Biological Markers of Intermediate Outcomes in Studies of Indoor Air and Other Complex Mixtures

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Biological markers of intermediate health outcomes sometimes provide a superior alternative to traditional measures of pollutant-related disease. Some opportunities and methodologic issues associated with using markers are discussed in the context of exposures to four complex mixtures: environmental tobacco smoke and nitrogen dioxide, acid aerosols and oxidant outdoor pollution, environmental tobacco smoke and radon, and volatile organic compounds. For markers of intermediate health outcomes, the most important property is the positive predictive value for clinical outcomes of interest. Unless the marker has a known relationship with disease, a marker response conveys no information about disease risk. Most markers are nonspecific in that various exposures cause the same marker response. Although nonspecificity can be an asset in studies of complex mixtures, it leads to problems with confounding and dilution of exposure-response associations in the presence of other exposures. The timing of a marker’s measurement in relation to the occurrence of exposure influences the ability to detect a response; measurements made too early or too late may underestimate the response’s magnitude. Noninvasive markers, such as those measured in urine, blood, or nasal lavage fluid, are generally more useful for field studies than are invasive markers. However, invasive markers, such as those measured in bronchoalveolar lavage fluid or lung specimens from autopsies, provide the most direct evidence of pulmonary damage from exposure to air pollutants. Unfortunately, the lack of basic information about marker properties (e.g., sensitivity, variability, statistical link with disease) currently precludes the effective use of most markers in studies of complex mixtures. — Environ Health Perspect 101(Suppl 4):193–197 (1993).

Key Words: Biological markers, assays, environmental exposure, air pollution, complex mixtures, pulmonary disease, health outcomes

Introduction

Most studies of health effects in humans exposed to complex air-pollutant mixtures have used such outcome measures as hospital admission rates during air pollution episodes, symptom reports from questionnaires or diaries, and disease prevalence or mortality rates in communities with different levels of air pollutants. A few studies in controlled exposure settings have attempted to assess the combined as well as separate effects of mixture components. Such approaches provide useful information; but studies of overt disease often are insensitive to low-dose exposure effects, and they focus on the extreme end of the disease spectrum, where only a small proportion of the exposure-related disease burden occurs.

Biological markers of intermediate health outcomes (i.e., early pathologic changes detected by markers do not progress to symptomatic conditions, more people will show positive marker responses than overt disease; so studies using markers can potentially have increased statistical power due to the more numerous outcomes. However, the markers are useful only to the extent that they have a known relationship to clinical diseases of interest.

In general, biological markers are indicators of events occurring in the body that are difficult to measure directly. Markers can indicate that an exposure, a response to exposure, or an early pathologic change has occurred; other markers, often enzyme phenotypes, indicate an individual’s increased susceptibility to disease from a particular exposure. Such markers differ from genetic markers, which are usually defined in the current genetic literature as discrete phenotypes controlled by genes that occur in close proximity on chromosomes to other genes of interest. A good genetic marker will be correlated highly with the presence of the gene of interest, which may be undetectable.

The following presentation discusses some methodologic issues associated with the use of biological markers of intermediate health outcomes arising from exposures to complex pollutant mixtures. In most instances, this article focuses on outcomes related to four examples of complex mixtures: a) environmental tobacco smoke (ETS) and nitrogen dioxide (NO2), b) acid aerosols and oxidant outdoor pollution, c) ETS and radon, and d) volatile organic compounds (VOCs). No standard definition exists for the term biological marker. For example, the National Research Council’s (NRC) 1989 book *Biologic Markers in Pulmonary Toxicology* (1) defines biological markers as “indicators of events in biologic systems or samples,” while others [e.g., Hulka et al. (2)] limit the definition to indicators measured in biological specimens obtained from a person. The NRC definition includes, for example, spirometry, which involves no assays of biological samples. This discussion uses the more restrictive definition of Hulka et al. (2). These two references provide additional information about markers in general and about the specific markers discussed here.

Methodologic Issues

Link with Disease

The most important property of any biological marker of effect is its link with the health outcome of interest. Although markers in a target tissue (i.e., tissues that give rise to the disease of interest) usually provide the best early indicator of an adverse event, markers...
outside of the pathogenic pathway can be superior for a variety of reasons. For example, target tissues such as the lung are relatively inaccessible, markers in the target tissue may require higher exposures to show a response than do markers in a nontarget tissue, and markers in a target tissue may have less persistence due to rapid cell turnover or other mechanisms of marker loss.

