

**COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD,
CONSUMER PRODUCTS AND THE ENVIRONMENT**

Mechanisms contributing to the synergistic effects of asbestos and tobacco in human lung cancers

1) Following the discussion paper on the synergistic interaction between alcohol and smoking on certain cancer endpoints presented at the COC meeting in July 2008, COC members suggested a number of other known interactions which could be considered in this way. This paper examines the synergistic interaction between asbestos and tobacco smoking in causing lung cancer and the mechanisms that might underpin this synergism.

2) Cigarette smoking and asbestos exposure can both cause lung cancer in exposed individuals. Combined exposure results in a synergistic effect on lung cancer induction. Both are complex carcinogens and can affect multiple steps in the multistage process of carcinogenesis. The first report that asbestos and smoking may interact to enhance lung cancer was by Selikoff et al (1968) in an occupational study in the US. This study led to a series of epidemiological publications to further assess and validate the relationship between smoking and asbestos and lung cancer risk. Investigations were also initiated to uncover the biological basis of this epidemiologically observed interaction.

3) The exact nature of the interaction between asbestos and tobacco smoking in the induction of lung cancer has been debated among researchers. From the published literature, most systematic reviews have found a marked heterogeneity in the magnitude of the joint effect, with the interaction ranging from less than additive in some studies to more than multiplicative in other studies. Saracci et al (1977) reviewed five epidemiological studies and concluded that although the data did not allow a definitive discrimination, the multiplicative model appeared to be more plausible. Liddell et al (1977) assessed the relationship between smoking and lung cancer in a cohort of 11,000 Quebec miners and millers of asbestos. Analysis of the data, which included attempts to fit an additive or multiplicative model to the matched data, revealed that both models fitted the data, with the multiplicative model fitting slightly better. Berry et al. (1985) combined the data from six epidemiological studies to investigate the interaction between asbestos

and smoking on lung cancer and found the evidence for the nature of interaction was inconclusive. Steenland and Thun (1986) compared four epidemiological studies and although their analysis revealed that asbestos and smoking interact with respect to cancer risk, they found no consistency between the studies as to whether the interaction was additive or multiplicative. Saracci et al (1987) reviewed thirteen epidemiological studies that investigated the interaction between asbestos and tobacco smoking and the risk of lung cancer. The authors reported that a variable pattern of interaction was observed for asbestos and tobacco smoking between the studies. However, the authors did report that for the majority of studies the size of the interaction was more consistent with a multiplicative effect and that the variation in the strength of interaction may reflect real differences stemming from the fact that both asbestos and smoking act at various stages of the carcinogenic process.

4) Vainio and Boffetta (1994) reviewed 16 epidemiological studies and the evidence from their paper indicated an interaction in the multiplicative region, although the magnitude of the interaction was not uniform. They commented that an additive interaction was observed for Canadian miners and millers (Liddell et al., 1984), while for Australian underground crocidolite miners a more than multiplicative interaction was seen (Baker et al, 1986). They also commented that studies of highly exposed insulators (Selikoff and Hammond, 1975 and Blot et al., 1980) and case-referent studies of any type of asbestos exposure (Kjuus et al., (1986) and Pastorino et al., (1984)) showed a more uniformly multiplicative pattern. Overall, Vainio and Boffetta (1994) found that the epidemiological evidence for an interaction between asbestos and tobacco is clearest in studies of workers exposed to high levels of asbestos and that evidence points to an interaction that approximates the multiplicative model. Erren et al (1999) explored the strength of synergy between asbestos and smoking tobacco on lung cancer risks using data from 12 epidemiological studies. The authors found that the synergy index (S), defined as the ratio of the combined effects to the sum of the separate effects of smoking and asbestos, varied from 1.2 to 5.3, with a weighted summary value of 1.64 -1.66 and the RERI (Relative Excess Risk due to Interaction) varied from 0.88 to 30.22; AP (the attributable proportion of risk due to interaction) defined as the fraction of total lung cancer risk among those exposed to asbestos and tobacco smoke and which is attributable to the combined effects of these two factors, as opposed to separate effects, varied from 0.16 to 0.67. The authors estimated that the excess lung cancer risk arising from simultaneous exposures to

particular levels of asbestos and tobacco smoke is higher than the sum of the separate excess risks by a factor of about 1.64 (95% CI 1.33-2.03). They reported that 33 % of lung cancers among smokers exposed to asbestos were attributable to the interactive effect of the two carcinogens as opposed to their separate effects.

