Chapter 2

Introduction

Exposure to asbestos is well-known causative factor in the development of malignancies like bronchogenic carcinoma and mesotheliomas of the pleural and peritoneal cavity respectively (Mossman et al., 1990) Asbestos is well recognized environmental carcinogen (WHO, 1986) yet the mechanism is not fully understood. The several alterations at the chromosomal levels are known to play key role in the initial stages of neoplastic progression (Barrett et al., 1990). Asbestos does not cause gene mutation but is known to induce cytogenetic alterations in variety of cells e.g., lung epithelial cells, Syrian hamster embryo (SHE) fibroblasts (Dopp et al., 1995), rat mesothelial cells in culture (Jaurand et al., 1986), lymphocytes of occupationally exposed workers (Rahman et al., 2000) and human amniotic cells (Dopp et al., 1997). There are two known possible causes of these cytogenetic changes, either by direct physical interaction with spindle fibers or indirectly with the generation of reactive oxygen species (ROS) (Kamp and Weitzman, 1999). The mechanical interaction of asbestos fibers with chromosome is dependent upon the involved cell and fiber type. Previous studies, have suggested, that the production of reactive oxygen species (ROS) such as superoxide anion (O$_2^-$), hydroxyl radical (OH) as well as reactive nitrogen species such as nitric oxide (NO) are related to asbestos genotoxicity (Kamp and Weitzman, 1999).
The ROS production induced by asbestos exposure is suggested by two probable mechanisms (Kamp and Weitzman, 1999). The first pathway is the participation of redox active iron (Fe$^{2+}$, Fe$^{3+}$) which the formation of OH$^-$ radicals through Fenton's reaction and the presence of iron is considered to be as a surface impurity on asbestos fiber and also as an integral constituent in the crystalline structure of asbestos. The chain of reactions in which H$_2$O$_2$ is converted to HO is called the iron catalyzed Harber-Weiss reaction. The second probable pathway involves the interaction of asbestos fibers with various leukocytes including neutrophils and macrophages. The elicitation of these phagocytic cells and its subsequent interactions with fibrous particulate are an important response reported in lung and mesothelium after asbestos exposure (Brody et al., 1981; Maolli et al., 1987). The phagocytic cells can produce whole array of RO intermediates associated with respiratory burst mechanism which is often associated with phagocytosis. Asbestos is known to stimulate the release of O$_2^-$ and H$_2$O$_2$ from PAMs and neutrophils (Kamp and Weitzman, 1999).

Cells from bronchi alveolar lavage (BAL) of animals exposed to asbestos and other airborne particle have been used to report the cytotoxicity and genotoxicity of fibers and particle (Mossman and Begin, 1989). The persistence of inflammatory cells is known to be a pivotal in the progression of asbestosis and releases several cytokines after asbestos exposure (Driscoll et al., 1996). Macrophages are the most abundant in the BAL fluid and are known to be the first line of defense against inhaled particles and are known to mount up
in the alveoli when exposed to inflammatory genotoxic airborne agents. In addition, macrophages phagocytise, metabolise and detoxify the inhaled toxic substances (Au et al., 1988; Evans et al., 1973; White and Garg, 1981)). The cells that are exposed to inhaled agents in high concentration can more accurately determine the possible genotoxicity induced by inhaled toxic agents and pulmonary alveolar macrophages (PAM) are such cells. Macrophages are not known to divide under normal conditions until unless they are confronted and thus, PAMs can serve to be responsive indicator cells for sensing inhaled mutagens and carcinogens (Evans et al., 1973).

