Complex Mixtures of Air Pollutants: Characterizing the Cancer Risk of Polycyclic Organic Matter

by Joellen Lewtas

Complex mixtures of polycyclic organic matter (POM) are used to illustrate the scientific problems and issues associated with characterizing the comparative risk of related complex mixtures. The complexity of mixtures in which the active components are not wel characterized present special challenges, which include identifying the critical components of mixtures, their sources, and the appropriate biomarker(s) of exposure and dose; developing the appropriate experimental models for dose-response assessment; species extrapolation; and developing a scientific basis for predicting from one mixture to another. Strategies for addressing these issues include bioassay-directed chemical characterization of bioactive components of complex mixtures, apportionment methods to determine the source of biological activity and risk, DNA adduct methods to determine tissue exposure and target dose of mixtures, and comparative approaches to determining the relative similarity, potency, and risk of complex mixtures. Epidemiological data are available for humans exposed to POM from coke ovens, coal roofing tar, coal smoke, aluminum smelters, and cigarette smoke. These emissions are characterized and compared to POM from automotive emissions (diesel and gasoline), woodstove emissions, residential oil furnace emissions, and ambient air particles. The tumor potency and estimated cancer risks for these POM mixtures ranges over nearly three orders of magnitude.

Historical Perspective

In the early 1900s, the first chemicals recognized to be human and animal carcinogens were complex mixtures of coal tars and coal soot from chimneys (1). Fractionation and animal bioassay of these mixtures resulted in the identification of carcinogenic polycyclic organic matter (POM) (2,3). POM is a general term referring to a complex mixture of polycyclic aromatic compounds including many diverse classes of hydrocarbons (e.g., polycyclic aromatic hydrocarbons, PAH), substituted aromatic hydrocarbons (e.g., nitrated-PAH), heterocyclic aromatic compounds (e.g., aza-arenes). The earliest recognized sources of carcinogenic POM were derived from coal combustion and pyrolysis. In addition to coal related processes, POM are emitted from the combustion of petroleum (e.g., diesel and gasoline fuel), wood, and synthetic chemicals (e.g., plastics). Several carcinogenic PAH species are known to account for a significant portion of the cancer risk associated with POM from coal tar soot (1,2) and some petroleum combustion emissions. PAH, however, do not account for all the carcinogenic activity of several other POM sources [e.g., cigarette smoke, diesel emissions, and urban aerosol (3)]. Although benzo[a]pyrene (BaP) has been used as a marker for PAH, BaP alone is not a good surrogate for the cancer risk from POM (4). Recent improvements in quantitative chemical analytical detection methods for measuring POM species have shown that BaP is not always well correlated with total PAH content and that many other carcinogenic chemicals such as nitrated PAH, aromatic amines, and aza-arenes are present in complex POM mixtures.

The primary source of POM in air pollution is from products of incomplete combustion (PICs). The cancer risk from PICs are thought to arise primarily from the POM associated with the carbonaceous particle (often referred to as soot) component of the PIC. These particles emitted from combustion sources contain most of the POM that induces tumors in animals, mutations in cells, and has been clearly implicated in epidemiological studies as a human carcinogen (5,6). Incomplete combustion products, however, also contain gaseous chemicals that are carcinogenic, such as benzene, aldehydes, and alkenes (e.g., 1,3-butadiene), and semivolatile organic compounds that have not been well characterized either chemically or toxicologically.

The complexity of the POM emissions, estimated to contain thousands of chemicals, has generally precluded risk assessment of these emissions based on analysis of the components. Because human exposure to these POM emissions occurs as the whole complex mixture, both qualitative assessments (6,7) and quantitative assessments of the human cancer risks 7 have been based on either the whole emissions or the POM component rather than using additivity of the components (8).

