

Cytogenetic Markers and Air Pollution

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Introduction

In the field of environmental toxicology, initial attention was given to occupational exposures and their connection with acute toxic effects and diseases in humans. The development of new methods of molecular biology and genetics has shifted scientific focus to the mechanisms of interaction of these occupational factors with target tissues, cells and molecules of the human body and, eventually, to prevention of adverse health effects. Meanwhile, public and scientific awareness has grown concerning the consequences of environmental exposures which may potentially affect much larger populations. A new discipline, known as molecular epidemiology, is evolving, which employs different biological markers (biomarkers) to assess exposure, early adverse health effects, and differential susceptibility of individuals in exposed populations. Biomarkers are indicators of molecular and cellular events in biological systems which may allow epidemiologists to better examine relationships between environmental hazards and human health effects. The main advantage of biomarker studies is the ability to detect effects long before clinical manifestation of disease, which allows for intervention and prevention. Further, a far smaller population is needed for study and mechanistic information can be obtained including factors which affect interindividual variability. The evolution of cytogenetic markers that will be briefly described here has contributed greatly to the establishment of the connection between human exposure and adverse health outcomes. We have performed molecular epidemiological studies using several cytogenetic markers, including fluorescence *in situ* hybridization (FISH) and micronucleus assay (MN), to assess the effects of aerial occupational exposure to formaldehyde and benzene and of environmental exposure to urban traffic pollution. Using examples from our studies, we will describe the advantages of employing cytogenetic markers in epidemiology and toxicology and identify areas that require additional work and clarification.

Traditional Cytogenetic Biomarkers

Since studies in the 1960s which connected radiation exposure with cytogenetic damage and changes in the number and structure of chromosomes, tremendous progress has been made both in the development of new methods of cytogenetic analysis as well as in their application to the monitoring of effects of air pollution caused by occupational exposure and environmental factors. The first generation of cytogenetic biomarkers includes chromosome aberrations (CA), sister chromatid exchanges (SCE), and micronuclei (MN) (Fig. 1).

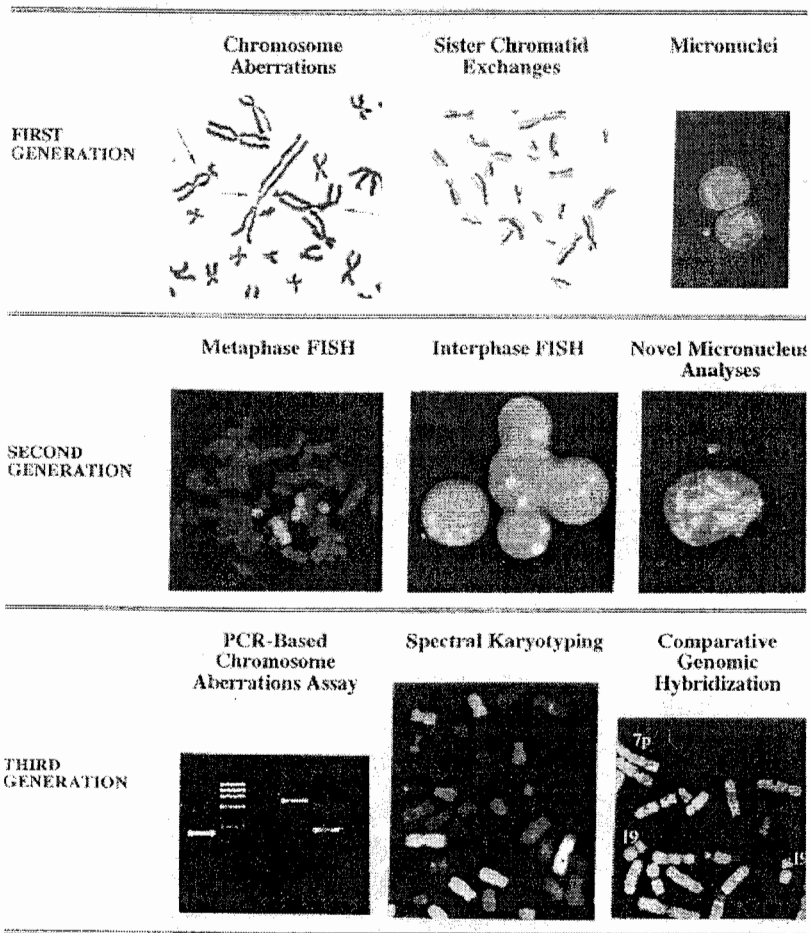


Figure 1. Evolution of cytogenetic biomarkers. Note: We are grateful to Dr. Lee Moore, Environmental Health Division, School of Public Health, UC Berkeley, for providing the image depicting comparative genomic hybridization in metaphase chromosomes of human bladder cancer. Image depicting spectral karyotyping is courtesy of Dr. Ilse Chudoba, MetaSystems, Inc., Boston, MA. All other images used in the figure were obtained in the laboratory of the authors.

