Identification of Subpopulations That Are Sensitive to Ozone Exposure: Use of End Points Currently Available and Potential Use of Laboratory-Based End Points under Development

Robert B. Devlin*

Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711

A number of epidemiological studies have attempted to assess the effect of recurrent ozone exposure in humans. For the most part, they have failed to document convincingly an association between chronic ozone exposure and differences in lung function performance or respiratory symptoms. This is not surprising given the small respiratory effects observed in animals chronically exposed to ozone and assuming that people with abnormal respiratory function resulting from other occupational or environmental exposures, such as tobacco smoke, would make up a much larger percentage of the population than people with respiratory effects attributable to ozone. Therefore, either more sensitive end points must be developed to detect subtle changes due to chronic ozone exposure, or ways of selecting subpopulations that are especially sensitive to ozone must be devised. It has been well documented that there are large and reproducible differences in the acute response of individuals to ozone as measured by pulmonary function tests. Recently, it has also been shown that there are large differences in the acute response of individuals to ozone as measured by inflammatory and other biochemical parameters. This paper discusses the problems of selecting individuals who are sensitive to ozone depending on the end point chosen. It also describes potential new sensitive end points that might be available for ozone epidemiology studies in the near future. — Environ Health Perspect 101(Suppl 4):225-230 (1993).

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Introduction

It is widely accepted that humans exposed to known concentrations of ozone under controlled conditions exhibit reversible changes that affect the large and small airways as well as the alveolar region of the lung. These changes include reduction in pulmonary function performance, narrowing of small airways, increased nonspecific airway reactivity, alveolar inflammation, damage to pulmonary epithelial cells, and increased leakage of vascular components into the lung. In addition, acute reduction of pulmonary function performance following ozone exposure has been documented in epidemiological studies in which small groups were followed over a short period (1–6).

However, it is not known if recurrent exposure to ozone results in induction of chronic disease. Some studies of chronic animal exposure suggested that rats and nonhuman primates exposed to levels of ozone below 0.5 ppm for up to 2 years exhibit permanent or slowly reversible lesions in the distal airway and proximal alveolar region of the lung. An ongoing low-level inflammatory process with increased numbers of neutrophils was accompanied by increased numbers of type II alveolar cells, thickening of the basement membrane, and increased numbers of collagen fibers in the interstitium (7–9). Measurement of pulmonary function in these animals suggested very small changes in some variables, including total lung capacity and the diffusing capacity of carbon monoxide (10,11).

A number of epidemiological studies have attempted to assess the effect of recurrent ozone exposure in humans; they are summarized by Ostro in this volume. By and large, these studies have failed to document convincingly an association between chronic ozone exposure and differences in lung function performance or respiratory symptoms. In a discussion of why chronic effects have not been observed, McDonnell (12) noted that it is not surprising that epidemiologists have experienced difficulty in observing respiratory effects due to chronic ozone exposure, given that small respiratory function changes could be found only in chronically exposed animals that had developed advanced cellular and biochemical lesions. If similar lesions occur in humans, and only a small proportion of individuals with lesions go on to develop frank disease, it would be difficult to identify such individuals in an unselected population, particularly given the rate of frank disease and abnormal respiratory function resulting from other occupational or environmental exposures. Thus, it would seem that either more sensitive end points must be developed to detect chronic effects, or ways of selecting subpopulations that are sensitive to ozone must be devised.

There is some evidence that individuals sensitive to ozone can be identified. As mentioned above, acute decrements in forced expiratory volume in 1 sec (FEV1) have been reported to occur in a concentration-dependent manner in both controlled exposure studies and some field studies. In these studies, the magnitude of response to
similar concentrations of ozone varies considerably among individuals. For example, decrements in FEV₁ that range from 2 to 48% have been reported in subjects exposed to levels of ozone between 0 and 0.4 ppm (13–16). The variation in magnitude of response is also reproducible when individuals are reexposed to the same level of ozone up to 13 months later (17,18). These findings suggest that differences in individual response to ozone are not due to random experimental error nor to different environmental conditions between exposures, but rather to factors that are inherent in individuals and that are stable over time. If this suggestion is true, it may be possible to screen large numbers of individuals in a controlled setting and to select those with the largest decrements in pulmonary function for inclusion in epidemiological studies of chronic health effects.

