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Necrosis of Nasal and Airway Epithelium in Rats Inhaling Vapors of Artificial Butter Flavoring

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As the result of a high prevalence of fixed airways obstruction in workers at a microwave popcorn manufacturing plant, we examined the hypothesis that vapors of butter flavoring used in the manufacture of microwave popcorn and other foods can produce airway injury in rats. Rats were exposed to vapors liberated from heated butter flavoring. Rats were exposed for 6 h by inhalation and were necropsied 1 day after exposure. The exposure was found by GC–MS analysis to be a complex mixture of various organic gases with the major peaks consisting of diacetyl (2,3-butanedione), acetic acid, acetoin (3-hydroxy-2-butanone), butyric acid, acetoin dimers, 2-nonanone, and δ-alkyl lactones. Diacetyl was used as a marker of exposure concentration. In the lung, butter flavoring vapors containing 285–371 ppm diacetyl caused multifocal, necrotizing bronchitis, which was most consistently present in the mainstem bronchus. Alveoli were unaffected. Butter flavoring vapors containing 203–371 ppm diacetyl caused necrosuppurative rhinitis, which affected all four levels of the nose. Within the posterior two nasal levels (T3 and T4), necrosis and inflammation was principally localized to the nasopharyngeal duct. Control rats were unaffected. Therefore, concentrations of butter flavoring vapors that can occur during the manufacture of foods are associated with epithelial injury in the nasal passages and pulmonary airways of rats.

Key Words: **flavoring; diacetyl; butter; popcorn; lung; nose; bronchitis; airway obstruction.**

Individuals in the food industry work with products that are considered safe to eat. Among the agents used in the food industry are substances used to flavor foods and many of these have been evaluated for safety. However, the designation of a flavoring as "generally recognized as safe" (GRAS) is intended to protect the consumer, not the worker. Indeed, the GRAS designation applies to exposure to the flavoring by the oral route, in low concentrations, by the consumer (Woods and Doull, 1991; Munro *et al.*, 1998). This designation is not intended to imply safety for a different kind of exposure, such as inhalation exposure in workplaces producing flavorings or using flavorings to produce commercial foods.

The National Institute for Occupational Safety and Health (NIOSH) has conducted Health Hazard Evaluations involving lung disease in workers exposed to inhaled flavorings (Kreiss *et al.*, 2002). In one of these studies, several compounds were detected in the workplace, including diacetyl (2,3-butanedione), acetoin (2-hydroxy-3-butanone), 2-nonanone, and methyl ethyl ketone (2-butanone). The major ketone present was diacetyl, a volatile ketone that imparts the odor and flavor of butter to food and is on the GRAS list (Food and Drug Administration, 2001). The apparent source of the diacetyl and acetoin in the workplace was butter flavoring used in microwave popcorn packaging (Kreiss *et al.*, 2002). Due to limited data on the respiratory toxicity of inhaled butter flavorings or diacetyl mixtures, we have investigated the hypothesis that butter flavoring vapors (BFV) cause respiratory injury when inhaled in concentrations that may occur in the workplace.

MATERIALS AND METHODS

Characterization of the butter flavoring vapors. The composition of the BFV was assessed using a Perkin–Elmer ATD automatic thermal desorption system containing an internal focusing trap that was directly connected to an HP6890A gas chromatograph with an HP5973 mass selective detector (TD—GC–MSD). For vapor analysis, the butter flavoring, which is a solid at room temperature, was heated to 50°C for 10 min in the ATD and the headspace was analyzed for composition using the TD—GC–MSD. Peaks were identified by comparison with the gas chromatograph–mass spectrometry (GC–MS) library.

Experimental animals. Male, specific pathogen-free, Sprague–Dawley rats [Hla:(SD)CVF], 200–250 g, were obtained from Hilltop Laboratories (Scottdale, PA). Rats were housed in HEPA-filtered, individually ventilated, shoebox cages containing autoclaved Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, which were placed in animal racks (Thoren Industries, Hazelton, PA). The NIOSH Animal Facility is an environmentally controlled, barrier facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. During the expo-

[1] Mention of company or product names does not constitute endorsement by the National Institute for Occupational Safety and Health.

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TABLE 1
Clinical and Anatomical Pathology Specimens from Rats Exposed to Different Concentrations of Butter Flavoring Vapors

	Diacetyl concentration (ppm)	VOC meter reading[a]	Total rats	Rats necropsied	Nasal histo-pathology	Pulmonary histo-pathology (left lung)	Transmission electron microscopy	Broncho-alveolar lavage (right lung)	Nasal lavage
Controls[b]	0	0	19	19	19	16	13	16	4
Low exposure group	203	298	6	6	6	6	6	6	ND
Middle exposure, group[c,d,e]	285	445–447	4	3	3	3	3	2	3
High exposure, constant group[b]	352	578	6	6	6	3	3	3	ND
High exposure, pulsed group	371 (range 72–940)	609	3	3	3	3	0	3	ND
Total butter flavoring vapor exposed (all groups)[b,d,e]	203–371	298–578	19	18	18	15	12	14	3

Note. Vapor concentration was measured both by the diacetyl concentration and the volatile organic compound meter reading. ND, not done; VOC, volatile organic compound.

