extent, founded on the use of biomarkers of individual dose, susceptibility and/or effects as recently reviewed, see e.g., Bartsch et al. (1992) and Sculte and Perera (1992). This approach, termed molecular epidemiology, is based on the concepts of the mechanism of the disease under study. In case of carcinogen

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exposure and cancer development the multistep carcinogenesis theory is prevailing as depicted in Fig. 1 (Wogan, 1992).

2. Identification of single important human carcinogens

Experimental rodent carcinogenicity studies are designed to test the specific hypothesis of whether a particular xenobiotic is a carcinogen. In analogy, the human epidemiological studies of carcinogens usually focus on exposure to a single factor. The pioneering studies began in the end of the 19th century and were based on clinical observations of bladder cancer patients from the German dye industry (Case, 1966), which gave important evidence that occupational and environmental chemicals are related to cancer development in humans.

Today, it is acknowledged that in the human situation exposure to a single carcinogen is not the common situation. Studies have established estimates of the contribution of various factors for cancer deaths (Doll and Peto, 1981) (Table 1). With regard to identification of single important carcinogens, large efforts are being made by the IARC to classify an increasing number of chemicals with regard to whether they are carcinogens or not (Vainio et al., 1991; Vainio and Wilbourn, 1993). The classification depends on all available evidence, particularly on human and non-human etiological and epidemiological evidence.

Studies that aim at identifying a single potential carcinogen naturally put emphasis on methods of estimating exposure (external dose, internal dose and estimation of the extent of absorption and distribution). Within this context identification of a dose-response relationship is an important and strong indication of causal relationship. With molecular epidemiology, the progress in knowledge of chemical carcinogenesis has advanced the development of methods to estimate the biologically effective dose, i.e., amount or concentration of active xenobiotic available at critical or surrogate targets (e.g., DNA and protein adducts, as described by K. Hemminki et al. in this issue, or later oxygen adducts). Similarly, substantial advances have been made in the methodology to establish early biological effects, comprehensive reviews can be found elsewhere (Bartsch et al., 1992).

The number of single individual agents so far documented to be associated with human cancers remains small compared to the vast number of existing chemicals. Whereas some carcinogens are clearly associated with certain cancer cites such as lung, bladder, nasal sinus, skin, bone marrow and liver, few compounds are associated with cancers of the breast, colon, prostate or ovary (Vainio and Wilbourn, 1993). Among the documented associations, the proportion of the cancer cases that actually can be attributed to exposure

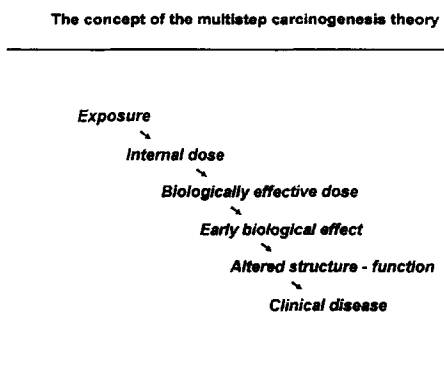

Fig. 1. The concept of the multistep carcinogenesis theory.

Table 1
Ranking of factors for human cancer (adapted from Doll, R. and Peto, R. (1981)

Factor	Contribution %	Range
Diet	35	10–70
Tobacco	30	25–40
Infection	10	?
Reproductive and sexual behavior	7	1–13
Occupation	4	2–8
Alcohol	3	2–4
Geophysical factors	3	2–4
Pollution	2	1–5
Industrial products	1	1–2
Medicine and procedures	1	−5–2
Food additives	1	0.5–3
Other	3	?

of the chemical, especially in the low dose range, is rarely high and it is frequently unknown.

3. Oncogene activation

Oncogenes are defined as genes that code for proteins involved in cell growth that unless properly regulated will result in inappropriate growth (Sculte and Perera, 1992). Proto-oncogenes are expressed during a variety of physiological conditions, e.g., embryogenesis, wound healing, liver regeneration, and their protein products are important for cellular growth and differentiation. Increasing evidence suggests that neoplasias in many cases may require changes in at least two classes of cellular genes: proto-oncogenes and tumor suppressor genes (Weber and McClure, 1987; Anderson et al., 1992).

