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Biochemical Alterations of Lung Structure as Predictors of Chronic Lung Disease

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This presentation reports on newer animal models and techniques that we and others have developed. These models attempt to predict in a reasonable experimental time frame the occurrence of structural changes in the lungs that are the basis of various chronic diseases. Since the major chronic diseases of the lung parenchyma involving what we think of as well-understood structural changes are pulmonary fibrosis and emphysema, I will discuss in detail ways of looking at effects of air pollutants on lung collagen and lung elastin metabolism.

Less than 10 years ago techniques for studying protein biosynthesis *in vitro* by tissue minces were first applied to the analysis of lung collagen synthesis by Crystal and his colleagues at the National Institutes of Health (*1*). We have adapted these methods for the study of collagen biosynthesis by lung minces from rats, mice and monkeys that have been exposed to various pneumotoxins, including air pollutants, that are known to cause pulmonary fibrosis. We study collagen synthesis by lung parenchymal minces cultured for a few hours *in vitro*, during which time the amount of collagen synthesized is a linear function of time of incubation (Fig. 1). This type of assay presumably tells us what the rate of lung collagen synthesis was *in vivo* at the moment the animal was killed. There actually is evidence that this presumption is true in another system, in which the *in vitro* rate of collagen synthesis by lung minces was quantitated and shown to be similar to the *in vivo* rate, determined by injection of labeled precursor several hours before killing the rats (*2*). When we exposed rats continuously to levels of ozone between 0.5 and 2 ppm for 1, 2, or 3 weeks, the observed collagen synthesis rates by their lungs was increased (Fig. 2). We have shown (*3*) that the

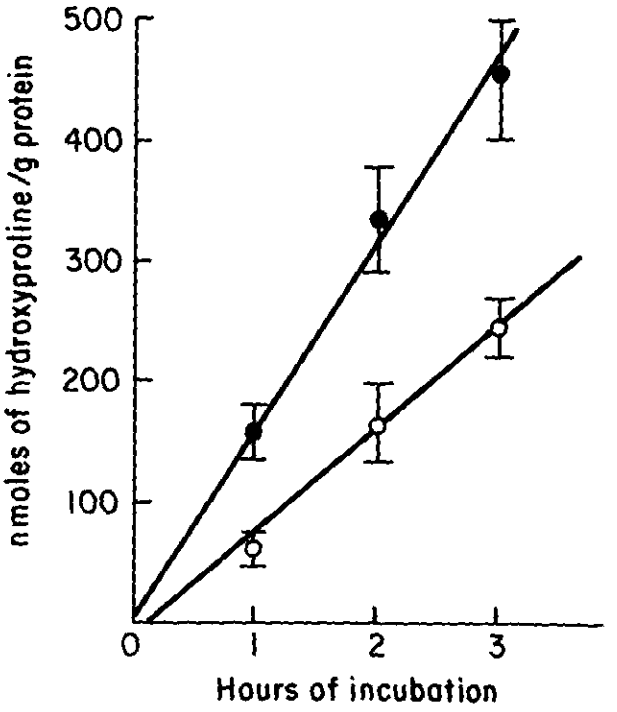

FIGURE 1. Time course of incorporation of [^{3}H]proline into collagen [^{3}H]hydroxyproline by lung minces from rats that had breathed only filtered air (○) or had breathed 0.8 ppm of ozone for 21 days (●). Data points are means ± SD of values from three different rats eat each time; the lines are fitted to the data by least-square analysis. The correlation coefficient (r^2 values) for each line is 0.99