Complex Mixture Issues in Risk Assessment

Complex mixtures present special problems for toxicological studies (9) and cancer risk assessment (10). The issues unique
to complex mixtures (Fig. 1) are related to the complexity of the exposures, bioavailability and dosimetry of the active components, and the predictability of the effects from one mixture to another. The complexity of mixtures presents problems in sampling and chemical analysis. Most of the chemical components of POM are unidentified and difficult to quantify. The biologically active species are rarely well characterized. In addition to these problems, the composition of many complex mixtures changes as a result of process changes, fuel variations, operational conditions, etc. Mixtures that are derived from multiple sources, such as air pollution, may have varying contributions from these sources over time. For example, woodstove emissions will often increase in the wintertime at night while automotive emissions will be highest during the peak traffic hours of early morning and late afternoon (11). Several strategies are discussed below to address these complexities and elucidate the biologically active components of mixtures and their sources.

The cancer risk of complex mixtures can be estimated without identifying the specific chemicals in the mixture responsible for causing cancer. However, identifying the specific causative agents or their chemical class is critical to several aspects of risk assessment and risk management. First, knowledge of the active or causative agent(s) facilitates exposure and dosimetry assessment. Second, this information is necessary to determine the similarity of related mixtures. Third, identification of the agents responsible for the risk of a complex mixture will usually facilitate monitoring emissions and human exposure as part of a public health or environmental regulatory measure to reduce risks. Bioassay-directed fractionation and chemical characterization has been the most successful approach to identifying biologically active agents in complex mixtures (12).

Assessment of total human exposure and dosimetry of chemical air pollutants is often difficult. Complex mixtures, however, present a challenge beyond the problems encountered with individual chemicals. The simplest approach to the exposure assessment is to measure the total mass of exposure. In the case of POM, the extractable organic matter (EOM) from inhalable or respirable particles has been used as a surrogate exposure measure. One approach to estimating the annual dose for cancer risk assessment is based on estimating the annual exposure concentration of EOM from the time spent in different microenvironmental activity zones and the inhalation rate for each zone (13). Such an approach does not take into account the biologically effective dose or dose to the target receptor, such as DNA. Complex mixtures, which are often only partially characterized with respect to chemical composition, rarely have the biologically active components quantified at the receptor. Recent advances in the application of new biomarker methods for measuring macromolecular adducts are now being used to measure DNA and protein adducts after animal and human exposure to complex mixtures (10).

The predictability of effects from one mixture to another presents one of the most difficult problems in cancer risk assessment of complex mixtures. Source categories of POM mixtures, such as coke ovens or diesel exhaust, have been assessed based on the assumption that the individual sources are sufficiently similar within a source category to consider all of the data on one source (e.g., coke ovens) in assessing the cancer risk to humans (5, 6, 14). The specific chemical composition of these mixtures typically changes over time or between individual sources (e.g., industrial plants or vehicles). The issue of how similar the different individual mixtures must be to meet the criteria of "sufficiently similar" will always continue to be a difficult issue as technologies change.

**Characterization of POM**

Bioassay-directed chemical characterization led to the identification of PAHs as cancer producing substances in coal tars (15), nitro PAHs as mutagenic substances in diesel emissions (12), and mutagenic hydroxylated nitro PAHs in ambient air (16). The first such studies (1, 2) used animal tumor bioassays, however, the development of short-term genetic bioassays provided a more rapid method to identify mutagens and potential carcinogens in complex mixtures. The Ames *Salmonella typhimurium* assay has been extensively used to identify mutagens in POM associated with both ambient air and source emissions (17), as illustrated in Figure 2 for ambient air. This approach, combined with the use of bacterial tester strains selectively sensitive to certain classes of chemicals (e.g., nitroarenes), led to the identification of nitratated polycyclic aromatic hydrocarbons (nitro-PAH) as potent mutagens in POM in carbon black (18), diesel exhaust (12) and kerosene heaters (19). Methodological advances in coupling microsuspension mutation assays to HPLC separation methods have been applied to improving bioassay-directed fractionation studies to more effectively identify compounds (20).

