# Chapter 3 – ASSESSMENT OF GENOTOXIC HAZARDS IN OPERATIONAL ENVIRONMENTS

by

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## 3.1 ABSTRACT

Key assay methods for detection and identification of genotoxic hazards are outlined and appropriate applications are discussed.

## 3.2 INTRODUCTION

Most carcinogenic chemicals are genotoxic, but not all genotoxic compounds are carcinogenic for humans. Monitoring of genotoxic effects of chemicals or their mixtures which are present in the environment may be used for the hazard identification and risk assessment. Therefore, the estimations of different endpoints of the genotoxicity are studied to assess the risk of the exposure of humans to particular chemical compounds or their mixtures.

Generally, we have two choices to study the possible genotoxic effect of the environment: we can follow the incidence of genotoxic compounds in the environment by testing the genotoxicity of water, food, air or soil by using different tests for genotoxicity (mostly *in vitro*, as the Ames test, test on cell cultures, etc.); and, the identification of genotoxic agents by chemical analysis may also be useful.

Genotoxicity endpoints are followed directly in exposed humans. The most frequently used endpoints (measured usually in isolated peripheral lymphocytes) are: chromosome aberrations, micronuclei, DNA adducts measured by $^{32}$P-postlabelling, DNA damage detected as single or double strand breaks, or alkali-labile sites in the DNA, DNA cross-links, and hypoxantine-guanine phosphoribosyltransferase (HGPRT) mutations.

Methods for the estimation of environmental genotoxicity and methods for the measurements of genotoxicity endpoints in exposed humans are described later in this chapter.

The useful endpoints of genotoxicity are not solely based on biological effects of genotoxins. In some cases, we are not able to measure direct mutagenic effects. However, we can monitor parameters reflecting the exposure to known or unknown mutagens. Thus, we do not measure the genotoxicity itself, but biomarkers of exposure to mutagens. For example, the number of protein adducts may reflect the exposure to a known mutagen that we monitor, but this parameter does not demonstrate anything about the quantitative mutagenic (genotoxic) response in the sense of a biological response.

In some cases it is quite difficult to divide the parameters strictly between the biomarkers of effect and the biomarkers of exposure. DNA adducts are more relevant to biological (genotoxic) effect than are protein adducts; however, they still do not represent the real genotoxic effect because of their possible processing by

DNA repair mechanisms. Similarly, DNA breaks, which we can consider as an effect of mutagens on DNA (from the biochemical point of view), need not necessarily cause mutations.

Other endpoints representing clear parameters of biological effects (chromosome damage or HGPRT mutation induction) can also be regarded as markers of exposure to a mutagen.

The distinction between biomarkers of exposure and genotoxic effects is not definitive. The interpretation of studies using biomarkers depends on the type of biomarker measured. The closer the monitored endpoint is to the biomarker of effect, which is directly related to the disease, the higher the predictive value of the biomarker for the risk assessment. On the other hand, if the biomarker reflects the exposure to a mutagen more than the real effects of the mutagen, it has lower predictive value for the risk assessment, because the exposure to this mutagen may not cause the same effect in all humans due to their different individual susceptibility.

Biomarkers of exposure are usually more specific for certain chemical compounds than are biomarkers of their effects. For example, protein or DNA adducts are measured for specific chemical compounds. On the other hand, biomarkers of effect are not chemical-specific and are less related to the exposure. Gene mutations or chromosome aberrations are not specific for individual chemical compounds.

Various genotoxicity endpoints in tested cells may disappear within different periods of time. This fact may depend on the lifespan of analysed molecules (protein adducts), repair processes (DNA adducts, DNA breaks and cross-links), or on the lifespan of cells (chromosome aberrations). Optimal time after the exposure should be selected for the particular genotoxicity endpoint measurements, depending on the chemical exposure duration and the type of the biomarker followed.

Peripheral lymphocytes isolated from blood samples of humans represent the only biological material which we can obtain from exposed (living) humans in keeping with all ethical rules for work with human tissues. The technique for the blood sampling depends on the particular technique of the assay, requiring special rules for collecting the blood samples. For example, although mostly heparinized blood is used for karyological examination and also for the isolation of lymphocytes for the comet assay, heparin must be avoided in samples used for genotyping. Therefore, separate sample preparations are required for different assays. The type of storage and transport of the sample must be considered from the point of view of the stability of the biomarker (endpoint) measured.