[a] Volatile organic compound meter readings reflect relative but not specific (ppm) concentrations of organics in mixtures.

[b] Three control and three high exposure constant rats were used for plethysmography prior to pathology submission and data from pulmonary pathology and bronchoalveolar lavage on these rats is excluded.

[c] Ligation failed in one middle exposure rat and lavage data from that rat is excluded.

[d] One middle exposure rat died and was not submitted to necropsy.

[e] An additional four control and four middle exposure rats were used for nasal resistance measurements and were not submitted to pathology.

sures, rats were individually housed in wire cages within the 20 × 16 × 14 inch whole-body exposure chamber. The overall experimental design for clinical and anatomical pathology specimens is provided in Table 1.

Exposures. An inhalation exposure system was designed and built to deliver butter flavoring effluents to small laboratory animals. Butter flavoring contained in a glass vessel was continually stirred and maintained at 55°C with a water bath. Generator air was conditioned and blown across the heated flavoring, diluted as required, and delivered to the exposure chamber. The system was designed for exposing animals to either pulsed or constant concentration. For pulsed exposure, bulk portions of flavoring were added to the vessel at the beginning and halfway through the experiment. Concentration output exhibited large peaks and valleys similar to the exposure experienced by workers who periodically check the conditions inside the holding tanks. For constant concentration, a syringe pump provided for adjustable flow of flavoring to the vessel throughout the duration of exposure. Output from a direct reading photo ionization detector positioned inside the exposure chamber was used for feedback control of syringe pump output and diluent air flow to maintain concentration at specific levels. GC–MS analysis of the exposure chamber environment yielded a complex organic vapor spectrum similar to that seen from workplace samples. Diacetyl concentrations were measured in the exposure chamber using GC with flame ionization detection. We exposed animals for 6 h at the following exposure conditions; values are average diacetyl concentration estimates: low exposure (LE) group (constant), 203 ppm; middle exposure (ME) group (constant), 285 ppm; high constant exposure (HCE) group, 352 ppm; and high pulsed exposure (HPE) group, 371 ppm (range 72–940 ppm).

Bronchoalveolar and nasal lavage fluid collection and analysis. Rats were euthanized the day after exposure initiation (approximately18 h after completion of exposure) and bronchoalveolar lavage (BAL) was conducted with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) as previously described (Hubbs *et al.*, 2001). The right lungs of 16 control, 6 LE, 2 ME, and 3 HCE rats exposed to BFV were lavaged. The identification and quantitation of BAL cells, collection of acellular BAL fluid, quantitation of lactate dehydrogenase (LDH) activity and albumin levels in acellular BAL fluid, and the measurement of zymosan-stimulated alveolar macrophage chemiluminescence were conducted as previously described (Hubbs *et al.*, 2001). Nasal lavage fluid was collected from 3 ME and 4 control rats by flushing 10 ml of PBS from the nasopharynx to the external nares. BAL and nasal lavage cell counts and BAL differentials were determined using an electronic cell counter/sizer (Hubbs *et al.*, 2001).

Collection and assessment of histopathologic specimens. The noses of 19 control, 6 LE, 3 ME, 3 HCE, and 3 HPE rats were immersion fixed in 10% neutral buffered formalin. The left lungs of 16 control, 6 LE, 3 ME, 3 HCE, and 3 HPE rats were airway perfused with 3 ml of fixative. The fixative used for the lungs was 10% neutral buffered formalin for 3 HPE and 3 control rats; the lungs of remaining rats were perfused with Karnovsky's fixative, a formalin-containing fixative that permits both light microscopy and ultrastructural evaluation of tissue (Karnovsky, 1965). Nasal tissue was decalcified in 13% formic acid for 5 days, rinsed in water for approximately 4 h, and then sectioned at four levels as previously described (Young, 1981). Lung tissues and decalcified nasal sections were processed overnight, embedded in paraffin, and stained with hematoxylin and eosin. Slides were interpreted by a board-certified veterinary pathologist blinded to the exposure status of the individual animal. Numerical scoring of severity and distribution was performed as previously described (Hubbs *et al.*, 1997). Briefly, slides were scored for distribution (0, none; 1, focal; 2, locally extensive; 3, multifocal; 4, multifocal and coalescent; and 5, diffuse) and severity (0, none; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe). The pathology score is the sum of the distribution and severity score.

