Correlation of Regional and Nonlinear Formaldehyde-induced Nasal Cancer with Proliferating Populations of Cells

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ABSTRACT

Formaldehyde induces nonlinear, concentration-related increases in nasal epithelial cell proliferation and squamous cell carcinomas (SCC) in rats. A formaldehyde carcinogenicity study was conducted in which a major end point was correlation of cell proliferation indices with sites of formaldehyde-induced SCC. A poor correlation in certain sites led to incorporation of the number of cells in each site into the correlation. Rats were exposed (6 h/day, 5 days/week) to formaldehyde (0, 0.7, 2, 6, 10, or 15 ppm) for up to 24 months with interim sacrifice time points at 3, 6, 12, and 18 mo. A unit length labeling index (ULLI; S-phase nuclei/mm basement membrane) was determined for specific nasal regions in addition to a population-weighted ULLI (PWULLI). The PWULLI was defined as the product of regional ULLI and total number of nasal epithelial cells in the respective site. Nasal SCC sites of origin were mapped. Formaldehyde induced SCC in a highly nonlinear fashion, with no observed effect at the level of 2 ppm, a minimal response at 6 ppm, and a sharp increase at 10 and 15 ppm. The tumor incidence was 1, 22, and 47% at 6, 10, and 15 ppm, respectively. ULLI was significantly (P < 0.05) increased at 10 and 15 ppm but not at the lower concentrations. There was a good correlation between PWULLI and regional tumor incidence (R² = 0.88), while the correlation of regional SCC with ULLI was relatively poor (R² = 0.46). We conclude that target cell population size and sustained increases of cell proliferation in these populations, determined by differences in regional airflow-driven formaldehyde dose to these sites, coupled with the known nonlinear kinetics of formaldehyde binding to DNA, can together account for the nonlinearity and site specificity of formaldehyde-induced nasal SCC in rats.

INTRODUCTION

Formaldehyde is an extensively used industrial chemical that requires accurate risk characterization and assessments for optimal risk management. The number of individuals occupationally exposed to formaldehyde in the United States exceeds 1.5 million, with an additional 11 million people potentially exposed in their homes by the offgassing from building materials or from other indoor air pollutants such as tobacco smoke (1—4). The finding that formaldehyde is a nasal carcinogen in rats (5—8) provoked concern that this chemical also may pose a cancer risk for humans. Federal regulatory action in response to the positive rodent cancer bioassay included estimating the low exposure risk to formaldehyde from the high-exposure animal data using a linearized multistage model that did not take into account the nonlinearities in the exposure concentration-response relationship (9). A recent review of formaldehyde data by the IARC concluded that there is sufficient evidence for carcinogenicity in experimental animals but only limited evidence in humans, with an overall conclusion that formaldehyde is probably carcinogenic to humans (10). Collectively, however, it has been concluded elsewhere that evidence from epidemiological data for exposed individuals fail to provide credible evidence of a causal association between formaldehyde exposure and human cancer incidence (11—15). Because of these uncertainties concerning the human risks associated with formaldehyde exposure, it is important that mechanistic data be incorporated into the human risk assessment process (16—21).

A potentially important feature of formaldehyde-induced nasal SCC in rats is their regional site specificity (22, 23). Previous work in our laboratory determined that the distribution of nasal lesions induced by toxic chemicals is attributable to regional dosimetry, local tissue susceptibility, or a combination of these factors (24, 25). The regional localization of formaldehyde-induced neoplastic and nonneoplastic lesions in rodents (22, 26—28) and nonhuman primates (29) has been evaluated to assess the role of regional airflow dosimetry in formaldehyde toxicity. During inhalation exposure, the intranasal deposition of formaldehyde is not uniform throughout the nasal lining, and it was concluded that airflow-driven dosimetry plays a major role in the location of nasal responses to formaldehyde (30, 31). This hypothesis was supported by recent molecular dosimetry studies measuring formaldehyde-induced, DNA-protein cross-links in rat and nonhuman primate nasal epithelium (16, 21). Formaldehyde-induced nonneoplastic responses, such as inhibition of mucusciliary function, epithelial degeneration, inflammation, squamous metaplasia (27), and increased cell proliferation (32) correlate well with these site-specific uptake patterns for formaldehyde. However, not all nasal sites receiving a high dose of formaldehyde develop tumors, with the medial aspect of the maxilloturbinate being a notable exception (22).