The mechanisms by which oncogenes are involved in carcinogenesis include several possible routes (Cline, 1994). A single point mutation at a critical site may be one mechanism, however, the mutation spectrum in lung, colon and pancreas tumors shows a distinct difference in K-*ras* codon 12/13 (Anderson et al., 1992). Furthermore, the K-*ras* oncogene mutational spectrum shows considerable variability between individual studies. Other mechanisms for activation include amplification, i.e., the repeating of DNA sequences by up to 50–100 times, and chromosomal translocation.

It is still debated how early in the process oncogenes are activated, and several of them can be activated on top of inactivation of tumor suppressor genes (Cline, 1994). Late in the carcinogenic process, such alterations may play an important role, as may oncogene amplification and chromosomal translocation.

The sequence and importance of the activation of oncogenes and inactivation of tumor suppressor genes are understood to an increasing extent (Harris and Hollstein, 1993), including the considerable variability between individuals and sites. There is general agreement, however, that they can occur spontaneously, from oxidative stress and from activation of xenobiotics and that, in animals, mutational spectra are reproducible. Oncogene activation and inactivation of tumor suppressor genes may emerge as markers of the early changes in malignant transformation: surely they are important late events (Cline, 1994).

4. Identification of multiple carcinogens and endogenous factors

One intriguing observation that is continuously reported, and now well recognised, is the huge interindividual variation in susceptibility to chemical carcinogens. Many genetically determined factors appear, or are inferred to be, of importance for risk of developing cancer without a clear relationship to any particular carcinogen. Moreover, exogenous factors, including life-style factors, appear to be of great importance, e.g., the dietary intake of antioxidant vitamins and related compounds apparently modulates the risk of a variety of epithelial cancers (Dorgan and Schatzkin, 1991).

More than 10 years ago it was pointed out that a vast number of (pro)mutagens are naturally present in the environment (Ames, 1983; Gold et al., 1992). The abundant and ubiquitous dioxygen radical (atmospheric oxygen), suggested to be involved in degenerative and carcinogenic processes (Ames and Shigenaga, 1992; Gold et al., 1992), appears to be equally important. Ranking carcinogens according to estimated importance reveals that, viewed against the large background of naturally occurring carcinogens in typical portions of common foods, the residues of synthetic or environmental pollutants rank low (Gold et al., 1992). Most importantly, such analyses pinpoint that the human situation is characterized by exposure to a vast number of mutagens/carcinogens, as opposed to the experimental setting where a single mutagen/carcinogen is given in controlled and huge doses. Accordingly, individual susceptibility factors, such as the capacity of the metabolic (in)activation enzymes and cellular repair mechanism, may be more important for the extent of DNA modification than the extent of exposure to a few selected carcinogens. Moreover, completely different carcinogens can induce the same biological changes, e.g., G–T transversion mutations can result from oxidative insults to DNA (Cheng et al., 1992), from exposure to nickel

(Higinbotham et al., 1992), aflatoxin (Chang et al., 1991), or benzoe[*a*]pyrene (Kcohavong et al., 1992). Again, this signifies that the total sum of insults, e.g., to DNA, and the individual capacity of repair and regeneration may be more important for the risk of disease than the insults from a single substance. If one accepts the hypothesis of humans being exposed to multiple xenobiotics (Fig. 2), rather than to the single massive exposure used in animal studies, it is more relevant and, in fact easier, to examine the question: what is the activity range of the most important activating enzymes? than the question: what is the range of exposure to the total amount of xenobiotics that potentially are converted into electrophiles and other reactive metabolites.

Consequently, we also would like to draw attention to the most massive exposure man is subjected to, i.e., oxygen, and to man's ability to activate xenobiotics. In the following two examples of biomarkers, a combined environmental and genetic regulation of susceptibility and early biological effects are given: biomarkers of cytochrome *P*-450 (CYP) activities and oxidative DNA damage.