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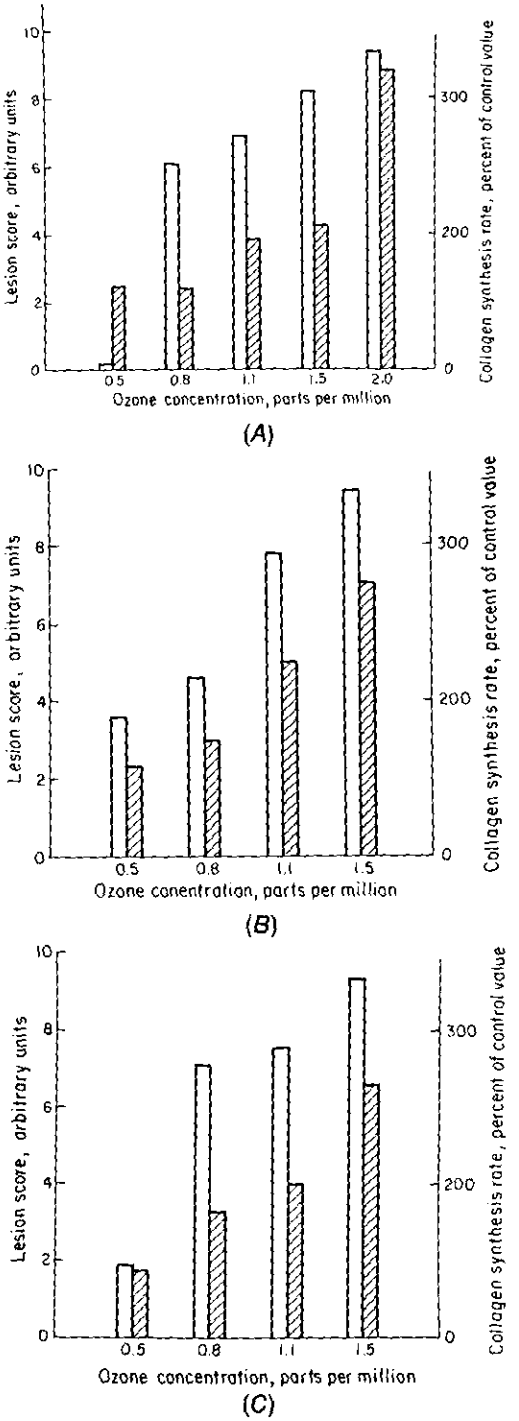

FIGURE 2. Biochemical and histological responses of rat lungs to exposure of rats *in vivo* to various concentrations of ozone for (*A*) 7, (*B*) 14, or (*C*) 21 days. Biochemical data are expressed as the percentage of control value (nmol of [^{3}H]hydroxyproline synthesized per hour per gram of protein) of collagen biosynthesis rate by lung minces. Histological responses are expressed as net lesion score. Open bars, histological lesion scores; hatched bars, collagen synthesis rates.

increase observed in the collagen synthesis rate is a linear function of the level of ozone to which the rats were exposed. We have also shown (*3*) that there is an excellent correlation between pulmonary fibrosis as defined by lung collagen synthesis rates and the extent of fibrosis as scored semiquantitatively by histological indices. Since the biochemically defined fibrosis is a linear function of the concentration of ozone that the rats breathe, we can linearly extrapolate from our data to determine "threshold" levels of ozone that cause elevations in lung collagen synthesis rate after continuous exposure for 1–3 weeks (Fig. 3); such linear extrapolations probably overestimate exist at all. Interestingly enough, we find the threshold level in these experiments to be less than about 0.10–0.13 ppm of ozone, approximately the former ambient air quality standard (0.08 ppm) for this pollutant (the present standard is 0.12 ppm). We have also performed experiments in nonhuman primates by examining open-chest biopsies before and after exposing them to high levels of ozone; such methods allow us to use each animal as its own control. In these experiments with cynomolgus monkeys, we have found them to be more sensitive than rats to high levels of ozone by this criterion (*4*). Obviously most humans are not rats, nor do they breathe ozone continuously at these levels for weeks at a time. The relationship of such threshold values obtained in experiments of this type to the setting of air quality standards remains a matter of judgment.

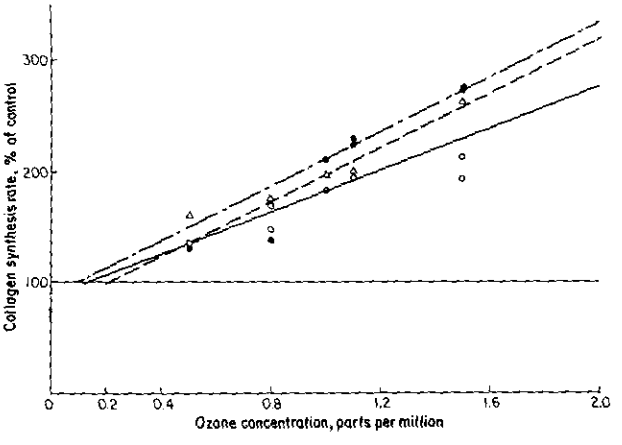

FIGURE 3. Collagen biosynthesis rates of rat lung minces as a function of concentration of ozone to which the rats had been exposed *in vivo*. Lines are fitted to the data by linear regression analysis (least-squares program): (○) 7 day exposures; (●) 14 day exposures; (Δ) 21 day exposures. Apparent "threshold" values are determined by linear extrapolation to zero effect levels (i.e. values of 100% of control).