5) Gustavsson et al (2002) also investigated the lung cancer risk associated with occupational exposure to asbestos and its interaction with tobacco smoke. In this case-referent study, they reported that the joint effect of asbestos and smoking was estimated to be 1.15 (CI 95% 1.19 – 1.87) [lower CI > central estimate, 1.19 cf 1.15] times that predicted from the sum of their individual effects and 0.31 (CI 95% 0.11-0.86) times that predicted from their product, indicating a joint effect between additivity and multiplicity. In a meta-analysis of 31 datasets across 23 epidemiological studies, Lee (2001) showed that exposure to asbestos increases the risk of lung cancer in non-smokers, and that the joint relationship between asbestos and smoking to risk is much better described by a multiplicative than by an additive model, with the fit to the multiplicative model being generally good. However, other researchers (Liddell, 2001 and 2002) do not agree and argue that the information from case-referent studies in support of a multiplicative relationship between asbestos and smoking is “unreliable” and that the multiplicative relationship is “not generally satisfactory”. The additive model is not generally applicable either (Liddell et al 2002) and the interactive effect may not conform to any simple hypothesis. Lee (2002) responded to these comments stating that the existing data “do not clearly reject the simple multiplicative relationship between asbestos and smoking”. Lee (2002) also commented that, although more complex models may fit the data better, he doubts whether more detailed statistical analysis would shed any greater insight. Wraith and Mengersen (2007) explored a Bayesian approach to assess evidence of an interaction between asbestos exposure and tobacco and their analyses showed, using estimates of S (synergy index) and V (multiplicativity index), that the relationship is closer to multiplicative than additive.

Potential Mechanisms of interaction

6) Despite extensive investigations exploring the interactive effects between cigarette smoke and asbestos, the precise mechanisms involved at the cellular and molecular level

are unclear. Asbestos and tobacco both are complex carcinogens affecting more than one stage of carcinogenesis and may have interdependent effects on the multistage process of lung cancer (Vainio and Boffetta, 1994). A number of mechanisms have been proposed as the potential explanation for the synergy between cigarette smoke and asbestos. These include the following –

- a) Cytotoxic, genotoxic and clastogenic nature of asbestos and tobacco smoke
- b) Generation of oxidative damage, ROS and DNA damage
- c) Tobacco enhances the penetration and accumulation of asbestos in the lung
- d) Asbestos serves as a vehicle to deliver concentrated doses of carcinogens present in tobacco smoke into the cells
- e) Somatic mutations in k-ras, *FHIT* and *p53* genes

Cytotoxic, genotoxic and clastogenic nature of asbestos and tobacco smoke

7) In vitro studies have shown asbestos to be cytotoxic, clastogenic and, although it is not an Ames test mutagen, it is mutagenic in systems that detect large deletions in DNA (Nelson and Kelsey, 2002). Both et al (1994) reported that asbestos fibres can induce mutations in human lymphocytes that result in loss of heterozygosity. Loli et al. (2004) investigated the mutagenic potential of asbestos in combination with the tobacco smoke carcinogen benzo[a]pyrene (B[a]P) in vivo in the rat lung and found the combined action of amosite and B[a]P caused a synergistic (supraadditive) increase of mutation frequency in the lung, as compared to groups treated with only asbestos or B[a]P. Lohani et al (2002) found the number of micronuclei to be significantly greater ($p < 0.05$) in the lymphocytes of smokers, asbestos-exposed non-smokers as well as asbestos-exposed smokers, as compared with those from non asbestos-exposed non-smokers.

