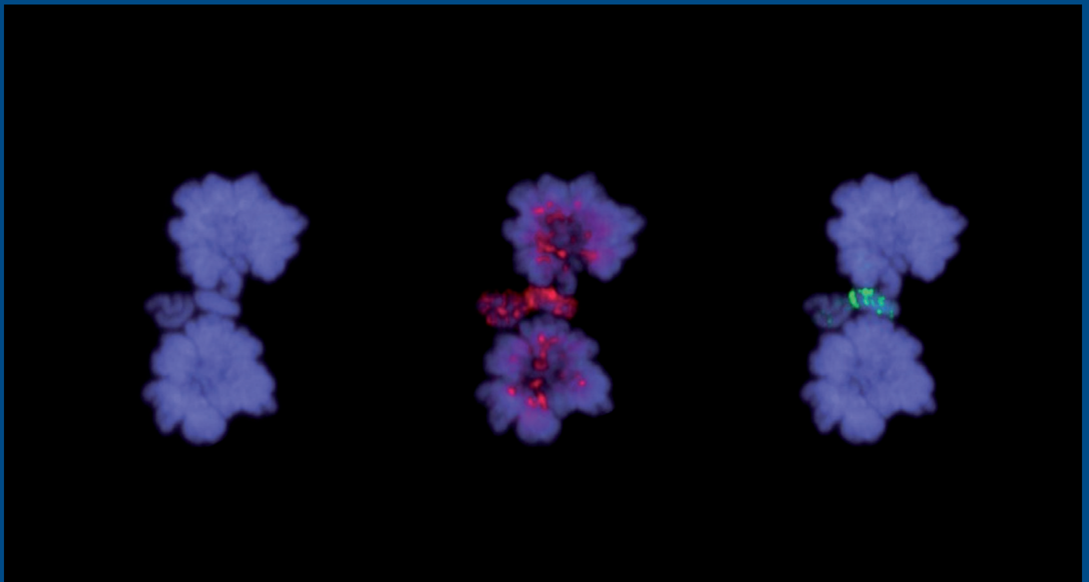
Ghita Falck

Micronuclei in Human Peripheral Lymphocytes – Mechanistic Origin and Use as a Biomarker of Genotoxic Effects in Occupational Exposure



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Address Finnish Institute of Occupational Health
Topeliuksenkatu 41 a A
FI-00250 Helsinki
Tel. +358-30 4741
Fax +358-9 477 5071
www.ttl.fi

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Helsinki 2014

GHITA FALCK

Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki, Finland

Health and Work Ability
Finnish Institute of Occupational Health
Helsinki, Finland

ACADEMIC DISSERTATION
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Supervisor:	Research Professor Hannu Norppa, PhD Systems Toxicology, Health and Work Ability Finnish Institute of Occupational Health Helsinki, Finland
Reviewers:	Dr. Maija Wessman, PhD Institute of Genetics Folkhälsan Research Center Helsinki, Finland
	Dr. Michael Fenech, PhD Health Sciences & Nutrition Commonwealth Scientific Industrial Research Organisation Adelaide, SA, Australia
Opponent:	Dr. Ilse Decordier Department of Biology Faculteit Wetenschappen en Bio-Ingenieurswetenschappen Vrije Universiteit Brussel, Belgium
Custos at the dissertation:	Professor Minna Nyström, PhD Division of Genetics, Department of Biosciences, Faculty of Biological and Environmental Sciences University of Helsinki, Finland

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ABSTRACT

The formation of micronuclei (MN) in peripheral blood lymphocytes represents one of the earliest cytogenetic effects of exposure to genotoxic carcinogens. An increased frequency of MN and of other cytogenetic alterations, such as chromosomal aberrations and sister chromatid exchanges, in a well controlled study of occupationally exposed people, is usually considered a strong indication of exposure to genotoxic carcinogens, requiring improvements in protective measures. Such considerations are further supported by epidemiological studies, suggesting that a high frequency of chromosomal aberrations and MN is predictive of an increased risk of cancer.

This study was performed to obtain comprehensive information about the suitability of the lymphocyte micronucleus assay (MN assay) in examining the effects of occupational exposure to genotoxic carcinogens. MN, defined as small, additional nuclei, are formed in mitosis from acentric chromosomal fragments or chromosomes that lag behind in anaphase and eventually fail to be included in either of the daughter nuclei. The primary objectives of this dissertation were to clarify factors affecting the mechanistic origin of MN and to assess the influence of some methodological aspects in the MN assay. The differentiation of MN harbouring fragments from those containing whole chromosomes was considered a main goal, in order to estimate whether these two classes of MN should be analyzed separately. Methodologically, the influence of cell culture time and the use of the cytokinesis-block method on the frequency and contents of MN were assessed, as well as the possibility to analyze MN *in vivo* from uncultured T-lymphocytes. In addition, the suitability of bromodeoxyuridine (BrdU) labeling as an alternative method to the cytokinesis-block technique was investigated in green-

house workers occupationally exposed to pesticides. Furthermore, some genetic polymorphisms (*GSTM1*, *GSTT1* and *NAT2*) were also studied in the greenhouse workers to reveal their possible role as modifiers of individual response to the occupational exposure. Finally, mechanistic aspects concerning the formation of MN from chromosomes and fragments lagging behind in anaphase, with a special reference to the X chromosome, were assessed.

By using pancentromeric fluorescence *in situ* hybridization (FISH), considerable proportions of MN were found to contain whole chromosomes or chromatids. The frequency of this type of MN was both age- and culture-time-dependent. Micronucleus analysis using the anti-BrdU staining technique showed that the pesticide-exposed greenhouse workers, in particular exposed smokers, had a higher micronucleus frequency than their respective controls. Subjects with the *GSTM1* positive and *NAT2* fast acetylator genotypes had higher frequencies of MN, but this effect was not related to the pesticide exposure. Comparison of the proportion of various types of MN in cultured and uncultured T-lymphocytes of women, using a pancentromeric and a centromeric X-chromosome specific DNA probe, indicated that *in vivo* MN in T-lymphocytes primarily consist of whole chromosomes, especially the X-chromosome. In general, the X chromosome was highly over-represented in female MN, and cell culture further increased the frequency of X-chromosome-positive MN. The high micronucleation of the X-chromosome appeared to be due to its frequent lagging behind in anaphase. Reciprocal chromosome gain and loss in lymphocytes was more common for the X-chromosome both in men and women than for the Y-chromosome in men. The results of this dissertation strongly suggest that, when MN are used as a biomarker of human genotoxic effects, the recognition of the contents of MN is of utmost importance and is expected to improve the specificity and sensitivity of the assay. Furthermore, the direct analysis of MN in uncultured peripheral T lymphocytes, combined with centromeric FISH, may provide a new possibility for the assessment of chromosome breakage and numerical chromosome alterations occurring *in vivo*.

ABSTRACT IN FINNISH – TIIVISTELMÄ

Mikrotumien lisääntyminen perifeerisen veren lymfosyyteissä on ensimmäisiä sytogeneettisiä vaikutuksia altistuttaessa syöpää aiheuttaville genotoksisille tekijöille. Työntekijöillä tehdyissä tutkimuksissa mikrotumien ja muiden sytogeneettisten muutosten, kuten kromosomiaberraatioiden ja sisarkromatidivaihdosten, määrän kohoamista kontrolliryhmään verrattuna pidetään selkeänä osoituksena altistumisesta genotoksisille karsinogeeneille ja perusteena korjaaville toimenpiteille. Tätä näkemystä tukevat epidemiologiset tutkimukset, jotka ovat osoittaneet, että kromosomiaberraatioiden ja mikrotumien määrän lisääntyminen ennakoi kohonnutta syöpäriskiä.

Tämän tutkimuksen tarkoituksena oli saada uutta tietoa lymfosyyttien mikrotuma-analyysin käyttökelpoisuudesta tutkittaessa työperäistä altistumista genotoksisille karsinogeeneille. Mikrotumat ovat pieniä, ylimääräisiä tumia, joita muodostuu, kun kromosomifragmentit tai kokonaiset kromosomit jäävät tuman jakautuessa jälkeen muista kromosomeista eivätkä kulkeudu tytärtumiin. Tämän väitöskirjan päätavoitteena oli selventää mikrotumien syntymekanismeja ja eräiden menetelmällisten tekijöiden merkitystä mikrotuma-analyysissä. Keskeinen päämäärä oli erottaa fragmentteja sisältävät mikrotumat kokonaisia kromosomeja sisältävistä mikrotumista, jotta voitaisiin arvioida, tulisiko näitä mikrotumien pääryhmiä tarkastella erikseen. Menetelmällisistä kysymyksistä keskityttiin selvittämään, miten lymfosyyttien viljelyaika ja sytokineesiblokki-menetelmän käyttö vaikuttavat mikrotumien sisältöön ja esiintymistiheyteen ja voidaanko mikrotumia tutkia T-lymfosyyteistä ilman soluviljelyä. Lisäksi pestiseideille työperäisesti altistuneilla kasvihuonetyöntekijöillä tutkittiin mahdollisuutta käyttää sytokineesiblokki-

menetelmän sijasta bromodeoksiuridiini (BrdU) -leimausta vaihtoehtoisena metodina. Työntekijöiltä tutkittiin myös, vaikuttaako eräiden vierasaineenvaihduntaan liittyvien geenien (*GSTM1*, *GSTT1* ja *NAT2*) geneettinen monimuotoisuus henkilökohtaiseen mikrotumavasteeseen työperäisessä altistumisessa. Lopuksi selvitettiin, miten mikrotumia muodostuu tuman jakautumisen aikana jälkeen jäävistä kokonaisista kromosomeista ja kromosomien fragmenteista.

Kun mikrotumien sisältöä tutkittiin fluoresenssi *in situ* hybridisaatio (FISH) -menetelmällä, valtaosan mikrotumista havaittiin sisältävän kokonaisia kromosomeja tai kromatideja. Tämän tyyppisten mikrotumien esiintymistiheys lisääntyi iän ja viljelyajan myötä. Käyttämällä antibromodeoksiuridiini-värjäystekniikkaa osoitettiin, että pestisideille altistuneilla kasvihuonetyöntekijöillä, erityisesti altistuneilla tupakoitsijoilla, oli korkeampi mikrotumafrekvenssi kuin kontrollihenkilöllä. *GSTM1*-positiivinen genotyyppi ja nopean asetylaation *NAT2*-genotyyppi liittyivät korkeaan mikrotumafrekvenssiin, mutta niillä ei ollut yhteisvaikutuksia pestisidialtistumisen kanssa. Kun T-lymfosyyttien mikrotumien sisältöä tutkittiin käyttäen pansentromeeristä ja X-kromosomin sentromeerin DNA-koetinta ennen soluviljelyä ja sen jälkeen, ilmeni, että *in vivo* -mikrotumat sisältävät pääosin kokonaisia kromosomeja. Erityisesti X-kromosomi oli selvästi yliedustettuna tutkittujen naisten mikrotumissa, ja solujen viljely lisäsi vielä X-kromosomin sisältävien mikrotumien esiintymistiheyttä. X-kromosomin yleisyys mikrotumissa näytti selittyvän sillä, että X-kromosomi jää odotettua useammin jälkeen muista kromosomeista tuman jakautumisen anafaasi-vaiheessa. X-kromosomin virheellinen segregaatio tytärtumien välillä oli odotettua yleisempää sekä naisilla että miehillä, ja miehillä virhe koski useammin X-kromosomia kuin Y-kromosomia. Väitöskirjan tulos viittaa vahvasti siihen, että mikrotumien sisällön tunnistaminen on erityisen tärkeää menetelmän spesifisyyden ja herkkyyden kannalta, kun mikrotuma-analyysiä käytetään genotoksisen vaikutuksen biomarkkerina työntekijätutkimuksissa. T-lymfosyyttien suora analyysi ilman soluviljelyä yhdistettynä pansentromeeriseen FISHiin tarjoaa uuden mahdollisuuden mitata kromosomikatkoksista ja kokonaisista kromosomeista *in vivo* syntyneiden mikrotumien esiintymistiheyttä.

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to in the text by the Roman numerals I–VI[1] as indicated below.

I Falck G, Catalán J, Norppa H. Influence of culture time on the frequency and contents of human lymphocyte micronuclei with and without cytochalasin B. Mutat. Res. 1997; 392: 71–79.

II Surrallés J, Falck G, Norppa H. *In vivo* cytogenetic damage revealed by FISH analysis of micronuclei in uncultured human T-lymphocytes. Cytogenet. Cell Genet. 1996; 75: 151–155.

III Catalán J, Surrallés J, Falck GC-M, Autio K, Norppa H. Segregation of sex chromosomes in human lymphocytes. Mutagenesis 2000; 15: 251–255.

IV Catalán J*, Falck GC-M*, Norppa H. The X chromosome frequently lags behind in female lymphocyte anaphase. Am. J. Hum. Genet. 2000; 66: 687–691.
* These authors contributed equally to this study.

V Falck GC-M, Catalán J, Norppa H. Nature of anaphase laggards and micronuclei in female cytokinesis-blocked lymphocytes. Mutagenesis 2002; 17: 111–117.

VI Falck G, Hirvonen A, Scarpato R, Saarikoski ST, Migliore L, Norppa H. Micronuclei in blood lymphocytes and genetic polymorphism for *GSTM1*, *GSTT1* and *NAT2* in pesticide-exposed greenhouse workers. Mutat. Res. 1999; 441: 225–237.

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ABBREVIATIONS

AF	Acentric fragment
B_{12}	Cyanocobalamin
BrdU	Bromodeoxyuridine
CA	Chromosomal aberration
C-	Centromere negative
C+	Centromere positive
CBMN	Cytokinesis-block micronucleus
COLO 320DM	Colorectal carcinoma tumor cell line
CREST	Calcinosis, Raynaud's phenomenon, esophageal dysmotility, Sclerodactyly and Telangiectasia
CYP	Cytochrome P-450
CYP1A1	Cytochrome p-450 1A1
Cyt-B	Cytochalasin B
DAPI	4',6-Diamidino-2-phenyl-indole-dihydrochlorid hydrate
DEB	1,2:3,4-Diepoxybutane
DNA	Deoxyribonucleic acid
DSB	Double-strand DNA break
DXZ1	X-chromosome-specific centromeric α-satellite probe
DYZ3	Y-chromosome-specific centromeric α-satellite probe
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
GST	Glutathione *S*-transferase
GSTM1	Glutathione *S*-transferase M1
GSTT1	Glutathione *S*-transferase T1

HOD	Hypodiploidy
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRD	Hyperdiploidy
HUMN	HUman MicroNucleus project
K-	Kinetochore-negative
K+	Kinetochore-positive
MN	Micronuclei
MN assay	Micronucleus assay
MN index	Micronucleus index
MNBNC	Micronucleated binucleate cell
MNC	Micronucleated cell
NAT1	N-acetyltransferase 1
NAT2	N-acetyltransferase 2
OECD	Organisation for Economic Co-operation and Development
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PI	Propidium iodide
PRINS	Primed *in situ* labelling
RGL	Reciprocal gain and loss
RPMI	Roswell Park Memorial Institute
RR	Rate ratio
RT	Room temperature
SCE	Sister chromatid exchange
SSB	Single-strand DNA break
TRITC	Tetramethylrhodamine isothiocyanate
WDLPS	Liposarcoma cell line
X	The X chromosome
XMEs	Xenobiotic-metabolizing enzymes
Y	The Y chromosome

INTRODUCTION

Human exposure to chemicals is basically an inevitable part of life in this day and age. In general, upon exposure, the chemical agent is often absorbed into the receptor bloodstream via three primary routes – inhalation into the lungs, oral ingestion, and dermal/skin contact (Asante-Duah 2002). Exposure to various chemical and physical agents both in the working environment as well as during the free time can damage DNA (deoxyribonucleic acid). Many genotoxic chemicals are known to act by binding to DNA, forming covalent complexes (adducts) with its constituent DNA nucleotides. Unless repaired, these adducts can lead to cell death or mutation by interfering with cell replication and other cellular processes (Vuyyuri et al. 2006). Such DNA-damaging genotoxic agents are known to have serious biologic effects, most importantly, they may eventually cause cancer. Although chemical exposures are in general considered to be decreasing in workplaces of the industrial society, there are still many examples of notable occupational genotoxic exposures, often to complex chemical mixtures containing a high number of different genotoxic components. Due to this complexity, exposure assessment may be difficult by traditional chemical techniques.

Most human carcinogens are genotoxic but not all genotoxic agents have been shown to be carcinogenic in humans. In monitoring of genotoxic effects of carcinogens in humans, the discovery of suitable biological markers is very important for hazard identification and risk assessment. In exposure to genotoxic carcinogens, a biological marker (i.e. biomarker) can be described as any event or component that is detectable or measurable in the human body and may influence or predict the incidence or outcome of the genotoxic insult. Using traditional criteria, biomarkers can be divided into markers of exposure, effects, and

susceptibility (Fenech and Morley 1985; Tucker et al. 1997; Salama et al. 1999). Distinction between these classes, especially between biomarkers of exposure and effect, is not always clear. The former serves as a measure of exposure and the latter as an indicator of cancer risk. Inter-individual biological variability affects biomarkers of both exposure and effect. This variability may be assessed using biomarkers of susceptibility which can be used to detect subjects who have higher probability of an adverse effect.

Many biomarkers have been developed to estimate exposure and to assess the risk of adverse effects in an early phase. Monitoring of biological effects as a measure of the internally effective dose may especially be relevant for the assessment of the ultimate health risks, namely cancer. Structural and numerical chromosomal alterations assessed with traditional cytogenetic methods, the analyses of chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and micronuclei (MN), are among the genotoxicity effect markers most often used (Carrano and Natarajan 1988; Albertini et al. 2000; Norppa 2004a). Other widely used markers of genotoxicity include covalent binding products (adducts) to DNA, other types of DNA damage (strand breaks, crosslinks, alkali-labile sites), and somatic gene mutations (hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and glycophorin A genes) (Albertini et al. 2000; Lander et al. 2000). The use of these methods is based on the fact that most established human carcinogens are genotoxic in short-term tests and capable of inducing chromosome damage (Waters et al. 1999). The mechanistic basis varies for different genotoxicity endpoints, and for some it has not fully been elucidated. Although exposure to a variety of different genotoxic carcinogens can be detected using these biomarkers, the causative agent cannot always be identified unambiguously, except in the case of specific DNA adducts.