To identify a sensitive subpopulation, however, one must define the criteria used to select the population. This is not necessarily straightforward, since there are a number of parameters that have been shown to be altered in humans exposed to ozone. In addition to changes in lung function tests that primarily measure inability to take a deep breath (e.g., FVC), changes in airway constrictions, demonstrated by an increase in specific airway resistance (SRₑₑₑₑ), have been reported in animals and humans exposed to ozone. Increases in nosophoric airway reactivity have also been observed in humans exposed to levels of ozone as low as 0.08 ppm (19,20). Bronchoalveolar lavage (BAL) has been used to sample both cells and fluid removed primarily from the alveolar region of the lung of humans and animals exposed to ozone. An influx of neutrophils increased permeability; decreased macrophage phagocytic ability; and increases in arachidonic acid metabolites, some cytokines, fibronectin, lactate dehydrogenase (LDH), coagulation factors, elastase, and plasminogen activator have been reported in humans exposed to levels of ozone as low as 0.08 ppm (21–24). As is the case in pulmonary function tests, there is a wide range of individual responses to ozone as measured by these assays, particularly to low levels of ozone (21–23). It is not known if the magnitude of change in these cellular and biochemical end points is reproducible over time for a given individual, as is the case with more traditional markers of lung function, such as FEV₁.

Preliminary analysis of some of the end points listed above suggests that none of them is strongly correlated with another; individuals who show large decrements in FEV₁ following ozone exposure do not necessarily have the largest neutrophil influx or the most nonspecific airway reactivity. Furthermore, drugs that block formation of cyclooxygenase products appear to ablate partially the pulmonary function decrements seen after human exposure to ozone (25,26), but they do not appear to affect neutrophil (PMN) influx, cell damage, leukotriene production, or increased permeability reported in humans exposed to ozone (27). Moreover, there does not appear to be a strong correlation between the cellular and biochemical assays when they are compared with each other. For instance, rats depleted of PMNs still show lung damage, production of inflammatory cytokines, and increased permeability of vascular components across the epithelial barrier after ozone exposure (28). Airway hyperresponsiveness may also occur prior to and in the absence of PMN influx in rats (29) and guinea pigs (30,31). More basic research into the underlying mechanism of ozone damage probably will be needed to understand fully the apparent lack of coherence of different end points.

The above discussion does not designate which end point(s) should be used to select sensitive individuals. Traditionally, lung function measurements derived from forced expiratory maneuvers have been used to define such people, probably because these tests are noninvasive, inexpensive, and performable on large numbers of individuals in the field. However, it might be the case that end points that measure airway hyperreactivity, inflammation, production of fibrogenic compounds, or some combination of these may be better suited for the selection of individuals who may be at high (or higher than average) risk for developing frank disease from chronic ozone exposure.

Additional studies are needed to define more precisely both the range and reproducibility (over the long and short term) of individual responses measured by the various end points discussed above. Such studies will need to combine a number of end points, including symptoms (e.g., cough, minor respiratory irritation), pulmonary function changes that reflect lavage and small airway function, and assays that measure cellular changes involved in inflammation and lung damage. Ozone concentration–response curves for each end point will also be needed to determine if different doses of ozone alter the response profile. In addition to defining the range and reproducing the response, it will be necessary to determine which, if any, of these end points are concordant (i.e., which ones are consistently elevated in responders but not in nonresponders). If both invasive and noninvasive concordant end points are identified, it will be important to know if noninvasive measures such as symptoms, standard lung function measurements, or measurements of airway resistance can be used as surrogates for more invasive procedures that measure inflammation, lung injury, reduced levels of antioxidants, or reduction in host defense capability. If concordant end points are not found, the relevance of pulmonary function changes to chronic outcomes should be more fully explored before using this end point to select responders for longitudinal epidemiology studies.