Chromosome Aberrations

A standard method of assessment of CA requires culturing cells in the presence of mitogen to obtain metaphases. The number of chromosome breaks and rearrangements is evaluated under bright field microscope in at least 100 cells to estimate whether there is an increase over the background level, which is usually close to 1% of aberrant metaphases or 1 break per 100 cells. An additional advantage is provided by chromosome banding, which allows symmetrical rearrangements, including inversions and translocations, to be studied (Caspersson et al. 1971). There is an extensive literature going back to the early 1960s which includes thousands of published papers and books describing analysis of CA in numerous applications related to occupational and environmental exposure (for review see Tucker and Preston 1996, Schwartz 1990). Importantly, CA are included in required methods of genotoxicity testing by both the United States FDA and EPA, as well as by most environmental protection agencies around the world. In two large prospective cohort studies, increased CA were shown to be a reliable marker for increased risk of cancer (Bonassi et al. 1995; Hagmar et al. 1994).

Sister Chromatid Exchanges

SCE were introduced in the mid-70s as a promising method of genotoxicity assessment. This method involves culturing cells in the presence of the DNA precursor bromodeoxyuridine (BrdU) during one or more cell cycles and counting switches between sister chromatids unequally labeled with BrdU (Wilcosky and Rynard 1990, Perry and Wolff 1974). The background level of SCE in human cells is 5-8 per cell, and can be increased to more than 70 per cell as a result of exposure. Compared to CA, scoring for SCE requires less training and time, since only 20-50 cells need be scored to reveal the difference between control and treated cells. Although SCE initially appeared to be a very sensitive method, significant shortcomings have become apparent, including selectivity to certain chemicals and environmental factors and lack of understanding of the mechanism of SCE (Tucker et al. 1993). The most plausible mechanism may be connection with recombination and DNA-repair process, but this hypothesis has not been conclusively proven. Nevertheless, some labs still employ this assay, and numerous papers have been published connecting exposure to air pollution with SCE (e.g. Hornberg et al. 1998, Bolognesi et al. 1997, Hadnagy et al. 1989).

Micronucleus Assay

MN are whole chromosomes or small pieces of chromosomes without centromeres left outside of the main nucleus as a result of abnormal mitosis or damage to the structure of chromosomes. MN reflect both numerical and structural damage (reviewed in Kirsh-Volders 1997). There is a significant

overlap (60-75%) between assessment of cytogenetic damage by CA and by MN (Heddle et al. 1983). An important advantage of MN analysis is that this method can be performed on non-dividing cells and thus does not require cell culture. It is less demanding of technical training on the part of the scorer and is less time-consuming than CA and SCE. The normal background level of MN in human nonstimulated lymphocytes and exfoliated cells is 0.5-3 per 1000 cells. Several factors are known to affect the MN assay *in vivo*, including age, smoking, and the sex of the subject (Tucker and Preston 1996). The MN assay has been effectively used for monitoring numerous environmental exposures and occupational groups (recently reviewed in Fenech et al. 1999). One variation of the MN assay in human lymphocytes employing cytochalasin B, a chemical which arrests the division of cytoplasm while allowing nuclear division, is widely used because it allows additional information on cell proliferation (Fenech and Morley 1985).

Fluorescence *In Situ* Hybridization (FISH)

Interphase FISH

A new approach in analyzing the integrity of human chromosomes was developed in the mid-1980s. This approach was based on hybridization of cells attached to the slides with DNA sequences complementary to certain chromosomes or parts of chromosomes (Gray and Pinkel 1992, Sandberg 1990). These probes have a fluorescent dye attached which allows visualization of the selected part of karyotype, including centromeres and telomeres, specific chromosomes, and genes. These principles of FISH were later developed into different forms of analysis of interphase and metaphase chromosomes (Gray and Pinkel 1992). FISH can be applied in interphase cells, which eliminates the need for cell cultures, and metaphase spreads. Initially, only single-color probes were used, and scoring was mostly limited to the detection of hyperdiploidy without separation of aneuploidy and polyploidy. Analysis of hypodiploidy was more questionable because of the high frequency of artifactual probe overlap (Eastmond et al. 1995). When the method was improved to allow two or three probes labeled with different fluorescent colors to be used simultaneously, precision and power of analysis were significantly increased.