8) Increased frequencies of sister chromatid exchanges (SCE) and chromosome aberrations in blood lymphocytes have been observed in asbestos workers (Rom et al., 1983; Lee et al., 1999 and Fatma et al, 1991). It has also been reported that cigarette smokers have a higher mean SCE frequency per cell than non-smokers (Murthy, 1979). A synergistic interaction was reported between asbestos and tobacco smoke exposure in the induction of sister-chromatid exchanges in exposed humans (Kelsey et al., 1986). Among asbestos-exposed workers, it was found that lymphocytes from those who smoked cigarettes were significantly more susceptible to the induction of SCE by in vitro exposure

to benzo(a)pyrene ($P = 0.01$) than were lymphocytes from non-smokers. Dusinská et al. (2004) found a significant increase in percentage of aberrant cells (or in numbers of aberrations per cell) in asbestos-exposed workers compared with that of the non asbestos-exposed workers and found that there was a higher level of aberrations, not statistically significant, in exposed workers who smoked compared with non-smokers.

9) Jung et al (2000) demonstrated that co-exposure of bronchiolar epithelial cells in vivo to asbestos and tobacco caused an increase in DNA strand breaks and necrosis. Using the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-biotin nick end labeling (TUNEL) method to evaluate DNA strand breaks, increases in the number of TUNEL-positive epithelial cells in distinct membranes were observed in smoke and asbestos-exposed rat lungs compared to control animals ($p=0.054$). Transmission electron microscopy (TEM) verified that TUNEL-positive cells reflected primarily necrotic death of bronchiolar epithelial cells in asbestos and smoke exposed lungs. Wang et al (2000) showed DNA strand breaks could be increased significantly when human embryo lung (HEL) cells were exposed to chrysotile (CH) and cigarette smoking solution (CSS) separately for 1 hour and increased in a dose-dependent relationship when cells were exposed to CH and CSS simultaneously. Cerna et al. (2004) evaluated the effect of inhaled asbestos fibres and smoking on cytotoxicity parameters in bronchoalveolar lavage fluid in rats. They reported that smoking significantly depressed the phagocytic activity of alveolar macrophages and amplified the asbestos-induced increase of lysosomal enzyme activities such as lactate dehydrogenase and alkaline phosphatase activity and especially the activity of cathepsin D.

Generation of oxidative damage, ROS and DNA damage

10) Several lines of evidence strongly implicate reactive oxygen species (ROS) generated by cigarette smoke and asbestos as an important mechanism causing synergistic lung damage. Both cigarette smoke and asbestos can generate significant levels of ROS. The formation of hydroxyl radicals appears to be one mechanism by which tobacco smoke and asbestos can induce ROS production. Cigarette smoke can generate superoxide anions and hydrogen peroxide (Nakayama et al., 1984) and the hydroxyl radical is thought to be responsible for cigarette smoke-mediated DNA damage (Nakayama et al 1985). Ferrous iron in asbestos has an important role in the generation of the reactive hydroxyl radical via a Fenton reaction with hydrogen peroxide (Weitzman and Graceffa, 1984). Asbestos can

synergistically increase the amount of damage seen in DNA exposed to cigarette smoke and this increased damage might be due to the stimulation of hydroxyl radical formation (Weitzman and Graceffa, 1984). Similarly, cigarette tar can accumulate iron that promotes the formation of hydroxyl radicals (Pryor, 1997; Cosgrove et al., 1985). Jackson et al (1987) exposed isolated bacteriophage PM2 DNA to cigarette smoke and/or asbestos and found that $78 \pm 12\%$ of the DNA exposed to both cigarette smoke and asbestos developed strand breaks, while only 9.8 ± 7.0 or $4.3 \pm 33\%$ of the DNA exposed to cigarette smoke or asbestos, respectively, developed strand breaks. They also found significant amounts of hydroxyl radical (detected by electron paramagnetic resonance (EPR)) in DNA mixtures containing both cigarette smoke and asbestos, but no hydroxyl radicals were detected in mixtures containing cigarette smoke alone or asbestos alone. Iron chelators and hydroxyl radical scavengers prevented both the production of hydroxyl radicals and the formation of DNA strand breaks. Kamp et al. (1998) found that the combination of cigarette smoke extracts (CSE) (0.01–0.1%) and asbestos (5 mg/cm^2) caused synergistic injury to two alveolar type I-like cell lines (WI-26 and RAEC) as assessed by ^{51}Cr -release. Higher doses of each agent caused primarily additive effects on ^{51}Cr -release. CSE augmented asbestos-induced DNA damage synergistically in an ATII-like cell line (A549 cells) but additively or less in ATI-like cells. CSE plus asbestos also reduced alveolar epithelial cell (AEC) levels of GSH and ATP in a manner that was cell type dependent and not synergistic. The iron chelator, phytic acid and catalase ameliorated DNA damage in A549 cells, indicating that damage was probably mediated by the hydroxyl radical.