The use of PAMs to monitor Cytogenetic damage by inhaled toxic agents was introduced by Conner et al, (1979). Further the technique was improved for CA in PAMs where 50 metaphase plates cells per animal were attained in cigarette smoke exposed rats (Rithidech et al.,1989). CA in PAMs was also done in rats exposed to formaldehyde and mosquito coil smoke (Scott and J.B.1983; Das et al., 1994)). A simple and short term micronucleus (MN) test in PAMs of rats was developed to study the cytogenetic alteration induced by mosquito coil smoke and also mosquito mat vapor (Sahu and Das, 1995). Intratracheal instillation of silica was also seen to induce MN in PAMs of silica exposed rats (Leigh et al., 2000). Thus, in the present study PAMs are investigated for cytogenetic alterations such as chromosomal aberrations (CA) and micronucleus (MN) induced by intratracheal instillation (i.t.) of chrysotile in rats.
Earlier studies done using iron chelators, ROS scavengers, antioxidants like deferroxamine, phytic acid, ascorbic acid, rutin have shown to attenuate asbestos induced genotoxicity (Fubini et al., 1995; Hardy and Aust, 1995; Kamp et al., 1995; Korkina et al., 1992). Glutathione (GSH) and glutathione redox system play important role in the defense against oxidative damage in mammalian cells and previous studies in our laboratory have reported that, there is depletion in the level of glutathione upon asbestos exposure (Khan et al., 1990; Arif et al., 1997; Afaq et al., 1998; Abidi et al., 1999). Organosulphur (OSC) compounds like garlic and onion have shown protective effects against cancer, which have been shown in many experimental studies with animal models (Sparnins et al., 1988; Reddy et al., 1993 ; Harber-Mignard et al.,1996). An important constituent of garlic is diallyl sulphide (DAS) and our laboratory has reported that it protects mesothelial cells against asbestos induced genotoxicity (Lohani et al., 2003). The organosulphur compounds anticarcinogenic properties are suggested to be mediated through inactivation of oxygen species by radical-scavenging sulphur-contaning compounds (Bianchini and Vainio, 2001). One of the suggested mechanisms by which these organosuplhur compounds protect from cancer is probably by modification of carcinogen metabolism via the modulation of drug metabolizing enzymes (Guyonnet et al., 2001). Organosuplhur had shown to alter cytochrome P450 (CYP) and phase 11 detoxification enzymes in many tissues in rodents (Guyonnet et al., 1999; Brady et al., 1988; Haber et al., 1995; Siess et al., 1997; Munday and Munday, 1999).
CYP plays pivotal role in the metabolism of chemical carcinogen because they catalyze either detoxification reactions or chemical activation resulting in the formation of toxic metabolites (Guyonnet et al., 2001). The resultant of this metabolism depends on CYP isoenzymes and on the type of carcinogen. In the contrary, phase 1 enzymes are commonly involved in detoxification pathways by catalyzing the elimination of reactive intermediates of carcinogens (Brady et al., 1988; Haber et al., 1995; Siess et al., 1997; Haber et al., 1994). Thus, the phase 1 enzymes are considered as a chief mechanism underlying in the protection against carcinogenesis.

In the phase 1 enzymes, glutathione S-transferases (GST) plays important role in the cellular protection against carcinogens by conjugating their electrophile metabolites with GSH (Seidegard and Ekstrom, 1997; Eaton and Bammler, 1999). Experimental studies have reported that OSCs tremendously enhances GST activity in various organs in rodents (Spanins et al., 1988; Reddy et al., 1993; Guyonnet et al., 1999; Munday and Munday, 1999). Thus, in the present study, analysis of BAL inflammatory cells types from chrysotile exposed rats is performed to examine the inflammatory status of the lower airways using differential cell count. PAMs are investigated for cytogenetic alterations such as chromosomal aberrations (CA) and micronucleus (MN) induced by chrysotile exposed rats. Further the study was extended to study the effect of aqueous extract of garlic (Allium sativum) and one of its constituents DAS against asbestos (chrysotile) induces genotoxicity in PAMs where MN is the parameter.
Material and method

Dust

Indian chrysotile asbestos was obtained from a local asbestos factory mined at Cuddapah, Andra pradesh, India. Particle size below 30μm of the chrysotile was prepared by Zaidi (1969).

Chemicals

All the Chemicals and reagent used were purchased from either Sigma (ST Louis, MO) or Sisco Research Laboratory (Bombay, India) and were of analytic Grade.