In the last decade, it has become clear that genetic pre-disposition may affect individual susceptibility to cancer and carcinogen-induced biomarkers of exposure and effect. Genetic susceptibility can be due to variation in genes coding for carcinogen-metabolizing enzymes, such as cytochrome P450 (*CYP*) and glutathione S-transferases (*GST*) (Wormhoudt et al. 1999; Norppa 2004a). In practice, biomarkers of exposure (substance or its metabolites in biological fluids, urinary mutagenicity, protein and DNA adducts) and of effects (CA, SCE, MN, mutations) may be affected by the genotype (and phenotype) of several activating/

detoxifying metabolic activities. Furthermore, biomarkers of effect may be influenced by genetic polymorphisms of DNA repair genes and other genes important for genomic integrity.

The present dissertation concentrates on one cytogenetic genotoxicity biomarker, namely MN in peripheral lymphocytes. MN are formed from both whole and fragmented chromosomes that lag behind in cell division and are eventually left outside the daughter nuclei (see (Norppa and Falck 2003). In general, micronucleus analysis is utilized in both genotoxicity testing and biomonitoring of genotoxic exposure and effect in humans. MN are particularly considered a useful biomarker of genotoxic effects in populations exposed to genotoxicants in occupational settings. In principle, the micronucleus assay (MN assay) allows the detection of both aneugenic agents (inducing numerical chromosome alterations) and clastogenic agents (inducing chromosome breakage) and can be applied, in addition to peripheral lymphocytes, to cells exfoliated from buccal, nasal and urothelial mucosa (Albertini et al. 2000; Pastor et al. 2001; Norppa and Falck 2003; Holland et al. 2008). When peripheral lymphocytes are examined, the cytokinesis-block method (Fenech and Morley 1985) is most often used. In comparison with other cytogenetic techniques, the cytokinesis-block micronucleus (CBMN) assay is a relatively rapid and simple test in which cells that have divided once in culture are identified by the use of cytochalasin B (Cyt-B) (Fenech 1993). This dissertation especially addresses the applicability of the MN assay as a biomarker in occupational exposure. Factors affecting the baseline level of MN and mechanisms of micronucleus formation are also evaluated, and the feasibility of some new methodological approaches is assessed.

REVIEW OF THE LITERATURE

Cytogenetic biomarkers of chromosomal damage

Cytogenetic biomarkers such as chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and MN have for many years been used for surveillance of human genotoxic exposure and cytogenetically visible early effects (Albertini et al. 2000). The use of these biomarkers is based on the fact that most established human carcinogens are genotoxic in short-term tests and capable of inducing chromosome damage (Waters et al. 1999). Furthermore, epidemiological studies have suggested that a high frequency of (structural) CAs in lymphocytes (Hagmar et al. 1998; Bonassi et al. 2000; Bonassi et al. 2008) and MN in peripheral blood lymphocytes (Bonassi et al. 2007) is predictive of an increased cancer risk lending support to the relevance of using the cytogenetic methods.

Chromosomal aberrations

Chromosomal aberrations (CAs) can be defined as microscopically visible changes in normal chromosome structure in cells. They can be formed spontaneously during the cell cycle or after chemical (or radiation-) treatment as a result of misrepaired or unrepaired DNA lesions (Russell 2002). CAs are mostly scored in proliferating cells – arrested after culturing at the metaphase stage using a tubulin polymerization inhibitor (e.g., Colcemid®), fixed, spread on microscope slides, and subsequently stained with solid Giemsa-stain (Dean 1984) (see Figure 1 A). The CA assay is primarily used for the analysis of structural CAs which, based on morphological criteria, are classified into two main

categories, chromosome-type (involving both chromatids of one or multiple chromosomes; see Figure 1 A) and chromatid-type aberrations (involving only one of the two chromatids of a chromosome or several chromosomes) (Albertini et al. 2000; Hagmar et al. 2004). In both categories, breaks and various types of rearrangements are distinguished (examples of chromosome-type aberrations illustrated in Figure 1 A). Structural CAs may be induced by direct DNA damage, by replication on a damaged DNA template, by inhibition of DNA synthesis and by other mechanisms (e.g. topoisomerase II inhibitors) (Albertini et al. 2000). In the formation of chromosome-type CAs; double strand DNA breaks (DSB) are considered to be the primary lesions (Pfeiffer et al. 2000). However, the analysis of CAs is not suitable for the evaluation of numerical CAs because artefactual chromosome loss may occur during slide preparation (Mateuca et al. 2006).

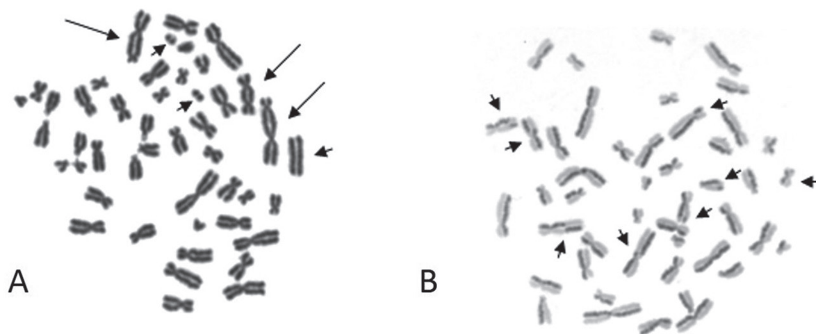

Figure 1. Microscopic images of human lymphocyte metaphases. A. An aberrant male metaphase stained with Giemsa. The aberrations consist of three dicentric chromosomes (long arrows) and three acentric fragments (short arrows). B. A female metaphase stained with the fluorescence-plus-Giemsa technique, showing eight sister chromatid exchanges. Arrows mark the sites of exchange. Photographs kindly provided by Hilkka Järventaus.

In occupational and environmental contexts, the analysis of structural CAs in peripheral blood lymphocytes has been used for more than 30 years as a biomarker of early effects of genotoxic carcinogens (Hagmar et al. 2004). The frequency of CAs, determined by ordinary metaphase analysis, has in several studies been shown to be associated with a higher cancer risk (Hagmar et al. 1994; Bonassi et al. 1995; Hagmar et al. 1998; Liou et al. 1999; Bonassi et al. 2000; Smerhovsky et al. 2001; Smerhovsky et al. 2002; Rossner et al. 2005; Bonassi et al. 2008). Because of these findings, the traditional CA assay is still much used in studies of human exposure to chemical genotoxins and ionizing radiation.

Newer techniques for the analysis of translocations and intrachromosomal rearrangements, based on fluorescence *in situ* hybridization (FISH painting), have been applied in radiation studies (Obe et al. 2002).

Sister chromatid exchanges

During S-phase of the cell cycle, DNA is replicated so that each chromosome becomes duplicated into two closely associated daughter chromatids joined at the centromere. SCEs are formed during the S-phase, sister chromatids breaking and rejoining with one another, physically exchanging regions. SCEs thus result from symmetrical DNA exchanges occurring during replication between the sister chromatids of the same chromosome, at homologous loci (Wolff 1975; Wolff et al. 1977; Carrano et al. 1979; Wilson and Thompson 2007). The exchange process has been suggested to represent homologous recombination repair of DNA double-strand breaks (Sonoda et al. 1999; Johnson and Jasin 2000; Wilson and Thompson 2007) or of single-strand breaks or other lesions converted into double-strand breaks at the replication fork (collapsed replication fork) (Helleday 2003). DNA interstrand cross-link has been suggested to be a major lesion leading to SCEs (Wojcik et al. 2004). Presumably, there may be more than one molecular pathway responsible for SCEs (Wilson and Thompson 2007).

In humans, SCEs are scored from second-division metaphases of PHA-stimulated lymphocytes after cell culture in the presence of 5-bromodeoxyuridine (BrdU) which enables differential staining of sister chromatids. BrdU is a DNA base analogue which is incorporated into the DNA during replication replacing thymine. If cells incorporate

BrdU during at least two cell cycles, the second mitotic division contains chromosomes where one sister chromatid is bifilarly and the other unifilarly substituted with BrdU (see Figure 1 B). Detection of SCEs can be achieved after differential staining with a combination of Hoechst 33258 and Giemsa (Perry and Wolff 1974) (fluorescence-plus-Giemsa technique; see Figure 1 B). A new technique for SCE analysis, based on the use of biotin-16-2'-deoxyuridine-5'-triphosphate was described by Wojcik et al. (2004).

As the number of SCEs is obtained for each cell scored, the assay is inherently sensitive and has been popular both in experimental and human biomarker studies. Since the biological significance of the SCE event is still elusive (Bonassi and Au 2002; Norppa et al. 2006), further studies are needed, e.g., to judge whether SCE analysis could be used as a marker of homologous recombination. Although SCE frequency is a useful parameter in well-controlled investigations of exposure to chemical genotoxins, it has not turned out to have cancer risk predictivity (Hagmar et al. 1998; Bonassi et al. 2000; Bonassi et al. 2004). One of the reasons for this finding is probably technical variation in SCE frequencies which makes it difficult to combine SCE data from different studies (Norppa et al. 2006). The usage of SCEs has progressively disappeared from the scientific literature (Bonassi et al. 2005).

Micronuclei

MN are small, extranuclear bodies additional to the main nucleus in a cell. They are formed when whole chromatids, chromosomes and acentric fragments are left behind and eventually excluded from the daughter nuclei at mitosis (Heddle et al. 1983; Ford et al. 1988; Lindholm et al. 1991; Ford and Correll 1992). Laggards cannot move towards the poles, because they are detached from the mitotic spindle or, alternatively, have bipolar (merotelic) kinetochore orientation (e.g. one kinetochore is attached to microtubules from both poles instead of just one), as described by Cimini et al. (2001). Besides these fundamental mechanisms, some MN may have their origin in fragments derived from broken anaphase bridges (Cornforth and Goodwin 1991; Saunders et al. 2000; Huang et al. 2011) formed due to chromosome rearrangements (dicentric chromatids, concatenated ring chromosomes or union of sister chromatids)

(Norppa and Falck 2003). In the microscope, a micronucleus is visualized as an extra small nucleus beside the main nucleus of an interphase cell as shown in Figure 2.

The MN assay can be described as a variation of the CA assay. Instead of detecting an aberration at the metaphase stage of the cell cycle, MN are first detected after the completion of nuclear division (Bonassi and Au 2002) (see Figure 2). In contrast with the CA test, the MN assay/test is able to detect aneugenic events, one of the major advantages of the MN assay (Albertini et al. 2000). In addition, MN can be assessed much more easily, quickly, and objectively than CAs in metaphase cells using the traditional CA assay. In practice, it is possible to score thousands instead of hundreds of cells per treatment or subject, which leads to a greater accuracy of the test. It is nowadays possible to use automated methods for micronucleus analysis (Decordier et al. 2011; Rossnerova et al. 2011). Furthermore, structural and numerical chromosome mutations are implicated in many human genetic diseases and are involved in the induction or progression of cancer (Kirsch-Volders et al. 2000). On the other hand, since cells need to survive at least one nuclear division for micronucleus formation, some damaged cells may not be detected because they have been lost or unable to divide (Bonassi and Au 2002).

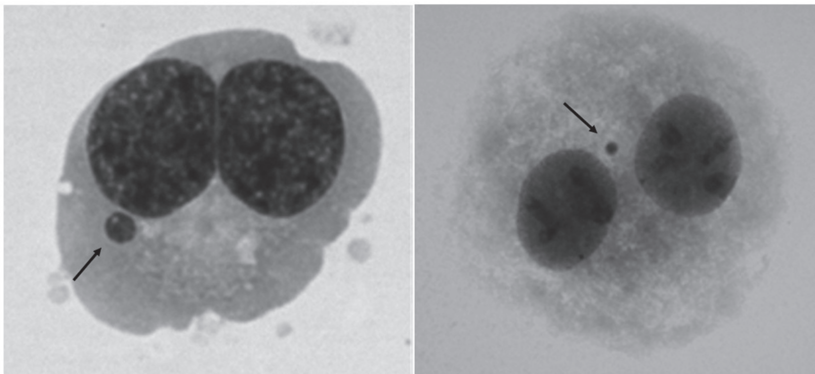

Figure 2. Examples of micronuclei (arrow) in Giemsa-stained binucleate human lymphocytes. The size of the micronuclei varies. Photographs kindly provided by Hilkka Järventaus.

The analysis of MN has been used to quantify exposure to genotoxic chemicals and radiation in a large number of studies. MN can be observed in almost any cell type, and for this reason many variations of the assay exist. Micronucleus analysis is employed in studies with humans and laboratory animals following *in vivo* or *in vitro* exposures (Tucker and Preston 1996). One could say that the use of MN as an indicator of (Kirsch-Volders et al. 2000) chromosomal damage has become a standard assay in both genetic toxicology and human biomonitoring studies.

Although the association between the frequency of MN and long-term biological consequences such as cancer has not fully been elucidated yet, a potential predictive role for this biomarker has been suggested on theoretical grounds (Fenech et al. 1999). Early investigations of micronucleus frequency (MN frequency) and cancer risk suggested no association (Brøgger et al. 1990; Hagmar et al. 1998; Bonassi et al. 2000). However, these data were based on heterogeneous methods for micronucleus detection, young cohorts, and relatively short follow-up. Recent studies, based on a single micronucleus technique (CBMN assay) have indicated that MN frequency may predict overall cancer in a similar way as CA frequency does (Bonassi et al. 2006; Bonassi et al. 2007; Bonassi et al. 2011). The baseline frequency of MN appears to be elevated in untreated cancer patients (Iarmarcovai et al. 2008).

Micronucleus assay in genotoxicity testing

The MN assay was used for the first time *in vitro* in radiation experiments with roots of *Vicia faba* in 1959 (Evans et al. 1959). Micronucleus tests in plants, such as in root tips cells of *Allium cepa* and *Vicia faba* or in pollen mother cells of *Tradescantia* has been used especially in environmental monitoring (Grant 1994; Rodrigues et al. 1997).

In the early seventies, Schmid (1975) used micronucleus analysis in bone marrow erythrocytes of mice as a technique to study the genotoxicity of various exposures *in vivo*. Since 1983, the erythrocyte micronucleus test (MN test) has been included in OECD test guidelines and is the most frequently used *in vivo* assay for routine genotoxicity screening of chemicals (OECD 1997). Depending on exposing agent, the erythrocyte MN test is indicative of exposure during the last 24–48 h in bone marrow and 36–72 h in peripheral blood.

Countryman and Heddle (1976) were the first to introduce a MN assay for cultured human cells. The *in vitro* MN test has been validated for genotoxicity testing (Corvi et al. 2008), and a guideline on "*In vitro* Mammalian Cell Micronucleus Test" was recently adopted among OECD test guidelines (OECD TG 487) (OECD 2010). The application of the CBMN assay for *in vitro* genotoxicity testing has been discussed extensively (Kirsch-Volders, Elhajouji et al. 1997; Kirsch-Volders, Sofuni et al. 2000; Kirsch-Volders, Sofuni et al. 2003; Corvi, Albertini et al. 2008).

Micronuclei in biomonitoring studies

The employment of the MN test for human biomonitoring studies was first described by Meretoja and Vainio (1979) who examined MN in ordinary cytogenetic preparations of cultured lymphocytes of styrene-exposed subjects. A few years later, Stich and co-workers (Stich et al. 1982) proposed that MN could be used to study genotoxic effects in all human tissues from which it is possible to obtain exfoliated cells. Since then, investigations with cells from buccal and nasal mucosa, esophagus, bronchi, urinary bladder, and cervix have been performed (Majer et al. 2001; Huvinen et al. 2002; Thomas et al. 2011). Most studies on exfoliated epithelial cells have though utilized buccal and nasal mucosa (Sarto et al. 1987; Lucero et al. 2000).

Nevertheless, the most common cell types examined for MN are those of the haematopoietic system, especially peripheral blood cells, because they are readily attainable in humans. The examination of bone marrow and other human internal tissues is technically possible but, because of the invasive sampling, not commonly utilised (Tucker and Preston 1996). Thus, due to the easy availability of peripheral lymphocytes, they are frequently used in biomonitoring. Peripheral differentiated lymphocytes that circulate everywhere in the body are regarded to imitate exposure in general but are not considered as immediate targets for carcinogenesis. They are in a way examined as a surrogate tissue for other tissues where cancer can develop. This assumption is based on the theory that similar genotoxic damage occurs in cancer-prone tissue and in the circulating peripheral lymphocytes. Lymphocytes may not well illustrate exposure

to substances of which the effect is at the point of entry into the body, for example at the upper airways, or which have special target tissue, e.g., due to organ-specific metabolism (Norppa 2004a).

The last few years have seen a strong increase in the use of the MN assay as a tool to biomonitor human genotoxic exposure and effects. A recent overview covering the last 25 years and reviewing population studies performed utilizing cytogenetic biomarkers (Bonassi et al. 2005), found 434 papers where MN had been used as an endpoint. A wide variety of exposures to chemicals and ionizing radiation were covered in these papers. Organic chemicals were by far the most commonly studied exposure. The most frequently examined compounds of this group were styrene, polyaromatic hydrocarbons (PAHs), benzene, ethylene oxide, and formaldehyde. Among inorganic chemicals chromium, arsenic, lead, and mercury were mostly studied. Several papers also addressed radiation exposure.

Furthermore, occupational exposure of pesticides has been examined in a number of papers. The use of pesticides in agriculture usually involves complex exposure to mixtures of pesticides which may contain genotoxic ingredients. A comprehensive review of the literature dealing with genotoxic effects in human groups exposed to pesticides (Bolognesi 2003) indicated that such a work is often associated with an increase in genotoxic damage depending on the degree and type of exposure. A positive association between occupational exposure to complex pesticide mixtures and an increase in MN, CAs and SCEs has been detected in the majority of studies, although a number of studies also failed to detect cytogenetic damage. An increase in MN frequency in workers with occupational exposure to pesticides was observed by several investigators (Bolognesi et al. 1993a; Bolognesi et al. 1993b; Joksic et al. 1997; Bolognesi et al. 2002; Garaj-Vrhovac and Zeljezic 2002). Others reported negative micronucleus results (Scarpato et al. 1996a; Davies et al. 1998; Venegas et al. 1998; Lucero et al. 2000; Pastor et al. 2003).

The ability to detect both clastogenic and aneugenic effects is an advantage of the micronucleus technique (Kirsch-Volders et al. 1997; Fenech 2000). The distinction between the two phenomena, by identifying the origin of MN, is important, whether micronucleus analysis is used for genotoxicity testing or biomonitoring of genotoxic exposure and effect in humans (Thomson and Perry 1988; Eastmond and Tucker

1989; Becker et al. 1990; Norppa et al. 1993; Norppa et al. 1993). For an optimal sensitivity of the assay, the MN deriving from chromosomal fragments and the whole chromosome-containing MN needs to be identified (Norppa and Falck 2003).

