



**HEALTH EFFECTS INSTITUTE**

## **Mechanisms of Aldehyde-Induced Bronchial Reactivity: Role of Airway Epithelium**

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**Includes the Commentary of the Institute's  
Health Review Committee**

**Research Report Number 49**

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### Mechanisms of Aldehyde-Induced Bronchial Reactivity: Role of Airway Epithelium

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#### ABSTRACT

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To investigate the relative irritant potencies of inhaled aldehydes, guinea pigs were exposed to formaldehyde or acrolein and specific total pulmonary resistance and bronchial reactivity to intravenous acetylcholine were assessed. The mechanisms associated with these responses were investigated by analyzing morphologic and biochemical changes in airway epithelial cells after *in vivo* and *in vitro* exposures. Immediately after exposure to formaldehyde or acrolein, specific resistance increased transiently and returned to control values within 30 to 60 minutes. Bronchial hyperreactivity, assessed by the acetylcholine dose necessary to double resistance, increased and became maximal two to six hours after exposure to at least 9 parts per million<sup>2</sup> (ppm) formaldehyde or at least 1 ppm acrolein for two hours. The effect of exposure to 3 ppm formaldehyde for two hours was less than the effect of exposure to 1 ppm formaldehyde for eight hours; thus, extended exposures produced a disproportionate heightening of bronchial reactivity. Bronchial hyperreactivity often persisted for longer than 24 hours.

Increases in three bronchoconstrictive eicosanoids, prostaglandin F<sub>2α</sub>, thromboxane B<sub>2</sub>, and leukotriene C<sub>4</sub>, occurred immediately after exposure, whereas an influx of neutrophils into lavage fluid occurred 24 hours later. Histological examination of the tracheal epithelium and lamina propria also demonstrated a lack of inflammatory cell infiltration. Treatment with leukotriene synthesis inhibitors and receptor antagonists inhibited acrolein-induced hyperreactivity, supporting a causal role for these compounds in this response. Acrolein also stimulated eicosanoid release from bovine epithelial cells in culture. However, the profile of metabolites formed differed from that found in lavage fluid after *in vivo* exposure. Similarly, human airway epithelial cells did not produce cysteinyl leukotriene or thromboxane B<sub>2</sub>. However, cysteinyl leukotrienes were mitogenic for human airway epithelial cells in a concentration-dependent manner and exhibited a structure-activity relationship; leukotriene C<sub>4</sub> was more potent than its sequential metabolites D<sub>4</sub> and E<sub>4</sub>. The potency of leukotriene C<sub>4</sub> was

striking, stimulating colony-forming efficiency in concentrations as low as 0.01 pM. Together, these findings suggest that environmentally relevant concentrations of aldehydes can induce bronchial hyperreactivity in guinea pigs through a mechanism involving injury to cells present in the airways during exposure (rather than from subsequently recruited migratory cells) and that this response is dependent on leukotriene biosynthesis.

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#### INTRODUCTION

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Oxidation of alcohols in a wide number of environmental processes (including internal combustion) can lead to the formation of stable chemical intermediates that may have greater toxic potentials than their parent compounds (Marnett 1988). A noteworthy example is the formation of aldehydes of low molecular weight, such as formaldehyde (HCHO) and acrolein (CH<sub>2</sub>=CHCHO) when methanol and ethanol are used as additives to alternative fuels.

On the other hand, alternative fuels show promise in reducing the concentration of certain components of smog because methanol has a lower atmospheric chemical reactivity than other hydrocarbons. Thus, the need to reduce ozone formation (which results from the photochemical reactions of hydrocarbons with nitrogen oxides) must be balanced against any additional potential health consequences associated with increased aldehyde exposure; this requires a complete understanding of the relative toxicity of ozone and low-molecular-weight aldehydes. Although ozone toxicity has received extensive study (Lippmann 1989), less is currently known about the possible noncarcinogenic pulmonary effects of low-level ambient exposures to aldehydes (Leikauf 1991).

The extensive sources of aldehydes in the environment are reviewed elsewhere (Leikauf 1991). The contribution of these compounds to indoor air pollution presents a major concern. For example, concentrations of both formaldehyde and acrolein in mainstream cigarette smoke can exceed 5 to 30 ppm, and levels as high as 1 to 4 ppm formaldehyde have been measured in homes with urea foam insulation. In ambient outdoor air, aldehyde levels are typically much lower than indoor levels; aldehydes are a part of the general hydrocarbon burden, and are derived from stationary sources (incineration and home wood fires) or mobile sources (diesel, internal combustion, and jet engine emis-

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<sup>2</sup> A list of abbreviations appears at the end of this report for your reference.

sions). In urban areas, aldehyde levels, like hydrocarbons, exhibit diurnal variation with peak levels preceding ozone peaks. Ambient aldehyde levels are not measured routinely (Marnett 1988), but total ambient aldehyde levels have occasionally reached 150 to 300 parts per billion (ppb) and are more commonly no greater than 50 ppb. Approximately 50 percent of the total aldehyde burden is formaldehyde, which can range from 10 to 30 ppb in heavily contaminated air masses. (Formaldehyde levels in urban areas have been measured at 90 to 150 ppb, whereas levels as low as 0.8 ppb have been measured in rural environments.) Estimates for other aldehydes indicate that 5 to 10 percent of the total aldehyde burden is acrolein, (which ranges from 5 to 30 ppb during peaks) and the remaining 35 to 40 percent is made up of other aliphatic aldehydes such as acetaldehyde. The possibility that these levels may increase with the acceptance of alternative fuels containing methanol or ethanol is based on tests of engines burning these fuels. Such studies have shown that emissions of formaldehyde, acrolein and acetaldehyde can be 75 to 200 times greater with alcohol fuels than with conventional gasoline even when the same internal combustion engine is used (Marnett 1988). This report addresses certain aspects of the pulmonary toxicology of aldehydes.

Aldehydes are respiratory irritants capable of inducing airway epithelial damage (Lyon et al. 1970; Dalhamn and Rosengren 1971; Feron et al. 1978; Beauchamp et al. 1985; Grafström et al. 1988) and bronchoconstriction (Amdur 1960; Murphy et al. 1963; Costa et al. 1986). Because the aqueous solubility of various aldehydes differ, the dosimetry of inhaled aldehydes also differs. Formaldehyde is principally deposited in the upper respiratory tract, and acrolein, having a lower aqueous solubility, can penetrate the nasal passages and be deposited in the bronchial airways (Egle 1972). Previous animal studies with acrolein reported acute injury to both the proximal (Lyon et al. 1970; Costa et al. 1986) and distal airways (Beauchamp et al. 1985; Kutzman et al. 1985; Costa et al. 1986), accompanied by alveolar macrophage dysfunction and increased susceptibility to infection (Astry and Jakab 1983). Hence, scientists have been concerned about the action of these compounds as airway irritants and about their potential as proliferative and carcinogenic agents (Svenberg et al. 1980; Albert et al. 1982; Berke 1987; Edling et al. 1988; Monticello et al. 1989).

Information on the comparative irritant potency of these compounds is important to our understanding of the toxicity of aldehydes. Bronchoconstriction has been examined with both formaldehyde and acrolein in the guinea pig. Increases in total pulmonary resistance and decreases in breathing frequency, indicative of airway narrowing, have been observed with exposures of at least 1.0 ppm formaldehyde (Amdur 1960) and at least 0.3 ppm acrolein (Murphy

et al. 1963). These responses were rapid in onset, remained constant during exposure, and were readily reversed after the cessation of exposure. Several other respiratory irritants that are bronchoconstrictive, most notably ozone (Holtzman et al. 1983; Fabbri et al. 1984, 1985; Aizawa et al. 1985; Seltzer et al. 1986) and toluene diisocyanate (Thompson et al. 1986; Fabbri et al. 1987), can also induce bronchial hyperreactivity, a feature characteristic of asthma (Boushey et al. 1980; Barnes 1986; Barnes et al. 1989). Because of the irritant properties of these aldehydes, an overall objective of this study was to determine whether or not these compounds produce increased bronchial reactivity in response to intravenous administration of acetylcholine in guinea pigs.

Although a topic of extensive research, the cellular mechanisms and specific sources of bronchoconstrictive mediators thought to be responsible for hyperreactivity are still largely unknown. Holtzman and associates (1988) proposed a causal relationship between neutrophil infiltration into the pulmonary epithelium and hyperreactivity in dogs after ozone exposure. Evidence supporting this hypothesis in dogs has been obtained by O'Byrne and colleagues, who found that the depletion of circulating neutrophils by hydroxyurea inhibited ozone-induced bronchial hyperreactivity (O'Byrne et al. 1984b), and that a nonbronchoconstrictive chemotactic factor, leukotriene B<sub>4</sub> (LTB<sub>4</sub>), induced hyperreactivity (O'Byrne et al. 1985). However, other recent studies examining this phenomenon in guinea pigs have found discordance between the temporal sequence of neutrophil infiltration and hyperreactivity (Hulbert et al. 1981, 1985; Murlas and Roum 1985b), and that neutrophil depletion with another agent, cyclophosphamide, did not inhibit the induction of hyperreactivity (Thompson et al. 1986). Such discrepancies only became apparent when sampling was conducted at several time points after acute exposure.

Data from several different experimental approaches, nonetheless, suggest an important role for epithelial injury and the expression of inflammatory mediators in the modulation of bronchial reactivity (Laitinen et al. 1985; Barnes 1986). Persons with asthma, for example, are more reactive to numerous bronchoconstrictive eicosanoids, such as prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) (Mathe et al. 1973; Newball and Lenfant 1977). Several nonimmunologic stimuli that induce hyperreactivity also stimulate eicosanoid release during periods of bronchial hyperreactivity in dogs (Aizawa et al. 1985; O'Byrne et al. 1985) and humans (Seltzer et al. 1986). However, these studies did not examine the possible temporal relationships among eicosanoid release, cellular infiltration, and hyperreactivity. To examine these relationships, we exposed guinea pigs to acrolein and sampled the airways with bronchoalveolar lavage to assess mediator release; we conducted histological examinations at selected

times after exposure to measure epithelial injury. Particular emphasis was placed on the early time points to investigate whether or not augmentation of eicosanoid release or inflammatory cell infiltration accompanied the onset of hyperreactivity.

Another objective of this study was to determine whether or not airway epithelial cells could be responsible for the formation of bioactive lipid mediators in response to acrolein. To this end, epithelial cells from the airways of different species were isolated, grown in cell culture, and exposed to each aldehyde. We then focused on eicosanoid metabolism in these cells and the possible physiological significance of such compounds within the epithelium.

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## SPECIFIC AIMS

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The purpose of this study was to determine whether exposures to environmentally relevant concentrations of two aldehydes of low molecular weight were associated with impaired airway function. Specifically, the study addressed questions of the relative irritant potency of formaldehyde and acrolein on the induction of increased bronchial reactivity to acetylcholine in guinea pigs. The relationship of bronchial reactivity to epithelial damage and inflammation were also examined after both *in vivo* and *in vitro* exposures.

*In vivo* exposures were designed to determine the dose-response relationships for inhaled acrolein and formaldehyde by measuring the following endpoints: (1) changes in bronchial reactivity induced by acetylcholine; (2) infiltration of neutrophils and other inflammatory cells into the airways; (3) release of augmented levels of eicosanoids into bronchoalveolar lavage fluid; and (4) the extent to which the inhibition of eicosanoid metabolism altered the induction of bronchial hyperreactivity.

*In vitro* exposures focused specifically on the effects of these compounds on an important target site, the airway epithelium. To accomplish this, airway cells were grown in culture and exposed to aldehydes, and the following endpoints were measured: (1) augmentation of eicosanoid metabolism, and (2) the elevation of the mitogenic effects of eicosanoids.

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## METHODS

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### EFFECTS OF FORMALDEHYDE AND ACROLEIN ON BRONCHIAL REACTIVITY

#### Experimental Design

We first investigated the onset and time course of in-

creases in specific total pulmonary resistance ( $sR_T$ ; measured as  $\text{mL} \times \text{cm H}_2\text{O}/[\text{mL}/\text{sec}]$ ; hereafter referred to as specific resistance) and bronchial reactivity to intravenous acetylcholine in guinea pigs exposed to selected concentrations of formaldehyde and acrolein. Specific resistance was measured at 5, 7.5, 10, 15, 20, 25, 30, and 60 minutes after exposure. In all tests, time zero refers to the end of the exposure period. Bronchial reactivity was assessed in each guinea pig twice before exposure and then 1, 2, 6, and 24 hours after the cessation of exposure. Using this measurement sequence, groups of five to seven guinea pigs were exposed either to 0.31, 0.67, 0.91, or 1.26 ppm acrolein, or to 0.86, 3.4, 9.3, or 31.1 ppm formaldehyde for two hours each, or to 0.11, 0.31, 0.59, 1.05 ppm formaldehyde for eight hours. After the eight-hour exposure, the six-hour reactivity measurement was reduced to three hours. Other groups of guinea pigs serving as controls were sham-exposed for either 2 or 8 hours (acrolein and formaldehyde concentrations of less than 0.01 ppm). Two measurements of baseline reactivity obtained before exposure were used to determine the day-to-day variability within each group. These measurements were made 18 to 24 hours before exposure and again 0.5 to 2 hours before the initiation of exposure.

#### Measurements of Specific Resistance and Bronchial Reactivity to Acetylcholine

Bronchial reactivity was assessed in conscious Hartley guinea pigs (male, 300 to 590 g) by measuring the change in specific resistance induced by increasing doses of intravenous acetylcholine chloride. Specific resistance was determined using the inverse of specific pulmonary conductance as measured by the method described by Agrawal (1981). One day prior to acrolein exposure, each animal was anesthetized (50 mg/kg ketamine hydrochloride and 10 mg/kg acepromazine maleate, intramuscular injection), and a polyethylene cannula (polyethylene 10, 0.61 mm o.d., 0.25 mm i.d.) was inserted into the jugular vein and externalized through a dorsal cervical incision. Animals were allowed to recover from anesthesia, and four to six hours later they were placed in a constant-volume body plethysmograph. Rebreathing was prevented by passing warmed (38°C), humidified air near the nares (0.03 L/minute).

Box pressure was measured with a differential pressure transducer connected to a carrier demodulator (Models MP45-2 and CD-19, respectively, Validyne Instrument, Northridge, CA). Pulmonary air flow was measured with a pneumotachograph (No. 0, Gould-Godart-Fleisch, Pres Lausanne, Switzerland) and a differential pressure transducer connected to a carrier demodulator (MP45-3 and CD-19, respectively, Validyne). Box pressure (abscissa) and air flow (ordinate) were displayed on a storage oscilloscope

(Model 5115, Tektronic, Gaithersburg, MD), and the angle ( $\tan \Theta$ ) of the ascending limb during the transition of expiration to inspiration was obtained with an electronic protractor (B.B. Ruktin, University of California, San Francisco, CA). The electronic output of the protractor was sampled using a microcomputer (Model RT11, Digital Corp., Maynard, MA) with software (developed by A. Vinegar, University of Cincinnati, Cincinnati, OH) designed to record  $\tan \theta$  at a rate of 10 to 35 measurements within two minutes. All values were corrected for temperature, barometric pressure, and water vapor pressure at a body temperature of 38°C.

Baseline  $sR_t$  (mean of 8 to 20 measurements) was determined, then acetylcholine chloride (73.5  $\mu\text{g}/\text{mL}$  in saline containing 10 U/mL heparin sulfate) was infused at a constant rate starting at 0.014 mL/minute (0.99  $\mu\text{g}$  acetylcholine/minute), and  $sR_t$  was measured again approximately 30 seconds later. Once the measurement of  $sR_t$  became constant, as determined by five equivalent consecutive determinations, the rate of infusion was increased in stepwise increments to 0.037, 0.101, 0.193, 0.389, 0.763, and 1.490 mL/minute, until the  $sR_t$  was twice the level of baseline. Values of the log acetylcholine dose rate ( $\mu\text{g}/\text{kg}/\text{min}$ ) and  $sR_t$  ( $\text{mL} \times \text{cm H}_2\text{O}/[\text{mL}/\text{sec}]$ ) were plotted, and the dose of acetylcholine sufficient to double specific resistance (effective dose 200 percent,  $[\text{ED}_{200}]$ ) was determined by log-linear interpolation. The total procedural time for this method was less than 10 minutes.

### Inhalation Exposure

Formaldehyde, acrolein, or sham exposures lasted two or eight hours. Formaldehyde was generated by passing nitrogen ( $\text{N}_2$ ) (15 mL/minute) over a reservoir of warmed paraformaldehyde (50°C). The aldehyde-laden  $\text{N}_2$  was diluted and mixed with air (CGA grade D, 500 mL/minute). Depending on the desired concentration, a portion of this mixture was introduced into a 0.32- $\text{m}^3$  stainless-steel chamber, and the remainder was exhausted to a fume hood. Exposures were to the whole body in air passed through a high-efficiency particulate air (HEPA) filter with a flow rate of 161 L/minute. Concentrations were sampled with two glass-fitted impingers in series filled with 10 mL of distilled water. Samples were collected at a rate of 0.5 L/minute for 5 to 10 minutes. One milliliter of the sample was mixed with 25  $\mu\text{L}$  of 1 percent chromatographic acid and 1.0 mL sulfuric acid, and the absorbance was read at 580 nm (Altshuler and McPherson 1963; Green and Kulle 1986). Formaldehyde concentrations in solution from each sample were determined separately and corrected for breakthrough (no more than  $0.3 \pm 0.5$  percent, mean  $\pm$  SD, at 3.4 ppm).

Acrolein was generated by passing  $\text{N}_2$  (15 mL/minute) over a 5-mL reservoir of liquid acrolein. The acrolein-laden  $\text{N}_2$  was diluted and mixed with air (CGA grade D, 500

mL/minute). Depending on the desired concentration, a portion of this mixture was introduced into a 0.32- $\text{m}^3$  stainless-steel chamber, and the remainder was exhausted to a fume hood. Exposures were to the whole body and the chamber air passed through a HEPA filter at a flow rate of 161 L/minute. Concentrations were sampled with two glass-fitted impingers in series filled with 10.3 mL of 49.4 percent ethanol in water containing 50 mM hexylrescorcinol, 2.1 mM mercuric chloride ( $\text{HgCl}_2$ ), and 29.7 M trichloroacetic acid (Cohen and Altshuler 1961). Samples were collected at a rate of 0.5 L/min for 5 to 10 minutes, heated to 60°C for 15 minutes, allowed to cool for 15 minutes, and the absorbance at 636 nm was determined with a spectrophotometer (Coleman Model 124, Perkin-Elmer Corp., Maywood, IL). Acrolein concentrations in solution from a bubbler were determined separately and corrected for breakthrough (less than  $3.3 \pm 1.2$  percent, mean  $\pm$  SD, at 1.3 ppm).

### ACROLEIN-INDUCED CHANGES IN CELLULAR AND EICOSANOID COMPOSITION OF BRONCHOALVEOLAR LAVAGE FLUID

#### Experimental Design

The lavage fluid was evaluated for changes in the cell distribution and in eicosanoid concentrations after acrolein exposure. Five groups of five to seven guinea pigs each were exposed for two hours to  $1.31 \pm 0.11$  ppm acrolein. The animals were then anesthetized and bronchoalveolar lavage was performed immediately or 1, 2, 6, or 24 hours after the end of exposure. A group of seven unexposed guinea pigs served as controls. Differentials of recovered cells and the concentration of selected eicosanoids were determined in the lavage fluid. To confirm the changes in lavage fluid by direct histological examination of the airways, another set of tests was conducted with guinea pigs (six per group) anesthetized at the same times after exposure to  $0.92 \pm 0.10$  ppm acrolein. A portion of the trachea was removed and placed in glutaraldehyde for histological examination with light and electron microscopy. Controls for these studies included six unexposed (lot controls; i.e., some animals from each lot from the supplier that were housed for longer than one week but not placed in an exposure chamber) and 12 sham-exposed guinea pigs (six of the sham-exposed animals were killed immediately after exposure and six were killed 24 hours after exposure).

#### Bronchoalveolar Lavage

To assess the infiltration of inflammatory cells into the lung and mediator release, bronchoalveolar lavage was conducted either before (control) or at various times after a two-hour acrolein exposure. Bronchoalveolar lavage was performed *in situ* with anesthetized animals (50 mg/kg Keta-

mine hydrochloride and 10 mg/kg Acepromazine maleate administered intraperitoneally). An incision was made in the abdomen, the dorsal aorta was severed, and the diaphragm was punctured. The trachea was cannulated with a polyethylene tube (polyethylene 260, 1.77 mm i.d., 2.8 mm o.d.). Calcium-magnesium-free phosphate-buffered saline (10 mL, 38°C) was injected slowly into the trachea, withdrawn, and then reinjected two additional times. The recovered fluid (volume measured) was placed in ice-cooled, sterile centrifuge tubes (50 mL). The lungs were lavaged with four more 10-mL volumes (each injected and removed three times; total injected volume was 50 mL) and the lavage fluid was pooled. An aliquot (100  $\mu$ L) of each sample was collected, and the total cell count was determined with a hemocytometer (Brightline, American Optical, Buffalo, NY). The remainder of the fluid was centrifuged (150  $\times$  g for 15 minutes at 4°C), and the supernatant was removed and frozen (-70°C) until further analysis. The cell pellet was resuspended in 5 mL of phosphate-buffered saline (PBS), and an aliquot (100  $\mu$ L) was placed on a microscopic slide with a cytocentrifuge (300 rpm for 3 minutes, Shandon Cytocentrifuge, Pittsburgh, PA). Slides were fixed in methanol (containing 1.8 mg/L triarylmethane dye) for 30 seconds and stained with modified Wright stain (Diff-quick Stain Set, American Scientific Products, McGaw Park, IL). Cell differential counts were performed by counting 500 to 1,000 cells per sample, and values were expressed as percentages of the total cells recovered.

Concentrations of four cyclooxygenase metabolites of arachidonic acid were determined by radioimmunoassay. Thromboxane B<sub>2</sub> (TxB<sub>2</sub>), the stable metabolite of thromboxane A<sub>2</sub> (TXA<sub>2</sub>), PGF<sub>2 $\alpha$</sub> , prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and 6-keto prostaglandin F<sub>1 $\alpha$</sub>  (PGF<sub>1 $\alpha$</sub> ), the stable metabolite of prostacyclin, were measured in 100- $\mu$ L aliquots of lavage fluid. In previous studies with physiologic buffer and lavage fluid (O'Byrne et al. 1985; Leikauf et al. 1986; Seltzer et al. 1986), the cross reactivity of each antiserum was determined to be less than 0.3 percent, except for PGE<sub>2</sub>, which had a cross-reactivity of 3.7 percent with prostaglandin E<sub>1</sub> (PGE<sub>1</sub>).

Concentrations of leukotriene C<sub>4</sub> (LTC<sub>4</sub>) were determined by radioimmunoassay. Preliminary determinations indicated that concentrations of leukotrienes in unextracted lavage fluid from a single animal were below detectable levels. Therefore, the lavage fluid samples from seven sham-exposed controls, six guinea pigs exposed to acrolein, or five animals exposed to acrolein that was pretreated with the 5-lipoxygenase inhibitor L-651,392 were individually concentrated for radioimmunoassay analysis. Lavage fluid (25 mL) from each animal was concentrated by silicic acid chromatography (Sep-pak, Waters Associates Division, Millipore Corp. Milford, MA), as described by Metz and associ-

ates (1982). After each sample was applied, the column was washed serially with 10 mL of hexane, 5 mL of methylene chloride, and with 10 mL of methanol twice. The methanol collections containing leukotrienes were dried in N<sub>2</sub> (30°C). Samples then were resuspended in assay buffer for radioimmunoassay. At least 95 percent of the <sup>3</sup>H-LTC<sub>4</sub> was recovered, added to the lavage fluid, and applied to the silica columns.

### Histological Analysis

To microscopically assess the time course of airway damage, inflammation, and repair, portions of the mid-trachea and mid-right main-stem bronchus were fixed in 2.5 percent glutaraldehyde, 2.0 percent paraformaldehyde in PBS (pH = 7.3), post-fixed in 1 percent osmium tetroxide (4°C for 2 hours, pH = 7.3), dehydrated stepwise to 100 percent ethanol with two changes of propylene oxide, and embedded in Spurr plastic. Tissues then were cut into 1.0- $\mu$ m sections and stained with toluidine blue. Then, 15 fields per section were read at a magnification of  $\times$  1,250 without knowledge of the exposure treatment. Sections were viewed sequentially. The areas examined included the epithelium, defined as all cells above the basement membrane, and the lamina propria, defined as the tissue below the basement membrane and above the airway cartilage. Cells were classified by the following characteristics: (1) ciliated epithelial cells were cells with an apical membrane contiguous with the airway lumen and containing cilia; (2) non-ciliated epithelial cells were cells that had an apical membrane reaching the lumen and did not contain cilia (this included intermediate and secretory cells); (3) mitotic figures were cells with segregated chromosomes and no nuclear membrane; (4) neutrophils had a lobular nucleus with fine cytoplasmic granules; (5) macrophages were mononuclear cells with abundant cytoplasm and inclusions; (6) eosinophils had lobular nuclei with cytoplasmic granules that were large and greenish; (7) mast cells had a rounded nucleus with large metachromatic granules; (8) small mononuclear cells had rounded nuclei with few or no pale granules; (9) plasma cells had a round clock-faced nucleus with abundant agranular cytoplasm. In each field the lumen of blood vessels were also examined for marginating leukocytes.

## EFFECTS OF AGONISTS AND INHIBITORS ON ACROLEIN-INDUCED BRONCHIAL HYPERREACTIVITY

### Experimental Design

The role of eicosanoid formation in acrolein-induced hyperreactivity was assessed further by examining the effects of a putative LTC<sub>4</sub> and leukotriene D<sub>4</sub> (LTD<sub>4</sub>) receptor

antagonist and two 5-lipoxygenase inhibitors. The onset and time course of increased bronchial reactivity to intravenous acetylcholine was assessed in seven guinea pigs exposed to 1.2 to 1.4 ppm acrolein. Specific resistance was monitored 5, 7.5, 10, 15, 20, 25, 30, and 60 minutes after exposure. Bronchial reactivity was assessed twice before the administration of either an antagonist or an inhibitor and once afterward. Bronchial reactivity was assessed 1, 2, 6, and 24 hours after acrolein exposure. The measurements obtained after treatment with an antagonist or inhibitor and before the acrolein exposure allowed us to determine the effect of each compound on acetylcholine reactivity. To measure mediator release in lavage fluid in an accompanying set of tests, six guinea pigs were exposed for two hours to 1.3 ppm acrolein, five guinea pigs were exposed to 1.4 ppm acrolein after treatment with a lipoxygenase inhibitor, and seven unexposed guinea pigs were used as controls. The guinea pigs were anesthetized and bronchoalveolar lavage was performed within 20 minutes after the end of exposure. The concentration of LTC<sub>4</sub> was determined in the lavage fluid by radioimmunoassay.