In the characterization of complex ambient air mixtures of POM, it is crucial to determine the extent to which different sources contribute to the whole mixture. Source apportionment of air pollution was initially conducted using dispersion methods that relied on the use of emission factors for each source and dispersion models to estimate ambient concentrations of pollutants and the contribution from each source. Advances in source apportionment of air pollution have used new receptor
modeling methods or combinations of receptor and dispersion models (21). The apportionment of mutagenic activity in a complex mixture of ambient air to the sources of this activity was reported for the first time by Lewis et al. (22). In this study, we used lead as a tracer for automotive emissions and potassium as a tracer for woodsmoke. A multiple linear regression form of receptor modeling was used to determine the sources of mutagenicity in the POM from particles of ambient air in Albuquerque, New Mexico. Wood smoke and motor vehicle emissions together accounted for over 90% of the mutagenicity in the presence of metabolic activation. Estimation of the mutagenic potency (revertants/µg extractable organic matter) of the POM traced to the motor vehicles was three times greater than the potency of wood smoke (Fig. 3).

**Exposure Dosimetry of Complex Mixtures Using DNA Adducts**

Biomarkers of human exposure to complex mixtures have only recently been developed. In the past, analysis of tracer compounds (e.g., BaP and nicotine) have typically been used as surrogates for the entire mixture. The development of 32P-postlabeling methods (23,24) for detecting DNA adducts covalently bound to DNA has had a dramatic impact in facilitating the measurement of exposures to complex mixtures at the DNA level (25). This method allows hundreds of bulky aromatic DNA adducts to be detected simultaneously at extremely low detection limits without structural knowledge of the specific adducts being detected.

We have conducted human and experimental studies of DNA adducts formed following *in vitro* and *in vivo* exposures to specific complex mixtures of POM using 32P-postlabeling methods (26). DNA adducts derived from complex mixtures of POM emitted from tobacco smoke were compared to industrial pollution sources (e.g., coke ovens and aluminum smelters), vehicle exhaust, and urban air pollution. Exposures to coke oven emissions and smoky coal, both potent rodent skin tumor initiators and lung carcinogens in humans, result in comparatively high levels of DNA adducts compared to tobacco smoke in an *in vitro* calf thymus DNA model system, in cultured lymphocytes, and the mouse skin assay (26). Using tobacco smoke as a model in human studies, we have compared relative DNA adduct levels detected in blood lymphocytes, placental tissue, bronchoalveolar lung lavage cells, sperm, and autopsy tissues of smokers and nonsmokers. Adduct levels in DNA isolated from smokers were highest in human heart and lung tissue, with smaller but detectable differences in placental tissue, blood lymphocytes, and lung lavage cells (26). No smoking-related adducts were detectable in sperm cell DNA isolated from smokers. DNA isolated from lung cells lavaged from individuals exposed to smoky coal showed substantially higher adduct levels than tobacco smokers. These studies suggest that humans exposed to these complex combustion mixtures will have higher DNA adduct

**FIGURE 2.** Bioassay-directed fractionation and characterization of highly mutagenic fractions from ambient air particulate organic matter. This illustration is adapted from Nishioka et al. (16), who identified hydroxylated nitro PAH and hydroxylated nitro azaarenes in urban air particle extracts.

**FIGURE 3.** The mutagenic activity of air particle samples plotted versus the fraction of the organic matter in the sample from wood smoke. The airshed contained only automotive emissions and wood smoke; therefore the 0.0 fraction of organics from wood smoke represents nearly 100% automotive emissions, and the 1.0 fraction represents 100% woodsmoke. Extrapolation of the regression line may be used to estimate the mutagenic potency of either automotive emissions or wood smoke from ambient mixture data. Adapted from Lewis et al. (22).
levels in target cells (e.g., lung) as compared to nontarget cells (e.g., lymphocytes) and that the adduct levels will be dependent on the genotoxic potency of the mixture.

