CHAPTER 5

DISCUSSION

A major goal for human biomonitoring is to provide precise information on exposure and health risk assessment for effective prevention of the health problems in populations that are potentially exposed to environmental toxic chemicals. The study requires accurate, sensitive, easy and less time-consuming methodologies to assess mutations. Various genetic endpoints have been used for monitoring human populations exposed to environmental mutagens. Blood cells has been frequently used as sentinel cells type to detect biological effects and to provide early warning signals for the health risk (Salama *et al.*, 1999).

In this study, the biomonitoring was performed using three genetic endpoints, namely chromosome aberration (CA), micronucleus (MN) and DNA damage detected by comet assay. The studied populations are from Saraphi and Chom Thong districts, Chiang Mai province, the areas with high and low incidence of lung cancer, respectively. There were differences between effects in cytogenetic tests (chromosomal aberration and micronucleus tests) and comet assay which were basically due to variation of DNA alteration that each technique detected. Therefore, data analysis was separately divided into the comparison of the basal level of CA and MN frequencies and the comparison of DNA damage detected by comet assay, between the studied populations.

Chromosome aberration test is the most extensively used and best validated biomarker of early biological effects in population studies (Albertini *et al.*, 2000; Kirland, 1998). The assay has been successfully used to detect exposure to ionizing radiation. Ionizing radiation specifically induces chromosome-type aberrations such as dicentric and ring chromosomes that are easily detected (Heimers, 2000; Stephan and Pressl, 1999). Exposure to chemical mutagens usually leads to the induction of chromatid-type aberrations such as breaks and exchanges. Cells with certain chromosome aberrations can survive and can lead to the development of cancer as indicated by the presence of various chromosome abnormalities described for different types of cancer (Smerhovsky *et al.*, 2001; 2002).

Lung cancer risk is known to be strongly correlated to exposures towards environmental carcinogens. However, no correlations were observed between lung cancer risk and CA frequency in our study. The aberration frequency in the high-risk area, the Saraphi district, was low and the distribution of the various types of aberrations did not indicate anything specifically. There is no characteristic difference between the two studied populations besides the fact that the aberration frequencies are higher in Chom Thong population. However, even in Chom Thong population, the aberration frequency of about one percent was within the normal range found in our laboratory and others (Michalska *et al.*, 1999; Scarpato *et al.*, 1996). Although there was statistically different in mitotic index between the two studied groups, the difference in cell proliferation was not much. However, the mitotic index differences might explain differences in aberration frequencies.

The lower chromosomal frequency observed in the area supposing to expose to higher environmental pollutants, Saraphi district, might be explained by the various

independent events. The chromosome damage rate in population with extensive genotoxicant exposure might be declined over time due to an adaptation mechanism being produced to the pollutants or an equilibrium being reached between chromosome damage (Vaglenov *et al.*, 1998). Moreover, lymphocytes with high DNA damage could die because of high genomic instability and/or the DNA damage might be entirely repaired in the cells (Heimers, 2000; Meng and Zhang, 1997; Zeljezic and Garaj-Vrhovac, 2001). Additionally, the increasing of chromosome aberration frequency in subjects who smoked cigarette and chewed fermented tea leaves and/or betel nut was observed in the present study, and such subjects were higher in Chom Thong. Therefore, this might be the reason of the elevated chromosomal aberrations shown in the population residing in Chom Thong area.

Because the analysis of chromosome aberrations is labor-intensive and needs an extensive experience, alternative assays are under investigation. The micronucleus test is an alternative of the chromosome aberration test. Micronuclei are detected in G_1 cells after the cells have progressed pass the first cell cycle. The major advantages of this assay over the traditional analysis of chromosome aberrations are that it is less labor-intensive and besides the clastogenic events it can also detect aneuploidy (Fenech, 1993; 1997). Moreover, the micronucleus test has more statistical powers due to higher number of cells analyzed (Miller *et al.*, 1997). In our study, the MN test indicates the increased MN frequency in the Saraphi population. The data for the NDI also indicates that the proliferation of lymphocytes is rather similar between the two populations. Thus, the differences in micronucleus frequencies cannot be due to different growth of the blood cultures.

With regard to the confounding factors, cigarette smoking and chewing of fermented tea leaves affected CA frequency, while only gender affected micronucleus induction. Nevertheless, the different influence of the confounding factors on both of CA and MN endpoints have also been reported in several studies, for example, age and gender (Bukvic *et al.*, 2001; Fenech *et al.*, 1997; 1998; Huber *et al.*, 1992), pesticide exposure (Carbonell *et al.*, 1995; De Ferrari *et al.*, 1991; Gómez-Arroyo *et al.*, 2000; Titenko-Holland *et al.*, 1997), smoking habit (Fenech, 1998; Huber *et al.*, 1992; Perara and Whyatt, 1994; Tompa *et al.*, 1994), alcohol drinking (Maffei *et al.*, 2000; 2002) and chewing of fermented tea leaves or betel nut (Mahimkar *et al.*, 2001; Nakachi *et al.*, 1999; Simarak *et al.*, 1977).