5. Metabolic activation of carcinogens: CYP activities from biomarkers

Among the enzyme systems involved in metabolic activation of xenobiotics to proximate or ultimate carcinogens, the cytochrome *P*-450 superfamily is the most important. Particularly, the CYP1A-family (PAHs, aromatic amines), the CYP2E1 (nitrosamines, halogenated hydrocarbons) and the CYP3A family (mycotoxins, PAHs)

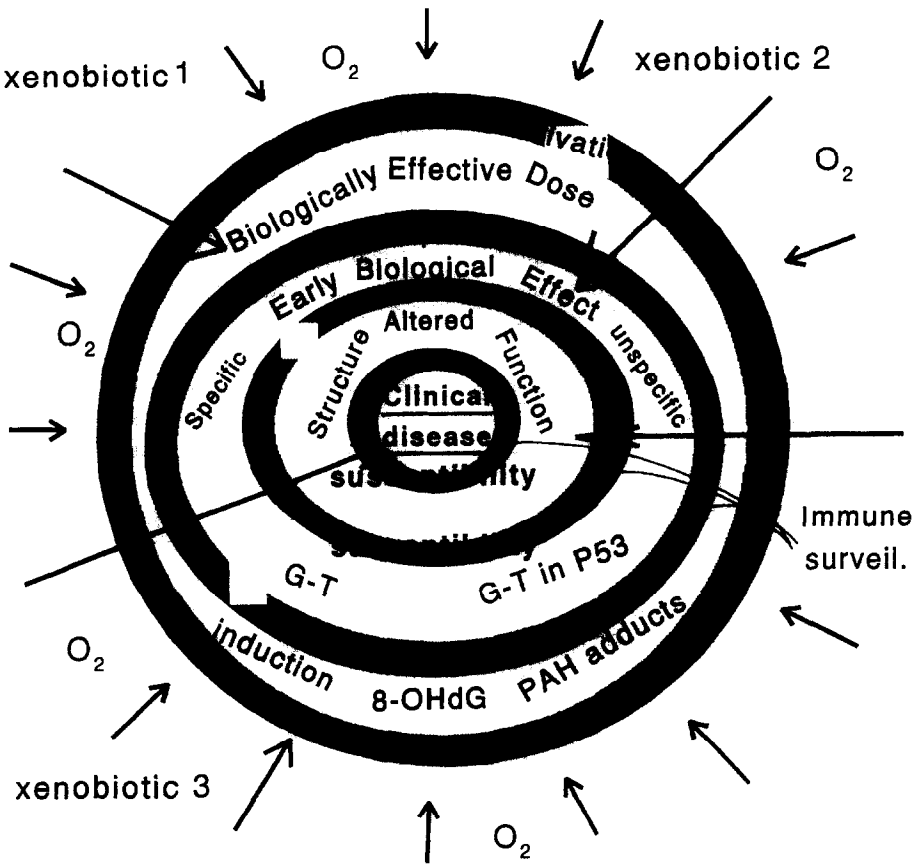

Fig. 2. Multi-exposure, multi-step, multi-effect hypothesis for carcinogenesis.

show preference for converting xenobiotics into highly reactive electrophiles that bind covalently to DNA (Guengerich et al., 1991). With regard to the importance of genetically controlled enzymes (Table 2) involved in metabolic (in)activation and their assessment by means of biomarkers, evidence of associations with diseases has recently been reviewed in, e.g., Wolf (1991), Kadlubar et al. (1992) and Poulsen et al. (1993). Many of the enzymes showing genetic polymorphism have been associated with the risk of various cancers without any relation to a particular carcinogen; for example, the CYP2D6 enzyme has a very poor affinity for most known carcinogens.

Genetic polymorphisms of the important carcinogen-activating CYPs have not yet been clearly identified, with the exception that RFLPs of CYP1A1 and CYP2E1 have been described and associated with lung cancer in Japanese but not in Caucasian populations (Kawajiri et al., 1990; Tefre et al., 1991; Uematsu et al., 1991; Hirvonen et al., 1993; Persson et al., 1993). More importantly, the enzyme activities appear unimodally distributed and are modified by environmental factors, e.g., CYP1A1/2 induction by smoking (Sesardic et al., 1988; Bartsch et al., 1991; Vistisen et al., 1992) and from environmental exposure to polybrominated biphenyls (Lambert et al., 1990), CYP2E1 induction by ethanol and acetone, and CYP3A4 inhibition by naringenin, the aglycone of the bitter principal of grape fruits (Guengerich et al., 1990; Bailey et al., 1991). In fact, a large proportion of the interindividual variation in, e.g., CYP1A2 activity in a population (Fig. 3), may be ascribed such factors (Vistisen et al., 1992). The individual activity appears quite stable in an unperturbed environment and can easily by manipulated by a factor of 2 by known inhibitors and inducers (Døssing et al., 1983). Accordingly, these CYP activities may be regarded as biomarkers of susceptibility as well as of important biologically effective doses which alter susceptibility.