An increasing amount of evidence suggests that chemically induced cell proliferation plays a key role in chemical carcinogenesis (33—35). Cell proliferation is required to convert promutagenic DNA damage to mutations and to clonally expand initiated (mutant) cells to a large enough population that the probability of an additional critical mutation is high. Moreover, cell proliferation is necessary to enhance the progression of neoplasia (36). Some chemicals that induce cancer only at high doses in animal bioassays often fail to fit the traditional characterization of mutagens. A threshold exposure concentration for cancer induction has been proposed for such chemicals that induce cancer as a consequence of chemically induced cell proliferation (33—35). Whether such a threshold exists for weakly genotoxic chemicals such as formaldehyde (37) remains to be determined and was, therefore, a major impetus for conducting the present study. Formaldehyde is a respiratory cytotoxicant at concentrations ≥6 ppm (26, 32), and it increases airway epithelial cell proliferation following short-term exposure in the rat (32), nonhuman primate (29), and xenotransplanted human nasal epithelium (38). This proliferative response is believed necessary to replace the damaged epithelium and to

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A principal objective of the present study was to assess the role of regional increases in nasal epithelial cell proliferation in the pathogenesis of formaldehyde-induced nasal cancer. We previously reported regional epithelial cell proliferation data following 6 weeks of formaldehyde exposure (32). Although the concentration-response for cell proliferation mimicked the original bioassay tumor response, these short-term studies failed to demonstrate a direct correlation between sites susceptible to formaldehyde-induced nasal cancer and sites with increased cell proliferation (32). In this report, cell proliferation indices over subchronic and chronic exposures were determined, and we addressed the site specificity of nasal tumor responses using improved lesion-tumor mapping procedures. It has been proposed that chance may play a major role in carcinogenesis, with the number of cells in a target population playing a role in the degree of risk associated with exposure to a given dose of a carcinogen (40). This issue was incorporated into the present studies through determination of an index of the number of cells at risk of mutation. The index developed for this work we termed the PWULLI, which was defined as the product of: (a) the site-specific ULLI; and (b) the total number of nasal epithelial cells in the respective site of interest. The size of the population of cells undergoing formaldehyde-induced replicative DNA synthesis is probably an important parameter in formaldehyde carcinogenesis since this chemical is mutagenic, binds covalently to DNA and associated proteins, and reacts preferentially with single-stranded DNA (18). Thus, mutation frequency may increase during replicative DNA synthesis in proportion to the number of dividing cells present in the exposed population, with a given dose of formaldehyde per cell resulting in more mutated (and thus potentially cancerous) cells in a larger cell population. The cell proliferation end points were also selected for future use in the development of a biologically based mathematical model and risk assessment strategy for formaldehyde nasal carcinogenesis (20).

Our study was based on the design and exposure concentrations of the original formaldehyde bioassay (7), with two additional concentrations, 0.7 and 10 ppm, included to better define the concentration-response curve for tumors and other responses. Results from this study confirm the sublinear, concentration-dependent tumor response curve of the original bioassay and demonstrate a marked increase in tumor response at both 10 and 15 ppm. Thus, the marked increase in slope of the original bioassay (7) and the present studies may account, at least in part, for the formaldehyde tumor response at both 10 and 15 ppm. Previous studies on DNA-protein cross-links have shown a similar sublinear concentration-response curve to that observed with SCC, i.e., at low exposure concentrations of formaldehyde the responses are less than would be predicted by a linear extrapolation from the response at the highest exposure concentrations. Furthermore, the quantity of cross-links was higher in the lateral meatus, the most frequent site of SCC in the present and a previous study (21) than in sites with lower tumor incidence. These observations, combined with studies of nasal airflow in rats (31), suggest that it is a high local dose of formaldehyde that leads to high local cell proliferation rates and DNA-protein cross-links. Sustained increases of cell proliferation in these cell populations, coupled with the known kinetics of formaldehyde binding to DNA, can account, at least in part, for the sublinearity of formaldehyde-induced SCC in rats. Furthermore, in the present study, it was found that regional correlation of SCC with ULLI was poor, while correlation with PWULLI was high, indicating a role for target (proliferating) cell population size in the regional incidence of formaldehyde-induced SCC.