Under the assumption that suitable end points are identified, a combination of two approaches would be most useful for the identification of subpopulations sensitive to ozone. One approach would be to select subjects for an epidemiological study on the basis of their response to ozone in controlled chamber studies. This would allow the selection of individuals who respond to precisely known ozone concentrations and exposure regimens that could mimic those found in the geographical area in which the individual resides. One could also be certain that selected individuals truly are responsive to ozone rather than other occupational or environmental agents. The alternative approach would be to select responders from a cross-sectional study and attempt to duplicate their response in a controlled environment. A combination of both approaches would allow a better understanding of the relative contribution of ozone and other environmental agents to individual range of response.

One problem faced by epidemiologists attempting to carry out these studies is the selection of suitable end points. Traditionally, lung function assays have been used to assess pulmonary changes in humans exposed to ozone. This approach is suitable for detecting acute changes, but it is not likely to detect subacute changes in small airways that have been demonstrated in animals chronically exposed to ozone. It is possible to monitor changes in inflammatory or fibrogenic compounds in the lungs of humans exposed to ozone with biochemical and molecular assays by using BAL to obtain cells and fluid lining the airways and alveolar region of the lung. However, the procedure is relatively invasive and probably not applicable for studies involving more than several dozen people. Techniques that measure changes in the lung that currently detected by standard lung function assays,
yet applicable to large-scale epidemiology studies are required. Summarized below are four promising approaches that are currently being developed in laboratories for potential use in epidemiology studies.

**Tests that Measure Changes in Small Airways**

There is much evidence that humans exposed to ozone develop reversible decrements in FEVi and FVC, which have been attributed to a reduction in total lung capacity (TLC) that is mediated by neural mechanisms. There also are obstructive changes in the large airways that are characterized by small increases in specific airways resistance (SRaw) and airways reactivity (32–35). These measurements, however, do not provide convincing evidence of functional changes in the small airways of the lung. Yet, studies of animals acutely and chronically exposed to ozone show that tissue damage occurs first in the small airways, particularly in the bronchioleolar duct region (36–38). It is likely that ozone perturbation of this region also would be reflected by measures that detect functional change in the airways. To detect small airways functional changes, tests are needed that do not rely on vital capacity maneuvers. This is because any acute small airways change from ozone that might be detectable from forced expiratory flows at low lung volumes will probably be obscured by the reduction in FVC.

Several potential tests of small airway function have been described. One such test involves the measurement of the dispersion of an inhaled bolus of a small (0.5–1 mm) aerosol. This assay has been used to demonstrate differences between a group of healthy, nonsmoking individuals and a group of asymptomatic smokers with otherwise normal lung function; it suggests the presence of small airways abnormalities in the latter group (39). Healthy, nonsmoking volunteers exposed to 0.4 ppm ozone also show differences in the dispersion of an aerosol bolus, which suggests that ozone exposure results in functional changes in the small airways (40). This bolus technique is relatively noninvasive, easy to perform, and can be done on large numbers of people. It has the potential to measure functional changes in the region of the lung known to be most sensitive to ozone inhalation, which may allow for detection of changes too small to be detected with traditional functional measurements derived from forced expiratory maneuvers. However, there needs to be more research comparing the aerosol bolus dispersion measurement with other putative small airways tests, such as multiple-breath nitrogen washout or radioactive gas bolus, and examining functional tests of the large and small airways derived from forced expiratory maneuvers. Experiments also need to be designed to understand better the underlying structural and functional changes in the lung that can induce dispersive processes. Finally, the sensitivity and specificity of this test needs to be assessed in relation to other small airways tests.

**Tests that Measure Changes in Nasal Passages**

The nose is the primary portal of entry for inspired air in humans, and therefore it is the first region of the respiratory tract that is in contact with airborne pollutants such as ozone. If these pollutants are respiratory irritants capable of causing cellular damage, as ozone is, then effects should be detected in the nasal passages. Since many of the cell types found in the nasopharyngeal region are the same or similar to cells found in the trachea and bronchi, the responses of nasal cells to ozone may be similar to the response of airways and alveolar cells.

