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Detection of Paracrine Factors in Oxidant Lung Injury

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Includes the Report of the Institute's Health Review Committee

Research Report Number 22

HEALTH EFFECTS INSTITUTE

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ABBREVIATIONS

ddH_2O	distilled deionized water
dH_2O	distilled water
EDTA	ethylenediaminetetraacetic acid
EPA	U.S. Environmental Protection Agency
^3H	tritium
HBSS	Hank's balanced salt solution
HPLC	high-performance liquid chromatography
medium	Eagle's minimal essential culture medium
NO_2	nitrogen dioxide
O_2	oxygen
O_3	ozone
pH_I	isoelectric point
serum	fetal bovine serum

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A. Keith Tanswell

ABSTRACT

It is well recognized that the oxidant gases ozone and nitrogen dioxide cause lung injury at high concentrations and, as such, are considered to be "hazardous" air pollutants. What is not known is the upper limit of their concentration in ambient air that can be tolerated without causing lung injury. This uncertainty is due, in major part, to a lack of sensitive and noninvasive tests that can identify the presence of early lung injury after exposure to oxidant gases.

The hypothesis underlying the studies reported in this document was that the changes in cell populations that occur in the lung after oxidant lung injury are due to the local generation of lung-cell-specific growth factors, and that these factors should leak into the blood stream in sufficient quantities to allow their identification. Once identified, such factors could be used as early markers of oxidant injury.

The question asked in the design of these studies was: "Is oxidant lung injury associated with the appearance in blood of factors that enhance lung cell growth, as tested in a cultured lung cell bioassay?" Groups of rats were exposed to either 1 ppm ozone, 85 percent oxygen, or air, and samples of plasma, lung washings, and lung tissue were collected at intervals over a two-week period. These samples were tested for their effect on the DNA synthesis of purified populations of three major lung cell types (pneumocyte, fibroblast, and endothelial cell) in culture. The concentrations of the two oxidant gases used in these studies were selected on the basis of their known effect on these cell types in the intact animal. The collected samples were tested either whole or after separation into various fractions, determined by the preference of the sample's constituents for an alkaline or acidic environment. This fractionation procedure was included because simple testing of crude samples may not always reveal the presence of biologically active material because both growth stimulators and growth inhibitors may be present in the same sample.

The results from testing whole samples confirm that factors that enhance DNA synthesis by all three lung cell types do appear in blood and lavage after exposure to 1 ppm ozone or 85 percent oxygen.

These factors appear to be distinct for each lung cell type, in that the timing of maximal activity in the collected samples differs for each cell type. The time at which they appear in blood bears a close temporal relationship with cellular changes reported to occur in the whole lung of similarly exposed animals.

A fairly simple set of experiments is required to characterize these factors further; this should allow for the development of a two-step procedure for processing samples from a more extensive study in order to determine the specificity of such factors for oxidant lung injury, and whether or not they are present with very low dose exposures.

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INTRODUCTION

The U.S. Environmental Protection Agency (EPA) establishes National Ambient Air Quality Standards for "criteria" air pollutants, and has broad authority to adopt emission standards for any source of "hazardous" air pollutants. These include the oxidant gases nitrogen dioxide (NO_2) and ozone (O_3). Nitrogen dioxide is a common environmental pollutant derived from a variety of sources, including automotive and other combustion engine emissions, cigarette smoke, gas and wood-burning stoves, as well as coal-burning plants (Horvath et al. 1970; Shy 1973; Kaldor et al. 1984). Nitrogen dioxide acts as a major precursor for the formation of O_3, a photochemical oxidant that results from the reaction of nitrogen oxides and hydrocarbons in the presence of sunlight. A major difficulty for the EPA in setting ambient air quality standards for oxidant gases such as NO_2 and O_3 is the lack of sensitive markers of oxidant lung injury applicable to large populations. Disturbance of pulmonary function, to a degree that can be detected by routine pulmonary function tests, requires a significant degree of lung injury. Ideally, standards should be set to prevent the early cellular alterations that precede detectable alterations of lung function. In some instances, such alterations might be detected very early in the course of oxidant injury to the lung by the use of radioactive probes for detection of altered alveolar epithelial and endothelial lining transport (Jefferies et al. 1984). The complexity of the required equipment, the use of radioactive isotopes, and the invasiveness of the procedure preclude the use of this technique in the large populations necessary to define optimal ambient air quality standards.

Inhalation of sublethal concentrations of NO_2 and O_3, under well-controlled experimental conditions, causes damage to pulmonary alveolar architecture. The cellular changes observed have been well documented and contrasted (Crapo et al. 1984; Evans 1984) to the changes observed after the inhalation of a third oxidant gas, oxygen (O_2). Oxygen is not an environmental pollutant, as such, but is a well-documented cause of destructive lung injury in laboratory animal models and in human patients who require O_2 supplementation for various pneumopathies. Of these three oxidant gases, O_2 has been studied most extensively in small animal experiments; this provides a data base against which the effects of other oxidant gases can be compared and contrasted (that is, it can act as a "gold standard" for dosimetric studies of O_3 and NO_2). The intracellular events that underlay cellular O_2 injury are better understood and documented (Freeman and Tanswell 1985) than are the events of either O_3 or NO_2 injury. In vitro studies suggest that O_3 damage occurs not only as a consequence of the oxidation of membrane constituents, but also results from the inhibition of several key intracellular enzymes and the depletion of the intracellular glutathione pool (Freeman and Mudd 1981; Van der Zee et al. 1987). It is probable that many, although not all, of the mechanisms of cellular injury, defense, and repair are shared by all three oxidant gases.

Inhalation of sublethal concentrations of all three oxidant gases (O_2, O_3, NO_2) results in type I pneumocyte destruction, followed by type II pneumocyte hyperplasia and interstitial thickening, with an increase of fibroblasts (Crapo et al. 1984; Evans 1984). Oxygen, and to some extent O_3 (but not NO_2), causes capillary damage, followed by endothelial cell hyperplasia during the repair process. These differences in effect on the capillary bed reflect the chemical reactivities of the three gases, with the most reactive (NO_2) tending to target areas closer to the point of inhalation than does the least chemically reactive gas (O_2), which exerts its major effects more distally along the respiratory tract. Were sufficient unreacted NO_2 to reach the capillary bed, it would be expected to react with endothelial cells, and to cause significant physical and metabolic changes, as indicated by the in vitro studies of Patel and Block (1986).

The biological control mechanisms by which type II pneumocyte and lung fibroblast hyperplasia occur after oxidant lung injury have not been established. It is even unclear if the stimulus to cellular hyperplasia comes from local events within the lung, or is a result of some systemic response to oxidant inhalation. Experiments of nature, duplicated in laboratory animals, suggest (at least for O_2) that the stimulus for cell division arises locally, since vascular shunts leading to systemic cyanosis do not reduce the proliferative changes observed (Miller et al. 1970; Jentzen et al. 1984). Potential local mechanisms include the release of growth factors by lung cells in response to oxidant injury, or from lymphocytes and phagocytes attracted to the lung by the injury. It is also possible that there is chronic inhibition of cell division, which is lost after oxidant injury. Lastly, oxidant-injured lung cells may secrete basement membrane of a modified composition that encourages cell division. There is good evidence from the work of others (Bitterman et al. 1982; Elias et al. 1985) that alveolar macrophage-derived polypeptide paracrine growth factors may influence fibroblast hyperplasia after oxidant injury. My colleagues and I have recently demonstrated the release of apparently autocrine growth factors by lung fibroblasts exposed to oxidant stress in vitro (Tzaki et al. 1988), and had previously demonstrated the in vitro release of paracrine growth factors by oxidant-stressed lung fibroblasts that were active against tumorous pneumocyte cell lines and primary cultures of adult lung pneumocytes (Tanswell 1983; Freeman and Tanswell 1985).

Since type II pneumocyte and fibroblast hyperplasia are early events in oxidant lung injury, the detection of any paracrine or autocrine growth factors that control such cellular changes would be an excellent marker of early oxidant lung injury. There is experimental evidence of the early release of such factors from an ipsilateral oxygen-exposed lung, which affects an air-exposed contralateral lung in double intubation experiments (Motlagh et al. 1969). To be of practical value, they would have to be detectable in serum, rather than in lung lavage, to maintain a low level of invasiveness. The early increase in epithelial and endothelial layer permeability that is observed with some oxidant injuries suggests that water-soluble polypeptide growth factors would have access to the circulation, and should thus be detectable in the blood with a sufficiently sensitive assay.

AIMS

The specific aim of this project was to test four hypotheses:

1. That growth factors affecting type II pneumocytes, lung fibroblasts, and, possibly, lung endothelial cells are detectable in blood after exposure to O_3.
2. That the appearance of any such growth factors shows a temporal correlation with expected proliferative changes that occur in the lung parenchyma.
3. That similarly conducted studies with another oxidant gas (O_2) reveal qualitatively, though not quantitatively,

similar findings; that is, that growth-factor release is determined by the degree and duration of oxidant injury, rather than by the specific oxidant involved.

4. That any such growth factor can be characterized by preparative electrofocusing, molecular-weight sizing, and target cell type specificity. This would allow a simple two-step procedure to be developed for handling multiple samples from a more extensive analysis.

The initial proposal was to collect both plasma and lung lavage from exposed animals. The lavage was included in the study because growth factors derived from the lung parenchyma might be expected to appear in the lavage shortly before, or at the same time as, their appearance in plasma. At the request of the HEI Health Research Committee, lung homogenate was also collected from all exposed animals for concurrent assay.

This modified original proposal predicted that a three-year time frame would be necessary for the complete investigation of this specific aim. Unfortunately, a misperception between the investigator and the granting agency resulted in a two-year funding period for a three-year project. The investigator was unaware of this time restriction until late in the second year of funding. Consequently, the project is only two-thirds completed, and testing of the fourth hypothesis listed above could not be done. Unfortunately, the late date at which the misperception became evident did not allow sufficient time to restructure the experimental design. A two-year time frame would have meant limiting the study to a single oxidant gas, and performing a more restricted analysis. Such an approach would not allow an evaluation of the specificity of observed responses to a single oxidant gas, and would significantly reduce the potential value of such studies. Since each cell preparation yields all three cell types tested, no time would have been saved by restricting the analysis to a single cell type.

Two years into the project, progress was essentially as predicted in the original application. The initial screening of crude samples for growth factors had been virtually completed, as had the majority of the electrofocusing studies. Samples had been fractionated by high-performance liquid chromatography (HPLC), through a sizing column, but they awaited assay. A summary of the progress during the term of these studies is shown in Table 1.

Table 1. Summary of Progress in Assay of Samples from Ozone- and Oxygen-Exposed Animals Against Three Lung Cell Types

		Cell Type		
Analysis	Sample	Pneumocyte	Fibroblast	Endothelial
Bioassay (O_3)	Plasma (C)[a]	X[b]	X	X
	Plasma (D + HI)[c]	X	X	X
	Lavage	X	X	X
	Homogenate	X	X	X
Bioassay (O_2)	Plasma (C)	X	X	
	Plasma (D + HI)	X	X	X
	Lavage	X	X	
	Homogenate	X	X	
Preparative electrofocusing fractionation (O_3)	Plasma	X	X	X
	Lavage	X	X	Z[d]
	Homogenate	X	X	Z
Preparative electrofocusing fractionation (O_2)	Plasma	X	X	Z
	Lavage	X	X	
	Homogenate	X	X	

[a] C = crude.
[b] X = all groups completed.
[c] D + HI = dialyzed and heat inactivated.
[d] Z = most groups completed.

METHODS

OVERALL PLAN

The experiments intended for these studies followed a plan of four successive steps:

Step 1. Determine if exposure to the oxidant gases results in the detectable release, into the collected samples, of factors that enhance DNA synthesis by lung cells.

a. Expose adult male rats to 1 ppm O_3, 85 percent O_2, or air for two weeks and collect plasma, lung lavage, and lung homogenate on days 3, 5, 7, and 14 of exposure.
b. Test all crude samples for effect on DNA synthesis by isolated lung fibroblasts, endothelial cells, and pneumocytes in vitro. Also test plasma samples after heat inactivation and dialysis.

