Multifactorial Hamster Respiratory Carcinogenesis with Interdependent Effects of Cannula-induced Mucosal Wounding, Saline, Ferric Oxide, Benzo[a]pyrene and N-Methyl-N-nitrosourea

Kevin P. Keenan, Umberto Saffiotti, Sherman F. Stinson, Charles W. Riggs, and Elizabeth M. McDowell

ABSTRACT

The carcinogenic response induced in the respiratory tract of Syrian golden hamsters by repeated intratracheal instillations of benzo[a]pyrene (BP) adsorbed to ferric oxide (Fe$_2$O$_3$) particles suspended in saline, is shown to result from the interactions of these factors and cannula-induced tracheal wounding. Previous acute studies of intratracheal cannulation (ITC) versus intralaryngeal cannulation (ILC) showed that tracheal cell proliferation increased significantly in ITC-induced mucosal wounds. Only mild increases in intrapulmonary cell proliferation were produced only additive, but also interactive, including synergistic as well as inhibitory interactions. It is, therefore, appropriate to consider the interplay of multiple causative factors in the etiology of respiratory cancers, rather than attempt to attribute each cancer to a single factor (1).

The animal model that most closely reproduces the morphology of human respiratory cancers has been obtained by administering to Syrian golden hamsters a series of intratracheal cannulations with instillation of a saline suspension of fine particles of an inorganic carrier dust, such as Fe$_2$O$_3$, to which a carcinogen such as BP is crystallized and adsorbed by nucleation (2–4). The resulting tumors closely resemble human bronchogenic carcinomas, including squamous cell (epidermoid) carcinomas, adenocarcinomas and their poorly differentiated variants (5–8), but not neuroendocrine carcinomas. Papillomas and adenomas are also induced in this model. Combined exposure studies in the hamster showed the synergism of repeated instillations of BP-Fe$_2$O$_3$-saline with systemically administered DEN (9) or topical MNU (10).

In recent studies, we focused our attention on the role of cell injury and epithelial regeneration in the hamster, in order to characterize the cell types involved in the proliferative response and their role in the process of carcinogenesis (11–15). Epithelial proliferation is considered essential for the fixation of initiation events, as well as for the expression of preneoplastic and neoplastic changes in many organs (16–18). Reactions, involving cell injury, death, removal, and replacement with new proliferating cells, recall the process of epithelial wounding and wound healing, known from the early literature to enhance carcinogenesis (19–21). Wound healing, a subject of interest since antiquity (22), remains poorly understood at the molecular level (23–25).

Both epithelial regeneration and mesenchymal repair are prominent features of most physical and toxicological injuries to the respiratory epithelium (11–16, 26–34). If injury to the respiratory epithelium is mild and acute, an orderly progression of localized cell death and inflammation is followed by epithelial cell migration, proliferation and transient hyperplasia, with subsequent cellular differentiation and a return to normal structure and function (11–14, 31). This epithelial regenerative response is accompanied by an orderly sequence of subjacent mesenchymal repair events. However, if the injury is severe or chronic, there is a disruption of the normal temporal sequence of events in regeneration and repair. An injury to the respiratory tract that compromises normal reepithelialization will be accompanied by an abnormal subjacent mesenchymal response that can result in persistent submucosal fibroplasia in the conducting airways and pulmonary fibrosis in the distal lung parenchyma (13, 32, 33). This fibroplasia may be accompanied by abnormal states of overlying epithelial differentiation, either in the form...
of chronic atrophy or hyperplasia and metaplasia. These pathological states of epithelial and mesenchymal cell differentiation are frequently associated with neoplastic changes in the respiratory epithelium (5, 6, 15, 34). The current interest in epithelial and mesenchymal repair, inflammation, growth factors, and other cellular mediators in relation to carcinogenesis (23–25, 35–37), suggests that the role of wounding in the carcinogenic process warrants further consideration.