Collection and assessment of ultrastructural specimens. Sections were collected from the left lung for transmission electron microscopy from all rats except those in the HPE group and three corresponding controls (Table 1). Sections of the mainstem bronchus and alveoli were preserved in Karnovsky's fixative, postfixed in osmium tetroxide, mordanted in tannic acid, and stained *en bloc* in uranyl acetate. The tissues were then dehydrated in alcohol and embedded in Epon. The sections were stained with uranyl acetate and lead citrate. The mainstem bronchus of each rat was examined ultrastructurally using a JEOL 1220 electron microscope as outlined in Table 1. In addition, sections of alveoli were examined from the left lung of three HCE rats and three controls.

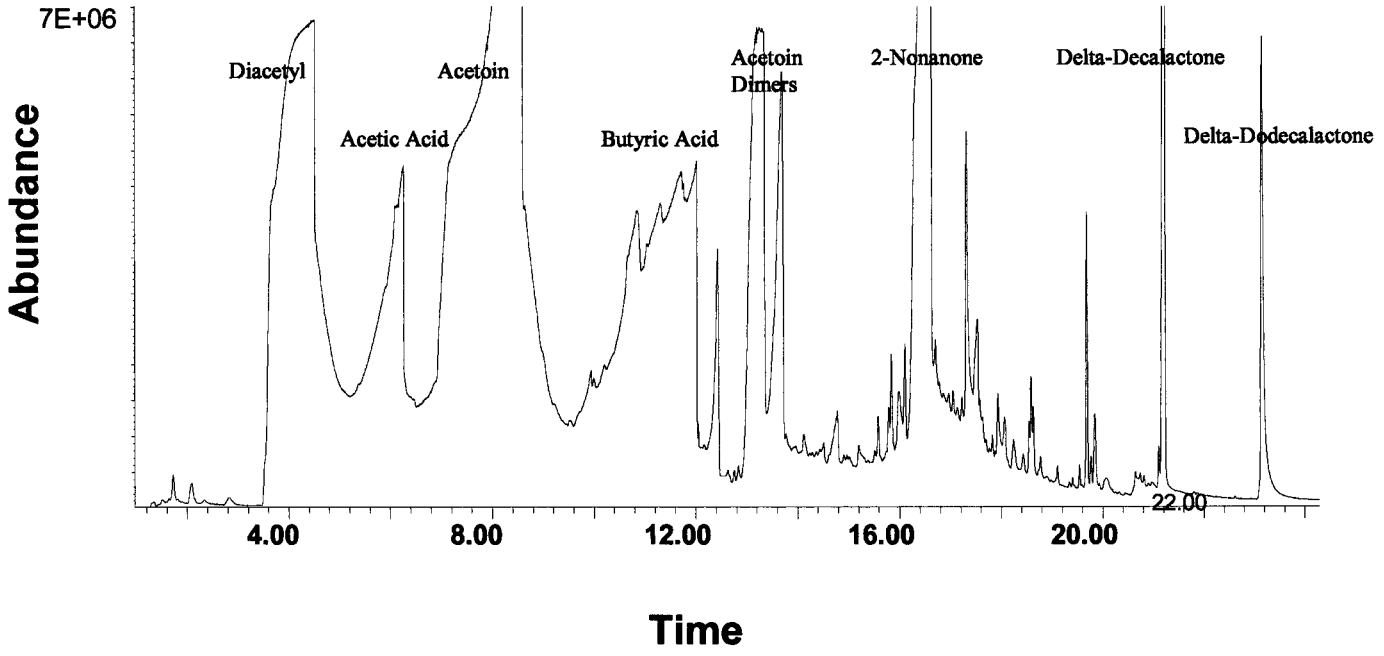

FIG. 1. Thermal desorption–gas chromatography–mass spectrometry chromatogram of volatile compounds present in butter flavoring. The major peaks are identified on the chromatogram.

Functional measurements. Nasal resistance was measured as previously described (Dalbey *et al.*, 1998). To assure accuracy of the resistance measurement, the esophagus was ligated during the procedure.

Statistics. Data were analyzed using commercial software (SigmaStat; SPSS Science, Chicago, IL).

RESULTS

Characterization of the Butter Flavoring Vapors

The TD–GC–MS chromatogram of the headspace (vapor phase) from the butter flavoring revealed a complex mixture of organic vapors (Fig. 1). The compounds associated with major peaks in the chromatogram were, in order of their retention times, diacetyl (2,3-butanedione), acetic acid, acetoin (3-hydroxy-2-butanone), butyric acid, acetoin dimers, 2-nonanone, and δ-alkyl lactones. Additional compounds identified from peaks on the GC–MS chromatogram were methanol, ethanol, acetone, methyl acetate, methylene chloride, meso-2,3-butanediol diacetate, caproic acid (hexanoic acid), dimethyl or ethyl resorcinol, caprylic acid, and capric acid. Two minor peaks did not have an exact match in the GC–MS library. One of these appeared to be comprised of aliphatic esters/diols/acid esters and the other may have been dihydroxytetramethyl dioxane.