MATERIALS AND METHODS

Animals and Husbandry. Male F344 [CDF(F344)/CrI Br] rats, 6–7 weeks old, were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were quarantined for 2 weeks upon arrival. All housing and handling of animals conformed to NIH guidelines (Publication No. 86–23, 1985) and were approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee. Animals were housed individually in stainless steel mesh cages with a 12-h light and dark cycle and had free access to pelleted chow (NIH-07 diet) and filter-purified tap water, except during exposure periods. Animals were free of virus titers by standard rat virus antibody determinations (Microbiological Associates, Bethesda, MD).

Exposures. Animals were randomized by body weight, divided into six groups, and whole-body-exposed to nominal formaldehyde concentrations of 0.0, 0.7, 2, 6, 10, or 15 ppm for up to 24 months (6 h/day, 5 days/week). Exposure procedures and analytical methods have been described (26, 32). Briefly, formaldehyde exposure atmospheres were generated in a oven by thermal depolymerization of paraformaldehyde (Aldrich Chemical, Milwaukee, WI), a solid polymer. Nitrogen gas carried the formaldehyde gas from the oven into a tangential inlet at the top of stainless steel and glass, Hinners-style, 8-m³ inhalation chambers. The concentration of formaldehyde was controlled by adjusting the nitrogen flow through the oven. Chamber concentrations were monitored continuously using a calibrated IR spectrophotometer (Miran 1A; Wilkes Scientific) and were recorded at least once per hour from each of the six inhalation chambers.

DNA Labeling. Labeling methods have been described previously (41). In brief, six animals per group were labeled continuously during the last 5 days of exposure by administration of [methyl-3H]thymidine with osmotic pumps (2ML1; Alza Corp., Palo Alto, CA). Five days prior to each interim sacrifice at 3, 6, 12, and 18 months, six rats/group were anesthetized with ketamine and xylazine, and pumps containing 2 μCi of [methyl-3H]thymidine (~20–50 CPM mmol, Amersham Corp., Arlington Heights, IL) were surgically implanted s.c. over the dorsal thoraco-lumbar area. The pumps remained in the animals until sacrifice 5 days later.

Histoautoradiography and Histopathology. On the day of each sacrifice, animals were deeply anesthetized with sodium pentobarbital (i.p.) and exsanguinated. The nasal cavities were flushed, immersion-fixed in formalin, and subsequently decalcified in 5% formic acid with ion-exchange resin. Cross-sectional blocks of the nasal cavity were prepared at six levels (42), embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For histoautoradiographic detection of cells in S phase, adjacent sections were cut from each block and mounted on glass slides, dipped in Kodak NTB2 emulsion, exposed at −15°C for 10 weeks, developed, fixed, washed in water, and stained with hematoxylin and eosin (32). The nasal cavities from all unscheduled death animals, in addition to animals euthanized at the terminal sacrifice following 24 months of exposure, were routinely processed for histopathology. Nasal sections from every animal were evaluated by light microscopy. The distribution of nasal tumors was recorded on nasal diagrams as outlined below.

Mapping of Nasal Tumors. Diagrams of the nasal passages, at 30 selected levels designed to permit accurate localization of nasal lesions (43), were used to record the location of nasal tumors. A set of these diagrams was prepared for each animal, and lesions were recorded using colored markers. The data generated by these maps were then tabulated in a spread sheet using a coding system for each location and lesion type. The location code permitted rapid preparation of computer files for subsequent statistical analyses. Lesion tabulation data were combined with additional information, including animal identification number and date of sacrifice or death.

Scoring of Labeled Cells. Quantitative methods for determining cell proliferation in nasal epithelia have been described previously (32, 41). Histoautoradiographic cell proliferation data were expressed as the number of labeled cell profiles/mm basement membrane, i.e., ULLI (41). Based on earlier studies determining the distribution of formaldehyde-induced lesions (22, 24, 27), a ULLI was determined for seven specific locations in the nasal passages (Fig. 1): anterior lateral meatus, posterior lateral meatus, anterior mid-septum, posterior mid-septum, anterior dorsal septum, medial maxilloturbinate, and maxillary sinus (excluding the ostium). The basement membrane length for each specific nasal location was determined by tracing the magnified projected image onto a digitizing tablet using a Zeiss Videoplan image analyzer. Cell
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Fig. 1. Sites selected for detailed analyses of cell proliferation indices and total cell number in each site (see "Materials and Methods"). These sites were mapped onto a finite element fluid dynamics mesh from data derived from studies of nasal airflow in the F344 rat. The surface area and total number of cells in each site, for one side of the nose, of control animals is presented.

proliferation indices were determined separately for putative preneoplastic lesions and are not reported here.