The Syrian golden hamster model of respiratory carcinogenesis induced by instillations of carcinogens carried by inorganic particles suspended in saline (2, 4, 15) was chosen for the present analysis of the interdependent effects of cannula-induced mucosal wounding and instillation of saline, Fe₂O₃ particles, BP or MNU. The mucosal wounding produced by the insertion of a metal probe into the tracheal lumen and the ensuing cellular reactions have been described in detail (11–14). The present study was preceded by an investigation (38) of the acute morphological and cytokinetic effects of cannula-induced mucosal wounding and instillation of saline, or of Fe₂O₃-saline in the hamster. Both studies compared the reactions induced by ITC and ILC, i.e., cannulation along the length of the trachea or limited to the larynx. The ILC avoids mechanical injury to the tracheal mucosa while allowing instillation of saline and particulates to the peripheral lung. The previous (38) and present studies showed that the instilled suspension is drawn into the lungs by inspiration and that the lung distribution of the instilled particles is the same by both methods, thus allowing analysis of the effects of tracheal wounding as an independent variable. The potential enhancing effects of ITC-induced mechanical wounding on respiratory carcinogenesis were suggested in earlier studies (15, 39), but no direct experimental evidence was provided. In experiments involving the instillation of BP-Fe₂O₃-saline by ITC, the enhanced carcinogenic response was attributed directly to the Fe₂O₃ carrier particles influencing the distribution and the retention rate of the carcinogen in the respiratory tract (2, 4, 15, 40). One recent study reported no evidence of respiratory carcinogenic enhancement in hamsters which received a single severe local tracheal wound by electrocoagulation, 48 h prior to six weekly ITC instillations of BP-Fe₂O₃-saline, compared to hamsters given only the six ITC instillations (41). However, in both groups, considerable ITC-induced mechanical wounding would have occurred at each weekly instillation and this is likely to have overwhelmed the effects of the preceding electrocoagulating wound. Moreover, cell proliferation, hyperplasia, and metaplasia following a single mechanical injury in the hamster trachea are known to rapidly increase and then decrease, as differentiation returns to normal in 5–7 days (11–14, 42).

The major potential enhancing effect of ITC-induced mechanical wounding was also overlooked in the carcinogenesis model which uses the direct acting nitrosamide MNU instilled locally in the hamster trachea by a special recirculating cannula, resulting in the rapid induction of a high incidence of carcinomas locally in the trachea (43–47). Early hyperplastic and regenerative epithelial lesions were attributed to the cytotoxic effects of the MNUs. However, no observations were made of lesions induced by cannulation alone, and thus no consideration was given to the enhancement by mechanical wounding from repeated intubation with the large intratracheal cannula.

The synergistic role of MNU with BP-Fe₂O₃ instillations was previously demonstrated following repeated MNU instillations by ITC (10). The present study now reports on the enhancing effects of a single pretreatment with MNU, instilled by ILC (thereby sparing the trachea from mechanical injury) in hamsters at 5 weeks of age, when the mitotic rate in the respiratory epithelium is about 25 times that of adults (48).

This multifactorial long-term carcinogenesis study was undertaken to identify the individual and interactive effects of all the components: pretreatment with a single dose of MNU by ILC; 15 repeated treatments (once weekly) by either ILC or ITC, with or without instillation of saline alone, Fe₂O₃ in saline or BP-Fe₂O₃ in saline. Statistical analysis of all the permutations in the 14 experimental groups showed the effects and interactions of all treatments.

### MATERIALS AND METHODS

**Animals.** Male Syrian golden hamsters (CR:RGH), 4 weeks of age, were obtained from the Animal Production Branch, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. The hamsters were housed four per cage on hardwood chip bedding in Illinois Isolation Cubicles of a biohazard containment area that was environmentally controlled and had a 12-h light/dark cycle. Hamsters had access to pelleted food (Ralston Purina Rodent Lab Chow No. 5001) and water ad libitum. At regular intervals throughout the study, serum samples were collected and tested for antibodies against reovirus type 3, lymphocytic choriomeningitis virus, pneumonia virus of mice, Sendai virus, and simian virus 5. All samples tested were consistently negative for antibodies to these agents.

**General Procedures and Treatments.** Hamsters were anesthetized at each treatment (Table 1) with a mixture of oxygen and methoxyflurane (Pittman-Moore, Inc., Washington Crossing, NJ) and were placed on their backs on a metal board slanted at 60°; as previously described (2, 38). A 19-gauge stainless steel blunt cannula was used for cannulations, with or without instillations. A plastic stop was fitted on the cannula to prevent its insertion beyond the cricoid cartilage for ITC, and beyond the tracheal carina for ITC. When ITC was performed, the cannula unavoidably scraped against the laryngeal and tracheal wall during insertion and withdrawal, thus producing mechanical wounding of the mucosa, as previously described (38). ILC limited this injury to the larynx and left the trachea undamaged. Instillations were given during a spontaneous inspiration. No loss of suspension by reflux was noted by either ITC or ITC.