Mortality

No rats died during the exposures. One ME rat and one HPE rat died after exposure.

Pulmonary Alterations, Light Microscopy

Inflammatory and/or necrotizing changes associated with airways were observed at the light microscopic level after inhaling BFV. These changes were not seen in control rats ($n = 16$) or LE rats ($n = 6$) but were seen in all nine rats exposed to higher concentrations, including ME ($n = 3$), HCE ($n = 3$), and HPE rats ($n = 3$) (Fig. 2). With the exception of one HCE rat (where the mainstem bronchus was absent from the section), the principal morphologic change was necrotizing bronchitis with decreasing severity of necrosis in smaller airways. The one rat in the two high exposure groups that did not have necrotizing bronchitis on light microscopic examination where the mainstem bronchus was absent from the section did have necrotizing bronchitis of the mainstem bronchus in sections collected for ultrastructural studies and additionally had suppurative inflammation centered around alveolar ducts and terminal bronchioles (bronchiolocentric inflammation). Morphologic changes were similar in the involved large airways of all affected rats. Necrosis and/or inflammation had a multifocal to multifocal and coalescent distribution with moderate to marked severity. Pulsed and constant exposures produced similar lesions in the mainstem bronchus but necrosis was localized to the mainstem bronchus in HCE rats in contrast to involvement of the mainstem bronchus and midsize bronchioles in HPE rats. The severity of airway and bronchiolocentric changes was significantly increased in ME and high-exposure (HCE and HPE) rats compared with control rats ($p < 0.001$, ANOVA; $p < 0.05$, Dunnett's intragroup test).

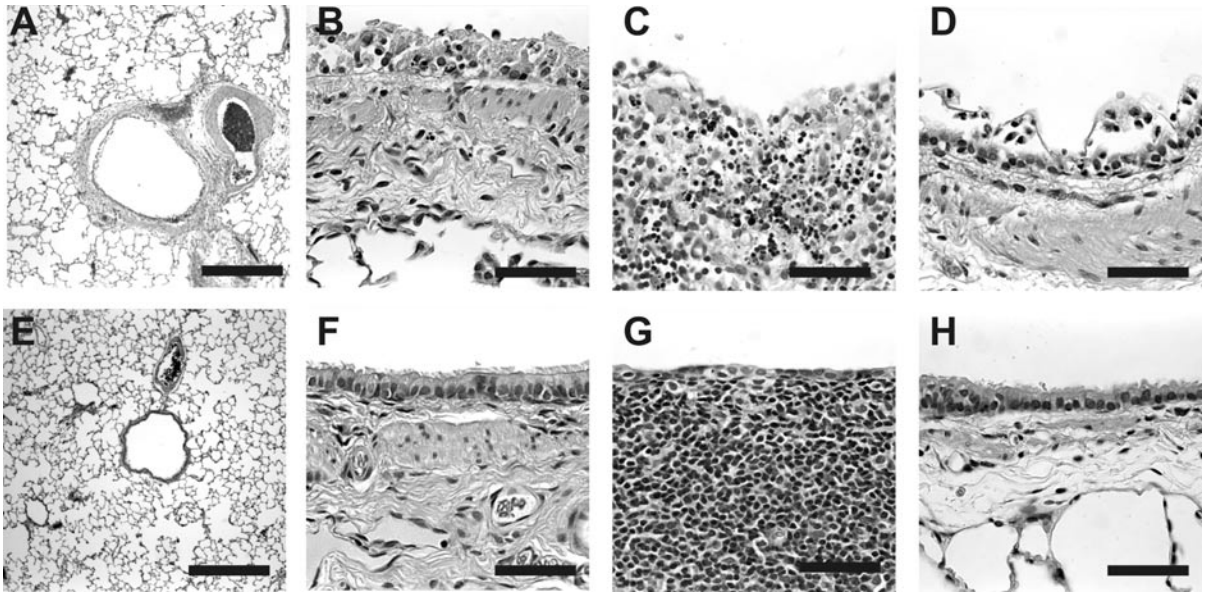

FIG. 2. Pulmonary histopathology of rats in the pulsed high exposure group (A–D) and control (E–H) rats. The control sections represent the normal tissue that has been damaged in the exposed group. (A) Necrotic bronchiolar epithelium lifting off the basement membrane of a rat exposed to butter flavoring vapors. Bar represents 500 μm. (B) Complete necrosis of the bronchiolar epithelium in a rat exposed to butter flavoring vapors. Bar represents 50 μm. (C) Necrosis extending beneath the epithelium and into bronchus-associated lymphoid tissue of an exposed rat. Bar represents 50 μm. (D) Necrosis and loss of individual airway epithelial cells in a smaller bronchiole. (E) A normal bronchiole in a control rat. Bar represents 500 μm. (F) Bronchiolar epithelium of a large bronchiole in a control rat. Bar represents 50 μm. (G) Mucosal epithelium and subjacent bronchus-associated lymphoid cells in a control rat. Bar represents 50 μm. (H) The epithelium of a smaller bronchiole in a control rat. Bar represents 50 μm.