Metaphase FISH

When applied to metaphase spreads, FISH allows visualization of the structural changes as well as numerical changes caused by exposure. Now up to 8 different spots on three pairs of chromosomes can be analyzed using a combination of centromere and telomere probes and painting probes to analyze both numerical and structural aberrations (Zhang et al. 1998, Eastmond et al. 1995).

Metaphase FISH is a more sensitive method than interphase FISH but is more difficult to perform.

MN Assay Using FISH or Immunolabeling

The MN assay has been improved by the introduction of antikinetochore-antibody staining, which allows differentiation between two major mechanisms of MN formation, chromosome lagging and breakage (Eastmond and Tucker 1989). Cells are incubated with special antiserum binding with kinetochores adjacent to the centromeres. In the second cycle of hybridization staining is enhanced by a second antibody labeled with fluorescent dye. This method has been successfully applied to human lymphocytes, transformed cell lines, etc.

Similar results with differentiating MN induced by chromosome breakage or aneuploidy could be obtained using FISH with a pancentromeric probe, which labels the centromeres of all human chromosomes. This procedure can produce reliable results in types of cells that are not susceptible to antikinetochore-antibody staining because of the characteristics of their cellular membranes. Human exfoliated epithelial cells of the mouth (buccal cells), nose or bladder (urothelial cells) require preliminary treatment to increase permeability of their membrane, which destroys cellular targets for antikinetochore labeling (Moore et al. 1993).

Novel Molecular Methods of Cytogenetic Damage Assessment

In the last several years, new methods of measuring cytogenetic damage have emerged, among which the most promising are PCR (polymerase chain reaction)-based methods of analysis of structural chromosome rearrangements, comparative genome hybridization (CGH), and spectral karyotyping.

PCR-Based Methods of Analysis of Structural Chromosome Aberrations (SCA)

This method bridges the gap between cytogenetics and molecular biology (see Smith and Zhang 1998, Natarajan et al. 1996). Fig. 1 shows that changes in chromosomes are represented by bands in the gel after chromosome rearrangements of DNA were analyzed by PCR (inversions) and reverse transcriptase PCR (translocations). This approach has several advantages, including the ability to study large numbers of samples quickly and at low cost, and the possibility of registering very rare events (1 copy/ 10^7 cells vs. $1/10^4$ by FISH). However, there is a risk of false-positive results due to sample contamination. More work is needed to validate this method for the purposes of molecular epidemiology. This method is especially promising in light of the development of real-time PCR analysis (Higuchi et al. 1993), especially when used in combination with FISH.

Comparative Genome Hybridization (CGH)

Unlike FISH, CGH involves the comparison of total DNA extracted from a normal with total DNA from cancer cells or other cells with an aberrant karyotype (Reid et al. 1997, see Gray and Pinkel 1992). Two DNAs labeled with different fluorochromes are mixed at hybridization conditions, and after hybridization is complete, scanning is performed along the length of each chromosome. Sites which appear as having an excess of red or green color (mostly used for labeling) are either deletions or duplications of chromosome material. Available software can automatically identify all abnormalities and produce a complete karyotype, sometimes with more than a dozen different cytogenetic abnormalities.

Spectral Karyotyping

Spectral karyotyping can be accomplished by differentiating all chromosomes of the karyotype using a combination of fluorescent dyes (Spector et al. 1998, Reid et al. 1997). While the human eye has a limited ability to differentiate colors, a computerized scanner is much more sensitive. Assigning pseudocolors to different chromosomes produces images readily analyzed by the human eye (Fig. 1). At present, several modifications of this approach are being developed, including FISH-banding for all human chromosomes.

Occupational Exposure via Inhalation

While occupational exposure may occur by several routes, exposure by inhalation is the most prevalent. However, in the opinion of members of regulatory agencies, "there is currently very little information to estimate the risk of exposure to chemicals by inhalation" (Pour 1999).