Micronuclei in lymphocytes – methodological aspects

MN are expressed in cycling eukaryotic cells that have completed nuclear division, which makes the MN assay quantitatively unusable in non-dividing cell populations. In order to control the effect of cell division kinetics on MN frequency, development of a method that makes it possible to distinguish non-dividing cells from mitotic (dividing) cells in a cell population was necessary. In the case of cultured lymphocytes, MN should be analyzed from cells that have completed only one *in vitro* division after the *in vivo* exposure studied. The main reason for this is that most induced MN are assumed to be formed in the first division following the genotoxic exposure. Although reappearance of MN in subsequent division has been observed in live cell imaging studies (Huang et al. 2011), it is unclear how frequently this could occur.

The CBMN assay, originally described in 1985 for human lymphocytes by Fenech and Morley (Fenech and Morley 1985; Fenech and Morley 1986) is a method that enables the identification of cells that have divided once. The method has widely been used as a sensitive and reliable technique for assessing chromosome damage in humans (Fenech 2000; Fenech et al. 2003). The assay has been utilized for both monitoring chromosome damage in human populations and *in vitro* testing of genotoxicity (Fenech 1993; Kirsch-Volders 1997; Kirsch-Volders et al. 1997).

In the CBMN assay, cytokinesis is blocked by adding Cyt-B to the cell cultures (Fenech and Morley 1985). Cyt-B, an inhibitor of actin polymerization, inhibits the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis (Carter 1967). In the presence of Cyt-B, the stimulated lymphocytes that have divided are identifiable as binucleate cells which are easily distinguished from mononucleated, non-dividing cells. MN are scored

in binucleated cells only (see Figure 2) and the number of micronucleated binucleate cells (MNBNCs) is assessed. The concentration of Cyt-B should generally be minimized but still allowing a maximum amount of binucleated cells: 6 µg/ml of Cyt-B is recommended for whole-blood lymphocyte cultures and 3–6 µg/ml for cultures of isolated lymphocytes (Kirsch-Volders et al. 2000). At 72 h after initiation of culture and PHA stimulation, optimal culture conditions should give 35–60% or more binucleates as proportion of viable cells (i.e. all cells excluding necrotic and apoptotic cells) (Fenech 2000). The CBMN technique enables comparisons of chromosome damage between cell populations that may differ in their cell division kinetics (Fenech 2000). Technically, binucleate cells can be visualized by the traditional Giemsa staining or with DNA fluorochromes (Norppa et al. 1993; Surralles et al. 1995; Fenech 2000; Norppa and Falck 2003); the fluorescence techniques can be complemented by fluorescence *in situ* hybridization (FISH) (Norppa et al. 1993) or anti-kinetochore staining (Eastmond and Tucker 1989) for the identification of MN contents. The advantages of the two latter methods are clear when the mechanism of MN formation is assessed (Kirsch-Volders et al. 1997; Norppa and Falck 2003).

Another possibility to identify DNA synthesizing cycling (e.g. dividing) cells in culture is to use 5-bromodeoxyuridine (BrdU) labeling of DNA. BrdU is a DNA base analogue BrdU which is incorporated in the place of thymine when the cell divides. The presence of incorporated BrdU can be detected with immunofluorescence or immunocytochemical detection methods (Norppa et al. 1990; Montero-Montoya et al. 1997). BrdU-labeling followed by Giemsa staining (without using BrdU antibodies) has earlier been used for human lymphocytes by Pincu et al. (1984), Fenech et al. (1988), and Brenner et al. (Brenner, Jeffrey et al. 1991). As this technique does not exclusively identify cells that have completed nuclear division, some BrdU-labelled cells may still be in S or G2 phase.

Mechanisms of micronucleus formation

For several years already, it has been well established that several different mechanisms can be involved in the formation of MN (Heddle

et al. 1983). A general assumption is that MN are formed from whole chromosomes or chromosomal fragments that lag behind in anaphase, and that cell division is prerequisite for MN formation. A MN containing chromosome fragments may result from direct double-strand DNA breaks (DSB), conversion of single-strand DNA breaks (SSBs) to DSBs after cell replication, or inhibition of DNA synthesis. Misrepair of DNA lesions in two chromosomes may result in an asymmetrical chromosomal rearrangement forming a dicentric chromosome and an acentric fragment (AF); in cell division, the centromeres of the dicentric chromosomes may be pulled towards opposite poles, forming a nucleoplasmic bridge (NPB) between the daughter nuclei with the AF that laging behind and becoming a micronucleus (Thomas et al. 2003; Fenech 2005; Mateuca et al. 2006; Pampalona et al. 2009). MN may also be created from (multiple) breaks in anaphase bridges originally formed from dicentric chromosomes, concatenated ring chromosomes, union of sister chromatids, unresolved sister chromatid connections, or chromosomes joined by telomere associations (Gisselsson et al. 2000; Hoffelder et al. 2004; Pampalona et al. 2009).

Defects in the chromosome segregation machinery, such as deficiencies in cell cycle controlling genes, spindle disturbances, kinetochore, and dysfunction of other components of the mitotic apparatus, or damage to chromosomal substructures, mechanical disruption (Albertini et al. 2000), and hypomethylation of centromeric DNA (Crott et al. 2001; Fenech 2001; Fenech et al. 2005; Mateuca et al. 2006) are also thought to be the essential events in micronucleus formation.

This may not, however, apply to all situations. MN may also derive from gene amplification through breakage-fusion-bridge cycles when amplified DNA at the nuclear periphery is eliminated by nuclear budding during the S-phase of the cell cycle (Fenech 2002). In tumor cells, e.g. liposarcoma (WDLPS) (Pedeutour et al. 1994) and colorectal carcinoma (COLO 320DM tumor cell line) (Shimizu et al. 1998; Shimizu et al. 2000), MN has been suggested to derive from nuclear buds, without cell division. Using transformed cell lines, Shimizu et al. (Shimizu et al. 1998; Shimizu et al. 2000) also showed that amplified DNA is localized selectively to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MN during the S phase of the cell cycle. Nuclear buds, as potential precursors of MN, are morphologically simi-

lar to MN with the exception that they are joined to the nucleus by a thin nucleoplasmic connection (Fenech and Crott 2002) (see Figure 3). According to the conventional model of micronucleus formation, nuclear buds, may also derive from anaphase laggards that independently form a nuclear envelope before fully integrating into the nucleus (Serrano-Garcia and Montero-Montoya 2001) or from remnants of broken anaphase bridges (Gisselsson et al. 2000; Lindberg et al. 2007; Pampalona et al. 2009).

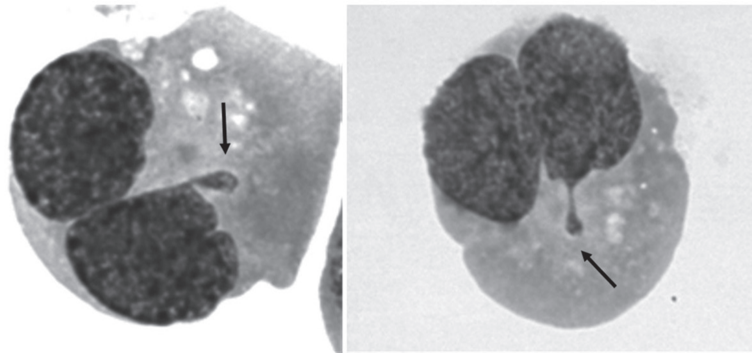

Figure 3. Examples of nuclear buds (arrow) connected by thin treads to the main nuclei in human lymphocytes blocked with Cytochalasin-B. Photgraphs kindly provided by Hilkka Järventaus.

Detection of micronucleus contents

Previously, analysis of MN provided little or no information concerning the type of damage involved in their formation. Some researchers considered that the size of MN could tell about the type of damage. Larger MN were more likely to be caused by spindle damage and to contain whole chromosomes or chromatids, while smaller MN could consist of one or more chromosome fragments (Yamamoto and Kikuchi 1980; Tucker and Preston 1996). MN containing entire chromosomes have also been characterized by C-Banding (Verschaeve et al. 1988; Van Hum-

melen and Kirsch-Volders 1992) and by measurement of DNA content (Vanderkerken et al. 1989; Grawe et al. 1994) of the MN.

One solution to this problem has been to use molecular methods to identify the contents of MN. The techniques are of two general types: those that use antikinetochore antibodies and those that use centromeric DNA probes. Unique sequence DNA probes and probes detecting repetitive DNA for a single chromosome can also be utilized to examine the contents of MN.

Centromere and kinetochore identification has successfully been used in numerous *in vitro* and *in vivo* studies to examine the contents of MN (Norppa et al. 1993; Norppa and Falck 2003). Information on the more detailed composition of MN is limited, but there are clear indications that different chromosomes are micronucleated nonrandomly. Interestingly, several studies have shown that the X chromosome appears in MN of females and the Y in MN of males far more often than expected by chance (Guttenbach et al. 1994; Hando et al. 1994; Richard et al. 1994). It is important to realize that our knowledge of the contents of MN is still scarce. Yet, understanding what MN actually consists of is a basic requirement for the correct use of the MN assay (Norppa and Falck 2003).

Antikinetochore antibodies, deriving from the serum of scleroderma CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly and Telangiectasia) patients (Moroi et al. 1980), bind to chromosomes at the site of spindle fiber attachment, and thus serve as a marker for the centromeric region. The bound antikinetochore antibodies can be detected by immunofluorescence or immunohistochemistry (Hennig et al. 1988; Thomson and Perry 1988; Eastmond and Tucker 1989; Fenech and Morley 1989). MN with a kinetochore signal (K+) are considered to contain whole chromosomes or chromatids and are thus indicative of events that disturb the mitotic apparatus and result in chromosome lagging at anaphase (Hennig et al. 1988; Thomson and Perry 1988; Eastmond and Tucker 1989). Kinetochore-negative MN (K-), on the other hand, indicate clastogenic processes (Eastmond and Tucker 1989).

Centromeric DNA probes have been used to make similar determinations (Becker et al. 1990; Norppa et al. 1993; Titenko-Holland et al. 1994). The occurrence of an entire chromosome (or chromatid) in MN

can be shown by a DNA probe that recognizes complementary centromeric DNA sequences (Ford et al. 1988; Becker et al. 1990; Migliore et al. 1993; Norppa et al. 1993) in a centromere-positive (C+) MN. MN with acentric fragments are not expected to contain centromeric DNA and are centromere-negative (C-).

The two main classes of MN are usually distinguished from each other by DNA probes hybridizing to alpha satellite DNA present on all human chromosomes. Alpha satellite DNA is characterized by a diverged 171-base-pair motif repeated in a tandem fashion (Schueler et al. 2001; Tsuchiya et al. 2001). The pancentromeric DNA probes used for MN characterization have been cloned alphoid probes (Becker et al. 1990; Thierens et al. 1999), oligonucleotides with an alphoid consensus sequence (Norppa et al. 1993; Elhajouji et al. 1995; Darroudi et al. 1996), commercially available probes for all human centromeres (Migliore et al. 1993; Titenko-Holland et al. 1994; Doherty et al. 1996), or alpha satellite probes prepared by polymerase chain reaction (Huber et al. 1996). Fluorescence *in situ* hybridization (FISH) with digoxygenin- or biotin-labeled DNA probes detected by antibodies conjugated with fluorescein or other appropriate fluorescence labels has commonly been used in several studies. Nowadays, DNA probes directly labeled with a fluorochrome are mostly used. Immunohistochemical detection by, e.g., alkaline phosphatase or peroxidase has been used in some papers (Guttenbach et al. 1994; Nardone 1997; Vral et al. 1997). In principle, centromeric DNA can also be detected by primed *in situ* labeling (PRINS) (Russo et al. 1996; Basso and Russo 2000), but this technique appears not to have been applied for human MN.

Schuler et al. (1997) have reviewed the advantages and disadvantages and technical aspects of the CREST and FISH methods in characterizing MN. Fragments with break point at the centromere may form C+ MN if the breakage destroys centromere function. True centric fragments with a complete centromere are expected to be rare in MN of non-cancerous cells, as they should normally be attached to the spindle, unless they lack a functional kinetochore. It is also possible that a MN contains both one or more fragments and one or more chromosome; in this case, the detection of centromeric DNA would only suggest the presence of the latter. In general, MN deviating from the two simple categories (acentric fragment vs. chromosome) should in most cases be relatively rare in non-

cancerous cells. They would either require an inducer with a very specific mode of action or the simultaneous occurrence of two or several (more or less) independent events whose combined probability should be low.

MN formed from entire chromosomes with a disrupted or detached kinetochore may result in MN with no kinetochore signal (Schuler et al. 1997). This appears to be a real problem for the use of kinetochore identification in human biomonitoring and studies of spontaneous MN, since a considerable portion of MN harboring the centromere of X or Y chromosome seem to be kinetochore-negative (K-) (Hando et al. 1994; Nath et al. 1995; Tucker et al. 1996; Hando et al. 1997). Kinetochore defects may be common for the micronucleation of other chromosomes as well, but even the X chromosome alone is expected to give rise to errors in the assessment of MN contents using kinetochore antibodies, since this single chromosome may account for 70% of MN in binucleate lymphocytes of women (Hando et al. 1994; Hando et al. 1997). In practice, centromere and (with some reservation) kinetochore detection can be expected to be accurate enough in distinguishing the two main types of MN in experimental studies, but it appears that centromeric FISH should be recommended for studies of human exposure and spontaneous MN levels, due to the low prevalence of kinetochore label in MN harboring entire sex chromosomes (Norppa and Falck 2003).

Chromosome-specific centromeric DNA probes have been used to identify the presence of specific chromosomes in MN by FISH (Guttenbach and Schmid 1994; Hando et al. 1994; Richard et al. 1994; Catalán et al. 1995; Tucker et al. 1996; Hando et al. 1997; Acar et al. 2001; Bakou et al. 2002). The use of a pancentromeric probe, an X-chromosome-specific centromeric probe, and (in men) a Y-chromosome-specific centromeric probe allows one to estimate the contribution of acentric fragments, X-chromosomes, Y-chromosomes, and autosomes in MN (Cafiero et al. 1989; Catalán et al. 1995; Surralles et al. 1996; Catalán et al. 1998).

The involvement of all human chromosomes in MN has been studied by chromosome painting (Fauth et al. 1998; Fauth and Zankl 1999; Fauth et al. 2000) and spectral karyotyping (Komae et al. 1999; Leach and Jackson-Cook 2001); coupled with centromeric probes this approach can distinguish between MN harboring centric and acentric DNA de-

rived from specific chromosomes (Lindberg et al. 2007; Lindberg et al. 2008; Pacchierotti and Sgura 2008; Attia 2009; Pampalona et al. 2009).

If micronucleation of chromosomes is random, each single human chromosome should be present in 2.17% (1/46) of chromosomes-containing MN. However, studies using the FISH method have shown that different human chromosomes are non-randomly micronucleated. Especially, in cultured human lymphocytes, the increase in centromere-containing micronuclei with ageing has primarily been attributed to micronucleation of the X and Y-chromosomes due to increasing age (Guttenbach et al. 1994; Hando et al. 1994; Richard et al. 1994; Catalán et al. 1995; Zijno et al. 1996; Zijno et al. 1996; Bakou et al. 2002). These findings are supported by age-dependent loss of X and Y chromosomes (Stone and Sandberg 1995; Catalán et al. 1998; Song et al. 2009).

Key factors affecting the baseline level of micronuclei in lymphocytes

MN frequency in lymphocytes is known to be affected by age, gender and multiple dietary and lifestyle factors (Fench and Bonassi 2011). Many studies have shown that chromosomal damage increases progressively with age (Fenech et al. 1994; Ramsey et al. 1995; Bolognesi et al. 1997; Bolognesi et al. 1997; Wojda and Witt 2003). The reasons for the increase are unclear. In addition to age, several other factors may affect the baseline level of chromosome alterations. By understanding the contribution of biological variation, diet, lifestyle, and genetic background to spontaneous chromosomal damage rate, it would presumably be easier to detect the effect of environmental exposures (Fenech 1998).

Biological variation

It is important to recognize the variables that can affect the frequency of MN when selecting donors for a study on MN in an occupationally exposed group. It is known that biological factors such as age, gender, and genetic background can influence the baseline frequency of MN. Lower spontaneous MN frequencies are found in healthy, nonsmoking

young adults (Kirsch-Volders et al. 2000). A twin study indicated more similar MN frequencies in monozygotic twins than dizygotic twins, indicating a strong influence of genetic factors have on baseline MN frequencies (Surowy et al. 2011).

The most consistent biological variables influencing the micronucleus index (MN index) in lymphocytes are gender and age. According to Fenech (Fenech 1998), MN frequencies are generally 1.2 to 1.6 times higher in females than in males. Furthermore, MN frequencies increase steadily and significantly with age in both sexes, but the linear regression slope is steeper in females (Fenech 1998). In order to verify the influence of age, gender and several other variables (e.g. experimental factors) affecting the observed micronucleated cell (MNC) frequency in experiments performed with the CBMN technique, a database of almost 7000 subjects, from 25 laboratories from 16 several countries was analyzed in context with the HUMN project (HUman MicroNucleus project, on the web: http://ehs.sph.berkeley.edu/holland/humn/; Fenech et al. 2011). An increase in MNC frequency with age was evident in almost all laboratories. The effect of gender was also present - females had higher levels of MNCs (19%). The analysis of the pooled data from the laboratories using the CBMN assay indicated that MNC frequency increases with age in both genders, with the steepest increase after 30 years of age, and to a higher level in females (Bonassi et al. 2001).

Besides age, there may be other sources of inter-individual and intra-individual variation over time. In most studies, only one MN measurement is performed for each individual. It can be argued how well MN level obtained from a single measurement can reflect the overall genetic damage rate for the individual, because it is not clear if the MN index varies with season. Investigations for CAs have suggested that chromosome damage can be influenced by season (Anderson et al. 1991). It has been suggested that CA and MN frequencies fluctuate over time depending on the exposure pattern of the subjects (see Bonassi et al. 2005). This might particularly concern chromatid-type CAs and MN derived from CAs that arise *ex vivo* (when the lymphocytes are cultured) from DNA damage pre-existing in the peripheral lymphocytes (Albertini et al. 2000). However, MN determinations repeated every three months showed a strong correlation between the repeat measurements and no significant seasonal effect (Fenech 1998). In principle, many of

the MN visible in cultured lymphocytes are expected to be formed in the first *in vitro* division. It was recently reported that cancer patients who had received varying regimens of cytostatics, including aneugens, showed a clearer increase in micronucleted binucleate and mononucleate lymphocytes after 72 h of culture than in mononucleate cells after 24 or 48 h of culture (Arsoy et al. 2009). Most of the induced MN were, however, large and contained several C+ signals. These findings appeared to suggest that the therapy-induced MN were primarily formed ex vivo from persistent damage through a complex mechanism.