Step 2. Use pooled aliquots of samples, from individual time points, to identify the fractionation procedures most likely to enrich the yield of any such factors.

a. Subject pooled samples from each time point of each exposure group to broad-range preparative electrofocusing. Test each fraction in triplicate against each of the above lung cell types for effect on DNA synthesis.
b. Use the bioactivity profiles obtained to determine that fractions from preparative electrofocusing should be combined for further separation by HPLC through a siz-

ing column. Test all resultant fractions in triplicate against the relevant cell types.

Step 3. Separate aliquots of individual original crude samples by sequential preparative electrofocusing and HPLC. Assay predetermined fractions for the presence of growth factors against the three lung cell types.

Using the information obtained from the two fractionation steps above, subject all the original samples to sequential preparative electrofocusing and HPLC. Only fractions identified as bioactive in the first round of studies (step 2) would be used for these definitive bioassays. Confirm by statistical analysis that growth factors of specific molecular weights and isoelectric points appear in blood in response to inhalation of oxidant gases at concentrations above those observed in blood after inhalation of air alone.

Step 4. Determine further physical and biological characteristics of any growth factors identified as active against one or more of the three lung cell types.

Further characterize fractions that show significant effects on lung cell DNA synthesis by sensitivity to proteolytic enzymes, solvents, heat treatment and dithiothreitol. Examine specificity against a variety of cell types.

ANIMAL EXPOSURES

All exposures were conducted with Charles River's CD strain male rats of 400 ± 25 gm at the onset of exposure. Rats were selected as the animal model for these studies because of their documented sensitivity to both O_3 and O_2, and because of the detailed descriptions of rat lung architectural changes after inhalation of both oxidant gases (Crapo et al. 1980; Evans 1984) that are available from the literature.

Ozone exposures were conducted under the auspices of Dr. Elaine Grose at the Health Effects Laboratory of the Environmental Protection Agency, Research Triangle Park, NC. Animals were exposed to either air or 1 ppm O_3 for up to 14 days, and 10 animals from each group were killed by inhalation of excess halothane for sample collection at three, five, seven, or fourteen days from the onset of exposure.

Oxygen exposures were conducted on site in the animal facilities of the Lawson Research Institute. Animals were exposed to either air or 85 percent O_2 for up to 14 days, and 10 animals from each group were killed by inhalation of excess chloroform for sample collection at three, five, seven, or fourteen days from the onset of exposure.

The choice of a 14-day exposure period was based on comparative morphological data from rats exposed to both gases (Crapo et al. 1980; Evans 1984); these data demonstrated that hyperplasia of pneumocytes and fibroblasts had occurred by this time point for both gases. The choice of three-, five-, and seven-day intermediate time points was arbitrary, but a minimum of four time points was felt to be essential. Microscopic examination of lung tissues identifies when a cellular response has occurred, but does not identify the timing of the signal that initiated that cellular response. To further complicate prediction of optimal timing for sample collection, the detection of growth factors in plasma depends not only on the timing of peak tissue concentrations, but also on when the capillary bed is sufficiently permeable to allow such factors to enter the blood stream.

The selection of O_3 as the oxidant pollutant for these studies was based on its greater reactivity, at the alveolar level, than NO_2. Since the sensitivity of the assay system was unknown at the start of these studies, the best chance of growth-factor detection lay with the gas producing the greatest pneumocyte and fibroblast hyperplasia at levels close to those currently experienced during peak periods of atmospheric pollution. The use of O_2 as the second oxidant gas was based on the desire to have a gold standard against which to compare future experimental data. The sublethal doses of 85 percent O_2 and 1 ppm O_3 were selected based on morphologic and morphometric reports (Crapo et al. 1980; Evans 1984) that suggested that these doses would produce similar degrees of type II pneumocyte hyperplasia over a two-week exposure period.

The decision to have the O_3 exposures conducted off-site had the disadvantages of using animals from different gene pools and transporting samples over long distances across a national border. These disadvantages seemed to be outweighed by the benefits of having an established, well-controlled exposure chamber immediately available, rather than having to set up a system on site. The size of the exposure facility allowed all animals to be exposed to filtered air or O_3 during a single two-week period. Animals were housed individually in stainless-steel Rochester-type inhalation chambers of 0.33 m^3. Exposure to 1 ± 0.1 ppm O_3 (1,960 ± 0.01 $\mu g/m^3$) occurred for 23 hours per day, with the other hour each day used for cage cleaning and so forth. Chamber air was maintained at 76.9° ± 1.9°F and relative humidity at 43.5 ± 6.9 percent, with a flow rate of 1 chamber volume per minute. Oxygen was supplied from stock gas cylinders, previously analyzed and verified, to a silent arc generator (model 0345–0, Orec, Phoenix, AZ) set at 0.25 A. The resulting O_3 was injected into the chamber supply air upstream of a metering orifice. The turbulent flow through the orifice provided good mixing and a stable distribution of the O_3 in the chamber. Ozone flow was monitored and regulated by a mass flow controller (model FC 260, Tylan, Carson, CA) sized for midrange flow at the

desired chamber concentration, and located in the injection line just upstream of the orifice plate. An O_3 monitor (model 8002, Bendix, Lewisburg, WV) was calibrated prior to the exposure. The analyzer sampled the air in the breathing zone of the animals. Output from the analyzer was recorded by a strip-chart recorder and was simultaneously provided to a reverse-feedback chamber-concentration control circuit. The control circuit compared the measured O_3 concentration to the output of the variable potentiometer, which represented the desired O_3 concentration. It then adjusted the voltage to the mass flow controller to vary the O_3 flow as required. Response time for the circuit was set to provide control at the desired O_3 level while providing for rapid startup and shutdown.

The O_2 exposures were conducted on site at the Lawson Research Institute. The exposure system used has been described in detail in a previously published report (Tanswell and Freeman 1987). Each animal was housed in a 30 × 30 × 15 cm plastic chamber with inlet and outlet gas ports. All exposures were conducted in pairs, with one chamber receiving air and the other receiving 85 percent O_2. Each pair of chambers was housed in a thermostatically controlled incubator to maintain chamber temperature at 22° ± 1°C. Chambers were bored to allow insertion of a thermocouple thermometer and a hygrometer. The relative humidity within the chambers was 85 ± 2 percent, and flow rates were maintained to keep chamber carbon dioxide concentrations below 0.05 percent. The 85 percent O_2 concentration was set using a commercial gas blender, but chamber carbon dioxide and O_2 concentrations were confirmed at regular intervals using an ABL gas measurement system (Radiometer, Copenhagen, Denmark). The commercial gas blender had to be introduced into the system because of wall-pressure fluctuations, which led to marked variability in O_2 concentrations from a simpler blending system, as identified by periodic gas sampling. This led to some delay in completion of the O_2 exposures, sample collection, and analysis.

SAMPLE COLLECTION AND PREPARATION

At sacrifice all animals were weighed, then assigned a code number that was used to identify collected samples of plasma, lung lavage, and lung homogenate. A detailed description of the methods used for sample collection and preparation for bioassay is given in Appendix A.

BIOASSAY

All samples were tested against purified preparations of fetal rat (day 20 of gestation, with term at 22 days) lung type II pneumocytes, lung fibroblasts, and lung vascular bed endothelial cells. Fetal cell types were used for bioassay in preference to adult cell types for a number of reasons, including:

1. Adult rat type II cells do not divide in culture, and only relatively small numbers can be obtained from each animal. Since thousands of bioassays were involved in this project, the use of primary adult rat cultures would be prohibitively expensive and time-consuming. Any factors identified with fetal target cells could ultimately be selectively tested against adult cells at a later date.
2. Adult rat pulmonary endothelial cells have been very difficult to obtain and maintain in culture in most investigators' hands.
3. Having used fetal and adult lung fibroblast cultures on a routine basis over many years, I have found that adult lung cultures are associated with a much greater inter- and intraassay variability than are fetal lung cell cultures.
4. The fetal lung is obtained from a sterile environment within the amniotic sac, and culture loss or bioassay artifact due to contamination is infrequent.

Details of the preparation techniques used for each of the purified cell types are provided in Appendix B.

EFFECT OF SAMPLE ADDITION ON LUNG CELL DNA SYNTHESIS

The effect of sample addition on DNA synthesis by isolated fetal rat lung cell types was assessed by incorporation of ^{3}H-thymidine into DNA. The fetal lung cells were initially grown from plating in Eagle's minimal essential culture medium (subsequently called medium) with 10 percent fetal bovine serum (subsequently called serum), then growth-arrested during exponential growth by removal of serum (for plasma samples) or a reduction to 2 percent serum (for samples of lavage or homogenate). The sample was added for a 48-hour time period in the presence of 0.5 μCi/ml ^{3}H-thymidine (New England Nuclear, Lachine, Quebec). This relatively prolonged incubation period was designed to provide a gross screening assay of the integrated response to added samples (Tzaki et al. 1988). At the end of this incubation period the cells were washed with fresh medium, and ^{3}H-thymidine incorporation into DNA was measured by the method of Greenstein and associates (1984). The cell monolayer was washed twice with cold phosphate-buffered saline, then twice with cold 10 percent trichloracetic acid and once with ethanol:ether (3:1). The culture well was then air-dried and the precipitated cellular residue (including DNA) was solubilized with 0.2 N so-

dium hydroxide. After 15 minutes an aliquot was removed for addition to a counting vial, with 5 ml of Aquasol-2 (New England Nuclear) and 50 µl of glacial acetic acid, for beta-counting (Tracor Analytic, Elk Grove Village, IL).

INITIAL SCREENING BIOASSAYS

Each sample (plasma, lavage, or homogenate) from the 10 animals, diluted as described in Appendix A, at each time point, for each of the four exposure groups, was assayed against each cell type (pneumocyte, fibroblast, and endothelial) as an initial screen for activity. Plasma samples were also tested after dialysis across a membrane with a molecular weight exclusion limit of 3,500 and heat treatment at 56°C, for 30 minutes. This choice of temperature and time for heat inactivation of the plasma was based on the observation (Miyazaki et al. 1985) that an inhibitor of epithelial cell growth, normally present in rat serum, can be inhibited by such treatment. The use of both crude and treated plasma samples was intended to overcome problems with dialyzable and heat-labile growth inhibitors normally present in adult rat serum, without missing potentially heat-labile or very low molecular weight mitogens present in the samples. Ideally, the samples would have had the effects of heat treatment and dialysis examined separately as well as in combination, but this was not feasible given the large number of samples handled in the limited time available. The ^{3}H-thymidine incorporation into DNA for each sample was expressed as a percentage of the incorporation (cpm) into DNA by control cells to which an equal volume of suspension buffer, but no sample, had been added.

PREPARATIVE ELECTROFOCUSING

After fractionation by preparative electrofocusing, collected samples were also studied for bioactivity against each of the lung cell types. With 240 samples, it was not feasible in the time available to electrofocus every sample, and to bioassay each of the resultant 7,200 fractions against each of the cell types. Aliquots of the original plasma, lavage, and homogenate samples were, therefore, pooled for animals exposed to each gas for each time point. To further reduce sample numbers, day-5 and day-7 samples were combined for these fractionation studies. These 48 pooled samples were fractionated individually, and tested in triplicate against each cell type for effect on ^{3}H-thymidine incorporation into DNA in the presence of 2 percent serum. Results were expressed as a percentage of the incorporation by control cells, not exposed to any sample, as described earlier. The methodological details of sample fractionation by preparative electrofocusing are provided in Appendix C.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Bioactive preparative electrophoresis fractions were further separated through a sizing column using HPLC, as described in Appendix C. Bioassay of these fractions awaits completion.