Garlic Preparation

Garlic extract 10% (w/v) of garlic bulbs (*Allium sativum* single clove variety) was prepared from freshly sliced cloves, grounded into paste, and dissolved in deionized water. The dose (intragastric, i.g.) given to experimental rat was 1% body weight (v/w), 6 days per week throughout the experimental phase.

Animals and their treatment for Differential cell count, chromosomal aberrations and Micronucleus

Female albino rats weighing 150± 10 g from the Industrial Toxicology Research Center animals colony were used for the study.

Chromosomal aberrations

Animals were divided into two groups. The first group received single chrysotile asbestos (5mg/0.5ml normal saline) intratracheally as describe by Zaidi (1969). The second group received normal saline only, which served as control. The rats
of both the groups were fed on standard pellet diet supplied from (Amrut feeds, Pune, India) and tap water ad libitum. They were housed in an air-conditioned room with the arrangement of 12-hr dark and light cycles. After 8th and 15th day the first and the second group of rats were sacrificed to obtain their lung lavage.

Total and differential cell count

Animals were divided into two groups. The first group received single chrysotile asbestos (5mg/0.5ml normal saline) intratracheally as describe by Zaidi (1969). The second group received normal saline only, which served as control. The rats of both the groups were fed on standard pellet diet supplied from (Amrut feeds, Pune, India) and tap water ad libitum. They were housed in an air-conditioned room with the arrangement of 12-hr dark and light cycles. After 1st, 8th and 15th day the first and the second group of rats were sacrificed to obtain their lung lavage.

Micronucleus induction

Animal were divided into four groups. First group received a single i.t. inoculation of 0.5 ml normal saline and served as control. Second group received a single i.t. inoculation of 5mg chrysotile (in 0.5ml saline), the third group received single intratracheal (i.t.) inoculation of 5 mg chrysotile (in 0.5ml saline) as well garlic (i.e., 1% body weight (v/w), 6 days per week. Fourth group received chrysotile i.t. same as third group as well as DAS at a 400mg/kg body weight. The animals
were sacrificed on 1st, 8th and 15th day in all the groups to study the MN induction.

**Isolation of alveolar macrophages for total and differential cell count**

The animal’s lungs were lavaged three times with 5ml Ca²⁺ and Mg²⁺ phosphate-buffered saline (PBS) (0.1M; pH 7.4) maintained at 37°C in situ using a closed technique (Brain, 1970). The recovery of lung lavage fluid was 85± 5%. The recovered BAL was centrifuged at 400 x g for 10 min at 4°C in order to separate the cells from supernatant. Then the cells were washed twice with PBS and the total cell count was done using hemocytometer as described earlier (Arif et al., 1997). The viability of cells was done by trypan blue exclusion test (> 90% viable cells). According to the total cells count an aliquot of cell suspension was diluted with normal saline solution to give an end concentration of approximately 1 million cells/ml. Smear was made and stained with Wright –Giemsa to performed differential cell count. At least 500 cells per smear were counted by light microscopy. Cells differentially counted were macrophages, neutrophils, lymphocytes, and eosinophils. Results were reported in percentage.

**Isolation of alveolar macrophages for MN**

Their lungs were lavaged three times with 5ml KCl (0.56%) solution which was maintained at 37°C in situ using closed Chest technique (Brian). The collected
fluid was immediately centrifuged at 1000 rpm for 2 minutes and the supernatant was decanted off. A thick homogenous suspension of cells was prepared in a leftover drop of KCl. A small drop of the final suspension was put on a clean slide and smear was drawn with folded margin of thick aluminum foil instead of a vertical slide. The rest protocol was same as Sahu and Das et al., 1995. PAM was identified by their large size, vacuolated cytoplasm and non ciliated margin. A minimum of 1000 PAM was scored to determine the proportion of micronucleated cells. The following criteria were used to identify micronuclei: color the same as main nucleus; diameter less than half of main nucleus; location within the cytoplasm as a round body completely separated from the main nucleus (Fenech, 1993).