11) There is evidence to indicate that oxygen radicals (particularly the hydroxyl radical) can produce lipid peroxidation (Church and Pryor, 1985). Asbestos fibres by themselves cause peroxidation of lipids when added to phospholipid emulsions (Weitzman and Weitberg AB, 1985) and Goodglick et al (1989) showed that crocidolite asbestos produces measurable increases in malondialdehyde, a product of lipid peroxidation, in lavage fluid or in macrophage cultures exposed to the fibre. Churg et al (1989) suggested that additional damage to cell membranes in the form of lipid peroxidation produced by active oxygen species either in or derived from cigarette smoke may be the cause of smoke-induced enhanced fibre uptake.

12) Fibres may cause a large area of lung tissue damage and this damage involves chronic inflammation, where inflammatory cells secrete a number of agents (including growth

factors) which result in chronic proliferation of epithelial cells (Davis et al., 1986). Vainio and Boffetta (1994) reported that asbestos can cause inflammatory reactions, oxygen radical bursts and after long term exposure, fibrosis of the lung. Smoking also induces cell proliferation, inflammation and the production of ROS (Nelson and Kelsey, 1994). Haugen et al (1982) demonstrated that asbestos fibres induce cell proliferation in cultured human tracheobronchial tissues and cells. Sekhon et al. (1995) found that the combination of amosite asbestos and cigarette smoke caused a synergistic increase in the number of proliferating cells in the small airways. Lillis et al (1991) has shown that the combination of smoking and asbestos can increase the occurrence of lung fibrosis.

Tobacco enhances the penetration and accumulation of asbestos in the lung

13) Another mechanism proposed for the synergistic effect is the ability of tobacco smoke to facilitate the penetration of asbestos into bronchial walls. Experimentally, cigarette smoke has been shown to increase pulmonary retention of asbestos in intact animals (McFadden et al 1986a) and to increase the number of asbestos fibres entering into tissue (McFadden et al., 1986b). Tissue penetration by asbestos fibres can also occur in tracheal explant systems (Mossman and Craighead, 1979) and Hobson et al. (1988) demonstrated that brief exposure to cigarette smoke, followed by exposure to amosite asbestos and maintenance in culture, greatly increased the number of asbestos fibres penetrating the tracheal epithelium over a period of several days. In a follow up study, Churg et al (1989) demonstrated that catalase and superoxide dismutase (enzymatic scavengers of active oxygen species) abolish the enhanced fibre penetration effects caused by cigarette smoke. This suggests that cigarette smoke augments the penetration of asbestos fibres by an oxygen radical mediated mechanism. Tobacco smoke may also interfere with the clearance of asbestos from the lungs and enhance the lifetime accumulation of asbestos fibres. In 1995, Churg and Stevens demonstrated that cigarette smoke increases the pulmonary retention of asbestos in human subjects. They recorded elevated concentrations of asbestos fibres in the airway tissues of smokers in comparison to non-smokers for both amosite (~ 6 fold, $p < 0.02$) and chrysolite (~ 50 fold; $p < 0.006$).

Asbestos serves as a vehicle for delivery of tobacco carcinogens into the lung and enhances the metabolism of tobacco carcinogens

14) One hypothesis that has received attention involves an asbestos-mediated enhanced delivery of mutagenic polyaromatic compounds found in tobacco smoke to the respiratory

epithelium. This mechanism requires asbestos fibres to act as “carriers” mediating the transfer of carcinogens into tissues by holding them in situ for a longer period of time than would be the case for the carcinogen alone. It was demonstrated by Lakowicz (1980) that asbestos fibres can catalyse the transfer of the hydrocarbon benzo(a)pyrene from the microcrystalline state into a lipid solution. It was also hypothesised by Gerde and Scholander (1987) that surfactant phospholipids coating the lung epithelium could adhere to the asbestos fibres creating a lipid bilayer. This would allow lipophilic carcinogens from tobacco smoke to diffuse within an all lipid environment across the aqueous regions of the bronchial lining layer into the cellular membranes of the bronchial epithelium. This hypothesis seems not to be very likely since Gerde et al. (1994) proved that on most alveolar or bronchial surfaces once deposition occurs, liberation of bound PAH from fibres is extremely fast.