A study of micronuclei measured in exfoliated epithelial cells in sputum of uranium workers exposed to radon and tobacco smoke illustrates how markers outside of the pathogenic pathway can show a stronger statistical association with the disease under consideration. Micronuclei, which are caused by agents that damage DNA, are small secondary nuclei formed during mitosis when whole chromosomes or chromosome fragments fail to become incorporated into daughter nuclei. As summarized by Loomis et al. (3), the micronucleus assay in exfoliated buccal cells is sensitive to ionizing radiation as well as tobacco smoke; uranium miners show a clear radon-related excess of lung cancer, but neither radon exposure nor cigarette smoking was associated with a higher prevalence of micronuclei in Loomis’s study of sputum cells from the miners. In contrast, other studies of uranium miners (4) and persons with residential radon exposure (5-6) show increased levels of structural chromosome aberrations in blood lymphocytes. Although chromosome aberrations in lymphocytes have no direct role in lung cancer, their association with radon exposure suggests that this marker, compared to micronuclei in sputum cells, has a stronger statistical link with lung cancer arising from radon exposure.

Other markers of premalignant changes with potential interest for studies of carcinogenic exposures (e.g., VOCs or radon and ETS) include sputum cytology, the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) assay for in vivo mutations, and assays of oncogene activation. To date, human studies using the HGPRT assay primarily have examined peripheral lymphocytes, but the assay could be adapted for pulmonary macrophages obtained through bronchoalveolar lavage (BAL) (1). By using macrophages, the marker would detect mutations occurring in the lung, although lung tumors do not arise from macrophages themselves. Theoretically, activated oncogenes could be detected in exfoliated cells in lavage fluid to characterize developing lung tumors (1). The use of almost any marker of intermediate outcomes would increase the number of exposure-related outcomes (compared to the number of cancers) in the study while reducing the necessary time interval between exposure and measurement of the outcome. At this point, however, most markers of intermediate outcomes have an unknown relationship to clinical disease; so their value is limited.

The rationale for using nontarget tissues and cells for assessing evidence of early disease is that pathologic changes observed in nontarget tissues often occur in the target tissues. For example, exposure to acid aerosols and oxidants can exacerbate airway hyperreactivity in asthmatics (1), with a resulting increase in pulmonary and blood eosinophils. Chronic deposition of eosinophils in the lung may cause airway inflammation, but eosinophils are much easier to measure in blood than in the lung. Even if markers in target tissues compared to nontarget tissues do show a stronger association with the ultimate outcome of interest, their inaccessibility may preclude their use in observational studies.

In general, the selection of markers involves a tradeoff between a marker’s positive predictive value for the disease and such practical issues as specimen availability, marker sensitivity, and assay cost. Unfortunatley, many potentially useful markers have an unknown relationship to lung disease. For example, the relationship between changes in constituents of BAL fluid (a potential source of myriad markers of intermediate outcomes) and pollutant-induced injury requires further study (1). Markers of events that occur further down the disease pathway, such as fibrosis, moderate air-space enlargement, or mutations, have clearer biological links with clinically apparent disease. In the absence of important advantages associated with other markers, biologically plausible markers having obvious biological links with disease are preferable to markers without such links. The validity of markers that occur in nontarget tissues or early in the pathogenic process would be assessed ideally in prospective studies, where their positive predictive value for subsequent overt disease can be estimated. Such studies would be difficult, especially when cancer is the outcome of interest, because of the need for large study populations and long follow-up periods.

Studies of associations that can be measured cross-sectionally are more feasible. One could, for example, ascertain whether a marker measured in blood has a high positive predictive value for inflammation in the lung. A marker with a high predictive value for inflammation could be used as an outcome variable in a study to determine whether exposure to a complex mixture causes inflammation.

Specificity and Confounding

Some markers respond to specific environmental exposures, while others respond to a wide variety of agents. For studies of complex mixtures, nonspecificity can be an asset, because the marker response will reflect the combined effects of multiple, sometimes unidentified, agents. Furthermore, the airways can react to inhaled toxic materials in a limited number of ways, so a wide variety of exposures lead to a small number of health effects. For example, many inhaled toxicants, such as acid aerosols and oxidant gases like NO_2 and ozone, cause inflammation in the respiratory tract. Exposure to ETS and NO_3 in children increases the risk of respiratory infections, which also cause inflammation. Chronic or repeated inflammation may in turn lead to irreversible lung injury and, eventually, clinically apparent diseases such as emphysema. Therefore, indicators of inflammation or early loss of elasticity can serve as markers of intermediate outcomes from numerous exposures, the effects of which converge on a common pathologic pathway.