STATISTICAL ANALYSIS

All values are shown as the mean ± SEM, unless otherwise stated. Significance ($p < 0.05$) was determined by analysis of variance, followed by both Dunnet's two-sided test and Duncan's multiple-range test. The Dunnet test was generally the more sensitive, and significant differences shown below have been determined with this method unless otherwise stated.

A flaw in the design of these studies, from a statistical perspective, was the use of animals from different gene pools for exposure to O_3 and to O_2, as discussed above. This precluded direct comparison between animals exposed to the two oxidant insults. While the appearance of apparent growth factors, with common physical characteristics, in samples from animals exposed to either oxidant gas would suggest involvement of a common biological mechanism, the timing and degree of such responses could certainly be modified as much by the characteristics of the animals as by the characteristics of the oxidant gas.

The results from crude samples obtained from air-exposed animals showed no significant differences between groups from the four different time points, and these values have been combined for statistical analysis and presentation. All results from the assay of crude samples are shown as percent differences from control cells not exposed to sample.

The only definitive analyses proposed in the original proposal were comparisons between groups at step 1 and step 3 described in the Overall Plan section. Such an analysis would include results from all individual animals. It had not been the original intent to conduct a statistical analysis at step 2. The use of pooled material, for the first preparative electrofocusing and HPLC fractionations, was not only intended as a mechanism to reduce the total number of assays conducted, but also as a means of minimizing the effects due to variations among animals. Aberrant peaks of activity from any one animal would be diluted 10-fold in the pooled sample. Any peaks of activity observed after fractionation would, therefore, be more likely to represent a general response in the majority of animals. The fractionations conducted during step 2 were only designed to produce qualitative information from which apparently bioactive fractions could be selected for a second fractionation

through a sizing column. However, the results of the preparative electrofocusing fractionations have been subjected to a statistical analysis for the purposes of presentation. Such an analysis is certainly subject to criticism for the very reason that it was chosen as a screening tool; that is, the use of pooled material will minimize error due to individual variation among animals.

Samples for electrofocusing studies were batched for bioassay, analysis, and data presentation by sample type and target cell. Each batch consisted of 576 assays, of which 36 assays were of control cells. All samples from animals exposed to air were fractionated separately for each exposure period. The profiles obtained at bioassay were similar for all air groups tested against one cell type. The mean of each triplicate analysis for each fraction was, therefore, calculated and used to derive an overall mean for any particular fraction for the combined air groups. Once again, all results were expressed as a percentage of the activity observed in control cells not exposed to added sample. A few batches of assays were not completed (see Table 1), and no statistical analysis is presented for these batches. These instances have been identified in the respective figure legends.

RESULTS

OZONE EXPOSURE

The effect of O_3 exposure on body weight, left lung weight, and left lung weight:body weight ratio $\times 10^3$ are shown in Table 2. As would be expected, the animals exposed to air gained weight over the two-week exposure period, while those exposed to O_3 had a significantly reduced weight gain. The left lung weight did not change significantly over two weeks for the air-exposed group. The O_3-exposed animals had significantly heavier lungs, and greater lung weight:body weight ratios, than did the air-exposed animals as early as three days from the onset of exposure, and throughout the exposure period. Visual inspection of the lungs on removal resulted in one animal from the air-exposure group being discarded from the analysis due to a blotchiness of the lung surface.

INITIAL SCREENING OF THE SAMPLES FROM THE AIR AND OZONE EXPOSURES

The results of the initial screening of all samples from the O_3 and air exposures against all three lung cell types is shown in Figure 1, with the air-exposed group containing samples from all 39 animals.

Crude plasma caused a profound inhibition of ^{3}H-thymidine incorporation into DNA of all three cell types, irrespective of the group from which the plasma was obtained. This was reversed by the dialysis and heat treatment of plasma samples.

Pneumocyte DNA synthesis (as indicated by an increase of ^{3}H-thymidine incorporation) was significantly increased by the addition of plasma from animals exposed to 1 ppm O_3 for five days and for seven days, compared with the effect of plasma from air-exposed control animals.

Lung fibroblast DNA synthesis was also significantly increased by the addition of plasma from animals exposed to 1 ppm O_3 for seven days and for 14 days, compared with plasma from air-exposed control animals.

Lung endothelial cell DNA synthesis was not significantly different in the presence of plasma samples from air- or O_3-exposed animals.

A small, but significant, stimulatory activity for pneumo-

Table 2. Effect of 1 ppm Ozone or Air Exposure over Two Weeks on Rat Left Lung Weight, Body Weight, and Left Lung Weight:Body Weight (LLW:BW) Ratio[a]

		Days of Exposure to Ozone or Air			
Parameter	Group	Day 3	Day 5	Day 7	Day 14
Body weight (gm)	Air	382 ± 29[b,c]	397 ± 26[b]	388 ± 13[b,c]	433 ± 19
	O_3	363 ± 18[c]	379 ± 23[b,c]	391 ± 28[b]	402 ± 22[b]
Left lung weight (gm)	Air	0.97 ± 0.10[d]	0.94 ± 0.12[d]	0.92 ± 0.08[d]	1.08 ± 0.16[d,e]
	O_3	1.20 ± 0.15[e]	1.19 ± 0.19[e]	1.18 ± 0.30[e]	1.44 ± 0.14
LLW:BW Ratio ($\times 10^3$)	Air	2.54 ± 0.22[f]	2.37 ± 0.29[f]	2.38 ± 0.23[f]	2.49 ± 0.31[f]
	O_3	3.29 ± 0.29[g,h]	3.16 ± 0.68[g,h]	3.03 ± 0.80[h]	3.58 ± 0.26[g]

[a] Data represent mean ± SD (n = 10, except for the day-7 air group, where n = 9).
[b] Matching superscripts (b through h) indicate no significant difference by Duncan's multiple-range test.

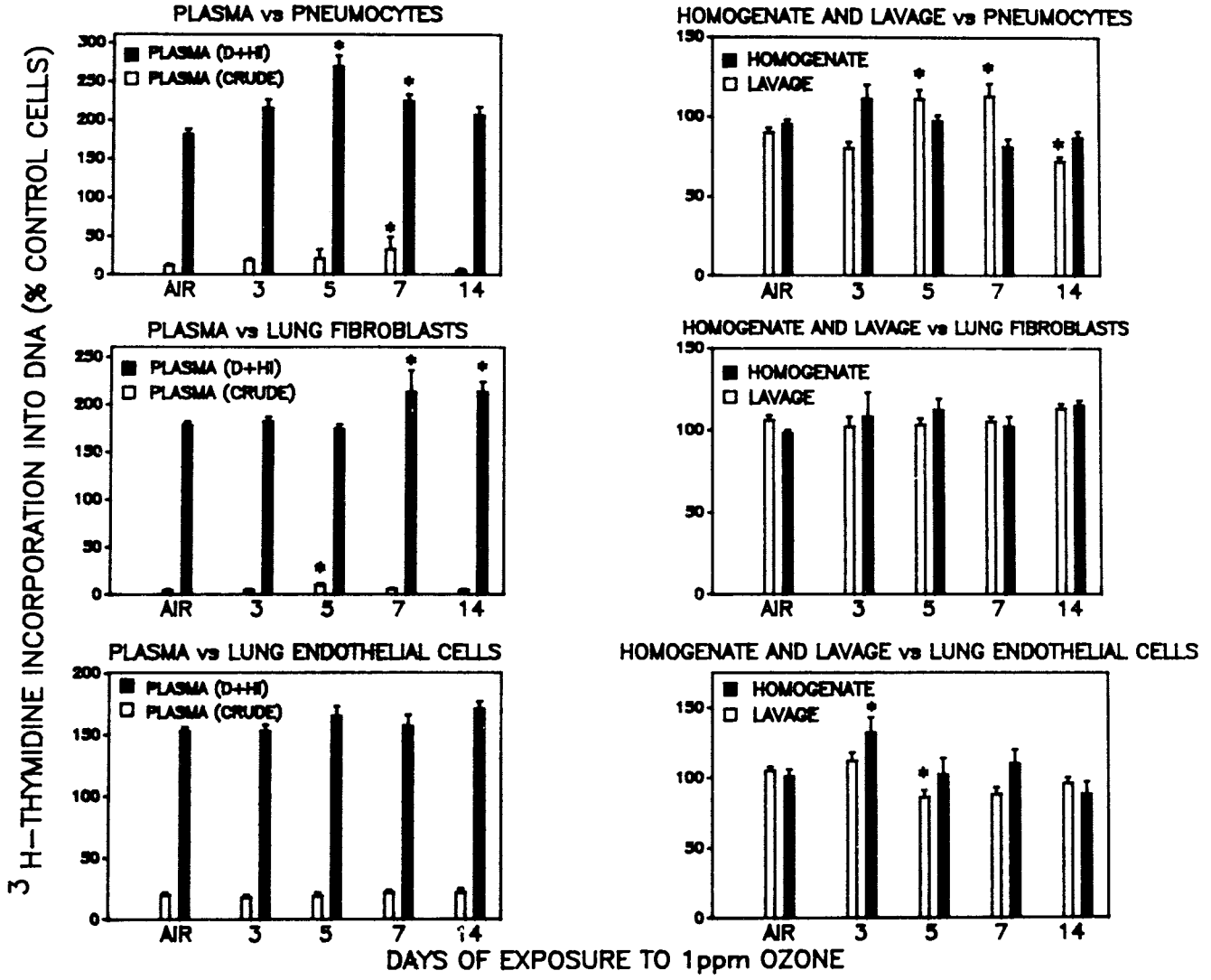

Figure 1. The effect of added plasma, lavage, or homogenate from animals exposed to air (n = 39) or O_3 (n = 9 or 10) for 3, 5, 7, or 14 days on DNA synthesis by lung fibroblasts, endothelial cells, or pneumocytes in vitro. All results are shown as a percentage of the activity observed in control cells not exposed to added sample, and expressed as the mean ± SEM. * = $p < 0.05$, for estimation of differences from the air group.

cytes was present in lavage from O_3-exposed animals at the same time points that stimulatory activity was present in plasma. However, after 14 days of O_3 exposure, the lavage contained pneumocyte inhibitory activity. Analysis of unfractionated lavage and homogenate samples did not otherwise reveal the presence of any O_3-related stimulators of DNA synthesis for any of the cell types, except for small amounts of endothelial cell stimulatory activity in homogenate from animals exposed to O_3 for three days, and inhibitory activity in lavage from animals exposed to O_3 for five days.