## 3.3 METHODS FOR THE ASSESSMENT OF GENOTOXIC HAZARDS

### 3.3.1 Protein Adducts

Mutagenic compounds are known to react with nucleic acid bases in RNA and DNA because of their electrophility. However, not only nucleic acids but also amino acids in proteins contain nucleophilic sites, especially cysteine and histidine. The adducts are estimated mainly in haemoglobin or albumin [1]. N-terminal valine of haemoglobin is very important for the monitoring of protein adduct formation. Because the lifetime of a human erythrocyte is about 4 months, the amount of adducts formed with haemoglobin gives relatively precise information about the exposure of the human to the mutagenic compound [2].

Unlike DNA adducts, there is no repair of protein adducts. Therefore, the kinetics of disappearance are the same as the turnover of the respective protein. For this reason, the optimal collection of blood samples is in

steady state, i.e. balanced between formation and loss of these adducts formed during the chronic exposure. Samples of blood must be transported and stored frozen in tightly closed ampules to prevent any contamination.

The relative advantage of this method is the fact that the extracted dry globulin can be stored for months before the analysis of adducts. S-alkylcysteines and N-alkyhistidines are estimated [3], or the terminal N-alkylvaline is measured by the modified Edman procedure [4, 5].

Albumin adducts are detected in albumin extracted from serum either immunochemically, or using analysis of species released by hydrolysis of the adducted chemical [6]. A statistically different amount of adduct in the exposed population compared to the group of unexposed humans is considered a positive result.

### 3.3.2 DNA Adducts

A DNA adduct is a chemical entity bound covalently to DNA. Most genotoxic human carcinogens are known to form such adducts with DNA bases. The stability and persistence of adducts in the DNA under physiological conditions is very important and is proportional to their predictive value [7]. The amount of adducts primarily correlates with the exposure, however, the adducts may not correlate with the induction of tumours in a particular tissue [8]. DNA adducts could undergo the process of degradation either spontaneously (as a chemical process), or they can be removed from the DNA by special enzymes involved in the particular pathway of DNA repair. Another reason of a decrease of adducts detected may be the cell loss due to cell turnover, or due to apoptosis or necrosis of heavily damaged cells. The stability of adducts may differ substantially. Adducts formed by aflatoxin B1, for instance, can be highly stable for many days, as well as adducts of $O^6$-guanine-styrene. Examples of relatively unstable adducts are $N^7$-guanine or $N^3$-adenine adduct, whose half-life is only 3 – 5 days [9]. The rapidity by which the particular adducts are removed from the DNA is also different. The cyclobutane-pyrimidine dimers are counted as short-lived, with a half-life of about 15 hours [10], while adducts formed by polycyclic aromatic hydrocarbons have a half-life of >1 month.

Sample collection is optimally made during chronic exposure, similar to sampling for protein adducts. During this exposure, a steady state is achieved between the adduct formation and adduct removal from the DNA. As the removal of adducts can be a relatively rapid process, the sampling should be accomplished within hours after an exposure.

$^{32}$P post-labelling is the widely used method for the detection of DNA adducts. The advantage of the method is the capability to detect and to quantify modifications of nucleotides. Mass spectrophotometry can be used for the identification of the adduct structure.

Highly purified DNA isolated from the tested tissue or cells (predominantly lymphocytes in the case of human studies) is hydrolyzed to 3′ mononucleotides using micrococcal endonuclease and spleen exonuclease. Because of the vast excess of normal nucleotides in the hydrolysate, the content of adducts is enriched by special methods using P1 nuclease, which cause dephosphorylation of nucleotides at the 3′ end. The dephosphorylation at the 3′ end by P1 nuclease is used to enhance the resolution of adducts.

Most radioactively labelled nucleoside 3′, 5′ diphosphates are separated by polyethyleneimine (PEI) cellulose thin-layer chromatography or high performance liquid chromatography (HPLC). Adducted nucleotides are labelled at the 5′ position with $^{32}$PγATP and T4 polynucleotidekinase and the labelled nucleoside 3′, 5′ diphosphate are separated by PEI cellulose chromatography. The resolved adducts are located on PEI cellulose chromatograms using X- ray film and adduct spots are quantified by scintillation counting. Prior to

post-labelling, HPLC is used for the enrichment of small adducts, while bulky adducts are enriched by N-butanol extraction.