Chromosomal aberration
Isolation of alveolar macrophage
Their lungs were lavaged three times with 5ml Ca$^{2+}$ and Mg$^{2+}$ free phosphate Buffered saline (PBS) (0.1M; pH 7.4) maintained at 37°C in situ using a closed Chest technique (Brain, 1970). The recovery of the lung lavage fluid was 25ml per animals. At 4 hr before sacrifice rats were injected intra peritoneally with colchicine dissolved in saline at a concentration of 6mg/ml. The injection volume was adjusted (0.2-0.3ml) to result in a final dose per animal of 6mg/kg-body weight. Conventional cytogenetic methods were used to prepare metaphase cells from the cell suspension. Briefly, the cell suspension was centrifuged at 1,000
rpm for 10 minutes. The supernatant was discarded, 5ml of 0.075M KCL which
was prewarmed at 37°C were slowly added and the cells were mixed thoroughly
before incubation at 37°C for 20 minutes. Following further centrifugation, cells
were fixed by resuspending in freshly prepared fixative (1:3 v/v acetic acid and
methanol). Centrifugation and resuspension of the cells in fixative were repeated
three times before slides were prepared three times before slides were prepared
using air-dried technique. All Slides were stained in 4% Giemsa (pH 6.8) for 8
minutes. The slides were coded for chromosome aberration analysis. Fifty
metaphase cells per rats were analyzed for structural changes.

Statistical analysis
In the present study Student t-test (two-tailed) was performed for the statistical
analysis and p<0.05 was considered as significant.

Result
Total cell and differential cell count

Total cell count increased significantly on the 1st day (p<0.05), 8th day
(p<0.001) and 15th day (p<0.001) after exposure (Table-2.1). There was a sharp
rise of neutrophils on the 1st day after chrysotile exposure (p<0.001) and was
high up to 8th day (p<0.001) after exposure but showed decline on 15th day after
exposure. Macrophage on the other hand showed a significant decline on the 1st
day (p<0.001) but slowly rose near to the control value on 15th day. On the 8th
day few binucleated macrophages were seen and on the 15th day some
multinucleated giant cells were also seen (~5 %). Eosinophil did not show any
significant change as compared to the control but lymphocytes showed a significant increase on the 15th day (p<0.05). The Indian chrysotile exposure led to the significant increase in the mitotic index on the 8th day (p<0.001) and 15th day (p<0.001) (Table-2.2).

**CA and MN induction**

Further the Indian asbestos fibers also induces Indian asbestos fibers induces significantly higher number of CA on the 8th day (p<0.001) and on the 15th day (p<0.001) after chrysotile instillation (Figure-2.1). Most of the chromosomal aberrations were chromatid breaks type and some were chromatid gaps. Some of the chromosomes were pulverized too. There was a higher induction of MN on the 1st day (p<0.05), 8th day (p<0.05) and on the 15th day (p<0.05) after chrysotile exposure (Figure-2.2).