#### **Effect of Leukotriene Receptor Antagonist and 5-Lipoxygenase Inhibitors on Bronchial Reactivity**

To assess the effects of the newly-developed leukotriene receptor antagonist L-649,923, six guinea pigs were pretreated with L-649,923 (10 mg/kg in saline administered intravenously) and exposed to  $1.21 \pm 0.19$  ppm acrolein. Bronchial reactivity to acetylcholine was measured one day before the exposure, immediately before intravenous administration of L-649,923, and again 10 to 15 minutes later. Each animal was then exposed to acrolein for two hours. Specific resistance was measured for 0 to 30 minutes after exposure, and bronchial reactivity was determined 1, 2, 6, and 24 hours after exposure. A second dose of L-649,923 (5 mg/kg of body weight) was administered four hours after the initial dosage of L-649,923.

To assess the effects of a 5-lipoxygenase inhibitor, another group of six guinea pigs was pretreated with L-651,392 (10 mg/kg in 0.5 percent polyoxyethylene sorbiton mono-oleate, Tween 80, in water, administered orally) and exposed to  $1.35 \pm 0.06$  ppm acrolein. The same protocol as that just described was followed, except the interval between the oral administration of L-651,392 and the preexposure measurement of bronchial reactivity was one hour instead of 10 to 15 minutes.

Another inhibitor of leukotriene synthesis, U-60,257 (6,9-deepoxy-6,9-(phenylimino)- $\Delta$ 6,8-prostaglandin I<sub>1</sub>, or Piri-prost) also was tested to determine its possible effects on acrolein-induced bronchial hyperreactivity in a group of seven guinea pigs. In these tests, guinea pigs were pretreated

with 5 mg/kg U-60,257 intravenously, bronchial reactivity was assessed 10 to 15 minutes later, and each animal then was exposed to  $1.35 \pm 0.26$  ppm acrolein for two hours.

#### **EFFECTS OF ACROLEIN ON EICOSANOID METABOLISM IN AIRWAY EPITHELIAL CELLS IN CULTURE**

##### **Experimental Design**

To investigate the effects of acrolein on eicosanoid metabolism in epithelial cells, airway cells were exposed in culture. To assess the effect of time in culture on eicosanoid release, freshly isolated (two hours after dissociation), subconfluent (24 hours in culture), and confluent bovine cells (day four of culture) were stimulated with either calcium ionophore (A23187) or bradykinin, and the release of PGE<sub>2</sub> was measured by radioimmunoassay. To assess the characteristics of <sup>3</sup>H-arachidonic acid uptake and labeling, cells were incubated with radiolabeled arachidonic acid, and lipids were separated and quantified. The effects of acrolein then were tested on confluent epithelial cell monolayers. Radiochromatography was used to identify the profile of eicosanoids released from the epithelial monolayers after control and acrolein exposures. Monolayers were prelabeled with <sup>3</sup>H-arachidonic acid, and the released <sup>3</sup>H-products were separated by reverse-phase high-performance liquid chromatography (HPLC). Finally, the dose-response relationship for acrolein-induced release of selected eicosanoids was determined using radioimmunoassays.

##### **Cell Culture**

Samples of guinea pig trachea were obtained from anesthetized animals (30 mg/kg pentobarbital sodium administered intravenously) and cannulated with polyethylene tubing (polyethylene 260, 1.77 mm i.d., 2.80 mm o.d.). A neutral protease enzyme solution containing 2 percent dispase and 5 mM dithiothreitol was placed in the lumen and incubated at 4°C for 12 to 24 hours. This solution was composed of (in mM) sodium ion (Na<sup>+</sup>) 123.9, potassium ion (K<sup>+</sup>) 5.6, chloride ion (Cl<sup>-</sup>) 113.8, acetate<sup>-</sup> 5.6, bicarbonate ion (HCO<sub>3</sub><sup>-</sup>) 5.0, gluconate<sup>-</sup> 3.8, monobasic phosphate ion (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) 1.3, glucose 5.6, and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) 20.0, and supplemented with 10<sup>5</sup> U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamycin, and 2.5 mg/L fungizone, pH 7.3. The preparation was then incubated for one hour at 37°C, and the solution was removed and replaced with fresh enzyme solution. The removed solution was mixed 1:1 with a culture medium containing Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient medium, and fetal bovine serum (FBS) (DMEM:F-12:FBS, 45:45:10), centrifuged

(150 × *g* for 10 minutes), and the resulting cell pellet was resuspended in DMEM:F12:FBS. The cell suspension was then added to 60-mm plastic dishes coated with type IV human placental collagen. After 24 hours the nonadherent cells were removed and the medium was changed every 48 hours. Typically, 1 million to 2 million cells were obtained per trachea and seeded into two 60-mm culture dishes.

Bovine tracheal epithelial cells were grown in primary culture using a method similar to that previously reported (Leikauf et al. 1988). Tracheas obtained at a local slaughterhouse first were cut longitudinally along the ventral side and opened to expose the luminal surface. Adherent blood and mucus were rinsed off the mucosal surface with Dulbecco's PBS (calcium-magnesium-free) containing 10<sup>5</sup> U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamycin, and 2.5 mg/L fungizone. Next, the epithelium was separated from the cartilaginous rings, minced, and placed into a protease enzyme solution for 12 to 24 hours as described above. The enzyme solution was warmed and stirred, and the decant was centrifuged at 150 × *g* for 10 minutes. The cell pellet then was resuspended in a culture medium consisting of 45 percent DMEM medium, 45 percent Ham's F-12 nutrient medium, 10 percent FBS, 5 mg/L insulin, 105 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamycin, and 2.5 mg/L fungizone at pH 7.3. When cells were observed to be in multicellular aggregates after this digestion period, further dissociation was achieved with an additional treatment with buffer solution containing 0.02 percent trypsin, 0.02 percent ethylene glycol bis(beta-aminoethylether)-N,N,N',N'-tetraacetate (EGTA), and 1.0 percent polyvinyl pyrrolidone. Cells were incubated for 40 minutes at 37°C in the trypsin solution, then centrifuged and resuspended in culture medium. The 60-minute digestion period was repeated three or four times, with the entire procedure yielding approximately 125 million cells per trachea. The freshly isolated cells were seeded on collagen-coated 60-mm plastic petri dishes at a density of 5 × 10<sup>4</sup> cells/cm<sup>2</sup> and placed in a 37°C (5 percent CO<sub>2</sub>) incubator. At this seeding density, cell confluency was obtained by the fifth day of culture. Cell viability after the replating period was at least 87 percent by trypan blue exclusion, and cells with beating cilia were frequently observed. The culture medium was changed after the first 24 hours and then every 48 hours thereafter.

Normal human tracheobronchial tissue was obtained by surgery or at autopsy (within 12 hours of death) and maintained in culture by the method described by Lechner and coworkers (1981, 1982; Lechner and LaVeck 1985). Tissue was placed in an L-15 medium containing penicillin G (100 U/mL), streptomycin (100 µg/mL), amphotericin B (1.0 µg/mL), and gentamicin (50 µg/mL); the external fascia was

removed, and each specimen was cut into 1.0-cm<sup>2</sup> pieces. Each piece was placed with its mucosal surface up in a 100-mm culture dish containing CMRL-1066 medium (Gibco, Grand Island, NY), and supplemented with insulin (5 µg/mL), hydrocortisone (0.36 µg/mL), beta-retinyl acetate (0.1 µg/mL), penicillin (50 µg/mL), streptomycin (50 µg/mL), amphotericin B (0.5 µg/mL), gentamicin (50 µg/mL), and 1 percent FBS.

These explants were placed in a plexiglass chamber (Bellco Glass, Vineland, NJ) and gently agitated in a mixture of 50 percent oxygen (O<sub>2</sub>), 45 percent N<sub>2</sub>, and 5 percent carbon dioxide (CO<sub>2</sub>) (at 37°C) on a rocker platform. The medium was changed each day for 7 to 10 days. After this time each explant was dissected into four to eight pieces that were placed in a 100-mm culture dish coated with a mixture of fibronectin (10 µg/mL), type I collagen (30 µg/mL), and bovine serum albumin (BSA) (10 µg/mL). These pieces were incubated in 5 percent CO<sub>2</sub> in air at 37°C. Cells were grown in MCDB-153-based medium (Boyce and Ham 1983; Wilke et al. 1988) supplemented with 0.2 µM hydrocortisone, 0.9 µM insulin, 0.1 µM transferrin, 0.5 µM phosphoethanolamine and ethanolamine, 0.8 nM epidermal growth factor, 10 nM 3,3',5-triiodo-L-thyronine, 0.5 percent bovine pituitary extract, and trace-elements (see Lechner and LaVeck 1985 for concentrations and commercial sources of medium components). Primary epithelial cells were allowed to grow from the explant for six to seven days and then were removed enzymatically by 0.025 percent trypsin and 0.02 percent EGTA in HEPES-buffered saline solution containing 1.0 percent polyvinylpyrrolidone (40 kDa). The resulting cell suspension was mixed with an equal amount of medium containing 1.0 percent FBS. It then was centrifuged (150 × *g* for 10 minutes), and the cell pellet was resuspended in a serum-free MCDB-153 medium supplemented with growth factors. In each test, cells were seeded into 60-mm plastic dishes coated with fibronectin, collagen, and BSA. Cell preparations obtained with this method are free of fibroblasts, retain human karyotype, express epithelial keratin and blood-group antigens, possess specialized characteristics of plasma membranes, and are capable of differentiating into ciliated epithelium (Lechner et al. 1984).

#### **Eicosanoid Metabolism in Cultured Epithelial Cells from Human Airways**

Several specific tests were conducted to characterize eicosanoid metabolic pathways in human cells. We examined whether or not airway epithelial cells could produce cysteinyl leukotrienes from the precursor molecules arachidonate or leukotriene A<sub>4</sub> (LTA<sub>4</sub>). Cells were seeded at high density (10,000 cells/cm<sup>2</sup>) and grown to confluency. To as-

say for cysteinyl-leukotriene formation from arachidonate, cells ( $10^6$ /trial, for three trials) were incubated overnight in a serum-free medium containing 0.8  $\mu\text{Ci}$   $^{14}\text{C}$ -arachidonic acid (specific activity 0.7 Ci/mmol) (New England Nuclear, Boston, MA). The medium was removed, the monolayer was washed three times in PBS containing 0.5 percent BSA, and the cells were incubated for one hour at  $37^\circ\text{C}$ . The medium then was removed, and 1.5 mL of PBS containing 10  $\mu\text{M}$  arachidonic acid and 3.0  $\mu\text{M}$  A23187 were added, and the cells were placed on a rocking platform for 20 minutes (at  $37^\circ\text{C}$ ). To terminate each test, the supernatant was removed and mixed with 7.5 mL of ice-cold ethanol, and the monolayer was washed with 2 mL of a 1:5 mixture of PBS and ethanol. The medium was mixed and centrifuged ( $500 \times g$  for 15 minutes), the supernatant was evaporated (rotary evaporation), and the water phase was placed onto an octadecyl silane column (Sep-pak- $\text{C}_{18}$  cartridge; Waters). The cartridge was washed serially with 10 mL each of water, hexanes, and ethanol. The methanol eluate was evaporated to dryness with a rotor evaporator, redissolved in 50  $\mu\text{L}$  methanol and quantitatively injected onto a 100-mm  $\times$  3.0-mm Nucleosil 120 column (3  $\mu\text{m}$ -spherical  $\text{C}_{18}$ ; Berkeley, CA).

To analyze the leukotrienes, samples were resolved with HPLC by isocratic elution with acetonitrile:methanol:water (28:18:54.1, by volume, pH = 5.6 adjusted with 30 percent sodium hydroxide [NaOH]) at a flow rate of 0.4 mL/minute. To analyze the monohydroxy acids, samples were eluted with methanol:water:acetic acid (72.5:27.5:0.01, by volume). The eluted substances were monitored and quantified using a programmable diode array spectrophotometer (Model 8451A, Hewlett Packard, Sverige AB, Vastra Frolunda, Sweden). Coelution of radioactive  $^{14}\text{C}$ -material was determined by collecting the eluate (1 minute/sample), mixing it with 5 mL of scintillation fluid, and counting the particles. To examine whether airway cells enzymatically hydrolyze  $\text{LTA}_4$  (5,6-*trans*-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) to  $\text{LTB}_4$  or convert it into  $\text{LTC}_4$  by the addition of glutathione, epithelial cells were incubated with 60 nmol of  $\text{LTA}_4/10^6$  cells for 20 minutes. Leukotriene  $\text{A}_4$  was prepared by alkaline hydrolysis of methylester  $\text{LTA}_4$  (Barnes et al. 1989). The incubation was terminated by adding ethanol, and samples were analyzed using the method described above.

Next, to examine whether or not airway epithelial cells can convert  $\text{LTC}_4$  to its sequential metabolites  $\text{LTD}_4$  and leukotriene  $\text{E}_4$  ( $\text{LTE}_4$ ), cells ( $10^6$  cells/trial, for 4 trials) were plated and grown in supplemental MCDB-153 as in the method described above. The cells then were washed three times and placed in Krebs-Henseleit buffer solution (KHBS) containing  $10^5$  cpm  $^{14}\text{C}$ - $\text{LTC}_4$  (with specific activity of 40 Ci/mmol; New England Nuclear) for 20 minutes at  $37^\circ\text{C}$ .

The medium was removed and centrifuged ( $150 \times g$  for 15 minutes), 50 ng of prostaglandin  $\text{B}_2$  ( $\text{PGB}_2$ ) was added to it to monitor recovery, and the medium was placed on a silica column (Sep-pak silica cartridge). The column was washed with 10 mL of hexane, 5 mL of methylene chloride, and with 10 mL of methanol twice. The methanol fractions were dried in 100 percent prepurified  $\text{N}_2$  at  $30^\circ\text{C}$ , resuspended in 100  $\mu\text{L}$  of solvent 1 (a mixture of methanol:water:acetic acid, 63:36:1, pH = 3.7), and quantitatively transferred onto a 5- $\mu\text{m}$  spherical  $\text{C}_{18}$  column (150  $\times$  3.9 mm; Resolve; Waters). The sample was eluted (1 mL/minute) with a gradient that initially started with 90 percent solvent 1 and 10 percent solvent 2 (an equivalent mixture of solvent 1 with a pH adjustment to 5.6 by ammonium hydroxide [ $\text{NH}_4\text{OH}$ ]) and was linearly changed to 100 percent solvent 2 within 15 minutes. Ultraviolet absorbance was monitored at 280 nm, fractions were collected every 30 seconds, and beta-activity was determined by scintillation counting.

To resolve other cyclooxygenase and lipoxygenase products, epithelial cells ( $2 \times 10^6$  cells/trial for 5 trials) were incubated overnight with 1  $\mu\text{Ci}/\text{mL}$   $^3\text{H}$ -arachidonic acid (specific activity = 100 Ci/mmol; New England Nuclear) in supplemented MCDB-153. After 24 hours, the medium was removed, and the cells were washed once with 50  $\mu\text{M}$  fatty acid-free BSA in KHBS and twice more with KHBS alone. Krebs-Henseleit was added, and monolayers were incubated for one hour in buffer as a control, then the monolayers were exposed to 3  $\mu\text{M}$  A23187 for 20 minutes. The medium was removed and centrifuged, and an aliquot (100  $\mu\text{L}$ ) was counted and stored frozen ( $-70^\circ\text{C}$ ). The supernatant was acidified with formic acid (pH = 3.5),  $\text{PGB}_2$  (50 ng) was added to monitor recovery, and then the supernatant extracted three times with two volumes of ethyl acetate. The ethyl acetate phase was evaporated with prepurified  $\text{N}_2$  ( $30^\circ\text{C}$ ) and resuspended in 50 to 100  $\mu\text{L}$  solvent 3 (68:32 water:acetonitrile containing 2.5 mM  $\text{H}_2\text{PO}_4$ ). Chromatography was performed as described previously (Leikauf et al. 1988) by quantitative injection onto a 75-mm  $\times$  4.6-mm Ultrasphere (3.0  $\mu\text{m}$   $\text{C}_{18}$ -silane; Altex Division, Beckman Instruments, San Ramon, CA) and by using a gradient elution from solvent 3 to solvent 4 (60:40 methanol:water containing 2.5 mM  $\text{H}_2\text{PO}_4$ ) at 1.0 mL/minute. Ultraviolet absorbance was monitored at 205, 280, and 240 nm for prostaglandins, dihydroxy and monohydroxy acids, respectively. In separate tests, immunoreactive eicosanoids were determined by radioimmunoassay, and values were expressed as picograms released per  $10^6$  cells. Cell counts were performed after each experiment by removing cells with trypsin:EGTA (in the method described above) and by counting with a hemocytometer.

### **<sup>3</sup>H-Arachidonic Acid Labeling**

To determine the optimal incubation period for <sup>3</sup>H-arachidonic acid incorporation into epithelial monolayers, cells were incubated in a culture medium containing 2  $\mu$ Ci of <sup>3</sup>H-arachidonic acid (6.7 nM) for 1, 2, 4, 8, 12, 24, or 48 hours at 37°C in an incubator with 5 percent CO<sub>2</sub>. Following the incubation period, cells were washed first with KHBS containing 1  $\mu$ m of fatty acid-free BSA. The composition of KHBS was (in mM): Na<sup>+</sup> 143.9, K<sup>+</sup> 5.6, calcium ion (Ca<sup>+2</sup>) 1.9, magnesium ion (Mg<sup>+2</sup>) 1.2, Cl<sup>-</sup> 117.6, HCO<sub>3</sub><sup>-</sup> 25.0, acetate<sup>-</sup> 5.6, gluconate<sup>-</sup> 3.8, monobasic phosphate ion (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) 1.3, sulfate ion (SO<sub>4</sub><sup>-2</sup>) 1.2, and glucose 5.6, at pH 7.3. The cells were washed two additional times with KHBS without albumin. Incorporation of <sup>3</sup>H-arachidonic acid into the epithelial cells was expressed as the percentage of the originally added radioactivity that was not recoverable in the washes and culture medium removed at the end of the incubation period.

### **Thin-Layer Chromatography of Radiolabeled Phospholipids**

The phospholipids incorporating <sup>3</sup>H-arachidonic acid at the various incubation periods were resolved using thin-layer chromatography. After indicated incubation periods, ice-cold methanol was added to each dish, and monolayers were scraped with a rubber policeman into a glass test tube. Dishes were washed two additional times with fresh methanol. Chloroform and water were added (the final ratio v:v:v was 2.0 chloroform:2.0 methanol:1.8 water), and lipids were extracted as described by Bligh and Dyer (1959). To inhibit oxidation, 0.5 mg/mL of butylated hydroxytoluene was added to each sample. The tube was flushed with 100 percent N<sub>2</sub> and then stored at -70°C before thin layer chromatography separation.

Two aliquots (25  $\mu$ L) of each extract were placed on separate silica gel G plates with authentic phospholipid standards; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA) were placed on neighboring lanes (3 to 5  $\mu$ L of a 1-mg/mL stock). The radiolabeled phospholipids were separated using two different solvents. One plate was treated with solvent 5, which consisted of 56.6 percent chloroform:34.0 percent methanol:7.5 percent water:1.9 percent methylamine (v:v:v:v). Solvent 5 effectively separated PC, PE, and PS; however, PI and PA co-eluted. The second plate was separated with solvent 6, which consisted of 50.6 percent chloroform:38.0 percent methanol:51 percent water:6.3 percent NH<sub>4</sub>OH (v:v:v:v) and effectively resolved PC, PE, and PA with PS and PI co-eluting. The locations of phospholipids were identified

by brief exposures of the gel plates to ultraviolet light, then sample lanes were scraped into scintillation vials and radioactivity was determined by scintillation spectrometry. The amount of each phospholipid was expressed as a percentage of the total radioactivity associated with the phospholipids resolved within each sample.

### **Extraction of Released <sup>3</sup>H-Eicosanoids**

Epithelial monolayers (in 60-mm dishes) labeled with <sup>3</sup>H-arachidonic acid were equilibrated in KHBS for one hour at 37°C and 5 percent CO<sub>2</sub>. Each dish then was treated sequentially to KHBS (control), to 10, 30, or 100  $\mu$ M acrolein, and to 3  $\mu$ M A23187, each for 20 minutes. The recovered samples were centrifuged for 10 minutes at 150  $\times$  g and aliquots (50  $\mu$ L) of the supernatants were counted in a liquid scintillation counter.

<sup>3</sup>H-Eicosanoids were extracted from recovered samples containing released <sup>3</sup>H-activity by acidifying the sample with 8.8 percent formic acid (53  $\mu$ L/mL), adding ethyl acetate at two times the sample volume, and mixing vigorously. After the aqueous phase settled, the upper ethyl acetate phase was removed, and the procedure was repeated two additional times with fresh ethyl acetate. The ethyl acetate samples were pooled and dried in 100 percent N<sub>2</sub> at 30°C. The dried residue was then resuspended in 100  $\mu$ L of a 50:50 mixture of solvent 3:solvent 4 (described below). Before each extraction, PGB<sub>2</sub> (50 ng) was added to the sample to monitor the recovery efficiency of the extraction procedure. The recovery efficiency was calculated as the ratio of the integrated area of the PGB<sub>2</sub> ultraviolet absorbance (280 nm) peak measured in the sample to the integrated area measured from a 50-ng PGB<sub>2</sub> standard. Extraction efficiencies were greater than 90 percent in all samples analyzed.

### **Reverse-Phase High-Performance Liquid Chromatography**

Separation of eicosanoids by reverse-phase HPLC was performed using a modification of a method previously described (Leikauf et al. 1988). The 100  $\mu$ L samples containing extracted <sup>3</sup>H-eicosanoids were injected onto a 3- $\mu$ m ultrasphere octadecyl saline 75-mm  $\times$  4.6-mm column (Altex Division, Beckman, San Ramon, CA). Temporal elution of eicosanoids was achieved with a programmed solvent gradient delivered at a flow rate of 1 mL/minute. The solvent gradient was generated using a two-solvent (3 and 4) delivery system (model 680 gradient controller; Waters).

Solvent 3 consisted of 70 percent acetonitrile and 30 percent H<sub>2</sub>O, with 2.5 mM phosphoric acid (H<sub>3</sub>PO<sub>4</sub>); solvent 4 was composed of 60 percent methanol, and 40 percent acetonitrile, with 2.5 mM H<sub>3</sub>PO<sub>4</sub>. The solvent gradient for

the first 10 minutes was held constant at 100 percent solvent 3. From 10 to 16 minutes, solvent 3 was reduced linearly to 44 percent, and solvent 4 was increased to 56 percent. From 16 to 24 minutes, the gradient was held at 56 percent solvent 4, and from 24 to 35 minutes, solvent 4 was increased linearly to 100 percent. Solvent 4 then was held constant at 100 percent for the remainder of the separation. Before each sample analysis, retention times for authentic standards of selected eicosanoids were determined by ultraviolet absorbance (model 490 multiwavelength detector; Waters). From 0 to 13 minutes, ultraviolet absorbance was monitored at 205 nm where the retention times for  $\text{PGF}_{1\alpha}$ ,  $\text{TxB}_2$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ , and prostaglandin  $\text{D}_2$  ( $\text{PGD}_2$ ) were 3.0, 6.3, 7.9, 9.3, and 11.0 minutes, respectively. From 13 to 27 minutes, ultraviolet absorbance was monitored at 280 nm, with  $\text{PGB}_2$  eluted at 22.3 minutes and  $\text{LTB}_4$  eluted at 25.7 minutes. At 27 minutes, 240 nm was monitored for the rest of the separation, and elution times for 12(s)hydroxy-5,8,14-eicosatetraenoic acid (12-HETE), 15(s)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid (15-HETE), and 5(s)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) were determined to be 31.1, 36.1, and 39.5 minutes, respectively. Eluate was collected every 0.5 minute, and radioactivity was counted in a liquid scintillation counter. After correction for  $\text{PGB}_2$  recovery, radiochromatograms were constructed, and radioactive peaks were correlated with retention times of the authentic standards.  $^3\text{H}$ -activity peaks lagged the ultraviolet absorbance peaks of authentic standards by approximately 0.5 minute, due to dead space existing between the wavelength detector and the fraction collector.

### Radioimmunoassay

The levels of eicosanoids identified by reverse phase HPLC also were measured from unlabeled cells using commercially obtained radioimmunoassays. In each test, duplicate dishes obtained from the same trachea were exposed to 0 (control), 1, 3, 10, 30, or 100  $\mu\text{M}$  acrolein for 20 minutes. After acrolein treatment, recovered KHBS samples were centrifuged at  $150 \times g$  for 10 minutes at  $4^\circ\text{C}$ , and the supernatants were stored frozen at  $-70^\circ\text{C}$ . Radioimmunoassays then were performed on unextracted aliquots of the samples. Following each experiment, cell numbers were determined by first incubating the cells in buffer solution containing 0.05 percent trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) at  $37^\circ\text{C}$ . After 15 to 20 minutes, dishes were observed under a microscope to confirm complete dissociation of cells. Cell counts were conducted with a hemocytometer, and viability was determined by trypan blue exclusion. The amounts of eicosanoids released were expressed as nanograms per  $10^6$  cells. The cross-reactivities of  $\text{PGE}_2$

and  $\text{PGF}_{2\alpha}$  antibodies with other eicosanoids were no greater than 3.7 percent and 1.1 percent, respectively.

### Lactate Dehydrogenase Release

To determine the effects of acrolein exposure on cellular viability, released lactate dehydrogenase (LDH) activity was assayed. Epithelial monolayers (in 60-mm dishes) were equilibrated for one hour in KHBS, and then fresh KHBS containing 100  $\mu\text{M}$  acrolein was added. The KHBS then was removed 10, 20, or 30 minutes after addition, or 1, 2, 4, 8, 18, or 24 hours after addition. Lactate dehydrogenase activity was measured in each sample using a commercially obtained kit. Briefly, 1 mL of 0.75  $\mu\text{M}$  pyruvate solution was added to 1 mg of reduced nicotinamide adenine dinucleotide (NADH) in a 15-mL vial and warmed to  $37^\circ\text{C}$ . Aliquots (100  $\mu\text{L}$ ) of recovered samples (some diluted 10:1) were added to the pyruvate and NADH solution and incubated for 30 minutes at  $37^\circ\text{C}$ . At the end of the 30-minute incubation period, 1 mL of 2,4-dinitrophenylhydrazine (2 mg/mL) in 1 N hydrochloric acid was added to the vial and incubated at room temperature for 20 minutes. Finally, 10 mL of 0.4 N NaOH was added to the vial. Pyruvate not converted to lactic acid during the 30-minute incubation period reacted with 2,4-dinitrophenylhydrazine under acidic conditions to form a product that absorbs light at 455 nm. Absorbance for each sample was measured with a spectrophotometer (Coleman model 124, Perkin-Elmer, Maywood, IL). The amount of reacted pyruvate was derived from a standard curve constructed from absorbance values obtained from control vials containing known concentrations of pyruvate. Subtracting the amount of pyruvate present at the end of the 30-minute reaction period from the amount originally added yielded the amount converted to lactate. Lactate dehydrogenase activity was expressed as units of activity ( $\mu\text{mol}$  of pyruvate converted to lactate per minute) per liter of the sample. Total LDH activity per cell monolayer was obtained from samples taken from dishes sonicated for 15 minutes.