BAL polymorphonuclear leukocytes (PMNs) were significantly elevated in HCE and ME rats ($p \leq 0.001$ ANOVA, $p \leq 0.05$ Dunnett's intragroup test). However, the number of BAL PMNs represented only a fraction of the potential inflammatory capacity of the lung (Table 2). Consistent with the BAL findings, a mild neutrophil infiltrate was seen histologically in airways of all rats exposed to the middle and high concentrations of BFV, but adjacent alveolar spaces were involved in only one of these rats. BAL albumin concentration and macrophage chemiluminescence were also significantly increased in HCE and HPE rats ($p \leq 0.001$ ANOVA, $p \leq 0.05$ Dunnett's intragroup test). The number of alveolar macrophages harvested by BAL and the BAL fluid LDH activity were not significantly changed after BFV inhalation.

Pulmonary Alterations, Ultrastructure

The major change seen by transmission electron microscopy was necrosis of the bronchial epithelium. The bronchial epithelium was severely necrotic in all ME and HCE rats (Fig. 3).

TABLE 2
Bronchoalveolar Lavage Parameters, Bronchial Histopathology Scores, and Nasal Histopathology Scores (mean ± SE) after Butter Flavoring Vapor Inhalation

	BAL macrophages	BAL neutrophils	BAL albumin	BAL LDH	Macrophage chemiluminescence	Bronchial pathology[a]	Nasal (T1) pathology[a]	Nasal (T2) pathology[a]	Nasal (T3) pathology[a,b]	Nasal (T4) pathology[a,b]
Controls	1.25 ± 0.14	0.35 ± 0.03	0.10 ± 0	28 ± 4	0.98 ± 0.13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LE group	1.12 ± 0.09	0.48 ± 0.09	0.11 ± 0.01	45 ± 7	3.66 ± 1.87	0 ± 0	7.8 ± 0.17*	6.17 ± 0.48*	6.2 ± 0.54*	6.0 ± 0.52*
ME group	1.80 ± 0.72	3.55 ± 1.92*	0.17 ± 0.03	33 ± 4	0.80 ± 0.55	6.3 ± 0.33*	8.67 ± 0.33*	8.0 ± 0.0*	10 ± 0*	8.67 ± 0.88*
HCE group	1.54 ± 0.28	3.19 ± 0.89*	0.23 ± 0.08*	34 ± 15	12.66 ± 4.41*	5.33 ± 2.67*	8.17 ± 0.17*	7.8 ± 0.17*	10 ± 0*	10.0 ± 0*
HPE group	0.88 ± 0.18	1.08 ± 0.36	0.27 ± 0.06*	38 ± 12	8.02 ± 2.82*	7.0 ± 0[3]*	7.0 ± 0.58*	6.7 ± 0.33*	7.7 ± 1.45*	7.7 ± 1.45*

Note. Values are means ± SE.

[a] Sum of severity and distribution scores.

[b] For sections T3 and T4, these are the scores for the ventral air passageways of the nose (nasopharyngeal duct and septal window).

[c] In the HPE group, bronchial lesions appeared to extend into smaller airways than in the other groups.

* Significantly different from controls ($p < 0.05$, ANOVA and Dunnett's).