15) Brown et al (1983) investigated the effects of various asbestos dusts on the metabolism of B(a)P *in vitro* in an attempt to study the events post enhanced uptake into the target cell. Their results suggest that asbestos can mediate 1) the increased uptake of xenobiotics and 2) the inhibition of glucuronide conjugation. These two events could lead to an increased DNA binding and biological activity of the hydrocarbon. Further, asbestos catalyzes the oxidation of 6-hydroxybenzo[a]pyrene, a metabolite of benzo[a]pyrene, to the 6-oxobenzo[a]pyrene radical as determined by electron spin resonance spectroscopy and affects the aryl hydrocarbon hydroxylase enzyme system involved in metabolism of PAHs and other xenobiotics (Graceffa and Weitzman, 1987). Overall, this mechanism could increase the levels of toxic xenobiotic metabolites in asbestos-damaged tissue and increase the probability of malignant transformation in these areas.

Somatic mutations in k-ras, *FHIT* and *p53* genes

16) DNA damage is critical to the process of initiation in the multistage model of carcinogenesis and the joint effect of exposure to tobacco smoke and asbestos depends on the relative magnitude of the effects on mutation rates and on the rate of clonal expansion of mutated cells (Vainio and Boffetta, 1994). Somatic alterations in primary human lung tumours from asbestos exposed and unexposed patients may be important in delineating the underlying mechanisms of the asbestos/smoking synergy. In lung tumours, loss of a fragile region known as FRA3B in the short arm of chromosome 3 (3p14),

containing the tumour suppressor *FHIT* gene has been associated with asbestos–exposure and tobacco smoking (Nelson et al, 1998). Using logistic regression modelling, Nelson et al. (1998) examined the relationship between *FHIT* gene alteration and patient exposure. They found that asbestos exposure and >50 years of smoking were significantly associated with *FHIT* exon deletion. Results from a study by Pylkkanen et al. (2002) support these findings where reduced *FHIT* protein expression was common in both asbestos-exposed (67%) and non-exposed cases (59%); [OR 1.4, 95% CI 0.4-4.9]. Increased loss of heterozygosity (LOH) was observed in the lung tumours of asbestos-exposed patients compared to non-exposed cases (OR 1.8, 95% CI 0.5-5.9). They also reported that absent or reduced expression of *FHIT* was common in smokers, with no significant difference found between current smokers and non smokers (mainly former smokers) (OR 1.4, 95% CI 0.5 – 4.5).

17) Mutations at K-ras are primarily G to T transversions at codon 12 and this type of alteration has been associated with polyaromatic hydrocarbons (Nelson and Kelsey, 2002) Mills et al. (1995) demonstrated a significant association between adenocarcinoma of the lung and K-ras mutation. Husgafvel-Pursiainen et al (1993) reported a clear association of K-ras mutations with heavy life-time smoking (> or = 50 pack-years of cigarette smoking; odds ratio (OR) 4.9, 90% CI 1.2-19.5). In addition, occupational asbestos exposure showed an elevated, but non-significant, OR of 2.2 (90% CI 0.6-8.7) for these mutations. Vainio et al (1993) investigated the association of asbestos exposure and K-ras mutation in a cohort of Finnish lung cancer patients. They found that 57% of the adenocarcinoma cases and 11% of the non-adenomatous lung cancers were K-ras positive, the odds ratio for adenocarcinoma in K-ras positive patients? being significantly elevated (11.1; CI 95% 2.54 – 45.3). They also reported that asbestos exposure was predictive of K-ras mutation, though not significantly so, among the adenocarcinoma cases (odds ratio 4.9; 95% CI 0.7-34.3). For smokers, they found that the proportion of all smokers who were heavy smokers was 60% among people with K-ras mutations and 35% among K-ras-negative subjects, with a corresponding non-significant OR of 2.8 (CI 95% 0.6-13.0). Overall, their results suggest that patients with higher asbestos exposure are more likely to develop K-ras mutant cancers. Nelson et al (1999) also reported that the prevalence of K-ras mutations were higher among those with a history of occupational exposure to asbestos (Crude OR, 4.8; 95% CI 1.5-15.4) compared to those without asbestos exposure. The association also remained significant after adjustment for age and pack-years smoking