Nasal lavage (NL) is simple and economical to perform, relatively noninvasive, and allows multiple sequential sampling of both nasal secretions and cells from the same person. This procedure has been used to study nasal inflammation (as determined by an influx of PMNs) in humans exposed to rhinovirus (41) as well as to study mediators produced in human nasal fluid during allergic reactions (42). It has also been used to study changes in the cells and nasal fluid of humans exposed to tobacco smoke (43) and workers exposed to cotton dust (44). In a recent study, an increase in PMNs has been demonstrated in the NL fluid of humans exposed to volatile organic compounds (VOCs) in equivalent concentrations to those present in a new house (45). These studies clearly show the utility of using this technique in a variety of settings.

Work with animals has demonstrated that ozone causes damage to the epithelial cells of the nasal passages and ultimately results in an influx of PMNs (46). Two studies with humans have shown that an acute exposure to 0.5 or 0.4 ppm ozone results in a large increase in PMNs in the NL fluid (47), as well as increased levels of albumin and trypsin (a marker of mast cell degranulation). In the latter study, BAL was performed on the same individuals, and a qualitative correlation was found between the numbers of PMNs in the NL fluid and in the BAL fluid (48). Thus, there is a possibility that the noninvasive NL procedure can be used as a surrogate for the more costly and invasive BAL in epidemiological studies with some assurance that inflammation seen in the nasal passages is mirroring inflammation present in the alveolar region of the lung.

More work is needed to strengthen this assertion, particularly when considering low ozone concentrations. It is not known if NL is as sensitive as BAL in the detection of inflammation or production of other mediators. Work is also needed to extend the range of mediators that can be detected in the NL and to determine if a correlation exists between BAL and NL for them. This would include arachidonic acid metabolites, cytokines, and measurements of cell injury such as LDH, fibronectin, etc.

In addition to measuring NL fluid for the presence of mediators, it is also possible to perform nasal brush scarpings in which several thousand cells are removed. These cells can be analyzed by recently developed techniques such as RNA in situ hybridization and polymerase chain reaction (PCR), which are capable of analyzing mRNAs present in only a few hundred cells. Recently mRNAs coding for cytokines and other relevant mediators have been quantified in human upper airway cells removed by brush scraping (49,50). This approach may extend both the sensitivity and the range of end points that can be assayed. Clearly this newly emerging area needs more developmental work to validate the very preliminary results described to date.

**Noninvasive Methods of Imaging the Lung**

The 1973 discovery that nuclear magnetic resonance (NMR) signals could be spatially encoded through the use of magnetic gradients has spawned an entire field of research and application in magnetic resonance imaging (MRI). A subject is placed in a strong magnetic field that causes hydrogen protons to align synchronously about the direction of the applied field. Radio frequency pulses are then applied to stimulate the protons and to generate a radio frequency echo in the tissue. This signal is spatially encoded, which permits the construction of a two-dimensional image of the selected plane through the subject. Contrast is achieved by changes in the number of protons per cubic centimeter, the spin–lattice relaxation time, and the spin–spin relaxation time. These parameters reflect how much water is present in a tissue and how the water is bound to various other
molecules. The technique is completely noninvasive, involves no ionizing radiation, and can be performed repeatedly on the same individual over time. MRI has been used as a diagnostic tool for only the past 6 years, and its current applications include detection of pathologic changes in a number of soft tissues, including hepatitis and fatty liver, that are associated with chronic liver disease, infarction of skeletal muscle, renal lesions, soft tissue tumors, and joint lesions (51–55).

Recently a number of researchers have exploited the potential of this tool to develop MR microscopy, in which sections through soft tissues can be viewed at <50 mm resolution to permit noninvasive microscopy on live animals (56–59). This approach has been used to quantify edema and fibrosis in the lungs of animals exposed to paraquat (60), as well as edema in animals exposed to hyperoxia and ozone (J. Crapo, personal communication). This technique has the potential to become a powerful tool with the ability to detect noninvasively ozone-induced lesions in humans. MRI microscopy can focus on the whole lung or on a small portion such as the bronchoalveolar duct region, where ozone-induced lesions are first visualized by conventional microscopy.