## MITOGENIC EFFECTS OF EICOSANOIDS

### Experimental Design

We investigated the mitogenic effects of a specific group of eicosanoids, the cysteinyl leukotrienes, on epithelial cells of the human airways. This subclass of eicosanoids was elevated in lavage fluid obtained from guinea pigs exposed to acrolein in the present study. In previous studies with aldehydes, alterations in cysteinyl leukotriene levels have been reported in conjunction with observations of proliferative changes in the airways. In this study, cells were grown from tissue explants and passaged into 60-mm dishes to

measure colony-forming efficiency, clonal growth rate, and thymidine incorporation.

### Colony-Forming Efficiency and Clonal Growth Rate

To assay colony-forming efficiency, cells were seeded at 250 to 500 cells/cm<sup>2</sup> for 24 hours and grown in supplemented MCDB-153 medium without (control) or with selected concentrations of LTC<sub>4</sub>, LTD<sub>4</sub>, or LTE<sub>4</sub> for seven to nine days (with one medium change at day 4). In one series of tests, 1.0 μM indomethacin, a cyclooxygenase inhibitor, was added one hour before the LTC<sub>4</sub> addition. At the end of the incubation period cultures were fixed in PBS containing 10 percent formaldehyde. The cultures were stained in 0.25 percent crystal violet, and colonies were counted with a dissecting microscope (magnification × 7). Values of colony-forming efficiency (based on colonies of 16 or more cells) were averaged for determinations from duplicate trials from each donor (*n* = 6) and normalized to appropriate paired controls (80 colonies/dish) of either 4 μL of 50:50 ethanol:water (leukotriene vehicle) in 4 mL of culture medium (with a final volume percent of 0.05 ethanol) or to the same vehicle with 1.0 μM indomethacin (86 colonies/dish).

To assay clonal growth rate, the number of cells in 18 randomly selected clones from a pair of dishes (nine each) were counted with a Zeiss-IBAS (Oberlocken, Germany) image analyzer. Clonal growth rate (population doubling/day) was derived from the log<sub>2</sub> of the average number of cells per clone by dividing by the number of days in culture.

### Thymidine Incorporation

To assay for leukotriene effects on thymidine incorporation (DNA synthesis), cells were seeded 5,000 to 10,000/cm<sup>2</sup> on coated 60-mm dishes. A dual labeling method was used with an initial incubation in <sup>14</sup>C-thymidine for normalization of total DNA, followed by an incubation in <sup>3</sup>H-thymidine to measure rapid incorporation stimulated by each agonist. Twenty-four hours after the initial seeding, cells were washed and placed in supplemented MCDB-153 containing 10 nCi/mL thymidine(methyl-<sup>14</sup>C; specific activity 52 mCi/mmol; New England Nuclear) for 48 hours. After cells were prelabeled with <sup>14</sup>C-thymidine, each dish was rinsed three times and equilibrated for three hours. Cells were then treated with indomethacin (1.0 μM) for one hour. Next, cells were treated with either the vehicle alone (controls), or the vehicle containing either 0.001 or 1 pM LTC<sub>4</sub>, and grown for 48 hours.

After this, thymidine (methyl-<sup>3</sup>H) (specific activity 54 Ci/mmol) was added, cells were incubated for an additional 24 hours. The medium was removed, the culture was

washed four times with PBS (4 mL each wash), and 0.8 mL of 0.2 M NaOH containing 40 μg/mL DNA (calf thymus) was added for one minute to lyse the cells. The lysate was acidified (1 mL of 1.0 M hydrochloric acid) and resuspended by pipetting, and the dish was scraped with a rubber policeman. The suspension was poured onto a 25-mm glass-fiber filter (Whatman GF/A; Kent, UK) prewetted with 1.0 M hydrochloric acid. The dish and filter were washed twice with 2.5 mL of 1.0 M hydrochloric acid and twice with 2.5 mL of ice-cold ethanol. The filter was placed in a glass vial that then was filled with 10 mL scintillation fluid (Instagel, New England Nuclear), and radioactivity was counted using a dual-labeled program (Beckman Instruments, Fullerton, CA). Results were expressed as the ratio of <sup>3</sup>H-thymidine (in disintegrations per minute [dpm]):<sup>14</sup>C-thymidine (dpm); this ratio was at least 3:1 in control tests. It should be noted that this method relies on an assumption that <sup>3</sup>H-thymidine is uniformly introduced into a nucleotide precursor pool that is not affected by treatment, and that increases in the rates of precursor incorporation into DNA may reflect an increase in initial labeling of the precursor pools and an increase in DNA synthesis.

### MATERIALS

The following materials were obtained from the specified sources: acepromazine maleate [10-(3-dimethylamino)-propyl-(phenothiazine-2-yl methyl ketone)maleate] (Fort Dodge Laboratory, Fort Dodge, IA); ketamine hydrochloride [Vetalar, dl 2-(*o*-chloro-phenyl)-2-(methylamino) cyclohexane hydrochloride] (Parke-Davis, Division of Warner-Lambert Co., Morris Plains, NJ); sodium pentobarbital (Butler Co., Columbus, OH); heparin sulfate (Elkins-Sinn, Cherry Hill, NJ); acrolein (0.05 to 0.15 percent hydroquinone inhibitor) (Eastman-Kodak Co., Rochester, NY); 4-hexylresorcinol and mercury (II) chloride (Aldrich Chemical Co., Milwaukee, WI); acetylcholine chloride and trichloroacetic acid (Sigma Chemical Co., St. Louis, MO); and PBS (without calcium and magnesium) (GIBCO Laboratory, Grand Island, NY). Radioimmunoassays included PGE<sub>2</sub>, F<sub>1α</sub>, TxB<sub>2</sub> (NEK-020, -025, and -024, New England Nuclear), and PGF<sub>2α</sub> (SG-6002, Seragen, Cambridge, MA); N<sub>2</sub> and breathing air in cylinders were purchased from Welco Medical Division, Cincinnati, OH, and guinea pigs from Murphy Breeding Labs, Plainfield, IN. Radioimmunoassay antiserum for LTC<sub>4</sub> (NEK-303, New England Nuclear) had 11.6 and 3.3 percent cross-reactivity with LTD<sub>4</sub> and LTE<sub>4</sub>, respectively and no more than 1.0 percent cross-reactivity with other eicosanoids and arachidonic acid. Other materials included <sup>3</sup>H-LTC<sub>4</sub> (39.0 Ci/mmol; NET-714, New England Nuclear); authentic leukotriene standards LTB<sub>4</sub>, LTC<sub>4</sub>, and LTD<sub>4</sub> (J. Rokach, Merck

Frost Canada, Pointe Claire-Dorval, Quebec, Canada); leukotriene receptor antagonist L-649,923 (sodium( $\beta$ S\* $\gamma$ R\*)-4-(3-(4-acetyl-3-hydroxy-2-propylphenoxy)-propylthio)- $\gamma$ -hydroxy- $\beta$ -methylbenzene butanoate); 5-lipoxygenase inhibitor L-651,392 (4-bromo-2,7-dimethoxy- $^3$ H-phenothiazine-3-one) (AW Ford-Hutchinson, Merck Frost Canada); and 5-lipoxygenase inhibitor U-60,257 (Piriprostat; 6,9-deepoxy-6,9-(phenylimino)- $\Delta$ 6,8-prostaglandin  $I_1$ ) (MK Bach, Upjohn Co., Kalamazoo, MI). All solvents (HPLC-grade) were obtained from Fisher Scientific (Cincinnati, OH) except methanol, which was obtained from American Burdick and Jackson (Muskegon, MI).

Other materials included sodium chloride, potassium chloride, magnesium sulfate, sodium monophosphate, sodium bicarbonate, glucose, HPLC-grade  $H_3PO_4$ , formic acid, and ethyl acetate (Fisher Scientific, Cincinnati, OH); calcium chloride, sodium acetate, HEPES, EGTA, insulin, type IV human placental collagen, fatty acid-free BSA, penicillin, streptomycin, trypsin, dithiothreitol,  $TxB_2$ ,  $PGB_2$ ,  $PGD_2$ ,  $PGE_2$ ,  $PGF_{2\alpha}$ , and  $PGF_{1\alpha}$  (Sigma Chemical Co., St. Louis, MO);  $LTB_4$ , and 5-, 12-, and 15-HETE (Merck Frost Canada); acrolein in 0.05 to 0.15 percent hydroquinone inhibitor and sodium gluconate (Eastman-Kodak Co., Rochester, NY); dispase (Boehringer-Mannheim Biochemicals, Indianapolis, IN); calcium-magnesium-free Dulbecco's PBS, DMEM, Ham's F-12, gentamicin, fungazone, and trypan blue (GIBCO Laboratories); FBS (Hyclone Laboratories, Logan, UT); A23187 (Calbiochem, Behring Diagnostics, La Jolla, CA); HPLC grade acetonitrile and methanol (American Burdick & Jackson, American Hospital Supply Corp., Muskegon, MI); and polyvinyl pyrrolidone PVP-30-K (United States Biochemical Corp., Cleveland, OH).

## STATISTICAL METHODS

In studies examining temporal aspects of physiological and biochemical responses, multiple comparisons of mean values between the control group and each exposed group, or comparison within each group (for example, bronchial reactivity [ $ED_{200}$ ] and specific resistance [ $sR_t$ ]) were made by a general linear model procedure of repeated measures analysis of variance using a univariate test for within-subject effects (Winer 1971). Post-hoc single comparisons were conducted using Scheffe's test (Winer 1971) or the paired  $t$  test (Winer 1971), as indicated. Single comparisons between one exposed group and one control group (for example,  $LTC_4$  analysis in lavage fluid) were conducted with paired or unpaired  $t$  tests, using a Welch approximation procedure to obtain the degrees of freedom and variance for comparisons between means of two groups with unknown and unequal population variance (Remington and Schork

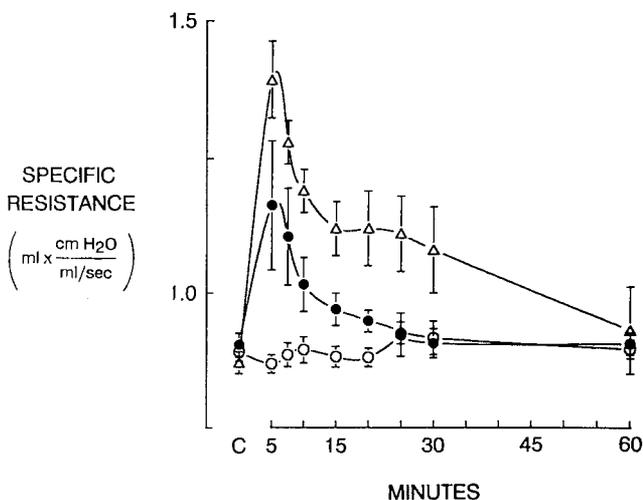
1970). Differences between means were considered statistically significant when  $p < 0.05$  by two-tailed  $t$  tests.

## RESULTS

### EFFECTS OF FORMALDEHYDE AND ACROLEIN ON BRONCHIAL REACTIVITY

#### Specific Resistance After Acrolein Exposure

Two-hour exposures to acrolein (at least 0.3 ppm) produced transient increases in  $sR_t$  that rapidly returned to baseline levels after cessation of exposure. A typical example of the time course for the mean response of guinea pigs exposed to the lowest (0.31 ppm) and highest (1.26 ppm) concentrations of acrolein is shown in Figure 1. Specific resistance was measured first five minutes after the two-hour exposure so that there was time to transfer each animal to the plethysmograph and to allow for thermal equilibration. The changes in  $sR_t$  for each group measured after exposure are shown in Table 1. The overall mean baseline  $sR_t$  for all animals tested was determined from values measured 15 to 30 minutes before exposure and was  $0.88 \pm 0.02 \text{ mL} \times (\text{cm H}_2\text{O}/[\text{mL}/\text{sec}])$  (mean  $\pm$  SE,  $n = 28$  animals, upper 95 percent confidence interval =  $0.92 \text{ mL} \times (\text{cm H}_2\text{O}/[\text{mL}/\text{sec}])$ ). After exposure to  $1.26 \pm 0.07$  ppm acrolein, the  $sR_t$  in-



**Figure 1.** Time course of the increase in specific resistance after a two-hour sham exposure (○), or a two-hour exposure to  $0.31 \pm 0.04$  ppm (●) or  $1.26 \pm 0.07$  ppm (△) acrolein. Baseline specific resistance (C) was assessed before exposure in unanesthetized guinea pigs after equilibration in a whole-body plethysmograph. Values were elevated immediately after exposure and returned to baseline values within 25 minutes (0.31 ppm) or 60 minutes (1.26 ppm) after cessation of exposure. Values are means  $\pm$  SE for five to six guinea pigs.

**Table 1.** Specific Resistance in Guinea Pigs Exposed to Various Concentrations of Acrolein<sup>a</sup>

Acrolein (ppm)	Control		Minutes After Exposure							
	Day 1	Day 2	5	7.5	10	15	20	25	30	60
Sham	0.89 ± 0.02	0.87 ± 0.02	0.89 ± 0.02	0.90 ± 0.02	0.88 ± 0.02	0.88 ± 0.02	0.88 ± 0.02	0.92 ± 0.04	0.92 ± 0.02	0.88 ± 0.02
0.31	0.89 ± 0.02	0.90 ± 0.02	1.16 ± 0.12 <sup>b</sup>	1.11 ± 0.09 <sup>b</sup>	1.02 ± 0.05 <sup>b</sup>	0.97 ± 0.03 <sup>b</sup>	0.95 ± 0.02	0.93 ± 0.03	0.92 ± 0.07	0.90 ± 0.02
0.67	0.82 ± 0.03	0.81 ± 0.02	1.07 ± 0.03 <sup>b</sup>	0.98 ± 0.03 <sup>b</sup>	0.97 ± 0.03 <sup>b</sup>	0.92 ± 0.04 <sup>b</sup>	0.90 ± 0.04	0.88 ± 0.05	0.85 ± 0.02	0.82 ± 0.02
0.94	0.92 ± 0.03	0.93 ± 0.03	1.34 ± 0.11 <sup>b</sup>	1.39 ± 0.20 <sup>b</sup>	1.12 ± 0.05 <sup>b</sup>	1.01 ± 0.04 <sup>b</sup>	0.99 ± 0.05 <sup>b</sup>	1.01 ± 0.06 <sup>b</sup>	0.90 ± 0.07	0.90 ± 0.03
1.26	0.87 ± 0.02	0.88 ± 0.02	1.39 ± 0.07 <sup>b</sup>	1.28 ± 0.04 <sup>b</sup>	1.19 ± 0.04 <sup>b</sup>	1.11 ± 0.05 <sup>b</sup>	1.12 ± 0.07 <sup>b</sup>	1.10 ± 0.07 <sup>b</sup>	1.08 ± 0.08 <sup>b</sup>	0.93 ± 0.02

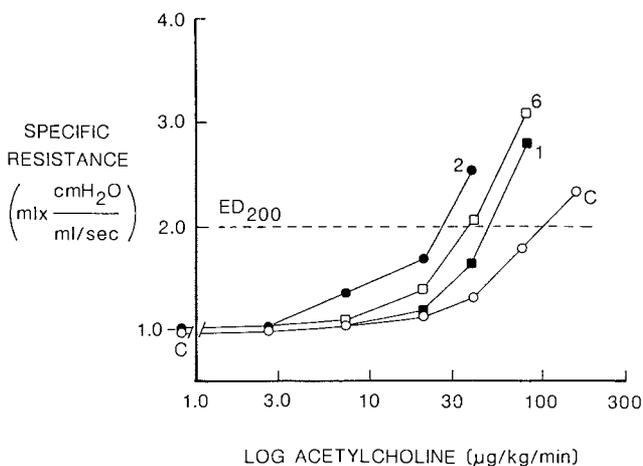
<sup>a</sup> Values are means ± SE; *n* = 5 to 7 unanesthetized guinea pigs. Guinea pigs underwent sham exposures (≤ 0.01 ppm acrolein for controls) or were exposed to the indicated concentration of acrolein for two hours. Specific resistance (mL × (cm H<sub>2</sub>O/[mL/sec])) was determined one day before exposure (control day 1) and the day of exposure (control day 2), and again at the times indicated after exposure.

<sup>b</sup> Significantly different from control (*p* < 0.05) as determined by analysis of variance followed by post-hoc Scheffe's test.

creased from 0.87 ± 0.02 (control) to 1.39 ± 0.07 mL × (cm H<sub>2</sub>O/[mL/sec]) (60 percent increase, *n* = 5 animals). This effect declined rapidly after exposure (30-minute sR<sub>t</sub> = 1.05 ± 0.08, a 21 percent increase) and was not significantly different from preexposure baseline levels one hour after the end of acrolein exposure (60-minute sR<sub>t</sub> = 0.90 ± 0.03 mL × (cm H<sub>2</sub>O/[mL/sec]), a 6 percent increase). Based on these results and the previous findings of Amdur (1960) and Murphy and associates (1963), bronchial reactivity was first assessed one hour after exposure when the increase in sR<sub>t</sub>, indicative of an increase in resting bronchomotor tone, had returned to baseline values.

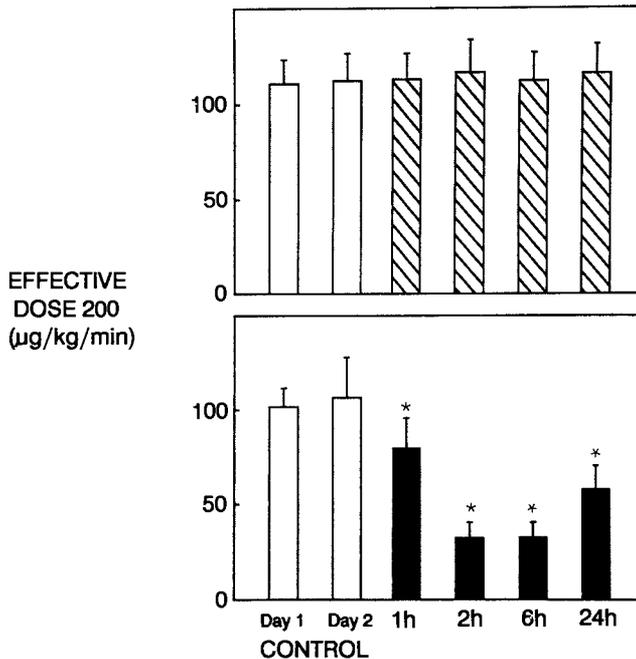
### Bronchial Reactivity to Acetylcholine After Acrolein Exposure

In contrast to the transient increase in baseline sR<sub>t</sub>, two-



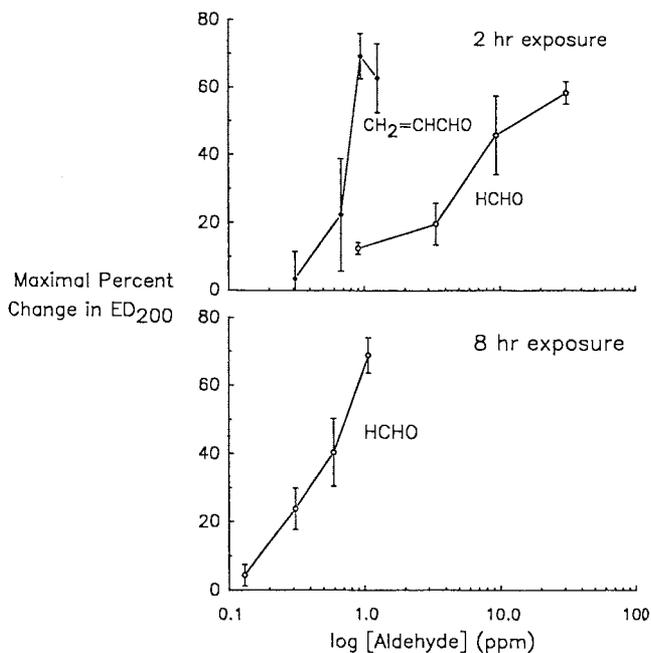
**Figure 2.** Time course of acrolein-induced bronchial hyperreactivity in a guinea pig after a two-hour exposure to 0.94 ppm acrolein. Specific resistance was measured during intravenous administration of acetylcholine either before exposure (control, ○), or 1(■), 2(●), 6(□), or 24 hours after exposure. (For the sake of clarity, values for 24-hour exposures are not shown.) The ED<sub>200</sub> was determined by log-linear interpolation and decrease from control ED<sub>200</sub> = 100 to maximal response at two hours of 25 µg/mL/minute. Each point is the mean of 10 to 15 measurements from a single guinea pig.

hour acrolein exposures of at least 0.94 ppm produced a persistent change in bronchial reactivity to intravenous acetylcholine (Figure 2). On the day before exposure, the baseline mean of the acetylcholine infusion rate (normalized to body weight) sufficient to produce a doubling in sR<sub>t</sub> (ED<sub>200</sub>) for the first group of animals tested was 108.8 ± 5.4 µg/kg/min (mean ± SE, GSEM = 1.06, *n* = 26). On the day of exposure, ED<sub>200</sub> was 107.3 ± 6.0 µg/kg/min; thus, the day-to-day variability was 1.3 ± 5.7 percent (coefficient of



**Figure 3.** Acrolein-induced bronchial hyperreactivity in guinea pigs exposed to 0.94 ppm acrolein for two hours. Open bars indicate controls measured one day before exposure and on the day of exposure; hatch bars indicate ED<sub>200</sub> after a two-hour sham exposure (*n* = 6); closed bars indicate ED<sub>200</sub> at 1, 2, 6, or 24 hours after a two-hour acrolein exposure (*n* = 5). Asterisks (\*) indicate values that are significantly different from control values (*p* < 0.05), as determined by analysis of variance followed by Scheffe's test.

variation). In the sham-exposed animals,  $ED_{200}$  remained constant with time (top panel of Figure 3), with a mean group variation of  $12.8 \pm 0.4$  percent ( $n = 6$  animals). The within-animal coefficient of variation with this method, as noted previously (Agrawal 1981), was far lower, at  $1.9 \pm 0.5$  percent ( $n = 6$  animals). Following 0.94 ppm acrolein (bottom panel of Figure 3), the effective doubling dose ( $ED_{200}$ ) decreased from  $104.2 \pm 7.3$  to  $79.6 \pm 15.9$   $\mu\text{g}/\text{kg}/\text{min}$  at one hour and to  $32.5 \pm 7.9$   $\mu\text{g}/\text{kg}/\text{min}$  at two hours (24 and 69 percent decreases, respectively). This effect persisted, with significant increases in reactivity also occurring at 6 and 24 hours after exposure. In four of these five guinea pigs, reactivity was measured one week after the exposure and was not significantly different from baseline values (1 week  $ED_{200}$  was  $106.4 \pm 10.7$ , mean  $\pm$  SE). Thus, bronchial hyperreactivity occurred either during the immediate acrolein-induced bronchoconstriction or shortly after it had resolved and was significantly different from controls as early as one hour after exposure. Significant decreases in  $ED_{200}$  also occurred after exposure to 1.26 ppm acrolein, with changes noted as early as one hour after exposure and becoming maximal two to six hours after exposure. In the two groups exposed to 0.31 and 0.67 ppm acrolein, the mean  $ED_{200}$  measured at 1, 2, 6, or 24 hours after exposure was



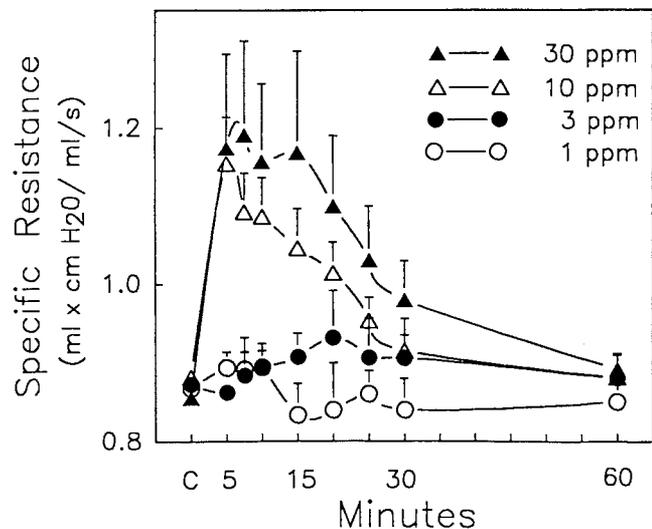
**Figure 4.** Effect of acrolein ( $\text{CH}_2 = \text{CHCHO}$ ) or formaldehyde (HCHO) on bronchial hyperreactivity in guinea pigs. Top panel: Two-hour exposures to acrolein or HCHO produced a maximal decrease in the  $ED_{200}$  at concentrations higher than 0.9 and 30 ppm, respectively. Bottom panel: When exposures were extended to eight hours, a dose of 1.0 ppm HCHO produced an effect equivalent to that after a two-hour exposure to 30 ppm HCHO. Values are means  $\pm$  SE of groups of five to seven guinea pigs.

not statistically different from that for the control group. After all exposures, the maximal increase in reactivity occurred two to six hours after exposure; the acrolein dose-response relationship for maximal percent change in  $ED_{200}$  is shown in Figure 4.

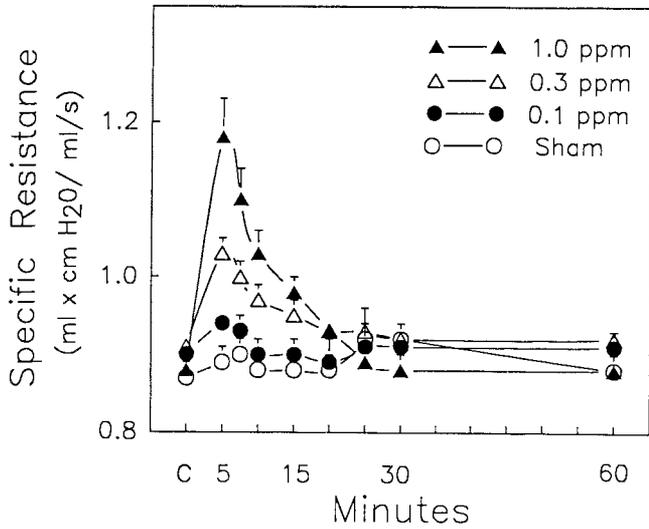
#### Specific Resistance After Formaldehyde Exposure

Specific resistance also increased after formaldehyde exposure at selected concentrations. Immediately after the two-hour exposure to 9.4  $\pm$  0.2 ppm formaldehyde (Figure 5),  $sR_t$  increased from  $0.88 \pm 0.01$  (control) to  $1.15 \pm 0.06$   $\text{mL} \times (\text{cm H}_2\text{O}/[\text{mL}/\text{sec}])$  (5 minutes after exposure,  $n = 8$  animals, mean  $\pm$  SE). This effect, like that of acrolein in concentrations of 0.3 to 1.3 ppm, was rapidly reversible, with measurements returning to near control values within 30 minutes after exposure ( $sR_t$  at 30 minutes was  $0.92 \pm 0.02$   $\text{mL} \times (\text{cm H}_2\text{O}/[\text{mL}/\text{sec}])$ ). Exposures of 3.0 ppm formaldehyde for two hours produced only a small change in specific resistance ( $sR_t$  control was  $0.88 \pm 0.01$   $\text{mL} \times (\text{cm H}_2\text{O}/[\text{mL}/\text{sec}])$  and maximal  $sR_t$  after exposure was  $0.95 \pm 0.07$   $\text{mL} \times (\text{cm H}_2\text{O}/[\text{mL}/\text{sec}])$ ,  $n = 5$  animals). Thus, acrolein is 10 to 30 times more potent than formaldehyde in inducing immediate bronchoconstriction.