(adjusted OR, 6.9; 95% CI 1.7-28.6). Stratification of the study groups by smoking status and asbestos exposure also suggested that the association between asbestos and K-ras mutation was not the result of confounding by heavy smoking. They also found that those patients with the K-ras mutation were exposed to asbestos much earlier than those whose tumours did not have the K-ras mutation and that time from initial asbestos exposure to lung cancer diagnosis was significantly longer in the asbestos exposed group with the K-ras mutation. In an extension of the 1993 report, Husgafvel-Pursiainen et al. (1999) reported that the frequency of K-ras mutations showed an increasing trend with increasing daily smoking in males (13% in smokers who smoked < 20 cigarettes/d, 21% in those smoking 20 to 29 cigarettes/d, and 36% in those who consumed \geq 30 cigarettes/d, but not statistically significant (P for trend, 0.07). They also reported that the mutation frequency was 33% in cases with occupational asbestos exposure, and 17% in the nonexposed cases ($P = 0.07$).

18) Mutation of the p53 gene is a frequent event in carcinogenesis and as a tumour suppressor gene it is thought to act as either a transcriptional activator of multiple genes or a factor in the assembly of the initiation-replication complex. Mutational spectra of the p53 gene in lung cancer have shown cigarette smoke to be associated with induction of lesions or classes of lesions (Ryberg et al., 1994 and Suzuki et al., 1992). In a study of patients with lung cancer, Wang et al (1995) found that 29% of the patients had somatic alterations in exons 5-9 of the p53 gene. They also found that those patients with p53 mutation who were current smokers were also older and had smoked longer than those who did not have the p53 mutations. They also reported that patients with the p53 mutation were significantly more likely to have had an occupational asbestos exposure. Nuorva et al (1994) reported that asbestos exposure (measured by the microscopic detection of asbestos bodies from histologic samples of peripheral lung) and smoking were associated with p53 accumulation. However, this association with p53 was not observed by Husgafvel-Pursiainen et al (1999). They found that lung cancer patients with p53 mutation were not more common among cases with occupational asbestos exposure (39%) than in the non exposed cases (54%). Similarly, Pairon et al (1997) in a study of asbestos body measurements and questionnaire-based exposure classifications as indicators of exposure did not find increased accumulation of p53 protein among the asbestos-exposed lung cancer cases.

Discussion

19) The precise mechanism of interaction between asbestos and tobacco remains unknown but many hypotheses have been put forward in the literature. Further advances in molecular epidemiology may improve our understanding of the asbestos-smoking interaction. A difficulty is the complex and as yet unknown mechanisms involved in lung cancer caused by both agents, particularly cigarette smoking. This makes it difficult to develop testable hypotheses, and also to judge the contribution of the demonstrated joint effects of the compounds. Cancer is a multistage process, involving initiation and sustained proliferation which results in further genetic and non-genetic changes. Cigarette smoke contains a number of initiating chemicals and also acts as a powerful promoter. Hence, interactions could plausibly occur from the earliest steps of initiation, such as uptake or metabolism of mutagens, to much later stages such as autonomous growth and invasiveness. A particularly difficulty in studying possible mechanisms for the interaction is the long persistence of asbestos in the lung, which makes epidemiological studies of temporality of effects very difficult, if not impossible. Of the various hypotheses proposed, some are more plausible than others. It seems less likely that toxicokinetic interactions play a major role in the interaction, whereas genotoxic/cytotoxic effects and generation of reactive oxygen species appear more plausible. It is also likely that there are related, in that ROS are likely to contribute to both genotoxic and cytotoxic effects.

Questions for the committee

The committee is asked to consider the following questions:

1. What are members' opinion of the nature of the interaction between asbestos and tobacco, i.e. is it additive, multiplicative, other?
2. What are members' opinions of the mechanisms that have been proposed to explain the synergistic effect of asbestos and tobacco?
3. Do members consider that any other mechanisms may be of importance?
4. Do members have suggestions on experimental or epidemiological research that might shed more light on the mechanism(s) for this interaction?

COC Secretariat, October, 2008

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