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Effects of Formaldehyde on Xenotransplanted Human Respiratory Epithelium

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

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HEI Statement

Synopsis of Research Report Number 51

Effects of Formaldehyde on Human Airway Epithelial Cells Exposed in a Novel Culture System

BACKGROUND

Whether or not the air pollutant formaldehyde is injurious to human health is controversial. Results from human epidemiological studies, animal inhalation studies, and cell culture assays suggest that formaldehyde has the potential to cause adverse human health effects, including cancer. Based on these findings, the 1990 Amendments to the Clean Air Act specifically defined formaldehyde as a toxic air pollutant.

Motor vehicle emissions currently contain formaldehyde. An increased use of alcohols, particularly methanol, as alternative fuels and in fuel blends, may increase outdoor levels of formaldehyde because their combustion yields greater formaldehyde emissions than conventional fuel combustion. Whether such emissions will increase the risk of cancer for humans is unknown.

Cells lining the airways (epithelial cells) are a frequent site of cancer origin in humans. This study, sponsored by the Health Effects Institute, employed a novel exposure system to explore the capacity of formaldehyde to cause cancerous changes in human epithelial cells. This exposure system provided special growth conditions for the cells within a laboratory animal (in vivo) that closely mimicked the cells' usual environment in the human trachea.

APPROACH

Dr. Klein-Szanto and colleagues obtained autopsy samples from human infant airways and from adult nasal tissue. They propagated the epithelial cells in culture and inoculated them into isolated segments of rat tracheas from which the epithelium had been removed. The tracheas then were placed under the skin of mice to allow the cells to attach and grow in the tracheas. Unlike most other cell culture systems, this exposure system provided a support structure similar to that of the human trachea, as well as many substances important for cell growth. Another advantage was that cells from one tissue sample were propagated and used for many experiments; this reduced the variability caused by using cells from different samples for different experiments. For the exposures, the investigators inserted into the tracheas small silicon tubes containing different amounts of powdered formaldehyde. After 2, 4, 8, and 16 weeks, they evaluated the epithelial cells' appearance and determined the formaldehyde dose that produced the most cell changes. They also exposed the cells to formaldehyde alone and in combination with a potent carcinogen (a metabolite of benzo[*a*]pyrene) for 6 or 12 months and looked for cell changes suggesting cancer.

RESULTS AND IMPLICATIONS

The investigators demonstrated that, unlike other cell culture systems, their in vivo cell culture system was suitable for prolonged maintenance and exposure of human epithelial cells. However, technical difficulties compromised completion of all of the experiments using cells from all of the samples and, thus, precluded a broader interpretation of the results. The investigators reported that none of the formaldehyde exposures, either alone or in combination with the benzo[*a*]pyrene metabolite, produced any cancerous changes in the epithelial cells in their exposure system. The cells exhibited changes indicating injury, increased replication, and repair; however, the irritant effects of the silicon tube alone on these changes were not defined clearly. Because the benzo[*a*]pyrene metabolite, the putative control for this system, did not produce any cancerous changes in the human epithelial cells, the question remains whether or not any carcinogen can elicit cancerous changes within this system. Another important question is whether the response of infant cells to formaldehyde is similar to that of adult cells.

To maximize this system's potential for future studies with other pollutants, investigators should clarify the differences in response characteristics between infant and adult epithelial cells, better define the relationships among the cells from the three species (human, rat, and mouse), and include other analytical approaches, such as molecular biology techniques. Such studies are worthwhile because exposure systems using human cells can yield results relevant to the human health effects caused by toxic and potentially carcinogenic environmental pollutants.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1986 to 1990. This study was conducted by Dr. Andres J.P. Klein-Szanto and associates at the Fox Chase Cancer Center at the Institute for Cancer Research. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

A laboratory animal model that permits the exposure of xenotransplanted human respiratory epithelium to formaldehyde was used to study the effects of formaldehyde alone or in combination with the ultimate carcinogenic metabolite of benzo[*a*]pyrene, benzo[*a*]pyrene diol epoxide. Epithelial cells obtained from autopsies of full-term human fetuses or infants less than one year old were isolated, amplified in primary cultures, and then inoculated into rat tracheas from which the epithelial layer had been removed. These tracheas then were sealed and transplanted subcutaneously into irradiated athymic nude mice. Four weeks after transplantation, the tracheal lumen was completely covered by epithelium, most of which was of the mucociliary respiratory type. At this stage, tracheal transplants containing tracheobronchial epithelium from 20 different human infant donors were exposed to silastic devices containing 0, 0.5, 1, or 2 mg of formaldehyde. The tracheal transplants were examined histologically 2, 4, 8, or 16 weeks after transplantation. Before being killed, all animals were injected with a single pulse of tritiated thymidine. Important epithelial alterations were seen in the transplants treated with formaldehyde, with a maximum effect visible two weeks after exposure. In most cases, the highest dose of 2 mg produced numerous areas of epithelial erosion and inflammation; however, this effect was not as evident with the lower doses. All doses produced areas of hyperplastic epithelium alternating with areas of atrophic epithelium. Although the differences in predominance of different types of epithelium were not clearly dependent on dose, the labeling index showed dose dependence between two and four weeks after the initiation of exposure. The maximum mean labeling index was three to four times higher than normal, although in some focal hyperplastic-metaplastic lesions the labeling index increased up to 20 times. These studies show that formaldehyde, although toxic at higher doses, is able to elicit at lower doses a proliferative response of the human infant tracheobronchial epithelium that is not preceded by a massive toxic effect.

Similar studies were performed using xenotransplanted human adult nasal respiratory epithelium (Study 2). The response pattern was very similar to that of the xenotransplanted human tracheobronchial epithelium from human infants (Study 1).

In Study 3, using cells obtained from 11 human infant tracheobronchial epithelia, the formaldehyde applied simultaneously or sequentially with benzo[*a*]pyrene diol epoxide did not induce epithelial alterations different from those observed with formaldehyde treatments alone. This indicated that under the doses and exposure conditions used in this study, especially the time frames, which could not exceed the life span of the nude mice (12 months), it was not possible to observe any cocarcinogenic or promoting effects of formaldehyde on the human infant tracheobronchial epithelium.

INTRODUCTION

An important objective of biomedical research is to advance further our understanding of the mechanisms of human disease in order to implement more effective preventative measures and to improve therapeutic methods. Toward this end, a number of experimental models designed to approximate the intact human subject have been developed. The most widely used and sensible approach to studying the general mechanisms of noxious agents, which are basically the same for most mammals, has been the development of experimental models using laboratory rodents. However, because of the metabolic peculiarities of different mammalian species and because of immunological, genetic, and other differences, it is often difficult to extrapolate reliably from the laboratory animals to humans. This is especially a problem in the fields of chemical carcinogenesis and toxicology, in which some substances are apparently innocuous or less effective to some species and very toxic or carcinogenic to others (Albert 1968; Kennedy and Little 1979). A number of experimental models using human tissues have been developed to evaluate adequately the susceptibility of human tissues and compare it to that of other

This Investigators' Report is one part of the Health Effects Institute Research Report Number 51, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Andres J.P. Klein-Szanto, Fox Chase Cancer Center, Institute for Cancer Research, 7701 Burholme Avenue, Philadelphia, PA 19111.

mammals. Several investigators have employed organ cultures of human tissues, such as lung, colon, pancreas, and esophagus, to study the effects and metabolism of polycyclic aromatic hydrocarbons (Webber 1988). Other studies using human cell cultures have demonstrated malignant transformation after in vitro treatment with chemical carcinogens. The most recent approach has been the in vivo exposure of xenotransplanted human tissues to xenobiotics. The availability of immunosuppressed athymic nude mice, which usually do not reject transplanted normal human cells (Reed and Manning 1978), has made this approach possible.

Several laboratories have shown that human bronchus can be transplanted to nude mice and maintained subcutaneously for many months (Okamoto et al. 1980; Shimosato et al. 1980; Valerio et al. 1981). By introducing 1 to 2 mg of 3-methylcholanthrene into the lumens of bronchial grafts, Shimosato and associates (1980) induced squamous metaplasias in many bronchial transplants. Similar findings were also described by Okamoto and coworkers (1980). These authors inserted bar-shaped beeswax pellets containing 3.2 mg 7,12-dimethylbenz[*a*]anthracene (DMBA)* into the lumen of the bronchial graft. Both dysplastic (3 of 11 bronchial grafts) and neoplastic (3 of 11 bronchial grafts) lesions appeared 9 to 32 weeks after insertion of the DMBA pellet; these were absent from untreated human grafts. One of the major complications of these studies, which used human adult tissues from surgical specimens (primarily tuberculosis or cancer patients), is that it is impossible to ascertain whether the transplanted airway segments were normal. To avoid this difficulty, we used epithelia derived from newborns or human infants that had died from hereditary or respiratory disease and had not been exposed to any known carcinogen.

Another obstacle in most experimental models thus far devised for studying normal human epithelial cell populations is the difficulty in finding a reliable and homogeneous supply of viable human tissues. This seriously limits the size and nature of experiments that can be realistically carried out. The tissue culture techniques developed by Lechner and associates (Lechner et al. 1982, 1986; Lechner and LaVeck 1985) at the National Institutes of Health permit marked amplification of the number of available human tracheobronchial cells. Using this method, we were able to repopulate a large number of tracheas and utilize cells from the same donor in different experimental conditions (Klein-Szanto et al. 1982, 1986). The development of this experimental in vivo model using epithelial cells from human organs provides a reliable and ethically acceptable method to study the basic mechanisms of action of chemicals and other noxious agents, for interspecies comparisons as well as for assay purposes.

Formaldehyde (HCHO) is a known respiratory irritant and hyperplasiogenic agent (International Agency for Research on Cancer 1982; Rusch et al. 1983; Swenberg et al. 1983) and has produced nasal tumors in rodents (Swenberg et al. 1980; Albert et al. 1982). Although the experimental findings are quite convincing, the epidemiological evidence for HCHO carcinogenicity in humans is still controversial (Walfrath and Fraumeni 1983; Olsen et al. 1984). In addition, the irritant acute and subacute effects of HCHO on the human respiratory mucosa have never been studied with histopathology techniques.

Conversely, the effect of HCHO on the rodent epithelium has been investigated by several groups. In collaboration with Drs. A. Marchok and M. Shiba, we have studied the effect of HCHO on syngeneic rat tracheal transplants. We used an open-ended rat tracheal implant model that permitted quantitative and repetitive exposure to HCHO solutions (Shiba et al. 1983, 1984). The open-ended rat tracheal implants were exposed to HCHO solutions twice a week, for up to 12 weeks. At the higher concentrations (1.0%, 0.6%, and 0.3%), the tracheal lumens became obstructed by granulation tissue. At the lower concentrations (0.2%, 0.1%, and 0.01%), flattened and atrophic epithelium, as well as hyperplasia and squamous metaplasia, were observed. For the most part, these epithelial alterations were dependent on dose. In addition, we studied the effect of the aldehyde on previously initiated tracheal transplants by exposing the epithelia to 800 µg benzo[*a*]pyrene. The carcinogen was released from beeswax pellets during a four-week period. After this initiation period, open-ended rat tracheal implants were exposed twice a week to 0.1% HCHO in phosphate-buffered saline for up to 16 weeks. Under these conditions, we observed several lesions ranging from mild to severe dysplasias. The number and severity of lesions as well as the labeling indices of the open-ended rat tracheal implants treated with both benzo[*a*]pyrene and HCHO were significantly higher than in those treated with either benzo[*a*]pyrene or HCHO alone (Shiba et al. 1987).

SPECIFIC AIMS

The objectives of this study were to investigate the possible toxic, carcinogenic, cocarcinogenic, and promoting effects of HCHO on normal human xenotransplanted tracheobronchial epithelium. The epithelium was obtained from previously unexposed cells from immediate autopsies, grown in deepithelialized rat tracheas transplanted subcutaneously into nude mice.

The specific objectives of the study were:

* A list of abbreviations appears at the end of this report for your reference.

1. To determine the acute and subacute effects of HCHO on the xenotransplanted human tracheobronchial epithelium using different aldehyde doses. This study focused on the structure and cell kinetics of the injured epithelium and was aimed at establishing the HCHO dose necessary for a maximal proliferative response of the tracheobronchial cells. Because human nasal epithelium is a putative target for HCHO, the study of the effect of HCHO on xenotransplanted nasal respiratory epithelium was added for comparative purposes.
2. To study the response of the epithelium to repetitive HCHO exposure, including the possible production of preneoplastic and neoplastic lesions.
3. To expose repetitively to HCHO the xenotransplanted epithelia previously initiated with benzo[*a*]pyrene in order to establish the promoting and enhancing capacity of the aldehyde. In addition, the cocarcinogenicity of HCHO was explored by simultaneously exposing the xenotransplanted epithelia to benzo[*a*]pyrene and HCHO.