ELECTROFOCUSING OF SAMPLES FROM THE AIR AND OZONE EXPOSURES

Our experience from in vitro studies of lung-cell-derived growth factors has been that the assay of crude biological materials in initial screening studies, such as those above, can give false-negative results from the masking of stimulatory activity by inhibitors that are also present in the material (Tanswell 1983; Tzaki et al. 1988). This problem is overcome by sample fractionation, which can be achieved in a variety of ways. In the next step of this study, pooled samples from each exposure time point were fractionated by

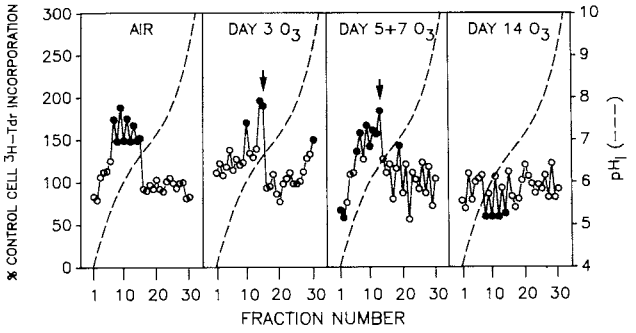

Figure 2a. The effect of preparative electrofocusing fractions from pooled plasma of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by type II pneumocytes in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

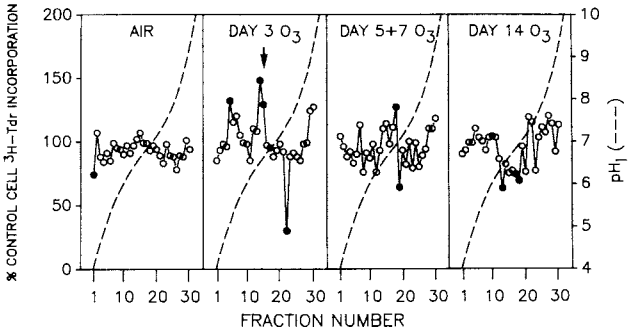

Figure 2b. The effect of preparative electrofocusing fractions from pooled lavage of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by type II pneumocytes in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

preparative electrofocusing. This technique has the following advantages: it is unlikely to denature biologically active samples, it characterizes active fractions in terms of their isoelectric point (pH_I), and it simultaneously "cleans up" active fractions for subsequent molecular-weight estimations by removal of many unwanted proteins. Fractionation has the disadvantage that a stimulatory factor may not only appear more active after the removal of inhibitory factors, but it may also appear less active because of its separation from cofactors for growth or from other synergistic growth factors. A widely held theory of growth factor activity is that cell division occurs in response to a (progression) growth factor only after the cell has been primed to respond by another (competence) growth factor. One factor, separated by fractionation, may appear inactive in the absence of the

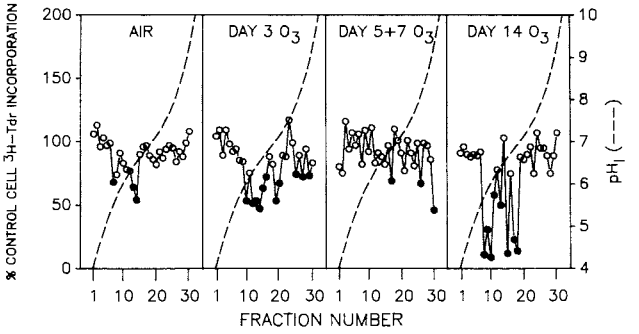

Figure 2c. The effect of preparative electrofocusing fractions from pooled homogenate of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by type II pneumocytes in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

other. It is for this reason that 2 percent serum is included in the assay medium, since it will generally supply sufficient competence and progression factors to allow activity of an unknown factor to be recognized. For these reasons, it is rarely possible to calculate the net effect of a fractionated material and come up with the same effect seen with unfractionated material.

Alterations of pneumocyte DNA synthesis by fractionated plasma, lavage, and homogenate from air- and O_3-exposed animals are shown in Figures 2a, 2b, and 2c, respectively. Control (air-exposure) plasma has stimulatory activity in fractions 7 through 15 that represents a pH_I range of 5.3 to 6.7. Neither control lavage nor homogenate fractions has any stimulatory activity. The profiles of preparative electrofocusing fractions shown in Figures 2a through 2c suggest that the stimulation of pneumocyte DNA synthesis observed in the original screening studies can be attributed to activity that electrofocuses to fraction 14 ± 1 (identified by the arrow in the figures), representing a pH_I of 6.6. This activity can be seen in day-3 and days-5 + 7 plasma and in day-3 lavage from the O_3-exposure groups. Control homogenate fraction 14 ± 1 has inhibitory activity that disappears by days 5 + 7 of O_3-exposure; this could reflect a superimposed stimulatory activity. All samples from day-14 O_3-exposed animals showed a wide range of fractions that were inhibitory to pneumocyte DNA synthesis. This was compatible with the results observed in the initial screening procedure.

Alterations of lung fibroblast DNA synthesis by fractionated plasma, lavage, and homogenate from air- and O_3-exposed animals are shown in Figures 3a, 3b, and 3c, respectively. Control plasma has stimulatory activity in fractions 7 through 17. This is lost by three days of O_3 ex-

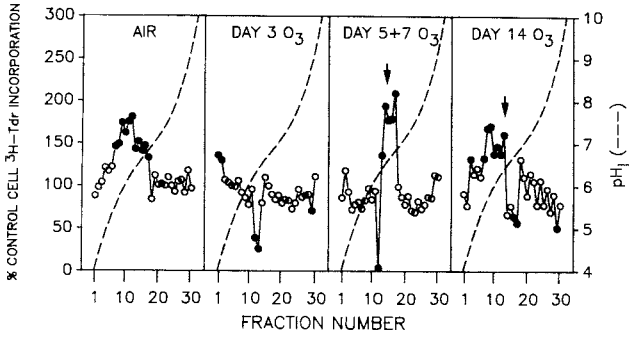

Figure 3a. The effect of preparative electrofocusing fractions from pooled plasma of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by lung fibroblasts in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

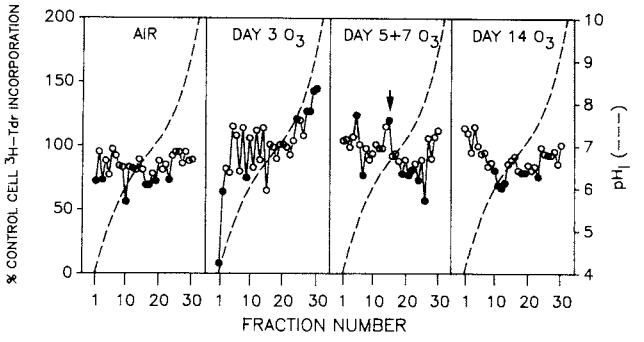

Figure 3b. The effect of preparative electrofocusing fractions from pooled lavage of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by lung fibroblasts in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

posure, and is replaced by an inhibitory activity in fraction 13 ± 1, representing a pH_I of 6.5. This activity is widely distributed among the samples from the O_3-exposed animals, being present in lavage at day 14 of exposure and in homogenate on days 3, 5 + 7, and 14. The stimulatory activity that was observed in the initial screening of plasma from animals exposed to O_3 for seven and 14 days must derive from the stimulator of DNA synthesis that is evident in fractions 15 ± 2 (arrow in figures) of the days-5 + 7 O_3-exposure plasma and lavage collections. While there is also a suggestion that this activity may be present in the day-14 O_3-exposure plasma, showing stimulatory activity in fraction 13, this is difficult to distinguish from the control plasma profile because of overlapping inhibition. This is most evident in fractions 17 and 18.

Studies of endothelial-cell DNA synthesis (Figures 4a,

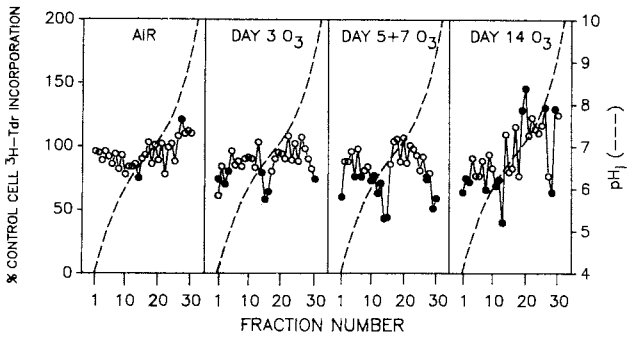

Figure 3c. The effect of preparative electrofocusing fractions from pooled homogenate of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by lung fibroblasts in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

4b, and 4c) have not been completed for all study groups, since the endothelial cell yields from each preparation are less than the other cell types and grow more slowly than do the fetal lung fibroblasts or pneumocytes. Initial screening studies did not reveal any O_3-related stimulatory activity in the plasma collections, but the completed plasma fractionation analyses (Figure 4a) show the presence of stimulatory activity in the plasma from animals exposed to O_3 for five and seven days. This occurred in fractions 6 through 13, corresponding to a pH_I range of 5.3 to 6.4. The partially completed analyses of fractionated lavage and homogenate samples are shown in Figures 4b and 4c.

OXYGEN EXPOSURE

The effects of 85 percent O_2 exposure on body weight, left lung weight, and left lung weight:body weight ratio × 10^3 are shown in Table 3. Body weights were lighter, and lung weights heavier, for animals exposed to O_2 at all time points. Because the size of the exposure system did not allow all O_2 exposures to be conducted simultaneously, as had been the case for O_3, it was not possible to evaluate the effect of exposure on growth. As with the O_3 exposures, the exposure to 85 percent O_2 resulted in significant increases in the left lung weight:body weight ratio at all time points.

INITIAL SCREENING OF SAMPLES FROM THE AIR AND OXYGEN EXPOSURES

The results of the initial screening are shown in Figure 5.

Crude plasma was again seen to cause a profound inhibition of ^{3}H-thymidine incorporation into DNA, which was reversed by dialysis and heat treatment.

Pneumocyte DNA synthesis was significantly increased

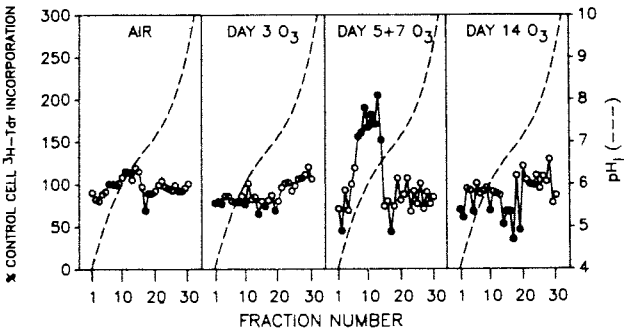

Figure 4a. The effect of preparative electrofocusing fractions from pooled plasma of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by lung endothelial cells in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

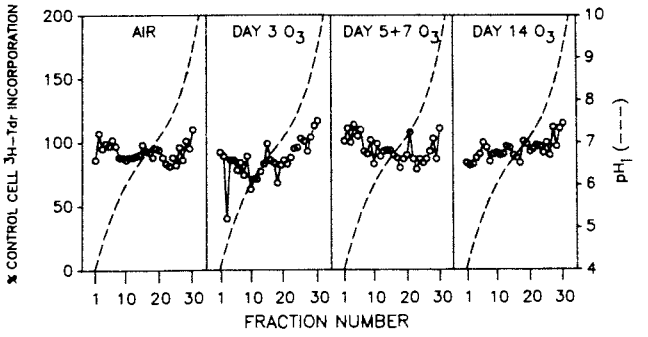

Figure 4b. The effect of preparative electrofocusing fractions from pooled lavage of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by lung endothelial cells in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Statistical analysis has not been conducted since all assays of this batch are not yet complete. ^{3}H-Tdr = ^{3}H-thymidine.

by the addition of heat-inactivated and dialyzed plasma from animals exposed to O_2 for 14 days, or of lavage from animals exposed to O_2 for seven or 14 days.

Fibroblast DNA synthesis was not significantly affected by the addition of O_2-exposure plasma or homogenate samples, but addition of lavage from animals exposed to O_2 for seven days caused a significant increase in fibroblast DNA synthesis.

Endothelial cell DNA synthesis was significantly increased by the addition of heat-inactivated and dialyzed plasma from animals exposed to O_2 for 14 days. The effects of unfractionated lavage and homogenate await analysis.

ELECTROFOCUSING OF SAMPLES FROM THE AIR AND OXYGEN EXPOSURE

Alterations of pneumocyte DNA synthesis by fractionated plasma, lavage, and homogenate are shown in Figures 6a,

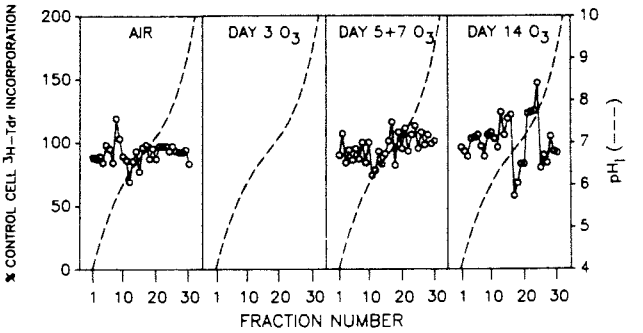

Figure 4c. The effect of preparative electrofocusing fractions from pooled homogenate of animals exposed to air or to O_3 for 3, 5 + 7, or 14 days on DNA synthesis by lung endothelial cells in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Statistical analysis has not been conducted since all assays of this batch are not yet complete. ^{3}H-Tdr = ^{3}H-thymidine.