Hamsters instilled with sterile saline received 0.2 ml (0.9% NaCl solution, USP). Those instilled with Fe₂O₃ particles in saline (Fe₂O₃-saline) received 3 mg of Fe₂O₃ (Pfizer Chemical, Type R3098R) suspended in 0.2 ml of sterile saline. The particle size (by number) of the Fe₂O₃ preparation was: 94% < 1 μm; 4% between 1 and 3 μm; 1% between 3 and 15 μm; 1% > 15 μm. Hamsters instilled with BP-Fe₂O₃-saline received 3 mg of BP (Sigma Chemical Co., St. Louis, MO) adsorbed to 3 mg of Fe₂O₃ and suspended in 0.2 ml of sterile saline,

### Table 1 Treatment protocols: 5–21 weeks of age

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* Initial number of hamsters per group, 40; Total number, 560.
* 0.2 ml of 1% MNU in sodium citrate given by ILC at 5 weeks of age.
* Metaphane in oxygen gas anesthesia at the 16 treatment times.
* Larynx wound from ILC (19 gauge) weekly × 15.
* Trachea wound from ITC (19 gauge) to carina weekly × 15.
* 0.2 ml saline (alone or with particles) weekly × 15.
* 3 mg Fe₂O₃ particles in 0.2 ml saline weekly × 15.
* 3 mg BP added to the 3 mg Fe₂O₃ particles in 0.2 ml saline weekly × 15.
prepared by nucleation. Briefly, a concentrated solution of BP in a volume of acetone was added drop-wise to 50 volumes of cold distilled water containing Fe$_2$O$_3$ particles dispersed by sonication (the carcinogen crystallizes into very fine particles adherent to the surface of the carrier dust). The suspension was filtered and dried and aliquots were suspended in saline at the time of treatment (3, 4). The particle size (by number) of the BP/Fe$_2$O$_3$ preparation was: 92–96% < 3 μm; 4–6% between 3 and 15 μm; 1–2% > 15 μm. Hamsters instilled with MNU (Ash-Stevens, Detroit, MI) received a single dose of 0.2 ml of a 1% solution of MNU prepared in 0.015 N sodium citrate solution (pH 6.5) and used within 30 min of preparation, as previously described (10).

Preliminary MNU Toxicity Study. To determine the potential acute toxicity of MNU, 50 5-week-old male hamsters were divided into three groups and were given a single instillation by ILC of 0.2 ml of either a 0.5% MNU solution or a 1.0% MNU solution or sodium citrate solution alone. Four to six hamsters from each group, and age-matched untreated controls were examined at 24 h, 48 h, and 14 days posttreatment. The larynx, trachea, bronchi, and lung lobes were fixed in formalin, cross-sectioned, and processed in glycol methacrylate and paraffin for high resolution and routine light microscopy.

Long-term Study. The experimental design is shown in Table 1. A total of 560 male hamsters, 5 weeks old at the start of the study, were divided into 14 groups of 40 animals each. Group 1 was an untreated control. Group 2 was an anesthesia control (anesthetized 16 times along with the treatment groups). Groups 3–11 received a single ILC instillation of 0.2 ml of a 1% MNU solution at 5 weeks of age. At 7 weeks of age, the hamsters of Groups 4–11 were begun on 15 weekly treatments of various combinations of ILC or ITC and instillations of saline alone, Fe$_2$O$_3$-saline, or BP-Fe$_2$O$_3$-saline (Table 1).

At 25 weeks of age, 1 month following the final treatment, 15% of the animals were killed (interim kill). The experiment was terminated when the hamsters were 78 weeks of age (terminal kill). Hamsters were observed daily and animals moribund or found dead were necropsied. Hamsters were weighed, anesthetized, and killed by exsanguination. Tissues were fixed with 4% formaldehyde in a 300-millimolar phosphate buffer. The respiratory tract was rapidly exposed and the lungs gently inflated with fixative to normal distention via a needle inserted between the cricoid and first tracheal cartilage. Then the upper trachea was ligated near the first or second cartilage and the entire respiratory tract was removed en bloc and immersed in fixative. Complete necropsies were performed and other tissues were fixed as appropriate by intraluminal perfusion or immersion in fixative. Up to 25 weeks of age, multiple cross-sections of the larynx, trachea, each of the five lung lobes and any gross lesions found in other organs were examined histologically. Following 25 weeks of age, other thoracic and abdominal organs, plus any additional gross lesions were evaluated histologically. Tissues from this long-term study were processed and embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin & eosin. Special stains were performed as required. The respiratory tract was evaluated by examination of three cross-sections of the larynx, and six to eight cross-sections of the trachea, carina, and extrapulmonary bronchi, all embedded in a single block. Each of the five lung lobes was separated and serially cross-sectioned into 2-mm slices. Each lobe was embedded in a separate block for evaluation of intrapulmonary airways and pulmonary parenchyma.