Formaldehyde

We will illustrate the dynamics of applying different types of cytogenetic biomarkers and main findings using the example of formaldehyde. The genotoxicity of formaldehyde has been demonstrated in several biological models, including human cells in vitro (Migliore et al. 1989, Auerbach et al. 1977). Nevertheless, many people are occupationally exposed to aerial concentrations of formaldehyde close to or exceeding safe levels. Formaldehyde is used in the manufacture of plywood, paper, iron, resins and herbicides. It is also widely used as a component of embalming solutions as well as in other areas of science and medicine. Exposure to formaldehyde occurs mainly through inhalation. All traditional cytogenetic biomarkers have been used to monitor genetic damage in different occupational groups (Table 1). Reports on the increased frequency of CA aberrations in human lymphocytes are more con-

Table 1. Cytogenetic Effects of Formaldehyde Exposure *In Vivo*

Exposure	Method of analysis	Cell type	Response	Reference
Epoxy resins	CA	Lymphocytes	+	<i>Mitelman et al., 1980</i>
Furniture manufacturing	CA	Lymphocytes	+	<i>Chebotarev et al., 1986</i>
	SCE		—	
Plywood manufacturing	MN	Nasal	+	<i>Ballarin et al., 1992</i>
Embalming solution: anatomy students	MN	Lymphocytes	±	<i>Suruda et al., 1993</i>
		Buccal	+++	
		Nasal	±	
Furniture manufacturing	MN	Buccal	+	<i>Norppa et al., 1993</i>
		Lymphocytes	—	
Embalming solution	MN-FISH	Buccal	+++	<i>Titenko-Holland et al., 1996</i>
		Nasal	±	
Manufacturer of nitrogen fertilizer	CA	Blood	++	<i>Kitaeva et al., 1997</i>
	MN	Buccal	+	
Embalming solution: faculty and students	CA	Blood	—	<i>Kitaeva et al., 1997</i>
	MN	Buccal	+	
Anatomy students	MN	Buccal	+	<i>Ying et al., 1997</i>
		Nasal	++	
		Lymphocytes	±	

+ = Significant increase; ++, +++ = large increase; ± = slight increase approaching significance; — = no effect.

sistent (Kitaeva et al. 1996, Chebotarev et al. 1986, Bauchinger and Schmid 1985) than are those examining SCE (Yager et al. 1986). In several other studies no changes in CA (Norppa et al. 1993, Fleig et al. 1982, Mitelman et al. 1980) or SCE (Chebotarev et al. 1986) were observed.

In humans, peripheral blood lymphocytes are the type of cell most frequently studied. Epithelial tissues are in most cases the real targets of carcinogens, as indicated by the sites of cancers related to the exposures, and this is certainly true in the case of formaldehyde. Exfoliated epithelial cells from the mouth and nose can be easily collected and hold strong potential as a tool for biomonitoring human populations exposed to air pollution. In the last several years, half a dozen of papers have been published on the MN assay in epithelial tissues (mostly nasal and buccal epithelia) of individuals exposed to formaldehyde via inhalation (Table 1). Most of the data shows a very strong response ranging from a 2 to 3-fold increase to a 10 to 12-fold increase in the frequency of MN in buccal cells. A less pronounced effect has been observed in nasal cells, and probably depends on the concentration of formaldehyde in the breathing zone, length of exposure, and combination with other factors (e.g., wood dust or other chemicals).

We have developed a procedure that improves the MN assay in exfoliated cells by using FISH with a pancentromeric probe (Titenko-Holland et al. 1994). This approach allows the mechanism of MN formation to be readily identified. Using this technique, a significant increase in MN was observed in the buccal cells of mortician students exposed to formaldehyde, and the MN were found

to be primarily induced by chromosome breakage (10-fold increase) (Titenko-Holland et al. 1996). This finding is consistent with known clastogenic properties of formaldehyde, the component of embalming fluid most likely responsible for MN formation.

Benzene

Benzene is a chemical associated with both occupational and environmental exposure via inhalation. More than 2 billion gallons of benzene is produced annually in the US (30% of worldwide production) (Smith and Zhang 1998). In developing countries, benzene continues to be widely used as a solvent. Significant amounts of benzene are added to the atmosphere via automobile emissions and contribute to urban pollution. Another source of inhalation exposure to benzene is associated with smoking. The average daily intake of a "moderate" smoker is 1800 μg , and through second-hand exposure, individual intake may reach 180-1300 μg (Smith and Zhang 1998).