Table 3. Effect of 85 Percent Oxygen or Air Exposure over Two Weeks on Rat Left Lung Weight, Body Weight, and Left Lung Weight:Body Weight (LLW:BW) Ratio[a]

		Days of Exposure to Oxygen or Air			
Parameter	Group	Day 3	Day 5	Day 7	Day 14
Body weight (gm)	Air	414 ± 23[b]	385 ± 32[b,c]	398 ± 38[b,c]	421 ± 47[b]
	O_2	363 ± 31[c]	357 ± 54[c]	355 ± 65[c]	351 ± 61[c]
Left lung weight (gm)	Air	1.17 ± 0.13[d]	1.23 ± 0.28[d,e]	1.25 ± 0.26[d,e]	1.29 ± 0.21[d,e,f]
	O_2	1.39 ± 0.14[d,e,f]	1.44 ± 0.21[e,f]	1.88 ± 0.32	1.51 ± 0.31[f]
LLW:BW Ratio ($\times 10^3$)	Air	2.85 ± 0.37[g]	3.24 ± 0.71[g,h]	3.18 ± 0.75[g,h]	3.12 ± 0.79[g]
	O_2	3.84 ± 0.41[h]	4.09 ± 0.56[i]	5.36 ± 0.83	4.32 ± 0.66[i]

[a] Data represent mean ± SD (n = 10).
[b] Matching superscripts (b through i) indicate no significant difference by Duncan's multiple-range test.

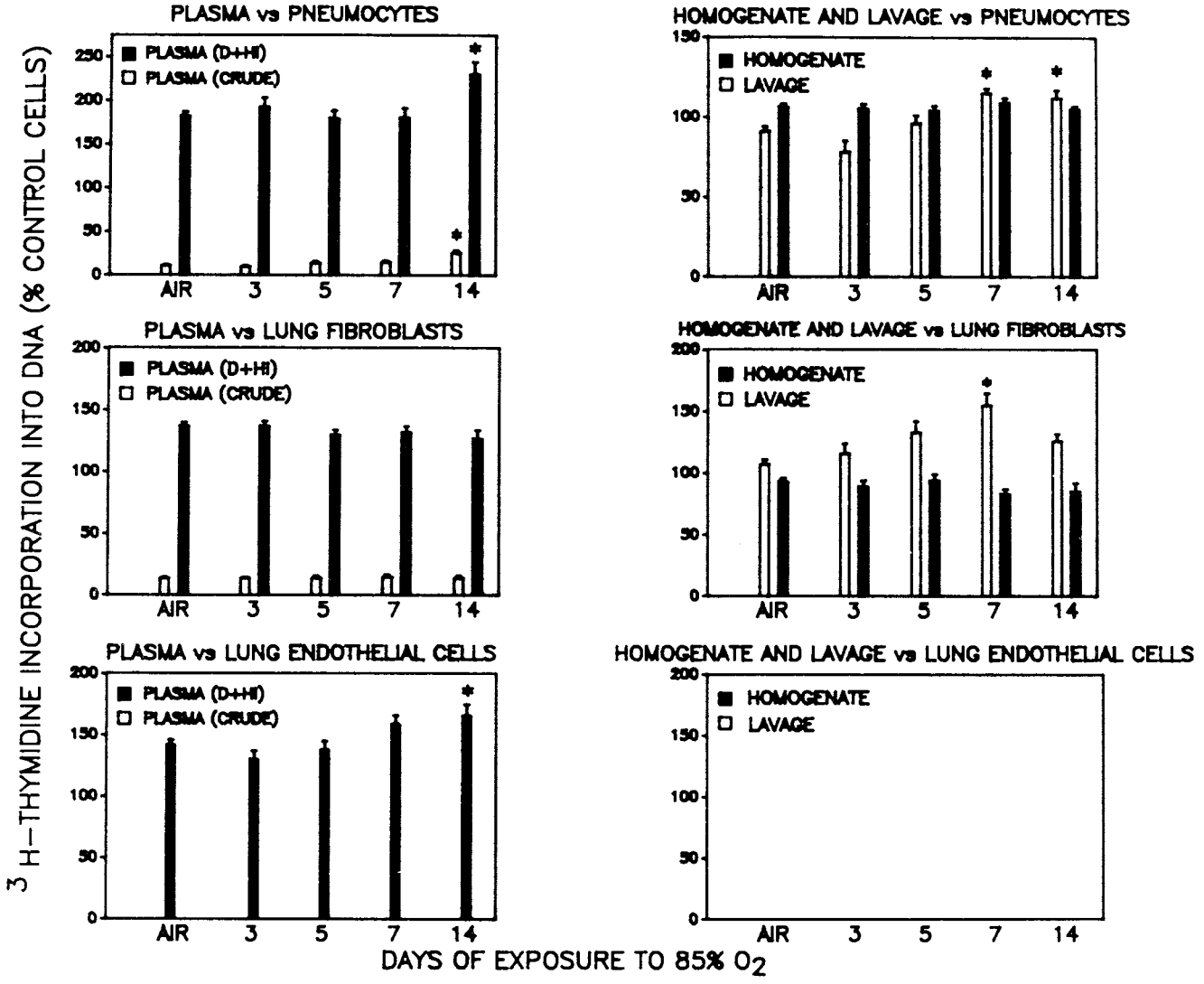

Figure 5. The effect of added plasma, lavage, or homogenate from animals exposed to air (n = 40) or O_2 (n = 10) for 3, 5, 7, or 14 days on DNA synthesis by lung fibroblasts or pneumocytes in vitro. (Results from studies with endothelial cells are not all available.) All results are shown as a percentage of the activity observed in control cells not exposed to added sample, and expressed as the mean ± SEM. * = $p < 0.05$, for estimation of differences from the air group.

6b, and 6c, respectively. Plasma from the air-exposed Canadian animals (used in the O_2 study) was less potent than was observed in the plasma from air-exposed U.S. animals (used in the O_3 study), presumably reflecting gene pool differences. The profile was, however, qualitatively, if not quantitatively, similar. The appearance of the stimulatory activity in fraction 14 ± 1 that was observed in the O_3 studies was again seen in the O_2 samples, but it appeared later in the time course of the exposure, as would be suggested from the initial screening studies. It was not evident until day 14 in plasma, but was evident in days-5 + 7 and day-14 lavage samples (arrows in figures).

Alterations of fibroblast DNA synthesis by fractionated plasma, lavage, and homogenate are shown in Figures 7a, 7b, and 7c, respectively. As with the pneumocyte studies, plasma from the Canadian control animals was less potent than plasma from their U.S. counterparts, though the control profiles are, again, qualitatively similar. As with the O_3 studies, there is the appearance of stimulatory activity at fraction 15 ± 1 in plasma, though somewhat earlier, at day 3 (arrow in figure). The days-5 + 7 O_2 profile could almost be overlaid on the day-14 O_3 profile, with possible stimulatory activity being masked by inhibitory activity (most evident in fractions 17 and 18). The day-14 O_2-plasma profile

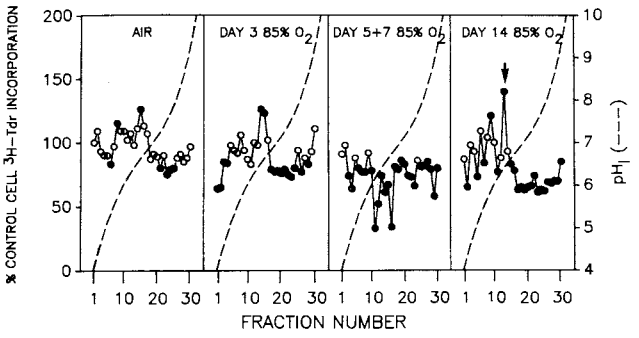

Figure 6a. The effect of preparative electrofocusing fractions from pooled plasma of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by type II pneumocytes in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

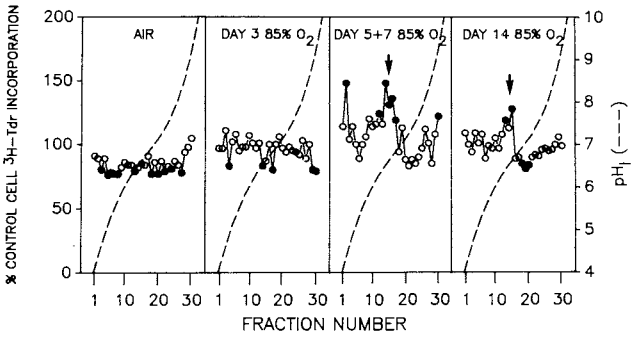

Figure 6b. The effect of preparative electrofocusing fractions from pooled lavage of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by type II pneumocytes in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

appears to have returned to the pattern observed in samples from air-exposed animals. No stimulatory activity was observed in fractionated lavage or homogenate.

Alterations of endothelial cell DNA synthesis by fractionated plasma are shown in Figure 8. The control (air-exposure) fractions show no significant stimulatory activity, as was seen with the O_3 study.

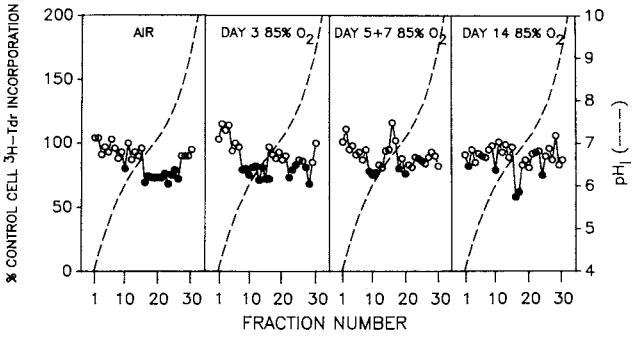

Figure 6c. The effect of preparative electrofocusing fractions from pooled homogenate of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by type II pneumocytes in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

DISCUSSION AND CONCLUSIONS

FIRM CONCLUSIONS

Because the project was only two-thirds completed, firm conclusions can be drawn only from the initial screening of crude samples (Figures 1 and 5). These are:

1. Exposure to both 1 ppm O_3 and 85 percent O_2 results in the appearance of factors in rat plasma that modify DNA synthesis by lung cells in vitro. Specifically, pneumocytes and fibroblasts respond to plasma from O_3-exposed animals, and pneumocytes and endothelial cells respond to plasma from O_2-exposed animals.
2. The appearance of these factors in plasma is related to the duration of exposure to the oxidant gas, and bears a temporal relationship to the morphologic changes in the lung reported by others using the same exposure protocols (Crapo et al. 1984; Evans 1984).
3. Stimulatory activity for pneumocytes is also evident in lavage from both O_2- and O_3-exposed animals, occurring at the same time (or slightly earlier) in the course of exposure as stimulatory activity in plasma. Lavage from O_2-exposed animals also contains stimulatory activity for lung fibroblasts, which was not seen with crude lavage from O_3-exposed animals.
4. The factors that stimulate DNA synthesis by pneumocytes, fibroblasts, and endothelial cells have apparent molecular weights exceeding 3,500, and the plasma factors are heat-stable at 56°C.

INTERPRETATION

While these preliminary results are exciting, in that there may indeed be circulating growth factor markers of early oxidant lung injury, there are, inevitably, a number of uncertainties remaining.