An obvious question that comes to mind from experiments of this type is what relationship, if any, exists between data on elevations in collagen synthesis rate of lung minces from rats exposed to air pollutants and changes in actual lung collagen content, especially over a long-term exposure regimen. We have attempted to answer this question by analyzing lung collagen synthesis rates and total lung collagen content in rats exposed continuously to 0.5 ppm of ozone for up to 6 months (*5*). Lung collagen synthesis rates were elevated above control values (Fig. 4) at all times studied (3, 30, 50, 90 and 180 days of continuous exposure). Lung collagen content (estimated as milligrams of hydroxyproline per lung) was significantly greater in the exposed rats for up to 3 months of exposure (Fig. 5). Thus, elevated lung collagen synthesis rates seemed to be associated with elevated lung collagen content in these rats. These changes could be correlated with mild centriacinar fibrosis observed in other rats from the same study (*6*).

We have also been able to show (*7*) that elevations in lung collagen synthesis rate in rats exposed to high levels of ozone (1.5 ppm for 1 week) are associated with a qualitative shift in the types of collagen being made and deposited in their lungs. In mammalian lungs (including those from rats) the normal ratio of collagen types being synthesized is about 66% Type I collagen and 33% Type III. In rats exposed to high levels of ozone the ratio of collagen types being synthesized is about 80-85% Type I and 15-20% Type III. It is certainly relevant to point out that Type I collagen is stiffer and less compliant than Type III; it is also what pathologists call collagen based on its histological appearance and staining properties, as opposed to Type III collagen, which may be what is called reticulin. Presumably such long-term structural changes are also occurring in the lungs of our rats exposed to 0.8 ppm of ozone for several months, although we have not as yet tested this possibility experimentally. Such an experiment is currently being performed in monkeys exposed for 1 yr to 0.8 ppm of ozone.

I would like to emphasize that collagen synthesis rates by lung minces from rats exposed to ozone for up to 6 months remain elevated over control values; as evaluated by this parameter, rats do not show a decreased response to continued ozone exposure. There are long-term structural changes occurring in these lungs from chronically exposed rats that make them different from "normal" lungs from control animals;

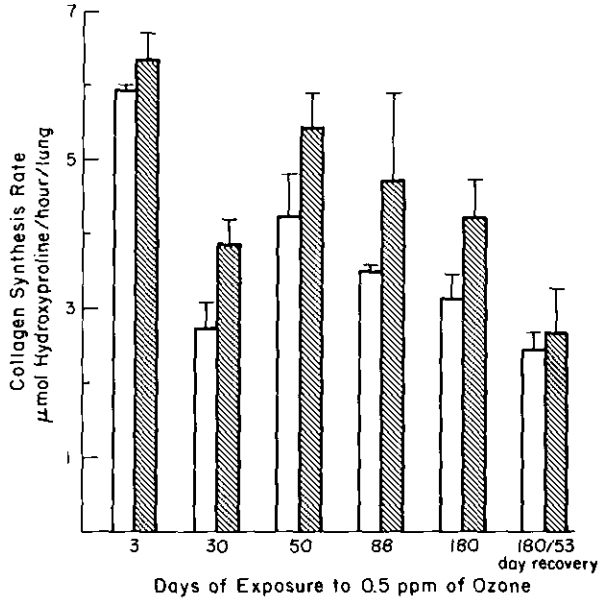

FIGURE 4. Collagen synthesis rates of lung minces prepared from rats after exposure to 0.5 ppm ozone for various times (as indicated). Results are means ± 1 SE for the slopes of the least-squares fitted lines prepared from 3 time points for each lung mince. Correlation coefficients for such straight lines ranged from 0.90 to 1.00, with an average value of 0.965 (24 data sets). Open and hatched bars are from control and ozone-exposed rats, respectively.

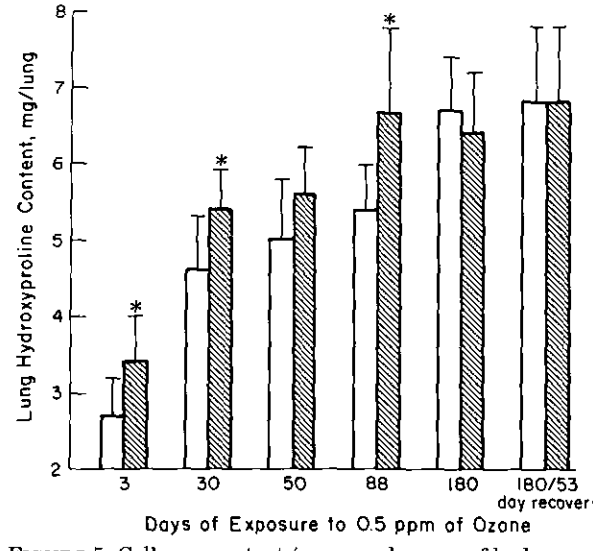

FIGURE 5. Collagen content (expressed as mg of hydroxyproline) of rat lung homogenates from the same rats evaluated in Fig. 4. Open bars: control rats that had breathed only filtered air; hatched bars: rats exposed to ozone. All results indicated are means ± SD of data from groups of six rats. Asterisks indicate significant differences ($p < 0.05$) as evaluated by Student's one-tailed t test.

whether these structural changes are considered to be good, bad or neutral for these rats depends more at our current levels of knowledge upon one's philosophical beliefs than upon any rigorous scientific comprehension of the relationship of such structural changes in the lungs to their functional capacity.