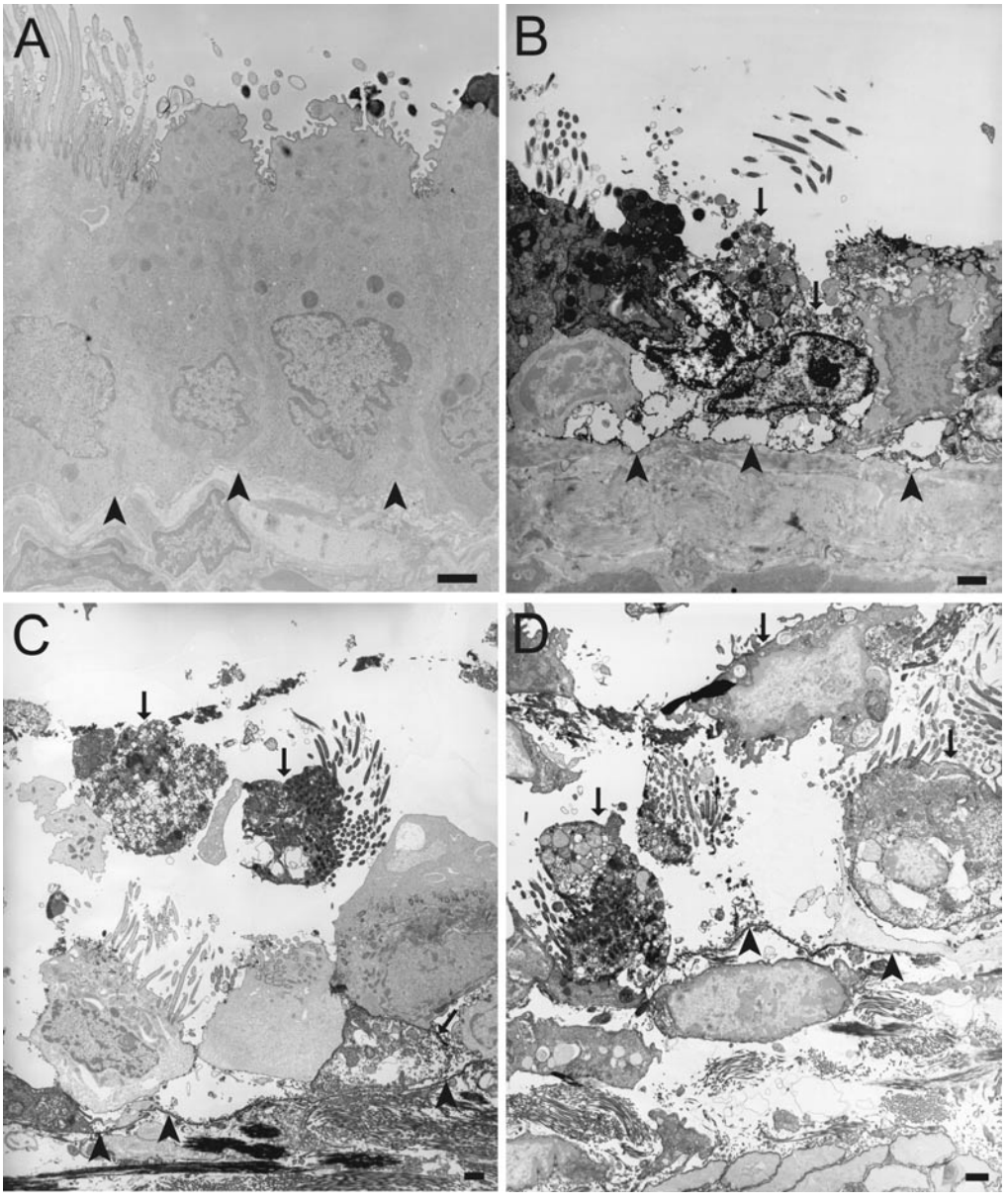

FIG. 3. Ultrastructural changes in the bronchus of rats inhaling BFV. (A) Normal bronchus of a control rat. Epithelial cells are firmly attached to the subjacent basement membrane (arrowheads). (B) Marked epithelial necrosis in a LE rat. Bronchial epithelial cells are detaching from the basement membrane (arrowheads) and the cytoplasmic membrane is disrupted consistent with necrosis (arrows). (C) Severe necrosis in an ME rat. Necrotic bronchial epithelial cells (arrows) are free in the lumen. Basement membrane (arrowheads) is multifocally denuded. (D) Severe epithelial necrosis in a HCE rat. Necrotic bronchial epithelial cells (arrows) are free in the lumen. Beneath the basement membrane (arrowheads), collagen fibers and fibrils are widely separated, consistent with edema of the lamina propria.

In addition, the bronchial epithelium of one LE rat was markedly necrotic. In ME and HCE rats, bronchial injury extended beneath the basement membrane and was characterized by edema of the lamina propria. Necrosis of the bronchial epithelium was not seen in controls.

Nasal Morphologic Alterations

Suppurative inflammation and necrosis of the epithelium lining nasal passageways characterized the response of the nose to inhaled BFV (Fig. 4). Nasal lavage fluid analysis revealed hypercellularity with a PMN predominance in ME rats. Histopathologic alternations were not seen in the noses of control rats but were present in the noses of all rats exposed to the low, middle, or high concentrations of BFV. Foci of necrosuppurative rhinitis frequently coalesced and necrosis sometimes extended beneath the epithelial basement membrane. Respiratory, transitional, and olfactory epithelium all had coalescing foci of necrosuppurative rhinitis. In the ventral