MATERIALS AND METHODS

TISSUE AND CELL CULTURES

Study 1 and Study 3: Tracheobronchial Epithelium from Human Infant Donors

Normal human bronchial tissues were obtained from autopsies of 31 human infants 1 to 12 months of age who had died from sudden infant death syndrome. The bronchi and tracheas were cut into 2- × 3-cm fragments and placed in rocking chambers for three days in Hank's balanced salt solution (Biofluids, Inc., Rockville, MD) containing penicillin (100 μg/mL), streptomycin (100 μg/mL), amphotericin B (0.5 μg/mL), and gentamicin (50 μg/mL) to promote reversal of ischemic damage (Lechner et al. 1982). The tissues then were cut into 2- × 2-mm pieces, and explanted with the epithelium side up on a 60-mm tissue culture dish coated with a mixture of human fibronectin (10 μg/mL), collagen (30 μg/mL), and bovine serum albumin (10 μg/mL) that had been dissolved in MCDB-151 medium. Tissues were incubated in LHC-9 medium at 36.5°C in an atmosphere of 5% carbon dioxide in air. (LHC-9 is serum-free medium developed by Lechner and LaVeck [1985] for culturing normal human epithelial cells.)

Study 2: Nasal Respiratory Epithelium from Human Adult Donors

Normal human nasal mucosa was obtained from autopsies of eight young human adults who had died accidentally and had no detectable disease of the respiratory tract. The mucosa fragments were cut into 1- × 2-mm pieces and explanted on a 60-mm tissue culture dish coated with a mixture of human fibronectin (10 μg/mL), collagen (30 μg/mL), and bovine serum albumin (10 μg/mL) that had been dissolved in MCDB-151 medium and incubated in ACB-1 medium at 36.5°C in an atmosphere of 5% carbon dioxide in air. ACB-1 medium is a mixture of F-12 nutrient mixture and MCDB-151 medium supplemented with serum and growth factors to a concentration of 1.25% of the final solution (Klein-Szanto et al. 1986).

PREPARATION OF TRACHEAL TRANSPLANTS

After one to two weeks of incubation, the explants derived from 31 different human infant donors for the tracheobronchial studies and from eight human adult donors for the nasal epithelium studies were removed, and the epithelial cells (5×10^5) were inoculated into deepithelialized Fischer-344 rat tracheas (Charles River Breeding Laboratory, Wilmington, MA). The number of cells obtained from each donor varied markedly from 3×10^6 to 3×10^7. Nevertheless, the average donor yielded 1×10^7 cells, which was enough for 17 to 20 transplants. Deepithelialization was achieved by repetitive freezing (−20°C) and thawing of the tracheas for three hours (Terzaghi et al. 1978).

KINETICS OF FORMALDEHYDE RELEASE FROM INTRATRACHEAL SILASTIC DEVICES

To test the kinetics of HCHO release from the silastic devices, 36 devices, each containing one pellet of 0.5 mg ^{14}C-paraformaldehyde (^{14}C-HCHO) (specific activity 100 mCi/g; Amersham, Arlington Heights, IL) were inserted into xenotransplanted tracheas with and without human infant tracheal epithelium. All HCHO experiments used the powdered form of this compound, paraformaldehyde. The silastic devices were recovered after two days, four days, and after one, two, four, or eight weeks. The remaining ^{14}C-HCHO inside each silastic device was assayed by dissolving the remaining pellet in 10 mL 2N sodium hydroxide. A 20-μl aliquot of this mixture plus scintillation liquid (Fisher Scientific, Fair Lawn, NJ) were used in a Rack Beta II scintillation counter (LKB, Wallac, Finland). Six unused silastic devices containing 0.5 mg [^{14}C]-HCHO also were processed in the same fashion.

TRACHEAL TRANSPLANTS AND FORMALDEHYDE EXPOSURE

Study 1: Effects of Formaldehyde on Human Infant Tracheobronchial Epithelium

Two tracheas that had been inoculated with epithelial cells derived from infant donors were transplanted into the

back (dorsum) of each four- to six-week-old female BALB/c nude mouse obtained from the Fox Chase Cancer Center Laboratory Animal Facility (Philadelphia, PA). Four weeks later, each tracheal transplant was first exposed through a small skin incision, and then a 16-mm hollow silastic tube (Stroz Instrument Co., St. Louis, MO) containing 0, 0.5, 1, or 2 mg HCHO powder (Fisher Scientific) was placed within the lumen of each transplant. Both ends of the silastic device were sealed with silastic glue (Stroz Instrument Co.) to avoid a massive leak of HCHO. After a device containing HCHO was placed within each tracheal lumen, the trachea was sealed with Hemoclips (Weck, Research Triangle Park, NC) and the incision was closed with surgical clips. Tracheal transplants from the three dose groups were exposed for either 2, 4, 8, or 16 weeks. The two tracheal transplants implanted in each mouse contained the same amount of formaldehyde and remained in the mouse for an identical exposure period. All mice were killed at the end of their exposure period. A total of 54 tracheal transplants from 20 different donors were used in these experiments.

Study 2: Effects of Formaldehyde on Human Adult Nasal Epithelium

Two tracheas that had been inoculated with epithelial cells derived from adult donors were transplanted into the back of each four- to six-week-old female BALB/c nude mouse obtained from the Fox Chase Cancer Center Laboratory Animal Facility. Four weeks later, each tracheal transplant was exposed through a small skin incision, and then a 16-mm hollow silastic tube (Stroz Instrument Co.) containing 0, 0.5, or 1 mg HCHO powder (Fischer Scientific) was placed within the lumina of each transplant. Both ends of the silastic device were sealed with silastic glue (Stroz Instrument Co.) to avoid a massive leak of HCHO. After a device containing HCHO was placed within each tracheal lumen, the trachea was sealed with Hemoclips (Weck), and the incision was closed with surgical clips. Tracheal transplants from the two dose groups were exposed for either two or eight weeks. A total of 38 tracheal transplants from 8 different donors were used in these experiments.

Study 3: Effects of Combined Formaldehyde and Benzo[*a*]pyrene Diol Epoxide on Human Infant Tracheobronchial Epithelium

Tracheal transplants that had been inoculated with epithelial cells derived from 11 different infant donors were prepared and transplanted into BALB/c nude mice in the method described for Study 1. Four weeks after transplantation, the tracheal transplants were divided into five groups (see Table 1). Group I was treated with a beeswax pellet containing 50 μg BPDE (benzo[*a*]pyrene diol epoxide); this was followed four weeks later by a silastic pellet containing 0.5 mg HCHO. Eight weeks after this first HCHO pellet was administered, a second identical pellet was inserted in the tracheas to replace the depleted pellet and left in for the remainder of the experiment. Group II was treated with a 50-μg BPDE pellet alone, which was replaced by a blank silastic device four weeks later. Group III received a single beeswax pellet, which was not replaced during the whole experiment, containing 50 μg BPDE and 0.5 mg HCHO. Group IV received a single treatment consisting of a beeswax blank pellet, which remained in place until the end of the experiment. Group V received a beeswax blank pellet for four weeks, followed by two silastic devices containing 0.5 mg HCHO, each of which remained in the tracheas for eight weeks. Unfortunately, only three donors could be treated with this last protocol because of a shortage of available transplants. Groups I and V originally were designed

Table 1. Study 3 Protocol: Tumor Promotion and Cocarcinogenesis Experiments with Xenotransplanted Human Infant Tracheobronchial Epithelium[a]

	Weeks 1–4	Weeks 5–12	Weeks 13-End of Experiment
Group I: Two-stage Carcinogenesis	50 μg BPDE in beeswax pellet	0.5 mg HCHO in silastic device	0.5 mg HCHO in silastic device
Group II: BPDE alone	50 μg BPDE in beeswax pellet	Blank silastic device	Blank silastic device remained in place
Group III: Cocarcinogenesis	50 μg BPDE, 0.5 mg HCHO in one beeswax pellet	Beeswax remained in place	Beeswax remained in place
Group IV: Beeswax controls	Beeswax blank pellet	Beeswax remained in place	Beeswax remained in place
Group V: HCHO alone	Beeswax blank pellet	0.5 mg HCHO in silastic device	0.5 mg HCHO in silastic device

[a] The first HCHO pellet was inserted four weeks after the BPDE pellet. A second pellet containing 0.5 mg HCHO was inserted eight weeks later and left in place for the duration of the experiment.

to receive three successive HCHO pellets. Unfortunately, because the tracheal transplants shrank due to fibrotic changes, it was not possible to implant more than two HCHO pellets within the tracheal lumens. In all groups, animals were killed and studied at 6 and 12 months. A total of 64 tracheal transplants from 11 different donors were used in these experiments.

AUTORADIOGRAPHY

Each mouse with tracheal transplants was injected intraperitoneally with 2 μCi of [^{3}H-methyl]thymidine (100 Ci/mmol; NEN Research Products, Boston, MA) per gram of body weight 45 minutes before it was killed with an overdose of methoxyfluorane (Pitman-Moor, Inc., Washing Crossing, NJ). Tracheal transplants were removed and sectioned into 3-mm-thick rings. Tracheal rings were selected from the middle section of the transplants that had been in contact with the middle portion of the device containing HCHO. The rings were fixed in neutral buffered formalin and then embedded in paraffin. For autoradiographic studies, 5-μm-thick sections of the embedded rings were dipped in Kodak NTB 2 photographic emulsion (Eastman Kodak Co., Rochester, NY) and exposed in the dark for four weeks. Autoradiograms obtained from this method were used to calculate the labeling index, expressed as a percentage of labeled cells in the total epithelial population covering the tracheal lumen. Cells with more than five silver grains over the nucleus were counted along the entire tracheal lumen. A total of 279 tracheal rings (two to three rings per human infant or adult donor and time point) and 129,000 cells were counted.

HISTOLOGY

Tracheal transplants were prepared and fixed in neutral buffered formalin as described in the Autoradiography section. Several 5-μm-thick sections from paraffin-embedded tissues were stained with hematoxylin and eosin for histologic studies. The percentage of the tracheal luminal surface covered by different types of epithelium was calculated in all mice exposed to HCHO by using cross sections from each trachea.

Each section was directly projected with a Zeiss projecting microscope attached to the screen of a Video-Plan graphic digitizer and interfaced with a computer (Zeiss, Oberlochen, Germany). The following epithelial categories were identified on each section: (1) normal mucociliary epithelium; (2) columnar hyperplastic epithelium; (3) atrophic or cuboidal epithelium; and (4) squamous metaplasia. The luminal surface covered by each epithelial type was measured with the digitizer. Lesions smaller than 100 μm could not be resolved clearly with this projection method and were not included in this study. This analytic system permitted a precise planimetric determination of each type of epithelium. Percentages of the tracheal surface occupied by each type of epithelium were calculated for each trachea. To estimate the pathologic status of each experimental condition for each donor, the mean percentages of each epithelial type were calculated from two to three tracheal rings from two to three tracheal transplants. A total of 589 tracheal rings containing tracheobronchial epithelia from 31 human infant donors were studied in Studies 1 and 3. A total of 148 tracheal rings containing nasal epithelia from eight human adult donors were studied for Study 2.

DETERMINING THE HUMAN ORIGIN OF TISSUE

The phenotype of the xenotransplanted epithelia was determined by in situ hybridization with biotin-labeled ^{35}S-labeled Alu 2 probes from human DNA according to a previously described method (Obara et al. 1986).

STATISTICAL METHODS

All possible pairs of treatment combinations were compared using both cell labeling index data and histometric data. Histometric comparisons between treatment combinations were based on the nonparametric Mann-Whitney test. The data used were from the fraction of each ring that was abnormal. Thus, the abnormal ring fractions in one group were compared with the same fractions in another group.

In addition to the nonparametric analysis described above, general linear models were fitted to the cell labeling index data and to the percentage of abnormal epithelia data in the study of the effect of HCHO on human infant and adult epithelial cells. Dose, dose squared, time, time squared, and dose-time interaction effects were tested in various models. In separate analyses, dose and time were also modeled as categorical variables. The outcome variable was a logistic transformation of the proportion of labeled cells or the proportion of abnormal cells. Nesting of trachea within dose-time combinations was also analyzed. The human donor factor is partially crossed with dose and time. The General Linear Models procedure (Statistical Analysis Systems, Cary, NC) was used for modeling the data.

Cell labeling index data pairs were compared by testing the hypothesis that the number of marked cells in each member of a pair is proportional to the total number of cells counted in the corresponding member. Thus, if n_1 and n_2 cells were counted in the members of a pair to be compared, and m_1 and m_2 were the corresponding numbers of marked

cells, then the proportion $p = n_1/(n_1 + n_2)$ was used as a null hypothesis to see whether m_1 was a member of either extreme tail of the binomial distribution $b(k, m_1 + m_2, p)$. This test approximates Fischer's exact test when the numbers n_1 and n_2 are large. Because there were 105 such comparisons, the Bonferroni bound (Miller 1966) for significance is 0.0005 (0.05/105) for a 5% test. We can be 95% confident that all contrasts significant at this level are simultaneously significant.

RESULTS

STUDY 1: EFFECTS OF FORMALDEHYDE ON HUMAN INFANT TRACHEOBRONCHIAL EPITHELIUM

Kinetics of Formaldehyde Release from Intratracheal Silastic Devices

The kinetics of HCHO release from silastic devices containing 0.5 mg ^{14}C-labeled HCHO powder in tracheal transplants with or without human infant tracheobronchial epithelium showed no significant differences. In transplants with and without epithelium (Figure 1), approximately 65% of the HCHO was released during the first week, and the remainder was released more slowly. Even after eight weeks, radioactivity representing approximately 10% of the HCHO still was retrieved in the silastic devices placed intratracheally.