Quantitating Target Cell Populations. The number of cell profiles in each of the seven locations listed above was determined. Target cell populations were obtained in control rats sacrificed at 3 months since those animals had no evidence of background nasal pathology and this interim sacrifice time point precedes the point at which formaldehyde-induced neoplasms occur. For each of the locations studied, cell profiles were quantitated from two representative 0.5-mm sites. Cell counts from the two representative sites were averaged and recorded as the number of cell profiles/mm basement membrane. Cells were assumed to be uninnucleate, and total counts were corrected for tissue section thickness using the procedure of Barry and Crapo (44). The surface area of each site was then determined from a finite element mesh of the nose of a male F344 rat (31) using a fluid dynamics software package (FIDAP, Version 7.0, Fluid Dynamics Analysis Package; Fluid Dynamics International, Evanston, IL). The locations studied in cross-sections were identified in the mesh (Fig. 1). Node numbers in the mesh that geometrically define these locations were used to determine the surface area of each location using the FIDAP software. For each location, the total number of cell profiles was estimated as the product of the surface area and the number of cell profiles/mm basement membrane.

PWULLIs. An index of the number of cells at risk of mutation in each of the locations studied was then estimated from the total cell population in each site (see above) and the ULLI. The ULLI was found previously to be highly correlated with the true labeling index (proportion of cells in S phase; Ref. 41). Because the ULLI value was adjusted on the basis of population size in each location, a new term was developed, the PWULLI, to capture the underlying principal of this approach. PWULLI data were calculated by multiplying the respective ULLI at each time point by the total number of target cells measured at each site.

Statistical Analyses. The probability of survival was estimated using the product-limit procedure of Kaplan and Meier (45). Assessments of treatment-related effects on survival were based on Cox’s method for pairwise comparisons. Animals found dead of other than natural causes (e.g., scheduled
The incidence of SCC was calculated as the ratio of the number of animals bearing lesions at a site to the number of animals examined histologically. Logistic regression analysis was the primary statistical method used to determine significant differences in incidence of SCC among treated and control groups. This method assumes that the diagnosed tumor was discovered as the result of death from an unrelated cause (e.g., scheduled sacrifice). In applying the method, tumor incidence was modeled as a logistic function of exposure and time. Linear and quadratic temporal terms were included in the model initially, and the quadratic term was eliminated if it did not significantly enhance the fit of the model. Assessments of significance were based on the likelihood score test for the regression coefficient on concentration (46, 47). All reported Ps are one-sided under the alternative hypothesis of a treatment-related increase in tumor incidence. Comparisons of tumor incidence at different sites within the same animals were based on McNemar’s test. Reported Ps are two-sided.

The comparability of ULLIs among formaldehyde concentrations, nasal sites, and across time was assessed using ANOVA. The statistical significance of pairwise comparisons to controls was assessed with Dunnett’s test at α = 0.05 and α = 0.01. The relationships between formaldehyde concentration and ULLI and PWULLI at each nasal site were displayed graphically for comparison to the site-specific concentration-response function. The strengths of the associations between ULLI and observed incidence and between PWULLI and observed incidence were characterized with the square of the Pearson product moment correlation coefficient ($R^2$). Fitted linear relationships were depicted for log-transformed cells at risk and log-transformed ULLI to provide a clearer portrayal of all observations. These fitted lines appear as exponential curves on the graphs of transformed data.

RESULTS

Chamber Concentrations

Formaldehyde exposure atmosphere concentrations (± SD) were 0.0 (± 0.0), 0.69 (± 0.05), 2.05 (± 0.15), 6.01 (± 0.33), 9.93 (± 0.44), and 14.96 (± 0.80) ppm for the target concentrations of 0.0.7, 2, 6, 10, and 15 ppm, respectively.

Survival and Clinical Observations

Apart from the 15-ppm group, there was little, if any, evidence of treatment-related decreases in survival relative to that of controls (Fig. 2). Survival was higher in the 0.7-, 2.0-, and 6.0-ppm groups than in controls and was statistically comparable to controls in the 10-ppm group (35.7% versus 31.3%, respectively). There were numerous premature deaths in the 15-ppm group, resulting in significantly decreased survival relative to that of controls (18.8% versus 35.7%; P < 0.001). Yellow discoloration of the fur, a characteristic response to formaldehyde in rats (7), was observed in rats exposed to 10 and 15 ppm formaldehyde. Animals subsequently found to have nasal tumors or other severe nasal lesions frequently exhibited peri-orbital hematoporphyrin staining attributed to nasolacrimal duct occlusion (42) or decreased grooming. Large nasal neoplasms were associated with the presence of a mass over the anterior maxilla due to penetration of the nasal bones and subsequent local invasion of the subcutis. Smaller nasal tumors were often observed during trimming of tissues.