A number of problems must be solved before MRI microscopy is ready to apply to humans in an epidemiological study. Resolution is partly dependent on the size of the magnet through which the subject must pass, and current magnets that are large enough to accommodate humans yield a resolution of only a few millimeters. It is particularly difficult to create an image of the normal lung because it has fewer protons than other tissues, and the microscopic structure of the airwater interface in the alveoli produces distortions resulting in very long scan times. In addition, distortion due to breathing can reduce resolution. Fortunately, research in this field is progressing very rapidly. In the past several months many of the problems associated with imaging the lung have been solved, and new images have greatly increased resolution and decreased scanning time. If the progress in magnet technology continues at the same pace it has had the past few years, it can be anticipated that magnets suitable for visualizing the human lung with high resolution will be available sometime in this decade. Developments are currently underway in which three-dimensional reconstruction of the lung will allow any plane to be viewed from any angle without loss of resolution.

It is tempting to overstate the importance MRI microscopy because of its potential to provide the epidemiologist with a powerful tool that can visualize the interior of a human lung with microscopic resolution. Clearly there is a considerable amount of developmental research which must be done, much of it with animals, before this technique can be applied to epidemiology studies. However, most of the current developmental effort is focused on disease models, and research funds must be provided to encourage development of models appropriate to environmental epidemiology studies. There are at least two general areas of research that should be encouraged in the near future. The technology necessary to measure inflammation and edema and to visualize lesions in animals chronically exposed to ozone exists. Studies are needed in which MRI imaging, conventional quantitative histology, and BAL (which can measure inflammation and lung damage from a cellular and biochemical perspective) are all applied to animals exposed to ozone for varying periods of time. This would allow an assessment of the ability of a noninvasive technique such as MR microscopy to detect acute and chronic changes in the lung that have already been described using histology and BAL. Studies are also needed to assess the ability of MR microscopy to detect edema and inflammation in the lung of humans exposed acutely to ozone. Even if current MR technology does not permit visualization of small structures in humans, MR is capable of detecting more pervasive changes such as inflammation and edema. These studies would allow an assessment of whether MRI could be used as a surrogate for BAL in epidemiology studies.

Development of Biomarkers of Exposure and Predictive Biomarkers

An important quality for any epidemiological study would be the ability to perform a simple test that could quantify the exposure of an individual to a pollutant or perhaps even detect individuals who are susceptible to exposure to a specific pollutant. The molecular technology necessary to identify specific macromolecules that could serve as dosimeters of exposure to specific pollutants or to identify components that could serve as predictive biomarkers of sensitivity exists today. Furthermore, the technology needed when analyzing changes in a tiny number of cells (100–1000), which greatly expands the range and type of tissues that potentially can be sampled in an epidemiology study, also exists. Nearly all of the developmental work and most of the current applications are focused on the identification and quantification of biomarkers related to disease. Very little effort has been made to develop or apply this technology to problems related to exposure of humans to environmental pollutants. Those operating the laboratories most suited to the development of the kinds of biomarkers needed for epidemiology studies are usually not aware of nor appreciative of the utility or value of developing these kinds of biomarkers. In addition, very little funding has been provided by the epidemiology community for long-term research dedicated to developing suitable biomarkers. However, if such biomarkers are ever to be developed, funding must be provided and lines of communication must be opened between epidemiologists and molecular biologists.

Research, preferably in easily accessible tissue such as nasal epithelium or blood, is needed to define the range of proteins and mRNAs that are induced by exposure of humans to ozone and other pollutants. It is likely that different sets of macromolecules are induced by different pollutants, similar to the induction of different rat liver proteins by different toxicants (61). If such sets of proteins or mRNAs are found in animals or humans exposed to ozone, studies will be needed to determine if a quantitative relationship exists between induction of any protein or mRNA and the level of ozone exposure. ELISA or RIA assays could then be developed to quantify specific proteins (and PCR to quantify specific mRNAs) whose level of induction is dependent on the concentration of ozone exposure. This would allow these macromolecules to serve as molecular dosimeters of ozone exposure. If suitable criteria for sensitivity can be determined, similar approaches can be used to identify proteins or mRNAs present in humans judged sensitive to ozone. It is likely to take at least several years to develop and validate biomarkers suitable for epidemiological studies of ozone, and the difficulties and pitfalls in attempting to identify such markers have been well documented by Hatch and Thomas (62). However, the potential power and utility of these biomarkers suggest that research in this area should be pursued.

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