In addition, when exposure to formaldehyde was extended to eight hours, an increase in bronchoconstriction was measured five minutes after the end of exposure (Figure 6). After eight hours, however, a 1.0-ppm exposure

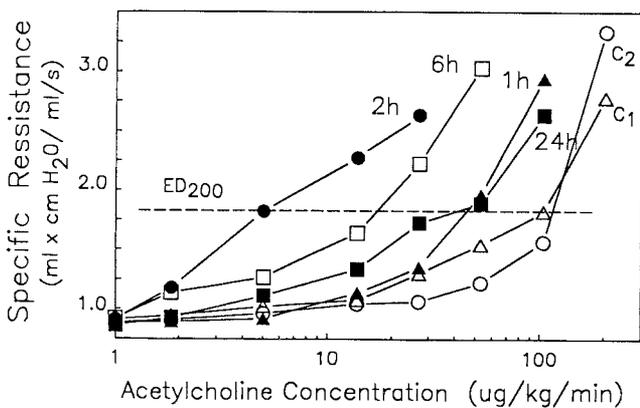


**Figure 5.** Specific resistance after a two-hour exposure to 1, 3, 10, or 30 ppm formaldehyde. Baseline specific resistance (C) was assessed before exposure in unanesthetized guinea pigs after equilibration in a whole-body plethysmograph. Values were elevated immediately after exposure and returned to control within 30 minutes (10 ppm) or 60 minutes (30 ppm) after cessation of exposure. Each data point represents the mean  $\pm$  SE for a group of six guinea pigs.



**Figure 6.** Specific resistance after an eight-hour exposure to 0.1 or 1.0 ppm formaldehyde. Baseline specific resistance (C) was assessed before exposure in unanesthetized guinea pigs after equilibration in a whole-body plethysmograph. Increases in specific resistance returned to baseline values within 25 minutes after cessation of exposure. Values are means  $\pm$  SE for five or six guinea pigs per group.

resulted in an increase in  $sR_t$  from  $0.83 \pm 0.01$  to  $1.13 \pm 0.05$  mL  $\times$  (cm H<sub>2</sub>O/[mL/sec]) (mean  $\pm$  SE,  $p < 0.05$ , paired  $t$  test). In contrast, two-hour exposures to 0.9 ppm and 3.4 ppm formaldehyde produced little change in  $sR_t$  (control was  $0.87 \pm 0.01$  and  $0.88 \pm 0.01$  mL  $\times$  (cm H<sub>2</sub>O/[mL/sec]), and exposed was  $0.89 \pm 0.01$  and  $0.95 \pm 0.02$  mL  $\times$  (cm H<sub>2</sub>O/[mL/sec]), respectively). Two-hour exposures to 9.3 ppm or 31.1 ppm did produce increases in  $sR_t$  from  $0.88 \pm$



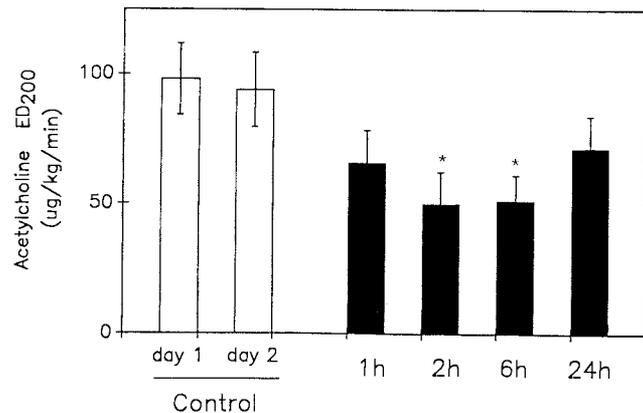
**Figure 7.** A typical time-course of increased bronchial hyperreactivity after a two-hour exposure to 10 ppm formaldehyde. In this guinea pig, the ED<sub>200</sub> was lower than the control value within one hour after the end of exposure, became maximal within two hours, and returned to baseline control values (C<sub>1</sub> and C<sub>2</sub>) within 24 hours. Each data point is the mean of 10 to 15 measures at a given concentration of acetylcholine.

0.01 and  $0.86 \pm 0.01$  to  $1.15 \pm 0.06$  and  $1.17 \pm 0.12$  mL  $\times$  (cm H<sub>2</sub>O/[mL/sec]), respectively. The  $sR_t$  increased 36 percent after eight hours, which is similar to the 29 percent increase in airway resistance measured by Amdur (1960) during exposure to 1.2 ppm.

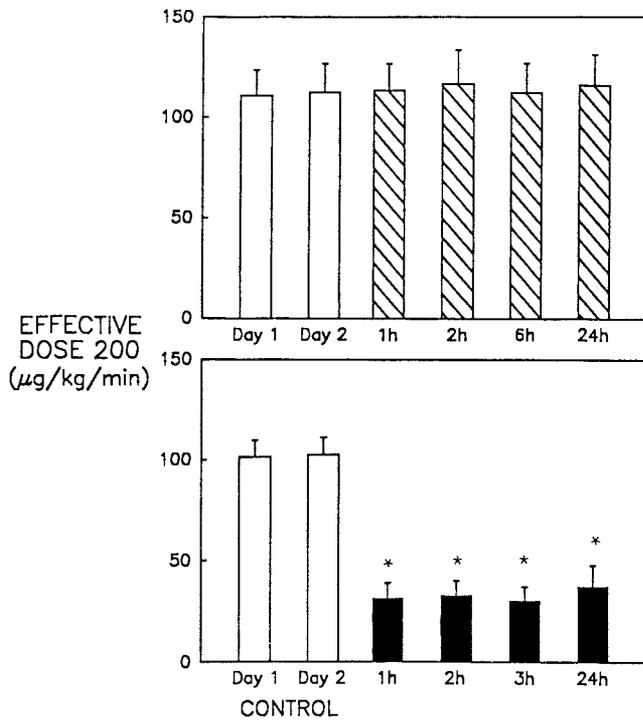
### Bronchial Reactivity to Acetylcholine After Formaldehyde Exposure

Two-hour exposures to formaldehyde (at least 9.4 ppm) altered the dose of acetylcholine necessary to double the baseline specific resistance (see Figure 7). The mean response for six guinea pigs is shown in Figure 8 and demonstrates a response similar to that produced by 1.0 ppm acrolein (Figure 3). The half-maximal doses for a two hour exposure were approximately 0.8 ppm acrolein and 8.0 ppm formaldehyde (Figure 4). In contrast to a two-hour acrolein exposure, the response to a two-hour formaldehyde exposure was only significantly different two and six hours after exposure (Figure 8).

When the exposure to formaldehyde was extended to eight hours,  $1.07 \pm 0.01$  ppm formaldehyde produced a significant increase in bronchial reactivity (Figure 9). Unlike the response after the two-hour exposure, which progressed with time, reactivity became maximal one hour after exposure, remained unchanged over the next two hours, and was still evident 24 hours after exposure. The magnitude of the response measured three hours after exposure, expressed as a percentage of change in ED<sub>200</sub>  $\{[(ED_{200} \text{ control} - ED_{200} \text{ exposed})/ED_{200} \text{ control}] \times 100\}$ , was  $67 \pm 4$  percent. This is equal to the maximal effects produced by



**Figure 8.** Formaldehyde-induced bronchial hyperreactivity in guinea pigs exposed to 9.4 ppm formaldehyde for two hours. Mean  $\pm$  SE ED<sub>200</sub> was reduced significantly in guinea pigs. Open bars indicate controls measured one day before exposure and on the day of exposure; closed bars indicate responses at 1, 2, 6, and 24 hours after a two-hour exposure to formaldehyde ( $n = 6$ ). Responses at two and six hours after exposure were significantly different from control responses.



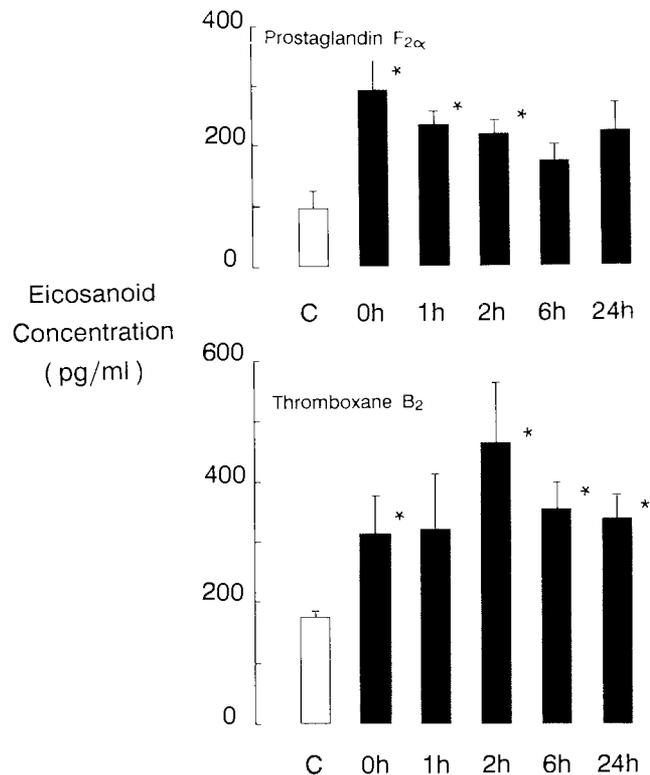
**Figure 9. Formaldehyde-induced bronchial hyperreactivity in guinea pigs exposed to  $1.07 \pm 0.01$  ppm formaldehyde for eight hours.** Mean  $\pm$  SE ED<sub>200</sub> was reduced significantly. Open bars indicate controls measured one day before exposure and on the day of exposure. Hatch bars indicate responses at 1, 2, 6, and 24 hours after an eight-hour sham exposure ( $n = 5$ ). Closed bars indicate responses at 1, 2, 3, and 24 hours after an eight-hour exposure to formaldehyde, with all values significantly different from control values ( $p < 0.05$ ) as determined by analysis of variance followed by Scheffé's test. An asterisk (\*) indicates significance at the level of  $p < 0.05$ .

exposure to at least 1.0 ppm acrolein or 30 ppm formaldehyde. This suggests that the estimated dose of the prolonged exposure did not follow the simple concentration  $\times$  time relationship (that is, an equivalent hyperreactivity response occurred after 1.1 ppm  $\times$  8 hours or 8.8 ppm $\cdot$ hours, and this dose is approximately six times less than what would be predicted by 31 ppm  $\times$  2 hours, or 62 ppm $\cdot$ hours [Figure 4]).

#### ACROLEIN-INDUCED CHANGES IN CELLULAR AND EICOSANOID COMPOSITION OF BRONCHOALVEOLAR LAVAGE FLUID

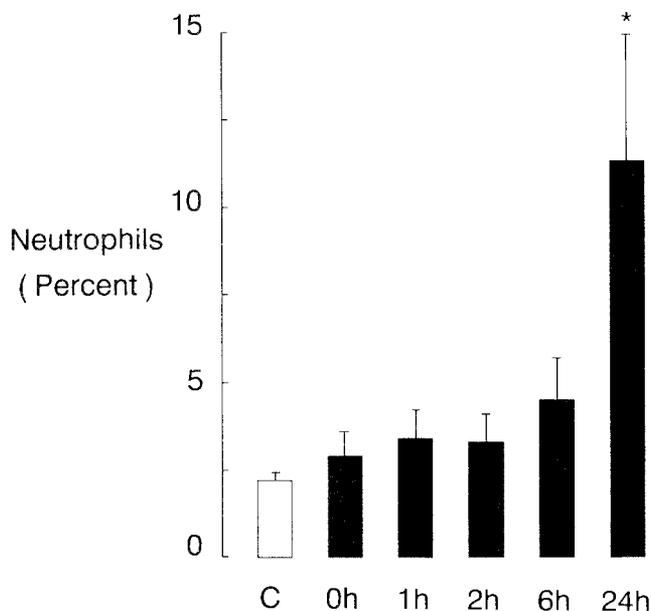
##### Bronchoalveolar Lavage After Acrolein Exposure

Inflammatory cell infiltration and the release of selected arachidonic acid metabolites into bronchoalveolar lavage fluid were determined before acrolein exposure and at various times after exposure. The concentrations of two eicosa-



**Figure 10. Time course of acrolein-induced eicosanoid release in bronchoalveolar lavage fluid obtained from guinea pigs at 0, 1, 2, 6, and 24 hours after exposure to 1.31 ppm acrolein.** Concentrations of two eicosanoids, prostaglandin F<sub>2α</sub> (top) and thromboxane B<sub>2</sub> (bottom), were determined by radioimmunoassay of cell-free aliquots of lavage fluid. Bronchoalveolar lavage was performed with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate-buffered saline (37°C). Recovered fluid was placed in ice-cold centrifuge tubes and centrifuged (150  $\times$  g for 15 minutes at 4°C), and the supernatant was stored at -70°C for later analysis. Values are means  $\pm$  SE for different groups of five to seven guinea pigs. Open bars indicate unexposed control group; closed bars indicate times when animals were killed after the end of a two-hour exposure to acrolein. An asterisk (\*) indicates significant increases ( $p < 0.05$ ) in prostaglandin F<sub>2α</sub> concentrations at 0 (immediately after exposure), one, and two hours, and significant elevations in thromboxane B<sub>2</sub> concentrations. Unexposed guinea pigs served as controls.

noids, PGF<sub>2α</sub> and TxB<sub>2</sub>, were increased immediately after exposure (Figure 10). The concentrations of two other eicosanoids, PGE<sub>2</sub> and PGF<sub>1α</sub>, were unchanged during the first six hours after exposure, but PGE<sub>2</sub> values became greater than those for controls at 24 hours after exposure (control PGE<sub>2</sub> =  $34 \pm 6$  pg/mL,  $n = 7$  animals; 24 hours PGE<sub>2</sub> =  $84 \pm 22$  pg/mL,  $n = 5$  animals). Resting concentrations of PGE<sub>2</sub> and PGF<sub>1α</sub> obtained from control animals were less than PGF<sub>2α</sub> and TxB<sub>2</sub> (control PGE<sub>2</sub> =  $34 \pm 6$ , PGF<sub>1α</sub> =  $46 \pm 13$  pg/mL; control PGF<sub>2α</sub> =  $98 \pm 20$ , TxB<sub>2</sub> =  $167 \pm 21$  pg/mL). The mean values of PGE<sub>2</sub> in lavage fluid were  $31 \pm 5$ ,  $25 \pm 10$ ,  $45 \pm 5$ ,  $20 \pm 6$ , and  $84 \pm 22$  pg/mL, and the mean values of PGF<sub>1α</sub> were  $67 \pm 29$ ,  $80 \pm 26$ ,  $33 \pm 5$ ,  $73 \pm 12$ , and  $51 \pm 11$  pg/mL at 0, 1, 2, 6, and 24 hours after exposure, respectively. These



**Figure 11.** Time course of acrolein-induced neutrophil infiltrations into bronchoalveolar lavage obtained from guinea pigs obtained at 0, 1, 2, 6, or 24 hours after a two-hour exposure to 1.31 ppm acrolein. Lavage fluid from each animal in groups of five to seven guinea pigs was centrifuged. A sample of the resuspended cell pellet (0.5 mL) was placed on a slide using a cytocentrifuge. Cells were stained with modified Wright's stain, and cell differential counts were performed by counting 500 to 1,000 cells. Percentage of neutrophils present in bronchoalveolar lavage fluid was significantly increased (\*) at 24 hours after exposure, as determined by analysis of variance followed by Scheffé's test. Open bar indicates controls; closed bars indicate samples.

values were not significantly different from control values (with the exception of PGE<sub>2</sub> measured at 24 hours) as determined by analysis of variance followed by Scheffé's test.

The percentage of neutrophils present in lavage fluid increased with time after exposure, but did not become significant until 24 hours after exposure (Figure 11). At one hour after exposure, when hyperreactivity was measurable

(Figure 2), the percentage of neutrophils was  $3.4 \pm 0.8$ , compared to a preexposure control value of  $2.2 \pm 0.2$  percent. At six hours, neutrophils were  $4.5 \pm 1.2$  percent of recoverable cells, and they reached  $11.3 \pm 3.6$  percent when measured 24 hours after exposure. This temporal relationship would suggest that neutrophil infiltration neither preceded nor accompanied the onset of heightened altered airway reactivity.

The measures of the percentages of other granulocytes (eosinophils and basophils) varied, and again, only the values at 24 hours were significantly different from the control values (Table 2). Identification of columnar epithelial cells in lavage fluid also varied, and changed from  $2.2 \pm 0.4$  percent (control) to  $7.6 \pm 3.2$  percent immediately after the two-hour exposure. This change was not statistically significant ( $p \leq 0.05$ , analysis of variance followed by Scheffé's test or unpaired Student's *t* test). The percentage of cells identified as macrophages and monocytes decreased at 24 hours. The total number of cells and the recovered volume of the injected 50-mL lavage fluid were unchanged and did not vary significantly among groups (Table 2).

#### Histopathology of 1.0 Part per Million Acrolein Exposure

Evidence of the temporal sequence of epithelial injury and inflammation was obtained through histological examination of the mid-trachea from four groups (six guinea pigs each) exposed to  $0.92 \pm 0.10$  ppm acrolein for two hours. One group was killed 0, 2, 6, or 24 hours after the two-hour exposure. Three groups served as control groups; these included an unexposed group, a group killed immediately after a sham exposure, and a group killed 24 hours after a sham exposure. Histological changes were as-

**Table 2.** Differential Cell Counts in Bronchoalveolar Lavage Fluid Obtained from Guinea Pigs After Exposure to Acrolein<sup>a</sup>

Time After Exposure (hours)	Total Cells ( $\times 10^6$ )	Recovery (mL)	Eosinophils and Basophils (%)	Epithelial Cells (%)	Macrophages and Monocytes (%)
Control	$34.1 \pm 6.2$	$48.1 \pm 0.7$	$3.0 \pm 0.9$	$2.2 \pm 0.4$	$92.3 \pm 2.3$
0	$30.6 \pm 2.8$	$47.6 \pm 0.8$	$5.2 \pm 2.0$	$7.6 \pm 3.2$	$80.5 \pm 4.5$
1	$34.2 \pm 5.8$	$47.7 \pm 0.9$	$3.7 \pm 2.4$	$2.4 \pm 0.7$	$89.9 \pm 3.4$
2	$33.0 \pm 2.7$	$48.4 \pm 0.9$	$7.4 \pm 1.6$	$3.0 \pm 0.8$	$86.3 \pm 3.0$
6	$46.4 \pm 8.3$	$47.2 \pm 1.2$	$4.3 \pm 1.0$	$1.5 \pm 0.5$	$91.1 \pm 1.9$
24	$30.6 \pm 2.0$	$47.9 \pm 2.0$	$11.9 \pm 1.9^b$	$3.8 \pm 1.3$	$71.2 \pm 4.9^b$

<sup>a</sup> Values are means  $\pm$  SE;  $n = 5$  to 7 guinea pigs in either control groups or exposed to  $1.31 \pm 0.11$  ppm acrolein for two hours. Animals were anesthetized, and bronchoalveolar lavage was performed at the times indicated after exposure. The volume of lavage fluid recovered (of 50 mL injected) was measured and centrifuged, and a sample of the resuspended cell pellet was placed on a slide with a cytocentrifuge. Samples from each animal were stained, and differential cell counts were obtained by counting 500 to 1,000 cells.

<sup>b</sup> Significantly different from control ( $p < 0.05$ ), as determined by analysis of variance followed by post-hoc Scheffé's test.

sessed with both the light and electron microscopes, and particular emphasis was placed on leukocytes in the epithelium and lamina propria.

As the previous lavage experiment (Figure 11 and Table 2), neutrophil or eosinophil margination in capillaries and infiltration into either the lamina propria or the epithelium were not extensive during the first 24 hours after exposure (Table 3).

In contrast to the lack of change in leukocytes, a small reduction in the number of ciliated cells was noted after acrolein exposure. This effect was transient, with apparent recovery within 24 hours, inasmuch as the group exposed

for two hours and killed 24 hours after exposure was not significantly different from the control group. The number of cilia and basal bodies per ciliated cell were also assessed semiquantitatively. The number of cilia per cell decreased immediately after exposure and again two and six hours later, but no change was noted 24 hours after exposure. A complementary increase in the number of free (not associated with the apical membrane) basal bodies was evident at six hours. The value at 24 hours was less than that at six hours and was not different from the control values, again suggesting that recovery may have occurred within 24 hours.

**Table 3.** Leukocytes Contained in the Tracheal Epithelium and Lamina Propria of Guinea Pigs Exposed to 0.9 ppm Acrolein for Two Hours<sup>a</sup>

	Number of Cells per Millimeter of Basement Membrane			
	0 hours <sup>b</sup>	2 hours	6 hours	24 hours
<b>Tracheal Epithelium</b>				
Sham-Exposed				
Neutrophils	1.0 ± 0.5	—	—	1.4 ± 0.6
Eosinophils	7.2 ± 1.9	—	—	6.3 ± 1.7
Mast cells	0.0 ± 0.0	—	—	0.7 ± 0.6
Macrophage	0.8 ± 0.3	—	—	1.0 ± 0.4
Small monocytes	29.7 ± 5.0	—	—	25.0 ± 3.3
Plasma cells	4.1 ± 1.5	—	—	2.1 ± 0.9
Acrolein-Exposed				
Neutrophils	0.6 ± 0.6	1.4 ± 0.6	0.8 ± 0.4	1.1 ± 0.3
Eosinophils	4.9 ± 2.5	5.4 ± 2.0	3.1 ± 1.2	3.7 ± 1.3
Mast cells	0.3 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.6 ± 0.3
Macrophage	0.9 ± 0.4	1.0 ± 0.7	0.5 ± 0.1	1.0 ± 0.4
Small monocytes	37.3 ± 6.3	19.8 ± 3.9	21.9 ± 4.8	20.8 ± 3.0
Plasma cells	4.7 ± 1.2	3.4 ± 1.8	2.1 ± 1.2	2.5 ± 0.9
<b>Lamina Propria</b>				
Sham-Exposed				
Neutrophils	0.5 ± 0.4	—	—	0.3 ± 0.1
Eosinophils	7.0 ± 2.5	—	—	5.1 ± 1.1
Mast cells	6.8 ± 0.7	—	—	6.1 ± 1.2
Macrophage	0.4 ± 0.3	—	—	0.3 ± 0.3
Small monocytes	19.2 ± 3.4	—	—	14.1 ± 3.8
Plasma cells	5.4 ± 2.8	—	—	1.8 ± 0.5
Acrolein-Exposed				
Neutrophils	0.4 ± 0.1	0.0 ± 0.0	0.3 ± 0.3	0.6 ± 0.3
Eosinophils	4.4 ± 2.1	3.7 ± 1.0	3.1 ± 1.1	5.9 ± 1.9
Mast cells	3.4 ± 0.8	6.0 ± 0.9	5.0 ± 0.7	5.0 ± 0.5
Macrophage	1.2 ± 0.8	0.6 ± 0.4	0.3 ± 0.1	0.3 ± 0.3
Small monocytes	16.9 ± 6.0	10.1 ± 4.8	11.0 ± 3.4	12.1 ± 3.7
Plasma cells	5.9 ± 3.7	0.9 ± 0.9	0.4 ± 0.2	1.3 ± 0.3

<sup>a</sup> Values are means ± SE for groups of six guinea pigs undergoing exposures or sham exposures to acrolein (0.92 ± 0.10 ppm) for two hours. Animals were killed, and the tracheas were removed, fixed, and sectioned (1.0-μm thickness). Each cell type was enumerated by an observer without knowledge of treatment condition. No value was significantly different from control values as determined by analysis of variance using a univariate test for within-subject effects followed by post-hoc unpaired *t* test.

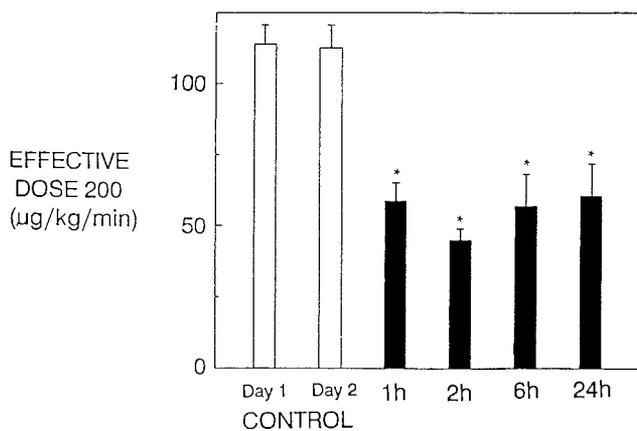
<sup>b</sup> Hours elapsed between exposures and time when animals were killed.

## EFFECTS OF AGONISTS AND INHIBITORS ON ACROLEIN-INDUCED BRONCHIAL HYPERREACTIVITY

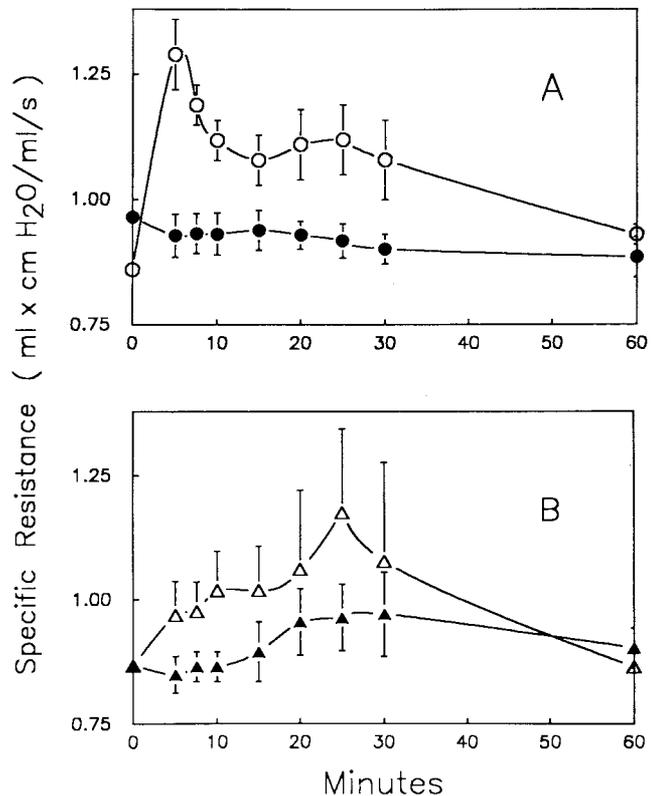
### Leukotriene Receptor Antagonist and 5-Lipoxygenase Inhibitors

To examine further the role of eicosanoid production in responses to aldehydes, specific resistance and bronchial reactivity to acetylcholine induced by acrolein were compared in four groups of animals. One group was treated with acrolein alone, two groups were treated with acrolein and leukotriene synthesis inhibitors, and one group was treated with an LTC<sub>4</sub>/LTD<sub>4</sub> receptor antagonist.