The influence of the confounding factors on the genetic endpoints can be modulated by different factors. It should be due to the differences on the intrinsic factors, such as age, gender and/or extrinsic factors, for example diet and an environment of the studied populations. It is accepted that gender and many dietary constituents markedly influence the adverse effect of the genotoxic substances on micronucleus frequency (Fenech and Rinaldy, 1994). Diet, in particular, will be a key factor in determining chromosome damage in humans. Many micronutrients, mineral and vitamins act as substrates and/or co-factor in DNA maintenance reactions (Fenech *et al.*, 1998).

Theoretically, the CA and MN tests similarly detect exposure to clastogens. The combination of the two tests has usually been observed in biomonitoring in populations exposed to mutagens/carcinogens. However, CA and MN tests reflect different genetic mechanisms and an association between both endpoints has been still uncertain, for example, the monitoring study in the floriculturists in Italy did not show

the correlation of the two tests (Scarpato *et al.*, 1996). Moreover, the results from studies involving workplace exposures and controls using the CA and MN tests were not consistent. Some studies showed inconclusive or negative results of chromosomal aberration with other endpoints on environmental occupational exposure (Carbonell *et al.*, 1993; Michalska *et al.*, 1999; Tompa *et al.*, 1994) while some studies showed significantly higher both on CA and MN frequencies in exposed workers than those from control (Meng and Zhang, 1997; Testa *et al.*, 2002). In addition, one study found that the difference between CA frequencies in the exposed and control was of borderline statistical significance (Carere *et al.*, 1995), whereas another study showed significantly higher CA frequency but there was no significantly difference on MN frequency between exposed workers and controls (Joksić *et al.*, 1997).

The main conclusion of this study is that, there are no biologically significant differences in exposure between the two studied groups which can be detected by these two cytogenetic tests. The association between the CA and/or MN frequencies and long term biological consequence has not been elucidated yet as described by Fenech *et al.*, 1999. The statistically different results measured in both assays might be by chance, which is not unlikely considering the low level of significance. It is also unlikely that the MN test detects either specifically or with higher sensitivity of an exposure, which is not detected by the chromosome aberration test.

The lack of consistency of cytogenetic damage related to the environmental exposure between the exposed and control populations could reveal that some of confounding factors and biological factors showed a relationship with the variable analyzed. Since the cytogenetic assays depend on many endogenous and exogenous factors including various environmental pollutants (Bukvic *et al.*, 2001; Pastor *et al.*,

2001). The damage will depend, not only on exposure to clastogenic substances, but also on the capacity of the system to metabolite the agent and the ability of DNA repair mechanism to maintain genomic integrity (Lucas *et al.*, 1999). Moreover, the experimental variables and biological factors should be impact on the cytogenetic results (Fenech, 1998).

The other biomonitoring study in Saraphi and Chom Thong populations was performed by comet assay. Peripheral whole blood and stimulated lymphocytes in the absence and presence of aphidicolin were comparatively investigated to determine possible differences in baseline DNA damage. The comet assay detects DNA strand breaks and DNA modifications which induce DNA labile under alkaline conditions, leading to additional breaks during the assay procedure (Kassie *et al.*, 2000). The sensitivity of our protocol was ensured in the preliminary experiments. The optimal combination of the duration of alkaline treatment and electrophoresis was defined because minor DNA migration was seen in control cells under the experimental conditions as recommended by Tice *et al.* (2000). The additional of internal standard in the electrophoretic step was also performed to minimize the experimental variations as suggested in IPCS guideline for monitoring of genotoxic effects of carcinogen in humans (Albertini *et al.*, 2000).

The overall of comet assay results did not reveal a clear difference between two populations living in areas with significantly different of lung cancer incidences. Only the marginal but statistically significant difference between the two studied populations was seen in peripheral blood when measuring the tail length. It might indicate a difference in nature of genotoxic exposure, since the residents of Saraphi had more DNA damage than the residents of Chom Thong. However, the biological

significance of this finding has to be questioned because it is not seen with the other image analysis parameters.