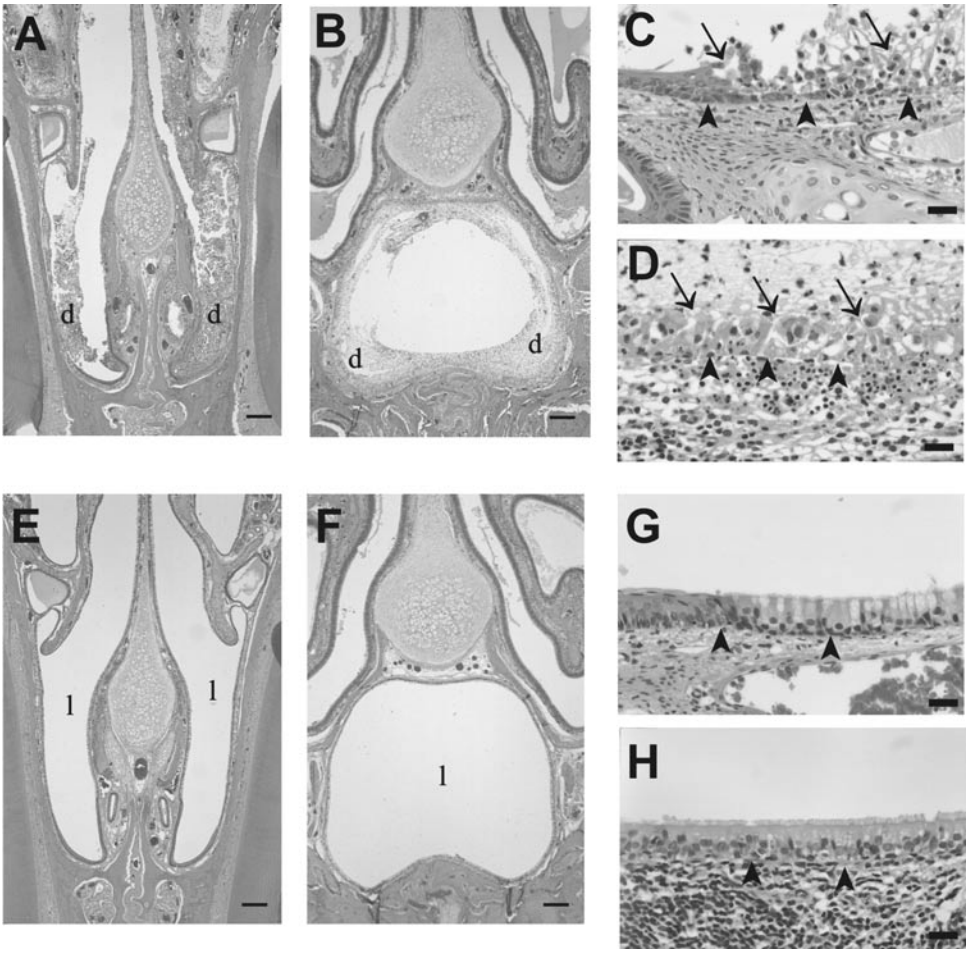

FIG. 4. Nasal histopathology of rats from the middle constant exposure (A–D) and control (E–H) groups. The control sections represent the normal tissue that has been damaged in the exposed group. (A) Debris and inflammatory cells (d) extending into the nasal lumen at level T1 of a rat exposed to butter flavor vapors. Bar represents 200 μm. (B) Debris and inflammatory cells (d) partially obstructing the lumen of the nasopharyngeal duct at level T4 of a rat exposed to butter flavoring vapors. Bar represents 200 μm. (C) Necrosis of the nasal epithelium (arrows) with less extensive involvement of the squamous epithelium and without extension beneath the basement membrane (arrowheads) at level T1 of a rat exposed to butter flavor vapors. Bar represents 20 μm. (D) Necrosis of the epithelium (arrows), which extends beneath the basement membrane (arrowheads) of the nasopharyngeal duct at level T3 of a rat exposed to butter flavoring vapors. Bar represents 20 μm. (E) Normal ventral nasal cavity at level T1 in a control rat. Bar represents 200 μm. The lumen (l) of the nasal passageway is unobstructed. (F) Normal nasopharyngeal duct at level T4 in a control rat. Bar represents 200 μm. The lumen (l) of the duct is unobstructed. (G) Normal junction of the squamous and respiratory epithelium at level T1 in a control rat. Bar represents 20 μm; arrowheads show basement membrane. (H) Normal respiratory epithelium lining the nasopharyngeal duct at level T3 of a control rat. Bar represents 20 μm; arrowheads show basement membrane.

portions of the anterior nose (T1 and T2), nasal tissue lined with squamous epithelium was less affected than adjacent nasal tissue lined with respiratory epithelium. Further back in the nose, dorsal portions of the nose were less affected than the ventral portions. In the most posterior two levels (T3 and T4), necrosis and inflammation was principally localized to the nasopharyngeal duct within the ventral portion of the nose. Severity of necrosis was significantly increased in all nasal levels and at all exposure concentrations relative to controls ($p < 0.001$ ANOVA, $p < 0.05$ Dunnett's intragroup test). Severe necrosis with luminal accumulation of inflammatory cells and cellular debris appeared sufficient to have impaired air flow in the obligate nasal breathing rat.