I would like to expand upon this point. We (*8*) and others (*9, 10*) have shown in another animal model of pulmonary fibrosis induced by intratracheal injection of bleomycin that pulmonary fibrosis as evaluated by lung compliance, a physiological measurement, is not the same as pulmonary fibrosis as evaluated by histological appearance and/or biochemical measurements of total lung collagen content.

For example, in rats or hamsters administered bleomycin, the lung compliance is decreased for 2–4 weeks after instillation of the drug, a change consistent with the definition of pulmonary fibrosis by physiological criteria. However, 5–6 weeks after instillation of bleomycin, the lung compliance returns to normal values and the animals do not have physiological manifestations of lung fibrosis. These same animals are fibrotic upon histological examination of lung sections (i.e., the pathologist would report their lungs to be fibrotic) and their lung collagen content (estimated as total lung hydroxyproline) continues to increase over the time interval between 2 and 6 weeks. Thus, the pathologist and the biochemist would agree that these animals had pulmonary fibrosis, while the physiologist would feel they were normal. Indeed, these studies suggest that we really do not understand as much as we think we do about the structural basis of lung function, especially about the relationship between lung collagen content, lung fibrosis, and lung compliance.

Similar lung mince systems can be used to probe for effects of air pollutant inhalation on lung elastin metabolism. Dubick and co-workers (*11*) have demonstrated using *in vivo* labeling techniques that three acute exposures of mice to 1.5 ppm of ozone for 4 days each do not seem to increase the turnover of mature molecules (fully crosslinked) of lung elastin. Since changes in lung compliance (elasticity) have been reported in rats (*12*) and rabbits (*13*) exposed to ozone, such short-term techniques for evaluating putative changes in lung elastin content or crosslinking are of obvious interest. Lung mince techniques for studying rates of elastin biosynthesis utilizing antibodies to soluble precursors of elastin are currently under development to allow us to look at synthesis independently of crosslinking reactions.

What are the implications of these experiments for those of you charged with setting ambient air quality standards? Clearly, the traditional data base used for such standard setting is heavily skewed in the direction of detecting acute, short-term effects such as reflex bronchoconstriction in human subjects undergoing controlled experimental exposures. It just is not practical to look at each pollutant, and every possible combination of pollutants, in long-term dose-response experiments that may require inhalation exposures for 6 months or a year. However, the potential adverse health effects of air pollution that I fear the most are chronic effects from intermittent, long-term low-level exposures—cancer, emphysema, pulmonary fibrosis and chronic obstructive lung disease. For assessment of these types of risks controlled human exposures are of absolutely no value. Epidemiological studies have also been of little value in assessing these risks (*14*), due in part to the overwhelming impacts of smoking and occupational exposures on the incidence of these diseases in our population. Thus, I would argue that short-term assays that probe for potential structural changes in the lung, such as those for collagen and elastin metabolism that I have described today, may see potential adverse health effects of air pollutants that are being completely missed by the conventional techniques currently being used to evaluate risk. I don't think we can afford to ignore the signals these types of assays can send us. Yet I sense a regulatory climate wherein animal inhalation toxicology experiments are, to all intents and purposes, being ignored in favor of data from controlled human exposures. I, for one, would rather be protected by air quality standards from putative long-term structural changes in my lungs than from transient increases in the resistance of my large airways. Such increased airway resistance may occur in response to release of stored histamine from airway mast cells in response to signals from my irritant receptors (*15*). Such an effect may have no long-term consequences to my health.

We also seem to be allowing ourselves to be lulled into a sense of security with the current ambient air quality standards based upon the belief that we "adapt" to pollutants upon continued exposure. Again, I would emphasize that the concept of "adaptation" comes from the attenuation of reflex bronchoconstriction in controlled human exposures to (for example) ozone upon continued exposure. I do not know of any data that suggest that the lung can adapt to continued exposure to ozone when the assay for effects is based upon structural changes in the lung rather

than being based upon transient responses such as reflex bronchoconstriction or localized inflammation. In my opinion it is only by means of designing experiments that correlate data between short-term assays for structural change (to evaluate dose-response characteristics of the lung) with selected long-term chronic exposures that include detailed examination of lung structure that we will eventually be able to evaluate properly the true risks of exposure to ambient air pollutants and their mixtures.

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