Individual hamster data on treatment, in-life observations, weights, survival, gross observations at necropsy, and histological lesions observed were recorded in a tabular format for analysis. Morphological criteria for the tumors and nonneoplastic lesions have been reported in detail elsewhere (2, 6, 8, 11-14, 42, 49) and diagnoses are listed in Tables 3–5. Carcinoma in situ was diagnosed when an epithelial lesion was composed entirely of malignant-appearing cells that had not invaded the stroma. Most in situ lesions had the cellular features of epidermoid carcinoma. Only malignant epithelium showing invasion of the stroma was diagnosed as invasive carcinoma. Probabilities of survival estimated by the product-limit procedure of Kaplan and Meier (50) were performed for the entire study. Animals were statistically censored at the time of death from other than natural causes (anesthetic deaths, interim kills, and terminal kills). Animals dying of natural causes or killed when moribund were included for statistical analysis.

The survival analyses were performed on all animals from 5 weeks through 78 weeks of age, and also for those animals remaining on the study from 17, 22, and 26 weeks of age through 78 weeks. Differences in survival patterns or curves were evaluated by both the generalized Kruskal-Wallis and the Cox life-table analyses (50). These nonparametric methods are appropriate for survival time studies when censored times of the type referred to above are present.

Statistical analyses of differences between treatment groups were performed using Fisher's exact test (51) for the incidences of nonneoplastic lesions and of tumors observed in the larynx, trachea, and lungs. All reported P values are one-sided, and the level of significance was set at P ≤ 0.05. These comparisons were made on differences of a single treatment factor between two groups for all treatment variables and for all the lesions observed in hamsters surviving from 17 to 78 weeks, 22 to 78 weeks, and from 26 to 78 weeks of age. These analyses were then compared with the appropriate survival analyses to identify lower incidences that might be due to reduced survival time. The tumor and lesion incidence data from the 22- to 78-week-old animals (including data from the 25-week interim kill hamsters) are shown in Tables 3, 4, and 5 (see "Results"). Lesions and tumors observed in nonrespiratory tissues will be reported in a separate publication.

RESULTS

The preliminary MNU toxicity study showed no mortality or histological evidence of cytotoxicity in the trachea or distal lung of hamsters given either 0.5% or 1.0% MNU solutions or the sodium citrate solution by ILC, when compared to controls at 24 h, 48 h, or 14 days posttreatment. Focal, superficial mucosal wounds to the laryngeal epithelium were induced by ILC. The 1.0% MNU solution was given to 5-week-old hamsters in the long-term study.

Fig. 1 shows the Kaplan-Meier survival curves and Table 2 shows a comparison of survival curves by both the Kruskal-Wallis and Cox analyses for all groups in the long-term study.

![Fig. 1. Kaplan-Meier survival curves for all groups with respect to death from all causes. Group 1, untreated controls (○); Group 2, anesthetic controls (●); Group 8 (△); Group 11 (□). Curves for all other groups lie within the shaded area. Deaths by interim kill at 25 weeks of age (20 weeks of survival) were treated as censored data and do not appear in these curves. See Table 2 for a comparison of the survival curves between each group by the Kruskal-Wallis and Cox analyses.](image-url)
from 5 to 78 weeks of age. A significant difference in survival was seen between Group 1 (untreated controls) and Group 2 (anesthesia controls) by the Kruskal-Wallis analysis, that gives more weight to earlier events in the survival curve. However, no difference was seen in the incidence or severity of the observed respiratory lesions between these two control groups. When Group 2 was compared with the 12 treatment groups, the only significant differences in survival were seen with Groups 8 (ITC) and 11 (ILC), which had received both carcinogens. These two groups had significant differences in the onset, incidence, and severity of observed respiratory lesions and/or tumors compared with Group 2. Group 8 had high mortality rates become higher). For example, in Group 8 (all treatments), the risk of tumor development because three hamsters in Group 8 were found to be similar regardless of whether the particles had been instilled by ILC or ITC. Furthermore, the distribution of the particles at the junctions of the terminal bronchioles and alveolar ducts, and the incidence of particle-laden macrophage accumulations and BAH at these locations, were also similar, irrespective of the instillation level. These observations were consistent throughout the study in hamsters killed at 25 weeks (interim kill), at 78 weeks (terminal kill), and in hamsters killed moribund or which died spontaneously at various times throughout the study.