As observed previously, specific resistance measured five minutes after the two-hour exposure to  $1.29 \pm 0.07$  ppm acrolein increased from  $0.86 \pm 0.01$  to  $1.29 \pm 0.07$  mL  $\times$  (cm H<sub>2</sub>O/[mL/sec]) (50 percent increase, mean  $\pm$  SE,  $n = 6$  animals). The increase in sR<sub>t</sub> declined with time and fell to  $1.08 \pm 0.08$  30 minutes after exposure and  $0.93 \pm 0.02$  mL  $\times$  (cm H<sub>2</sub>O/[mL/sec]) 60 minutes after exposure. Similarly, bronchial reactivity to intravenous acetylcholine increased within one hour after acrolein exposure and became maximal two hours after exposure (Figure 12). Before exposure to acrolein, the acetylcholine infusion rate (normalized to body weight) sufficient to double sR<sub>t</sub> (ED<sub>200</sub>) was  $114.0 \pm 6.6$   $\mu$ g acetylcholine/kg/min (mean  $\pm$  SE, GSEM = 1.05,  $n = 6$  animals). A subsequent measurement of ED<sub>200</sub> for this group made on the following day was  $112.7 \pm 8.0$   $\mu$ g/kg/min (the mean difference was  $1.3 \pm 4.2$   $\mu$ g/kg/min, or  $1.1 \pm 3.7$  percent). One hour after acrolein exposure, the ED<sub>200</sub> decreased to  $58.5 \pm 6.5$  (49 percent decrease,  $p < 0.002$ ), was  $44.7 \pm 4.2$  at two hours (61 percent decrease,  $p$



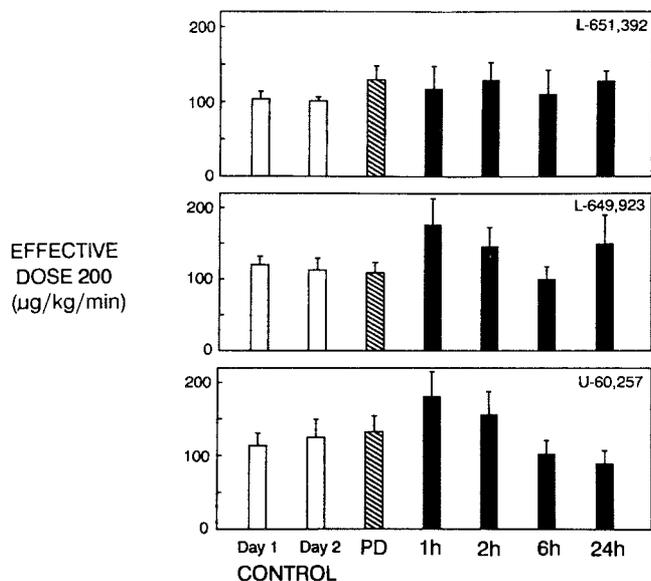
**Figure 12.** Acrolein-induced bronchial hyperreactivity in guinea pigs exposed to  $1.29 \pm 0.07$  ppm for two hours. Mean  $\pm$  SE ED<sub>200</sub> was less after acrolein exposure (closed bars) than in controls (open bars). Heightened reactivity became maximal two hours after the end of exposure and persisted for up to 24 hours after exposure. Values are means  $\pm$  SE for six guinea pigs. An asterisk (\*) indicates significance at the level of  $p < 0.05$ .



**Figure 13.** Attenuation of bronchial reactivity by pretreatment with either an LTC<sub>4</sub>/LTD<sub>4</sub> receptor antagonist or one of two 5-lipoxygenase inhibitors before guinea pigs were exposed to 1.2 to 1.3 ppm acrolein for two hours. A: Immediately after exposure to  $1.29 \pm 0.07$  ppm acrolein (○), specific resistance (mL  $\times$  (cm H<sub>2</sub>O/[mL/sec])) increased transiently. Treatment with 10 mg/kg L-649,923 administered intravenously (●) 10 to 15 minutes before acrolein exposure inhibited this response. B: Treatment with 5-lipoxygenase inhibitor (10 mg/kg L-651,392 administered orally [▲] one hour before exposure, or 5 mg/kg U-60,257 administered intravenously [△] 10 to 15 minutes before exposure) also diminished or delayed the acrolein-induced increase in specific resistance. Time zero is equal to the measurement of specific resistance obtained immediately before exposure. The second measurements were obtained five minutes after the two-hour exposure.

$< 0.001$ ), and  $60.2 \pm 11.6$   $\mu$ g/kg/min ( $p < 0.01$ ) 24 hours after exposure (Figure 12). Thus, the bronchial reactivity to intravenous acetylcholine in this second group of guinea pigs significantly decreased as early as one hour after exposure, was maximal within two hours after exposure, and persisted for 24 hours after acrolein exposure.

Treatment with either the receptor antagonist or the 5-lipoxygenase inhibitors diminished both the acrolein-induced immediate bronchoconstriction and the subsequent bronchial hyperreactivity. Figure 13 shows the changes in specific resistance that occurred in each of the four groups of six to seven guinea pigs exposed to acrolein. Treatment with L-649,923, the LTC<sub>4</sub>/LTD<sub>4</sub> receptor antagonist, completely abolished the expected increase in sR<sub>t</sub> (Figure 13). However, treatment with both of the 5-lipoxygenase inhibi-



**Figure 14.** Attenuation of bronchial reactivity to intravenous acetylcholine by pretreatment with either an  $LTC_4/D_4$  receptor antagonist or one of two 5-lipoxygenase inhibitors before guinea pigs were exposed to 1.2 to 1.3 ppm of acrolein for two hours. Marked decreases in  $ED_{200}$  were apparent one to two hours after exposure (see Figures 3, 4, and 11), an effect inhibited by 10 mg/kg oral L-651,392, or 10 mg/kg intravenous L-649,923, or 5 mg/kg intravenous U-60,257. The possible effect of each compound on baseline reactivity was assessed through the comparison of two control measurements (open bars) made the day before exposure (control day 1) and the day of exposure (control day 2) with those obtained after the administration of each compound. Hatch bars indicate postdrug (PD) measurements. Closed bars show the levels of reactivity 1, 2, 6, and 24 hours after acrolein exposure. Values are mean  $\pm$  SE;  $n$  = six or seven guinea pigs. No values were significantly different from control values.

tors appeared to diminish, and perhaps delay, the immediate bronchoconstriction (Figure 13).

Each compound also inhibited the acrolein-induced bronchial hyperreactivity. In contrast to the reduction of the effective acetylcholine dose necessary to double  $sR_t$  ( $ED_{200}$ ), as observed within one hour after exposure in the

control group exposed to acrolein, exposure of these groups failed to induce hyperreactivity (Figure 14). The effect of each compound on baseline acetylcholine levels was assessed by comparing the two pretreatment measurements made before exposure with the measurements made after the administration of each drug. If these compounds directly competed with acetylcholine, then treatment would decrease subsequent acetylcholine reactivity. Direct comparisons of the difference between day 1 (control)  $ED_{200}$  with the  $ED_{200}$  after drug administration were not significant ( $p > 0.10$ , paired Student's  $t$  test) regardless of the compound tested (Table 4). Overall, the variability in the two control measurements of  $ED_{200}$  obtained on separate days for 25 guinea pigs differed by  $0.92 \pm 3.82$   $\mu\text{g}/\text{kg}/\text{min}$  (day 1 control  $ED_{200}$  was  $112.7 \pm 6.0$  and day 2 control  $ED_{200}$  was  $113.3 \pm 7.7$  mean  $\pm$  SE). Thus, it appeared that these compounds did not directly affect baseline reactivity to acetylcholine when measured 10 to 15 minutes (L-649,923 and U-60,257) or 60 minutes (L-651,392) after intravenous or oral administration, respectively.

The  $ED_{200}$  for both the L-651,392 and U-60,257 increased slightly after acrolein exposure, but neither value was significantly different from preexposure control values (Figure 13). The minimal mean value of  $ED_{200}$  (and the time of occurrence after exposure) for each group was: for L-649,923,  $ED_{200} = 100.7 \pm 17.1$  (6 hours); for L-651,392,  $ED_{200} = 110.5 \pm 32.0$  (6 hours); and for U-60,257,  $ED_{200} = 89.43 \pm 17.7$   $\mu\text{g}/\text{kg}/\text{min}$  (24 hours). Thus, each compound was effective in inhibiting acrolein-induced bronchial hyperreactivity.

#### Effect of Inhibitors and Antagonists on Leukotriene $C_4$ in Bronchoalveolar Lavage Fluid

In the previous test, eicosanoid product formation was elevated by acrolein exposure, and inhibition of specific

**Table 4.** Effect of a Leukotriene Receptor Antagonist, L-649,923, and Two 5-Lipoxygenase Inhibitors, L-651,392 and U-60,257, on Bronchial Reactivity to Acetylcholine in Guinea Pigs<sup>a</sup>

Compound (Dose in mg/kg)	Effective Dose <sub>200</sub> ( $\mu\text{g}/\text{kg}/\text{min}$ )			$t$ Test <sup>b</sup>
	Control Day 1	Control Day 2	After Drug	
L-649,923 (10 iv)	119.8 $\pm$ 12.0	113.0 $\pm$ 16.5	109.2 $\pm$ 14.6	0.938 (NS)
L-651,392 (10 po)	103.7 $\pm$ 10.2	101.0 $\pm$ 5.7	129.5 $\pm$ 18.7	1.333 (NS)
U-60,257 (5 iv)	113.3 $\pm$ 17.0	124.7 $\pm$ 23.2	132.2 $\pm$ 21.9	1.780 (NS)

<sup>a</sup> Values are means  $\pm$  SE. Bronchial reactivity was assessed by interpolation of the dose rate of intravenous acetylcholine necessary to double specific airway resistance ( $ED_{200}$ ) in groups of six (L-649,923 and L-651,352, each) and seven (U-60,257) guinea pigs. Measurements were obtained twice before the administration of each compound (on separate days) and then 10 to 15 minutes (L-649,923 and U-60,257) or 60 minutes (L-651,392) after the administration of each compound.

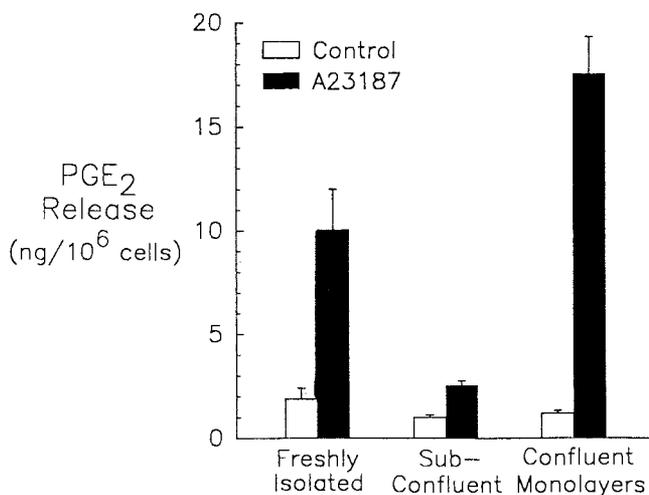
<sup>b</sup> Values of paired  $t$  test analyses by comparison of the differences between  $ED_{200}$  for day-1 control and  $ED_{200}$  after drug administration. Critical values at  $p \leq 0.05$  significance level for 5 and 6 degrees of freedom are 2.571 and 2.447, respectively. NS = not significant.

metabolites (lipoxygenase products) diminished acrolein's effect on bronchial reactivity. Therefore, the formation of an additional eicosanoid, LTC<sub>4</sub>, was determined as follows. Concentrates of 25-mL samples of bronchoalveolar lavage fluid from seven control guinea pigs and six guinea pigs exposed to acrolein (1.28 ± 0.13 ppm × 2 hours) were analyzed by radioimmunoassay to detect LTC<sub>4</sub> (which has an 11 percent cross-reactivity with LTD<sub>4</sub>). Concentrations of LTC<sub>4</sub> increased from 8.8 ± 0.3 to 15.9 ± 2.4 pg/mL (mean ± SE, *p* < 0.01, unpaired *t* test) after acrolein exposure. In addition, five guinea pigs exposed to acrolein (1.41 ± 0.06 ppm × 2 hours) and treated with the 5-lipoxygenase inhibitor L-651,392, demonstrated no elevation of LTC<sub>4</sub> (9.4 ± 2.4 pg/mL), when compared with control animals (*p* < 0.40, unpaired *t* test).

### EFFECTS OF ACROLEIN ON EICOSANOID METABOLISM IN AIRWAY EPITHELIAL CELLS IN CULTURE

#### Effects of Culturing Time on Eicosanoid Release

To prepare for tests of aldehyde-induced effects on eicosanoid metabolism in epithelial cells, the culture conditions first were optimized using freshly isolated bovine tracheal cells. Bovine tracheal epithelial cells at various stages of primary culture exhibited marked differences in their ability to release PGE<sub>2</sub>. Figure 15 shows the levels of PGE<sub>2</sub> released from freshly isolated cells (two hours after enzy-



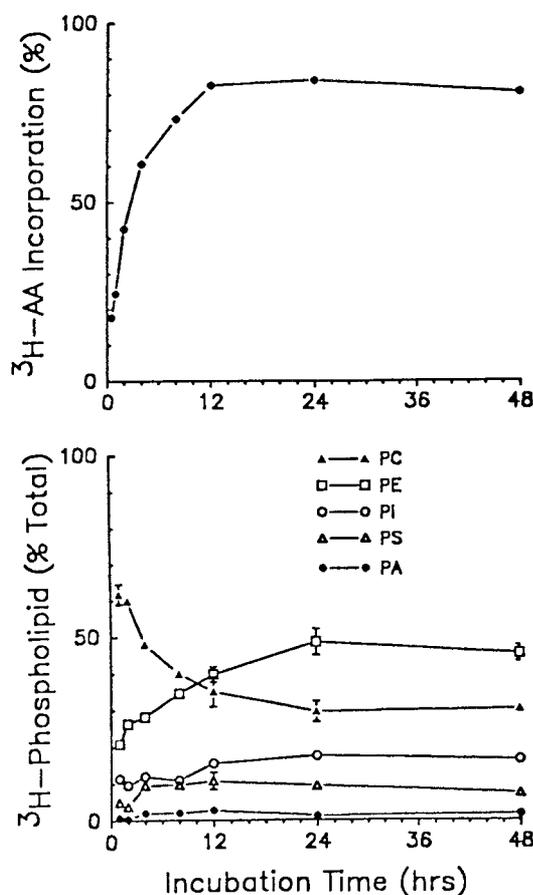
**Figure 15. Effects of time in culture on release of PGE<sub>2</sub> from bovine airway epithelial cells.** Cells were isolated freshly by three hours of enzymatic dissociation. This was followed by a one-hour equilibration in Krebs-Henseleit buffer and exposure either to buffer alone (control) (open bar) or 3.0 μM calcium ionophore A23187 (closed bar). Unexposed cell aliquots were seeded onto collagen-coated dishes and then tested 18 to 24 hours later (subconfluent) or tested at confluency (day 4 in culture). Values are means ± SE; *n* = 5.

matic dispersion), subconfluent cells (24 hours after plating), and confluent cell monolayers (day four of culture) after stimulation with 3 μM A23187. The ability of subconfluent cells to release PGE<sub>2</sub> (2.6 ± 0.3 ng/10<sup>6</sup> cells, mean ± SE) was reduced significantly compared to either freshly isolated (10.1 ± 2.3 ng/10<sup>6</sup> cells, *p* ≤ 0.05, paired *t* test) or confluent cells (17.5 ± 2.1 ng/10<sup>6</sup> cells, *p* ≤ 0.05, paired *t* test). A similar pattern was observed for receptor-mediated PGE<sub>2</sub> release after 10 μM bradykinin stimulation (freshly isolated, 5.8 ± 1.3 ng/10<sup>6</sup> cells; subconfluent, 0.9 ± 0.1 ng/10<sup>6</sup> cells; and confluent, 3.4 ± 0.6 ng/10<sup>6</sup> cells, *n* = 4 dishes). Because of confluent epithelial cell monolayers to release PGE<sub>2</sub> to an extent equal to or greater than freshly isolated cells, all subsequent experiments were done on cells at confluency.

#### Incorporation of <sup>3</sup>H-Arachidonic Acid into Epithelial Monolayers

Uptake of <sup>3</sup>H-arachidonic acid into bovine epithelial cell monolayers depended on time. Figure 16 (upper panel) shows the percentage of exogenously added <sup>3</sup>H-arachidonic acid (2 μCi) that was incorporated into the monolayers at the end of various incubation periods. At incubation periods of less than 12 hours, monolayers continued to incorporate exogenous <sup>3</sup>H-arachidonic acid. At periods of 12 hours and longer, incorporation was saturated and represented 82.5 ± 0.3 percent, 83.9 ± 1.4 percent, and 80.2 ± 1.0 percent (mean ± SE, *n* = 12 dishes) of the originally added radioactivity at 12, 24, and 48 hours, respectively.

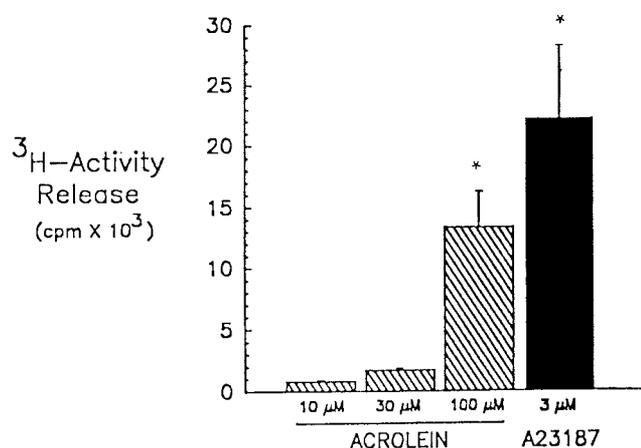
The distribution of the <sup>3</sup>H-arachidonic acid among the different phospholipid classes also depended on incubation time, as shown in Figure 16 (lower panel). At one hour, the radiolabel associated with the phospholipids was primarily located in PC pools, 62 ± 3 percent (*n* = 4 dishes). Between 1 and 24 hours however, as incorporation continued, the relative fraction of incorporated <sup>3</sup>H-arachidonic acid increasingly became esterified to PE, resulting in 49 ± 4 percent (*n* = 3 dishes) at 24 hours compared with 30 ± 3 percent (*n* = 3 dishes) for PC pools. Because incorporation was saturated between 12 and 24 hours, the changes in PC and PE labeling represented a redistribution of <sup>3</sup>H-arachidonic acid from PC pools to PE pools. This process reached an apparent equilibrium at 24 hours because the phospholipid labeling was unchanged at 48 hours. The fraction of radiolabel in PI, PS, and PA pools was relatively unchanging over the incubation periods tested. Because both <sup>3</sup>H-arachidonic acid incorporation and its distribution among phospholipid classes were saturated and stable 24 hours after exposure, subsequent experiments were conducted on monolayers radiolabeled for 24-hour periods.



**Figure 16. Time-course of  $^3\text{H}$ -arachidonic acid uptake and phospholipid distribution in bovine airway epithelial cells.** A: The percentage of added  $^3\text{H}$ -arachidonic acid that was incorporated at the end of varying incubation periods. Cell monolayers were incubated at  $37^\circ\text{C}$  with  $2.0\ \mu\text{Ci}$  of  $^3\text{H}$ -arachidonic acid in a 45:45:10 mixture of DMEM:Ham's F-12:fetal calf serum. At the end of each incubation period, monolayers were washed three times and the recovered radioactivity was counted. The percentage of incorporation equaled the unrecoverable radioactivity (total dpm added to dpm in washes) divided by the total radioactivity added  $\times 100$ . Values are means  $\pm$  SE,  $n = 12$ , with standard error bars often smaller than the symbol. B:  $^3\text{H}$ -Radioactivity in different phospholipid classes, expressed as a percentage of the total extracted  $^3\text{H}$ -radioactivity associated with phospholipids. Monolayers were extracted as described by Bligh and Dyer (1959), then separated by thin-layer chromatography. Values are means  $\pm$  SE,  $n = 2$  to 6 at each time.

#### Effects of Acrolein on $^3\text{H}$ -Arachidonic Acid Metabolism

Exposure of bovine epithelial monolayers prelabeled with  $^3\text{H}$ -arachidonic acid (24 hours) to  $100\ \mu\text{M}$  acrolein for 20 minutes caused a significant increase in the release of radioactivity (Figure 17). Monolayers were stimulated with KHBS alone (control), or with 10, 30, or  $100\ \mu\text{M}$  acrolein, or with  $3\ \mu\text{M}$  A23187, each for 20 minutes. The  $100\text{-}\mu\text{M}$  acrolein-induced release was  $13.4 \pm 2.9 \times 10^3$  cpm/dish (mean  $\pm$  SE), whereas the control release was  $2.7 \pm 0.4 \times 10^3$  cpm/dish ( $p \leq 0.05$ , paired  $t$  test). Stimulation with either 10 or  $30\ \mu\text{M}$  acrolein did not cause a significant in-



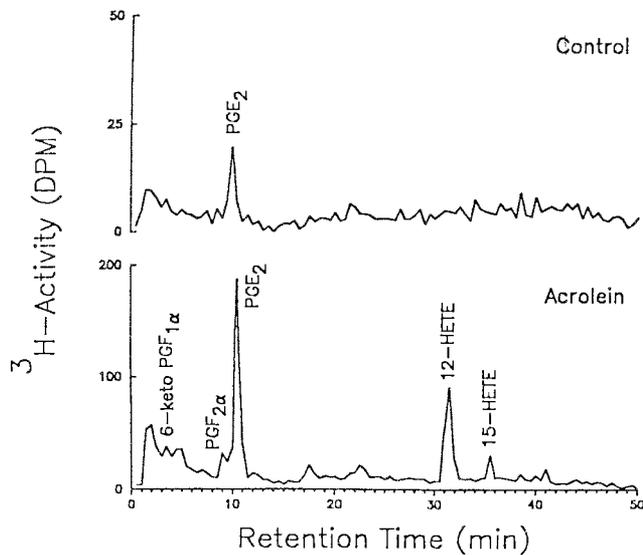
**Figure 17. Acrolein-induced release of  $^3\text{H}$ -arachidonic acid from bovine airway epithelial cells.** Confluent cell monolayers were equilibrated in Krebs-Henseleit buffer solution for one hour following a 24-hour  $^3\text{H}$  incorporation period. After equilibration, cells were stimulated with 10, 30, or  $100\ \mu\text{M}$  acrolein (hatched bars), or  $3\ \mu\text{M}$  calcium ionophore A23187 (solid bar), each for 20 minutes. Released radioactivity ( $^3\text{H}$ ) is expressed as counts per minute divided by each 60-mm dish with confluent cells. Values are means  $\pm$  SE. An asterisk (\*) indicates a significant difference ( $p < 0.05$ ) from paired control values as determined by paired  $t$  test.

crease in released radioactivity. A23187 ( $3\ \mu\text{M}$ ) further stimulated  $^3\text{H}$ -activity release ( $22.3 \pm 6.1 \times 10^3$  cpm/dish). For within-dish comparisons, the release induced by  $100\ \mu\text{M}$  acrolein was 27 percent of the A23187 response.

Reverse-phase HPLC separation of the  $^3\text{H}$ -activity released during control and  $100\text{-}\mu\text{M}$  acrolein exposures (20 minutes) revealed several  $^3\text{H}$ -activity peaks coeluting with authentic eicosanoid standards. Figure 18 shows radiochromatographs from a representative cell monolayer identifying  $^3\text{H}$ -activity released after control (top) and acrolein ( $100\text{-}\mu\text{M}$ ) exposure (bottom). Under control conditions, the major  $^3\text{H}$ -activity peak that was observed coeluted with  $\text{PGE}_2$  and was increased nine-fold in this monolayer after treatment with  $100\text{-}\mu\text{M}$  acrolein. Radioactivity coeluting with two other prostaglandins was also elevated after acrolein treatment;  $\text{PGF}_2\alpha$  showed a six-fold increase, and  $\text{PGF}_{1\alpha}$  exhibited a five-fold increase.  $^3\text{H}$ -activity peaks coeluting with two lipoxygenase products not predominantly expressed under control conditions also were substantially increased: 12-HETE showed a 181-fold increase and 15-HETE showed an eight-fold increase. Table 5 contains the mean results from the analysis of seven bovine epithelial monolayers.

#### Dose-Response Relationship for Acrolein-Induced Eicosanoid Release

Radioimmunoassay measurements showed acrolein-induced release of endogenous eicosanoids from monolayers of bovine epithelial cells to be dose-dependent (Figure 19). Duplicate dishes of confluent monolayers were stimulated



**Figure 18.** Radiochromatographs of <sup>3</sup>H-labeled eicosanoid released from a confluent monolayer of bovine airway epithelial cells prelabeled with <sup>3</sup>H-arachidonic acid (for 24 hours). Each monolayer (60-mm dish) was equilibrated in Krebs-Henseleit buffer solution for one hour at 37°C. Then the cells were stimulated for 20 minutes with control buffer (top), and then with buffer containing 100 μM acrolein (bottom). Released eicosanoids were extracted from the cell-free supernatant with ethylacetate and separated by HPLC. Eluates were collected every 30 seconds and radioactivity was counted. Radioactive peaks were correlated with retention times of authentic eicosanoid standards before each separation.

with either 0 (control), 1, 3, 10, 30, or 100 μM acrolein for 20 minutes, and the release of PGF<sub>2α</sub> and PGE<sub>2</sub> was measured by radioimmunoassay. The threshold dose for both PGF<sub>2α</sub> and PGE<sub>2</sub> release was 30-μM acrolein, with some of

the monolayers responding at this dose. The mean values, however, were not statistically different from control values (paired *t* test). Yet, exposure to 100-μM acrolein did cause a significant increase in the release of both eicosanoids (PGF<sub>2α</sub>, control 0.4 ± 0.2 ng/10<sup>6</sup> cells, 100-μM acrolein 2.1 ± 0.7 ng/10<sup>6</sup> cells, *p* < 0.05; and PGE<sub>2</sub>, control 2.3 ± 0.7 ng/10<sup>6</sup> cells, 100-μM acrolein 10.4 ± 3.8 ng/10<sup>6</sup> cells, *p* < 0.05). These values represent a 5.3-fold and 4.5-fold increase in PGF<sub>2α</sub> and PGE<sub>2</sub>, respectively. Treatment with 3-μM A23187 released 17.5 ± 2.1 ng of PGE<sub>2</sub>/10<sup>6</sup> cells.