As in the case of protein adducts, the optimal time of collection of samples is at the steady state, during long-term exposure. In the case of acute exposure or after termination of chronic exposure, samples should be withdrawn not later than hours or days of the event, because the number of DNA adducts decreases during time due to the repair process [9]. As mentioned above, the DNA level of adducts could be decreased not only due to the DNA repair, but also some adducts (e.g. $N^7$-guanine, $N^3$-adenine) are unstable and may be degraded spontaneously, with a halftime of about 3 – 5 days [10].

### 3.3.3 Chromosome Aberrations

Chromosome aberrations appear as a result of action of a genotoxin reacting with the DNA and causing DNA damage (DNA breaks, or alkali-labile sites – ALS). There are other mechanisms leading to chromosome structural aberrations such as a replication of DNA on the damaged template or inhibition of DNA synthesis. Topoisomerase inhibition may also result in chromosome aberrations. Chromosome aberrations may be followed in peripheral lymphocytes collected from individuals exposed to chemical mutagens.

Ionising radiation and bleomycin induce direct DNA breaks. This kind of DNA damage causes aberration of whole chromosomes (and chromatids) in cells in G0/G1 phase.

In cells treated with a mutagen during the S-G2 phase, aberrations formed involve only one chromatid [11 – 13]. This kind of the DNA damage is detected in lymphocytes after their isolation and stimulation, when they pass through S-phase and mitosis.

When the aberration is caused by DNA synthesis inhibition (without direct DNA damage), it only appears when the inhibitor is present during the S-phase (in vivo). We can barely monitor this type of aberration in stimulated lymphocytes because of the absence of DNA synthesis inhibitor.

Numerical aberrations may occur, leading to aneuploidy or polyploidy. These aberrations are consequences of abnormal cell division and the mechanism is not quite clear. These mechanisms are linked to the damage of mitotic spindle or chromosome structures involved in the mitotic apparatus, etc. [14].

To detect chromosomal aberrations, the dividing cells have to be arrested in mitosis using colchicine which inhibits the inhibition of tubulin polymerization. When lymphocytes are used, these cells have to be stimulated with phytohemagglutinin (PHA).

In the case of acute exposure of humans, it is important to withdraw the lymphocytes as soon as possible after the end of exposure because the persistence of the DNA damage is critical for the frequency of aberrations scored.

The frequency of chromosome aberrations in humans exposed to genotoxic compounds is strongly dependent on the dose, duration and frequency of exposure. The clastogenic mechanism of the tested compound is also important. For example, the frequency of aberrations induced by S-dependent agents can be influenced by the DNA repair, if the DNA damage is repaired before S-phase.

A new technique for the scoring of chromosome aberrations has appeared, enabling the scoring of specific damage in metaphase or interphase cells (including lymphocytes). It is the fluorescence *in situ* method [15 – 19]. This method is better for detection of certain aberrations compared to classic staining methods.

Both structural and numerical aberrations are known to be involved in the etiology of cancer [20, 21]. In general, an increased frequency of chromosome aberrations is associated with an increased risk of cancer [21 – 23].

Blood samples must be heparinised and should be processed within 24 hours; however, lymphocytes can be cultivated successfully even after several days. In the case of late processing, there is a possibility that the DNA damage is repaired prior to the stimulation of lymphocytes to the S-phase. This may explain a decline in the efficiency of recovery of aberrations. Therefore, the blood should be kept at <8$^{o}$C before processing, and optimally at 2$^{o}$C.

Whole blood is added to the cultivation medium containing PHA (phytohemagglutinin) and cultures are incubated for 1 generation (until the first mitosis), when the highest frequency of aberrations appears. During mitotic division, the appearance of aberrations is significantly decreased because the damage is diluted among daughter cells. The more damaged cells may be lost, and a decrease of the aberration frequency may also occur. Therefore, the cultures are harvested 48 hours after stimulation with PHA to obtain the first generation of lymphocytes.

As the proliferation of the first and second generations of lymphocytes may vary among individuals, the chromatids are labelled by adding BUdR into the culture medium to discern the first and the second mitosis [24]. Before harvesting, colcemide is added for the last 2 hours of cultivation. The routine processing of cells for the preparation of karyological preparations follows, using the hypotonization of cells (0.075 M KCL) and fixation 3:1 (methanol : acetic acid). Slides are prepared and cells stained with differential staining for metaphase cells based on the incorporation of BUdR.