A large-scale molecular epidemiological study of Chinese workers exposed to benzene via inhalation was undertaken in collaboration with the U.S. National Cancer Institute, the Chinese Academy of Preventive Medicine, and several other institutions (for overview of the program see Smith and Zhang 1998, Rothman et al. 1996). Here we will focus only on cytogenetic findings from this study with the MN assay and FISH analysis in the lymphocytes of exposed workers. Traditional cytogenetics using CA have clearly demonstrated an association between benzene exposure and increased levels of genetic damage in human cells (Table 2) (Forni 1971, Tough and Court Brown 1965). More recently, data has accumulated that show that cytogenetic changes to certain chromosomes may occur at concentrations below 10 ppm (Tompa et al. 1994) (Table 2).

It is important to emphasize that the effectiveness of cytogenetic analysis depends on the level of exposure, target tissue, and appropriateness and sensitivity of the method. Several negative findings following benzene exposure are likely to be caused by one or a combination of these factors (Table 2). MN were a sensitive biomarker of genetic damage after *in vitro* exposure to benzene metabolites (Zhang et al. 1993, Yager et al. 1990). However, no increase in MN was observed in the blood cells of workers occupationally exposed to benzene, although gender and age effects were clearly discernible (Holland et al. 1999). Furthermore, a negative result with the MN assay was reported in both blood lymphocytes and buccal epithelia of workers in an Estonian petrochemical plant (Surrals et al. 1997).

On the other hand, benzene exposure in Chinese workers was shown to be associated with changes in the number and structure of certain chromosomes as assessed by FISH (Smith and Zhang 1998, Zhang et al. 1998, Zhang et al. 1996). Though peripheral blood lymphocytes are not the real target of benzene metabolites *in vivo*, they are a useful surrogate target because they evolve from bone marrow; furthermore, being "long-lived," they also can ac-

Table 2. Examples of the Cytogenetic Effects of Benzene Exposure *In Vivo* by Traditional Cytogenetic Assays

Exposure	Benzene exposure level, ppm	Method of analysis	Response	Reference
Solvent	N/A	CA	+	<i>Tough and Court Brown, 1965</i>
Printing	125 - 532	CA	+	<i>Forni, 1971</i>
Benzene production	0.2 - 12.4	CA	+	<i>Sarto et al., 1984</i>
Refinery workers	1 - 10	SCE	—	<i>Yardley-Jones et al., 1990</i>
		CA	+	
Printing	0.2 - 5	SCE	—	<i>Nise et al., 1991</i>
		CA	—	
Benzene production	0.3 - 15.3	MN (PHA-)	—	<i>Major et al., 1994</i>
		MN (PWM-)	+	
Benzene production	1 - 21	CA	+	<i>Tompa et al., 1994</i>
		SCE	++	
Solvent	4 - 60	CA	+	<i>Gao et al., 1994</i>
		MN	++	
Solvent	1.6 - 328.5	MN	—	<i>Holland et al., 1999</i>

+ = Significant increase; ++ = large increase; ± = slight increase approaching significance; — = no effect; PHA- = pokeweed mitogen; PWM = phytohemagglutinin.

cumulate genotoxic changes. FISH analysis is well suited for investigation of possible increases in chromosome rearrangements and numerical changes of specific chromosomes. The hypothesis which was explored in our studies of Chinese workers was whether chromosome changes induced by exposure to benzene are the same ones associated with leukemia in humans. A summary of our cytogenetic findings by FISH associated with benzene exposure is presented in Table 3. Interphase FISH was used to show that dose-dependent increases in hyperdiploidy of chromosomes 7, 8 and 9 was observed in workers exposed on average to 31 ppm benzene (Zhang et al., 1995). Interphase cytogenetics do not allow for a reliable assessment of monosomy or chromosome translocations, the most typical changes associated with benzene-induced leukemia (Eastmond et al. 1995). Therefore a special method, which combines chromosome painting and region-specific fluorescent probes to ex-

Table 3. Specific CA Detected by FISH in Benzene-exposed Workers

FISH	Chromosome number	Cell type	Response	Reference
Interphase	9	Lymphocytes	+	<i>Zhang, 1996</i>
Metaphase	8, 21	Lymphocytes	+	<i>Smith, 1998</i>
Metaphase	1, 5, 7	Lymphocytes	++	<i>Zhang, 1998</i>
Inter/Metaphase	7, 8	Lymphocytes	++	<i>Zhang, 1999</i>

+ = Significant increase; ++ = large increase.

amine complete and partial deletions of chromosomes 5 and 7 and translocations of chromosomes 8 and 21, was developed and applied to metaphase spreads of control and benzene-exposed groups. Initial findings indicate not only that these specific changes are significantly elevated in otherwise healthy workers, but that this increase is dose-dependent. More studies are underway to explore changes in all chromosomes to determine whether a specific pattern of cytogenetic damage is associated with benzene exposure.