Tail length has been the most common parameter used for DNA damage measurement. It relates directly to fragment size of DNA and would be expected to be proportional to the extent of DNA damage. It increases rapidly with dose at low levels of damage, but soon reaches its maximum. It is therefore the most sensitive parameter at near background levels of damage (Tice, 1995). Recently, tail moment is an increasingly popular method of comet evaluation incorporates both amount of damaged DNA in the tail and the distance it migrates (Olive *et al.*, 1990; Singh, 2000; Speit and Hartmann, 1999). Tail intensity presents the percentage of DNA in the tail. Tail moment bases on the multiplication between tail length and tail intensity, or with percentage of migrated DNA. However, tail moment has not been approved as the most appropriate parameter. Therefore, the use of computerized image analysis systems to collect comet data should be investigation by various parameters. The fact that there was no difference in all parameters between the two studied populations leads us to the conclusion that an unequivocal difference cannot be stated.

Besides the DNA damage itself, elimination of induced damage by DNA excision repair activity can also contribute to the DNA effects seen in the comet assay. The contribution of excision repair to the comet assay effects can be measured by using inhibitors of DNA repair, aphidicolin; a DNA repair inhibitors which inhibits the repair synthesis but not the incision step. If more DNA damage is present, more repair activity should occur, and as a consequence, enhanced DNA effects in the comet assay should be expected. Thus, the additional application of repair inhibitors has been suggested for the use of the comet assay in human biomonitoring to increase

its sensitivity (Mayer, *et al.*, 2002; Schmezer *et al.*, 2001; Tice, 1995). However, no difference was revealed with this approach in our study. It might be concluded that biomonitoring study, using peripheral blood and measuring by the parameter tail length, was appropriate for detecting the difference on DNA damage between the two populations by comet assay.

The lack of consistency of DNA damage measured by comet assay, between the two populations was considered. The result was not unexpected. Even so DNA damage between lung cancer patients and controls were not different. Nevertheless, the results revealed that lung cancer patients had significantly higher sensitivity to the mutagen-induced DNA damage than that in controls (Rajaee-Behbahani *et al.*, 2001; Schmezer *et al.*, 2001). There was a potential that the environmental genotoxic substances these two populations had exposed, was not at the level of causing their DNA damage. Moreover, it could be indicated that comet assay can measure identified hazard rather than risk (Albertini *et al.*, 2000).

It might be suggested that, although the comet assay may be generally considered as a more sensitive method to assess DNA damage, it could not be concluded that chromosome aberrations and micronucleus assays would likely be much less sensitive than the comet assay because they detected the damage with different mechanisms. Chromosome aberration is widely accepted as an early effect biomarker in the surveillance of human exposure to carcinogen. Micronucleus assay can detects the chromosome damage from chromosome breakage (clastogenesis) and spindle disruption (aneugenesis) but it does not require metaphase cells. Comet assay is a method for evaluation of DNA damage (strand break, alkaline labile sites, DNA cross-linking and incomplete excision repair sites) in individual cell.

With regard to the health risk assessment, three genetic endpoints were still not generating the type of reliable information needed for precise risk assessment. Some of the problems are due to an inconsistent observation of biological effects from similarly exposed populations, lack of predictable dose-response relationship and existence of inter-individual variation in response to exposure. Studies using inappropriate experimental protocol and/or existence of polymorphic genes which influence biological effects from environmental mutagens may account for some problems (Au *et al.*, 1998).

Furthermore, the traditional approach usually uses the readily available blood cell, e.g. lymphocytes and red blood cell to document biomarker of effect. However, such approach still cannot be reliably used for developing strategies in risk assessment and disease prevention. Moreover, the confounding factor, such as smoking, on the CAs, MNs, DNA damage by comet assay, in lymphocytes are not consistent. The relationship between expression of biomarker effect in blood cell and health outcome have not been well documented (Salama *et al.*, 1999).

The usefulness of available non-blood cells for biomonitoring, in our case the nasopharyngeal cells, was suggested (Brittebo, 1997; Flato et al., 1996; Zhang, 1994). It is obvious that the decision in using non-blood cells for biomonitoring is based on exposure condition and the experimental design. For example, it is necessary to elucidate whether the dose-response to specific exposure conditions are reproducible, whether these non- and/or blood cells are high sensitivity and specificity to the exposure and whether especially, they can provide the precise prediction on health effect.

In summary, our chosen three short-term assays cannot precisely monitor and assess an anticipated high environmental toxic substances exposure in our studied populations. The results show that Saraphi population did not associate with the lung cancer incidence or any adverse outcomes. The associations between genotoxic evaluations and the exposure to complex mixtures were uncertain, since the characterization of risk could be confounded by several factors (Paster *et al.*, 2001). In addition, the assessment will be more problematic when the number of the compounds in the mixtures increased and when the empirical data are insufficient.