Individuals may differ in their sensitivity to Cyt-B and to the concentration of Cyt-B used which is usually between 3 and 6 μl/ml. Reports from different laboratories also show that binucleate cell frequencies vary considerably, which may reflect both biological and technical variation. Much of this variability is probably due to inter-individual variation in lymphocyte cultures as concerns mitogen stimulation and cell growth (Bonassi et al. 2001); age is one of the key variables associated with reduced number of dividing cells in lymphocyte cultures.

Dietary variables

Diet is a presumptive key factor in determining genomic stability and probably more important than has been believed before. Nutrition antioxidants are also believed to play an important role in cellular antioxidative defence. The impact of diet and vitamin status on MN index has been examined in a couple of cross-sectional and randomized placebo-controlled intervention studies (Fenech 1996). When vegetarians/vegans and non-vegetarians were compared, no significant difference in the age-dependent slope of MN index between these groups was detected (Fenech and Rinaldi 1994). In men, MN index was significantly lower in the 20–40 years age group among non-vegetarians than in the 41–60 years age group among vegetarians.

The main nutritional factors influencing MN index in persons who were not folate deficient were the levels of vitamin B12 and homocysteine in the plasma (Fenech 1996). Folate is an essential B-vitamin that occurs naturally in miscellaneous food, such as broccoli, cabbage, cauliflower, fruits and nuts. Mammals are unable to synthesize folate de novo and are, therefore, obliged to receive it from dietary sources (Beetstra et al.

2005). Folate plays a critical role in the prevention of chromosome breakage and hypomethylation of DNA (Fenech and Ferguson 2001; Wang and Fenech 2003; Fenech 2005). Both *in vitro* and *in vivo* studies with human cells show that folate-, and vitamin B12-deficiency as well as elevated plasma homocysteine are associated with expression of chromosomal fragile sites, chromosome breaks, excessive uracil in DNA, MN formation, and DNA hypomethylation (Jacky et al. 1983; Everson et al. 1988; Blount and Ames 1995; Blount et al. 1997; Duthie and Hawdon 1998; Fenech 1998; Titenko-Holland et al. 1998; Ames 1999; Crott et al. 2001; Fenech 2002; Fenech 2002; Smolkova et al. 2004; Lindberg et al. 2007). Furthermore, DNA breaks in human cells are minimized when folic acid concentration in culture medium is greater than 180 nmol/l (80 ng/ml) (Jacky et al. 1983; Duthie and Hawdon 1998). The exact mechanisms by which low folate influences micronucleation rate is not known, but it probably occurs through increased incorporation of uracil into DNA or hypomethylation of DNA. It appears that folate deficiency increases MN harboring chromosomal fragments and MN harboring whole chromosomes (Lindberg et al. 2007). Besides low folate, low intake of calcium, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin were associated with increased frequencies of lymphocyte MN (Fenech et al. 2005). Intervention studies suggest that multiple micronutrient combinations are more effective (at normal levels of folate and B12) than individual micronutrients in reducing MN frequency (Thomas et al. 2011).

It can be expected that diet containing natural mutagens or those generated in the processing of food (e.g., heterocyclic amines) results in increased genotoxic damage that could possibly also be seen in the MN level. However, in mice, 3–6-month consumption of cooked meat did not increase the frequency of MN in spleen lymphocytes (Fenech and Neville 1993). Recent data suggest that the non-alcoholic fraction of red wine decreases gamma-radiation-induced micronuclei in human lymphocytes, apparently by protecting DNA from oxidative damage (Greenrod et al. 2005).

Experimental variation

One important factor assumed to affect the baseline level of MN is experimental variation. There seems to be some inter-laboratory variation in baseline MN frequencies, which could reflect methodological differences in cell culture techniques, specimen preparation, and staining among the laboratories. For instance, most laboratories use RPMI 1640 medium (Surralles and Natarajan 1997) in their experiments, while some groups use modified McCoy's culture medium. These culture media differ in composition; particularly with respect to some vitamins (Ascorbic acid, Folic acid and Vitamin B12, which are present at higher concentrations in McCoy's medium). However, scoring of MN in 23 healthy persons, cultured in the two different types of media did not reveal any significant effect of culture media on the baseline MN frequencies (Fenech 1998).

Slide preparation and staining of cells can be performed in several ways. Since the quality of the slide preparation and staining influences the analysis, it is important to use the most accurate methods. In fixation and slide preparation, the cytoplasm and the boundaries of the cells should be preserved and cell clumping minimized (Kirsch-Volders et al. 2000). Several staining methods have been used but fluorescence DNA dyes may be preferred (Kirsch-Volders et al. 2000), since they enable detection of very small MN more easily than for instance Giemsa (Surralles et al. 1995).

Many studies have shown that both sampling error and error in scoring the slides is a major source of variation for the MN assay. These errors can be reduced by allowing only experienced scorers to do the work. An important contributor to the scoring error is the inter-scorer variability. The difference between scorers has been noted to vary by a factor of 1.6 (Fenech 1998). Inter-scorer variability is naturally avoided if only one scorer is involved. If several scorers are used, the impact of inter-scorer variability can be minimized by each scorer analyzing the same number of (different) cells from all samples (the coefficient of variation among the scorers can be reported). Within an experiment, variation between cultures is usually reduced by including duplicate or triplicate cultures (Fenech 1998). Automation of MN analysis is expected to reduce variation in MN scoring (Decordier et al. 2011; Rossnerova et al. 2011).

Aneuploidy in somatic cells

An unequal distribution of the sister chromatids during the division of cell nuclei (as well as during mitosis and meiosis) produces aneuploid daughter cells (Cimini et al. 2001). Aneuploidy can thus be defined as a deviation from the normal (euploid) chromosome number of a cell or an individual. Euploidy can be described as the condition of having a normal number of structurally normal chromosomes. In humans, the normal chromosome number is 46 which consist of 44 autosomes and two sex chromosomes. Thus, euploid human females have two X chromosomes and males an X and a Y chromosome. To maintain genome integrity accurate chromosome segregation during mitosis and meiosis is crucial. An unequal distribution of chromosomes leads to a gain or loss of chromosomes in the daughter cells forming aneuploid cells (Hegarat et al. 2005).

Aneuploidy can be induced by different mechanisms: 1) failure of chromosome attachment to the mitotic spindle, 2) monotelic attachment (single kinetochore attachment to one pole), 3) merotelic attachment (single kinetochore attachment to both poles), 4) syntelic attachment (both sister kinetochores attach and migrates to the same mitotic pole) and 5) premature sister chromatid separation (Cimini et al. 2001; Cimini et al. 2003; Salmon et al. 2005). Aneuploid cells with hypoploid (loss) or hyperploid (gain) chromosome numbers arise when chromosomes fail to segregate properly during mitosis. Several studies have shown a significant increase in chromosome loss, primarily of the sex chromosomes, in peripheral blood lymphocytes and skin fibroblasts in both males and females of advanced age (Jacobs et al. 1961; Fitzgerald and McEwan 1977; Richard et al. 1993; Stone and Sandberg 1995). Some other studies have, however, suggested that the frequency of autosomal loss (hypoploidy) and autosomal gain (hyperploidy) is not necessarily a function of age or gender (Nowinski et al. 1990; Hando et al. 1994). Aneuploidy is the most frequently observed type of cytogenetic abnormality. It can be induced by chemical action upon the cell. The cellular targets can be those involved in cell division as well as the chromosomes themselves (Parry and Parry 1989). Many known chemical aneuploidogens (or aneugens) exist (Parry et al. 1996; Kirsch-Volders et al. 2002), and aneugenic effects are used as a criterion in carcinogen classification (Bolt et al. 2004).

Incorrect segregation of the chromosomes into the daughter cells is also a major cause of human reproductive failure and an important contributor to cancer. The two major causes of aneuploidy are chromosome loss and non-disjunction. The different mechanisms leading to chromosome loss and non-disjunction can be investigated by the analysis of morphological and structural changes in the cell division apparatus by the application of specific stains and antibodies for various cell division components (Parry et al. 2002). Non-disjunction of chromosomes at anaphase gives a trisomic daughter nucleus and a monosomic daughter nucleus. On the other hand, chromosome loss from the nucleus during cell division produces a monosomic daughter nucleus and a normal daughter nucleus. In the latter case, the lost chromosome may form a MN that can be cytogenetically detected. The micronucleus may also be randomly re-incorporated into either one of the daughter nuclei producing trisomic and monosomic daughter cells (Parry et al. 2002) or be dispelled from the cell.

Aneuploid lymphocytes increase with age. In women this mainly concerns the loss of the X chromosome (Jacobs et al. 1961; Fitzgerald and McEwan 1977; Richard et al. 1993).

Detection of aneuploidy in interphase nuclei

By the application of FISH with chromosome-specific centromeric DNA probes, direct analysis of aneuploidy in a large numbers of interphase nuclei can be performed. This approach can in principle be applied to any type of human cells, and the cells do not need to be cycling at the moment of the analysis to reflect chromosome loss or gain that have occurred when the cells divided. In mononucleate cells, it cannot usually be judged if the aneuploidy observed derived from non-disjunction or chromosome loss and if it occurred during the previous or an earlier division. However, if cultured cells are studied, the cytokinesis-block technique can be used. In binucleated cells, both of the daughter nuclei are retained, and the probable origin of a specific aneuploidy can be assessed (Eastmond and Pinkel 1990; Eastmond et al. 1995; Zijno et al. 1996). In many studies, this technique has been used to determine the frequency of aneuploidy as a function of age, cancer, folate deficiency, or *in vitro* treatments with suspected or known aneuploidogens (Eastmond

and Pinkel 1990; Eastmond et al. 1995; Zijno et al. 1996; Ramirez et al. 1997; Carere et al. 1999; Migliore et al. 1999; Leopardi et al. 2002; Wang et al. 2004; Camps et al. 2005).

Metabolic polymorphisms of importance in biomonitoring

A multitude of enzymes, capable of using foreign chemicals (xenobiotics) as substrates, carry out xenobiotic metabolism *in vivo*, leading to either decreased or increased toxicity. Most xenobiotics that enter the body are lipophilic, which enables them to penetrate lipid membranes and to be transported by lipoproteins in the blood. Xenobiotic metabolism consists of two phases. In phase one; a polar reactive group is introduced into the molecule, forming a suitable substrate for the phase-two enzymes. Cytochrome P-450 (CYP) monooxygenases are typical phase-one enzymes responsible for the metabolic activation of a number of indirect carcinogens. The phase-two enzymes bring about the conjugations of xenobiotics to various endogenous substrates such as sugars, amino acids, and so on, forming water-soluble products that are more easily excreted. In general, this process is a detoxification sequence, even if in some cases reactive intermediates may be formed that are much more toxic than the parent compound (Hodgson 1997).

Many xenobiotic-metabolizing enzymes (XMEs) functioning in the metabolic activation or detoxification of carcinogens have been shown to be polymorphic due to single nucleotide changes or other genetic alterations in the genes coding for the enzymes, resulting in, e.g., altered enzyme activity or stability. Several of such genetic polymorphisms have been associated with cancer risk (Bonassi and Au 2002; Au and Salama 2005; McIlwain et al. 2006; Agundez 2008; Dong et al. 2008), and some of them also appear to influence the level of cytogenetic biomarkers (Norppa 2004b; Iarmarcovai et al. 2008).

Glutathione S-transferases

GSTs are (usually) detoxification enzymes which protect against electrophiles and products of oxidative stress by conjugation with glutathione (Ketterer 1988; Hayes and Pulford 1995; Strange et al. 1998; Hirvonen 1999). The GST super family consists of at least eight different classes of various soluble enzymes (α, μ, π, σ, θ, κ, ω and ζ) with broad and overlapping substrate specificity, many of them known to be genetically polymorphic (Landi 2000).

In this thesis, the *GSTM1* (mu family) and *GSTT1* (theta family) polymorphisms are considered. They are both gene deletion (null alleles) polymorphisms (Hirvonen 1999; Norppa 2003; Parl 2005; McIlwain et al. 2006). The genotype with the homozygous deletion of the *GST* gene is called *GST* null, whereas the genotype having at least one copy of the gene is called *GST* positive. Almost all studies available on *GSTM1* and *GSTT1* polymorphisms have been based on genotyping techniques differentiating between the homozygous null genotype and the positive genotype (including both wild-type homozygotes and heterozygotes). More comprehensive *GSTM1* and *GSTT1* genotype assays, in which distinction between the various wild-type and null alleles is made, have also been described (Sprenger et al. 2000; Roodi et al. 2004; Parl 2005). There appears to be ethnic differences in the distribution of the *GSTM1* and *GSTT1* genotype frequencies (Landi 2000). In Caucasians, the *GSTM1* and *GSTT1* genes are lacking from about 50% (Hirvonen 1999; Garte et al. 2001) and 13–28% of the population, respectively (Pemble et al. 1994; Hirvonen 1999; Garte et al. 2001). Among the Asian population the frequency of the *GSTT1* null genotype is highest among the Chinese (64.4%) followed nearby by the Koreans (60.2%) (Nelson et al. 1995). The frequency of the *GSTM1* null genotype is around 50% among Asians, and a little bit lower (30%) among the African population (Dandara et al. 2002).

GSTM1 enzyme activity is found in various human tissues (including leukocytes and erythrocytes) in *GSTM1* positive individuals, who have at least one functional allele, but not in *GSTM1* null individuals homozygous for the gene deletion (Seidegard and Pero 1988; Seidegard et al. 1988; Wiencke et al. 1990). GSTM1 has an important role in the deactivation of carcinogenic intermediates of polycyclic aromatic hydro-

carbons (PAHs) (Bolt and Thier 2006). As the *GSTM1* null genotype is common and expected to increase individual sensitivity to many carcinogens, the effect of *GSTM1* polymorphisms on the risk of various types of human cancer has extensively been studied (Parl 2005; McIlwain et al. 2006; Dong et al. 2008). Although the results are somewhat conflicting, the *GSTM1* null genotype has been associated with an increased risk of lung, bladder, prostate, and head and neck cancer, and acute leukemia (Brockmoller et al. 1994; McWilliams et al. 1995; Rebbeck 1997; Strange et al. 1998; Hirvonen 1999; Houlston 1999; Strange and Fryer 1999; Benhamou et al. 2002; Engel et al. 2002; Hashibe et al. 2003; Parl 2005; Ye and Song 2005; McIlwain et al. 2006; Dong et al. 2008; Hiyama et al. 2008; Lacko et al. 2009; Mo et al. 2009). *GSTM* null genotype has been suggested to increase the incidence of lung cancer among nonsmokers exposed to environmental tobacco smoke (Bennett et al. 1999). A recent review systematically examined the results of 161 meta-analyses and pooled analyses for various genetic polymorphisms and cancer risk published through March 2008 (Dong et al. 2008). Assuming a very low prior probability (0.000001) and statistical power to detect an odds ratio of 1.2, the only associations remaining noteworthy were between *GSTM1* null and risk of bladder cancer and acute leukemia. For lung cancer, seven meta-analyses and pooled analyses have consistently found a modest but statistically significant increase in risk for persons with the *GSTM1* null genotype, with summary odds ratios ranging from 1.17 to 1.54 (Carlsten et al. 2008). The latest meta-analysis of 98 studies showed an odds ratio of 1.22 (95% confidence interval = 1.14, 1.30) when all studies were considered, but no association was seen when only the largest five studies were included; furthermore, the *GSTM1* null status conferred a significantly increased risk of lung cancer to East Asians but not to Caucasians (Carlsten et al. 2008).

Meta-analyses and pooled analyses were not, however, able to demostrate a clear association between *GSTM1* genotype, smoking, and lung or bladder cancer (Benhamou et al. 2002; Engel et al. 2002; Taioli and Raimondi 2005; Vineis et al. 2007; Carlsten et al. 2008) Vineis et al (2007) observed a cumulative effect of the combination of the expected at risk genotypes for genes involved in metabolism of tobacco-related carcinogens (*GSTM1*, *GSTT1* and *CYP1A1*); the risk of lung cancer increased with the combination *GSTM1* null, *GSTT1* null, and *CYP1A1*

2B or 4 alleles both in smokers and nonsmokers. For bladder cancer risk, no multiplicative interaction between the *GSTM1* null genotype and cigarette smoking could be demonstrated, but an additive interaction was seen (Engel et al. 2002). The *GSTM1* null genotype has been associated with increased risk of esophageal and gastric cancers in smokers (Huang 2004; La Torre et al. 2005).

Reviews have indicated that the risk of breast cancer for Caucasian women with the *GSTM1* positive genotype is higher compared with subjects lacking the GSTM1 enzyme (Roodi et al. 2004; Parl 2005).

The null genotype of the *GSTT1* gene shows no GSTT1 activity (Pemble et al. 1994). *GSTT1* positive individuals have at least one undeleted allele. GSTT1-mediated glutathione conjugation detoxifies, e.g., epoxide metabolites of butadiene and monohalomethanes, cytostatic drugs, hydrocarbons and halogenated hydrocarbons (Schroder et al. 1992; Norppa et al. 1995; Wiencke et al. 1995; Bolt and Thier 2006), but may also be part of a metabolic activation route for both groups of chemicals. The GST class theta is highly expressed in human adult liver (Mera et al. 1994; Juronen et al. 1996). Actually, liver and kidney are the organs that express the highest levels of GST theta in the human body (Landi 2000). GSTT1 is a particularly interesting susceptibility factor in studies assessing effect biomarkers examined from blood, since it is expressed in erythrocytes and could thus affect the local metabolism of its substrates (Schroder et al. 1992; Norppa et al. 1995). Nevertheless, no GSTT1 activity in lymphocytes has been detected (Juronen et al. 1996). The *GSTT1* null genotype has been connected with increased risk of lung cancer, colorectal cancer, meningioma, head and neck cancer, and acute leukemia (Hashibe et al. 2003; Ye et al. 2004; Chen et al. 2005; Lai et al. 2005; Taioli and Raimondi 2005; Ye and Song 2005; Lacko et al. 2009; Liao et al. 2009). Raimondi et al. (2009) observed a non-significant positive interaction between the *GSTT1* null genotype and smoking for colorectal adenoma risk.

N-acetyltransferases

In addition to glutathione conjugation, reactions catalyzed by N-acetyltransferases (NATs) are important metabolic pathways of xenobiotics in humans (Hirvonen 1999; Hein 2000). Cytosolic NATs metabolize

xenobiotics with primary amine and hydrazine structures, including carcinogens such as 2-aminofluorene, benzidine, 4-aminobiphenyl, 4,4-chloroaniline, and 2-naphtylamine either by activation (O-acetylation) or detoxification (*N*-acetylation) (Blum et al. 1990; Vatsis et al. 1995; Hirvonen 1999). In humans, two genes, *NAT1* and *NAT2*, are involved in the *N*-acetylation of xenobiotics (Kadlubar et al. 1992). The NAT2 enzyme is the predominant form in the liver, while the NAT1 enzyme dominates in other tissues. Both enzymes are also polymorphic.