Histopathology

Control Animals

Control animals had no histopathological evidence of disease in the nasal passages. Buccal cavity SCC, not associated with the nasal cavity, was present in 2 of 90 control animals. This tumor was interpreted as an incidental finding and within the spontaneous tumor incidence range reported for this strain of rat (48).
Table 1  Summary of nasal SCC incidence

<table>
<thead>
<tr>
<th>Formaldehyde concentration (ppm)</th>
<th>No. of nasal cavities examined</th>
<th>Anterior lateral meatus</th>
<th>Posterior lateral meatus</th>
<th>Anterior mid-septum</th>
<th>Posterior mid-septum</th>
<th>Anterior dorsal septum</th>
<th>Anterior medial maxilloturbinate</th>
<th>Maxillary sinus</th>
<th>No. of animals with SCC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>0</td>
<td>0</td>
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</tr>
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<td>4</td>
<td>0</td>
<td>69</td>
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</tbody>
</table>

*Total number of animals with SCC, including those too large to allocate and those located in a site not listed in this table.

**Formaldehyde-exposed Animals**

Nonneoplastic Responses. Formaldehyde-induced nasal lesions were primarily confined to the transitional and respiratory epithelial lined areas of the anterior nasal passages, were symmetrically distributed, and exhibited an anterior-posterior severity gradient. Treatment-induced lesions were most severe in the two highest concentration groups, 10 and 15 ppm, while lesions in the 6-ppm group were minimal or absent, limited to focal squamous metaplasia in the anterior regions of the nasal cavity. No formaldehyde-induced lesions were observed in the 0.7- or 2-ppm groups. Predominant nonneoplastic nasal lesions included epithelial hypertrophy and hyperplasia, squamous metaplasia, and a mixed inflammatory cell infiltrate. Additional treatment-related lesions observed in animals exposed to 10 or 15 ppm included nasal turbinate adhesions and olfactory degeneration, which was usually confined to the walls of the anterior dorsal medial meatus. Most of the formaldehyde-induced lesions were more frequent or more severe in the 15-ppm concentration group as compared with the 10-ppm group. Significant distortion and destruction of the nasoturbinate architecture occurred in many animals exposed to 15 ppm formaldehyde.

Neoplastic Responses. Buccal SCC were observed in three animals at 15 ppm and in one animal at 2 ppm. All other neoplastic responses in the respiratory tract were confined to the nose. The nasal neoplasms observed, which included SCC and polypoid (transitional) adenoma, had similar morphological characteristics to those described in the original chronic bioassay (6, 7) and were considered to have originated from the epithelium lining the nasal airways. Some of the larger SCC eroded through the nasal bones and invaded the subcutis of the dorsal aspect of the nose. In addition to SCC, a low incidence of other neoplasms was observed, including polypoid adenomas. The latter neoplasms have been reported previously in rats in association with formaldehyde exposure, and both their morphology (49) and location in the nose (22) have been described in some detail. Two animals exposed to formaldehyde had nasal rhabdomyosarcomas, one at 15 ppm and one at 10 ppm. The latter neoplasms, which are rare in the nasal passages, have been reported previously in rats (50). Furthermore, two animals had nasal adenocarcinomas, one at 15 ppm and one at 10 ppm. The latter neoplasms were also potentially a consequence of formaldehyde exposure. However, as the large majority of formaldehyde-induced neoplasms were SCC, combined with the relatively low incidence of polypoid adenomas, adenocarcinomas, and rhabdomyosarcomas, this report has focused on the correlation of cell replication and other factors with SCC and makes no attempt to further investigate the other tumor types.

Quantitation of SCCs

The incidence of SCC by location is summarized in Table 1, which demonstrates a clear concentration-response relationship, with a no-observed-effect level of 2 ppm.