The convenience of using a nonspecific marker that responds to a variety of complex mixtures is offset by the possibility of dilution and confounding from exposures other than those of interest. As discussed by Weiss and Liff (7), the problem of dilution, where an exposure–response association is obscured by other associations, arises when different causal pathways lead to the same end point, as is the case with nonspecific markers of intermediate outcomes. If two different exposures (or sets of exposures), E_1 and E_2, cause the same marker response through independent pathways, they increase the overall marker response rate in an additive manner; but relative measures of association (e.g., relative risk, odds ratio, etc.) are based on the assumption of a multiplicative model of association. As a result, the relative risk of the response due to E_1 will be influenced by the background incidence of the response due to E_2. In this situation, use of the risk difference rather than relative risk to compare marker responses in persons exposed and unexposed to E_1 helps avoid the problem of dilution from a high background incidence from E_2.

Another strategy for mitigating the problem of dilution is to stratify an overall group of end points into its more homogeneous components (7). Inflammation from different exposures, for example, may have slightly different manifestations detectable by different markers. Each marker would have greater specificity for a given exposure than would a marker that detected overall
inflammation. The feasibility of this approach for studies of complex mixtures is unclear until additional basic information on properties of markers of intermediate outcomes becomes available.

Weiss and Lifl (7) point out that studies of intermediate outcomes sometimes facilitate the identification of a particular causal pathway. For example, if exposure to one complex mixture leads to pulmonary disease through inflammation, while another exposure causes the same disease through a non-inflammatory process, the complex mixture would show a stronger association with inflammation than with the pulmonary disease. However, this approach is feasible only when the intermediate outcome has a known relationship to the clinical outcome of interest—a rare situation.

Confounding could arise in studies of inflammation due to exposure to acid aerosols and oxidants, for example, if exposed persons tend to be heavy smokers or have occupational exposures that also cause inflammation. Problems with confounding are essentially the same whether one uses nonspecific markers of intermediate outcomes or actual diseases as study end points. The usual epidemiologic approaches for controlling confounders (i.e., stratified analysis, matching, or restriction) can remove the effects of extraneous variables. Exposure-specific markers would be less prone to confounding than would nonspecific markers, but outcome markers that arise only from single agents would have limited value for studies of complex mixtures.

Sensitivity

In many instances, different markers can be used to detect the same outcome. Inflammation, for example, involves numerous physiological changes that can be used as markers of the inflammatory response. For a given degree of inflammation, however, some markers will be easier to detect than will others. Markers that detect the mildest inflammation (i.e., those that are positive with the lowest exposures) would have the greatest sensitivity.

An animal study (8) illustrates several issues associated with marker sensitivity for intermediate outcomes. The investigators evaluated different markers of connective tissue metabolism (a response to injury in the lung) in urine or BAL fluid. In one exposure protocol using 0.5-ppm NO2 exposure for 4 weeks, hydroxylysine urinary excretion increased significantly, but levels of hydroxylysine and angiotensin-convert-}

age. Compared to other markers of effects on connective tissue, urinary hydroxylysine apparently has greater sensitivity.

Although this controlled study of NO2 exposure in rats only has indirect relevance to free-living human populations exposed to complex mixtures, it does illustrate that different markers vary in sensitivity, and that the same marker measured in different biological materials also can have different sensitivities. For reasonably benign exposures, such as many commonly occurring complex mixtures, exposure chamber studies can characterize a promising marker's properties (e.g., sensitivity, dose-response, and interindividual and intrapersonal variability) in humans under controlled conditions. These studies can evaluate markers of acute outcomes but precise estimates of such properties will rarely, if ever, be available for marker responses from chronic exposures.