The tumor and lesion incidence data were analyzed with and without the interim kill animals (25 weeks). The results were similar but appear more impressive when the interim kill animals (25 weeks) were then matched with the appropriate survival curve analyses, as shown in Table 6, which shows only the comparisons for the one variable, tracheal wounding. Since the results obtained for the periods from 17 to 22 weeks onward were generally similar, the data from 22 weeks onward, shown in Tables 3, 4, and 5 reflect the overall trends observed and are described below for each treatment.

Fig. 2 reports the observed incidence of benign and malignant tumors in each segment of the respiratory tract, calculated as the percent of each tumor type in the tissues examined.

The results presented below first consider the major response patterns observed at each respiratory tract segment: larynx, trachea, and lung lobes (bronchi and peripheral airways). Then the effects of each treatment factor on the incidence of respiratory tumors and nonneoplastic lesions are reported with analysis of the appropriate comparisons among groups.

Overview of Tumor Responses by Site. In the larynx (Table 3), carcinomas (in situ and invasive) were present in eight out of the 11 groups (Groups 4–14) that received repeated cannulations; there was also a single carcinoma in Group 3 (MNU only). The highest incidences of carcinomas were found in Group 8 (all treatments) and in Group 12 (all except MNU). In the trachea (Table 4), carcinomas (in situ and invasive) occurred only in Groups 8 and 12 that received tracheal wounds by ITC and weekly instillations of BP-Fe2O3-saline, the highest incidence being in Group 8 that received MNU pretreatment.
### Table 3 Larynx lesions and tumors: 22–78 weeks of age

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<tr>
<th>Treatments</th>
<th>Untreated control</th>
<th>Anest. control</th>
<th>MNU Lx wd</th>
<th>MNU Lx T wd</th>
<th>MNU Lx wd T wd</th>
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1. number and percentage of hamsters with laryngitis (percent rounded to the nearest whole number); 2. epithelial hyperplasia and/or metaplasia; 3. carcinoma in situ; 4. epidermoid carcinoma (EC); 5. small cell carcinoma (SCC); 6. number and percentage of hamsters with carcinoma in situ, invasive carcinoma or both. Mean time to tumor (age in weeks): a = 78, b = 78, c = 78, d = 56, e = 56, f = 48, g = 48, h = 45, i = 45, j = 56, k = 56, l = 56, m = 48, n = 54, o = 50, p = 52, q = 51, r = 51, s = 78, t = 52, u = 39, v = 47, w = 66, x = 73, y = 71, z = 78, a1 = 73, b1 = 63, c1 = 78, d1 = 78, e1 in addition, one carcinoma in situ and one combined epidermoid-adenocarcinoma, both at 21 weeks.

### Table 4 Trachea lesions and tumors: 22–78 weeks of age

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1. number and percentage of hamsters with tracheitis; 2. epithelial hyperplasia and/or metaplasia; 3. carcinoma in situ; 4. epidermoid carcinoma (one with metastasis); 5. combined epidermoid-adenocarcinoma; 6. number and percentage of hamsters with carcinoma in situ, invasive carcinoma or both. Mean time to tumor (age in weeks): a = 78, b = 78, c = 78, d = 56, e = 56, f = 48, g = 48, h = 45, i = 45, j = 56, k = 56, l = 56, m = 48, n = 54, o = 50, p = 52, q = 51, r = 51, s = 78, t = 52, u = 39, v = 47, w = 66, x = 73, y = 71, z = 78, a1 = 73, b1 = 63, c1 = 78, d1 = 78, e1 in addition, one carcinoma in situ and one combined epidermoid-adenocarcinoma, both at 21 weeks.

#### Effect of Tracheal Wounding

The most conspicuous effects of tracheal wounding by ITC were found in the trachea itself, where only Groups 8 and 12, which received tracheal wounds and one or both carcinogens, developed tracheal cancers. In contrast, hamsters that had received the same multiple instillations of BP-Fe2O3-saline by ILC without tracheal wounds (Groups 11 and 13) did not develop tracheal or intrapulmonary cancers, providing direct evidence of the role of tracheal wounding in the induction of these cancers (Table 6). The effects of tracheal wounding are evident in the pairwise comparisons of all relevant groups and show that ITC extends its effects to the entire respiratory tract.