Incidence for All Nasal Sites Combined

The cumulative incidence of SCC in the nasal passages is shown with respect to exposure concentration in Fig. 3. There was a steep concentration-response curve from 6 to 15 ppm, with a no-observed-effect concentration of 2 ppm, demonstrating a distinctly sublinear response. Tumor rates in the 10 and 15 ppm groups were high (22 and 45%, respectively), while there was only a single animal in the 6-ppm
there was clear evidence of a higher tumor incidence rate in the anterior sample site ($P = 0.001$ and 0.02, respectively). Smaller numbers of SCC were observed on the medial aspect of the maxilloturbinate and on the posterior lateral wall and lining of the nasopharyngeal meatus (data not shown). The single SCC in the 6-ppm group was located in the anterior lateral meatus. No SCC were observed in the maxillary sinus, with the exception of one animal exposed to 15 ppm that had a small tumor located in the wall of the ostium of this sinus.

Tumor rates across the seven nasal epithelial sites are presented in Fig. 5. There was an increasing tumor response between the 10- and 15-ppm exposure groups in all sites, except the anterior region of the lateral meatus. In the latter site, 5CC rates for 10 and 15 ppm were virtually identical (13.3 and 11.6%, respectively). The absence of a response across these concentration levels is probably attributable to the occurrence of many large neoplasms in the lateral meatus site. Such neoplasms were not suitable for site-specific analyses, and their absence from the data set might have skewed this component of the analysis.

Quantitation of Polypoid Adenomas

Small numbers of polypoid adenomas were induced by formaldehyde exposure. These benign tumors formed acinar-like structures, similar in nature (7) and location (22) to those described in the original formaldehyde bioassay. No polypoid adenomas were present in control animals or animals in the 0.7-, 2.0-, or 6-ppm concentration groups. A clear concentration response was observed in the 10- and 15-ppm groups. Five of 90 animals in the 10-ppm group and 14 of 147 animals in the 15-ppm group had a polypoid adenoma (5.6 and 9.5% incidence, respectively). Most of the polypoid adenomas (79%) were located in or adjacent to the lateral meatus.

Cell Proliferation Indices

Cell proliferation data for the seven regions studied are presented in Table 2 and Fig. 6. There were no detected treatment-induced responses in cell proliferation indices in the 0.7-, 2-, or 6-ppm groups. Statistically significant increases in ULLI were present only in the two highest concentration groups, 10 and 15 ppm, with the magnitude of increases generally greater in the 15-ppm group. Clear patterns of cell proliferation responses were observed in specific regions of the nose, with characteristic effects of both exposure concentration and duration.
of exposure. The most marked increase in ULLI was observed on the anterior lateral meatus and the medial maxilloturbinate (Fig. 6). No significant changes in the maxillary sinus ULLI occurred over time in animals exposed to 10 or 15 ppm, in contrast to the ULLI of the anterior dorsal septum, which increased with increasing time of exposure. Elevated cell proliferation indices in the anterior dorsal septum were attributed to an increased formaldehyde dose to this region and consequent epithelial injury, due to significant distortion and destruction of the nasal turbinate, which normally protects the nasopharynx.

Regional Nasal Cell Populations

The location and surface area of the seven sites studied and the total cell profile count for each site are shown in Fig. 1. There were clear differences in the number of epithelial cells in each region, ranging from almost one million in the anterior lateral meatus to approximately one hundred thousand on the medial aspect of the maxilloturbinate.

Dose-Response Function Relative to Potential Explanatory Variables

The nonlinear SCC dose-responses for individual sites exhibited in Fig. 5 are generally similar in form to the nonlinear ULLI dose-responses at the 3-month time point in Fig. 6. For example, the anterior lateral meatus had large increases in both SCC and cell proliferation indices at 10 and 15 ppm. These responses were either not observed at the lower concentrations or were much smaller than a linear dose-response would dictate. Significant increases in ULLI and SCC were also observed in both the posterior lateral meatus and anterior mid-septum at 15 ppm but not at lower concentrations. Importantly, the cell proliferative response for the medial maxilloturbinate differed considerably from the tumor response. At 10 and 15 ppm.

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Table 2 Mean ULLI

<table>
<thead>
<tr>
<th>Formaldehyde concentration (ppm)</th>
<th>Exposure time (mo)</th>
<th>Anterior lateral meatus</th>
<th>Posterior lateral meatus</th>
<th>Anterior mid-septum</th>
<th>Posterior mid-septum</th>
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* AMS = 3
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* ALM = 3
* DS = 3
* MS = 3
* NS = 3

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* As described in "Materials and Methods," n = 5 or 6, unless otherwise indicated. ND, not determined.
* n = 4.
* n = 3.
ppm, the ULLI at this site was similar in magnitude to that of the anterior lateral meatus and yet had less than one-sixth as many tumors.