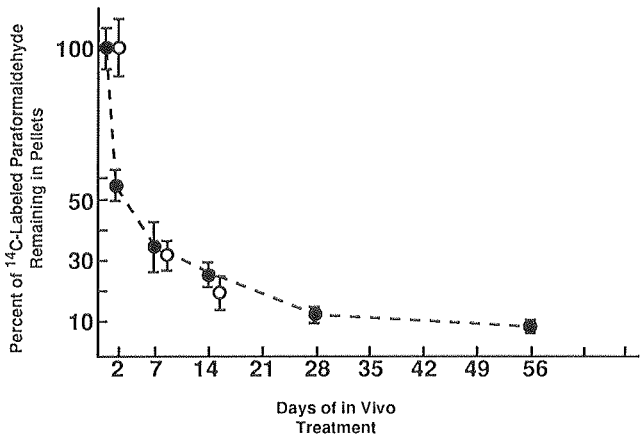


Figure 1. Formaldehyde release from silastic devices in tracheal transplants containing infant tracheobronchial epithelium (●) and tracheal transplants without epithelium (○). Points depict the mean ± SEM of four transplants.

Determining the Human Origin of Tissue

In situ hybridization with biotin-labeled or ^{35}S-labeled Alu 2 probes for human DNA confirmed the human origin of the respiratory epithelium (Obara et al. 1986). Silver grains on autoradiograms or positive histochemical reaction were observed only on the human infant tracheobronchial epithelium, and not on the rodent stromal cells.

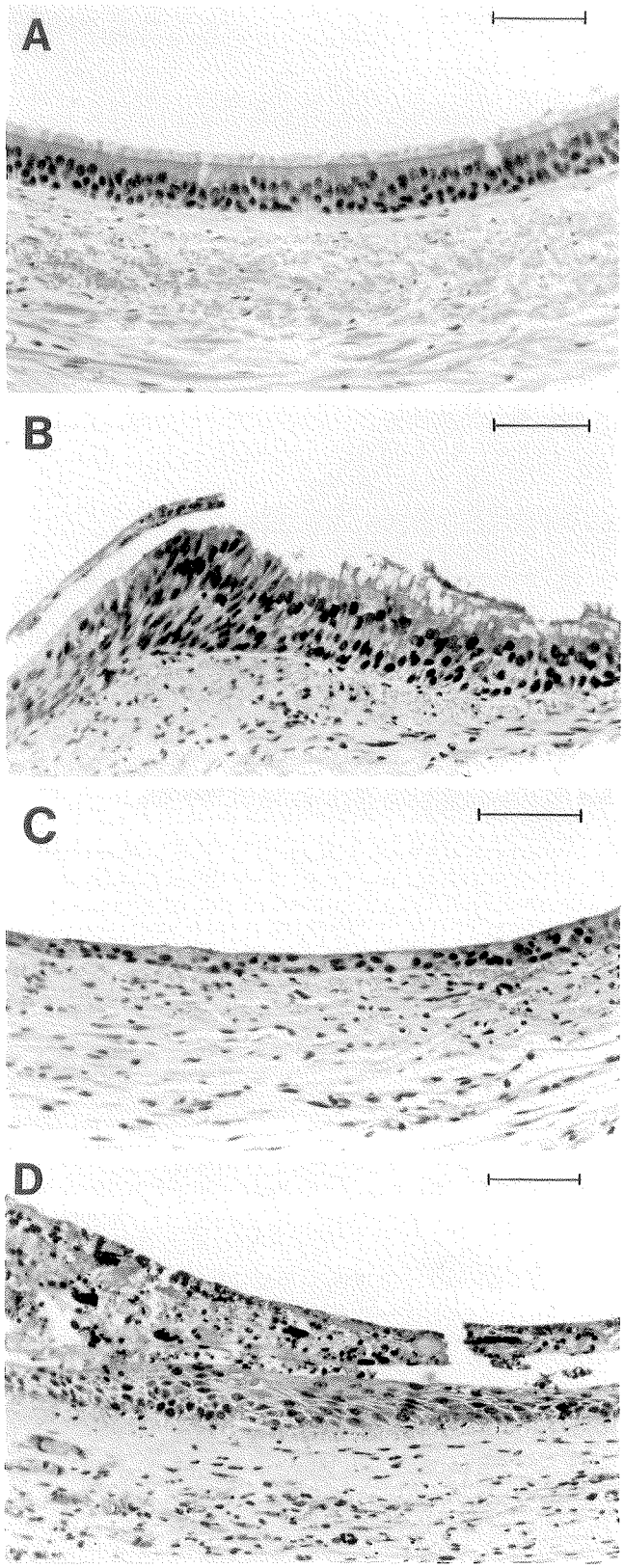
Epithelial Changes

Several changes in the epithelial structure and cellular composition were seen in human infant tracheobronchial epithelium exposed to HCHO. As early as two weeks after inserting the devices containing HCHO, the normal mucociliary epithelium (Figure 2a) either was eroded or was partially replaced by abnormal epithelia. These epithelia included: (1) hyperplastic epithelium composed of several layers of columnar or cuboidal epithelial cells (Figure 2b); (2) atrophic epithelium composed of one or two layers of cuboidal or flat cells (Figure 2c); and (3) squamous metaplastic epithelium, a stratified epithelium with superficial keratinization (Figure 2d). Normal mucociliary epithelium with numerous deciliated areas was evident in all transplants.

The epithelial changes reached a maximum at two weeks and were more obvious at higher doses. An appreciation of the changes that took place as a function of time and dose was obtained by planimetric evaluation of the percentage of the different types of epithelium covering the lumina of the tracheal transplants (Figure 3).

The most extreme change was the absence of epithelium. Although areas of erosion constituted less than 4% of the control tracheal transplants, 20% to 50% of the epithelial surface was eroded in transplants treated with 2 mg HCHO for two weeks. Treatment with 0.5 or 1.0 mg HCHO resulted in 5% to 30% erosion after two weeks. The erosion diminished markedly to less than 10% at later time points.

Mucociliary epithelium covered less than 50% of the luminal surface two weeks after HCHO exposure. At later time points, especially at eight weeks, a recovery was apparent, and values approached those of unexposed tracheal transplants. At 16 weeks, no major alterations were seen, and no histometric determinations were necessary. The most conspicuous change in all groups exposed to HCHO, and especially prevalent in transplants four weeks after treatment with 1 mg HCHO, was the presence of atrophic epithelium. Reactive, hyperplastic, and metaplastic epithelia were seen two to four weeks after treatment with all doses of HCHO. Although less conspicuous, transplants treated with control silastic devices also showed some of these changes. In situ hybridization confirmed the human derivation of these epithelia (Figure 4).



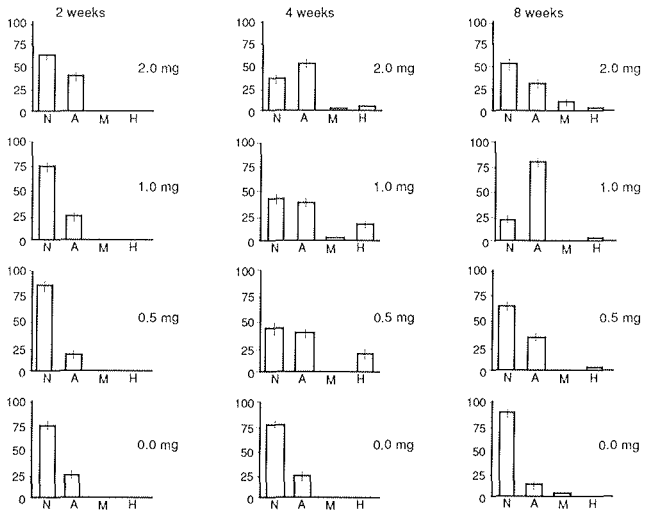


Figure 3. Percentage of different types of epithelium covering the lumina of tracheal transplants after HCHO exposure. Bars represent means of at least four infant donors per group. N = normal, A = atrophic, M = metaplastic, H = hyperplastic.

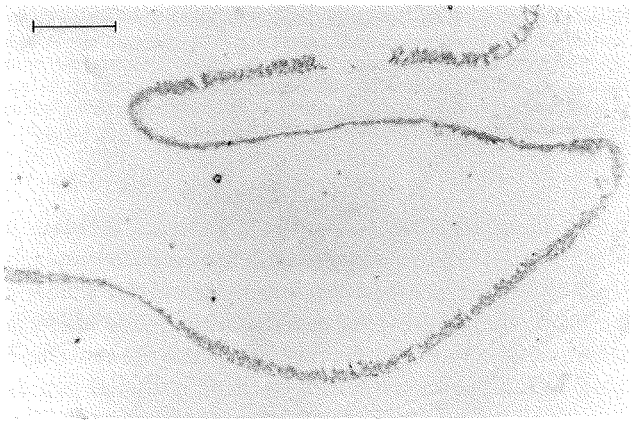


Figure 4. In situ hybridization of tracheal transplant showing xenotransplanted infant tracheobronchial epithelium treated with control pellets (unexposed group, eight weeks) (bar = 350 μm). Note that the biotin-labeled Alu 2 probe labeled only the epithelium.

Incorporation of Tritiated Thymidine (Labeling Index)

HCHO clearly increased the incorporation of DNA precursors in the human infant xenotransplanted epithelia. Figure 5 shows the dose dependency of this change. This is especially clear at two weeks, but the same tendency also can be seen at the other time points. Most epithelia exposed

Figure 2. Different types of infant tracheobronchial epithelium after HCHO exposure (hemotoxylin and eosin stain, bar = 50 μm). A: Normal mucociliary epithelium (unexposed group). B: Columnar hyperplastic epithelium (2 mg HCHO for two weeks). C: Atrophic epithelium (2 mg HCHO for two weeks). D: Squamous metaplasia (2 mg HCHO for two weeks).

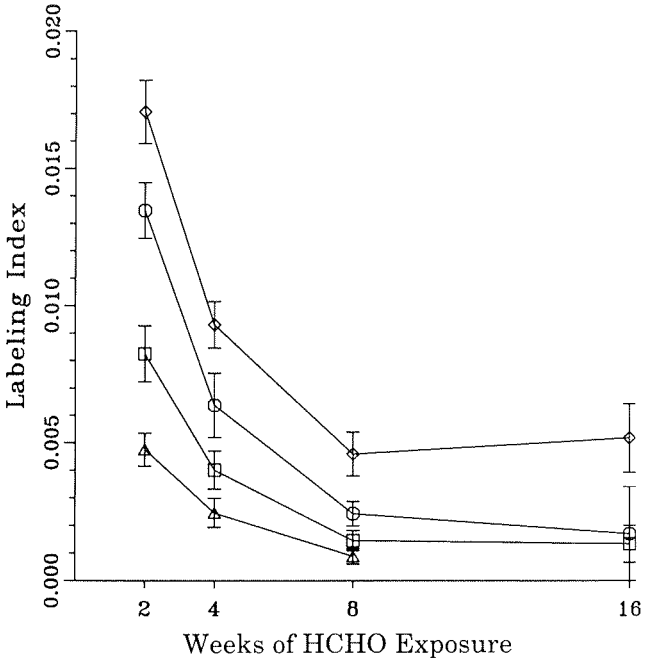


Figure 5. Labeling index versus duration of exposure for the four HCHO dose levels. ◇ indicates 2 mg; ○ indicates 1 mg; □ indicates 0.5 mg; △ indicates 0 mg (controls). Vertical bars give means ± 1 SEM. Except for the points representing 0 mg at 16 weeks, in which only three tracheal transplants from a single case were usable, all points represent means of two to three tracheal transplants from at least four different infant donors.

to HCHO exhibited a statistically significant increase in labeling index when compared with the untreated controls. Thymidine incorporation was prominent in the nuclei of the basal cells, whereas the suprabasal cells showed few labeled nuclei (Figure 6). Figure 6 illustrates the patterns of labeling indices for the whole tracheal epithelial population. The labeling index of normal mucociliary epithelium is less than 0.5%. Because the areas of cell proliferation were small and focal, the resulting labeling indices were relatively low. When atrophic, hyperplastic, or metaplastic lesions in transplants exposed to HCHO were considered individually, the labeling index of the focal lesions was 10 to 20 times higher than levels for normal mucociliary epithelium. Table 2 shows examples of these variations in tracheal transplants treated with 2 mg HCHO for two weeks.