The source of any growth factors appearing in plasma in

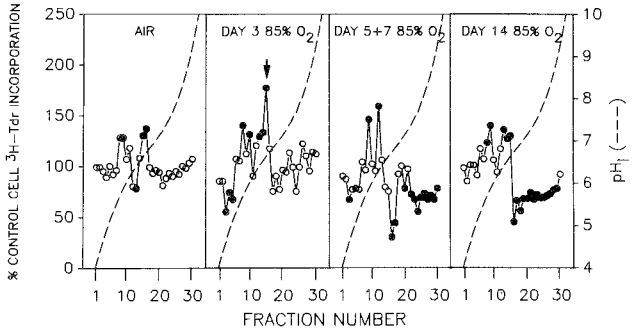

Figure 7a. The effect of preparative electrofocusing fractions from pooled plasma of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by lung fibroblasts in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

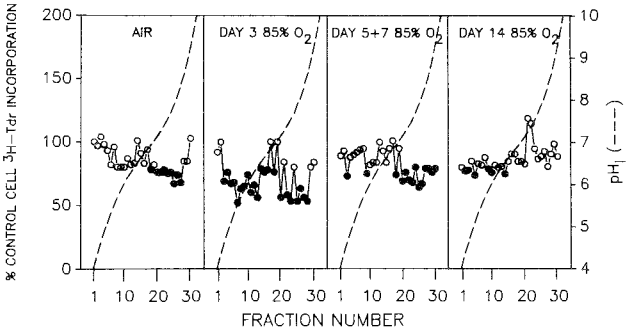

Figure 7b. The effect of preparative electrofocusing fractions from pooled lavage of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by lung fibroblasts in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

response to oxidant lung injury was not addressed in these experiments. This will have to await characterization of these factors; after that, lung tissue can be oxidant-injured in vitro and the release of the same factors studied. For the purposes of defining a circulating marker of oxidant injury, the source of a factor in blood is not important, as long as the appearance of the factor is a sensitive index of oxidant lung injury. Characterization is required before a protocol can be devised that will test the sensitivity of such a marker at low levels of exposure to inhaled oxidants.

The issue of whether the observed changes are a nonspecific response to stress, or a specific response to oxidant injury, was also not addressed by this protocol, although the appearance of activity in lavage samples at the same time as, or preceding, the appearance of activity in plasma samples makes a nonspecific response less likely. It is possible that any effects observed in lavage are due to leakage of blood into airways during oxidant injury. In biological terms their source may be unimportant, in that they are present at a potential site of action.

Analysis of homogenate samples was not helpful, and indeed had not been included in the original proposal because of (1) concerns that basement membrane proteins would exert a sufficient inhibitory effect to mask bioactivity in crude material; (2) difficulties of interpretation due to tissue-derived activity and activity present in contaminating blood; and (3) difficulties in designing an extraction protocol for uncharacterized factors. The only recognized mitogens, other than insulin, that enhance DNA synthesis by isolated adult type-II pneumocytes in vitro are epidermal growth factor and insulin-like growth factors I and II (A.K. Tanswell, unpublished observations). These growth factors, and many other peptides, can only be extracted under acid conditions, and it is for this reason that 1 M acetic

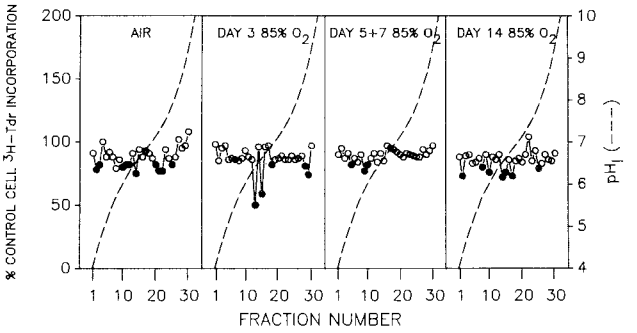

Figure 7c. The effect of preparative electrofocusing fractions from pooled homogenate of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by lung fibroblasts in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Values significantly different ($p < 0.05$) from sample-free control values are indicated by solid symbols. ^{3}H-Tdr = ^{3}H-thymidine.

acid was chosen for the extraction of lung homogenates. It is still possible that the use of an acid extraction technique failed to extract the factors evident in plasma and lavage, though it is more likely that stimulatory activity, which should have been present because of blood contamination, was masked by other inhibitory compounds present in the extract. Meaningful data from the homogenate samples may be generated once the molecular weight and pH_i of individual factors are known, so that samples can be appropriately fractionated prior to analysis.

INFERENCES FROM FRACTIONATION DATA

A stimulator of pneumocyte DNA synthesis, with an apparent pH_i of 6.6, appears in pooled plasma from animals exposed to either O_3 or O_2. The timing of the appearance

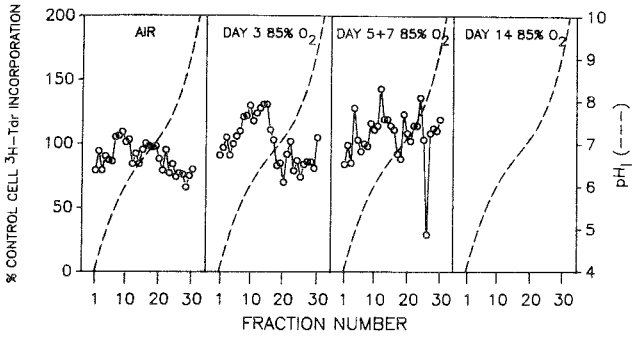

Figure 8. The effect of preparative electrofocusing fractions from pooled plasma of animals exposed to air or to O_2 for 3, 5 + 7, or 14 days on DNA synthesis by lung endothelial cells in vitro. Each data point represents the mean value for a triplicate assay of each fraction. All results are expressed as a percentage of the activity observed in control samples not exposed to added sample. Statistical analysis has not been conducted since all assays from this batch are not yet completed. ^{3}H-Tdr = ^{3}H-thymidine.

of this factor corresponds to the appearance of stimulatory activity in unfractionated plasma. Stimulatory activity, with the same pH_I, also appears in lavage at the same time as, or preceding, the appearance of stimulatory activity in plasma. No direct quantitative comparison can be made between animals exposed to different oxidant gases because they were derived from different gene pools, as evidenced by differences in the bioactivity of plasma from air-exposed control animals. In qualitative terms, however, there does seem to be evidence that the same factor appears in the airways and plasma of animals in response to both oxidant injuries.

The data from the fibroblast studies are more difficult to interpret. There was no activity against fibroblasts in the studies with crude plasma from O_2-exposed animals, yet it was present in plasma from animals exposed to O_3 for seven or 14 days. Conversely, there was stimulatory activity in crude lavage from O_2-exposed animals, but not from O_3-exposed animals. A stimulatory factor in fraction 15 ± 2 was evident in day-3 plasma from O_2-exposed animals, and in days-5 + 7 plasma and lavage from O_3-exposed animals. Interpretation of other groups was complicated by poor separation from the inhibitory materials present in fractions 17 and 18. Interpretation may become much simpler when results from fractionation in a second dimension (by size) become available. As stated earlier, studies of biological materials against lung fibroblasts can be confusing because of the presence of closely balanced inhibitory and stimulatory factors that may require complex separation techniques.

The results from the endothelial cell studies of fractionated samples are too preliminary to allow any inferences to be drawn.

REPRODUCIBILITY OF RESULTS

The assay system used here has highly reproducible results. For all three cell types, the 99 percent confidence limits for ^{3}H-thymidine incorporation into DNA into sample-free control cells were 95 to 105 percent of the mean incorporation. Time did not allow duplication of all sample assays, but all responses identified by asterisks in Figures 1 and 5, and those fractions identified by arrow in the other figures, have been reassayed, and the original responses were duplicated. The magnitude of responses observed are within an appropriate biological range, since a 10-fold increase in cell number over six days requires something less than a 50 percent daily increase in DNA synthesis.

COMPATIBILITY OF RESULTS WITH ORIGINAL HYPOTHESES

To date, the hypotheses in the first and second original aims have been confirmed by the initial screening studies. The incomplete fractionation data available so far are compatible with the hypotheses in the third and fourth original aims, particularly with respect to the pneumocyte studies.

Completion of these studies would first require analysis of the sizing column fractions. It would then be possible to address the question of whether a factor, of known size and pH_I, appears in each individual plasma sample in response to inhalation of O_3 or O_2. The fractionations have been completed, and only the bioassays remain outstanding. This same material could then be studied in a conventional fashion to determine whether it is a protein, as expected, and to provide additional physical characteristics. Lastly, any factors could be tested against a wide range of cell types for target cell specificity.

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APPENDIX A. Sample Preparation

SAMPLE COLLECTION

Plasma

The abdominal aorta was exposed and blood was withdrawn through an 18-gauge needle into a 10-ml syringe (prechilled to 4°C) containing 3.8 percent sodium citrate, to give approximately one volume of citrate to nine volumes of blood. This blood was gently transferred to a 15-ml centrifuge tube for centrifugation at 2,000 × g for 15 minutes at 4°C to separate plasma and erythrocytes. The plasma was aspirated and recentrifuged at 22,000 × g for 60 minutes to remove any residual red cell contamination, and then was recalcified with 20 mM calcium chloride. The final volume was recorded with the animal's code number, and the plasma was frozen at −70°C in siliconized glassware prior to lyophilization. Siliconized glassware was used to prevent the well-recognized problem of growth factor adherence to uncoated glass.

Lung Lavage

After the removal of blood, a neck incision was made and the trachea was identified. A wide-bore plastic tube was tied into the trachea, and the lungs were lavaged three consecutive times with 5 ml phosphate-buffered saline. The combined lavage fluids from each animal were centrifuged at 300 × g for 10 minutes at 4°C to remove contaminating cells. The supernatant was carefully decanted, labeled with the animal's code number, and stored frozen at −70°C in siliconized glassware prior to lyophilization.

Lung Homogenate

The left lung was excised, weighed, and made 20 percent with 1 M acetic acid for tissue disruption by homogenization on ice. The homogenate was centrifuged at 1,000 × g for 10 minutes at 4°C to separate insoluble material, and the supernatant was carefully decanted and stored frozen at −70°C in siliconized glassware prior to lyophilization.

Sample Transport

Samples from North Carolina were shipped to London, Ontario, on dry ice, and were placed at −70°C on arrival.

SAMPLE DILUTIONS

Assay of these samples for the presence of growth factor activity required that they be diluted with cell culture medium for application to cultured cells. The choice of an appropriate sample dilution was completely arbitrary, given the absence of similar previous experiments on which to base a selection. The final choices were determined by the volume of sample material available for study, and a need to have a sufficient amount of the sample available for resuspension at an increased concentration if necessary.

Plasma

Plasma was resuspended to its original volume (approximately 4.5 ml) in distilled water (dH_2O) and then filtered two times through a 0.22-μm syringe filter. It was then diluted to 10 percent with culture medium for bioassay.

Lavage

Lavage was resuspended in 5 ml dH_2O and dialyzed across a dialysis membrane, with a 3,500 molecular weight exclusion limit, against an excess of dH_2O. The dialysis was conducted over 72 hours, at 4°C with three changes of dH_2O. After dialysis the lavage was lyophilized and resuspended in 2 ml phosphate-buffered saline. The samples were diluted to 10 percent with culture medium containing 2 percent serum for bioassay.

Homogenate

Homogenate was resuspended in 5 ml 1 M acetic acid, sonicated for 30 seconds, and centrifuged at 1,000 × g for 10 minutes. The supernatant was dialyzed (as for lavage) against an excess of 1 M acetic acid. After dialysis the samples were lyophilized, then resuspended in 5 ml phosphate-buffered saline. This was diluted to 10 percent with culture medium containing 2 percent serum for bioassay.