The CYP1A2 activity can be determined by means of HPLC analysis of caffeine metabolites in spot urine sample some hours after ingestion of coffee or tea (Grant et al., 1984; Campbell et al., 1987; Kalow, 1993) or, alternatively, after

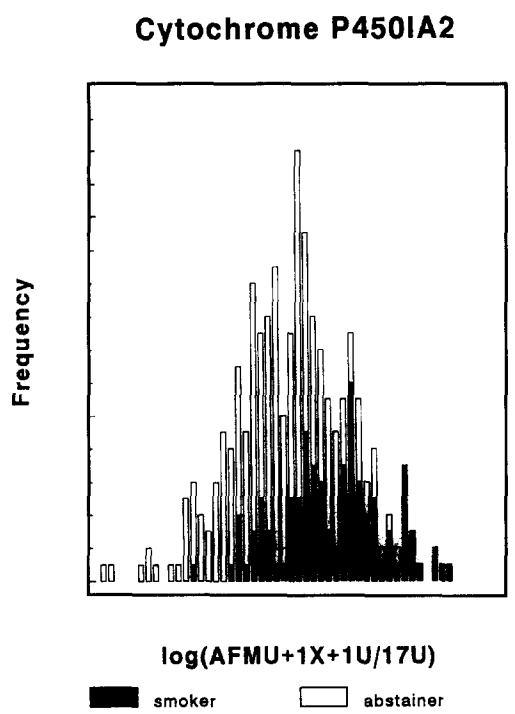

Fig. 3. Distribution of CYP1A2 activity, redrawn with permission from the authors (Vistisen et al., 1992).

administration of pure caffeine (Butler et al., 1992). This offers a non-invasive method for CYP1A2 activity determination that can be applied in large scale epidemiological studies. CYP1A2 activity appears unimodally distributed, although a genetic polymorphism concerning inducibility has been suggested (Butler et al., 1992). The methodology also gives information about the *N*-acetyltransferase phenotype and xanthine oxidase activity. A similar non-invasive method is available for the estimation of CYP3A4 activity by means of the urinary 6β-hydroxycortisol:cortisol ratio, whereas the estimation of CYP2E1 activity so far requires determination of the pharmacokinetics of an administered drug, chlorzoxazone (Peter et al., 1990).

For many of the polymorphic enzymes, molecular techniques that determine the genotype are replacing or supplementing measurements of the enzyme activity towards prototype substrates. Although genotyping may be simpler it does not allow determination of potentially important vari-

ation in activity within one genotype. Nevertheless, tools for study of constitutively and environmentally induced high (or low) activity of CYPs and other enzymes involved in carcinogen metabolism as risk factors are available. For the CYPs presently considered of greatest importance in the activation of xenobiotics (CYP1A, CYP2E1, CYP3A), there are no usable genotyping assays available.

6. Metabolic activation of oxygen estimated by urinary excretion of 8-hydroxy-deoxyguanosine (8-oxodeoxyguanosine, 8-oxodG)