Statistical Analysis

There were 105 comparisons of each dose and time combination with every other dose and time combination for

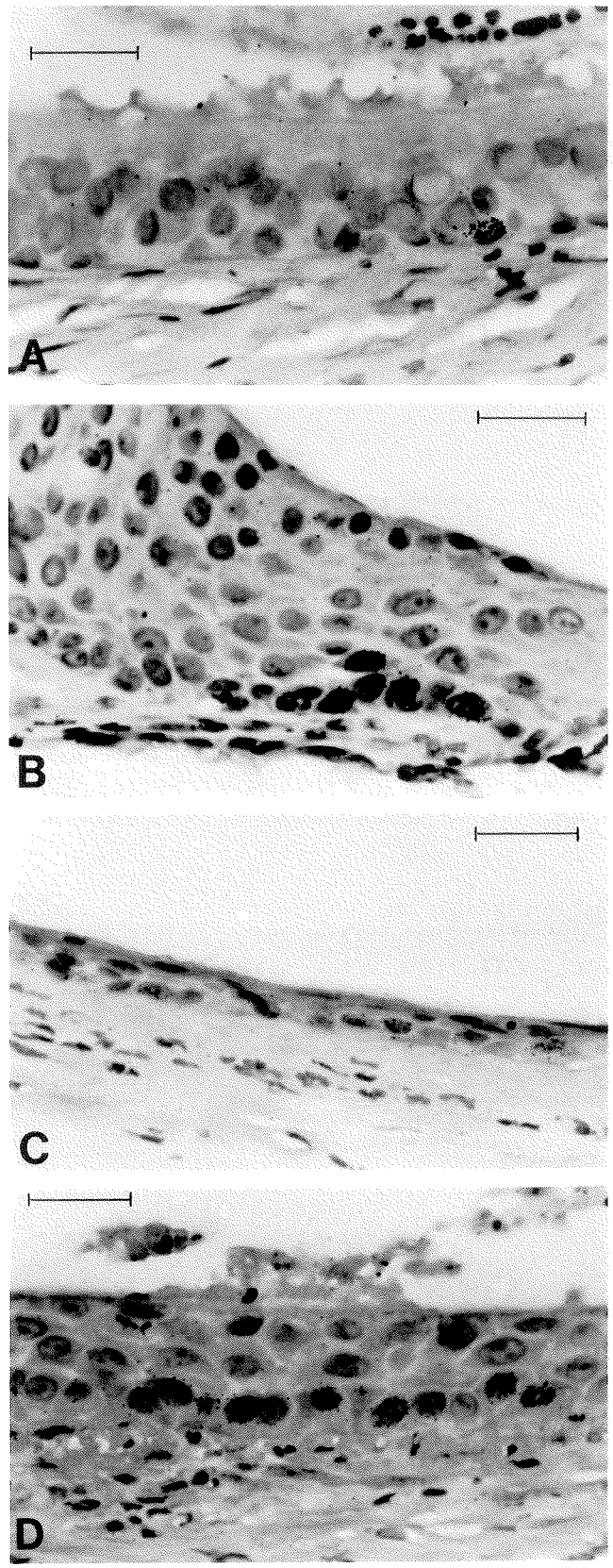


Figure 6. Autoradiographic features of different xenotransplanted tracheobronchial epithelia from infant donors treated with 2 mg HCHO for two weeks (hemotoxylin-eosin stain, bar = 50 μm). A: Normal epithelium (control group). Only one labeled cell is noted (arrowhead). B: Hyperplastic epithelium. C: Atrophic epithelium. D: Squamous metaplasia.

Table 2. Study 1: Labeling Indices of Xenotransplanted Human Infant Tracheobronchial Epithelium After Two Weeks of Formaldehyde Treatment[a]

	HCHO Dose	
Epithelial Type	0 mg	2 mg
Normal	0.38% (2,603)	0.34% (3,509)
Atrophic	0.19% (1,012)	3.30% (2,094)
Hyperplastic	1.08% (832)	3.15% (476)
Squamous metaplastic	0.11% (1,776)	6.11% (851)

[a] The labeling indices shown represent the percentage of labeled cells/total number of cells in each lesion type. Numbers in parentheses show the total number of cells counted.

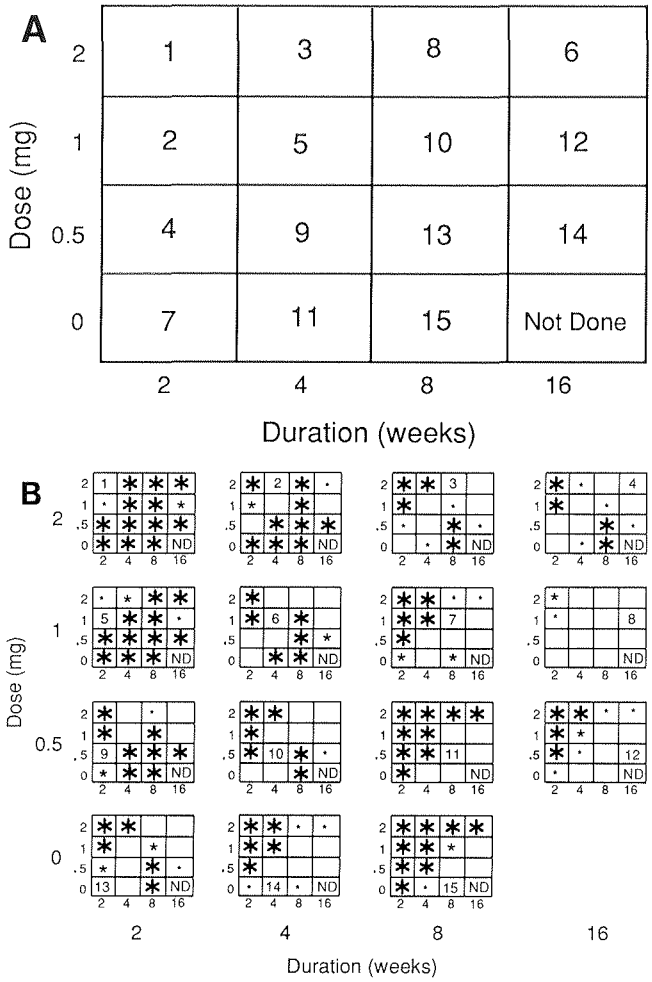


Figure 7. A: Representation of the 15 of the possible 16 treatment combinations from Study 1 for which cell labeling index data were obtained. Numbers within the large blocks are the rank order of labeling indices found for the combinations. Thus, the combination 2 mg, 2 weeks gave the highest labeling index (rank 1), and the combination 0 mg, 8 weeks gave the lowest index (rank 15). **B: Statistical comparison of cell labeling index data from various formaldehyde exposures.** Each large block represents a combination of dose and time, arranged as in Figure 7a. Small blocks within each large block, likewise arranged as in Figure 7a, represent the other combinations of dose and time with which statistical comparison is made. Asterisks represent, in decreasing order of size, statistically significant difference at p = 0.0005 (0.05/105), 0.001, 0.005, and 0.05. Note that in the two-weeks column, doses 1 mg and 2 mg are both significantly different from all combinations (except for 0 mg, 16 weeks, which was not used in this test because it was derived from a single donor).

the cell labeling index data (Figure 7). Therefore, the Bonferroni bound (Miller 1966) for simultaneous statistical significance was 0.0005 (0.05/105). There were 78 comparisons of each dose and time combination with every other dose and time combination for the percent of abnormal epithelium covering the tracheal lumina (Figure 8). Therefore, the Bonferroni bound for simultaneous statistical significance was 0.0006 (0.05/78).

One of the main goals of the study was to identify the dose-time combination that gave rise to the most aberrant cellular response. Figure 7a shows that the maximum labeling index was produced by the dose-time combination of 2 mg HCHO and two weeks; this is denoted as (2,2). The labeling index produced by this dose-time combination was simultaneously significantly higher than all other combinations except 1 mg HCHO for two weeks, denoted as (1,2). A pairwise comparison shows that (2,2) was significantly higher than (1,2), but this conclusion cannot be taken as unequivocal. A main result of the study was the establishment of (2,2) as the most effective dose-time combination among those tested.

Figure 8a shows similar results for the histopathologic data regarding the percent of abnormal epithelium covering the tracheal lumina. Here, (1,4) is the highest ranking dose-time combination for producing a high percentage of abnormal epithelial cells. The dose-time combinations of (2,2), (2,4), (1,2), and (0.5,2), however, do not differ significantly on a global level, that is, using the very stringent Bonferroni criterion. However, all the combinations, except for (2,2), differ on an individual basis. This corresponds to the conclusion reached for the labeling index.

The following general set of conclusions can be obtained from the analysis of the linear model:

1. Labeling index varies quadratically with both dose and time on a logit scale. It increases with increasing dose, with a diminishing return for higher doses, and decreases with time, diminishing fastest at early time points and more slowly at later time points. The proportion of abnormal epithelial cells varies with dose and time in the same way (coefficients significantly different from zero at the 0.05 level).
2. There is no interaction between dose and time for labeling index ($p > 0.2$, on a logit scale).
3. There is no interaction between dose and time for the percentage of abnormal epithelia, ($p > 0.2$, on a logit scale).

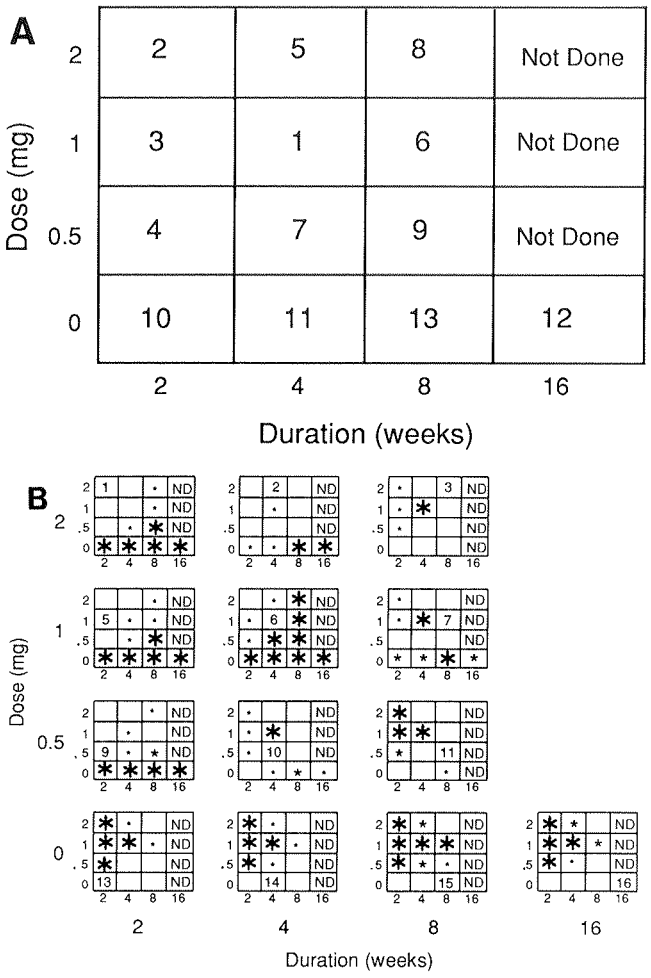


Figure 8. A: Representation of the 13 treatment combinations from Study 1 for which the percentage of abnormal epithelium covering the tracheal lumina was obtained using histometric techniques. The dose is expressed in milligrams of HCHO, and time is expressed in weeks. Numbers within the large blocks are the rank order of the percentage of abnormal epithelia found for 13 combinations. Thus, 2 mg, 2 weeks represents the second-highest proportion of abnormal epithelium, whereas 1 mg, 4 weeks (number 1 in rank order) shows the highest deviation from the norm. **B: Statistical comparison of histometric data from various formaldehyde exposures.** Each large block represents a combination of dose and time, arranged as in Figure 8a. Small blocks within each large block, likewise arranged as in Figure 8a, represent the other combinations of dose and time with which statistical comparison is made. Asterisks represent, in decreasing order of size, statistically significant difference at $p = 0.0006$ (0.05/78), 0.001, 0.005, and 0.05.

We also tested human infant donor as a partially crossed factor with dose and time. Two human infant donors contributed cells that excessively influenced the model. However, when the results from these donors were omitted from the data, the same conclusions as those noted above were reached. Human infant donor was not included as a partially crossed factor in the final model because it had a confounding effect with dose and time.

STUDY 2: EFFECTS OF FORMALDEHYDE ON HUMAN ADULT NASAL EPITHELIUM

All control transplants contained normal mucociliary epithelium as assessed by light microscopy. In most transplants, a small percentage of the luminal surface (usually less than 5%) was covered with atrophic epithelium. Hyperplastic epithelium also was observed in the foci of a few transplants.

Two weeks after exposure to HCHO, the main change was the presence of moderately hyperplastic respiratory epithelium, which sometimes had areas of basal cell hyperplasia (Figures 9a and 9b). This epithelium coexisted in the same tracheal transplants with focal areas of erosion or ulceration that were more evident in the transplants treated with 1 mg HCHO. After two weeks, the borders of the eroded areas were frequently covered by a simple, flat (squamous) epithelium that had migrated over the eroded areas (Figure 9c). This epithelium was frequently contiguous to a stratified epithelium composed of two, three, and sometimes more layers of cuboidal cells (Figures 9c, 9d), which is very similar to epithelial layers seen near wound edges during injury repair. Metaplastic epithelia of epidermoid or squamous type also were seen after two weeks (Figure 9e). After eight weeks, most tracheal transplants had a higher percentage of normal respiratory epithelium; the most abnormal epithelium observed was the simple flat or cuboidal epithelium (atrophic epithelium) (Figure 9f).