In this thesis, only the *NAT2* gene is considered. NAT2 is a polymorphic enzyme involved in the metabolism of arylamines. Individuals can be classified as either slow or rapid NAT2 acetylators. Several defective *NAT2* variant alleles have been described (Vatsis et al. 1995; Grant et al. 1997; Hein 2000). The *NAT2* slow acetylator genotype is conferred by any combinations of several mutant alleles (Vatsis et al. 1995; Grant et al. 1997; Hein 2000). 50–63% of Caucasians have two *NAT2* variant alleles with reduced enzyme activity (slow acetylators). Rapid acetylators are either homo- or heterozygous for the rapid (“wild-type”) allele (see (Hirvonen 1999); Japanese and Chinese populations are mostly (92% and 80%) rapid acetylators.

NAT2 rapid acetylators have been noted to have an increased risk of colon cancer, whereas slow acetylators have been described to have a higher risk of bladder cancer (Marcus et al. 2000; Marcus et al. 2000; Hein 2002; Chen et al. 2005; Struewing et al. 2005; Dong et al. 2008). Dong et al. (2008) who evaluated 161 pooled and meta-analyses on genetic polymorphisms and cancer risk, considered the influence of *NAT2* slow acetylator status on bladder cancer risk (odds ratio 1.46; 95% confidence interval, 1.26–1.68) among the four most noteworthy associations. There is evidence for a multiplicative interaction between smoking and *NAT2* slow genotype in determining bladder cancer risk (Marcus et al. 2000). The increased risk of colorectal cancer among *NAT2* rapid acetylators has been associated with smoking and red meat intake (Chan et al. 2005). In addition, the *NAT2* rapid acetylator genotype has been reported to increase the risk of lung cancer among Taiwanese women exposed to cooking oil fumes (Chiou et al. 2005), bladder cancer among benzidine-exposed workers (Carreon et al. 2006), and hepatocellular carcinoma among red meat consumers (Huang et al. 2003). *NAT2* polymorphisms have also been associated to malignant mesothelioma

development, even if the results have been contradictory (Gemignani et al. 2009). In a recent study, the *NAT2* fast acetylator genotype also showed an increased risk, although not significant, for malignant mesothelioma in asbestos-exposed subjects as well as in the entire studied population (Betti et al. 2009).

The influence of *GSTM1*, *GSTT1* and *NAT2* genotypes on cytogenetic biomarkers

Many studies have been conducted to clarify the possible influence of the presence or absence of a specific genotype both on the basal and induced level of cytogenetic biomarkers. Several of them failed to find an association between *GSTM1* genotype and the frequencies of CA's, SCEs or MN in nonexposed control persons (Norppa 2004b; Iarmarcovai et al. 2008). Nevertheless, the lack of glutathione S-transferase M1 *GSTM1* null genotype has been associated with increased sensitivity to genotoxicity of tobacco smoke. *GSTM1* null smokers also showed an increased frequency of CAs (Scarpato et al. 1997; Norppa 2001; Norppa 2004b; Tuimala et al. 2004) and SCEs (van Poppel et al. 1992; van Poppel et al. 1993) in their lymphocytes and benzo[a]pyrene diol epoxide adducts in lung cell DNA (Alexandrov et al. 2002). *GSTM1* null bus drivers, all nonsmoking, exposed to polluted city air had a higher frequency of lymphocyte CAs than *GSTM1* positive drivers, which may indicate sensitivity to the genotoxic effects of PAH-containing air pollutants in general (Knudsen et al. 1999). In a group of coke oven workers, MNBNC frequency was found to be lower in *GSTM1* positive subjects compared with the *GSTM1* null genotype, even if the difference did not reach significance (P = 0.12) (Leng et al. 2004). However, an association of *GSTM1* null genotype with lower baseline MN frequency was detected by Leopardi et al. (2003) (Leopardi et al. 2003; Iarmarcovai et al. 2006; Perez-Cadahia et al. 2008), and (for C- MN) (Iarmarcovai et al. 2006). Similarly, a possible protective role of *GSTM1* null genotype was found in arsenic-induced toxicity (skin lesions) through drinking water (Ghosh et al. 2006).

The *GSTT1* null genotype has been associated with an increased level of SCEs in lymphocytes, with no association to smoking or other

known exposure (Norppa et al. 1995; Schroder et al. 1995; Wiencke et al. 1995; Norppa 2001; Laffon et al. 2002a,b; Norppa 2004b). A couple of studies also suggest that the *GSTT1* null genotype is associated with higher CA level in lymphocytes (Landi et al. 1998; Sram et al. 1998; Sram et al. 2004; Tuimala et al. 2004). Induction of CAs and MN by diepoxybutane (DEB) was also seen to be higher in whole blood cultures of *GSTT1* null than *GSTT1* positive subjects (Landi et al. 1998; Vlachodimitropoulos et al. 1998). In a study performed on styrene-exposed workers, *GSTT1* null genotype was associated with higher frequencies of micronucleated lymphocytes (Migliore et al. 2006). In farmers exposed to pesticides, the *GSTT1* positive genotype was associated with increased frequencies of MN and SCE as compared with controls (Costa et al. 2006). However, most of the studies failed to show a significant effect of *GSTT1* genotype on the baseline level of MN (Laffon et al. 2002a,b; Leopardi et al. 2003; Godderis et al. 2004; Ishikawa et al. 2004; Leng et al. 2004; Silva Mdo et al. 2004; Teixeira et al. 2004; Migliore et al. 2006; Perez-Cadahia et al. 2008).

Several studies have indicated increased *in vitro* sensitivity to various genotoxins in blood cultures of *GSTM1* null and *GSTT1* null subjects. A good example is the 1,2:3,4-DEB sensitivity of *GSTT1* null individuals (Norppa 2001). DEB is an efficient inducer of SCEs (Wiencke et al. 1991), and this induction is much higher in whole-blood lymphocyte cultures of *GSTT1* null than positive subjects (Norppa et al. 1995; Wiencke et al. 1995; Norppa 2001). Induction of CAs and MN by DEB was also seen to be higher in whole blood cultures of *GSTT1* null than *GSTT1* positive subjects (Landi et al. 1998; Vlachodimitropoulos et al. 1998).

The possible effect of *NAT2* genotype on the basal level of chromosome damage has been evaluated in several studies. *NAT2* slow acetylation genotype seems to elevate the basal level of CAs and SCEs (Norppa 2001; Norppa 2004). The reason for this is probably the reduced capacity to detoxify some endogenous genotoxins. High baseline level of lymphocyte CAs has been associated with the *NAT2* slow acetylation genotype with no association to exposure (Knudsen et al. 1999; Norppa 2001), regardless of smoking (Norppa 2004). A slight increase in chromosome damage was also found in *NAT2* slow acetylator floriculturists with low levels of exposure (Scarpato et al. 1996b). On the other hand, Hernández et al.

(Hernandez et al. 2006) obtained results that indicated that rapid (or intermediate) acetylators had significantly more MNBNCs (P = 0.032) than slow acetylators after radioiodine treatment. The basal levels of MN in the same study were not significantly different between *NAT2* slow and *NAT* rapid genotypes.

No association of biomarker response (i.e. formation of CAs, SCEs and MN) with regard to *GSTM1* and *GSTT1* genotypes was observed in a study of automobile painters (Testa et al. 2005). Leng et al (2004) neither detected any influence of *GSTT1* and *NAT2* genotypes on MNBNC frequencies among coke-oven workers. In a recent review, *GSTM1*, *GSTT1* or *NAT2* genotypes did not clearly come up as variables affecting MN frequency (Dhillon et al. 2011). However, polymorphisms in *XRCC1* (X-ray repair complementing defective repair in Chinese hamster cells 1), *ERCC2* (excision repair cross-complementing rodent repair deficiency, complementation group 2), *CYP2E1* (cytochrome P-450 2E1) and *MTR* (methionine synthase) were consistently associated with MN formation.

AIMS OF THE STUDY

The main goal of this study was to examine the applicability of the lymphocyte MN assay as a biomarker in occupational genotoxic exposure. In order to optimize the sensitivity of the methods in detecting occupational exposure, it was necessary to clarify some methodological aspects and factors affecting the baseline level and the mechanistic origin of micronuclei.

The detailed aims were:

1. To differentiate MN containing fragments and whole chromosomes, in order to estimate if these two classes of MN should separately be assessed.
2. To examine the influence of cell culture time and use of Cyt-B on the frequency and contents of MN.
3. To evaluate the suitability of uncultured T-lymphocytes in MN analysis.
4. To assess the involvement of sex chromosomes in micronucleation and chromosome malsegregation.
5. To investigate the formation of MN from chromosomes and fragments lagging behind in anaphase, with a special reference to the X chromosome and use of Cyt-B.
6. To survey the suitability of BrdU labeling in analyzing MN as an alternative method to the cytokinesis-block technique.
7. To evaluate the effect of occupational pesticide exposure on lymphocyte MN frequency and the possible influence of the genetic polymorphisms of glutathione S-transferases M1 (*GSTM1*) and T1 (*GSTT1*), and N-acetyltransferase 2 (*NAT2*).

MATERIALS AND METHODS

Study subjects

Five healthy non-smoking male donors (age range 41–50 years) were examined in Paper I, and five healthy non-smoking women (47–60 years of age) were chosen as the blood donors of Paper II. The third study (Paper III) was based on two sets of experiments. For the first one, five non-smoking women, aged 47–60 years, were included. The second set was performed with four women and 10 men divided in two age groups (<30 years and >50 years). In the studies IV and V (Papers IV and V), lymphocytes from a 62-year-old woman were examined.

In the last paper the study population consisted of 34 pesticide-exposed greenhouse workers, 20 men and 14 women, employed in the production of flowers in Tuscany, Italy (Paper VI). The control group contained 33 healthy unexposed persons, 17 men and 16 women, from the same geographical region. At the time of drawing samples for the cytogenetic determination, a personal history questionnaire was filled-in. The questionnaire covered standard demographic questions as well as medical, lifestyle, and occupational questions. For the exposed group, a further questionnaire was completed including specific questions related to farming: pesticide application, use of protective measures, etc. The individuals examined included non-smokers, smokers, and ex-smokers. Table 1 in Paper VI shows the main characteristics of both groups.

Methods

Lymphocyte separation, cell culture, and slide preparation

In all studies, lymphocytes were cultured in tissue culture tubes containing RPMI 1640 medium with L-glutamine (Gibco Life Technologies, Paisley, UK) supplemented with 15% fetal calf serum (Gibco, UK), 1% phytohaemagglutinin (PHA; Murex Diagnostics, UK), 1% L-glutamine (200 mM; Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin solution (10 000 IU/ml penicillin, 10 000 μg/ml streptomycin; Gibco, UK) in 5% CO_2 atmosphere. Each experimental point was represented by duplicate cultures from each blood donor.

In Paper I, mononuclear cells were isolated from whole blood as described earlier (Lindholm et al. 1991; Norppa et al. 1993), and 5-ml cultures were established at a mononuclear cell density of 1.5–2.0 x 10^5/ml. The cells were cultured for total culture times of 52, 64 and 76 h. The cell specimens were prepared using a cytocentrifuge (Shandon, Cytospin 2, UK). The slides were fixed in absolute ethanol at -20 °C and were kept at -20 °C until *in situ* hybridization (Paper I).

In Paper II and in the first set of experiments in Paper III, purified T-lymphocytes were used. T-lymphocytes (CD3-positive cells) were immunomagnetically sorted from both cultured and uncultured cell suspensions by using the miniMACS (magnetic activated cell sorting) system (Miltenyi Biotec) as explained in Papers II and III. The 72-h cultures were established, with and without Cyt-B, as previously described (Norppa et al. 1993; Surralles et al. 1995). In the second set of Paper III, isolated mononuclear cells cultured for 65 h were used, with and without Cyt-B. The cell specimens were prepared using a cytocentrifuge (Shandon, Cytospin 2, UK), fixed in absolute methanol at -20 °C, and stored at -20 °C until *in situ* hybridization.

In studies of female anaphase aberrations and MN, isolated mononuclear leukocytes were cultured without Cyt-B (Paper IV) and with Cyt-B (Paper V) for 72 h. In order to identify the late-replicating X chromosome a pulse treatment with BrdU (10 μg/ml) for the last 7 h of culture was used. Anaphase-telophase cells were collected by applying a

modification (Lindholm et al. 1991) of the hypotonic technique of Ford and Congedi (1987). In brief, a 10% dilution of RPMI 1640 medium was used as a hypotonic solution followed by an immediate fixation. Slides were prepared after methanol-acetic acid fixation as described by Lindholm et al. (1991) and stored in room temperature until *in situ* hybridization.

In studies of the pesticide-exposed workers and their referents (Paper VI), whole-blood lymphocyte cultures were set up as described earlier (Luomahaara and Norppa 1994), 1994), except that a DNA base analog 5-bromodeoxyuridine (BrdU; Calbiochem, USA) was added to the culture medium at the start of the experiment. BrdU closely resembles thymine, and is efficiently incorporated into the elongating DNA strands during replication (Wilson and Thompson 2007). BrdU can be added to the cultures at different times but, to obtain the most efficient labeling of MN and chromatin buds, addition between 0 and 24 hr after initiation of the cultures has been considered the best (Serrano-Garcia and Montero-Montoya 2001). To obtain comparable data on the potential effect of BrdU concentration, two different concentrations (0.5 and 1 µg/ml) were used. BrdU and PHA were washed away after 42 hours of culture and culturing was continued for additional 20 h. The cells were harvested and, fixed, and prepared on microscopical slides by the air-drying method as described in detail in Paper VI.

Cytochalasin-B

Cytochalasin-B (Cyt-B) (Sigma, St. Louis, MO, USA;), used to induce cytokinesis block, was diluted in PBS (phosphate buffered saline) from a stock solution of 2 mg/ml (in dimethylsulfoxide) and stored in -70°C. When used, it was added at a final concentration of 3 µg/ml (Paper I) or 6 µg/ml at 24 hours (the 2nd set in Paper III) and at 44 hours (Papers II and V, the 1st set in Paper III).

DNA probes

A synthetic oligomer probe, SO-αAllCen, detecting a conserved region of the alphoid DNA monomer at the centromeres of every human chromosome (Meyne et al. 1989) labeled with Digoxigenin 3´End Labelling

Kit (Boehringer Mannheim, Germany) was used at a concentration of 2.5 µg/ml in Papers I and II. For the detection of the X chromosome in Paper II, a commercial X-chromosome-specific biotin-labeled centromeric α-satellite probe (DXZ1; Oncor) was used. In Paper III, the DXZ1 probe was used together with a Y-chromosome-specific centromeric α-satellite probe (DYZ3; Oncor). A biotin labeled X chromosome painting probe (1066-XB; Cambio) and a biotinylated pancentromeric probe (1141-B; Cambio) were used in Papers IV and V.

Fluorescence in situ hybridization

FISH was performed as described by Norppa et al. (1993) with modifications suggested by Catalán et al. (1995) and Surrallés et al. (1995) (Papers I, II, and III) and as explained in Papers IV and V. The labeling efficiency of the probes was checked, and the analysis was only performed if >90% of the nuclei on the slides showed appropriate labeling.

The slides were stained with 5 µg/ml DAPI (4',6-Diamidino-2-phenyl-indole-dihydrochloride hydrate; Aldrich) and 1 µg/ml propidium iodide (PI, Aldrich) and mounted in antifade solution (Johnson and Nogueira Araujo 1981) (Paper I). Figure 4 shows a summary of the FISH procedures.

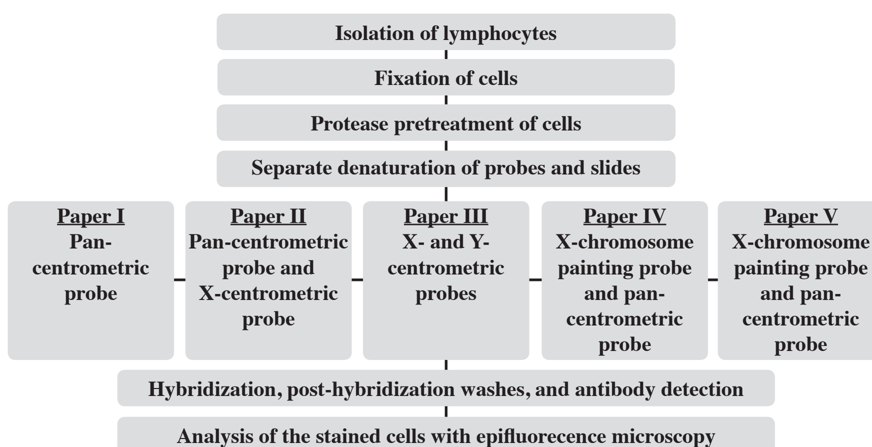

Figure 4. Illustration of the FISH staining procedure used in papers I–V.

BrdU detection

In Papers IV, V, and VI, the BrdU label was detected by immunofluorescence. Briefly (see Figure 5), following denaturation, a primary antibody (mouse anti-BrdU FITC*; Becton Dickinson) was applied, after which a secondary antibody (Paper VI: anti-mouse IgG whole molecule FITC conjugate; Sigma; Papers IV and V: FITC-conjugated rabbit anti-mouse antibody; Sigma) and (in Papers IV and V) a tertiary antibody (goat anti-rabbit FITC-conjugated antibody) were applied.

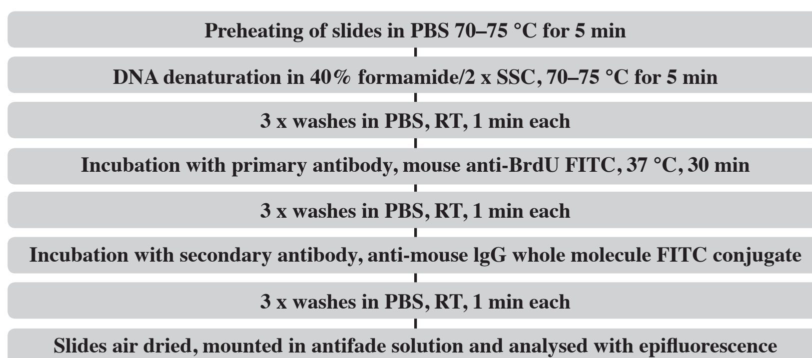

Figure. 5. Description of the principle of the anti BrdU-staining procedure (Paper VI).