In general, a marker's sensitivity and positive predictive value can be increased by studying susceptible populations. For example, exposure to acid aerosols—oxidants can exacerbate symptoms of asthma. Sensitized asthmatics compared to nonsensitized asthmatics and nonasthmatics are likely to show an inflammatory response at lower exposure levels, so markers of inflammation will have greater sensitivity in studies of sensitized asthmatics. Similarly, ETS—NO2 exposure may increase the risk of respiratory infections in children more than in adults, possibly through alterations in immune function; theoretically, markers of such alterations may have a greater sensitivity (i.e., occur at relatively low exposure levels) in children given their apparent increased susceptibility to infections compared to adults. Given the known susceptibility of such groups as asthmatics and children to some mixtures, they also may be susceptible to other pollutant mixtures, so that adverse health outcomes and associated markers could be detected at relatively low exposure levels.

Sensitivity also can be increased for a given ambient concentration by studying people with a relatively high internal dose of a pollutant mixture, such as those having a high rate of ventilation due to physical activity. Persons who spend a large proportion of time outdoors also have relatively high doses of ambient outdoor air pollutants. Thus, for a given exposure level, marker responses would probably be more pronounced in persons with biological susceptibility and in those with behaviors that increase either their internal dose or their contact with ambient pollutants.

Temporal Aspects

Markers can appear hours, days, or years after exposure. For example, nasal irritation is commonly associated with indoor air pollution (e.g., VOCs and other complex mixtures). Markers of cell and mediator changes in nasal lavage fluid could be useful for studies of such pollutants (1), and the markers would probably appear within hours of exposure. In contrast, several months or years of exposure to acid aerosols and oxidants may be necessary to detect airspace enlargement using morphometry, while changes in alveolar cell populations may appear after days or weeks of exposure.

For transient markers, the timing of measurements is especially crucial. The influx of neutrophils and eosinophils into the respiratory tract, for example, usually occurs during the first 3 to 7 days of an inflammatory response (1). Measurements of these markers of inflammation in BAL fluid immediately after exposure would underestimate the inflammatory response, as would measurements taken after the response subsides. Protein influx reflecting pulmonary epithelial damage, however, should be measured relatively soon after exposure. For sustained ongoing exposures, such as occurs with VOCs or residential radon exposure and ETS, transient markers will be replenished, and measurements can be made any time during seasons when buildings are likely to be poorly ventilated.

The timing of measurements is less important for markers of chronic exposure-related changes. Irreversible airspace enlargement, for example, can be measured long after exposure ends, and it will reflect cumulative exposure effects. Altered populations of alveolar epithelial cells due to oxidant air pollution exposure eventually revert to normal proportions, but these markers can probably be detected for at least several weeks after the end of exposure. Timing is still important in the sense that the exposure must be sufficiently long for the marker response to occur. Note that for some markers of chronic pathogenic processes, such as the markers of connective tissue degradation in the study by Evans et al. (8), the marker response diminishes after the exposure stops, even though the associated damage may be irreversible.

Approaches for Using Markers

The effective use of a marker in epidemiologic studies of complex mixtures depends not only on the marker's properties but also on the availability of suitable biological ma-
terials and moderately priced assays. Numerous markers of intermediate outcomes are inappropriate for field studies because of their invasive nature. The following section mentions some noninvasive markers with potential usefulness for studying complex mixtures, and it discusses strategies for using invasive markers.

Noninvasive Markers
In general, markers measured in such materials as urine, sputum, blood, and nasal lavage fluid are well suited for field studies because specimen collection involves relatively little inconvenience or risk for study participants. Urine could be valuable especially for studies of ETS and NO₂ exposure. ETS exposure can be estimated from urine samples, as can some markers of connective tissue metabolism associated with NO₂ exposure. Sputum cytology, a marker of disease that is non-specific with regard to exposure, may reveal early evidence of carcinogenic changes from such exposure as VOCs or ETS and radon gas. Standardization of sputum collection and preparation might alleviate the problems encountered by Loomis et al. (3) and allow detection of increased micronuclei from these exposures. Loomis et al., who used archived specimens, could not control the source of sputum and its cellular content; and laboratory manipulation of the old samples may have caused a loss of some cell structures.

Blood is a source of numerous and diverse markers. As noted earlier, radon exposure at levels that increase the risk of lung cancer are associated with chromosome abnormalities in blood lymphocytes. Markers of altered immune function, which increases the risk of respiratory infections, also can be measured in blood. Some studies suggest that markers of pulmonary hypertension, which apparently is caused by several toxic chemicals, also may be present in blood (1); possible markers include elevated plasma copper levels and ristocetin cofactor activity relative to plasma von Willebrand factor.