Functional Measurements

BFV at the middle exposure increased nasal resistance by 6.5 $\pm$ 1.3-fold (mean $\pm$ SE).

DISCUSSION

The major change associated with acute inhalation exposure of rats to BFV was necrosis of nasal and airway epithelium. The necrosis was seen in the pulmonary airways of all ME, HCE, and HPE rats and one LE rat. In addition, necrosis occurred in the epithelial lining of the nasal cavity in all rats exposed to BFV, regardless of exposure concentration. Thus,

the no-effect level for 6-h exposures in the rat lies below the levels that we investigated. Data are currently insufficient to determine whether the necrosis is dependent upon peak concentration or some combination of exposure concentration and time. However, we did see necrosis extending into more distal portions of pulmonary airways with a pulsed exposure that included higher peak exposures than the constant exposure pattern. Additional research will be needed to definitively establish the role of peak exposure on lesion distribution and severity.

The pattern of distribution of epithelial necrosis with lower concentrations producing more damage to nasal than airway epithelium is consistent with previous reports of greater uptake of many vapors in the nose (Dahl, 1989). In particular, water-reactive and water-soluble vapors tend to accumulate in nasal tissues (Heck, 1983; Dahl and Bechtold, 1985, Medinsky and Bond, 2001). Diacetyl and acetoin are major components of the butter flavoring used in this study and are highly water soluble (Food and Drug Administration, 2001). The ability of diacetyl to stimulate cellular replication in the pylorus after gavage administration is also consistent with the ability of diacetyl to play a role in cytotoxicity even in the relatively injury-resistant gastric epithelium (Furihata *et al.*, 1985). While the pattern of preferred distribution to the nose characteristic of many vapors potentially decreases the potential for BFV to cause damage to airways in the lung when individuals are nose breathing, humans are capable of mouth breathing (Ward *et al.*, 1993). If necrosis associated with BFV exposure is the result of the accumulation of water-soluble vapors in the mucous layer, the mouth-breathing worker would be expected to be at greatly increased risk of pulmonary airway damage after BFV exposure.

Importantly, the vapors emitted from butter flavoring are a complex mixture that produces necrosis that cannot be explained by the known toxicologic properties of any of its components.

The pulmonary lesions in workers exposed to vapors of butter flavoring have not been described. While the multifocal necrotizing changes present in the nasal and airway epithelium of acutely exposed rats suggest that the initial injury in these workers could be necrosis, there are no published pathology reports from exposed workers available for comparison. There is no animal model of bronchiolitis obliterans produced by toxic injury with which to compare our findings. However, the denuded rat heterotopic tracheal transplant has recently been reported to be a model of the form of bronchiolitis obliterans associated with lung transplantation–with obliterative airway disease resulting from a lack of epithelial cells during repopulation of denuded tracheas (Adams *et al.*, 2000). This has been interpreted to suggest that respiratory epithelial cells may release mediators that limit mesenchymal proliferation (Adams *et al.*, 2000). While large foci of necrosis exist in the airways of rats exposed to BFV, additional experiments will be needed to determine whether these morphologic lesions are predictive of bronchiolitis obliterans or other bronchial changes that may explain fixed airway obstruction in workers.

The concentrations used in this study are not extraordinary when compared with levels measured in the workplace. Diacetyl concentration measured inside the butter flavoring holding tank at the microwave popcorn plant was 1232 ppm (Kreiss *et al.*, 2002). A concentration of about 200 ppm was measured inside a vat that contained butter flavoring mixed with oil and salt. These measurements were made after installation of local exhaust ventilation for these tanks and previous concentrations were probably higher. The highest time-weighted average diacetyl concentration measured in a general work area was about 100 ppm (Kreiss *et al.*, 2002).

The necrosis of airway epithelium after exposure to BFV at concentrations similar to peak workplace levels is a major concern. The airway epithelium plays major roles in airway defense (Diamond *et al.*, 2000) and airway reactivity (Fedan and Frazer, 1992). In addition, airway epithelial cells are believed to play a protective role in preventing obliterative airway disease (Adams *et al.*, 2000). Since the general mechanism of healing of large tissue defects is not regeneration but scarring (Cotran *et al.*, 1989), the large foci of necrosis in the lungs of the MCE, HCE, and HPE rats cause concern for scarring of the conducting airways. While repair is possible in the injured epithelium (Puchelle, 2000), the extent of the nasal injuries seen in the rats and the general tendency for rats to be nasal breathers prevented our maintaining these rats long enough to determine the long-term sequelae to these exposures. A recently developed mouth-breathing rat model may permit investigation of the long-term sequelae to BFV exposures, particularly at lower concentrations (Dalbey *et al.*, 1998). Additional studies are planned to determine the active agent or agents in BFV.

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