Genotype analyses

The genotype analyses were performed as described in detail in Paper VI. The genotypes studied were *GSTM1*, *GSTT1*, and *NAT2*. For each polymorphism, the subjects were classified in two genotype groups. *GSTM1* null subjects were homozygous for a gene deletion and were, therefore, devoid of *GSTM1* activity, while *GSTM1* positive subjects were heterozygous or homozygous for the presence of the gene and consequently expected to express the *GSTM1* enzyme. A similar kind of deletion polymorphism also affected the *GSTT1* gene, with *GSTT1* null and positive genotypes. For *NAT2*, the subjects were classified as slow

acetylators, if they had two *NAT2* alleles associated with slow acetylation reaction; all other combinations were classified as fast acetylators.

Slide scoring

An outline of subjects studied and number of cells scored in the six papers are presented in Table 1. The scoring criteria for identification of binucleate cells and the criteria for identification and scoring of MN in binucleated cells performed in studies I, II and III, were those recommended in Bonassi et al. (2001). All analyses were performed on coded slides.

In the studies of the pesticide-exposed workers and their referents (Paper VI), the scoring was performed by one microscopist using coded slides with a Leitz Laborlux S microscope (Wetzlar, Germany) equipped with epifluorescence, using filter block I3. To restrict the scoring to cells that had divided once *in vitro*, MN were scored only in cells showing the fluorescent label. A total of 1000 FITC-stained cells were scored for each BrdU concentration and person for the number of MN in total 67 000 cells.

In Paper I, the scoring was performed on coded slides independently by two microscopists. Altogether 6000 cells were examined per subject and 1800 micronuclei were characterized, at a 1250x magnification for the presence of C+ signals using filter blocks I3 (and FITC).

At least 100 MN were analyzed for the presence of either any whole chromosome or the X chromosome by two scorers working independently in Paper II. The total number of MN scored was 1532. The frequency of MN was analyzed by scoring 3000–7965 cells per experimental point. In cultures containing Cyt-B, the analysis was restricted to binucleate cells. To accurately compare binucleate with mononucleate cells, the MN frequencies obtained in binucleate cells were divided by two (Fenech and Morley 1985). The MN frequencies were expressed per 103 nuclei.

In both sets of experiments of Paper III, the number of hybridization signals for the X and (in the men) Y chromosomes was analyzed from the nuclei of 2000 mononucleate (uncultured T cells and cell cultures without Cyt-B) or binucleate (cultures with Cyt-B) cells per donor and

cell type. The analyses were done by two trained microscopists working independently, each scoring half of the cells in each experiment.

From the different types of FISH signal distributions, the frequency of hyperdiploidy (% HRD) and the frequency of reciprocal gain and loss (% RGL) were calculated (Paper III). The % HRD reflects, both in mononucleate and binucleate cells, the frequency of nuclei with a number of FISH spots (X or Y) higher than normal. Non-disjunction in binucleated cells was categorized by the number of signals in nucleus 1 versus nucleus 2 (1/3 or 0/4) in the binucleated cell. The % RGL, which was only applied to binucleate cells where both daughter nuclei remain together, is the frequency of binucleate cells with X chromosome distributions of 3/1 and 4/0 cells in women and X or Y chromosome distribution of 2/0 in men. Thus, only those cells where the total number of spots was "correct" (i.e., 4 X-spots in women and 2 in men, and 2 Y-spots in men) were included in % RGL. Although some other distributions, such as 2/1 and 2/0 in women and 1/0 in men, might also result from reciprocal gain and loss – if two spots overlap (Eastmond et al. 1995) – they were not included in % RGL.

The frequency of apparent hypodiploidy (% HOD) – nuclei that showed less than 2 X-chromosomes in women and no X- or Y-chromosomes in men – was also evaluated. It was acknowledged that overlapping and fusion of hybridization signals may result in diploid cells being classified as hypodiploid (Eastmond and Pinkel 1990; Eastmond et al. 1995; Zijno et al. 1996); this phenomenon could be a problem especially when only single-color FISH (X chromosomes in women) is used. Hypodiploidy analysis was included to allow comparison with some earlier studies on X and Y loss in mononucleate interphase cells (Guttenbach et al. 1995).

To yield comparable hyper- and hypodiploidy data for cytokinesis-blocked and untreated cells, the unit of observation was in both cases the single nucleus. Without Cyt-B all intact nuclei were considered, whereas all nuclei belonging to intact binucleate cells were scored with Cyt-B.

In Papers IV and V, the slides were scored by two microscopists using a Leitz Laborlux S (Wetzlar, Germany) microscope equipped with epifluorescence including filter blocks A, I3, N2 and a triple band pass filter (Chroma, Brattleboro, USA) for simultaneous visualization of

rhodamine (red), FITC (green), and DAPI (blue) fluorescence. For the analysis of anaphases, we used the criteria of (Lindholm et al. 1991). Only cells with clearly separated poles, i.e. anaphase B according to Ford and Congedi (1987), were scored. The cells analyzed thus represented late anaphases and early telophases; for simplicity, they are all called anaphases in the present studies. Only cells showing both rhodamine and FITC signals were considered for the characterization of anaphase aberrations. The anaphases were classified as bipolar, indicating that the cell was in its first division after Cyt-B addition, or multipolar (mostly tri- and tetrapolar), representing second or further cell divisions.

An anaphase cell was considered aberrant if it contained lagging chromatin or bridge(s). The category lagging chromatin both included whole chromatids and chromosomes, as well as fragments lagging behind between the poles. A bridge could be either a chromatid stretching between the poles or a side-arm bridge, in which the sister chromatids were still partially connected. Laggards could represent a) an X chromosome, when the chromosome or the chromatid was totally painted red (see Paper V, Fig. 1 D and E), b) an autosome, when it showed a centromeric red signal (see Paper V, Fig. 1 A–C), and c) an autosomal fragment, when neither the red paint nor the red centromere signal was recorded (see Paper V, Fig. 1 A). In addition, both the active and inactive X chromosomes could be distinguished in some anaphases by considering their BrdU-incorporation patterns. The late-replicating inactive X chromosome was completely labeled green; whereas the active X was not at all or only partially green. Nevertheless, since the lymphocyte cultures were not synchronized, distinction between both X homologues was not always possible. About 2000 anaphases (1000 per scorer) were analyzed. 5000 cells were scored per cell type (i.e. binucleate and multinucleate cells) for MN frequency, and 200 MN were characterized per probe (X chromosome and pancentromeric probe). As the pancentromeric FISH was performed separately from X painting, C- MN contained acentric fragments originating from both autosomes and the X chromosome. Since the X chromosome is not known to be fragmented more often than other chromosomes, X fragments were probably rare among the C- MN. Thus, they were not expected to invalidate comparison of fragments in anaphases and in MN.

Table 1. Summary of subjects included in the studies and number of lymphocytes, micronuclei (MN), and laggards scored in studies I–VI.

Paper	No. subjects				No. cells scored for					No. MN scored in			
	Exposed		Unexposed										
	Men	Women	Men	Women	MN in uncultured cells	MN in cultured cells	MN in binucleate cells	X and Y segregation in binucleate cells	Laggards in anaphase	Uncultured cells	Mono-nucleate cells	Binucleate cells	No. laggards scored in anaphases
I	-	-	5	-		30000[a]	30000[a]				900	900	
II	-	-	-	5	23425	21959	18230			250	252	269	
III	-	-	-	5	10000[b]	10000[b]	10000[b]	10000[b]					
			10				20000[a]	20000[a]					
				4			8000[a]	8000[a]					
IV	-	-	-	1					2048				200
V	-	-	-	1	5000	5000	108		2000		400	400[d]	706
VI	20	14	17	16		134000[c]					1019		

[a] Isolated lymphocytes
[b] Isolated T cells
[c] Whole blood
[d] Also multinucleate cells

Statistical analyses

Logistic binomial regression model was used in Paper I to evaluate the differences in the frequency of MN and differences in C+ MN between mononucleate and binucleate cells. The Egret software package was used for the statistical analysis.

In Paper II, the one-tailed t-test for paired observations was used for the statistical comparison among uncultured cells and the two types of cultured cells.

Comparison among the different types of cells in the first set of experiments and between the sex chromosomes and age groups of men in the second set (Paper III) were performed by multifactorial analysis of variance. The sex effect in the second set was evaluated by using two-tailed t-test (Statview SE + Graphics, version 1.03).

In studies IV and V, all statistical comparisons were performed by the χ^2 test using the Statview SE + Graphics v. 1.03 program.

In the last study, for the assessment of pesticide exposure on the MN frequencies taking into consideration several confounding factors, multiple linear regression on log-transformed MN frequencies was used (Paper VI). For the detection of increase or decrease in MN frequencies for each subject class with respect to the reference class, rate ratio (RR) was computed by exponentiation of the regression coefficient of each variable after adjusting for the other factors (Bonassi, Ceppi et al. 1994). To study the influence of *GSTM1*, *GSTT1* and *NAT2* genotypes (separately and jointly) on the MN data as well as possible interaction among genotypes, analysis of variance was used. All statistical analyses in Paper I were carried out by using the STAT GRAPHICS package (Statistical Graphics, 1989; version 4.0).

RESULTS

Influence of culture time on the frequency and contents of micronuclei (Paper I)

In the first paper, the influence of three different culture times (52, 64 and 76 h) on the frequency of MN containing whole chromosomes and acentric fragments was assessed. We also wanted to investigate whether the presence of Cyt-B in the cell cultures had any impact on MN frequency and content.

The results showed a general culture time dependent increase in MN frequencies. This trend was noted irrespective of the presence of Cyt-B, both in mononucleate and binucleate cells. Furthermore, the results of the FISH analysis, visualizing centromeres in the MN, indicated that the higher MN frequencies were mainly due to C+ MN, i.e. MN with whole chromosomes, which were about 1.5-times more frequent at 64 and 72 h than at 52 h. The presence of Cyt-B influenced the frequency of MN with acentric fragments (C- MN). In binucleate cells the frequency of C- MN per 1000 nuclei was lower than in mononucleate cells.

In vivo cytogenetic damage in micronuclei (Paper II)

In Paper II, MN frequencies in T-lymphocytes were compared before and after cell culture in the presence and absence of Cyt-B. The mean frequency of MN per 1000 nuclei was consistently ($P < 0.01$) lower in the uncultured cells than in the cells cultured with or without Cyt-B (Table 1, Paper II). Cell culture increased the frequency of MN, but binucleate

cells formed by Cyt-B had less MN per 1000 nuclei than mononucleate cells cultured without Cyt-B. The distribution of C+ and C- MN was different ($P < 0.01$) in uncultured cells and cultured cells. In uncultured cells, the great majority of the MN was C+ (71.6%), whereas in cultured cells C+ MN (55.2% without Cyt-B, 53.3% with Cyt-B) only slightly outnumbered C- MN.

As in Paper I, fragment-containing (C-) MN were slightly more frequent in mononucleate cells (7.5/1000 nuclei; without Cyt-B) than binucleate cells (5.8/1000 nuclei; with Cyt-B), although the difference was not statistically significant. On the other hand, the frequency of (C+) MN with whole chromosomes was higher with Cyt-B (9.1/1000 nuclei) than without it (6.6/1000 nuclei) or in uncultured cells (6.5/1000 nuclei). This appeared to reflect primarily the high frequency of MN harboring the X chromosome in cells cultured with Cyt-B (5.2/1000 nuclei) in comparison with cells cultured without Cyt-B (3.9/1000 nuclei) or uncultured cells (2.6/1000 nuclei). In general, cell culture increased the frequency of MN with X chromosomes. In binucleate cells, as many as 42% of all MN were X-positive; this was clearly higher than in cells cultured without Cyt-B (24.2%) and in uncultured cells (28.5%). The respective percentages for the prevalence of the X chromosome in MN harboring whole chromosomes were 79.8% (with Cyt-B), 44.0% (without Cyt-B), and 39.6% (uncultured cells); all these figures are much higher than the 4.3% expected assuming equal contribution by all chromosomes. Thus, the X chromosome was highly over-represented in human T-cell MN *in vivo* and *in vitro*, and the use of Cyt-B further potentiated X chromosome micronucleation.

In addition to an increase in the frequency of X-positive MN in the presence of Cyt-B, the high prevalence of the X chromosome in binucleate cells was explained by a strong decrease in the frequency of MN harboring autosomes in these cells (Fig. 1, Paper II). The frequency of MN with autosomes (per 1000 nuclei) was significantly lower in binucleate cells in the presence of Cyt-B compared with both uncultured cells and cells cultured in the absence of Cyt-B.

Segregation of sex chromosomes in human lymphocytes (Paper III)

In Paper III, two sets of experiments were performed. The aim was to evaluate the effects of cell culture and cytokinesis block on the segregation of the sex chromosomes. In the first experiment, the frequency of X chromosome aneuploidy in female T lymphocytes cultured in the presence and absence of Cyt-B were compared with *in vivo* MN frequencies in uncultured T cells. Results on the distribution of the X chromosome signals in cultured and uncultured T cells of the five older women included in the first set of experiments are shown in Tables I and II in Paper III. Apparent X hypodiploid nuclei were clearly more common than X hyperdiploid nuclei. Multifactorial analysis of variance on the frequencies of hyperdiploid or "hypodiploid" nuclei showed no significant differences attributable to cell culture, individuals, or use of Cyt-B. Examples of the different types of abnormal segregation of the X chromosomes are shown in Figure 6.

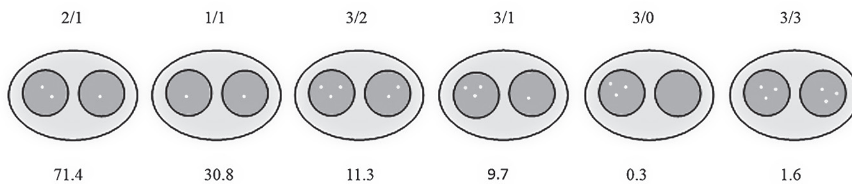

Figure 6. Examples of abnormal X chromosome segregation patterns deviating from the expected 2/2 distribution in cultured binucleate lymphocytes of five women in the first experiment of study III visualized with an X chromosome specific centromeric probe. The dots indicate the centromeres of the X chromosome. The frequency (per 1000 binucleate cells) of the type of abnormal segregation is indicated below the binucleate cell.

In the second set of experiments, both X and Y chromosome aneuploidy and segregation were analyzed in binucleate cells in two age groups of men (Table III, Paper III) (Figure 7). This approach also allowed us to evaluate the number of polyploid binucleate cells. Only 24 apparently polyploid cells were found in a total of 20,000 cells analyzed, which indicated a low polyploidy rate. Examples of the different types of abnormal distribution of the X and Y chromosomes among the male subjects analyzed are illustrated in Figure 7.

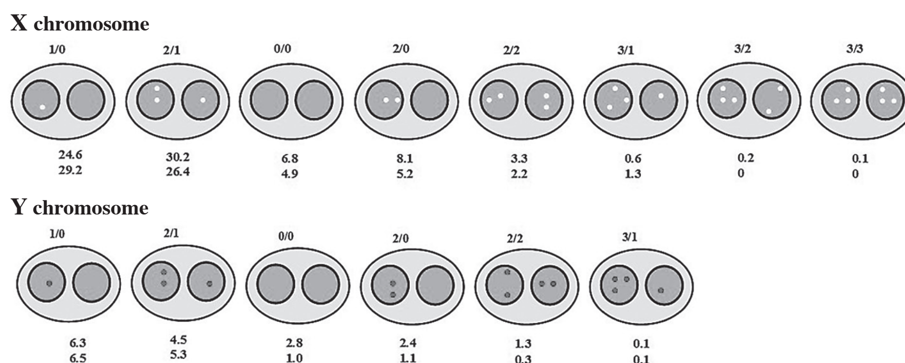

Figure 7. Examples of the most common types of abnormal segregation of the X chromosome and the Y chromosome in binucleate lymphocytes of two age groups of ten men in study III. The frequency (per 1000 binucleate cells) of the type of abnormal segregation is indicated below the binucleate cell.

The older group of men showed somewhat higher mean frequencies of X hyperdiploid nuclei, “hypodiploid” nuclei, and cells with reciprocal gain and loss of the X chromosome than the younger group (Table III, Paper III). However, no statistically significant age effects were observed for any class of X chromosome malsegregation in the men in the analysis of variance.

The mean frequency of binucleate cells with reciprocal gain and loss of the Y chromosome was 2.2 times higher ($P = 0.001$) in the older than the younger men, but the age difference was not statistically significant

for the mean frequencies of Y hyperdiploid or "hypodiploid" nuclei. In multifactorial analysis of variance, there was a statistically significant ($P < 0.01$) difference between the X and Y chromosomes for the frequency of hyperdiploid and "hypodiploid" nuclei as well as for reciprocal gain and loss, the X chromosome showing higher malsegregation rates.

In the four women studied in the second set, the mean frequency of X chromosome reciprocal gain and loss was similar as in the first series, and the 3/1 distribution of X chromosomes between the two nuclei of the binucleate cell predominated, although a few 4/0 cells were also seen. When the X aneuploidy frequencies of the women in the second set were divided by two, to take into account their two X chromosomes, men actually appeared to show slightly more X hyperdiploid nuclei, X hypodiploid nuclei, and cells with reciprocal gain and loss of the X chromosome than women. However, the effects were not statistically significant (two-tailed t-test).

X chromosome lagging in female lymphocyte anaphase (Paper IV)

In order to analyze 200 aberrant anaphases, over 1.5 million cells were screened. Of all cells, 2% were mitotic, and the frequency of anaphases was only 0.16% of which 7.96% were aberrant. Most of the aberrations found were laggards (71%). The rest were bridges, part of which had stretched and broken (19%). 25% of the aberrant anaphases contained two or more aberrations.

Half of the 200 laggards characterized by FISH were autosomes, 33.5% acentric fragments, and 17.5% X chromosomes (Table 2, Paper IV). The X chromosome represented 26% of all lagging chromosomes, i.e., was highly over-represented among anaphase laggards, assuming equal contribution (4.3%) by all chromosomes. BrdU detection indicated that both X homologues contributed equally to the X laggards. In five aberrant anaphases, both X chromosomes were lagging.

The relative distance of the laggard to the closer pole (distance to the closer pole/distance between poles) was significantly higher for the X chromosome than lagging autosomes ($P < 0.05$, Fisher's least significant difference).

The analysis of the contents of MN showed a clear increase, in comparison with anaphase laggards, in the prevalence of autosomal fragments (50%) and the X chromosome (31%), while whole autosomes were detected in MN at a lower rate (19%); the differences between MN and anaphase laggards were statistically significant ($P < 0.01$, χ^2 test).

Lagging chromosomes in cytokinesis-blocked anaphases (Paper V)

To be able to characterize 2000 anaphases, we had to go through almost 400,000 cells. Most of the anaphases were tetrapolar, but bipolar cells in their first division after Cyt-B addition were also frequent. Aberrations were very common in multipolar anaphases (34.5%) but fewer among bipolar anaphases (5.4%).