The mouse skin tumor initiation model has been widely used to evaluate the tumor initiating activity of complex mixtures containing POM (27). The relative tumor initiating potency of emissions from coke ovens, roofing (coal) tar, and cigarette smoke have been shown to highly correlate with the human lung cancer potency of these same three mixtures (28). Therefore, we have compared tumorigenicity in the mouse skin model with DNA adduct formation in the skin and lung for complex mixtures of POM (29). Figure 4 shows the correlation of tumor initiation potency with DNA adduct formation in both the mouse lung and DNA adduct formation in vitro. The DNA adducts as detected in mouse lung are often chromatographically similar to calf thymus DNA adducts for POM-derived DNA adducts (30). The relative ranking of the mixtures, with respect to DNA adduct formation in vitro and in the mouse skin, are similar to each other and to the relative ranking of tumor potency and human cancer unit risk estimates. Tobacco smoke is the weakest complex mixture we have evaluated with respect to formation of DNA adducts in vitro per unit exposure mass, and based on studies of tobacco smoke by Randerath et al. (31), tobacco smoke ranks as the weakest mixture with respect to DNA adduct formation in mouse skin and lung after skin application, as shown in Figure 4. The human exposure of smokers to tobacco smoke POM is, however, several orders of magnitude higher than for POM from environmental or occupational exposures.

Comparison of DNA adducts detected in cells and tissues of individuals exposed to tobacco smoke and coal-related emissions suggest that studies of target cells (e.g., lung and heart cells) will increase the sensitivity of these methods while providing DNA adduct dosimetry on target cell population. The high level of individual variation in DNA adduct levels in both blood cells and lung cells of individuals exposed to high levels of these complex mixtures is an important factor in these human studies that needs to be better understood with respect to predicting risk (10,26).

Dose–Response Assessment Using the Comparative Potency Approach

The comparative potency method has been the focus of a major research effort initiated in 1979 to develop a new approach to assess cancer risk from complex mixtures of POM from diesel and gasoline vehicle exhaust (32). This approach has been used in research to improve the estimation of human cancer risk when there are no human cancer data for the specific POM mixture being assessed but there are human cancer data for a similar POM mixture (33,34). The human cancer risk of the unknown mixture may be estimated by using the relative bioassay potency of the known mixture and known human carcinogen multiplied by the human potency of the known human carcinogen. The underlying assumption in this method is the constant relative potency hypothesis. This is the hypothesis that there is a constant relative potency across different bioassay systems (e.g., human and rodent) such that:

\[
\frac{\text{Human potency carcinogen}_1}{\text{Human potency carcinogen}_2} = k \frac{\text{Bioassay potency carcinogen}_1}{\text{Bioassay potency carcinogen}_2}
\]

In this method the relative potency is determined by the ratio of the slopes of the dose responses from the same bioassay, as shown below:

\[
\text{Relative potency} = \frac{\text{Bioassay potency of carcinogen}_1}{\text{Bioassay potency of carcinogen}_2}
\]
mined mutagenicity methods hypothesis (36,37).

Continuous linear emissions) carcinogens POM animals stant.

The constant-relative assumption is implicit in any comparison that uses the relative toxicity of two substances in animals to estimate their relative toxicity in humans. This constant relative potency assumption is an experimentally testable hypothesis, if the relative potency of two mixtures or components in one bioassay (e.g., humans) can be determined and compared to the relative potency in a second bioassay. The test of this hypothesis is whether there is a constant relationship (k) between the relative potencies in the two bioassays being compared. The current limitation to our testing of this hypothesis is the availability of human lung cancer data for quantitative estimation of the human cancer risk. Research is now in progress to expand the human database to include at least one additional human carcinogen, smoky coal combustion emissions (38). The human cancer potency estimate will be based on a highly exposed population of women in China who have a high lung cancer rate and are exposed indoors to smoky coal emissions (39).