The PWULLI (i.e., ULLI multiplied by local cell population) at different formaldehyde concentrations is presented in Fig. 8. A more consistent pattern between cell proliferation indices and tumor incidence is apparent when the total number of cells at each site was also considered. In particular, the greatest increase in PWULLI was observed in the anterior lateral meatus in the 10- and 15-ppm groups. The tumor rates in this site were also significantly higher than in any other site. Figs. 9 and 10 provide further evidence that tumor rate is more strongly associated with PWULLI ($R^2 = 0.88$; Fig. 9) than with the ULLI data alone ($R^2 = 0.46$; Fig. 10). Proliferative responses of the medial maxilloturbinate, which had the smallest total number of cells of any region, accounted for much of the weakness of the ULLI versus tumor rate relationship. The correlation coefficient for ULLI versus tumor rate increased to $R^2 = 0.75$ when the medial maxilloturbinate site was eliminated from the analysis (data not shown).

**DISCUSSION**

Due to the clear site-specificity of formaldehyde-induced nasal cancer, a regional approach was taken for the correlation of cell proliferation and tumor response. From the original formaldehyde bioassay, Morgan et al. (22) reported a high incidence of nasal SCC originating in the lateral meatus (57%), an intermediate incidence originating on the mid-septum (26%), and no evidence of neoplasms arising on the anterior medial maxilloturbinate. Cell proliferation data from the present study, but through the first 6 weeks of exposure only, were reportedly previously (32). Through 6 weeks, the concentration response relationship resembled that at the later time points; however, significant increases in cell proliferation were observed following exposure to 6–15 ppm formaldehyde (32) in all three sites listed above. Moreover, the same three sites evaluated in the present chronic study also had elevated cell proliferation indices at high concentrations (≥10 ppm), and yet tumors were located primarily in the lateral
meatus and on the septum, with only a few present on the anterior
medial maxilloturbinate.

The improved statistical correlation between local tumor incidence
and PWULLI over that observed for ULLI alone indicates that target
cell population size also contributes to the chance of a tumor occur-
ring in a given site. This observation is consistent with the proposed
stochastic nature of cancer (40, 51, 52), since a greater cell population
size, at a constant dose of formaldehyde/cell could lead to a greater
risk of a cancerous cellular transformation. Among the key determi-
nants in this theory are the number of target site cells that are
susceptible to events leading to tumor formation and the rates at which
these events occur. The cell proliferation index alone does not reflect
the number of susceptible cells at a target site. PWULLI more ade-
quately describes this quantity as the product of an index of the
number of cells in the target population and an index of the propor-
tion of susceptible cells, i.e., those cells in S phase. We, therefore, believe
that PWULLI is a theoretically grounded predictor of tumor incidence
for a weak mutagen like formaldehyde. The results of our analysis
from this study support this proposition.

Incorporation of the cell population size into our analyses indicated
that the apparently refractory nature of the medial maxilloturbinate to
SCC induction is a consequence of the small target cell population
size in this site. Initiated cells arise when induced or spontaneous
DNA damage is converted to a mutation at a critical site in the
genome during DNA synthesis (53). This event, and any related
cancer outcome, is probably dependent on the amount of DNA dam-
age/cell, the length of time available for DNA repair prior to DNA
synthesis, the extent of cell proliferation, the number of cells affected,
and the survival time of affected cells prior to their death and exfo-
liation from the surface. Therefore, the probability of initial and
subsequent mutations associated with neoplastic progression will be
largest in locations where the product of molecular dose, cell prolif-
eration, and number of cells affected is greatest. The results of the
present study, especially with respect to differences in tumor fre-
quency between specific regions of the nose, indicate that target cell
population size, cell proliferation, and local dosimetry are major
determinants of the cancer outcome of formaldehyde exposure. These
factors most likely play a significant role in the concentration-re-
sponse curve for formaldehyde-induced nasal cancer in rats. Further-
more, demonstration of the absence of a significant PWULLI in the
respiratory tract of formaldehyde-exposed humans would provide the
means to account for the apparent lack of formaldehyde-induced
respiratory tract cancer in this species (11, 12, 18).

Alterations of cell proliferation rates may play a pivotal role in

Fig. 9. Correlation of tumor (SCC) rate with PWULLI (product of number of cells and ULLI)
for each site (site not identified for each point) and exposure concentration. There was a high correla-
tion between these end points ($R^2 = 0.88$), indicating a role for both cell proliferation and number of
cells present in the site specificity of the tumor response.