The aberrant anaphases were classified into those containing a) laggards, b) bridges and c) both laggards and bridges. Most of the aberrant anaphases contained laggards. Laggards were found in 95.2% and 93.5% of aberrant bipolar and multipolar anaphases, respectively. In bipolar aberrant anaphases, the frequency of bridges was 0.25%. Over one third of the aberrant anaphases contained two or more aberrations, mostly laggards. A closer characterization of the laggards showed that most of them, both in binucleate and multinucleate cells, were whole chromosomes. In bipolar cells, 75% of the laggards were autosomes, whereas 15% were acentric autosomal fragments and 10% X chromosomes. These figures were similar as those observed in multipolar cells. Both in bipolar and multipolar anaphases, the X chromosome lagged more often than would be expected by chance, representing 12% (bipolar anaphases) and 7% (multipolar anaphases) of all lagging chromosomes The X chromosome was not observed to be involved in anaphase bridge formation. Activity status of the lagging X chromosome could be judged by BrdU label only in 13 cases (all multipolar anaphases), and 3 of the X laggards (23%) were considered inactive.

In comparison with lymphocytes cultured without Cyt-B, bipolar anaphases cultured with Cyt-B showed a higher frequency of lagging autosomes, but a lower frequency of lagging X chromosomes and fragments.

The frequency of MN per 1000 nuclei was 11 times higher in multinucleate cells than in binucleate cells. Considering that most multinucleate cells contain four genomes, while binucleate cells contain two, the difference in MN frequency between the two cell types remained 5.7-fold. The percentages of MN containing fragments, autosomes, and X chromosomes were, respectively, 34.9%, 22.3%, and 42.9% in binucleate cells, and 19.3%, 60.5%, and 20.2% in multinucleate cells. The high MN frequency in multinucleate cells was primarily due to autosomes, with a smaller contribution of fragments and X chromosomes. The frequency of X chromosome in MN was higher in binucleate cells with Cyt-B than in mononucleate cells without Cyt-B, while the frequency of MN with acentric fragments was higher in the latter; this time there was no difference in the frequency of MN harboring autosomes between binucleate and mononucleate cells.

The conversion of anaphase laggards into MN was examined by comparing the frequencies of anaphase laggards and MN (Fig. 2 in Paper V). Autosome laggards were very poorly micronucleated in bipolar divisions, clearly less efficiently than lagging X chromosomes and fragments. X laggards generally formed MN efficiently; without Cyt-B, 49% of them formed MN, and with Cyt-B practically all X laggards appeared to end up in MN. Thus, a lagging X chromosome appeared to be micronucleated more often in the presence of Cyt-B than in its absence. Also lagging acentric fragments formed MN effectively in the presence of Cyt-B (65% in bipolar divisions and 58% in multipolar divisions with Cyt-B, 48% without Cyt-B).

Influence of genetic polymorphisms of *GSTM1*, *GSTT1*, and *NAT2* on MN level (Paper VI)

The distribution of the *GSTM1*, *GSTT1*, and *NAT2* genotypes among the greenhouse workers and the controls is shown in Table 1 in Paper VI. The *GSTM1* null genotype (17/33 controls, 20/30 exposed) and the *NAT2* slow acetylator genotype (17/32 controls, 18/30 exposed) were reasonably well represented among the different groups, for a meaningful

statistical analysis, but the low prevalence of the *GSTT1* null genotype (3/33 controls, 6/30 exposed) restricted conclusions concerning this polymorphism.

GSTM1 positive genotype was associated with a slightly higher mean MN frequency than *GSTM1* null genotype, at both doses of BrdU used (Table 4, Paper VI). This was especially noted in smokers, although the difference was not quite statistically significant in smokers alone (Table 5, Paper VI). *NAT2* fast acetylator smokers had an elevated mean frequency of micronucleated cells in comparison with slow acetylators at 1 μg/ml but not at 0.5 μg/ml BrdU.

Influence of occupational pesticide exposure on micronucleus frequency (Paper VI)

In paper VI, the frequency of MN in cultured lymphocyte was analyzed by the anti-BrdU assay in greenhouse workers exposed to genotoxic components in pesticides. Two concentrations of BrdU were used in the anti-BrdU MN assay. A clear correlation of individual MN frequencies between both concentrations used was obtained.

The study indicated a statistically significant effect of pesticide exposure on the MN frequency. When comparing all subjects (Table 3, Paper VI), smoking greenhouse workers had a higher frequency of micronucleated cells than the controls at both BrdU concentrations. When controlling for confounding factors such as age, sex and smoking, the subjects classified as extensive sprayers showed a significant increase in MNCs ($P = 0.004$ at 1 μg/ml BrdU, $P = 0.052$ at 0.5 μg/ml BrdU) when *GSTM1*, *GSTT1* and *NAT2* genotypes were taken into account (Table 4, Paper VI) in the multiple regression analysis.

Subjects older than 43 years had a significantly elevated MN frequency at 0.5 μg/ml BrdU ($P = 0.036$; Table 4, Paper VI), whereas gender had no significant influence on MN levels. At 1 μg/ml BrdU, smokers who smoked less than 20 cigarettes per day had a significantly lower MN frequency than non-smokers or heavy smokers.

DISCUSSION

Fragments and chromosomes in spontaneous micronuclei *in vitro* and an *in vivo*

Most MN studies are nowadays based on the CB method in which cells that have divided only once *in vitro* are identified by using Cyt-B (Fenech and Morley 1985). However, both Cyt-B and culture conditions themselves may modify the frequency and contents of MN (Norppa, Renzi et al. 1993). This was demonstrated in the studies involved in this thesis (Papers I, II, III and V). For example, the use of Cyt-B increased the proportion of X chromosome MN in women radically (Paper II).

We showed that the baseline frequency of MN in cultured human lymphocytes increases with culture time (52–76 h) both in mononucleate cells without Cyt-B and binucleate cells with Cyt-B. This effect was clearly restricted to MN harboring whole chromosomes, as it only concerned C+ MN (containing centromeric alpha satellite DNA), with no influence on C- MN (containing fragments). Thus, MN due to errors of chromosome segregation became more prevalent at longer culture times. The generation of fragment-containing MN in 64-h and 76-h cultures did not markedly differ from the level at 52 h (Paper I). The frequency of MN harboring fragments was lower in Cyt-B-induced binucleate cells than in cells cultured without Cyt-B. If micronucleation of fragments is diminished in the presence of Cyt-B, the efficiency of the CB method to identify weak clastogenic effects might be reduced. It is not yet clear whether this effect has practical significance.

Our findings also highlight the benefits of using centromere detection in conjunction with the MN analysis. Centromere identification allows the identification of the mechanistic origin of MN. By using probes

identifying the centromere or telomere of the chromosomes/ chromatids it becomes possible to determine the mechanistic origin of the MN and distinguish clastogens from aneugens. This possibility increases the sensitivity of the assay in a major way. In various other studies concerning the spontaneous MN level in cultured human lymphocytes, the average proportion of whole chromosomes in MN has usually varied between 30% and 80%, as measured by CREST antibody or pancentromeric FISH (Fenech and Morley 1989; Yager et al. 1990; Norppa et al. 1993; Scarpato et al. 1996c; Surralles et al. 1996; Migliore et al. 1997; Calvert et al. 1998; Carere et al. 1998; Davies et al. 1998; Migliore et al. 1999; Migliore et al. 1999; Thierens et al. 1999; Maffei et al. 2000; Thierens et al. 2000; Leach and Jackson-Cook 2001; Bakou et al. 2002). In uncultured T lymphocytes of women 47–60 years of age, most MN were labeled positive with the centromere probe and were thus expected to contain whole chromosomes. The proportion of C+ MN was 71.6% compared with 53.3–55.2% in cultured cells (with and without Cyt-B) (Paper II). Compared with uncultured cells, the frequency of the C- MN per 1000 nuclei was 2.3–2.9 times higher in cultured cells. This could probably be due to an expression, after *in vitro* mitoses, of damage accumulated *in vivo* in the quiescent lymphocytes.

The X chromosome was, in general, represented in female micronuclei far more often than would be expected – not only in cultured cells but also in uncultured cells. This finding supports earlier reports of excessive overrepresentation of the X chromosome in MN of female lymphocytes; the involvement of the X in MN has been observed to increase with age (Guttenbach et al. 1994; Hando et al. 1994; Richard et al. 1994; Catalán et al. 1995). Our results showed, for the first time, that the X chromosome is also involved in T cell MN *in vivo* of women and that this phenomenon is not an *in vitro* artifact, although cell culture further increases the frequency of X chromosome positive MN. The findings suggest that X chromosome aneuploidy is a common event in the somatic cells of women.

The present study showed that the direct analysis of MN in resting G0 stage T lymphocytes in peripheral blood, combined with centromeric FISH, is a technically feasible possibility for the analysis of chromosome breakage and numerical chromosome alteration occurring *in vivo*. Considering biomonitoring of human genotoxic continuous

exposure, long-lived T lymphocytes could reflect *in vivo* genotoxic damage acquired during a longer exposure period. In conclusion, the *in vivo* technique may be useful not only for basic studies, but also applicable for monitoring studies. In accordance with many previous studies (see Fenech and Bonassi 2011), we also found an increase in the frequency of (binucleated) micronucleated cells with age – and in females compared to males. Furthermore, the age effect was observed as an increase in the percentage of C+ MN. Therefore, it appears that the age effect reflects an age-related micronucleation of the sex chromosomes (i.e. X and Y) (Hando et al. 1994; Zijno et al. 1996; Norppa and Falck 2003). The gender effect can probably be explained by the preferential loss of both homologues of the X chromosome (paper IV).

Involvement of sex chromosomes in micronucleation and chromosome missegregation

We found no significant differences in the frequency of nuclei hyperdiploid or hypodiploid for the X chromosome in women between uncultured T-cells and T-cells cultured with or without Cyt-B, which suggests that cultured lymphocytes reflect *in vivo* aneuploidy rates (Paper III). This finding agrees with that of Guttenbach et al. (1995), who could not find differences between resting and cultured lymphocytes in X and Y hypodiploidy. We did not however, observe a higher frequency of MN harboring the X chromosome in the cultured lymphocytes in comparison with uncultured T lymphocytes. This may indicate that cell culture affects X-micronucleation in women even if no significant effects are observed in the frequencies of hyper- or hypodiploid nuclei.

The X+ MN or other types of X malsegregation formed *in vitro* do not markedly affect X aneuploidy rates probably because aneuploidy of *in vivo* origin is so frequent. Binucleate cells with an illegitimate number of X chromosome signals (more or less than 4), which apparently are mostly derived from *in vivo* (Zijno et al. 1996a), were the most prevalent types of aneuploidy. Our data indicate that less than half of all X hyperdiploid nuclei and a minority of apparent hypodiploid nuclei seen in female lymphocyte cultures are formed by reciprocal gain and loss in the first

in vitro division, much of the rest being of *in vivo* origin. On the other hand, Carere et al. (1999) observed that, in binucleate lymphocytes of men, reciprocal gain and loss of the X chromosome (an *in vitro* event) is more frequent than balanced hyperdiploids (a probable *in vivo* event).

In Paper III, binucleate cells with reciprocal gain and loss of the X chromosome constituted about 1% of women's binucleate cells. These figures closely follow the frequencies of X+ MN reported earlier from the same experiments (Paper II and Catalán et al. 1998). Our results agree with those of Zijno et al. (1996a), who concluded that X chromosome loss (MN) and reciprocal gain and loss ("nondisjunction") occur at similar rates in binucleate female lymphocytes.

The correct interpretation of hypodiploidy data remains unclear. The high frequencies of hypodiploid interphase cells may partly be due to overlapping of FISH signals (Eastmond and Pinkel 1990; Eastmond et al. 1995), but the clear age-dependent increase in X and Y loss (Guttenbach et al. 1995; Mukherjee et al. 1996) also suggests that hypodiploidy analysis yields meaningful data. Accordingly, the present results showed a higher hypodiploidy rate for the X than the Y chromosome in men, which agreed with our data on hyperdiploidy and reciprocal gain and loss of these chromosomes.

We could not demonstrate any effects on sex chromosome segregation for Cyt-B-induced cytokinesis block (Paper III). Similarly, Zijno et al. (1996b) saw no increase of hyperdiploid nuclei in binucleate cells. These results contrast with our previous findings on MN in the same cultures, where a higher frequency of MN containing the X chromosome was seen when binucleate cells were compared with cells cultured without Cyt-B (Paper I; Catalán et al. 1998).

As the X chromosome was the only chromosome we labelled in women, we could not distinguish hyperdiploidy from polyploidy (Eastmond et al. 1995). However, polyploids in women do not explain our results on hyperdiploidy in men and women, since after correction for the number of X copies, the rate was higher (although not significantly) in men. Undetected polyploidy was not an important source of error probably because of the low frequency of polyploid cells (0.12% in men) in comparison with sex chromosome hyperdiploidy.

The lack of a significant difference between the sexes in the frequency of X chromosome reciprocal gain and loss was an unexpected finding. In

our earlier paper on MN of the same donors (Catalán et al. 1998), we found a clearly higher frequency of X+ MN in women than in men and, therefore, also expected an elevated rate of gains and losses. Metaphase studies have likewise indicated a higher total rate of X malsegregation in women than men (Fitzgerald et al. 1975; Fitzgerald and McEwan 1977), and Zijno et al. (1996) observed a five times higher frequency of X malsegregation ("nondisjunction" and MN combined) in women than men. Our findings seem to suggest that X chromosome micronucleation and reciprocal gain and loss are originated by different mechanisms. It would appear that the high X chromosome loss in women is primarily due to excessive micronucleation of the X chromosome rather than nondisjunction.

Paper III was the first study where the aneuploidy of both sex chromosomes was simultaneously analyzed in binucleate lymphocytes of men. The X chromosome showed higher frequencies of hyperdiploidy, reciprocal gain and loss, and apparent hypodiploidy than the Y chromosome. This is in agreement with our previous observation of a higher frequency of X+ MN than Y+ MN in the same men (Catalán et al. 1998). The different rates of reciprocal gain and loss of the X and Y chromosomes may be explained by chromosome-specific mechanisms of nondisjunction, as has been suggested for germ cells (Abruzzo and Hassold 1995; Abruzzo et al. 1996). In fact, our frequencies of reciprocal gain and loss of the Y chromosome were similar as the previously reported frequencies of Y-positive MN (Catalán et al. 1998), while reciprocal gain and loss was clearly more prevalent than MN for the X chromosome of the men. As these classes of X malsegregation showed similar rates in women, it could be that nondisjunction affects the homologous X chromosomes similarly, but micronucleation would preferentially concern the inactive X (which only women have). This hypothesis would explain why females have a clearly higher frequency of X chromosome loss in metaphase and X+ MN but similar rates of X reciprocal gain and loss in comparison with men. Although the preferential involvement of the inactive X in aneuploidy has been investigated in several studies (Fitzgerald et al. 1975; Abruzzo et al. 1985; Tucker et al. 1996; Surrallés et al. 1996b), conclusions have been contradictory and further experiments are thus required.

An effect of age was observed for Y chromosome reciprocal gain and loss, in accordance with the prevoius finding on a clear age-related

increase in Y+ MN in the same subjects (Catalán et al. 1998). Similar (nonsignificant) trends were seen in the frequencies of Y hyper- and hypodiploid nuclei.

Carere et al. (1999) observed a positive correlation with age for both hyperploidy and reciprocal gain and loss of the X chromosome. Also in our study, the older men had more X hyperdiploid nuclei and reciprocal gain and loss than the young, although the effect was not statistically significant. Previously, Zijno et al. (1996b) described an age-dependent increase in X malsegregation, combining X reciprocal gain and loss and micronucleation. They could not find an association between age and the rate of putative pre-existing monosomic and trisomic cells, in agreement with our finding of a lack of an age effect in X chromosome hypodiploidy. An age-dependent increase was described for the frequency of X+ MN in men (Hando et al. 1997; Catalán et al. 1998; Carere et al. 1999).

In conclusion, Paper III suggests that the baseline frequencies of X hyperdiploidy and apparent hypodiploidy in female lymphocytes are not markedly affected by cell culturing or by the use of Cyt-B. Malsegregation of the X chromosome is common in lymphocytes of both men and women and more frequent than Y-chromosome malsegregation. The reciprocal gain and loss of the Y chromosome shows an age effect, which together with the previously shown age-dependent formation of Y-positive MN indicates a chromosome-specific mechanism of malsegregation for the Y chromosome. There does not seem to be any clear sex difference for X chromosome hyperploidy or reciprocal gain and loss, which would suggest that the high loss of the X chromosome in women is due to micronucleation.

Characterization of anaphase laggards

The fact that the number of aneuploid lymphocytes increases with age has been known for years, in fact the X chromosome in women appeared to be the chromosome mainly responsible for this loss (Jacobs, Court Brown et al. 1961; Fitzgerald and McEwan 1977; Richard, Aurias et al. 1993). As we, along with others (Guttenbach et al. 1994; Hando et al. 1994; Richard et al. 1994; Catalán et al. 1995) detected an excess of X chromosome containing MN in our previous studies (Papers II and

III) we wanted to examine the formation of MN from chromosomes and fragments lagging behind in anaphase, with a special reference to the X chromosome and use of Cyt-B to get further information on the mechanisms of X chromosome loss. In order to do this, we had to go through more than 1.5 million cells to obtain ~200 classifiable aberrant anaphases. The majority (71%) of the aberrations found were laggards, the rest were bridges. In 25% of the cases, two or more aberrations were seen in one aberrant anaphase. With appropriate DNA probes using FISH technology we also classified the laggards into autosomes, acentric autosomal fragments and X chromosome. Half of the laggards were autosomes, while acentric autosomal fragments and the X chromosome constituted 33.5% and 17.5% of the laggards, respectively. Of all the lagging chromosomes, the X accounted for one-fourth, a fact that made us realize that the X chromosome, during the anaphase stage, is more prone to be lost than the autosomes. When we compared these results with the results of the MN analysis, 50% of the MN contained autosomal fragments and 31% contained the X chromosome. MN containing autosomes were found at a much lower frequency (19%) compared with what we found in anaphases, respectively. The reason for this difference could be lagging chromosomes with a functional centromere are more easily reincorporated in the reformed daughter nuclei, while the acentric fragments are left outside and eventually forms MN. Furthermore, the proximal position of the autosome laggards enables correct segregation of the chromosomes resulting in a lower micronucleation. When we measured the distance between each laggard and the closest anaphase pole we noted that the relative distance to the closest pole was significantly higher for X chromosome laggards than for autosomal laggards. Another explanation for the lower prevalence of autosomes in MN may be selection against cells that have lost autosomes, which carry genes important for cell survival (Marshall et al. 1996).

By using DNA probes that identify centromeric regions of chromosomes it is possible to use the CBMN assay to determine the rate of aneuploidy in nuclei of binucleate cells (and mononucleate cells) and determine malsegregation pattern in these cells (Wang et al. 2004).