APPENDIX B. Preparation of Cells for Bioassay

PREPARATION OF A MIXED LUNG CELL SUSPENSION

Timed gestation Sprague-Dawley rats were killed on day 20 of gestation by chloroform excess. Five litters were used for each preparation. The uterine horns were immediately exposed through an abdominal incision, and the fetuses were removed after intrauterine decapitation. The bodies were placed in calcium- and magnesium-free Hank's balanced salt solution (HBSS) at 4°C. The lungs were excised, dissected free of major airways, and pooled. These lungs were minced into 1- to 2-mm^2 fragments, washed in HBSS, and then stirred at 20°C in HBSS containing 0.05 percent trypsin, 0.02 percent ethylenediaminetetraacetic acid (EDTA), and 50 μg/ml DNAase, until the tissue was completely dispersed. The protease activity was neutralized with the addition of serum to 10 percent of total volume, and the suspension passed through 100-μm mesh nylon bolting cloth. The filtered cell suspension was then pelleted at 300 × g for 10 minutes. The characteristics of mixed cell cultures prepared in this way have been described in detail elsewhere (Tanswell et al. 1983).

PREPARATION OF ORGANOTYPIC CULTURES

The cell pellet was resuspended in medium with Earle's salts, 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, and 1 percent nonessential amino acids containing 10 percent serum and plated in a 25-cm^2 culture flask for a 60-minute differential adherence at 37°C. During this time lung fibroblasts, but few other lung cells, attached. The nonadherent cells were removed and pelleted, then allowed to stand for 60 minutes at 37°C before being resuspended in 1.3 ml fresh medium with 10 percent serum. This cell suspension was applied as 50 μl aliquots to 24 cm^3 × 1 cm^3 gelatin sponges saturated with medium with 10 percent serum. After one hour the culture flasks containing the sponges (8 per flask) had culture medium added to float the sponges, which were then left to incubate at 37°C overnight. The sponges were used the next day for type II pneumocyte purification. The original flask, containing cells that had passed through the sponge, was used for endothelial cell purification.

TYPE II PNEUMOCYTE PURIFICATION

Organotypic cultures prepared in this way in my laboratory contain primarily pneumocytes, with some residual fibroblast contamination (Simpson et al. 1985). The gelatin sponges were dissolved with 1 percent collagenase in HBSS. After the addition of serum to 10 percent by volume, the released cells were pelleted at 300 × g for 10 minutes. These were resuspended in fresh medium with 10 percent serum and incubated at 37°C for 15 minutes to allow any residual fibroblasts to attach. Nonadherent cells were plated in medium with 10 percent serum and then grown to confluence. The flask was split 1:3 with 0.05 percent trypsin:0.02 percent EDTA and replated after another 15-minute differential adherence step. These cells were grown to confluence in the second passage with medium and 10 percent serum; L-valine was replaced by D-valine in the medium to prevent fibroblast growth. The type II pneumocytes were then removed from the flasks with 0.05 percent trypsin:0.02 percent EDTA for use in the bioassay. This is a minor modification of the method described by Post and associates (1984). Purity of the type II cell population at the time of assay was 90 to 95 percent, as assessed by indirect immunofluorescent staining for surfactant apoproteins, and by light microscopy following tannic acid and polychrome staining for lamellar body identification.

ENDOTHELIAL CELL PURIFICATION

The flasks that had originally housed the organotypic cultures were used for endothelial cell purification, since these pass through gelatin sponges without adhering. They do, however, adhere to the bottom of the culture flasks. The flasks were washed with medium plus 10 percent serum to remove nonadherent lymphocytes. Adherent cells were removed from the flasks with 0.2 percent recrystallized trypsin in HBSS (without EDTA). The combined cell suspensions from five flasks (20 ml) were placed in a vertical 50-ml centrifuge tube and diluted 1:1 with medium containing 10 percent serum. After 30 minutes of unit gravity sedimentation at 20°C, the suspension was gently aspirated until only the bottom 2.5 ml remained. This was diluted 1:1 with 2.5 ml of medium containing 10 percent serum, and was allowed to stand for an additional 30 minutes of unit gravity sedimentation. The upper 2.5 ml was gently aspirated and discarded. The lower 2.5 ml was diluted 1:1 with medium and plated over one hour to allow any residual fibroblasts to adhere. The nonadherent cells were replated and grown to confluence. At confluence, the cells were split 1:3 after a further 15-minute differential adherence step, and then were maintained in D-valine medium, to eliminate fibroblasts, with 10 percent serum for the second passage. The endothelial cells were removed from the flasks with 0.05 percent trypsin:0.02 percent EDTA, and then replated in fresh medium with 20 percent serum until use in the bioassay. Characteristics of this endothelial cell population have been reported elsewhere (Olson and Tanswell 1987). The av-

erage purity of the cell population at the time of assay was 95 percent, as assessed by indirect immunofluorescent staining for factor VIII antigen and for angiotensin-converting enzyme.

LUNG FIBROBLAST PURIFICATION

Adherent cells from the differential adherence step with the mixed cell suspension were predominantly fibroblasts. The adherent cells were grown to confluence in medium with 10 percent serum, then trypsinized and split 1:3 with a 15-minute differential adherence step (only adherent cells being retained). At confluence, the fibroblasts were trypsinized for use in the bioassay. Contamination with type II pneumocytes and endothelial cells was less than 5 percent by the time of bioassay.

APPENDIX C. Preparative Electrofocusing

Aliquots of resuspended homogenate and plasma samples (100 µl), and of lavage samples (50 µl), were pooled for animals exposed to each gas for 3, 5 + 7, and 14 days. The pooled materials were dialyzed against three changes of an excess of 1 percent glycine for 72 hours at 4°C. After dialysis, the sample was aspirated and made up to 1 ml (days 3 and 14) or 2 ml (days 5 + 7) with distilled deionized water (ddH_2O) to which was added 95 ml of ampholine solution (ddH_2O containing 0.28 ml of each of the LKB ampholines pH 4 to 7 and pH 5 to 7, 0.55 ml of the LKB ampholine pH 9 to 11, and 3.8 ml of the LKB ampholine pH 3.5 to 10) and 4 g of LKB 2117–510 Ultrodex. Flat-bed electrophoresis in this granulated gel was conducted over 20 hours at 10°C at 5 watts constant power (with the voltage set at the maximal setting of 2500 V and the current set at the maximal setting of 250 mA) in an LKB Multiphor apparatus. Cooling was supplied by a Haake G thermostatic circulator.

At the end of the separation period, a fractionating grid was pressed into the slurry and each of the 30 compartments were collected with a spatula into a BioRad disposable minicolumn. Each column was washed overnight with 2 ml of phosphate-buffered saline at pH 7.0, and the eluate was dialyzed against ddH_2O and then lyophilized. Fractions were then taken up in 2 ml (plasma), 800 µl (lavage), or 1 ml (homogenate) of medium. These volumes were diluted 10-fold for bioassay. Each fraction of each sample was tested in triplicate for ^{3}H-thymidine incorporation into DNA, as described earlier.

The linearity of the gradient obtained in this system was ascertained by using three separate runs over the course of these experiments, and by determining the pH of individual fractions. Each of the 30 lanes was removed with a spatula, then suspended in 1 ml of ddH_2O adjusted to a pH of 7.0. Adjacent lanes were combined and their average pH recorded. Results are shown in Figure C.1. A fourth-order polynomial regression analysis was conducted by the method of least squares, and this regression line ($r = 0.998$) was included in all data plots.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Preparative electrofocusing fractions 12 through 16 from each exposure group were subjected to a further separation by size using a Waters (Milford, MA) HPLC with a Protein Pak 300SW column and a Protein Pak 125 column in series, and phosphate-buffered saline at pH 7.4 at 0.5 ml/minute as the mobile phase.

A sample separation of molecular weight standards by this column series is shown in Figure C.1, where the eluate

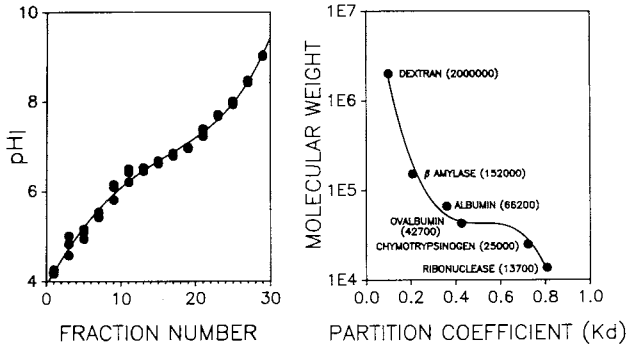

Figure C.1. The left figure shows the separation of isoelectric point standards by the separation system used in these experiments. Data points represent the values obtained for three separate runs over the course of these experiments. The linear regression line shown was calculated from these values by the method of least squares. The right figure shows an example of molecular weight standard separation by the HPLC column system used in these experiments. Standards were selected to demonstrate separation in the 20,000 to 40,000 range in which the putative growth factors observed in these experiments are expected to fractionate. The regression line was calculated by the method of least squares.

was monitored by absorbence at 280 nm. A third-order polynomial regression analysis was conducted by the method of least squares, and this regression line (r = 0.995) has been included in the figure.

ABOUT THE AUTHOR

A. Keith Tanswell graduated from St. Mary's Hospital Medical School of London University, London, England in 1969. He held a Canadian Cystic Fibrosis Foundation Fellowship during his research training at Queen's University, Ontario, and at Duke University, NC. Dr. Tanswell is currently Professor of Paediatrics at the University of Western Ontario and an attending neonatologist at the St. Joseph's Health Centre in London, Ontario. His research interests have focused on factors affecting oxidant injury of the immature lung.

PUBLICATION RESULTING FROM THIS RESEARCH

Tanswell AK, Fraher LJ, Grose EC. 1989. Circulating factors which modify lung cell DNA synthesis following exposure to inhaled oxidants: I. Effect of serum and lavage on lung fibroblasts following exposure of adult rats to 1 ppm ozone. J Toxicol Environ Health (in press).

INTRODUCTION

A Request for Applications (RFA 84–3), which solicited proposals for studies on "Mechanisms of Oxidant Toxicity," was issued by the HEI in the summer of 1984. In response to the RFA, Dr. A. Keith Tanswell, from The Research Institute of St. Joseph's Hospital in London, Ontario, Canada, submitted a proposal entitled, "Detection of Paracrine Factors in Oxidant Lung Injury." The HEI approved the two-year project and authorized a total expenditure of $128,093. The project began in October 1985, and the final report was accepted by the Health Review Committee in April 1988.

The Health Review Committee's Report is intended to place the Investigator's Report in perspective as an aid to the sponsors of the HEI and to the public.

THE CLEAN AIR ACT

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1977. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and provide the EPA with limited discretion to modify those requirements.

The determination of the appropriate standards for emissions of oxidants depends, in part, on an assessment of the risks to health that they present. Research on mechanisms of oxidant injury, including the possible role of lung-specific growth factors, can contribute to such risk assessment and, therefore, to informed regulatory decision-making.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards to protect the public health. The current standards include those for ozone and nitrogen dioxide. Research on mechanisms of oxidant injury can also contribute to the assessment of the appropriateness of the standards.

BACKGROUND

Oxidants represent a class of pollutants derived primarily from the combustion of fossil fuels and the atmospheric reaction of combustion products with oxygen, catalyzed by sunlight. In acute animal experiments, the inhalation of high concentrations of oxidants, such as nitrogen dioxide or ozone, results in pulmonary edema, inflammation, and hyperplasia of constituent cells (U.S. Environmental Protection Agency 1986). The relation of these oxidant-induced effects to either ambient levels of pollutants or to chronic pulmonary disease, such as fibrosis, has yet to be determined. Information on the cellular mechanisms and biochemical mediators of acute, oxidant-induced injury would contribute to our understanding of these relationships.

After exposure to sublethal concentrations of nitrogen dioxide, ozone, or another oxidant, oxygen, examination of lung parenchymal tissues reveals cellular damage followed by repair; type II epithelial cells, fibroblasts, and endothelial cells proliferate in response to the insult (Crapo et al. 1984; Evans 1984). The time course and the reaction of different cell types depend on the particular oxidant and its associated physicochemical properties.