Although still present in some areas, there were less hyperplastic-metaplastic lesions after eight weeks of exposure to HCHO than after two weeks of exposure. Labeling index changes indicated that epithelium exposed to HCHO underwent a proliferative burst that could be detected two and eight weeks after the beginning of aldehyde exposure. Overall, there was a two- to threefold increase in the labeling index of the xenotransplanted nasal epithelium. Although less remarkable, this increase was still evident after eight weeks of exposure in the group of transplants treated with 1 mg HCHO. It is important to note that the areas with increased labeling index were rather focal and that certain

Figure 9. Histopathology from Study 2. A: Normal appearance of xenotransplanted adult nasal respiratory epithelium two weeks after exposure to 0.5 mg HCHO. Although histologically this is a normal epithelium, the labeling index of this area was extremely high (0.04 vs. less than 0.01 for control epithelia). Arrow indicates labeled cells (hemotoxylin and eosin stain, bar = 40 μm). **B: Mild basal cell hyperplasia with two labeled cells two weeks after exposure to 0.5 mg HCHO** (bar = 40 μm). **C: Border of an eroded area partially covered by a flat simple epithelium.** Arrow shows labeled flat cell migrating over the eroded surface. Arrowhead indicates epithelial hyperplastic tongue at the border of the injured epithelium two weeks after exposure to 1 mg HCHO (bar = 36 μm). **D: Regenerated epithelium devoid of cilia near a recently eroded area two weeks after exposure to 1 mg HCHO** (bar = 36 μm). Note the loose subepithelial granulation tissue. **E: Epidermoid metaplasia of nasal epithelium two weeks after exposure to 0.5 mg HCHO** (bar = 70 μm). **F: Simple flat epithelium with a low labeling index seen eight weeks after exposure to 1 mg HCHO** (bar = 40 μm).

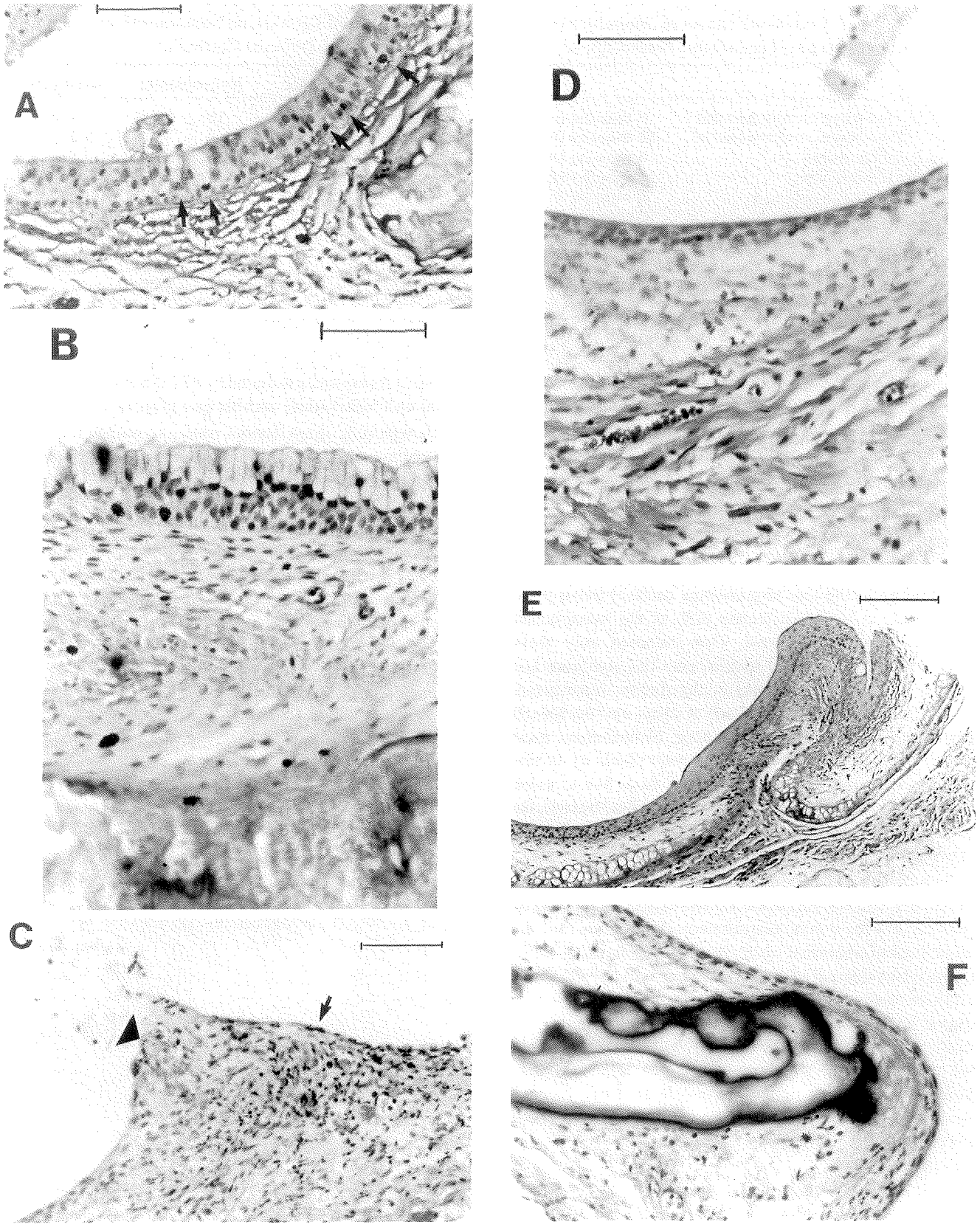

A
B
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Table 3. Study 3: Combined Effects of Formaldehyde and Benzo[*a*]pyrene Diol Epoxide on Xenotransplanted Human Infant Tracheobronchial Epithelium: Percentage of Tracheal Lumina Covered by Different Epithelia[a]

		Normal	Atrophic	Hyperplastic	Metaplastic
Group I: Two-stage carcinogenesis	6 months (n = 5)	54 ± 21	24 ± 21	18 ± 12	4 ± 4
Group I: Two-stage carcinogenesis	12 months (n = 5)	96 ± 2	3.6 ± 2	—	2.2 ± 2
Group II: BPDE alone	6 months (n = 5)	72 ± 16	3 ± 2	21 ± 19	4 ± 2
Group II: BPDE alone	12 months (n = 5)	90 ± 1	10 ± 11	—	—
Group III: Cocarcinogenesis	6 months (n = 7)	57 ± 18	35 ± 15	2.6 ± 1.4	5 ± 4
Group III: Cocarcinogenesis	12 months (n = 5)	84 ± 10	15 ± 10	1.4 ± 1.5	0.2 ± 0.2
Group IV: Beeswax controls	6 months (n = 3)	99 ± 1	1 ± 1	—	—
Group IV: Beeswax controls	12 months (n = 6)	90 ± 5	8 ± 5	0.8 ± 1	0.7 ± 0.5

[a] Histometric determinations were done on two to four cross-sections per tracheal transplant, with two to three tracheal transplants per donor (n = number of donors). A total of 296 cross-sections were used. No data are presented for Group V because the results were not different from Group IV. Data are the percentages of total luminal epithelium covered by the different types of epithelia expressed as means ± SEM.

small areas of hyperplastic-metaplastic epithelium exhibited labeling indices that were 10 to 20 times higher than control values.

STUDY 3: EFFECTS OF COMBINED FORMALDEHYDE AND BENZO[*a*]PYRENE DIOL EPOXIDE ON XENOTRANSPLANTED HUMAN INFANT EPITHELIUM

Several difficulties were encountered during this study that resulted in the loss of numerous tracheal transplants. As a result, only approximately 60% of the transplanted tracheas could be evaluated. This included only those transplants that contained epithelium. The rest were lost because of a lack of epithelial repopulation, intercurrent disease in the mice, cannibalism of mice, and accidental loss of animals due to cage flooding. Nevertheless, most groups could be evaluated comparatively (Table 3). In this table, most groups include transplants from five to seven donors, from whom at least two to three tracheal transplants each were examined. For the most part, each donor also was represented in at least three different treatment groups.

Histological and histometric analysis of these tracheal transplants showed that both the two-stage carcinogenesis protocol and the cocarcinogenesis protocol increased the percentage of abnormal epithelium in the tracheal transplants when compared with treatment with BPDE alone. All three treatments were significantly effective in eliciting altered epithelial morphology when compared with the minimal effect produced by the beeswax blank pellets (control treatment). Nevertheless, 12 months after the treatments began, most tracheal transplants were covered mainly by a normal mucociliary respiratory epithelium (Figure 10) that comprised approximately 90% of the epithelia.

All of the observed epithelial lesions were cytologically typical. No lesions showed cellular atypias, and even the squamous metaplasias induced by BPDE had a regular cytological and histological architecture (Figure 11).

Although only three donors were exposed to repetitive applications of HCHO (Group V), these tracheas showed no differences from the beeswax controls (Group IV) 6 and 12 months after the insertion of the pellets. Five tracheal transplants (two from Group III and one each from Groups I and II) were completely destroyed and invaded by spindle and giant cell tumors (Figure 12). In situ hybridizations with Alu 2 probes proved that the tumors were not of human derivation, although some scattered positive cells indicated that a few human cells were still recognizable inside the tumors.

Labeling indices were also determined in the different groups. Although the effective number of donors per group decreased even more because of premature deaths of animals, especially in the groups killed 12 months after the experiments were begun, no differences in labeling indices

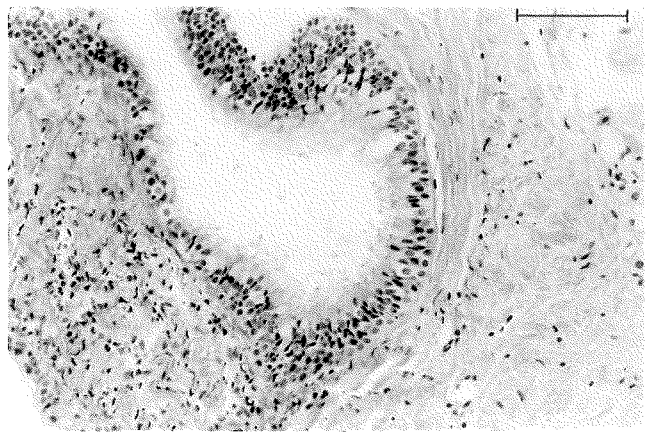

Figure 10. Normal mucociliary epithelium covering a tracheal transplant 12 months after simultaneous exposure to benzo[*a*]pyrene diol epoxide and HCHO (Study 3, Group III) (hemotoxylin and eosin stain, bar = 40 μm).

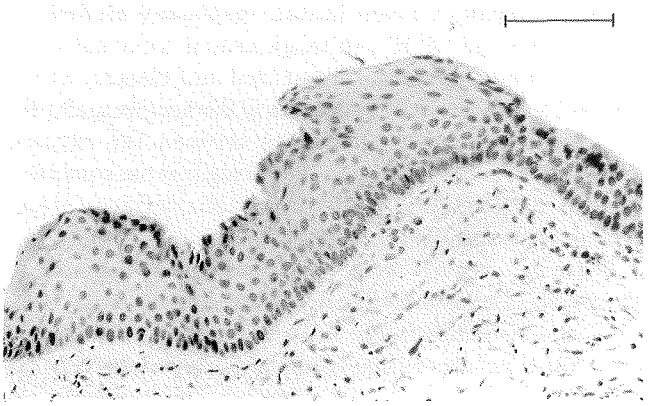

Figure 11. Squamous metaplasia without atypical cells in a tracheal transplant exposed to benzo[*a*]pyrene diol epoxide alone for six months (Study 3, Group II) (hemotoxylin and eosin stain, bar = 38 μm).

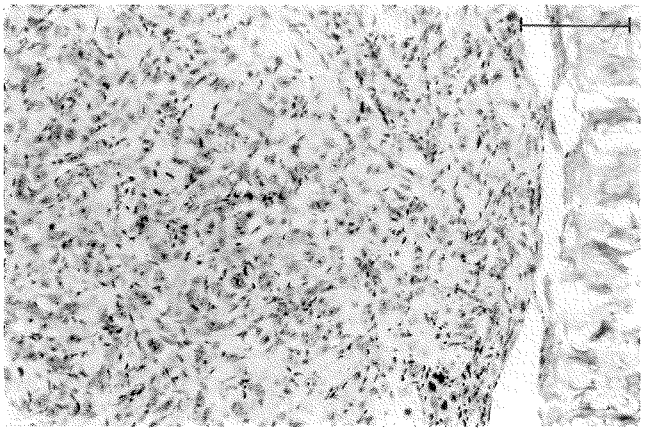

Figure 12. Intratracheal large cell sarcoma in a transplant treated with benzo[*a*]pyrene diol epoxide and HCHO (Study 3, Group I) (hemotoxylin and eosin stain, bar = 40 μm).

were seen. The labeling indices varied from 0.005 to 0.02 in all groups, indicating minimal proliferative activity.