### Eicosanoid Metabolism in Human Airway Epithelial Cells

Several aspects of eicosanoid metabolism were characterized before tests with acrolein were conducted. To examine whether or not epithelial cells in human airways could synthesize cysteinyl-leukotrienes, cells were incubated with either LTA<sub>4</sub> or radiolabeled arachidonic acid (and stimulated with A23187 and excessive arachidonate). Stimulation with 3-μM A23187 and 10-μM arachidonate yielded no detectable increase in radioactivity coeluting with LTC<sub>4</sub> or LTD<sub>4</sub>, and no more than 1.0 percent of the total radioactivity released coeluting with 5-HETE. This suggests that 5-lipoxygenase activity is not a predominant metabolic pathway in these cells. Incubation with a proximal precursor of LTC<sub>4</sub>, LTA<sub>4</sub> (60 nmol/10<sup>6</sup> cells), also produced no detectable LTC<sub>4</sub> or LTD<sub>4</sub> formation (the limit of detection was 25 pmol), but did produce 41 pmol LTB<sub>4</sub> (no more than 0.7 percent conversion). Incubation of cells with <sup>3</sup>H-LTC<sub>4</sub> yielded 1.2 ±

**Table 5.** <sup>3</sup>H-Labeled Eicosanoids Released from Bovine Airway Epithelial Cells in Response to Acrolein Exposure in Vitro<sup>a</sup>

Retention Time (minutes)	Coelution Standard	<sup>3</sup> H-Activity (dpm)	
		Control	Acrolein Exposure
3.0	6-keto PGF <sub>1α</sub>	25 ± 9	268 ± 34 <sup>b</sup>
6.3	TxB <sub>2</sub>	15 ± 4	56 ± 14
7.9	PGF <sub>2α</sub>	25 ± 8	322 ± 93 <sup>b</sup>
9.3	PGE <sub>2</sub>	339 ± 225	4,102 ± 1,555 <sup>b</sup>
11.0	PGD <sub>2</sub>	12 ± 6	114 ± 33
25.7	LTB <sub>4</sub> , 5,12-diHETE <sup>c</sup>	19 ± 7	15 ± 5
31.1	12-HETE	14 ± 4	2,645 ± 460 <sup>b</sup>
36.1	15-HETE	34 ± 13	187 ± 26 <sup>b</sup>
39.5	5-HETE	22 ± 5	110 ± 12
42.5	Arachidonic Acid	77 ± 45	162 ± 36

<sup>a</sup> Confluent bovine airway epithelial cells were incubated with 2 μCi <sup>3</sup>H-arachidonic acid per 60-mm dish for 24 hours, washed three times with Krebs-Henseleit buffer solution, then equilibrated at 37°C for 60 minutes. Each dish then was exposed sequentially to Krebs-Henseleit buffer (control) and Krebs-Henseleit buffer containing 100 μM acrolein, each for 20 minutes. <sup>3</sup>H-arachidonic acid products were extracted with ethyl acetate and then separated by reverse-phase HPLC. The eluate was collected every 30 seconds and radioactivity was determined by beta liquid scintillation. Peaks in <sup>3</sup>H-activity were correlated with retention times of authentic eicosanoid standards before each separation. Values are means ± SE (*n* = 7).

<sup>b</sup> Value significantly different from control as determined by paired *t* test (*p* < 0.05).

<sup>c</sup> 5,12-diHETE = 5(*S*),12(*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid.

0.2 percent conversion of LTC<sub>4</sub> to <sup>3</sup>H-LTD<sub>4</sub>. These findings imply that epithelial cells in the human airways retain only a vestigial enzymatic capacity to generate or metabolize cysteinyl leukotrienes (exhibiting little 5-lipoxygenase, LTC<sub>4</sub> synthetase-glutathione S-transferases, or glutamyl transpeptidase activity), with each of these activities being much less than that of leukocytes.

Because acrolein-stimulated bovine cell eicosanoid metabolism and the mitogenic activity of LTC<sub>4</sub> in human cells was greater in the presence of indomethacin, we also analyzed the ability of human cells to produce other eicosanoids that result from cyclooxygenase metabolism. Chromatographic analysis revealed two major cyclooxygenase metabolites coeluting with authentic PGF<sub>2α</sub> and PGE<sub>2</sub>. The mean results of the reverse-phase HPLC analysis of products extractable with ethyl acetate and expressed as the sum of radioactive material contained in two 30-second fractions of the elution solvent (at and immediately after the elution times of selected standards) are shown in Table 6. The amount of material coeluting with cyclooxygenase standards (prostaglandins F<sub>1α</sub>, F<sub>2α</sub>, E<sub>2</sub>, and D<sub>2</sub> or 12-hydroxy-5,8,10-heptadecatrienoic acid [12-HHT]) was increased after the addition of A23187. Significant increases in material coeluting with 15-lipoxygenase products were also noted.

Prostaglandin E<sub>2</sub> and F<sub>2α</sub> release was also measured by radioimmunoassay in control or A23187-activated conditions. A23187 at a concentration of 3 μM produced a marked increase in the release of PGE<sub>2</sub> (control value was 0.96 ± 0.08 pmol/10<sup>6</sup> cells, compared with A23187 value of 15.43 ± 1.93 pmol/10<sup>6</sup> cells) and PGF<sub>2α</sub> (control value was 0.54 ± 0.06 pmol/10<sup>6</sup> cells, compared with A23187 value of 5.08 ± 0.85 pmol/10<sup>6</sup> cells). Indomethacin inhibited this response (for PGE<sub>2</sub>: control + 10 μM indomethacin was 0.23 ± 0.11 pmol/10<sup>6</sup> cells, compared with A23187 + indomethacin was 0.83 ± 0.22 pmol/10<sup>6</sup> cells).

### Effects of Acrolein on Epithelial Cell Viability

Acrolein (100 μM) had no significant effect on the cellular viability of bovine cells after a 20-minute exposure period. Lactate dehydrogenase activity was not significantly elevated until two hours into exposure to 100-μM acrolein (18 ± 2 U/L, *n* = 6 dishes, *p* ≤ 0.05). After two hours, continued exposure led to increased LDH activity (91 ± 34, *n* = 4 dishes; 223 ± 18, *n* = 4 dishes; and 240 ± 1 U/L, *n* = 6 dishes, at 8, 18, and 24 hours, respectively). Sonication (15 minutes) of cell monolayers yielded LDH activity (237 ± 1 U/L, *n* = 6 dishes) equivalent to the 24-hour exposure to acrolein.

**Table 6.** <sup>3</sup>H-Labeled Eicosanoids Released From Human Airway Epithelial Cells Grown in Serum-Free Medium<sup>a</sup>

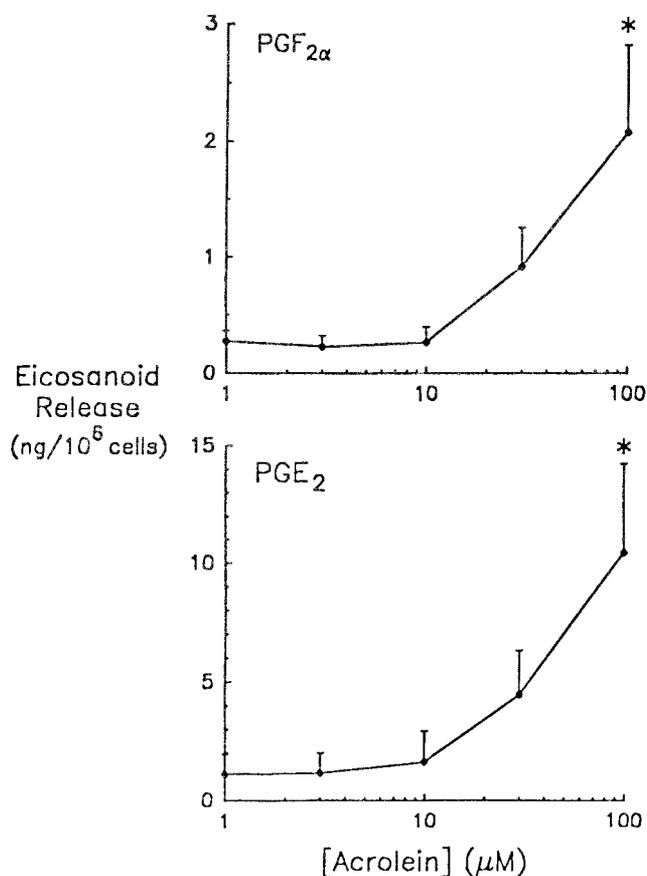
Retention Time (minutes)	Coelution Standard	<sup>3</sup> H-Activity (dpm)	
		Control	Calcium Ionophore
3.1	6-keto PGF <sub>1α</sub>	37 ± 14	269 ± 69 <sup>b</sup>
8.0	PGF <sub>2α</sub>	125 ± 23	700 ± 123 <sup>b</sup>
9.4	PGE <sub>2</sub>	331 ± 96	4,773 ± 855 <sup>b</sup>
11.0	PGD <sub>2</sub>	32 ± 13	297 ± 100 <sup>b</sup>
17.5	Unidentified	16 ± 6	259 ± 50 <sup>b</sup>
23.7	8( <i>R</i> ),15( <i>S</i> )-diHETE, 8( <i>S</i> ),15( <i>S</i> )-LT <sup>c</sup>	29 ± 7	170 ± 32 <sup>b</sup>
24.8	8( <i>S</i> ),15( <i>S</i> )-diHETE, 8( <i>R</i> ),15( <i>S</i> )-LT <sup>d</sup>	23 ± 8	88 ± 27
26.1	LTB <sub>4</sub>	57 ± 41	36 ± 3 <sup>b</sup>
31.5	12-HHT	58 ± 5	1,215 ± 205 <sup>b</sup>
39.5	15-HETE	326 ± 148	537 ± 157
40.5	12-HETE	241 ± 79	678 ± 174
41.2	5-HETE	206 ± 35	347 ± 114

<sup>a</sup> Epithelial cells were seeded at 10,000 cells/cm<sup>2</sup>, grown to confluency, and incubated with 1.0 μCi/mL <sup>3</sup>H-arachidonic acid for 24 hours. Monolayers were incubated in buffer as a control for one hour and then in 3 μM A23187 Ca-ionophore for 20 minutes. Samples were acidified (pH = 3.5), 50 ng prostaglandin B<sub>2</sub> (mean recovery = 89 ± 5 percent) was added, and ethyl acetate extracts were separated by reverse-phase HPLC. Counts represent two 30-second fractions collected during and immediately after the indicated retention times of coeluting standards. Compounds that eluted less than one minute apart from one another in this gradient system were combined for this analysis.

<sup>b</sup> Values (mean ± SE, *n* = 5) significantly different from control values (*p* < 0.05), as determined by paired *t* test.

<sup>c</sup> 8(*R*),15(*S*)-diHETE = 8(*R*),15(*S*)-dihydroxy-5-*cis*-11-(*cis,trans*)-9,13-*trans*-eicosatetraenoic acid. 8(*S*),15(*S*)-LT = 8(*S*),15(*S*)-dihydroxy-5-*cis*-9,11,13-*trans*-eicosatetraenoic acid.

<sup>d</sup> 8(*S*),15(*S*)-diHETE = 8(*S*),15(*S*)-dihydroxy-5-*cis*-11-(*cis,trans*)-9,13-*trans*-eicosatetraenoic acid. 8(*R*),15(*S*)-LT = 8(*R*),15(*S*)-dihydroxy-5-*cis*-9,11,13-*trans*-eicosatetraenoic acid.

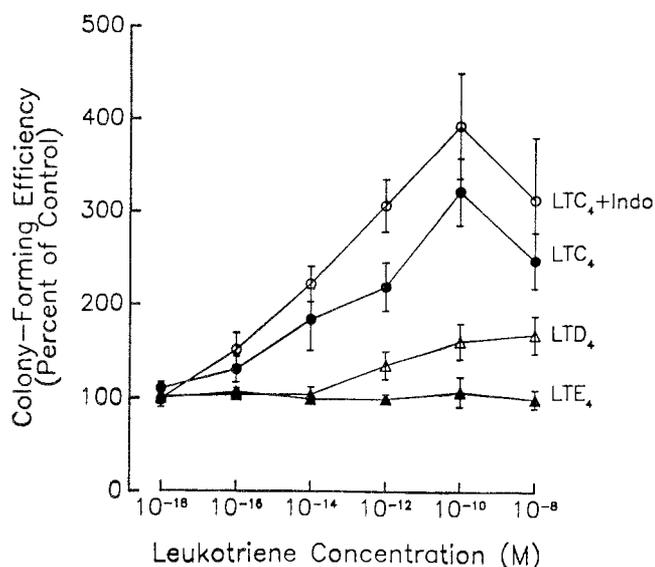


**Figure 19.** Dose-response of acrolein-induced release of immunoreactive PGF<sub>2α</sub> (top) and PGE<sub>2</sub> (bottom) from confluent monolayers of bovine airway epithelial cells. Monolayers were equilibrated for one hour in Krebs-Henseleit buffer, and duplicate dishes from 4 to 10 tracheas were incubated for 20 minutes at 37°C in 0 (control), 1, 3, 10, 30, or 100 μM acrolein. Radioimmunoassays were performed on duplicate aliquots taken from each sample of cell-free supernatant. Cell counts were performed after each experiment. Eicosanoid release is expressed as ng/10<sup>6</sup> cells. Values are means ± SE. An asterisk (\*) indicates a significant difference from control values as determined by paired *t* test ( $p < 0.05$ ).

## MITOGENIC EFFECTS OF EICOSANOIDS

### Effect of Leukotriene C<sub>4</sub> on Growth of Human Airway Epithelial Cells

With the demonstration of an increase in LTC<sub>4</sub> in lavage fluid following acrolein exposure, we also examined the effect of this compound and related cysteinyl leukotrienes on cell growth. Leukotriene C<sub>4</sub> stimulated colony-forming efficiency at concentrations as low as 0.01 pM (185 ± 33 percent of control), with the greatest mitogenic effect noted at 0.1 nM (323 ± 36 percent of control) (see Figure 20). Leukotriene D<sub>4</sub> also stimulated growth at concentrations of 1.0 pM to 10 nM, and LTE<sub>4</sub> produced an insignificant effect over a concentration range of 10<sup>-18</sup> to 10<sup>-8</sup> M. The effect of LTC<sub>4</sub> on colony-forming efficiency was potentiated by the addition of a cyclooxygenase inhibitor, 1.0-μM indomethacin.



**Figure 20.** Cysteinyl LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> effects on colony-forming efficiency of human airway epithelial cells. Leukotriene C<sub>4</sub> produced a greater mitogenic effect than its sequential metabolites LTD<sub>4</sub> and LTE<sub>4</sub>. Inhibition of cyclooxygenase by 1.0 μM indomethacin increased the LTC<sub>4</sub> effect on growth. Cells initially were seeded with 250 to 500 cells/cm<sup>2</sup> and grown in serum-free medium for seven to nine days. Values for LTC<sub>4</sub> and LTC<sub>4</sub> plus indomethacin were significantly ( $p < 0.05$ ) different from control values for doses of at least 10<sup>-14</sup> M, and for LTD<sub>4</sub> doses of at least 10<sup>-12</sup> M, as determined by paired *t* test. Values for LTE<sub>4</sub> were not significantly different from control values.

These results suggest a structure-activity relationship related to the sequential metabolism of these compounds, because LTD<sub>4</sub> is a metabolite of LTC<sub>4</sub>, and LTE<sub>4</sub> is a metabolite of LTD<sub>4</sub>.

Because the initial analysis of LTC<sub>4</sub>'s effect on growth was completed in a serum-free medium containing an extract from bovine pituitary gland (Lechner et al. 1982), we also examined the effect of LTC<sub>4</sub> on growth in the same medium without this extract. In these chemically defined conditions, LTC<sub>4</sub> again stimulated colony-forming efficiency (10-nM LTC<sub>4</sub> = 163 ± 10 percent of control), and this mitogenic effect was potentiated by the addition of indomethacin (10-nM LTC<sub>4</sub> + 1.0-μM indomethacin = 241 ± 46 percent of control). Thus, LTC<sub>4</sub> can stimulate growth by itself, but its effect is greater in a medium containing pituitary gland extract (see Figure 20).

The mitogenic activity of LTC<sub>4</sub> also was assayed by measuring thymidine incorporation (DNA synthesis) and clonal growth rate (population doublings per day). Consistent with the above findings, the rates of thymidine incorporation and clonal growth were stimulated by LTC<sub>4</sub> in the presence of indomethacin (Table 7). These two methods confirm that LTC<sub>4</sub> has a mitogenic effect on airway epithelial cells, and a comparison of these assays indicates that LTC<sub>4</sub> has a greater effect on colony-forming efficiency (a measure

**Table 7.** Leukotriene C<sub>4</sub> Stimulation of Human Airway Epithelial Cell Growth in the Presence of Indomethacin

Treatment	Colony-Forming Efficiency <sup>a</sup> (% control)	DNA Synthesis <sup>b</sup> (% control)	Clonal Growth Rate <sup>c</sup> (PD/D)
Vehicle control	100 ± 9	100 ± 10	0.76 ± 0.06
0.01 pM LTC <sub>4</sub>	223 ± 19 <sup>d</sup>	128 ± 12	0.97 ± 0.08 <sup>d</sup>
1.0 pM LTC <sub>4</sub>	307 ± 28 <sup>d</sup>	224 ± 39 <sup>d</sup>	1.13 ± 0.09 <sup>d</sup>

<sup>a</sup> Colony-forming efficiency was determined for six trials with cells seeded 250 to 500 cells/cm<sup>2</sup> and grown as described in Figure 1. Vehicle control values were obtained from cells grown in 0.5 μL of ethanol per mL of medium.

<sup>b</sup> DNA synthesis was evaluated in 4 to 5 trials using the ratio of <sup>3</sup>H:<sup>14</sup>C incorporation. For this assay cells were labeled for 48 hours with <sup>14</sup>C-thymidine followed by a 24-hour labeling period with <sup>3</sup>H-thymidine. Seeding density = 5,000 cells/cm<sup>2</sup>.

<sup>c</sup> Clonal growth rate (population doubling per day[PD/D]) was derived from the log base-2 of the average number of cells per clone (from 18 randomly selected clones), divided by the number of days in culture (5 to 9 days). Seeding density = 250 to 500 cells/cm<sup>2</sup>.

<sup>d</sup> Values significantly greater than control, as determined by paired *t* test (*p* < 0.05).

of the number of colonies) than on clonal growth rate (a measure of the number of cells per colony).

## DISCUSSION

### TRANSIENT BRONCHOSPASM

As noted previously by Murphy and colleagues (1963) with acrolein, and Amdur (1960) with formaldehyde, increases in baseline total pulmonary resistance occur rapidly after the onset of aldehyde exposure, remain constant during exposure, and are readily reversible after the cessation of exposure. The results from the present study confirm the transient property of this bronchoconstrictive response in guinea pigs. Two-hour exposures to acrolein (at least 0.3 ppm) or formaldehyde (at least 9.0 ppm) produced changes in specific resistance that readily returned to preexposure baseline values within one hour after exposure. (Because specific resistance was measured several additional times over the next 24 hours with each test of bronchial reactivity, these results also indicate that this return to control values remained constant, and a delayed secondary bronchoconstriction sometimes noted after irritant exposures did not occur.) Another noteworthy finding was that when exposures to formaldehyde were extended from two to eight hours, the threshold dose necessary to increase specific resistance decreased to 1.0 ppm.

Murphy and associates (1963) reported that acrolein-induced bronchoconstriction was inhibited by autonomic antagonists, suggesting that this immediate response involves occupancy of irritant receptors by the aldehyde leading to reflex bronchoconstriction (vagally-mediated). With the removal from such occupancy, this response is rapidly reversible, indicating that it is unlikely that this response involves a stable metabolite of formaldehyde, such as formic

acid, which has a clearance half-time exceeding 10 to 15 minutes.

In summary, our findings are in agreement with what has previously been reported by Amdur (1960) and Murphy and colleagues (1963) for formaldehyde and acrolein, respectively, and we predict a threshold dose for this response in guinea pigs between 0.1 and 1.0 ppm, with acrolein being more potent than formaldehyde.

### BRONCHIAL HYPERREACTIVITY

A principal finding of this study is that either acrolein or formaldehyde exposure can induce bronchial hyperreactivity in guinea pigs. Increased reactivity typically developed within one to two hours after exposure and reached maximum two to six hours after exposure, and persisted for 24 hours after each 2-hour exposure. The two-hour exposure doses of acrolein and formaldehyde necessary to induce a maximal change in reactivity were approximately 1.0 and 30 ppm, respectively (Figure 4). Again, the unsaturated aldehyde, acrolein, was more potent than formaldehyde.

The extension of the duration of formaldehyde exposure from two to eight hours resulted in a lowering of the dose necessary to induce hyperreactivity. A two-hour exposure to 31 ppm of formaldehyde produced the same magnitude of change in reactivity as an eight-hour exposure to 1.1 ppm of formaldehyde. Unlike the transient bronchoconstriction noted in the Results section above, this finding does not follow an application of a simple estimate of dosage (dose = concentration × time).

A possible reason for this difference is that much of the respiratory effects of formaldehyde are influenced by the high water solubility of this compound, which limits penetration through the upper respiratory tract (Egle 1972). For this reason, the dose to the airways may have been low

in guinea pigs breathing nasally. However, prolongation of exposure may serve to saturate the "uptake" sites of the upper airways and thus allow a greater percentage of airway deposition. Evidence for this possibility exists with another water-soluble gas, sulfur dioxide. In dogs and other species, sulfur dioxide-induced changes do not always follow concentration  $\times$  time dosimetry (Amdur 1991). This has led previous investigators to suggest that although solubility is a major determinant of the site of toxic effect, other influential factors of airway absorption could include the duration of exposure, respiratory flow rate, exposure concentration, and, of course, the ratio of nasal:oral breathing time (Gordon and Amdur 1991). Thus, further studies may seek to investigate how such physiologically-based variables interact to influence the regional dosimetry of inhaled formaldehyde and subsequent physiological responses.

#### **ASSOCIATION OF BRONCHIAL HYPERREACTIVITY WITH INFLAMMATION AFTER ACROLEIN EXPOSURE**

Two-hour exposures to acrolein also produced an increase in specific eicosanoids and inflammatory cells in bronchoalveolar lavage fluid. The relative roles in lipid mediators and the cellular basis of increased bronchial hyperreactivity to nonantigenic stimuli, including acrolein and formaldehyde, remain uncertain, although numerous investigations have implicated neutrophil infiltration as a necessary component in this response. Holtzman and coworkers (1983) were among the first to report a strong correlation between the number of neutrophils found in the airway epithelium and cholinergic reactivity in dogs exposed to 2.1 ppm ozone for two hours. Similarly, Fabbri and associates (1984) and Seltzer and colleagues (1986) have demonstrated marked neutrophil influx into bronchoalveolar lavage fluid shortly after ozone exposure with dogs and humans, respectively. More recently, Koren and associates (1989) have extended this finding in humans to more relevant concentrations of ozone.

One way to examine the role of neutrophil infiltration in this response is to deplete neutrophils and other granulocytes by cytotoxic agents. Pretreatment with hydroxyurea can diminish bronchial hyperreactivity induced either by ozone (O'Byrne et al. 1984b) or toluene diisocyanate (Thompson et al. 1986), which supports the theory of a causal role for neutrophil infiltration in this response. However, both ozone-induced and toluene diisocyanate-induced hyperreactivity are not preventable by pretreatment with another cytotoxic regimen involving cyclophosphamide (Thompson et al. 1986), although this latter treatment clearly inhibited neutrophil infiltration into the airway epithelium. Likewise, the onset of increased bronchial reactivity after exposure to ozone (Murlas and Roum 1985b) or cig-

arette smoke (Hulbert et al. 1985) preceded migration of neutrophils into the bronchial mucosa. Cigarette smoke, which contains a number of irritants including acrolein (Newsome et al. 1965; Ayer and Yeager 1982), produced a transient hyperreactivity that became maximal within 30 minutes after exposure but was not significantly different from control values when measured six hours after exposure (Hulbert et al. 1985).

The results from the present study also suggest that neutrophil infiltration may be coincidentally rather than causally related to the onset of bronchial hyperreactivity. As in a previous study with cigarette smoke (Hulbert et al. 1985), we found that acrolein-induced increases in bronchial reactivity were measurable as early as one hour after exposure, which is before substantial neutrophil infiltration. Increases in the percentage of neutrophils in lavage were only significantly greater than those for control at 24 hours after exposure. Likewise, neutrophil influx into the airway epithelium or lamina propria was not evident in histological samples obtained during early periods of hyperreactivity. A plausible explanation for previously reported correlations may be related to the high doses of irritants used in these studies (for example, more than 0.4 ppm ozone in humans) (Aizawa et al. 1985; Murlas and Lee 1985; Seltzer et al. 1986). Such exposures may have produced frank injury sufficient to accelerate neutrophil migration and thereby lead to the apparent simultaneous presence of epithelial neutrophils during periods of increased bronchial reactivity. Species difference may also have significance, because many of the apparent differences occur in two different species. Neutrophil infiltration was marked in random-bred dogs, whereas histological and lavage studies failed to find significant correlations in specific-pathogen-free guinea pigs.

#### **BIOACTIVE MEDIATOR RELEASE AFTER ACROLEIN EXPOSURE**

Because bronchial hyperreactivity can develop independently of a rapid influx of infiltrating granulocytes, other cells normally present in the lung during acrolein exposure are likely to have a role in mediating this response. With the diversity of precipitating factors, augmentation of the production of a wide array of bioactive mediators within the airways is probable. Furthermore, these mediators are likely to initiate multiple humoral interactions that may overlap with neurotransmitters and thereby have a prominent role in determining airway reactivity. Several groups of such mediators are conceivable, based on independent assays, and one probable group includes metabolites of arachidonic acid.

The present findings after acrolein exposure include elevations of  $\text{PGF}_{2\alpha}$ ,  $\text{TxB}_2$ , and  $\text{LTC}_4$  observed in lavage fluid

at times that precede or accompany hyperreactivity. The temporal aspect of these increases is consistent (unlike neutrophil influx) with a causal role for these bronchoactive metabolites. However, we recognize that an array of mediators derived from epithelial cells and other cells acting together will probably influence a number of complex cell-cell interactions that control reactivity (Nadel 1973; Barnes 1986; Stephens 1986). For example, the immediate bronchoconstriction observed during and shortly after exposure may have been controlled by a neurally mediated irritant effect, because Murphy and associates (1963) have demonstrated this possibility through inhibition by atropine pretreatment. Such a mechanism probably does not explain bronchial hyperreactivity, however. In a related study, Newball and Lenfant (1977) suggested that such a vagally mediated reflex component probably plays only a minor role in PGF<sub>2α</sub>-mediated bronchial hyperreactivity, because it is not inhibited by atropine or a mixture of atropine and cromolyn.

Increases in PGF<sub>2α</sub> and TxB<sub>2</sub> in this study parallel previous experience with both ozone exposure in humans (Seltzer et al. 1986) and LTB<sub>4</sub>-induced hyperreactivity in dogs (O'Byrne et al. 1985). Furthermore, inhalation of threshold doses of these cyclooxygenase products has been found previously to enhance subsequent muscarinic reactivity of the airways (O'Byrne et al. 1984a; Aizawa et al. 1985). Interestingly, in dogs, an experimental thromboxane synthesis inhibitor, OKY-046, prevented both ozone- and LTB<sub>4</sub>-induced hyperreactivity. Thus, cyclooxygenase product formation may be an important event in this species, although recent studies by Coffey and associates (1990) suggest a lesser role for this pathway in humans.