This hypothesis was initially tested for three complex POM emissions from a coke oven, roofing coal tar pot, and cigarette smoke by using the human lung cancer data from epidemiological studies of humans exposed to these emissions. The relative human cancer potency, as expressed by lung cancer unit risks, was compared to the potency of these emission sources in a series of bioassays (3,27,28). Human lung cancer unit risk estimates, animal tumorigenicity data, and short-term mutagenesis bioassay data were developed for each of these emission sources. The potency of these three POM emissions in the mouse skin tumor initiation assay resulted in the highest correlation across these three human carcinogens. Although further research on this methodology is continuing using additional human data (38,39), for current applications of the comparative potency method, the mouse skin tumor initiation assay is proposed as the only bioassay that produced a constant relative potency across the coke oven, roofing tar and cigarette smoke emissions adequate to support the assumptions in the comparative potency method.

Another approach to evaluate this method is to compare the cancer unit risk estimates obtained by the comparative potency method to risk estimates obtained by species extrapolation from chronic lifetime animal inhalation studies. The comparative potency method predicted a human lung cancer unit risk for
diesel emissions that is similar to the unit risk estimate for the same diesel vehicle used in a lifetime rodent inhalation carcinogenesis study (40), as shown in Figure 6. These two independent approaches to the cancer risk estimation of the POM from diesel emissions result in similar cancer unit risk estimates.

**Similarity and Critical Variables of POM Mixtures: Key to Predictability**

The validity of this constant relative potency hypothesis may depend on the chemical nature of the mixtures being compared, as well as on the similarity of those mixtures. Characterization of the POM from these three emission sources identified a wide range of polycyclic aromatic (PAH) compounds. In general terms, these POM mixtures are similar in their relatively high content of polycyclic aromatic compounds. All of the mixtures contain polycyclic aromatic hydrocarbons (PAH), however, the relative concentration of these PAHs differ substantially among the mixtures. The coke-oven emissions and cigarette smoke contain mutagenic basic constituents containing nitrogen; however, the specific nitrogen heterocyclic compounds are different in these two mixtures, and the roofing coal tar emissions do not contain these nitrogen bases.

Comparison of the mutagenic potency of a series of POM from the same source category, in this case diesel vehicle emissions in *Salmonella typhimurium*, with the tumorigenic potency shows high correlations ($r^2 = 0.90$ and 0.93 for $-S9$ and $+S9$, respectively) between the two bioassays, as shown in Figure 7. When gasoline emissions are added to the diesel emissions, the correlation is slightly decreased ($r^2 = 0.90$ and 0.72 for $-S9$ and $+S9$).
(17). Both the tumorigenic potencies and the mutagenic potencies of this series of diesel emissions is also highly correlated with the concentration of nitrated PAH and PAH in the POM mixture (17). As the similarity of the mixtures decreases, the correlations between these two bioassays decreases. An even wider range of POM mixtures is included in the correlation plot in Figure 8. Nitro-PAH, which do not always require microsomal activation, are not present in wood smoke emitted directly from airtight wood stoves (3). The addition of several POM samples from wood smoke emissions and a POM sample from a residential oil heater to diesel and gasoline POM emissions (Fig. 8) results in a decreased but still reasonably high correlation ($r^2 = 0.88$, +S9) between mutagenic activity and tumor initiation potency in Sencar mice.

There are no simple guidelines for establishing the similarity of mixtures (8). Our research suggests that within a source category, such as diesel emissions, the chemical composition and relative mutagenic activity of the emissions could provide evidence for similarity. The more similar the POM source mixtures, the better we are able to predict, for example, the relative tumor potency of the mixture based on the mutagenic activity or the chemical composition.

**Concluding Remarks**

Complex mixtures present unique problems beyond that normally encountered with pure chemicals or simple mixtures. These problems present additional challenges to research in exposure assessment, environmental toxicology, and risk assessment. Several of the strategies and approaches described here have allowed us to develop methods to a) assess exposure to complex mixtures of POM, b) identify the genotoxic components, c) determine the contribution of different sources to ambient mixtures, d) determine the DNA dosimetry at target organs, and e) predict the relative risk of related mixtures.

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