**Garlic and DAS supplementation on MN induction**

Treatment of garlic and DAS resulted in a clear reduction of MN induction (Figure-2.2 and 2.3). There was sharp percentage decrease in the MN induction on the 1st day (150 %), 8th day (207%) and 15th day (316%) after garlic administration. The administration of garlic was not only alleviated the MN induction but lowered the induction below the control value. On DAS treatment the percentage decrease was 57% on the 1st day, 90.476% on the 8th day and 100% on the 15th day after treatment and was not high as compared to garlic.
<table>
<thead>
<tr>
<th>Days after exposure</th>
<th>Control</th>
<th>Chrysotile</th>
<th>Control</th>
<th>Chrysotile</th>
<th>Control</th>
<th>Chrysotile</th>
<th>Control</th>
<th>Chrysotile</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td></td>
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<tr>
<td>1st day</td>
<td>3.338</td>
<td>6.566*</td>
<td>95.926</td>
<td>60.802*</td>
<td>0.574</td>
<td>25.808*</td>
<td>2.556</td>
<td>2.614</td>
<td>0.74</td>
<td>0.764</td>
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<td>SD</td>
<td>0.188</td>
<td>0.1936</td>
<td>0.983</td>
<td>0.49</td>
<td>0.171</td>
<td>0.422</td>
<td>0.525</td>
<td>0.266</td>
<td>0.195</td>
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<td>n</td>
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<td>5</td>
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<td>5</td>
<td>5</td>
<td>5</td>
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<td>3th day</td>
<td>3.424</td>
<td>8.014*</td>
<td>93.466</td>
<td>73.662*</td>
<td>2.29</td>
<td>22.736*</td>
<td>3.2</td>
<td>3.142</td>
<td>1.124*</td>
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<td>SD</td>
<td>0.088</td>
<td>0.105</td>
<td>0.664</td>
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<td>0.614</td>
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<tr>
<td>5th day</td>
<td>3.532</td>
<td>10.1*</td>
<td>95.4</td>
<td>86.632*</td>
<td>1.104</td>
<td>3.385*</td>
<td>2.494</td>
<td>6.24*</td>
<td>0.436</td>
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<tr>
<td>SD</td>
<td>0.203</td>
<td>0.233</td>
<td>1.338</td>
<td>0.702</td>
<td>0.265</td>
<td>0.389</td>
<td>0.447</td>
<td>0.426</td>
<td>0.148</td>
<td>0.107</td>
</tr>
<tr>
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<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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</tr>
</tbody>
</table>

SD, Standard deviation

n, number of animals

Table 2.1

Differential cell count in bronchialveolar lavage fluids on chrysotile exposure
Table-2.2

Effect of Indian chrysotile on the mitotic index in Rat Alveolar Macrophages

<table>
<thead>
<tr>
<th>Days after exposure</th>
<th>Groups</th>
<th>No of rats</th>
<th>No of cells scored</th>
<th>Mitotic index (%) (mean ± SE)</th>
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<tbody>
<tr>
<td>8(^{th}) day</td>
<td>Control</td>
<td>5</td>
<td>17689</td>
<td>0.2844± 0.010</td>
</tr>
<tr>
<td></td>
<td>Chrysotile</td>
<td>5</td>
<td>2434</td>
<td>2.059± 0.052*</td>
</tr>
<tr>
<td>15(^{th}) day</td>
<td>Control</td>
<td>5</td>
<td>17730</td>
<td>0.279± 0.008</td>
</tr>
<tr>
<td></td>
<td>Chrysotile</td>
<td>5</td>
<td>4097</td>
<td>1.4076± 0.200*</td>
</tr>
</tbody>
</table>

P<0.001; statistically significant when compared to controls.

SE, standard error.
Effect of Indian chrysotile on Chromosomal aberrations in Rat AMs

* = statistically significant as compared to chrysotile \( P < 0.001 \)

![Graph showing percentage of aberrations](image)

Days after exposure

Figure-2.1
Effect of Garlic and DAS on Chrysotile induced micronucleus

* = Statistically significant compared to Chrysotile P<0.05

Figure-2.2

% Decrease over Chrysotile with Treatment with garlic and DAS

Figure-2.3
Figure-2.4 Binucleated PAM cells 8th day after chrysotile exposure (100 x magnifications under oil immersion)
Figure-2.5 Chromatid breaks in PAM after 8th day Chrysotile exposure (100 x magnifications with oil immersion)
Figure-2.6 Chromatid gap in PAM after 15th day of chrysotile exposure (100 x magnifications with oil immersion)
Figure-2.7 PAM under apoptosis after 8\textsuperscript{th} day of chrysotile exposure and presence of chrysotile fibers adjacent to the cells in BALF.
Figure-2.8 MN induction in PAM after 8th day chrysotile exposure (100 x magnifications (under oil immersion)
Figure-2.9 Multinucleated giant cell after 15th day chrysotile exposure
Figure-2.10 Normal PAM cell in chrysotile exposed BALF on 1\textsuperscript{st} day after exposure.
Figure 2.11 Differential cell counts after 15th day of chrysotile exposure (40 x magnifications)