DISCUSSION

HCHO is a powerful irritant that causes profound changes in the respiratory tract mucosa of several laboratory animal species (Brusick 1983; Kerns et al. 1983; Shiba et al. 1983, 1984). Although HCHO is known to cause important alterations in human cells in culture (Grafstrom et al. 1983, 1984, 1985), for ethical reasons, no histologic evidence of these sublethal changes has been reported from in vivo human exposures. The model of xenotransplanted human respiratory tract epithelium developed in our laboratory (Klein-Szanto et al. 1982) allows the systematic histopathologic evaluation of the effects of HCHO on the human tracheobronchial epithelium. This xenotransplantation model is unique because it permits the study of the human epithelial cell populations in an experimental setting that simulates in vivo conditions. Although the technological problems were numerous and the final yield of usable transplants was lower than expected, this experimental model permits a good evaluation of human epithelial cells under almost physiological conditions. This model is amenable to experimental manipulation that can result, as was the case with HCHO exposure, in new information on the response of human cells to measurable doses of toxicants.

An additional advantage of this model is that it allows controlled and localized exposures of human epithelium to known amounts of HCHO. Although the use of intratracheal silastic devices containing HCHO is characterized by an initial rapid release or burst effect (Pal and Klein-Szanto 1983) that results in the delivery of approximately 50% of the total dose during the first week, it has the advantage of exposing the xenotransplanted epithelium to a slowly released low amount of HCHO during the following four to eight weeks. Although the burst effect produced necrosis and erosion, especially at higher doses, it is clear that it also produces reactive epithelial changes, such as hyperplasia and squamous metaplasia.

Increased cellular proliferation was also evident in all dose groups studied, and was clearly dependent on dose and time. The detailed study of the labeling indices in the specific types of epithelia showed that HCHO produced focal areas with increased epithelial proliferation, especially after two weeks of aldehyde exposure. Some foci of squamous metaplasia exhibited labeling indices that were more than 10 times higher than in the control mucociliary epithelium. Similarly, foci of hyperplastic epithelium and even atrophic epithelium showed increased labeling. The latter epithelia could represent proliferating areas of flat and cuboidal cells similar to those seen in hamster tracheal epithelium undergoing regeneration after mechanical injury (Keenan et al. 1982). Furthermore, although the epithelium of the control transplants treated with silastic devices without HCHO exhibited abnormal epithelia, including a few areas of squamous metaplasia, these focal lesions had a much lower labeling index. Although higher than the labeling index of untreated mucociliary epithelium, the latter lesions also had different morphologic features, namely, fewer cell layers and predominantly flat cells. These epithelial changes were interpreted as a reaction to the mechanical stimulus produced by the presence of the silastic devices, whereas the multilayered and highly proliferative metaplasias were produced exclusively by HCHO exposure.

It is important to point out that similar changes in morphology have been described in rats after exposure to HCHO

gas or formalin solutions (Shiba et al. 1983, 1984, 1987). Furthermore, Swenberg and associates (1983) showed a 10- to 20-fold acute increase in labeling index of the respiratory tract epithelium in rats and mice exposed once to a HCHO aerosol. These increments were dependent on dose and time. Shiba and coworkers (1987) also showed an approximately threefold increase in the labeling index of rat tracheal epithelium after protracted treatment (30 weeks) with HCHO solutions. In this same study, chronic HCHO treatment produced focal areas of atrophic, hyperplastic, and metaplastic epithelium.

A comparison between the data described in the present report for xenotransplanted human respiratory epithelium and those obtained from rodents clearly indicates the same response pattern. Although it can be argued that the exposure procedures, doses, and schedules of administration were different, and that the rat epithelium exhibited more exuberant changes, the general tendencies for increased proliferation and epithelial changes were similar in both human and rodent epithelia exposed to HCHO.

Our studies using xenotransplanted nasal respiratory epithelium exposed to HCHO (Study 2) showed similar results. As with the xenotransplanted tracheobronchial epithelium exposed to HCHO, the nasal epithelium responded to the abrupt release of HCHO by producing areas of erosion. However, HCHO also induced proliferative epithelial changes, such as hyperplasia and squamous metaplasia, sometimes directly adjoining areas of erosion and healing.

The coexistence of epithelial necrosis and hyperproliferative changes, including nasal cancer, have been emphasized repeatedly in several experiments using HCHO inhalation in rats (Swenberg et al. 1980; Feron et al. 1988). The similarities in the type of HCHO-induced reactions between the xenotransplanted human tracheobronchial epithelium and the xenotransplanted human nasal epithelium suggest that there is no particular regional sensitivity to the aldehyde, and that the localization of lesions in humans exposed to HCHO depends mainly on the air flow and deposition patterns. Similar conclusions were reached in the rat, which has airflow patterns that make the nasal mucosa the prime target for cancer development (Morgan et al. 1986a,b). In another investigation using rodent tissues (Swenberg et al. 1980), an increase in epithelial proliferation very similar to the one described herein was reported. The similarity between the responses of the rodent and human respiratory epithelia exposed to HCHO further supports the recommendation that caution be exercised in the handling of this aldehyde, which has clearly produced squamous carcinomas of the rat nasal mucosa.

In Study 3, we attempted the simultaneous (cocarcinogenesis) and sequential (two-stage carcinogenesis) treatment of xenotransplanted human respiratory epithelium with HCHO and BPDE. Although several technical problems decreased the expected survival and integrity of the tracheal transplants, the evaluation of the healthy grafts did not reveal any major differences between the exposed groups and the control group, indicating that the combined effect of HCHO and BPDE did not elicit the formation of epithelial preneoplastic or neoplastic lesions.

These experiments were conducted during a period of 12 months after exposure to the two chemicals; it is possible that a longer exposure time would yield different results. Because human lung tumors usually develop over the course of many years, it is very feasible that the present experimental system did not reproduce these conditions with the dose levels employed. Although it is very difficult, if not impossible, to extend further the useful life of the transplanted animals, it might be possible to increase the dosage of both BPDE and HCHO. Both doses employed in these experiments were selected because they produced a minimal toxic effect (erosion) of the epithelium during the first weeks of exposure, but elicited a recognizable reactive response (hyperplasia-metaplasia) after four to eight weeks of exposure. Increasing the BPDE dose to 100 μg or more, and the HCHO dose to 1 mg, would increase the reactive response markedly and might result in a tolerable toxic effect. However, because of practical constraints, we did not use these dose levels, and instead employed the less toxic doses of 50 μg BPDE and 0.5 mg HCHO, which elicited minimal effects.

Six months after exposure ended, the percentage of luminal epithelium covered by abnormal epithelium was definitely higher in the cocarcinogenesis (Group III) and two-stage carcinogenesis (Group I) groups than in the group of transplants exposed to BPDE alone (Group II). Although these three groups exhibited changes that were statistically significant when compared with the control group (Group IV), the differences between the effects in Groups I, II, and III were not statistically significant. This indicates that HCHO did not enhance the effect of BPDE under the conditions of the present experiments.

Twelve months after the exposures ended, the differences among the groups were even less obvious; practically all of the tracheal transplants exhibited predominantly normal mucociliary epithelium. Nevertheless, all groups contained patches or small foci of abnormal epithelium, especially atrophic epithelium and epidermoid metaplasias. The presence of small foci of regular metaplastic epithelium probably indicates potential preneoplastic alterations. In the model of syngeneic rat tracheal transplants exposed either to polycyclic aromatic hydrocarbons alone (Nettesheim and Marchok 1983) or in combination with HCHO (Shiba et al. 1987), hyperplastic metaplastic lesions, similar to the ones

observed late in the present research, were seen. Some of these lesions developed cell cultures with altered cell behavior, and in a more complete follow-up study (after exposure to DMBA), these late lesions were identified as precursors to squamous carcinomas that appeared some months later (Nettesheim and Marchok 1983).

As stated above, the relatively short life span of the nude mice made it impossible to ascertain whether these lesions in the xenotransplants could evolve further into undisputable, preneoplastic lesions. Conversely, it was clear in the present study that the effect of BPDE alone was not less effective than BPDE in combination with HCHO. It must be emphasized that no treatment produced real atypical preneoplastic lesions, and no neoplastic proliferation of human derivation was seen. Only four sarcomas of mouse origin were observed.

CONCLUSIONS

The model of xenotransplanted human infant epithelium was used to determine the dose- and time-dependent effects of HCHO during a subacute exposure for four to eight weeks. Clear hyperplastic and metaplastic responses were seen together with an increase in cell proliferation. The mean labeling index was three to four times higher than normal, although in some small foci of hyperplastic-metaplastic epithelium the labeling index increased up to 20 times. These studies showed that HCHO, although toxic at higher doses, can elicit at lower doses a proliferative response of the human tracheobronchial epithelium that is not preceded by a massive toxic effect. Similar responses were obtained when xenotransplants containing human adult nasal epithelium were exposed to the same levels of HCHO, indicating that respiratory epithelium from different sites in the airways exhibit the same reactive patterns after aldehyde exposure, and that eventual differences in incidence of lesions in humans probably depends on other factors, such as gross anatomy and airflow patterns. The combined effect of BPDE and HCHO applied either simultaneously or sequentially (cocarcinogenesis and two-stage carcinogenesis protocols) to xenotransplanted human infant epithelium did not elicit lesions of preneoplastic nature, indicating that under the present conditions and duration of the experiments, it is not possible to ascertain the eventual cocarcinogenesis or promoting effect of HCHO.

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ABBREVIATIONS

BPDE	benzo[*a*]pyrene diol epoxide
DMBA	7,12-dimethylbenz[*a*]anthracene
HCHO	formaldehyde

INTRODUCTION

A Request for Applications (RFA 85–1) that solicited proposals for "Health Effects of Aldehydes" was issued by the Health Effects Institute (HEI) in the summer of 1985. In response to this RFA, Dr. Andres J.P. Klein-Szanto, from the University of Texas System Cancer Center in Smithville, Texas, submitted a proposal to HEI, entitled "Effects of Formaldehyde on Human Xenotransplanted Respiratory Epithelium." Prior to the start of his four-year study in August 1986, Dr. Klein-Szanto changed his affiliation to the Fox Chase Cancer Center in Philadelphia, Pennsylvania. Total expenditures for the project were $423,957. The Investigators' Report was received in September 1990 and was accepted by the Health Review Committee in April 1991. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of HEI and to the public. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Health Review Committee's Commentary.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. 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), (b)(1), (g), and (h) and Sections 207(c)(4), (5), and (6) impose specific requirements for reduction in motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide the EPA with limited discretion to modify those requirements.

Formaldehyde has been regulated by the EPA under emission standards for organic material hydrocarbon equivalents produced in connection with the operation of various classes of vehicles using methanol fuel. 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 emission 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 301 of the 1990 Amendments, formaldehyde is one of the three emissions specifically mentioned in Section 202(l)(1).

SCIENTIFIC BACKGROUND

The respiratory system is a primary route of entry for environmental pollutants into the body. Humans are exposed in their homes and workplaces to a variety of gases, particles, and other inhalable compounds that can exert a broad range of toxic effects. The epithelial cells lining the airways of the respiratory tract are the first to interact with these inhaled materials and to be susceptible to their potentially toxic effects (Brain et al. 1977). Despite the presence of many different defense mechanisms in the lungs, airborne pollutants can damage epithelial cells and may initiate changes that lead to cancer. The present report describes the results from an in vivo cell culture system that was used to investigate the effects of formaldehyde, an indoor and outdoor air pollutant, on human airway cells. This system was used to propagate and sustain human airway epithelial cells and to evaluate the capacity of formaldehyde to act as a cancer-causing agent on these cells.

FORMALDEHYDE TOXICITY

Exposure to formaldehyde has been linked to the development of cancer in animals based on the results of long-term inhalation studies (Swenberg et al. 1980; Kerns et al. 1983). In these chronic studies, rats exposed to 6 or 14 parts per million (ppm)* formaldehyde for up to 24 months developed nasal tumors. Because of these findings, as well as data from human epidemiological studies, and in vitro genotoxicity assays (International Agency for Research on Cancer 1987; U.S Environmental Protection Agency 1987),

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

formaldehyde is classified as a probable human carcinogen. Formaldehyde's toxicity is related to its water solubility and its ability to form cross-links with a variety of cell macromolecules, including DNA, RNA, and proteins. Mutations produced by formaldehyde may be related to cross-links formed between DNA and proteins, leading to errors in DNA replication and subsequent development of cancer (Heck et al. 1990; Casanova et al. 1991).

The reports of cancer incidence in rats raised concerns about adverse human health effects of formaldehyde because many people can be exposed to 0.1 to 1 ppm formaldehyde and possibly higher concentrations at work and in their homes (Hart et al. 1984; U.S. Environmental Protection Agency 1987). Indoors, formaldehyde is released by outgassing from a variety of products, including building materials, plastics, and textiles; in some occupations, workers can be exposed directly to formaldehyde fumes (Feinman 1988). Formaldehyde also is a component of outdoor air pollution, partially because of incomplete combustion of gasoline and diesel fuels by motor vehicles (Marnett 1988). The projected use of methanol and ethanol as alternative fuels and in fuel blends may increase outdoor formaldehyde levels because their combustion, particularly that of methanol, yields more formaldehyde than conventional fuel combustion. Thus, formaldehyde is recognized not only as a significant indoor air pollutant, but also as a potentially important outdoor air pollutant that may affect human health.