Environmental Pollution and Biomarkers in Children

Children's exposure to environmental pollution is likely to be greater than that of adults because of differences in metabolism, sensitivity, and outdoor activity. However, few data are available on the background level of cytogenetic damage or the effects of environmental pollution in children. The few studies available indicate that in some cases children's exposures may be comparable with occupational exposures. In a study of inner city children in Baltimore, trans-trans-muconic acid was used as a biomarker of environmental exposure to benzene (Weaver et al. 1996). As many as 10% of 79 children had levels greater than the highest reported among adults not exposed by occupation.

Another indication that urban pollution, including ozone exposure, may cause increased DNA damage in children was obtained in a study performed in Mexico City (Calderon-Garciduenas et al. 1996). Using a "comet" assay which measured strand-breaks of DNA in respiratory epithelial cells, the authors showed that significantly higher levels of damage are present in epithelial cells of exposed individuals compared to newcomers who had never been exposed to the urban pollution of Mexico City.

Millions of people living near chemical waste sites around the world are vulnerable to high risks of exposure by inhalation, and biomarkers represent a promising approach in monitoring their exposure. In a study conducted in Belgium, MN and SCE were repeatedly analyzed in two villages located close to a chemical waste site. Initial testing was prompted by reports of damage to the site's container and high measurable levels of several chemicals including benzene, xylene, etc. (Vleminckx et al. 1997). Levels of MN were significantly elevated in children in the exposed groups. After remedial measures, the children's MN levels normalized. In this study, children were more susceptible overall to the effect of inhalation exposure to chemicals escaping from the waste site. All these data emphasize the importance of validating cytogenetic biomarkers in children, and of studying changes associated with traffic pollution, second-hand smoking, and proximity to industrial facilities and chemical waste sites.

Recent findings suggest that air pollution is associated with genetic damage in respiratory epithelia. However, few data are available on the background level of cytogenetic damage in children, and almost no studies have

been performed to assess the effects of air pollution in this most vulnerable segment of the population. While all children are vulnerable to environmental hazards, minority children and children of lower socioeconomic status are particularly at risk because they tend to live in less healthful environments. These groups also have an elevated risk of leukemia and other diseases. By establishing new monitoring and preventive biomarkers of air pollution, we can better protect the health of children.

We are developing an approach for the collection and experimental analysis of samples from inner-city children. African-American families have been recruited for this pilot study from the healthy child clinic at Children's Hospital Oakland in Oakland, CA. Samples of buccal epithelia, urine and blood were collected, and questionnaires were completed which addressed proximity to freeways and gas stations, second-hand smoke in the household, vitamin consumption, vital statistics, socioeconomic status and other factors which may contribute to the risk assessment. Provisions were made to measure levels of folate, vitamin B12, cotinine, and t-t-muconic acid among both children and adults. Analysis of cytogenetic damage will be performed by MN assay in three cell types: peripheral blood lymphocytes and buccal and urothelial cells. Preliminary data indicate a broad variability in the levels of MN in both lymphocytes and epithelial cells of children. Moreover, contrary to expectations, children had slightly higher levels of MN in both cell types than adults from the same area. Usually, there is an age effect on the level of chromosome damage, in which the lowest level is observed in newborns with a gradual increase in adults and especially in older individuals (Fenech et al. 1999; Tucker and Preston 1996). However, it is too early to conclude whether this inverted ratio in the MN frequencies between a small group of inner city children and adults will be confirmed after analysis of a larger group, and whether this phenomenon may be associated with increased exposure among some children to traffic pollution or other factors of their environment.

Summary and Conclusions

Cytogenetic biomarkers are sensitive and helpful tools in exploring adverse health effects of air pollution. More work is needed to validate novel methods, presenting exciting opportunities to establish mechanisms of diseases associated with inhalation of chemicals and possibly to prevent their impact on the population. The examples of formaldehyde and benzene show that many aspects of risk assessment of exposure to chemicals via inhalation may be improved by using adequate cytogenetic biomarkers of effect in relevant target tissues. There is a growing need to apply these methods not only to occupationally-exposed groups but also to environmentally-endangered or more susceptible populations. Information on cytogenetic biomarkers in children is nearly nonexistent, and more studies in this field are urgently needed.

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