Modifying effect of genetic polymorphisms in *GSTM1*, *GSTT1* and *NAT2* genes on the micronucleus frequency in human lymphocytes

Data on the association between the frequency of CAs and cancer risk assembled by Nordic (Hagmar et al. 1994), Italian (Bonassi et al. 1995; Bonassi et al. 2000) and later European collaborative studies (Hagmar et al. 1998; Bonassi et al. 2008) have shown an approximately two-fold risk of cancer in subjects with a high level of CAs. The risk was not significantly associated with exposure to tobacco smoke and occupational carcinogens, i.e. was seen in unexposed subjects and non-smokers alike (Bonassi et al. 2000). One of the explanations for these findings is that individual susceptibility factors affect both the level of CAs and cancer risk.

A large number of studies have suggested that genetic polymorphisms influence the risk of developing cancer (McIlwain et al. 2006; Dong et al. 2008). Combination of several nonbeneficial alleles is expected to increase the risk. It is important to remember that many polymorphisms are expected to function as effect modifiers: without exposure they have no consequences (Knudsen et al. 2001). The interaction of genotypes with tobacco smoking in determining cancer risk has been studied to some extent. However, few studies are available on genotype and cancer risk with respect to occupational and environmental exposures.

As GSTs play an important role in the metabolism of a wide variety of environmental carcinogens (Ketterer 1988), and the polymorphisms of both *GSTM1* and *GSTT1* involve a null genotype, these two GSTs are among the most studied low-penetrance cancer susceptibility genes so far (Rebbeck 1997; Parl 2005; Bolt and Thier 2006; McIlwain et al. 2006; Dong et al. 2008).

Several studies have been conducted to evaluate whether genetic polymorphisms in *GSTM1* and other xenobiotic-metabolizing genes affect the baseline level of cytogenetic alterations, as reviewed earlier (Norppa 2004b; Iarmarcovai et al. 2008). Most of them failed to find a clear association between *GSTM1* genotype and the frequencies of CAs, SCEs, or MN in unexposed control persons. However, the *GSTM1* null

genotype has been associated with increased sensitivity to genotoxicity of tobacco smoke in several studies. *GSTM1* null smokers showed an increased frequency of CAs (Scarpato et al. 1997; Norppa 2004b; Tuimala et al. 2004) and SCEs (van Poppel et al. 1992; van Poppel et al. 1993) in their lymphocytes and an increased level of benzo[a]pyrene diol epoxide adducts in their lung cell DNA (Alexandrov et al. 2002). These findings are consistent with the idea that the lack of *GSTM1*-mediated detoxification results in elevated DNA binding of the reactive metabolites of carcinogens in tobacco smoke and increased chromosome damage.

In the present study, we found slightly increased MN frequencies in *GSTM1* positive donors. Our result primarily concerned smokers and control subjects, although the genotype effect was not statistically significant for these groups separately. In agreement with our finding, also Leopardi et al (2003) and Pérez-Cadahía et al. (2008) saw an increased baseline MN frequency in *GSTM1* positive subjects. Iarmarcovai et al. (2006) observed a higher level of C- MN, harbouring chromosomal fragments, in lymphocytes of welders and control subjects with the *GSTM1* positive genotype; this effect was separately seen in smokers and control subjects, in accordance with our findings. A genotype difference of similar magnitude, although not statistically significant, was likewise observed by Pitarque et al. (2002) (in smoking controls); Laffon et al. (2002a), Ceu Silva et al. (2004), Teixeira et al. (2004), and (for C+ MN in controls) (Migliore et al. 2006). Five other studies did not, however, indicate a higher MN level for *GSTM1* positive subjects (Cafiero et al. 1989; Cheng et al. 1996; Ishikawa et al. 2004; Guven et al. 2005; Laffon et al. 2006). In one study (Godderis et al. 2004), reporting no statistically significant effect of *GSTM1* genotype on MN frequency, data on MN frequencies with respect to *GSTM1* genotype were not shown.

The possible reasons for the higher MN level of the *GSTM1* positive genotype are not known. Leopardi et al. (2003) suggested that the *GSTM1* null genotype is associated with greater efficiency in the repair of DNA damage, as an adaptive response to the enzyme deficiency; alternatively, they considered that the higher incidence of DNA damage in *GSTM1* proficient individuals could reflect the involvement of glutathione conjugation in the metabolic activation of some chemical carcinogens. One explanation could be that the combined conjugation activities of various GSTs lead to depletion of the glutathione pool in

chronic exposure to low levels of genotoxicants (Kirsch-Volders et al. 2006). The apparent discrepancy between increased CAs in *GSTM1* null smokers and increased MN in *GSTM1* positive donors may be related to the complex effect of smoking on MN level. While smoking seems to increase CAs in a dose-dependent manner, the frequency of MN appears to rise only in heavy smokers, low and moderate smokers showing a decreased MN level (Bonassi et al. 2003; Kirsch-Volders et al. 2006).

In our study, the numbers of *GSTT1* null individuals were unfortunately too low to draw any conclusions of *GSTT1* genotype on MN. In other studies, higher baseline levels of lymphocyte SCEs (Norppa et al. 1995; Schroder et al. 1995; Wiencke et al. 1995; Knudsen et al. 1999; Laffon et al. 2002a,b; Silva Mdo et al. 2004) and CAs (Landi et al. 1998; Sram et al. 2004; Tuimala et al. 2004) have been described in *GSTT1* null persons. Iarmarcovai et al. (2006) also reported that the baseline level of C+ MN, harbouring whole chromosomes, was elveated in lymphocytes of *GSTT1* null subjects. However, most studies have observed no significant effect of *GSTT1* genotype on the baseline level of MN (Laffon et al. 2002; Leopardi et al. 2003; Godderis et al. 2004; Ishikawa et al. 2004; Leng et al. 2004; Silva Mdo et al. 2004; Teixeira et al. 2004; Migliore et al. 2006; Migliore et al. 2006; Perez-Cadahia et al. 2008). Nevertheless, when data from the available studies were pooled, comprising 644 persons, *GSTT1* null subjects actually showed a lower average MN frequency than *GSTT1* positive subjects (Kirsch-Volders et al. 2006). This effect only concerned occupationally exposed subjects and was age-dependent, so that high MN frequency was associated with the *GSTT1* positive genotype in the youngest individuals (mean age ~20 years) but with the *GSTT1* null genotype in the oldest individuals (mean age ~60 years) – with no genotype effect in the middle age group (mean age ~40 years) (Kirsch-Volders et al. 2006).

In a study by Murgia et al. (2007), an association of the *GSTT1* positive genotype and cardiovascular disease was found. A significantly higher MN frequency was observed in the cardiovascular disease group than in the control group. Subjects with higher MN frequency and *GSTT1* positive genotype also showed a higher mortality rate (Murgia et al. 2007). The *GSTT1* null genotype was, however, found to be associated with an increased frequency of MN in styrene-exposed reinforced plastics workers (Migliore et al. 2006) and in rubber workers (Laffon et al. 2006).

Although the *NAT2* slow acetylator genotype has been associated with an increased baseline frequency of CAs, the *NAT2* genotype had no influence on baseline MN frequencies in our study. The *NAT2* genotype did not either affect MN induction by pesticide exposure. This is in agreement with our earlier studies using the CBMN assay (Scarpato et al. 1996a). However, we found an increased frequency of MN in *NAT2* rapid acetylators among smokers (exposed workers and controls combined). This finding is supported by Pluth et al. (2000) who observed that heavily smoking rapid acetylators had a higher frequency of stable CAs than those with the slow acetylator genotype.

Even though there are suggestions that lymphocytes from subjects with unfavorable metabolizing alleles are more susceptible than those with favorable alleles to radiation-induced chromosome damage *in vitro* (Au et al. 1999), there are no conclusive findings on whether metabolic polymorphisms could affect the level of chromosomal damage induced by pesticides *in vivo* (Bolognesi 2003). Apart from Paper I and the earlier study on the same population (Scarpato et al. 1996b), only one study has examined the possible effect of genotype (*GSTM1* and *GSTT1*) on MN in pesticide-exposed workers – with negative results. CYP1A1, *GSTM1*, *GSTT1*, and PON were not observed to affect the frequency of Cas in pesticide-exposed workers (Scarpato et al. 1996; Au et al. 1999; Gregio D'Arce and Colus 2000).

Individual exposure level may vary a lot, therefore a reliable estimate of the exposure is important for a correct interpretation of genotype-exposure interaction (Norppa 2004).

MN assay and occupational exposure to pesticides

Cytogenetic biomarkers have for long been used in monitoring human occupational genotoxic exposure to potential carcinogens. A number of biomarker studies have been performed to assess altered levels of MN in exposed persons. Several of the previous studies have indicated an increase of MN in peripheral lymphocytes of humans exposed to complex mixtures of pesticides (see reviews by Bolognesi, 2003, 2011) (Bolognesi 2003, 2011). Pesticide sprayers together with persons preparing pesticide

mixtures, represent the most exposed group of all agricultural workers, positive findings being found in 18 out of 27 cytogenetic biomarker (CAs, SCEs and MN) studies (Bolognesi 2003, 2011). Consequently, occupational exposure to pesticide mixtures was considered to be associated with a genetic risk including genetic damage at the chromosomal level (chromosomal number and -structure) in sprayers (reviewed in Bolognesi, 2003, 2011).

Some of the greenhouse workers examined in paper VI, had previously been analyzed for MN using the CB method (Scarpato et al. 1996a; Scarpato et al. 1996b; Scarpato and co-workers 1997), with negative results. However, in agreement with the majority of the earlier studies, we detected, by using the anti-BrdU MN assay, a statistically significant effect of pesticide exposure as well as of age on the frequency of MN. The multiple linear regression model showed an especially clear effect of the pesticide-exposure in sprayers after adjustment for age, gender, smoking, and the metabolic genotypes assessed. In general, occupational exposure of floriculturists is characterized by intense pesticide spraying seasons alternating with periods of reduced or no exposure (Scarpato et al. 1996a or b). The blood samples we processed were drawn during extensive pesticide application, while those of Scarpato and co-workers (Scarpato et al. 1996a; Scarpato et al. 1996b) were drawn both after pesticide spraying and during low exposure season. Thus, the discrepancy between the results of the earlier CBMN study and the present anti-BrdU assay probably reflected differences in exposure. Our results suggested that the workers had been exposed to the genotoxic components of the pesticides. Among the pesticides used by the workers, Acephate, Captan, Dichlorvos, Dimethoate and Metiram have been reported to be genotoxic (see refs in Paper VI).

In this project, the anti-BrdU MN assay was used for the first time in biomonitoring of human genotoxic exposure. In the MN assay of human exposure to genotoxins, using cultured human lymphocytes, the exclusive scoring of cells that have divided once in culture is of high importance for the accuracy of the analysis. The anti-BrdU method was chosen as an alternative to the well established cytokinesis-block method which is most often used to recognize MN in cells that have divided once *in vitro*. One reason for the choice were our previous findings suggesting that Cyt-B may interfere with MN formation (Lindholm, et al. 1991

and paper I; see discussion on paper I). It has to be recognized that also BrdU, as a thymine analogue incorporating into DNA, has genotoxic potential and may interfere with MN levels. In our study, we used very low and nonclastogenic concentrations of BrdU (0.5 and 1 µg/ml). BrdU started to have a dose-dependent effect on MN frequency only at 20 µg/ml (Roth et al. 1993). As a matter of fact, Montero et al. (1997) claimed that the concentration of BrdU in this assay can be set at a low concentration, since the affinity of the antibody is very high.

The inclusion of cells that have mostly divided once in the culture was achieved by using pulse-labeling with BrdU for 42 hrs, followed by washing BrdU and PHA away and further culturing for 20 h. The simultaneous use of BrdU and Cyt-B has shown that most BrdU-labeled nuclei (81–93%) are in binucleate cells (Autio et al. 1991). The agreement of the two methods was likely to be even higher, as some cells probably escaped the cytokinesis block at the concentration of Cyt-B used (3 µg/ml). The BrdU method is convenient to perform on whole blood samples as well as on isolated mononuclear cells. The labeled cells and the MN are very easily identified, especially if fluorescent dyes are used. As only labeled nuclei are scored, the analysis is fast and unambiguous, which enables analysis of a great number of cells, if desired. The contents of MN were not assessed in paper VI, but it should be possible to combine the BrdU method with DNA probes recognizing centromeres and telomeres.

CONCLUSIONS

The present thesis investigated various methodological aspects and biological factors that are relevant when MN in lymphocytes are used as a biomarker of genotoxic effects in occupational exposure. In particular, the studies provided new information on the mechanistic origin of MN, which is essential for the proper use of this cytogenetic endpoint in biomarker studies.

The main conclusions of the thesis are:

1. MN contain either chromosomal fragments or whole chromosomes, and both classes of MN represent a considerable share of all MN. The proportion of MN harboring whole chromosomes increases with age and is higher in women than men. Since the genotoxic exposure usually induces only one type of MN, it is important to distinguish MN deriving from chromosomal fragments from MN containing whole chromosomes. Fluorescent *in situ* hybridization with DNA probes that identify the centromeric α-satellite DNA of chromosomes is the method of choice for detecting both clastogenic and aneugenic effects in the MN assay (Papers I, II and III).
2. The baseline frequency of MN in cultured human lymphocytes is increased with culture time, in both mononucleate and binucleate cells. Especially MN harboring whole chromosomes become more frequent at longer culture times. The frequency of MN containing fragments is lower in Cyt-B-induced binucleate cells than without Cyt-B. This phenomenon may reduce the efficiency of the cytokinesis block method to identify weak clastogenic effects, unless

centromeres in MN are identified. The use of the cytokinesis block method is also associated with a reduced frequency of MN with autosomes (Papers I and II).

3. Analysis of MN in uncultured human T-lymphocytes provides a new possibility to assess structural and numerical chromosome damage *in vivo*. In women, the *in vivo* MN mainly contain whole chromosomes. Cell culture enhances the proportion of MN harboring chromosomal fragments (Paper II).
4. The X chromosome is represented in female lymphocyte MN far more often than expected by chance, not only in cultured cells but also in uncultured cells. Thus, the preferential micronucleation of the X chromosome also occurs in human lymphocytes *in vivo* and is not an *in vitro* artifact. Cell culture and use of Cyt-B increase the frequency of X-chromosome-positive MN but not the frequencies of X hyperdiploidy and hypodiploidy in female lymphocytes. Malsegregation of the X chromosome is common in lymphocytes of both men and women and more frequent than Y chromosome malsegregation. An age effect is detected for the reciprocal gain and loss of the Y-chromosome but not of the X chromosome. This suggests that the high loss of the X chromosome in women, documented in metaphase studies, is due to micronucleation rather than non-disjunction (Papers III, IV and V).
5. The high micronucleation of the X chromosome appears to be due to its frequent lagging behind during anaphase. Lagging X chromosomes and acentric chromosomal fragments form MN efficiently, which agrees with the idea that they lack a functional centromere and are not attached to the spindle. The lagging X chromosomes are distally located in the anaphases, further favoring micronucleus formation. All lagging X chromosomes appear to be micronucleated in the presence of Cyt-B, which may explain the high prevalence of the X chromosome in MN of binucleate cells. The lowered micronucleation of fragments in binucleate cells may be due to their reduced anaphase lagging in the presence of Cyt-B. Lagging autosomes seldom appear to form MN, suggesting that they are still attached to spindle and just delayed in anaphase movement (Papers IV and V).

6. Greenhouse workers showed an increased frequency of lymphocyte MN, indicating that the occupational exposure to pesticides involves genotoxic components. Higher micronucleus frequencies were associated with the *GSTM1* positive genotype in all subjects and with the *NAT2* fast genotype in smokers, which is opposite to what earlier studies have shown for chromosomal aberrations. Genetic polymorphisms might explain some of the discrepancies observed between the analysis of MN and chromosomal aberrations, e.g., in response to smoking (Paper VI).
7. The results of the greenhouse worker study suggest that the bromodeoxyuridine labeling technique could possibly be used as an alternative for the cytokinesis block assay in biomonitoring human genotoxic exposure and effects. Also the effect of age on MN formation, an important biological factor affecting the baseline level of MN, described in earlier studies using other micronucleus techniques, was confirmed with the bromodeoxyuridine assay (Paper VI).

The main practical recommendation deriving from the results of this thesis is that centromeric fluorescence *in situ* hybridization should routinely be applied when lymphocyte MN are used as a biomarker of human genotoxic effects. In exposure to clastogens, this approach offers the possibility to remove the potential sensitivity problem associated with the high background of MN with whole chromosomes. A specific analysis of centromere-negative MN will also abolish most of the effects of age and sex on MN frequency, since these factors are exclusively due to the preferential micronucleation of whole sex chromosomes. The use of centromere labeling would appear to be particularly important when the cytokinesis block method is used in women, as a considerable portion, even the majority, of MN in binucleate cells of females can contain the X chromosome. Also in exposure to aneugens, centromere identification is expected to increase the sensitivity of the MN assay, although it remains unclear if an *in vivo* exposure of humans to aneugens can really increase MN in resting peripheral lymphocytes.

Aneugen exposure may better express itself in MN formed in cells that have divided *in vivo*. Therefore, the second recommendation for future studies is that techniques for the analysis of MN formed *in vivo*

should further be evaluated. One alternative is the immunomagnetic separation of lymphocytes applied in this thesis. To choose the cell population that had the best possibilities to divide during the exposure, the leukocyte type to be studied could be selected taking into account the half-life of the cells.

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Micronuclei (MN) in lymphocytes represent early signs of exposure to genotoxic carcinogens. An increased frequency of lymphocyte MN is considered a strong indication of genotoxic exposure and predictive of an elevated cancer risk – a reason to improve protective measures.

This study was performed to obtain information on the suitability of the lymphocyte MN assay in examining the effects of occupational exposure to genotoxic carcinogens. It sheds light on the mechanistic origin of MN (including MN formation in anaphase) and the effect of methodological aspects such as MN content identification, cell culture time, cytokinesis block, analysis of MN in vivo, and (among pesticide-exposed workers) use of bromodeoxyuridine labelling.

Orders:
Finnish Institute of Occupational Health
Topeliuksenkatu 41 a A
FI-00250 Helsinki
Finland

Fax +358-9 477 5071
E-mail kirjakauppa@ttl.fi
www.ttl.fi/bookstore

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Cover picture:
Cover picture: microphotographs of a lymphocyte anaphase from a 62-year-old woman, showing lagging of both X chromosomes (DNA paint, red) – one of which is late-replicating (bromodeoxyuridine label, green); DNA is blue, centromeres red (Julia Catalán, Ghita Falck, and Hannu Norppa)

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