MECHANISMS OF CARCINOGENESIS

The development of cancer is a complex, multistage process involving several progressive stages that have been defined broadly as initiation, promotion, and progression (International Agency for Research on Cancer 1991). Exposures to chemical, physical, and biological agents, as well as individual variables such as genetics, hormonal levels, and nutritional status, are important factors that can alter cells and lead to the development of cancer. Cocarcinogens may also work in concert with these factors to enhance the development of cancer. Extensive research on the mechanisms of cancer has identified alterations in the genetic material as a fundamental early event in the cancer process. The role of genetic alterations is not, however, restricted to the early stages of the cancerous, or neoplastic, process.

Although continuing research on the mechanisms of carcinogenesis has blurred the distinctions among the three classic stages of initiation, promotion, and progression (Harris 1991), these stages remain useful for describing the overall process of cancer as it is currently understood. Initiation involves one or more genetic changes that confer upon the initiated cells an altered responsiveness to their environment and an advantage for selective clonal expansion, compared with neighboring cells. Promotion results in the proliferation of initiated cells compared with the surrounding normal cells. DNA replication during this proliferative phase may increase the probability of further genetic changes. Additional damage to the DNA at this stage may also activate or inactivate genes responsible for regulating cell proliferation and differentiation. During progression, proliferation of the altered cells continues, and further genetic alterations may also occur. Cells may express further changes in their appearance and metabolism that reflect more dramatically their altered, or transformed, state.

IN VITRO MODELS FOR STUDYING RESPIRATORY TOXICANTS AND CARCINOGENS

A variety of in vitro exposure systems has been developed to study the steps involved in neoplastic transformation, as well as the cell injury and the genotoxic damage caused by exposure to toxic agents. Several in vitro systems are available for studying these effects in lung tissue. Slices of lung lobes or tissue excised from tracheas can be maintained easily in an organ culture for biochemical or metabolic studies either before or after a toxic exposure (Fisher and Placke 1988). Lung explant systems can be used to propagate cells in vitro from a tissue piece and then to expose the cells to various agents in vitro. In vivo exposures can be combined with an in vitro system by exposing animals to a selected agent and then isolating and growing the cells of interest in culture (Nettesheim and Barrett 1984). Pulmonary alveolar macrophages, free lung cells residing in the airspaces and airways, can be obtained readily either before or after an exposure by rinsing lung lobes with saline (Fisher and Placke 1988). Relatively new methods are also available for isolating specific lung cells, such as airway epithelial cells (Van Scott et al. 1991), type 2 epithelial cells (Dobbs et al. 1986), and endothelial cells (Ryan et al. 1978) for in vitro metabolic and toxicity studies.

Because many human lung cancers originate from epithelial cells, in vitro systems that use human airway epithelial cells are helpful for understanding the cellular and molecular mechanisms of neoplastic transformation induced by compounds such as formaldehyde (Rhim 1992). Prior to this study on the effects of formaldehyde on human airway cells, Klein-Szanto and colleagues developed an in vivo cell culture system that combined techniques for in vitro propagation of human airway epithelial cells (Lechner et al. 1981) with an in vivo growth system that closely simulated the environment of a trachea (Terzaghi et al. 1978). The cell propagation methods described by Lechner and colleagues (1981) yielded epithelial cells from explants of adult human bron-

chi that were free of fibroblasts and would proliferate in vitro. The in vivo culture methods were based on those of Terzaghi and coworkers (1978), in which rat tracheal epithelial cells were inoculated into rat tracheas from which the epithelium had been removed to expose the underlying growth support surface. The inoculated tracheas then were implanted under the skin of rats in which the immune systems had been suppressed.

Klein-Szanto and coworkers adapted these techniques and developed a three-species system for studies of epithelial cell carcinogenesis (Klein-Szanto et al. 1982). They obtained tissue samples of airways from autopsies of human infants and then propagated the epithelial cells in vitro. The cultured human cells were xenotransplanted by inoculating them into deepithelialized rat tracheas; the tracheas then were implanted under the skin of mice that would not reject foreign tissues (female athymic mice of BALB background). This combined in vitro and in vivo system provided a unique growth environment for airway epithelial cells and for studies of the potential preneoplastic and neoplastic changes in the cells caused by exposure to toxic agents, such as formaldehyde.

IN VITRO SYSTEMS IN PERSPECTIVE

Interpretation of data obtained from any in vitro system relative to those changes that may occur in vivo should take into account several factors. For example, a close correlation exists between the phenotypic changes observed in the in vivo and in vitro models of tracheal cell carcinogenesis (Nettesheim and Barrett 1984). This correlation supports the argument that in vitro observations are relevant to the process of cancer development in vivo. However, the question remains regarding whether the various phenotypic changes that transformed cells undergo during neoplastic progression are the direct result of carcinogen exposure or whether they occur secondarily, as a result of genetic errors that arise from repeated cell replication. Furthermore, a basic difference between in vitro and in vivo systems is that in most in vitro systems, the normal cellular surroundings of blood supply, connective tissue support, and relationships among cells are absent. As a result, the expression of specific differentiation characteristics and the neoplastic behavior of many cell types may be markedly different in vitro than in an intact animal (Stoker et al. 1990). Finally, the in vitro conditions themselves, such as the absence of essential nutrients or the properties of the underlying growth surface, may cause cells to respond to test agents differently than cells in vivo.

Despite such limitations, systems in which cells are grown and sustained in an environment that differs from that of their origin are useful because they provide flexible exposure models. A variety of chemicals and exposure scenarios can be tested more easily in these systems than in intact animals. Responses of different types of cells to selected test agents can be evaluated, exposures to specific agents can be controlled rigorously, and findings can be replicated easily. These systems provide an experimental framework for testing the pulmonary toxicity and carcinogenicity of numerous compounds, such as formaldehyde. These exposure models also make possible many experiments that would not otherwise be feasible, cost-effective, or ethical.

JUSTIFICATION FOR THE STUDY

The HEI wanted to support studies that would evaluate potential adverse human health effects caused by chronic exposure to the low levels of aldehydes found in motor vehicle emissions. This interest stemmed in part from a projected increase in the use of methanol and ethanol as alternative fuels and in fuel blends. Although the combustion of both conventional (gasoline and diesel) fuels and alcohol fuels produces emissions containing aldehydes, the percentage of aldehydes is greater in emissions from alcohol fuels (Marnett 1988).

The HEI was interested in studies that would determine the individual and combined biological effects of aldehydes and their potential interactions with carbon particles. Three specific areas of interest outlined in RFA 85–1 were: (1) effects of aldehydes on susceptibility to respiratory infections; (2) interactions between aldehydes and genotoxic compounds in motor vehicle emissions that may produce cancer; and (3) studies to evaluate human hypersensitivity to formaldehyde and the development of new models to evaluate hypersensitivity in animals.

Dr. Klein-Szanto and his colleagues proposed to evaluate the effects of formaldehyde on human airway epithelial cells. Epithelial cells would be taken from samples of tracheobronchial tissues obtained immediately at autopsy of full-term fetuses or infants. The cells would be isolated, propagated in vitro, and xenotransplanted by inoculating them into segments of deepithelialized rat tracheas. The tracheas then would be sealed and transplanted into female BALB/c nude mice. After repopulation of the tracheal surface, the epithelial cells would be exposed to formaldehyde by implanting silastic devices containing powdered formaldehyde, called paraformaldehyde, within the tracheal lumens. The formaldehyde presumably would dissolve in the tissue fluids and be released slowly from the devices to the surrounding epithelial cells. The acute and chronic effects

of formaldehyde exposure on the epithelial cells would be assessed by morphological, histochemical, and cell kinetics techniques.

The HEI Research Committee regarded the use of human tracheobronchial cells for evaluating the effects of formaldehyde as a positive aspect of this proposal. Another positive aspect was the opportunity to look for both preneoplastic and neoplastic changes induced by formaldehyde in the xenotransplanted human airway epithelial cells. The Research Committee recommended funding for a revised version of the proposal.

SPECIFIC AIMS AND STUDY DESIGN

The primary objective of the study by Dr. Klein-Szanto and colleagues was to study the carcinogenic, cocarcinogenic, and promoting effects of formaldehyde on human airway epithelial cells. The core of the study was the xenotransplantation system that provided for long-term maintenance of human airway cells in rat tracheas. For Studies 1 and 3, the investigators used epithelial cells obtained at autopsy from the tracheas and large airways of human infants less than one year old who had died from causes unrelated to the lungs. For Study 2, they used epithelial cells obtained at autopsy from the nasal mucosa of normal young adults who had died of accidental causes.

A purported advantage of the in vitro propagation of epithelial cells before inoculation into the rat tracheas was that cells from a single donor could be used for many different experiments. Thus, a complete matrix of dose and exposure duration combinations could be tested on cells from the same donor. This protocol would minimize variability among the results that could be attributed to interdonor differences.

The first of the investigators' three specific aims, addressed in Study 1, was to evaluate the acute and subacute effects of formaldehyde on the xenotransplanted epithelial cells caused by different exposure protocols. The focus of this objective was to determine the optimal formaldehyde dose for producing the maximal proliferative response in the investigators' system. Four weeks after the xenotransplantation of airway epithelial cells from infants, Dr. Klein-Szanto and associates exposed the cells to formaldehyde by inserting silastic devices containing 0, 0.5, 1.0, or 2.0 mg paraformaldehyde into the tracheal lumens for two, four, eight, or sixteen weeks. For Study 2, they exposed xenotransplanted nasal epithelial cells from adults to 0, 0.5, or 1.0 mg paraformaldehyde for two or eight weeks.

After exposure, the tracheas were removed from the mice and prepared for evaluation by several methods. Using light microscopy, the investigators determined the percentages of the tracheal surfaces occupied by normal, hyperplastic, squamous metaplastic, and atrophic epithelium. These pathologic categories indicated the extent of alterations in the appearance of the epithelia caused by the formaldehyde exposures.

The proliferative activity of the epithelial cells was evaluated using cell-labeling indices, a technique that takes advantage of the fact that dividing cells incorporate radioactive materials into their DNA. To label the cells, mice were injected with radioactive tracers 45 minutes before they were killed. The investigators processed rings of tracheal tissue with autoradiographic techniques and then identified and counted cells that had divided recently. Control data were obtained from tracheas implanted into mice that contained silastic devices without formaldehyde. In addition, the investigators used in situ hybridization techniques to verify that the DNA, and consequently the airway epithelial cells themselves, were of human origin.

Specific aims two and three were addressed in Study 3. The second specific aim was to evaluate the airway epithelial cells' response to repeated exposure to silastic devices containing paraformaldehyde. The third specific aim was to evaluate the ability of formaldehyde to act either as a promoter or as a cocarcinogen when combined with a known carcinogen, benzo[*a*]pyrene. This carcinogen was selected as a positive control for inducing changes in the epithelial cells within this system. The protocol for these two specific aims is outlined in Table 1 of the Investigators' Report. It should be noted that during an early phase of the study, the Research Committee recommended that the investigators use a metabolite of benzo[*a*]pyrene, benzo[*a*]pyrene diol epoxide (BPDE). This substitution eliminated the necessity for the epithelial cells to metabolize benzo[*a*]pyrene to its most carcinogenic metabolite. The morphologic changes in the infant-derived epithelial cells and the percentages of tracheal surface occupied by each cytologic category were evaluated 6 and 12 months after the silastic devices were implanted.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators successfully completed the morphologic analyses and evaluation of the labeling index data for the single-dose formaldehyde exposure experiments (Study 1). The investigators applied their considerable expertise with the technical and analytical procedures to the entire matrix of dose and time exposures. Unfortunately, their pro-

posed plan to carry out multiple, sequential formaldehyde exposures was not entirely successful. This failure was largely attributable to the technical difficulty of reinserting a new silastic device into the tracheal lumen after the prior one was removed.

Experiments on nasal epithelium (Study 2) were partially completed. Results from the cell-labeling index experiments were discussed, but no data were presented. The initiation, promotion, and cocarcinogenesis experiments with formaldehyde and BPDE (Study 3) were partially completed; data were not obtained for all times, exposures, and replicates. As in Study 2, the results of the labeling index experiments of Study 3 were discussed, but no data were presented. Overall, the authors reported the findings of their studies with clarity and accuracy, and they acknowledged appropriately many of the deficiencies associated with their study.

STUDY DESIGN AND METHODS

The investigators utilized a novel and potentially useful experimental in vivo culture system that permits exposure of human airway epithelial cells to a variety of test drugs and other agents. Although this exposure system is technically challenging, it provides an environment that closely simulates many characteristics of the trachea in an intact animal. These investigators demonstrated that their in vivo culture system could maintain human airway epithelial cells for longer periods of time than other types of cell culture systems.

Under the conditions of this study, the cells maintained their differentiated morphology. However, the investigators did not evaluate whether key biochemical features of the epithelial cells, such as the synthesis of specific products or enzymes, were similarly maintained for the duration of the experiments. They also did not provide information about the appearance or biochemical characteristics of the airway cells before the insertion of the silastic device. One important parameter that was not addressed either in the original proposal or in this report was the impact of using tissues from three different animal species on the experimental end points.