Aberrations are scored using human cytogenetic nomenclature [25]. Chromosomal damage such as breaks are regarded as a consequence of breakage of DNA, while large rearrangements of chromosomes involve multiple breakages and misrepair. In addition to the classical scoring, the FISH method can be used for the evaluation of structural aberrations. Differential staining of 2 – 6 chromosome pairs is carried out by means of special DNA probes.

The frequency of aberrations measured must be compared with a control or reference population to determine meaningful differences for individuals.

### 3.3.4 Micronuclei

Micronuclei (MN) arise in mitotic cells from fragmented chromosomes. Some chromosomes may lag behind during anaphase and are not integrated in the nuclei of daughter cells. Such MN may appear as a consequence of DNA breakage, replication on the damaged DNA or inhibition of DNA synthesis. MN containing whole chromosomes also exist. They can be formed by the failure of mitotic spindle, kinetochore, or chromosome structures taking part in the chromosome movement. The frequency of MN can represent not only the active DNA breakage but also reflects an aneuploidogenic effect which is not linked to effects on DNA.

The frequency of MN in human studies is determined in stimulated lymphocytes, similarly to chromosome aberrations [26]. MN arising from lagging chromosomes may contain kinetochores which may be clearly identified by a specific antibody. They also contain centromeres which can be identified by selective DNA probes against centromere DNA sequences.

Like chromosome aberrations, the frequency of MN appearing in the studied population must be compared with control or reference populations. There should be a clear dose-response which helps support the significance of results.

Lymphocyte cultures are prepared by similar techniques to those used for chromosome aberrations scoring (see above) and stimulated by phytohemagglutinin. The analysis is limited to cells that have divided once *in vitro*. The technique uses cytochalasin B (cyt B) added to the culture to block cytokinesis in the telophase [27]. Therefore, cells containing 2 nuclei can be identified, representing cells which have divided once *in vitro*. The proliferation activity of cells can also be scored by this mechanism. Binucleated cells are only scored for MN with this cytochalasin B block-method.

### 3.3.5 Single Cell Gel Electrophoresis (SCGE) – Comet Assay

We can estimate the DNA damage in terms of single strand breaks (SSB) in any accessible cells which can be isolated or prepared as a single cell suspension by this technique [28 – 31]. In this assay, cells are embedded in agarose on microscopic slides and lysed in lysing solution containing detergent and high salt. There are neutral and alkaline versions of the method. In the alkaline version of this method, lysed cells are placed in alkaline solution (0.3 M NaOH) to unwind DNA. DNA is pulled to the anode during electrophoresis. If the DNA contains alkali-labile sites (ALS), it is released from the nucleoids and moves to the anode, forming a comet-like pattern in the fluorescent microscope after staining of the DNA with fluorescent dyes. In the case of neutral version of the method, the alkaline unwinding is avoided and the electrophoresis is carried out under the neutral pH. Under neutral pH, only double strand breaks can cause the formation of the comet. On the other hand, the single strand and double strand breaks may be analyzed at pH 12.1. At higher pH, both single strand breaks and alkali-labile sites (ALS) in the DNA molecule (which disrupt and form breaks under the high pH) are detected. Strand breaks may arise from direct strand breakage or as an intermediate stage during incomplete DNA repair.

Modifications of the technique have been published enabling the detection of specific DNA lesions (pyrimidine dimmers, oxidised bases, alkylated bases) using the cleaving of DNA in nucleoids by specific enzymes (like T4 endonuclease, endonuclease III, formamido- pyrimidine nuclease) [32, 33].

After the electropohoresis, cells are stained with fluorescent dyes staining the DNA such as DAPI, ethidium bromide or acridine orange and several parameters are evaluated using the image analysis software. The comet tail length (TL), % of DNA in tail (TD) and tail moment (TM) are the most important parameters [34, 35].

## 3.4 TESTING OF ENVIRONMENTAL CONTAMINATION WITH GENOTOXIC AGENTS

Generally, air, water and soil can be contaminated with different genotoxic agents. Some of these agents may be present naturally such as different heavy metals, etc. Other genotoxic contamination of environment may be a consequence of human activity, especially as products of industry. Many chemicals arising from industrial activity may contaminate the environment as air pollution or wastewater.