Currently, an active area of investigation in cell biology is the identification of growth factors, primarily polypeptides, which modulate cellular growth (reviewed in Gospodarowicz 1983; Wahl and Carpenter 1987). Cells synthesize and secrete these growth factors, which are utilized either locally by neighboring cells (paracrine action) or by the cell of origin (autocrine action) (Sporn and Todaro 1980). Not all factors, however, appear to be secreted, and cell disruption may be necessary for the release of some factors. Although growth factors can be found in the blood, they are often associated with other proteins; whether these binding proteins serve to inactivate, clear, or deliver growth factors to distant target sites is unknown.

Target cells have been identified by their biological responses and by the presence of specific cell-surface receptors (Wahl and Carpenter 1987). The receptor not only determines the sensitivity of the target cell, but also dictates the type of biological response. Often, the response of punative target cells is used to indicate the presence of a particular growth factor. However, because the specificity of the response resides, in part, with the receptor, care should be exercised in the interpretation of the results. For example, alpha-transforming growth factor and epidermal growth factor, which are two distinctly different factors, bind to the same receptor, and their effects are indistinguishable in a bioassay.

In addition to their mitogenic activity, growth factors can inhibit cell proliferation as well as influence cell functions unrelated to growth. For example, under different conditions, beta-transforming growth factor either stimulates or

inhibits the growth of fibroblasts (Roberts et al. 1985). What ultimately determines the response of a target cell to a particular growth factor is unknown, but it has been postulated that the context of the factor, in relation to other mediators, governs the outcome (Sporn and Roberts 1988). Thus, individual polypeptides represent characters in a code or "language," which cells translate (Sporn and Roberts 1988).

Several advances in cell biology have contributed to the rapidly expanding data base on growth factors. Highly purified preparations of polypeptides have been obtained using techniques such as affinity chromatography and high-pressure liquid chromatography. The purified molecules can be sequenced and structural characteristics analyzed. Such preparations can be used to generate antibodies, monoclonal in some cases, that can be used for detection and quantification by radioimmunoassay or for localization by immunocytochemistry. Finally, techniques of molecular biology have been used to generate greater amounts of peptide for study, to sequence genes, and to derive probes that have been used to detect cellular sites of synthesis.

Growth factors that stimulate epithelial, endothelial, or fibroblast cells could be active during oxidant-induced pulmonary hyperplasia. Epidermal growth factor (Carpenter and Cohen 1979), acidic- and basic-fibroblast growth factors (Thomas and Gimenez-Gallego 1986; Folkman and Klagsburn 1987), insulin-like growth factors (D'Ercole 1987), platelet-derived growth factor (Ross and Vogel 1978; Scher et al. 1979), alpha- and beta-transforming growth factors (Roberts and Sporn 1985), and interleukin 1 (Schmidt et al. 1982) have been shown to stimulate proliferation of one or more of these cells. Whether or not these factors have paracrine or autocrine roles during oxidant-lung injury is unknown. Alternatively, other, as yet unidentified, mediators of pulmonary cell growth may be present during this process.

Numerous studies have investigated proliferation of lung fibroblasts or pneumocytes after exposure to fibrogenic agents. Macrophages have been reported to release paracrine factors that either stimulate or inhibit fibroblast proliferation (Leibovich and Ross 1976; Bitterman et al. 1982; Lugano et al. 1984; Elias et al. 1985; Lemaire et al. 1986). Fibroblasts may also release autocrine factors after oxygen exposure (Jones et al. 1987; Tzaki et al. 1988). Oxygen-exposed fibroblasts also secrete a paracrine factor for pneumocytes (Tanswell 1983). However, these various factors have not been isolated to purity and, thus, are not fully characterized.

Despite all the information that has been compiled about specific growth factors, the physiological role of growth factors in the organism is not known (Gospodarowicz 1983). Most experiments have been conducted using cultured cells, and it has been extremely difficult to determine the role of these peptides in physiological or pathological processes. Problems encountered by researchers include the complexity of the mediator "language" and the likelihood that other factors that regulate growth have yet to be discovered. Sorting out the roles of growth factors during embryonic growth and differentiation, normal cell turnover, or repair following injury is difficult, but has important implications for understanding the pathological counterparts to normal processes.

JUSTIFICATION FOR THE STUDY

The HEI sought proposals that would improve our understanding of the relationship between oxidant injury and lung disease. One area of particular interest to the Institute was the identification of cellular mediators of oxidant injury. Growth factors were considered promising candidates for investigation because oxidant exposure is associated with parenchymal cell hyperplasia.

Dr. Tanswell proposed to determine if plasma and lung lavage fluid derived from rats exposed to atmospheres containing 85 percent oxygen possessed mitogenic activity against type II pneumocytes, lung fibroblasts, and pulmonary endothelial cells. Dr. Tanswell further proposed that mediators of cell growth, once identified, could ultimately be used as markers of oxidant-induced lung injury. The HEI Research Committee was impressed with the experience of the investigator in defining growth factors for type II pneumocytes and with the available cell culture facilities. There were, however, no preliminary data suggesting that in vivo hyperoxic exposure causes growth factor production. The committee thought that the proposal warranted a feasibility study. To address the goals of the RFA more effectively, the Research Committee requested that in vivo exposures to ozone and examination of lung tissue homogenates be added to the study.

OBJECTIVES AND STUDY DESIGN

The goal of the project was to test the hypothesis that hypertrophy of the lung after oxidant injury with ozone or oxygen is due to local generation of lung-specific growth factors. Specifically, the objectives were to:

1. Measure the putative growth factors affecting type II pneumocytes, lung fibroblasts, or lung endothelial cells in blood, bronchoalveolar lavage fluid, or lung tissue homogenates after exposure to ozone or oxygen.
2. Demonstrate a temporal correlation between the appear-

ance of any such growth factors and the expected proliferative changes occurring in the lung parenchyma.

3. Show that growth factor release is determined by degree and duration of oxidant injury, rather than by any specific oxidant.
4. Characterize any identified growth factor(s) by preparative electrofocusing, molecular weight sizing, and target cell specificity.

To test the hypothesis, rats were exposed to 85 percent oxygen or to 1 part per million ozone for up to two weeks. Blood plasma, lung homogenates, and lung lavages were examined for the ability to stimulate ^{3}H-thymidine incorporation into cultured fetal rat lung endothelial cells, fibroblasts, and type II pneumocytes. Crude plasma and dialyzed heat-treated plasma were tested for growth-promoting activity. The plasma, lung homogenates, and lung lavages were fractionated by preparative isoelectric focusing, and the ability of these fractions to stimulate ^{3}H-thymidine incorporation was determined.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

Animal exposures and the sampling of blood, lung lavage, and lung tissue were completed. The crude, as well as fractionated, samples from ozone- or oxygen-exposed animals were tested on pneumocytes and fibroblasts. The response of endothelial cells to crude or fractionated samples was not completely assayed. Not all of the objectives outlined in the original proposal to HEI were achieved due to the exploratory nature of the study.

METHODS AND STUDY DESIGN

The Investigator's Report provides the rationale for choosing particular experimental procedures. However, alternative approaches could have been used to address the hypothesis; these are discussed below under Remaining Uncertainties and Implications for Future Research.

STATISTICAL AND DATA ANALYSIS

A comparison between the responses to ozone and oxygen was not an objective of this study, and although the investigator makes some general statements combining the oxygen and ozone results, he is careful not to make direct comparisons between the responses to the two oxidants. Direct statistical comparison of the responses to oxygen and ozone could be made, but only if the differences between the two air-exposed control groups were adjusted.

Some of the laboratory analyses followed a protocol that precluded a complete estimate of the variance. For the electrofocusing, the samples were pooled in order to reduce to a manageable number the total number of assays. The pooling of samples should give approximately the same mean as the average of individual assays. However, it obscures the contribution of interanimal variation to the analytical error. Since the variation among animals dominates the analytical error, the analytical error may be a gross underestimate.

RESULTS AND INTERPRETATION

The investigator states that the results from unfractionated plasma, lavage, or tissue homogenate samples show that:

1. Exposure to ozone or oxygen results in the appearance of factors in rat plasma that modify DNA synthesis by type II pneumocytes, lung fibroblasts, and lung endothelial cells.
2. The appearance of these factors is related to the duration of exposure to the oxidant gas, and bears a temporal relation to the morphologic changes reported by others using the same exposure protocols.
3. Lavage from animals exposed to oxygen or ozone contains stimulatory activity for pneumocytes, whereas lavage from animals exposed to oxygen, but not ozone, contains activity for lung fibroblasts.
4. The factors that stimulate DNA synthesis by pneumocytes and fibroblasts have apparent molecular weights exceeding 3,500 and are heat-stable at 56°C.

Interpretation of these findings, as acknowledged by the investigator, requires additional experimentation. For example, no stimulatory bioactivity was detected in the lung homogenates. The absence of a response in the bioassay, however, does not necessarily imply the absence of a growth factor. The marked inhibition of ^{3}H-thymidine incorporation into all test cell types by crude plasma may be due to the presence of factors with inhibitory functions. This inhibition could also be due to a cytotoxic effect of complement, which is inactivated at 56°C.

With respect to the fractionated samples, specific growth factor activity related to oxidant exposure was not definitively demonstrated, although the investigator inferred that a particular fraction, corresponding to an isoelectric point (pH_I) of 6.6, promoted DNA synthesis. Additional fractions, however, also appeared to contain stimulatory activity, but their significance was not discussed by the investigator. Furthermore, in plasma from air-exposed animals, growth-promoting activity was apparent (see Figures 2a, 3a, 6a, and 7a), implying that the presence of activity was not specific to oxidant exposure.

The investigator's claim that the factors appear in the lavage prior to, or at the same time as, their appearance in plasma is not fully supported by the data. In the case of the ozone-plasma-fibroblast group, stimulatory activity is present, but it is not detected in the ozone-lavage-fibroblast assay. In contrast, in the oxygen-exposed animals, stimulatory activity for fibroblasts is present in the lavage sample, but not in the plasma sample. Furthermore, in the ozone experiment, the pH_I of the more acidic peak on day 3 differs for plasma and lavage (Figure 2a versus Figure 2b), suggesting the possibility of two different factors.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The hypothesis of this study is reasonable and testable. Both the completed work and the proposed high-pressure chromatography steps clearly indicate that the investigator realizes that the definitive proof of the hypothesis is the eventual isolation and characterization of the growth factor(s).

In future studies, alternative approaches can be used to assess the role of growth factors in oxidant-induced hyperplasia. Because a number of well-characterized factors are already known to have a mitogenic effect on the cell types of interest, experiments can be conducted to screen for the activity of these factors. Highly specific antibodies, receptor assays, and, in some cases, cDNA probes, are currently available and can be used to determine if any of these factors are present during oxidant injury.

Determining the presence of a new factor represents a greater challenge, but consideration should be given to the autocrine or paracrine modes of action in the experimental design. Although it may ultimately be desirable to use the presence of a growth factor in the circulation as a marker of oxidant exposure or early lung injury, isolating the factor from the blood and relating its presence specifically to oxidant exposure are both extremely difficult. Purification of growth-promoting factors from potential lung cell sources may be more efficient. As the investigator points out, the use of crude tissue homogenates creates difficulties in purification procedures. However, current or improved cell culture techniques can be used for short-term culture of cells derived from lung parenchymal tissue; these, in turn, can be tested for growth-promoting activity.

CONCLUSIONS

The investigator presents evidence that some stimulatory material is present in dialyzed, heat-treated plasma from rats exposed to high levels of oxidants. Some activity is also present in lung lavage. The investigator recognizes that, due to the presence of lung edema in the oxidant-exposed animals, it is not possible to deduce the exact source (that is, lung-derived or blood-borne) of any factor detected. The presence of inhibitors and the complexity of the design contribute to the difficulty of drawing conclusions about the specificity or temporal relation of a growth factor to oxidant injury. Although the data are preliminary and the biological significance of the observed changes is uncertain, the hypothesis is worthy of pursuit.

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Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research (Supplement)	January 1988

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