The investigators initially expected that they would propagate enough cells from each donor in vitro so that a sufficient number of tracheas could be inoculated with cells and tested for all of the proposed time and dose combinations. However, this goal was not achieved because of a variety of technical difficulties, including cell culture contaminations, animal infections, and animal deaths. Consequently, the data generated for each time-dose combination are averages calculated from assorted groups of donors. Whether combining data from different groups of donors influenced the experimental outcomes is uncertain because the investigators did not specify which donors contributed which cells to the various dose and time combinations. In addition, the effects of variability in pertinent characteristics among the groupings of different donors can not be readily tested because the investigators did not provide or apparently collect information about the age, sex, clinical status, or cause of death for the different donors.

Two factors clearly compromised interpretation of the data obtained from these xenotransplantation experiments. First, the experiments with BPDE alone, the putative positive control studies, did not produce neoplastic changes in the xenotransplanted epithelium. Although a carcinogen capable of acting as a positive control for this system has not yet been identified, such experiments could have established whether the human airway cells in this xenotransplantation system were capable of expressing neoplastic changes. Second, these studies were performed with the underlying, though unproven, assumption that cells derived from different donors are biologically equivalent with respect to their response to injurious agents, their ability to proliferate, and their capacity to mount protective responses.

A strength of this experimental design is that the cells were cultured in intact animals on biologically appropriate substrata. To some extent, this growth environment should mimic the environment that the cells encounter in an intact human trachea. Such an environment could supply a complete range of growth factors, cytokines, hormones, metabolites, and other unrecognized factors needed for appropriate cellular responses. It is unlikely that this environment could be matched as well with standard methods of cell or organ culture. However, if this model is to be used optimally in future experiments, much information needs to be acquired and considered regarding the cross-reactivity among species for growth factors, cytokines, integrins, integrin receptors, and other elements of signal transduction systems. All these factors could act as regulatory signals for cells treated with formaldehyde, a combination of formaldehyde and BPDE, or other agents. Thus, the experimental model has great potential, but it will require further detailed evaluation before it can provide mechanistic data at the cellular and molecular levels.

The analytic procedures, which were predominantly standard morphologic methods, were well-known to the investigators and appear to have been executed carefully. Because categorization of epithelial subtypes is subjective, the use of an observer who was blinded to the experimental protocols would have increased the confidence in the data. The investigators did not indicate whether the results of the cytologic analyses were verified by a second individual reading the same slides.

The cell-labeling index data relied on the incorporation of radiolabeled thymidine into DNA, a phenomenon that occurs during both cell replication and DNA repair. Although methods are available for discriminating between these two events, no such data were provided for this system. Because formaldehyde can act both as an irritant and as a potential carcinogen, either response could increase cell proliferation. In addition, the investigators suggested that the intratracheal silastic device may have acted as a mechanical irritant. The effects of this irritation on cellular metabolism, such as incorporation of thymidine into damaged DNA, were not addressed directly. Therefore, the labeling index data cannot be interpreted readily because no data analyses were performed to discriminate clearly between irritant effects of the silastic device and cellular proliferative events in response to the formaldehyde exposures.

STATISTICAL METHODS

A systematic experimental design was employed to assess the dose-response relationship between cellular proliferative events and formaldehyde exposure in Study 1. The two response variables were the cell-labeling index and the percentage of abnormal epithelium. The two dose variables, the amount of formaldehyde (0, 0.5, 1, or 2 mg), and the duration of exposure (two, four, eight, or sixteen weeks) were applied in a substantial subset of the 16 possible combinations.

The results were analyzed in two ways. The first method involved a nonparametric comparison between each pair of dose-time combinations. Analysis of the data for the cell-labeling index, which was measured for 15 dose-time combinations, produced 105 statistical comparisons (14 + 13 + . . . + 1) (Figure 7a). Analysis of the data for the percentage of abnormal epithelium, which was measured for 13 dose-time combinations, produced 78 statistical comparisons (12 + 11 + . . . + 1) (Figure 8a). The critical probability level for declaring statistical significance in each comparison was tightened to compensate for the increased probability of spurious results derived from multiple testing. The results of the pairwise tests are presented graphically in 4 × 4 grids, which are difficult to interpret because of the density of information and their emphasis on detail, as opposed to the broad scientific theme of response to the formaldehyde exposure dose and duration.

A second analysis, remedying most drawbacks of the first method, involved regression analysis. Each response variable was transformed logistically and analyzed as a function of dose and duration of exposure (Figures 7b and 8b). The two predictor variables were treated in a variety of ways, including discrete categories, linear predictors, and quadratic terms. This approach dealt with human airway cells' response to the important variables, dose and duration of exposure, in a more focused and useful way than the preceding approach, which covered every dose-time combination indiscriminately. The results from this second analysis are presented sketchily, but appear to be consistent with those of the nonparametric analysis.

RESULTS AND INTERPRETATION

The major results of these studies can be summarized briefly. In general, the investigators confirmed the findings of their earlier studies, which showed the feasibility of establishing long-term cultures of human airway cells in mice using the xenotransplantation culture system (Klein-Szanto et al. 1982). They also verified that the xenotransplanted cells were human in origin by examining the DNA with in situ hybridization techniques. Although the epithelia exposed to formaldehyde displayed morphologic changes that altered their normal appearance of predominantly mucous and ciliated cells, the investigators found no evidence of neoplasia with any treatment protocol using formaldehyde alone or in combination with BPDE. Their interpretation that, under the conditions of these experiments, neither formaldehyde nor combinations of formaldehyde and BPDE induced cytologic changes indicative of neoplasia was appropriate. However, this interpretation is tempered by the fact that the investigators provided no evidence that the human airway cells used in this culture system were capable of expressing neoplastic changes.

The cellular capacity to express neoplastic changes is particularly important because many signaling events can influence cellular behavior. Signals can come from growth factors, cytokines, and other molecules originating from cells other than the targeted epithelial cells, such as nearby fibroblasts. As a result, a definitive interpretation of this study and an understanding of cellular responses to formaldehyde require that two questions be answered. The first is whether such signals, if indeed produced by host mouse or rat cells, can influence the behavior of human cells. The second question is whether cells from human infants are capable of expressing receptors and responding to formaldehyde like fully differentiated adult cells. An absence of these types of basic information for this xenotransplantation system limits the usefulness of the data from this study.

Although all four major categories of epithelial subtypes (normal, hypertrophic, atrophic, and squamous metaplastic) were observed in tracheas from each of the experimental protocols, no consistent trend toward neoplastic changes was evident. Atrophic epithelium was more apparent in tracheas treated with formaldehyde, but it was also observed, to a varying extent, in control tracheas. Considerable vari-

ability existed among the percentages of the different epithelial categories for all experiments and all exposure protocols. The underlying reason for this variability is not well understood.

The data regarding changes in the percentages of epithelial cell types induced by formaldehyde on the xenotransplanted epithelium were interesting. However, the investigators did not include a category for tracheal surface area without epithelium, that is, denuded areas. Although the loss of epithelium may in fact have been an important response to formaldehyde, to the formaldehyde and BPDE combination, or to mechanical irritation caused by the silastic device, no data were collected to test these hypotheses. The investigators suggested that the percentage of denuded surface area was variable, but a cause for this effect was not determined.

The cell labeling index data from Study 1 showed a definitive relationship between the dose of formaldehyde and the duration of the exposure. These changes were most evident two and four weeks after the implantation of the silastic device containing paraformaldehyde. By 8 and 16 weeks, the cell-labeling indices of tracheal cells exposed to lower formaldehyde doses approached control values, whereas the cell-labeling index data for cells exposed to high formaldehyde doses remained above control values. Despite some technical difficulties, the investigators also clearly demonstrated the feasibility of using two agents in sequence, in this case formaldehyde and BPDE.

The investigators presented no data for the amount or concentration of formaldehyde to which the cells were actually exposed. Instead, they measured the amounts of formaldehyde remaining in silastic devices that had been implanted in tracheas with and without human airway cells for intervals ranging from 2 to 56 days. These two measurements cannot and should not be equated. The relevance of the observed cellular changes to the potential effects of formaldehyde in humans largely hinges on knowledge of the actual measured formaldehyde concentrations to which the cells in these experiments were exposed. The investigators' data indicated that approximately 65% of the formaldehyde was released during the first week; slower release of the remaining dose occurred during the subsequent seven weeks. This large release during a relatively short time span was compounded by the irritant and abrasion effects produced by the silastic devices on the tracheal epithelia. However, the contribution of each of these factors to the observed epithelial changes was not discussed in the report.

The actual formaldehyde concentrations to which the cells were exposed may have been affected by the rate at which formaldehyde was removed from the cellular environment, by the metabolism of formaldehyde, and by the diffusion of formaldehyde into the tissues and lymph fluid. In other experimental systems, investigators have measured directly the concentration of small molecules within very restricted tissue compartments (Eichele and Thaller 1987). Although such analyses were neither proposed nor conducted by the investigators, they would have been helpful for interpreting the results of this study.

Finally, an overall interpretation of this study is further restricted by the limited types of analyses used for studying the xenotransplanted airway cells. Although the experiments were performed expertly, the morphologic data obtained are static in nature; they provide no insight into the cellular or molecular biochemical processes induced by formaldehyde exposure. Although such processes may have been altered by formaldehyde alone, by combined formaldehyde and BPDE exposure, or by the xenotransplantation event itself, the reported observations of altered epithelial morphology are inadequate to deduce such changes accurately.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

Several key questions remain unanswered regarding the potential for formaldehyde to induce neoplastic changes in human airway epithelial cells. As a result, further studies using a variety of experimental approaches and designs, including the one selected by these investigators, are needed for testing the effects of formaldehyde at the molecular, cellular, and organismal levels. The further justified use of this xenotransplantation system as a test system for potential carcinogens depends on whether the xenotransplanted epithelial cells can exhibit neoplastic changes in this system following treatment with known carcinogens. Another important parameter that must be determined for this exposure system is the concentrations of formaldehyde or other toxic compounds that are released directly from the silastic device onto the epithelial cells. This dose information is critical for extrapolating any data obtained from this system to effects that may occur in humans due to exposures to environmental toxicants.

The experimental system used in this study presents promise for long-term studies with human airway epithelial cells. This promise is contingent on careful analyses of the relationship between cells and molecules of the host and those of the donor. When carefully characterized, xenotransplanted human airway cells should be able to serve as a test system for analyzing a wide spectrum of toxic agents, carcinogens, and drugs. Correlations between results from this exposure system and data from other experimental systems,

such as whole animals and isolated molecules, can be very useful. Such information could provide an understanding of molecular mechanisms induced by formaldehyde that, in composite, accurately reflect human health effects.

CONCLUSIONS

The major goal of this study was to evaluate the potential of formaldehyde to act as a carcinogen, cocarcinogen, or promoter for human airway epithelial cells. A novel feature of this study was the propagation and maintenance of human airway cells on the connective tissue stroma of rat tracheas. As demonstrated by these investigators, a significant advantage of their xenotransplantation system is that essential differentiated structural features of the cells can be maintained for periods ranging from weeks to months. These are very long time periods compared with those used in standard methods of cell culture, and thus provide an opportunity for prolonged toxicity testing. Unfortunately, technical difficulties encountered by the investigators during the study compromised collection of a complete data set for all of the proposed experiments.

The investigators reported that formaldehyde exposure induced the appearance of abnormal cellular features, including atrophic, hypertrophic, and squamous metaplastic epithelia. However, no neoplastic changes indicative of cancer were observed in the human airway epithelial cells, even after 6 and 12 months of exposure. Because no carcinogen has yet been identified as a positive control for this system, a significant uncertainty that follows from these results is whether this system is capable of demonstrating preneoplastic and neoplastic changes in human airway epithelial cells.

The relationship between the results of this study and any adverse effects that may occur in humans who inhale formaldehyde is not entirely clear. This is due to an incomplete understanding of the xenotransplantation system. An important question is whether the human airway cells maintained in this system are capable of responding to formaldehyde or other injurious agents with the full spectrum of cellular and molecular faculties that can be expressed in vivo. Certain data, such as the positive correlation between increased formaldehyde dose and an increased labeling index, suggest that formaldehyde exposure increased cell proliferation. This interpretation, however, is not definitive because the analyses of cell proliferation were static in nature; no dynamic tests were made to prove directly that the cells were proliferating.

Further use of this exposure system to test the toxicity of potential carcinogens, environmental pollutants, and other toxins would benefit from refinement of the analytical approaches to include more molecular biological techniques. Through such methods, the cellular and molecular relationships among human, rat, and mouse tissues and cells could be understood more clearly. Additional future directions for this system could include direct assessment of the biochemical, proliferative, and metabolic processes of the airway cells in response to a toxic exposure.

Because the test cells originated from humans rather than laboratory animals, the xenotransplantation system used by these investigators has the potential to yield results relevant to the adverse human health effects caused by environmental pollutants. This and other exposure systems using human cells can serve as valuable tools for testing the toxicity and carcinogenicity of environmental pollutants such as formaldehyde.

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