Any human activity in heavily polluted areas may represent a health risk. Military activities in such areas may also increase health risks to soldiers, especially during long-term missions. Therefore, the monitoring of genotoxicity of water, air and eventually soil may be very useful for the prevention of any influence of such pollutants on health of military personnel operating in polluted areas and will enable health risk assessment.

As a consequence of industrial activity, many chemicals may spread into the surroundings. When these chemicals are stable, such as DDT, PCBs or heavy metal salts, they may stay in the environment for years and so

they can influence organisms living in contaminated areas, including man. These chemicals may contaminate water or may get into the food chain; thus representing a risk in the case of the long-term exposure of humans.

Military members may be at special risk for certain chemical exposures, such as those coming from the use of chemical weapons or explosives, diesel or other military engines, and fires in or near the battlefield (e.g. as a consequence of enemy activity – see burning oil fields in Iraq during the Gulf war). Many other potentially hazardous chemicals may be encountered during missions, e.g. insecticides, defoliants, disinfection, detergents, etc.

All these agents, or environmental contamination by them, should be under control to minimise their harmful influence on the health of soldiers or inhabitants in the area, and to enable risk assessment in case of human exposure to them. Thus, testing the environment for genotoxicity should be conducted in the operational areas of deployed military troops, especially in areas in which a long-term stay is expected (e.g. military bases, camps, etc.). The testing should especially examine water, air and soil. Techniques and methods for such testing have been used in many cases of industrial pollution and chemical contamination of the environment.

### 3.4.1 Air Genotoxicity

Any combustion process may increase the genotoxicity of the air. A typical source is the coal-burning power plant, but local heating of houses by burning of contaminated fuels may also substantially pollute the air. Any type of dust is also important. The dust particles may absorb different compounds such as aromatic hydrocarbons. Results of many studies focused on the genotoxicity of the air have been published. These studies are focused on several types of air pollution. In these studies, the biological activities of complex mixtures of organic compounds adsorbed onto ambient airborne dust particles are evaluated. Particles are collected on special teflon-coated filters and later extracted with dichloromethane or other solvents. The genotoxicity of compounds contained in dichloromethane extractable fraction is then measured. Primarily, the following short-term in vitro assays are used [36, 37]:

- Ames test. Bacterial mutagenicity test using different Salmonella typhimurium strains TA98 and YG1041.
- DNA adducts estimation using 32P-postlabelling. The induction of DNA adducts is measured in target human or mammalian cells treated in vitro with extracts from air samples (primarily HePG2 cells or isolated rat hepatocytes are used, because they are able to activate phenylalanine hydroxylase (PAH)).
- DNA breaking activity of air extracts. In this case, target cells are treated with air sample extracts dissolved in DMSO for different period of time (usually 1 – 3 hours) and the resulting DNA breaks in cells are scored using the alkaline version of the comet assay.
- Plant-based tests are also used for the evaluation of air-genotoxicity. As an example, Tradescancia growing in the tested area and the induction of micronuclei (MN) is one type of assay [38].

### 3.4.2 Water Genotoxicity

The water may be also tested by similar methods [39]. First of all, the induction of bacterial mutation in the Ames test is suitable, supplemented with S9 fraction isolated from rat liver. The induction of the DNA damage in cultured mammalian cells detectable with comet assay is important information about the possible action of the genotoxic chemical compound. Similar to air testing, some assays based on the use of plants may also be used.

### 3.4.3 Soil Genotoxicity

The genotoxicity of soil is important from the point of view of long-term missions, when the soldiers may be accommodated in the confined geographical area of the base for a long time. Thus, they may be exposed not only to the effects arising from industrial pollution, but also to the contamination of water with different PAH and to hydrocarbons from fuel which may be quite highly concentrated in contaminated soil.

Tests are aimed at evaluating the genotoxicity of contaminated soils by means of an integrated chemical/ biological approach, using a short-term bacterial mutagenicity test (Ames test), a plant genotoxicity test (*Tradescantia*/micronucleus test), and chemical analyses. The genotoxicity of soil is measured mostly using water or dichlormethane extracts from the soil [40]. Similar to the testing of air and water, the induction of DNA damage (strand breaks) in cultured rat or human cells exposed to soil extract may be followed using the comet assay.

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