To clarify the importance of other products of arachidonic acid, specifically the 5-lipoxygenase metabolites LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, in this response in guinea pigs, we determined whether or not treatment with a leukotriene receptor antagonist and 5-lipoxygenase inhibitors could diminish acrolein-induced hyperreactivity. As stated above, LTC<sub>4</sub> was markedly increased in lavage fluid recovered from guinea pigs exposed to acrolein. Furthermore, a 5-lipoxygenase inhibitor L-651,392 inhibited this response. This compound and the other 5-lipoxygenase inhibitor U-60,257, and the LTC<sub>4</sub>/LTD<sub>4</sub>-receptor antagonist L-649,923, each successfully inhibited acrolein-induced hyperreactivity. Details on the selectivity and duration of action of each of the compounds used in the study are presented elsewhere (Leikauf et al. 1989).

It has been previously reported that one compound, U-60,257, prevented bronchial hyperreactivity in guinea pigs exposed to ozone (Murlas and Lee 1985). Similarly, dual inhibition of lipoxygenase and cyclooxygenase en-

zyme pathways by BW-755c attenuated ozone-induced hyperreactivity in guinea pigs (Lee and Murlas 1985) and dogs (Fabbri et al. 1985). In addition, Lee and Murlas (1985) found that a leukotriene-receptor antagonist, FPL-55712, inhibited responsiveness in guinea pigs. Subsequently, Gordon and colleagues (1988) failed to find similar effects of U-60,257 or BW-755c on toluene diisocyanate-induced hyperreactivity. In addition, initial attempts in clinical studies to attenuate antigen-induced bronchoconstriction in persons with asthma through the use of lipoxygenase inhibitors or receptor antagonists have yet to produce therapeutic results (Massicot et al. 1986; Page and Morley 1986). A possible explanation for the differences observed in guinea pigs may lie in the agents employed to induce hyperreactivity. It is conceivable that acrolein, an aldehyde, may act chemically in ways similar to ozone. Aldehydes are dominant products formed when ozone reacts via the Criegee mechanism with its primary cellular targets, the unsaturated fatty acids contained in the plasma membrane lipid bilayer. Thus, the subcellular mechanisms of toluene diisocyanate and antigens are likely to differ from these compounds. Such differences, and the determination of the underlying mechanisms mediating the effects of each agonist on smooth muscle in the airways, are likely to be useful in future studies regarding the relationships between acute airway injury and heightened bronchial reactivity.

One difficulty with these studies is the use of lavage fluid to assess eicosanoid release from the airways. First, lavage recovers a greater portion of cells from the alveolar and distal airways than from the larger airways. Thus, changes in eicosanoids in lavage could be dominated by regions of the lung with minimal participation in the regulation of bronchomotor tone. Second, eicosanoids are lipid-soluble, and given the mobility of these compounds, they may not be readily available for recovery by lavage.

#### LEUKOTRIENE EFFECTS ON MITOGENESIS OF EPITHELIAL CELLS IN HUMAN AIRWAYS

Because airway epithelial proliferation and hyperplasia are common sequelae to irritation and inflammation, and because LTC<sub>4</sub> concentrations were elevated in lavage fluid after acrolein exposure, we examined the effects of cysteinyl leukotrienes on the growth of epithelial cells in the human airways. Leukotrienes were mitogenic in a concentration-dependent manner and exhibited a structure-activity relationship, with LTC<sub>4</sub> being more potent than its sequential metabolites LTD<sub>4</sub> or LTE<sub>4</sub>. The potency of LTC<sub>4</sub> is striking, stimulating colony-forming efficiency in concentrations as low as 0.01 pM. In addition, growth was enhanced by indomethacin, which may relate, in part, to leukotriene-stimulated phospholipase or cyclooxygenase activity with forma-

tion of E-series prostaglandins that have been reported to inhibit cell growth or accelerate differentiation (Jimenez de Asua et al. 1975; Abdel-Malek et al. 1987; Baud et al. 1987).

Implicit in any comparison between *in vitro* and *in vivo* findings obtained in this study is the assumption that epithelial cells in culture retain functions that were present *in vivo* at the time of aldehyde exposure. Current cell culture methods do permit this approach, but it is important to note the possible limitations still inhibiting reliance on *in vitro* data alone. One disadvantage of analyzing arachidonic acid metabolism with isolated epithelial cells in culture is the possible effect of cellular dedifferentiation (Leikauf et al. 1988). Our approach of using primary cell cultures, rather than repeatedly passaged transformed cell lines, may serve to limit these effects. However, the loss of certain phenotypic expression (for example, cilia) is evident when these cells are grown in culture. Future studies addressing the influence of factors that control differentiation (for example, changes in the extracellular matrix) on eicosanoid biosynthesis may supply additional information on the regulation of enzyme expression in these cells.

Another disadvantage with our analysis of arachidonic acid metabolism is that epithelial cells were studied in isolation. This eliminates the detection of possible transcellular eicosanoid metabolism. For example, airway epithelial cells can metabolize  $LTA_4$ , and inflammatory cells can release this intermediate at the site of inflammation. This possible additional role for epithelial cells in response to environmental agents also awaits further study. Lastly, oxygenation of arachidonic acid is species-dependent, and the products formed by bovine and human cells are likely to differ from those produced by epithelial cells from guinea pig airways. Little information exists regarding eicosanoid metabolism by epithelial cells in guinea pigs. However, Adler and coworkers (1987) have reported that in response to the platelet-activating factor, isolated guinea pig tracheal explants release cysteinyl leukotrienes by a process dependent on the presence of an intact epithelium. Thus, conclusions about the importance of epithelial cell injury and subsequent eicosanoid metabolism must be viewed with caution. However, the results from these studies do indicate that the activation of eicosanoid metabolism in epithelial cells may be one possible outcome of aldehyde inhalation and that eicosanoid may play a role in the proliferation of airway epithelial cells.

Previously, the mitogenic effects of eicosanoids were studied in a number of human cell types, including epidermal keratinocytes (Kragballe et al. 1985) and glomerular epithelial cells (Baud et al. 1985). Cysteinyl  $LTC_4$  and  $LTD_4$  each mediated thymidine incorporation in these two cell types, and in both cases  $LTC_4$  was more potent than  $LTD_4$ .

Keratinocytes also appeared to be sensitive to  $LTB_4$ , with an estimated half-maximal dose of 0.1 pM for  $LTB_4$  and 10 pM for  $LTC_4$ . ( $LTB_4$  also produced a greater maximal effect than  $LTC_4$  in keratinocytes.) In contrast, human glomerular epithelial cells were more sensitive to  $LTC_4$  (half-maximal dose of 10 nM) than  $LTB_4$ , which had little effect on thymidine incorporation in these cells. Leukotriene  $B_4$  was also found to be mitogenic in airway epithelial cells, but less potent than  $LTC_4$  and not enhanced by indomethacin (Leikauf et al. 1991). Thus, these findings support a cell-type specificity and stereospecific mechanism of action for the 5-lipoxygenase product in mitogenesis. Growth enhancement of the airway epithelial microenvironment through lipid mediator release may function also in other epithelium (for example, rat nasal epithelium) in response to aldehyde exposure.

#### ROLE OF AIRWAY EPITHELIAL CELL INJURY AND MEDIATOR RELEASE

Within the first six hours after exposure, essentially the only significant change in airway histology was an alteration of epithelial cell structure. During this period, significant increases in eicosanoids released into lavage fluid were recorded. Epithelial cell injury with cilia aberrations and mucous granule depletion are common observations with other known inducers of bronchial hyperreactivity (Hulbert et al. 1985; Murlas and Roum 1985a; Fabbri et al. 1987). Asthma, which is characterized by bronchial hyperreactivity, has essentially two primary pathological features: epithelial cell involvement and chronic eosinophilic inflammation (Nadel 1973; Laitnen et al. 1985). Another feature common to irritants capable of inducing hyperreactivity is a high chemical reactivity with cellular macromolecules that causes a short biological half-life and a limited diffusional path length. This confines much of the irritants' toxicity to adverse events within the epithelium.

With the assumption that epithelial injury has etiologic significance in the initiation of aldehyde-induced bronchial hyperreactivity, two questions remain. The first is: Are epithelial cells themselves the source of the specific mediators released during and shortly after aldehyde exposure? Findings from epithelial cells in culture indicate that, although aldehydes can stimulate arachidonic acid release from the cellular membrane stores and activate cyclooxygenase (principally  $PGE_2$  and  $PGF_{2\alpha}$  formation) and lipoxygenase (principally 15-HETE in humans and 12-HETE formation in guinea pig and bovine cells) enzyme pathways, little 5-lipoxygenase activity was measurable in this cell type. Further analysis is needed to learn whether or not epithelial cells have other roles in 5-lipoxygenase or thromboxane product formation *in vivo* through transcellu-

lar metabolism. However, the cellular source of the major eicosanoids elevated in lavage fluid (PGF<sub>2α</sub>, TxB<sub>2</sub>, and LTC<sub>4</sub>) does not appear to be solely the epithelial cells. Thus, other cells normally present in the airways (eosinophils, macrophage, mast cells, or other leukocytes) are likely to make significant contributions to the 5-lipoxygenase products that play a role in the induction of airway hyperreactivity.

A second question is: What is the role of the subsequent infiltration of neutrophils and eosinophils? The inherently low enzymatic capability within the epithelial cell to produce cysteinyl leukotrienes would strengthen the concept that the secondary leukocyte (and monocyte) infiltration at local sites of epithelial inflammation could produce additional amounts of leukotrienes that contribute to the initiation of cell proliferation. Persistent activation of leukocytes at focal sites of injury could lead to the enhancement of a microenvironment that promotes growth of epithelial cells and thereby contributes to subsequent epithelial hyperplasia observed in patients with chronic asthma (for example, mucus gland hypertrophy). Proliferative epithelial responses subsequent to aldehyde exposure are well documented (Swenberg et al. 1980; Albert et al. 1982; Berke 1987; Edling et al. 1988; Monticello et al. 1989). In addition, enhancement of local leukotriene formation could also play a role in proliferative phases of multistage carcinogenesis by providing an environment that selectively stimulates the growth of preneoplastic cells.

In summary, inhalation of formaldehyde and acrolein can initiate a transient bronchoconstriction and sustain a persistent bronchial hyperreactivity in guinea pigs. Extending formaldehyde exposures from two to eight hours produced a greater effect than would be predicted by a simple concentration × time dose estimate. This suggests that responses induced by prolonged low-level exposures may not be detected adequately by single short (15- to 120-minute) exposures. Bronchial hyperreactivity was apparent at times preceding inflammation and was dependent on 5-lipoxygenase product formation. Although epithelial cells are affected by eicosanoids and release selected eicosanoids in response to aldehydes, they are unlikely to be the source of 5-lipoxygenase products. These products, however, are potent mitogens for human airway epithelial cells, suggesting a link between aldehyde-induced mediator release and focal inflammation with epithelial hyperplasia.

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#### ABOUT THE AUTHOR

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George D. Leikauf received his A.B. in biological sciences from the University of California at Berkeley and his M.S. and Ph.D. in environmental health sciences from New York University Medical Center. He conducted post-doctoral research at the University of California at San Francisco and is currently an Associate Professor at the University of Cincinnati Medical Center in the Department of Environmental Health, and the Department of Physiology and Biophysics. Dr. Leikauf's primary research interest is the role of epithelial injury in irritant-induced bronchial hyperreactivity.

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## ABBREVIATIONS

A23187	calcium ionophore
BSA	bovine serum albumin
Ca <sup>+2</sup>	calcium ion
CH <sub>2</sub> =CHCHO	acrolein
Cl <sup>-</sup>	chloride ion
CO <sub>2</sub>	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
dpm	disintegrations per minute
ED <sub>200</sub>	dose sufficient to double airway resistance (effective dose 200 percent)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis (beta-aminoethylether)-N,N,N'-tetraacetate
FBS	fetal bovine serum
GSEM	geometric standard error of the mean
<sup>3</sup> H	tritium
HCHO	formaldehyde
HCl	hydrochloric acid
HCO <sub>3</sub> <sup>-</sup>	bicarbonate ion
HEPA	high-efficiency particulate air (filter)
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
5-HETE	5-(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid
12-HETE	12-(S)-hydroxy-5,8,14-cis-10-trans-eicosatetraenoic acid
15-HETE	15-(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid
12-HHT	12-hydroxy-5,8,10-heptadecatrienoic acid
HPLC	high-performance liquid chromatography
H <sub>2</sub> PO <sub>4</sub>	monobasic phosphate

H <sub>3</sub> PO <sub>4</sub>	phosphoric acid	PBS	phosphate-buffered saline
K <sup>+</sup>	potassium ion	PC	phosphatidylcholine
KHBS	Krebs-Henseleit buffer solution	PE	phosphatidylcholine ethanolamine
L-649,923	an LTC <sub>4</sub> /LTD <sub>4</sub> receptor antagonist	PGB <sub>2</sub>	prostaglandin B <sub>2</sub>
L-651,392	a 5-lipoxygenase inhibitor	PGD <sub>2</sub>	prostaglandin D <sub>2</sub>
LDH	lactate dehydrogenase	PGE <sub>1</sub>	prostaglandin E <sub>1</sub>
LTA <sub>4</sub>	leukotriene A <sub>4</sub>	PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
LTB <sub>4</sub>	leukotriene B <sub>4</sub>	PGF <sub>1α</sub>	6-keto prostaglandin F <sub>1α</sub>
LTC <sub>4</sub>	leukotriene C <sub>4</sub>	PGF <sub>2α</sub>	prostaglandin F <sub>2α</sub>
LTD <sub>4</sub>	leukotriene D <sub>4</sub>	PI	phosphatidylcholine inositol
LTE <sub>4</sub>	leukotriene E <sub>4</sub>	ppb	parts per billion
Mg <sup>+2</sup>	magnesium ion	PS	phosphatidylcholine serine
N <sub>2</sub>	nitrogen	SO <sub>4</sub> <sup>-2</sup>	sulfate ion
Na <sup>+</sup>	sodium ion	sR <sub>t</sub>	specific total pulmonary resistance measured as mL × (cm H <sub>2</sub> O/[mL/sec])
NADH	beta-nicotinamide adenine dinucleotide	TxA <sub>2</sub>	thromboxane A <sub>2</sub>
NaOH	sodium hydroxide	TxB <sub>2</sub>	thromboxane B <sub>2</sub>
NH <sub>4</sub> OH	ammonium hydroxide	U-60,257	a 5-lipoxygenase inhibitor
O <sub>2</sub>	oxygen		
PA	phosphatidic acid		



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## INTRODUCTION

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In the summer of 1985, the Health Effects Institute (HEI) issued a Request for Applications (RFA 85-1) that solicited proposals for studies on the "Health Effects of Aldehydes." In response to this RFA, Dr. George D. Leikauf from the New York University Institute for Environmental Medicine submitted a proposal to HEI, entitled "Mechanisms of Aldehyde-Induced Bronchial Reactivity: Role of Airway Epithelium." After minor revisions to the proposal, the HEI Research Committee recommended funding for the study. Before starting his research in July 1986, Dr. Leikauf moved to the University of Cincinnati. Total expenditures for the three-year study were \$421,132. The Investigator's Report was received at the HEI in June 1990 and a revised report was received in January 1991. The revised report was accepted for publication by the Health Review Committee in January 1991. During the review of the Investigator's Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigator's Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigator's Report in perspective, as an aid to the sponsors of the HEI and to the public.

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## REGULATORY BACKGROUND

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The U.S. Environmental Protection Agency (EPA) sets standards for air 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.

Several changes in the Clean Air Act instituted by the 1990 Amendments to the Act deal with formaldehyde. Section 211(k) of the Act, as added by Section 219 of the 1990 Amendments, establishes a program for the use of reformulated gasoline. The program is designed, at least in part, to reduce the "emissions of toxic air pollutants" such as formaldehyde. Similarly, the "clean-fuel vehicle" program emis-

sion standards set out in Section 243, as added by the 1990 Amendments, require that certain formaldehyde emission targets be met.

Section 202(l) of the Clean Air Act, as added by Section 206 of the 1990 Amendments, requires the EPA to "complete a study of the need for, and feasibility of, controlling emissions of [certain] toxic air pollutants. . . ." Section 202(l) states that the study "shall focus on those categories of emissions that pose the greatest risk to human health or about which significant uncertainties remain. . . ." Defined as a "hazardous air pollutant" under Section 112(b) of the Act, as amended by Section 301 of the 1990 Amendments, formaldehyde is one of the three emissions specifically mentioned in Section 202(l)(1).

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## SCIENTIFIC BACKGROUND

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Aldehydes are normal constituents of the emissions from internal combustion engines that use diesel and gasoline fuels (Marnett 1988). Depending on concentration, short-term exposures to aldehydes produce effects in humans ranging from simple eye, nose, and throat irritation to increased breathing rates. Recent amendments to the Clean Air Act of 1970 (U.S. Congress 1991) address production and use of vehicles that use alternative fuels, such as methanol and ethanol. Although the ideal combustion of alcohol fuels within engines produces only carbon dioxide and water, actual combustion is an incomplete process that produces toxic aldehydes as by-products. As a result, ambient air concentrations of some aldehydes, particularly formaldehyde from methanol fuels and acetaldehyde from ethanol fuels, may increase in the near future. Gasoline and diesel engines burning conventional fuels emit formaldehyde, acrolein, and acetaldehyde and thus currently contribute to the aldehyde burden in the atmosphere.

Because of the current and the potentially increased levels of aldehydes in the ambient air, the possible adverse human health effects from chronic inhalation of these chemicals must be investigated. Studies should include the effects of inhaled aldehydes on airway reactivity, breathing patterns, lung cell functions, and the mechanisms that produce these effects. Optimally, these studies should evaluate effects produced by aldehyde concentrations relevant to current and potential levels in the environment.

The concentrations of aldehydes that are emitted into the atmosphere by motor vehicle exhaust vary with the mode of operation and the fuel. Using cruising speeds under test

conditions, gasoline-fueled vehicles can produce levels of 1.0 part per million<sup>1</sup> (ppm) formaldehyde, 0.2 ppm acrolein, and 0.1 ppm acetaldehyde (Swarin and Lipari 1983). Using ethanol-fueled vehicles in these tests, levels of formaldehyde and acetaldehyde can increase to 3.6 and 8.4 ppm, respectively (Marnett 1988). Combustion of 100% methanol fuel can produce formaldehyde concentrations that are ten-fold higher than those of conventional fuels. Humans are very sensitive to the pungent odors of aldehydes in the air and can easily detect them at concentrations as low as 0.1 ppm (Beauchamp et al. 1985; Marnett 1988). Because the present study focused on formaldehyde and acrolein, a more detailed discussion of these two aldehydes is presented.

### TOXICITY OF FORMALDEHYDE AND ACROLEIN

Formaldehyde is not only a prominent component of motor vehicle emissions, but also a major product of the chemical industry. It is used in the manufacture of a wide variety of products, including lumber, plastics, and textiles (Feinman 1988). During the past decade, human exposures to materials containing formaldehyde in homes and workplaces has received much media coverage. As a result, the potential long-term and short-term health effects related to these exposures have generated considerable debate, as well as an extensive data base for this chemical (U.S. Environmental Protection Agency 1987a).

Formaldehyde is a very water-soluble compound, and, when inhaled, is readily absorbed by the upper respiratory tract, the primary target of its irritant effects (Egle 1972). Humans exposed to 0.1 to 3 ppm formaldehyde, concentrations detectable by nose and likely to occur in many industrial and occupational settings, experience eye and respiratory tract irritation. Workers also can develop asthma symptoms due to chronic occupational exposure to aldehydes (Chan-Yeung and Lam 1986). Marbury and Krieger (1991) reviewed the findings from recent clinical studies in which humans were exposed to formaldehyde concentrations of up to 3 ppm. Although these reports indicated minimal changes in lung function as a result of exposure, evidence from some of the studies suggested that formaldehyde induced a temporary airway constriction in subjects during strenuous exercise.

Formaldehyde also has been recognized as a carcinogen in laboratory animals. Chronic exposure to 6 or 14 ppm formaldehyde produced nasal tumors in rats (Swenberg et al. 1980; Kerns et al. 1983). The fact that formaldehyde can cross-link with a variety of cell macromolecules, including

DNA, RNA, and proteins is evidence of its genotoxicity (U.S. Environmental Protection Agency 1987a). A review by Heck and colleagues (1990) reported that mutations produced by formaldehyde may be related to crosslinks formed between DNA and proteins that lead to subsequent errors in DNA replication. These errors present a plausible explanation for the observed carcinogenic effects.

Whether long-term human exposure to formaldehyde is associated with an increased risk of cancer for humans is not yet known. A Consensus Workshop on Formaldehyde (Hart et al. 1984) reported that there was insufficient evidence to classify formaldehyde as a human carcinogen. The U.S. Environmental Protection Agency (1987) and the International Agency for Research on Cancer (1987) have categorized formaldehyde as a probable human carcinogen, indicating that although there is limited evidence of carcinogenicity in humans, there is sufficient evidence of carcinogenicity in laboratory animals.

Ambient concentrations of acrolein in urban air can range from 0.002 to 0.007 ppm, but the percentage of this level contributed by motor vehicle emissions is not known (Beauchamp et al. 1985). Urban and forest fires add to these ambient levels; air near building fires can contain up to 3 ppm acrolein. Occupational exposures to acrolein include chemical manufacturing, smelting, and foundry work. The threshold limit value for acrolein is 0.1 ppm, a concentration that does not produce adverse health effects in workers who are exposed daily (American Conference of Governmental Industrial Hygienists 1990). As a respiratory irritant, acrolein is more potent than formaldehyde and penetrates more deeply into the lungs because it is less water soluble. Humans exposed to levels of 0.1 to 1.0 ppm acrolein experience extreme eye and nose irritation within a few minutes. Because of this severe reaction, there is little information from experimental studies on prolonged human exposures to acrolein.

Acrolein rapidly reacts with sulfur-containing groups that are critical for the functional integrity of many proteins and peptides, such as those in the mucus of the nose and airways (Beauchamp et al. 1985). Acrolein does not appear to react readily or rapidly with DNA, but its binding to DNA and RNA polymerases may account for its inhibitory effects on cell division. Because there is limited information about the carcinogenic effects of acrolein, the International Agency for Research on Cancer (1987) has listed it as an agent not classifiable with regard to its carcinogenicity to humans. The U.S. Environmental Protection Agency (1987b) classifies acrolein as a possible human carcinogen.

Cigarette smoke is also a significant source of exposure to formaldehyde and acrolein (Marnett 1988). Concentrations up to 12 ppm of both of these aldehydes, levels 100

<sup>1</sup> A list of abbreviations appears at the end of the Investigator's Report for your reference.

times greater than those normally present in ambient air, can be inhaled during smoking. Concentrations lower than these levels can harm the respiratory defense mechanisms that protect the lungs against infections (Astry and Jakab 1983; Witz et al. 1985).

### EFFECTS OF FORMALDEHYDE AND ACROLEIN ON LUNG FUNCTION

Pulmonary function tests can evaluate how inhaling toxic compounds, such as formaldehyde and acrolein, affects lung function. Researchers use these tests to measure a variety of parameters, such as breathing rate, volume of air breathed, the capacity to expel air rapidly from the lungs, and lung distensibility, in either human or animal subjects. Because the rate at which air flows depends on the size of the opening, pulmonary function measurements of maximal airflow and resistance to airflow reflect the diameter of airway openings (Boushey et al. 1980).

Amdur (1960) was the first investigator to study the effects of inhaled formaldehyde on lung function. She used guinea pigs to evaluate airway reactivity, which is the propensity of airways to constrict. Amdur selected these animals because they develop airway constriction in response to toxic agents more readily than other laboratory animals. She exposed the guinea pigs for one hour to formaldehyde concentrations ranging from 0.05 to 49 ppm and then measured total pulmonary resistance, which is the sum of the frictional resistance to airflow in the airways and the lung tissue (Murray 1986). These exposures produced airway constriction, a reduction in the size of airway openings that causes decreased airflow in and out of the lungs. As a result, there were statistically significant increases in total pulmonary resistance. At formaldehyde concentrations as low as 0.3 ppm, Amdur also measured decreases in the ability of the lungs to expand normally. All of these parameters rapidly returned to preexposure values within an hour after exposure completion.

Murphy and coworkers (1963) measured pulmonary function in guinea pigs exposed to 0.4 to 1.0 ppm acrolein for two hours. They noted increases in airway resistance, that is, the frictional resistance to airflow contributed by the airways alone. Like Amdur (1960), Murphy and colleagues noted a rapid return to control values within an hour after exposure cessation. Costa and coworkers (1986) reported that exposing rats to 0.4, 1.4, or 4 ppm acrolein for nine weeks produced functional changes, including a significant increase in total pulmonary resistance, principally in the 4-ppm group. This group also had severe cell damage in the region around the smallest airways.

Another method for testing the possible effects of toxic

agents in airway function is an airway challenge test. A subject first inhales a specific drug that can provoke bronchoconstriction, such as methacholine, histamine, or carbachol. Even in healthy subjects, these drugs cause the smooth muscle around the airways to constrict, thus limiting maximal airflow in and out of the lungs. Subjects with asthma or obstructive lung disease are usually more responsive, or reactive, to these drugs; they require a smaller dose of the drug to achieve the same extent of constriction exhibited by a healthy subject given the same drug. The data from this first phase provide baseline values for the effects of the drug alone. If exposure to a toxic substance increases the propensity of the airways to constrict, less of the drug will be needed to produce the same airway constriction and increase in pulmonary resistance. The changes in maximal airflow and airway resistance with increasing drug concentration can be plotted to determine a dose-response curve for the toxic agent's effects on airway reactivity.

Bronchoalveolar lavage is a useful technique for evaluating the cellular and biochemical events related to airway reactivity and alterations in breathing patterns caused by inhaling toxic substances. In this procedure, a small volume of saline is instilled into the lungs via the trachea and then promptly removed. This lavage fluid contains cells and factors representative of those in the airways and alveolar spaces. Bronchoalveolar lavage can be performed at different time points before, during, and after an exposure to determine the profile of changes associated with an exposure. For example, the numbers and types of cells in the airways and alveolar spaces can increase in healthy individuals after inhaling toxicants, such as ozone (Seltzer et al. 1986; Koren et al. 1989), as well as in persons with asthma (O'Byrne et al. 1987). Influxes of cells, such as neutrophils, macrophages, and lymphocytes, and markers of tissue injury can be evaluated by bronchoalveolar lavage.

### MECHANISMS OF AIRWAY REACTIVITY CAUSED BY INHALED TOXICANTS

The airway reactivity that occurs in response to inhaled toxicants is linked to the numerous sensory nerve endings, or receptors, in the lungs. During normal breathing, impulses from the the brain regulate the size, or tone, of airway openings via nerve pathways connected to smooth muscles surrounding the airways. When formaldehyde or acrolein is inhaled, these sensory nerve receptors in the airways are stimulated to send messages to the brain. The brain, in turn, sends messages to the lungs that cause changes in breathing patterns and constriction of the airways. After aldehyde inhalation, the decreases in maximal airflow, which are

reported as increases in airflow resistance, are indications of these airway alterations.

A response called reflex bronchoconstriction also may occur when noxious fumes or gases, such as aldehydes, are inhaled. This constrictive response by the airways can result without the involvement of the brain and spinal cord (Sant'Ambrogio and Sant'Ambrogio 1991). In reflex bronchoconstriction, impulses from the sensory receptors do not reach the brain; rather, they complete a reflex loop entirely within the lungs, causing smooth muscle contraction and airway narrowing.