Living organisms are continuously exposed to reactive oxygen species as a consequence of biochemical reactions as well as external factors. The internal formation of reactive oxygen species has been estimated to be 1–5% of oxygen consumption (Chance et al., 1979), deriving from a minor 4-single electron pathway metabolism of oxygen. The targets in the cells are multiple, including an estimated 10^5 oxidative modifications of DNA per cell per day (Adelman et al., 1988; Shigenaga et al., 1989; Loft et al., 1992). This rate is so high that extensive repair is necessary, yet the steady-state level of oxidatively modified bases in human DNA is about 25 per 10^5 bases (Olinski et al., 1992). Moreover, in brain and lung tumours, the cancerous tissue showed elevated levels of oxidatively modified bases compared to the surrounding normal tissue (Olinski et al., 1992). By comparison, xenobiotic DNA adducts in smokers determined by ^{32}P-postlabeling assays ranged from 2 to 13 per 10^8 nucleotides (Bartsch et al., 1991), i.e., oxidative modifications outnumber the xenobiotic adducts by three orders of magnitude. It should be noted that exact comparison of such numbers may not be of value; however, the order of magnitude is intriguing.

A complete pattern of oxidative DNA modification can be studied after acid hydrolysis of isolated DNA or chromatin, and trimethylsilylation by means gas chromatography/mass spectrometry with selected-ion monitoring (Dizdaroglu et al., 1991; Halliwell et al., 1992). The oxidative DNA modification of the guanine base always amounts to a two digit percentage of the total DNA modification (Olinski et al., 1992). Thus, the relatively simple determination of only 8-oxodG in DNA isolated from target or accessible tissue, by means of HPLC with electrochemical detection, probably represents or correlates with the most important oxidative DNA damage. In replicating DNA, 8-oxodG leads to G–T transversions, as well as other mutations and codon 12 activation of c-Ha-*ras* or K-*ras* oncogenes in mammalian systems (Shibutani et al., 1991; Cheng et al., 1992; Higinbotham et al., 1992; Kamiya et al., 1992). In vivo 8-oxodG may be repaired by both an enzyme complex similar to the *E. coli* formamidopyrimidine-DNA glycosylase (FPG) (Yamamoto et al., 1992) and by nucleotide excision (Czeczot et al., 1991). The exact contribution of each pathway and the relative distribution between resulting free base and nucleoside has not been determined. In addition, digestion of damaged DNA from cell renewal and mitochondrial turnover will liberate 8-oxodG to an unknown extent. The cellular pools of nucleosides and nucleotides may be oxidized and lead to mutations if incorporated into DNA (Kamiya et al., 1992; Shibutani et al., 1991). In fact, a human enzyme corresponding to the *E. coli* MutT protein hydrolyses the phosphates of 8-oxodGTP with high affinity (Mo et al., 1992). As a candidate for a non-invasive biomarker of oxidative DNA, modification 8-oxodG appears most attractive since it is readily excreted unchanged into the urine, whereas 8-oxodG in the diet, or oxidation of dG during excretion, is not a contributing factor (Shigenaga et al., 1989). By contrast, 8-oxoG in the diet ends up in the urine (Shigenaga et al., 1989).

Inter-species correlations show a strong dependence on metabolic rate, supporting the importance of oxidative modifications in ageing and degenerative changes, e.g., cancer (Adelman et al., 1988; Loft et al., 1993). In man, in vivo, we have found the major determinants for urinary excretion to be smoking and oxygen consumption (Loft et al., 1993). The 50% increased excretion in smokers is depicted in Fig. 4. It is notable that there is a seven-fold range in the individual excre-

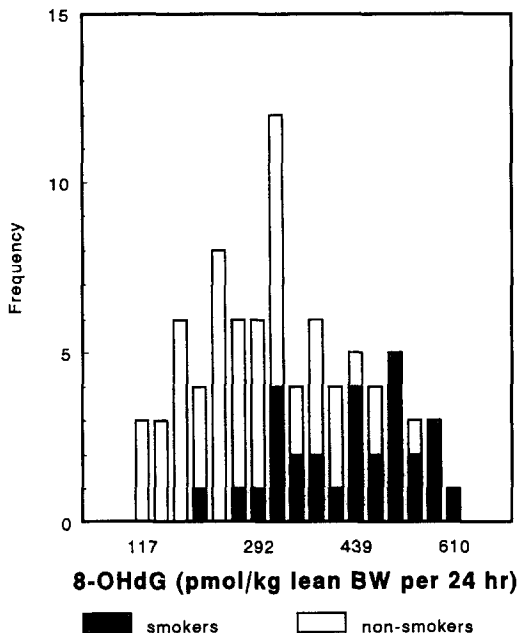

Fig. 4. Distribution of 8-oxodG (8-hydroxydeoxyguanosine, 8-OHdG) excretion in 24-h urine, redrawn with permission from the authors (Loft et al., 1992).