Fig. 10. Correlation of tumor rate and ULLI in specific nasal sites and exposure concentration. A
relatively poor correlation exists ($R^2 = 0.46$).
multistage carcinogenesis (33—36, 51). Results from our study indicate that sustained cell proliferation is an important feature in the carcinogenic process associated with formaldehyde exposure, since clear increases in ULLI were sustained only at the exposure concentrations yielding significant numbers of SCC (10 and 15 ppm). This hypothesis is further supported by another study that examined the role of tissue damage and repair in formaldehyde-induced nasal cancer (54). In that study, a tumor incidence 8-fold greater was found in rats chronically exposed to 10 ppm formaldehyde following nasal epithelial injury induced by electrocoagulation, as compared with rats similarly exposed but with an intact nasal mucosa, suggesting that tissue damage with consequent epithelial regeneration is important in formaldehyde-induced carcinogenesis (54). The molecular-biochemical-biological basis for disturbances in cell proliferation have yet to be elucidated but may involve the direct reactivity of formaldehyde with DNA or other macromolecules, autocrine or paracrine growth factors, mutations in growth regulatory genes, or an imbalance between cell proliferation and cell loss (36, 55, 56). Point mutations in the p53 gene have been demonstrated in formaldehyde-induced nasal SCC in the rat (57), indicating a common genetic alteration in certain rat and human squamous cell tumors of the respiratory tract.

The lack of increased cell proliferation over control levels in the 0.7-, 2.0-, and 6-ppm concentration groups indicates an apparent or experimental threshold response for formaldehyde-induced nasal cell proliferation. This observation suggests that the increased risk of malignant transformation observed at 10 and 15 ppm is greatly reduced at concentrations below the threshold. The one SCC in the 6-ppm concentration group occurred relatively late in the study and was located in the most sensitive site. Of interest is the observation that 6 ppm formaldehyde did not elicit a proliferative response following long-term exposure, although 6-week studies demonstrated increases in nasal cell proliferation at this concentration (32). In contrast, another study (21), in which a different method was used to detect cell proliferation, reported a significant increase in DNA synthesis after 3 months of exposure to 6 ppm but no increase after exposure to 0.7 or 2.0 ppm. It may be that 6 ppm is a borderline concentration, such that slight differences in concentration in this region can have a profound effects on the rate of cell proliferation. Thus, cell proliferation may be a critical factor also at 6 ppm, although it was not demonstrated in the present study.

Chemicals are rarely evaluated for carcinogenic potential in more than one chronic rodent study. Formaldehyde has been examined in at least three such studies: in the original bioassay (7), as a component of research on aldehyde carcinogenesis (5, 23, 54), and in the present investigations. All three studies revealed considerable consistency of outcome with respect to concentration-response relationships, tumor incidence, and location. The consistency of tumor response and site specificity, the increased knowledge of regional formaldehyde dosimetry (21, 31) and local metabolism (18), and the quantitation of potentially critical tissue responses such as PWWULI make formaldehyde a valuable model chemical for studies of interspecies extrapolation and human cancer risk assessment for weakly genotoxic carcinogens.

The occurrence of nonlinear responses for cell proliferation provides important data that should be considered during the interpretation of rodent bioassays and the assessment of risk for humans exposed to formaldehyde. Induction of nasal carcinomas in rats by formaldehyde requires long-term exposure to high concentrations that result in cell death followed by regenerative hyperplasia and metaplasia. Furthermore, formaldehyde reacts more readily with single-stranded DNA (18), which is present in increased amounts during cell division, with generation of DNA-protein cross-links providing a valuable indicator of site-specific DNA exposure (21). At high exposure concentrations, the probability of inducing heritable DNA damage is greater than at low concentrations due to the saturation of epithelial protective mechanisms (17, 58) and the sustained stimulation of the proliferative response. Based on results from the present study, exposure of humans to levels of formaldehyde that do not increase cell proliferation, and thus the PWWULI, would pose a much smaller risk of cancer than that currently estimated using a linearized multistage model for extrapolation from the cancer outcome at high exposure concentrations in the rat bioassays.

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Correlation of Regional and Nonlinear Formaldehyde-induced Nasal Cancer with Proliferating Populations of Cells

Thomas M. Monticello, James A. Swenberg, Elizabeth A. Gross, et al.


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