Murphy and associates (1963) proposed that reflex bronchoconstriction was the mechanism for the increased airway reactivity that they observed in guinea pigs after acrolein inhalation. These investigators suggested that acrolein bound to sensory receptors and mediated stimulation of nerves and an increase in airway resistance via the neurotransmitter acetylcholine. Murphy and colleagues based this proposal on experiments in which they pre-treated guinea pigs with atropine, an acetylcholine antagonist, and blocked the increase in airway resistance. They concluded that the constrictive effects of acrolein were mediated through nerves connected to bronchial musculature that used acetylcholine.

Certain parallels exist between the airway reactivity observed after the inhalation of toxicants and asthma. Researchers study both patterns of lung responses to understand better their similarities and dissimilarities. Asthma is characterized by episodic shortness of breath, wheezing, and cough, associated with an increased airway resistance and airway constriction, or hyperreactivity. These symptoms occur in response to many unrelated stimuli, including allergens, air pollutants, cold air, exercise, and certain provocative drugs, such as methacholine and histamine (Larsen 1991). As described earlier, bronchoalveolar lavage studies show that an influx of cells into the lungs is a common denominator for both of these airway responses, but the types of cells differ. Toxicant inhalation produces influxes of macrophages and neutrophils, whereas asthma usually involves eosinophils and lymphocytes.

#### **EICOSANOIDS, AIRWAY REACTIVITY, AND INFLAMMATION**

The biochemical and cellular factors that lead to airway reactivity, either in the case of toxicant inhalation or asthma, are currently under intense study (Barnes 1986; Drazen and Austen 1986; O'Byrne et al. 1987; Shore et al. 1989; Holtzman 1991). Research indicates that inflammation as well as airway reactivity plays an important role in some types of airway obstruction, particularly in asthma.

The metabolites of arachidonic acid, a fatty acid that is a component of cell membranes, are a potential common link for these two events. These metabolites, broadly termed eicosanoids, appear to be increasingly important in the airway reactivity and the cell influxes observed in response to inhaled toxicants and in asthma.

Controversy persists about whether the eicosanoids associated with airway reactivity originate from the initial damage to airway epithelial cells or from the cell influx that appears later. Definitive evidence shows that both epithelial cells and the cells that appear later can produce eicosanoids (Holtzman 1991). Regardless of the cell source, injury or lysis of membranes releases arachidonic acid from the cell membranes via the action of the enzyme phospholipase A<sub>2</sub>. The ensuing sequence of reactions between arachidonic acid and the enzymes cyclooxygenase and 5-lipoxygenase results in the formation of eicosanoids. Three broad groups within the eicosanoid family are prostaglandins and thromboxanes, which originate from the cyclooxygenase pathway, and leukotrienes, which originate from the lipoxygenase pathway. Eicosanoids can produce a wide spectrum of changes in the lungs, including effects on airway and pulmonary vascular smooth muscle, mucous glands, and bronchial epithelial and pulmonary endothelial cells. Eicosanoids also can act as mediators for inflammatory influxes into the lungs. The leukotrienes, one group of eicosanoids, have been reported to induce contractile activity in lung tissue both *in vitro* and *in vivo* (Drazen and Austen 1987). An extensive review of the *in vivo* and *in vitro* activities of eicosanoids in the lungs of diverse mammalian species is presented by Shore and colleagues (1989).

Although eicosanoids are probably involved in airway reactivity, the point at which these metabolites contribute to the response is unknown. Results from human and animal studies suggest that epithelial damage correlates with the airway reactivity observed after exposure to agents known to damage the airway mucosa (Laitinen et al. 1985). For example, healthy humans exposed to ozone or nitrogen dioxide exhibit increased airway reactivity in response to an aerosol of histamine (Golden et al. 1978). Because aldehydes are produced when ozone reacts with the polyunsaturated fatty acids in cell membranes (Mustafa and Tierney 1978), a mutual mechanism for the airway reactivity caused by inhaled aldehydes and by ozone may exist.

The specific eicosanoids or group of eicosanoids that may be responsible for airway reactivity and inflammation caused by inhaled toxicants is under investigation. In one experiment, treating dogs with the drug indomethacin before a two-hour exposure to 3 ppm ozone prevented an increase in the airway reactivity to inhaled acetylcholine (O'Byrne et al. 1984). Indomethacin treatment blocked the

synthesis of the cyclooxygenase products prostaglandins and thromboxanes, but it did not inhibit the recruitment of neutrophils into the airways. The results suggest that the influx may be mediated by leukotrienes, the synthesis of which is not affected by this drug. The authors suggest that cyclooxygenase products are responsible for airway reactivity by affecting either receptors in the airways, the airway epithelium, or the airway smooth muscle itself.

Mechanisms for the responses to inhaled toxic agents may differ greatly from those of asthma. However, asthma may provide instructive clues for the airway reactivity caused by toxic exposures. One current direction in the treatment of asthma is modifying airway reactivity by pharmacologic intervention of eicosanoid synthesis. The aim of a variety of new drugs is either to inhibit the synthesis of eicosanoids at selected points along their synthesis pathways or to block their receptors in the lungs. Studies in patients with exercise-induced asthma demonstrate that pretreatment with leukotriene receptor antagonists attenuate bronchoconstriction after exercise (Manning et al. 1990). Israel and colleagues (1990) reported that a 5-lipoxygenase inhibitor significantly reduced the response of asthma patients to cold, dry air, indicating that lipoxygenase products were involved in the response. Using drugs to modulate eicosanoid synthesis or to block specific receptors may help determine the mechanisms of airway reactivity caused by formaldehyde and acrolein.

In summary, inhaling toxic substances alters airway reactivity and causes influxes of inflammatory cells into the airways; however, these changes do not occur simultaneously. Increased airway reactivity and resistance usually begin immediately and subside quickly, whereas cell influxes occur later. This sequence presents several questions regarding the underlying mechanisms for airway reactivity in response to inhaled aldehydes. Is direct damage to the epithelial or other cells in the airways the first event in airway reactivity? What is the role of receptors in the response? What is the role of inflammatory cell influxes in airway reactivity and tissue injury? Do eicosanoids contribute to airway reactivity? If so, what is their origin and mechanism of action? These and many related questions continue to foster an active area of pulmonary research.

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## JUSTIFICATION FOR THE STUDY

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In 1985, the HEI was interested in supporting studies to evaluate the health effects of aldehydes at concentrations relevant to those present in automotive emissions. Part of this concern stemmed from the projected use of methanol as a fuel in motor vehicles. Several aldehydes, including

formaldehyde, acrolein, and acetaldehyde, are already present in the emissions from gasoline and diesel engines. However, the emissions from methanol-burning engines may significantly increase the levels of aldehydes produced, particularly formaldehyde. The HEI wanted to support studies to determine the individual and combined effects of these aldehydes and their potential interactions with carbon particles. Three specific areas of interest outlined in the RFA were (1) the effects of aldehydes on susceptibility to respiratory infections; (2) the interactive effects of aldehydes in producing cancer; and (3) human studies with aldehydes and the development of new animal models to evaluate hypersensitivity to formaldehyde.

Dr. Leikauf proposed to evaluate the relative potencies of acrolein, formaldehyde, and acetaldehyde to induce airway reactivity in guinea pigs. He wanted to use aldehyde concentrations similar to those that could occur in the environment but that had not yet received extensive study. The main focus of Dr. Leikauf's proposal was to examine whether aldehyde-induced airway reactivity was due to epithelial damage or to inflammatory reactions in the lungs. He also proposed inhalation exposures with aldehydes adsorbed to carbon particles. In support of his application, Dr. Leikauf cited his previous work in the area of airway epithelial cell biology and his extensive experience in pulmonary physiology related to the response of the airways to inhaled pollutants, such as acid aerosols and ozone.

The Research Committee was strongly enthusiastic about the proposal submitted by Dr. Leikauf, but was concerned that the levels of aldehydes proposed for the *in vivo* studies would be too low to produce changes in airway reactivity. If there were no positive results using these low concentrations, subsequent *in vitro* studies using the same concentrations would not be needed. The Committee, therefore, suggested that Dr. Leikauf provide positive results from the *in vivo* studies before proceeding to the *in vitro* studies. In accordance with this suggestion, Dr. Leikauf revised his proposal, and it was, in turn, approved by the Research Committee.

During the course of his study, Dr. Leikauf had the opportunity to collaborate with Dr. Roland Grafström, another HEI investigator funded under the same RFA. These studies were supported by the HEI and were conducted in Dr. Grafström's laboratories at the Karolinska Institute in Sweden. They resulted in the publication of several manuscripts related to the *in vitro* effects of aldehydes. Several of these manuscripts are listed in the Publications Resulting from This Research section of the report.

It also should be noted that, during the course of the study, Dr. Leikauf and the Research Committee agreed to delete the proposed inhalation exposures with aldehydes

adsorbed to carbon particles. In addition, they agreed that Dr. Leikauf should replace the proposed acetaldehyde studies with studies on longer exposures to lower levels of formaldehyde.

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## OBJECTIVES AND STUDY DESIGN

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This study was undertaken to investigate the relative capacities of the aldehydes acrolein and formaldehyde to irritate the respiratory tract after exposures to environmentally relevant concentrations. The exposures were followed by analyses of the functional changes in the airways and in the morphology and biochemistry of airway epithelial cells.

The specific aims of the *in vivo* studies were to determine the responses of guinea pigs to inhaled acrolein and formaldehyde by measuring (1) total pulmonary resistance and the airway reactivity produced by intravenous infusion of acetylcholine, a drug that causes airways to constrict; (2) infiltration of neutrophils and other inflammatory cells into the airways; (3) changes in levels of eicosanoids in bronchoalveolar lavage fluid; and (4) the effects of inhibition of eicosanoid metabolism on airway reactivity produced by inhalation of acrolein or formaldehyde.

For specific aim 1, guinea pigs were exposed to acrolein concentrations ranging from 0.3 to 1.3 ppm and formaldehyde concentrations ranging from 0.1 to 31 ppm. Although there are several methods to measure airflow resistance in the respiratory tract, Dr. Leikauf defined his measurement as total specific pulmonary resistance, which was abbreviated to specific resistance ( $sR_t$ ). He measured specific resistance at time points 5 to 60 minutes after the exposures ended. He also evaluated increases in airway reactivity in response to the bronchoconstrictive drug acetylcholine that were caused by exposure to acrolein or formaldehyde. Before an aldehyde exposure, Dr. Leikauf delivered increasing doses of acetylcholine intravenously and determined the effective dose of acetylcholine required to double the baseline value for specific resistance ( $ED_{200}$ ). He then repeated this procedure 1, 2, 6, and 24 hours after the end of the exposures and determined the concentration of acetylcholine required to reach this same value of specific resistance.

For specific aims 2 and 3, bronchoalveolar lavage samples were taken at selected times after acrolein inhalation to determine patterns of influxes of inflammatory cells and levels of specific eicosanoids in the lavage fluid. For specific aim 4, guinea pigs were treated before acrolein exposure with either a receptor antagonist for leukotrienes  $C_4$  and  $D_4$  or one of two 5-lipoxygenase inhibitors designed to inhibit leukotriene synthesis.

The specific aims of the *in vitro* studies were to determine (1) the effects of formaldehyde and acrolein on eicosanoid metabolism in tracheobronchial epithelial cells; and (2) whether an increase in the release of eicosanoids from tracheobronchial epithelial cells caused by exposure to either formaldehyde or acrolein stimulates proliferation of epithelial cells.

Both bovine tracheal and human airway epithelial cells were exposed in the *in vitro* experiments. Only acrolein was used for these studies. For specific aim 1, cells were exposed to acrolein concentrations up to 100  $\mu$ M, and the effects on eicosanoid metabolism and profiles of the eicosanoids released into the media were evaluated. For specific aim 2, the mitogenic effects of eicosanoids were evaluated by determining their effects on colony-forming efficiency. In this assay, known numbers of cells were plated onto a tissue culture dish, and the number of individual cells that successfully gave rise to cell colonies was tallied. Increases in the number of colonies formed following a specific treatment suggested that the treatment stimulated the cell division.

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## TECHNICAL EVALUATION

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### ATTAINMENT OF STUDY OBJECTIVES

The specific aims were addressed successfully and the findings of this study are definitive, well documented, and presented clearly.

### METHODS AND STUDY DESIGN

This study encompassed a wide variety of experimental approaches examining the effects of aldehydes on the lungs. The design was comprehensive, and, overall, well-conceived and well-executed. An unfortunate drawback of the experimental design was that, although both acrolein and formaldehyde were used for many of the *in vivo* inhalation studies, only acrolein was used for the bronchoalveolar lavage, the *in vivo* receptor antagonist, and the leukotriene inhibitor studies. All of the *in vitro* studies were done with acrolein alone. Although a large data base exists for the *in vitro* effects of formaldehyde, it would have been useful to compare the results for both acrolein and formaldehyde in Dr. Leikauf's experimental systems. Dr. Leikauf acknowledged that he simply did not have adequate time during the funded period to complete these matching studies.

## STATISTICAL METHODS

The data from this study were analyzed properly and presented clearly. Overall, the results from the five major groups of experiments were very consistent, suggesting that they would be readily reproducible.

For the studies of pulmonary resistance and airway reactivity to inhaled acrolein and formaldehyde, Dr. Leikauf correctly analyzed each endpoint and dose separately as a repeated measures of analysis of variance (ANOVA). The data for the bronchoalveolar lavage studies were appropriately analyzed using a one-way ANOVA with the time when animals were killed as a factor. For the studies on the effects of eicosanoid antagonists and inhibitors, Dr. Leikauf analyzed each treatment group by a repeated measures ANOVA. Alternatively, he could have analyzed the entire data set presented in Figure 13 as a four-group repeated measures ANOVA. Paired *t* tests were required and used to analyze the data from the studies of the effects of acrolein on epithelial cell metabolism of eicosanoids. Although this same analysis method was used for the data on the mitogenic effects of eicosanoids, a one-way ANOVA would have been appropriate as well.

## RESULTS AND INTERPRETATION

A notable new finding by Dr. Leikauf was that the *in vivo* exposures of guinea pigs to either acrolein or formaldehyde produced increases in airway reactivity that persisted for up to 24 hours after the end of an exposure. He reported that the ED<sub>200</sub>, the concentration of intravenous acetylcholine required to double the baseline value of specific resistance, was significantly less than preexposure values at 1, 2, 6, and 24 hours after the end of exposure. These results indicate that formaldehyde and acrolein produced a prolonged effect on airway reactivity that had not been detected previously.

Dr. Leikauf's measurements of specific resistance in response to either formaldehyde or acrolein exposure confirmed the findings of earlier investigators (Amdur 1960; Murphy et al. 1963). They reported that their measurements of resistance initially increased and then rapidly returned to baseline levels within an hour after the exposure had ended. Dr. Leikauf reported the same pattern for his specific resistance values. From his experiments, Dr. Leikauf also concluded that acrolein was 10 to 30 times more potent than formaldehyde in inducing increases in immediate airway reactivity.

Another noteworthy finding of this study was that formaldehyde exposure produced increases in specific resistance that were greater than the predictions based on estimates of

total exposure dose. Total dose is calculated as the simple product of the concentration multiplied by the duration of exposure. For example, an eight-hour exposure to 1 ppm formaldehyde (8 ppm•hours) produced changes in specific resistance comparable to 2 hour exposures to 10 ppm (20 ppm•hours) or 30 ppm (60 ppm•hours). Similarly, a 1-ppm formaldehyde exposure for eight hours (8 ppm•hours) produced an increase in the ED<sub>200</sub> measurement of airway reactivity equal to that of a two-hour exposure to 30 ppm (60 ppm•hours). Prolonging the exposure period at the 1-ppm concentration resulted in a disproportionate lowering of the total dose of formaldehyde needed to elicit the measured changes.

Dr. Leikauf suggested that one explanation for this result is that water-soluble formaldehyde may saturate its own binding sites, such as sensory nerve receptors, in the upper respiratory tract during the longer exposure period. Formaldehyde gas then could penetrate to potential binding sites in the lower airways and induce its prolonged effects on airway reactivity. This proposal is consistent with earlier observations that a similar dissociation exists between the effects of another water-soluble gas, sulfur dioxide, and its total exposure dose (Amdur 1991). These observations also suggest that other absorption factors, such as gas concentration, exposure duration, and nasal versus oral breathing patterns, may be important as well.

Analyses of the bronchoalveolar lavage fluid after *in vivo* exposures to acrolein indicated increases in the levels of certain eicosanoids, some of which have bronchoconstrictive effects (Shore et al. 1989). These eicosanoids included the cyclooxygenase products, prostaglandin F<sub>2α</sub> and thromboxane A<sub>2</sub>, and the 5-lipoxygenase product leukotriene C<sub>4</sub>. These eicosanoids were present immediately after exposure, whereas a significant increase in the number of inflammatory cells, including neutrophils, pulmonary macrophages, and monocytes, did not occur until 24 hours later.

The results of these bronchoalveolar lavage studies demonstrated that there was no temporal relationship between airway reactivity and an influx of neutrophils, eosinophils, and basophils. The observed changes in specific resistance occurred too rapidly to be attributable to an infiltration of inflammatory cells into the airways. A similar delay between airway reactivity and neutrophil influx has been reported in guinea pigs exposed to 3 ppm ozone for two hours (Murlas and Roum 1985). Dr. Leikauf suggested that eicosanoids were an important component of the immediate airway reactivity response that he observed in his studies. However, inflammatory cells were an unlikely source.

Pretreatment of the guinea pigs before acrolein exposure with either the receptor antagonist for leukotrienes C<sub>4</sub> and

D<sub>4</sub> or either of the two 5-lipoxygenase inhibitors decreased the extent of airway response measured by specific resistance and by airway reactivity to intravenous acetylcholine compared with no pretreatment. Although Dr. Leikauf demonstrated that these drugs were effective suppressors of acrolein-induced airway reactivity, he did not measure whether pretreatment alone with these drugs altered eicosanoid levels in the bronchoalveolar lavage fluid. These data would have been useful to substantiate the conclusion that eicosanoids were important mediators for the airway reactivity observed in these experiments. In addition, the effects of the receptor antagonist for leukotrienes C<sub>4</sub> and D<sub>4</sub> and the lipoxygenase inhibitors on airway reactivity without prior acrolein exposure were evaluated at only one time point. It would have been interesting to determine whether these drugs altered the baseline airway reactivity to acetylcholine for periods of up to 24 hours.

The *in vitro* studies with human tracheobronchial or bovine tracheal epithelial cells focused only on the effects of acrolein. Treatment of confluent monolayers of bovine tracheal epithelial cells with acrolein or the calcium ionophore A23187 significantly increased the release of radiolabeled arachidonic acid from the cells into the media. In these experiments, the calcium ionophore, a compound that causes an increase in intracellular calcium levels, was a positive control for an increase in the activity of phospholipase A<sub>2</sub> and the subsequent release of arachidonic acid from cell membrane phospholipids (Holtzman 1991).

One drawback of the presentation and discussion of the *in vitro* studies was that Dr. Leikauf did not correlate the concentrations of acrolein used for the *in vitro* studies with the doses inhaled by the guinea pigs during the *in vivo* studies. He also did not present a rationale for the selection of the specific concentrations used for these *in vitro* experiments. Finally, Dr. Leikauf did not explain why he did not choose guinea pig tracheal epithelial cells for his *in vitro* studies. He elected instead to use human and bovine cells, which complicated correlation of the *in vivo* acrolein studies with the *in vitro* results.

The profile of eicosanoids released by the airway epithelial cells after *in vitro* acrolein exposures did not match the eicosanoids found in the bronchoalveolar lavage fluid after an *in vivo* exposure. Analyses of the cell culture media showed that the predominant eicosanoid released was prostaglandin E<sub>2</sub>, whereas the lavage fluid contained predominantly prostaglandin F<sub>2α</sub>, thromboxane A<sub>2</sub>, and leukotriene C<sub>4</sub>. These findings suggest that the eicosanoids released from epithelial cells after acrolein exposure may not be responsible for the changes in airway reactivity. Some investigators are not convinced that damage to pulmonary epithelial cells triggers the release of the mediators for airway reactivity (Holtzman 1991). This doubt is based on an absence of evidence linking injury to lung epithelial cells

with an increase in phospholipase activity and a subsequent increase in eicosanoid levels.

Experiments on the effects of leukotrienes on human airway epithelial cell growth *in vitro* produced some surprising results. Leukotrienes, particularly leukotriene C<sub>4</sub>, were very effective stimuli for colony-forming efficiency at very low concentrations. Not only were levels of leukotriene C<sub>4</sub> increased in the lavage fluid of acrolein-exposed guinea pigs, but this eicosanoid also proved to be a powerful growth factor *in vitro*, producing a threefold increase in colony-forming efficiency. These results suggest that eicosanoids may be involved in epithelial hyperplasia, and possibly carcinogenesis, by stimulating the division and growth of preneoplastic cells. However, other *in vitro* experiments indicated that the airway epithelial cells were not the source of the lipoxygenase products observed in the bronchoalveolar lavage fluid after the *in vivo* exposures.

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#### REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

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The Clean Air Act of 1990 includes provisions for the use of clean alternative fuels, such as methanol and ethanol, and for vehicles that will burn these fuels (United States Congress 1991). As discussed earlier, this use implies an increase in the ambient levels of aldehydes, particularly formaldehyde and acetaldehyde, from the combustion of methanol and ethanol, respectively. The potential for aldehydes such as formaldehyde and acrolein to produce the persistent airway reactivity in guinea pigs that was reported by Dr. Leikauf, has important human health implications. This altered airway reactivity is particularly relevant to people with asthma and other susceptible individuals who may be more responsive to aldehyde inhalation. The results of this study emphasize the need for a clear understanding of the impact of inhaled aldehydes not only on healthy humans, but the young, the old, and those with existing lung disease.

Dr. Leikauf proposed one mechanism for the persistent airway reactivity that he observed after longer exposures to lower concentrations of formaldehyde. He reported that the longer exposures produced changes in airway reactivity that were not predictable, based on the total dose calculated as the product of the gas concentration multiplied by the duration of the exposure. He suggested that saturation of the formaldehyde binding sites in the upper respiratory tract during longer exposures to the 1-ppm concentration allowed the gas to penetrate to the lower airways and to cause the prolonged reactivity. His proposal is consistent with the reported effects of another water-soluble gas, sulfur dioxide, and with the roles that other absorption factors may play (Amdur 1991). This hypothesis has important implications

for healthy individuals as well as susceptible populations. Many individuals could be exposed to low levels of formaldehyde for prolonged periods as a result of being outdoors, living in a home built with formaldehyde construction materials, or working in certain occupational settings. The hypothesis that deeper formaldehyde penetration occurs during longer exposures to lower concentrations deserves further investigation as a mechanism for the observed persistent airway reactivity.

The contribution of the upper airways to the measured airway responses of guinea pigs to the inhaled aldehydes should receive further evaluation. For example, 75% to 80% of the total pulmonary resistance resides in the upper airways in laboratory rats (DiMaria et al. 1987). From the data presented by Dr. Leikauf, the fraction of the changes attributable to the upper airways of guinea pigs is not known. Future experiments could measure changes in pulmonary resistance in intubated or tracheostomized animals to verify that the observed changes in pulmonary resistance occur when the upper airways are bypassed.

Finally, the results indicate that leukotrienes are important for the airway reactivity induced by acrolein and formaldehyde exposure, but the sources of these eicosanoids remain unknown. Dr. Leikauf suggests that, rather than the epithelial cells, mast cells, eosinophils or cells that constitute the airways may be the source of the leukotrienes. The actual source of these mediators should be the focus of future animal studies and possibly experiments with human subjects.

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## CONCLUSIONS

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A major aim of this study was to evaluate and compare the irritant potential of two aldehydes found in motor vehicle emissions, formaldehyde and acrolein. Dr. Leikauf reported that, at levels comparable to those encountered by humans in the environment, both formaldehyde and acrolein produced not only transient increases in total pulmonary resistance, as previously reported, but also more persistent changes in airway reactivity. He also reported that the total dose of formaldehyde did not correlate with the measured airway responses. Dr. Leikauf noted that longer exposures to lower concentrations produced changes in airway reactivity comparable to those produced by shorter exposures to higher formaldehyde concentrations. From his data, he also concluded that at the same concentration, acrolein is approximately 10 times more potent than formaldehyde.

Dr. Leikauf observed that the changes in total pulmonary resistance and airway reactivity induced by acrolein and formaldehyde occurred too rapidly to be attributable to an infiltration of inflammatory cells into the airways. He pro-

posed that these responses were the likely result of the immediate release of eicosanoids. However, the source and identity of these biologically active compounds remains to be determined.

In summary, this study yielded significant findings related to the physiological and biochemical effects of formaldehyde and acrolein. The results contribute to our understanding of airway reactivity and demonstrate the capacity of these aldehydes, which are important oxidative pollutants in ambient air, to cause persistent changes in lung function. These findings have potential human public health significance, especially for persons with asthma and other susceptible individuals who have airways that are particularly responsive to inhaled toxicants. These data are especially relevant in light of a potential increase in the ambient concentrations of aldehydes if alcohols, particularly methanol, become significant as alternative fuels for motor vehicles.

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Title	Publication Date
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## **TWO-SECTOR FINANCIAL SUPPORT**

The Institute receives half of its funds from the United States government through the Environmental Protection Agency, and half from the automotive industry. Twenty-eight domestic and foreign manufacturers of vehicles or engines contribute to the Institute's budget in shares proportionate to the number of vehicles or engines that they sell in the United States.

## **THE HEI RESEARCH PROCESS**

The Institute is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is developed by the Health Research Committee, a multidisciplinary group of scientists knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific commu-

nity that research proposals are being solicited on a specific topic. Applications are reviewed first for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to HEI's mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, who review the merits of the study as well as the procedures, independence, and quality of the selection process.

## **THE HEI REVIEW PROCESS**

When a study is completed, a final report authored by the investigator(s) is reviewed by the Health Review Committee. The Health Review Committee has no role either in the review of applications or in the selection of projects and investigators for funding. Members are also expert scientists representing a broad range of experience in environmental health sciences. The Committee assesses the scientific quality of each study and evaluates its contribution to unresolved scientific questions.

Each Investigator's Report is peer-reviewed, generally by a biostatistician and three outside technical reviewers chosen by the Review Committee. At one of its regularly scheduled meetings, the Review Committee discusses the Investigator's Report. The comments of the Committee and the peer reviewers are sent to the investigator, and he or she is asked to respond to those comments and, if necessary, revise the report. The Review Committee then prepares its Commentary, which includes a general background on the study, a technical evaluation of the work, a discussion of the remaining uncertainties and areas for future research, and implications of the findings for public health. After evaluation by the HEI Board of Directors, the HEI Research Report, which includes the Investigator's Report and the Review Committee's Commentary, is published in monograph form. The Research Reports are made available to the sponsors, the public, and many scientific and medical libraries, and are registered with NTIS, MEDLINE, and Chemical Abstracts.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing of the release of an HEI Research Report is tailored to ensure that it does not interfere with the journal publication process.

**HEI** HEALTH EFFECTS INSTITUTE

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