tion. This might predict the individual risk for early ageing and development of degenerative diseases, e.g., cancer; a hypothesis that needs to be tested in prospective epidemiological surveys or in case control studies. A strong correlation between 8-oxodG excretion and oxygen consumption found in 33 smoking and non-smoking women supports the involvement of mitochondrial generation of reactive oxygen species in oxidative DNA damage and, by inference, the risk of cancer and rate of ageing (Loft et al., 1994).

Constitutively and environmentally induced oxidative stress may be a considerable modifier of the individual cancer risk. Biomarkers of oxidative DNA damage appear to be a valuable tool for studying this hypothesis.

7. Oxidative modification of LDL

Lipid peroxidation has been in focus as a result of oxidative damage and recent evidence points to oxidation of LDL as a major free radical mechanism of atherosclerosis (Steinberg et al., 1989; Esterbauer et al., 1991). The atherosclerotic process is characterized by accumulation of lipids and proliferation of smooth muscle cells. The early events appear as accumulation of lipid-laden foam cells in the arterial intima accumulating to fatty streaks and plaques (Stam et al., 1989). It is assumed that oxidized LDL is taken up by the macrophages, via the scavenger receptor, without down-regulation by the internalized cholesterol. This leads to loading of the cell with cholesterol and cholesteryl esters, and their irreversible transformation into foam cells (Steinberg et al., 1989; Esterbauer et al., 1992).

In epidemiological studies, an inverse correlation has been found between intake or plasma concentrations of vitamin E and mortality from ischemic heart disease (Gey and Puska, 1989; Gey et al., 1991; Rimm et al., 1993; Stampfer et al., 1993) and, in accordance with this, vitamin E appears to be the major antioxidant in LDL (Esterbauer et al., 1991, 1992). Yet, other antioxidants, such as vitamin C and β-carotene, may sustain the antioxidant effect of vitamin E in LDL by acting as plasma reductants to protect or recycle vitamin E (Kagan et al., 1992). The presence of autoantibodies against oxidized low-density lipoproteins has been claimed to predict progression of carotid atherosclerosis (Salonen et al., 1992); however, these findings have been disputed (Laakso et al., 1992).

It remains to be established whether registration of antioxidant intake, plasma vitamin E concentration or, possibly, LDL vitamin E concentration, measures of resistance of LDL to in vitro oxidation (Esterbauer et al., 1992), or measurement of autoantibodies against oxidized LDL (Salonen et al., 1992), is the best predictor of the risk of development and progression of atherosclerosis.

8. The future

Methodological advances have made it possible to identify individual factors of importance for the activation of chemical carcinogens, to estimate biologically effective doses, to determine early biological effects and alterations in cellular structure and function. Similarly, the possibilities for measuring early events in the atherosclerotic

process have made it feasible to determine factors of importance for atherosclerotic diseases. At the present stage, application of such measures into a large scale investigation on humans is emerging. In contrast to animal experiments, genetic variability and many environmental factors cannot be controlled. However, in future molecular epidemiological studies, the designs can utilize the huge variation and identify subpopulations with constitutively high susceptibility, allowing identification of environmental risk factors which are obscured in the population at large.

Identification and classification of single xenobiotics, such as carcinogens, is important and relates to situations where humans are exposed to one dominating carcinogen. However, we have to realize that the most massive xenobiotic dose man is exposed to is oxygen, which is activated to the mutagenic and atherogenic reactive oxygen species in the body. Furthermore, we have to realize that, besides this massive exposure, the human situation is characterized by life-long exposure to a huge number of naturally occurring potential carcinogens and oxidants, the sum of which may easily exceed that of a single massive exposure. In addition we have to realize that many different xenobiotics can lead to the same early biological effects, e.g., G–T transversion mutations.

Future efforts have to include more elaborate markers of oxidative modifications and markers of individual activation of xenobiotics, in addition to the biomarkers of early biological effects and altered structure and function.

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