Many changes in constituents of blood and nasal lavage fluid reflect the changes that occur in less accessible BAL fluid. For example, the distribution of lymphocyte subpopulations, a marker of air pollution effects, is similar in blood and BAL fluid (1). Nasal lavage fluid, which contains several markers that respond to a variety of constituents found in complex mixtures, may be especially useful in studies of indoor air pollutants that cause nasal irritation (1). Comparisons between markers in nasal and BAL fluid are necessary to ascertain the usefulness of nasal lavage markers as indicators of events in the lower respiratory tract.

Invasive Markers
Some of the most informative markers of intermediate outcomes occur in relatively inaccessible biological materials such as the lung. One approach to obtaining lung specimens is BAL, which uses a modified bronchoscope for collecting pulmonary cells and fluids. Lavage fluid contains a variety of biological materials in which to measure markers of intermediate outcomes. The method is used primarily for diagnostic purposes and in controlled-exposure chamber studies. Although its invasive nature precludes the routine use of BAL in field studies, small studies of individuals with exposures to naturally occurring complex mixtures could detect numerous potential exposure-related effects. The use of personal monitors in conjunction with lavage measurements would provide a high degree of precision in estimates of exposure-outcome associations. Retrospective collaborative studies using archived lavage specimens also may be feasible if unexposed controls from chamber studies in cities having different levels of air pollution could be identified. Differences in specimen collection and storage among research centers, however, may preclude such retrospective studies.

Much of the recent animal research and some human studies of air pollution have used morphometric measurements for assessing pulmonary damage. The morphometric approach uses an overlay of points or lines that is placed over an electron micrograph or other two-dimensional image of a lung section. By estimating the proportion of points or numbers of lines that fall on cell or airway structures, one can use a set of formulas to estimate various cell and tissue parameters, such as cell size and structure, proportions of cell types, or airway diameters (9).

Morphometric studies of lung tissue are limited to specimens obtained from surgery or autopsy. This severe constraint raises a host of methodologic problems, but the potential value of morphometric measurements argues in favor of further exploring this promising technique. For example, morphometry has been used to study postnatal lung growth (1), an outcome relevant to ETS–NO₂ exposure; and numerous morphometric studies have found pulmonary changes in animals exposed to single pollutants and pollutant mixtures (10). Limited studies of air pollution using morphometry with human autopsy specimens point to the feasibility of moving from animal to human tissues. The application of morphometric techniques to human lung specimens may eventually provide the most direct evidence of pulmonary damage from chronic low exposures to complex mixtures. However, basic work remains to be done. Studies are necessary to describe lung lesions in persons of different ages exposed to ubiquitous background pollutants (ETS, automobile exhaust, etc.) and to investigate the effects on lesion measurement of different protocols for collecting, handling, and storing lung specimens in autopsy settings.

Conclusion
Indoor and outdoor air pollutants potentially can affect the health of virtually everyone in the United States. Animal studies using controlled chronic exposures are important for identifying the pollutants responsible for adverse health effects, but epidemiologic studies address the effects of complex pollutant mixtures that actually occur in exposed humans. Biological markers of intermediate outcomes offer new opportunities for advancing the study of these pollutants.

Unfortunately, many research opportunities associated with markers remain theoretical. Much basic information critical for valid application of markers is lacking. For example, few markers have been characterized regarding their statistical properties, such as assay variability and variability in samples collected from the same individual at different times. Furthermore, protocols for collecting, storing, and analyzing biological specimens have not been standardized. More important, the relationship between markers of intermediate outcome and clinical disease remains a matter of speculation. Investigators studying exposures to complex mixtures therefore cannot interpret the health implications of a positive marker response, nor can they confidently attribute a lack of an exposure–response association to a true absence of a biological effect when a marker's sensitivity and variability are unknown. Also, the nonspecific nature of currently used markers of intermediate outcomes leads to potential problems with dilution and confounding of exposure-outcome associations. A combination of controlled exposure studies and systematically conducted epidemiologic studies could readily address the validity issues, but the high cost of many marker assays discourages their use in large-scale epidemiologic studies.

Progress in applying markers to studies of human exposures and diseases requires considerable effort from both toxicologists and epidemiologists. However, neither group has great incentive to undertake the mundane systematic studies necessary to characterize marker properties in a statistically valid manner: bench scientists usually prefer to investigate promising new techniques while epidemiologists are primarily interested in etiologic associations. Progress in the use of markers is likely to occur slowly until answers to basic questions become available.

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