Groups 8 (ITC) and 11 (ILC) each had a single MNU instillation followed by repeated BP-Fe2O3-saline instillations. Differences in survival over the entire study (Table 2) were seen by the Kruskal-Wallis test, but were not significant from 22 weeks onward (Table 6), reflecting early mortality from bronchopneumonia in Group 8. Group 8 had higher incidences of laryngeal epithelial proliferative lesions and laryngeal tumors than Group 11 (Table 3) and significantly increased incidences of inflammation, epithelial hyperplasia, metaplasia, atrophy, and fibroplasia in the trachea. Group 8 also had carcinomas in situ and invasive carcinomas in the trachea, in a total of 50% of the animals examined, whereas Group 11 had none (Table 4). In the intrapulmonary bronchi, 6 carcinomas were found in five animals of Group 8, but none were found in Group 11. No
**Table 5** Lung lesions and tumors: 22-78 weeks of age

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1. Number and percentage of hamsters with bronchiolar-alveolar hyperplasia; 2. alveolar metaplasia; 3. bronchopneumonia; 4. interstitial pneumonia; 5. bronchial adenocarcinoma; 6. carcinoma in situ; 7. adenocarcinoma; 8. epidermoid carcinoma; 9. combined epidermoid in situ, invasive carcinoma, or both. Mean time to tumor (age in weeks): a = 74, b = 78, c = 78, d = 33, e = 33, f = 65, g = 70, h = 60, i = 62.

**Table 6** Comparison of survival curves and incidence of lesions and tumors between groups with and without ITC-induced tracheal wounds

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a. Kruskal-Wallis and Cox analyses on survival curves from 22 to 78 weeks of age. SIG, both tests P < 0.05; NS, not significant; —, absent in both groups.
b. Incidence compared from Tables 3, 4, and 5 by Fisher's exact test, one-tailed probabilities given.
c. Abbreviations as in Tables 3-5.

differences in nonneoplastic intrapulmonary lesions or the distribution of intrapulmonary FeO₃ particles or particle-laden macrophages were seen (Tables 5 and 6).

Groups 12 (ITC) and 13 (ILC) each received repeated BP-FeO₃-saline instillations but no MNU. No difference was detected in their survival curves. Group 12 (ITC) had a signifi-
cantly higher incidence of laryngitis than Group 13, and showed 17 laryngeal tumors (12 carcinomas) in 11 hamsters versus only three tumors (one carcinoma in situ) in three hamsters in Group 13. Group 12 also had a significantly increased incidence of inflammation, hyperplasia, metaplasia, atrophy, and fibroplasia in the trachea compared with Group 13. Group 12 had a total of nine tracheal tumors (six carcinomas) in seven hamsters versus none in Group 13 (Tables 4 and 6). The incidence of intrapulmonary inflammatory lesions and alveolar metaplasia was higher in Group 12 than in Group 13, but no differences were seen between these two groups regarding the distribution of intrapulmonary Fe$_2$O$_3$ particle-laden macrophages or in the incidence of BAH (Tables 5 and 6).

Groups 6 (ITC) and 9 (ILC) each had a single MNU instillation and repeated saline instillations. Group 6 had a significantly shorter survival than Group 9. Group 6 had a significantly higher incidence of laryngitis and increased occurrence of hyperplasia/metaplasia. Group 6 also had four laryngeal tumors (three carcinomas in situ) in three hamsters versus none in Group 9 (Table 3). In the trachea, Group 6 had a highly significant incidence of hyperplasia, metaplasia, atrophy, and fibroplasia (Tables 4 and 6). These lesions were mostly absent in Group 9 and neither group had tracheal tumors. In the lungs, Group 6 had more severe inflammation and showed a single carcinoma (no tumors in Group 9) (Tables 5 and 6). However, comparison of Group 9 with Group 7, which received Fe$_2$O$_3$ in saline by ITC, showed no significant differences in tumor response.

Groups 5 (ITC) and 4 (ILC) each had a single MNU instillation and repeated cannulations (without instillations). Their survival curves were comparable. Group 5 had a higher incidence of laryngeal and tracheal inflammation, epithelial hyperplasia, metaplasia, atrophy, and fibroplasia (Tables 4 and 6). Tumor incidence was marginal in both groups.

From all the above comparisons it was clear that ITC-induced intratracheal wounding had a major influence on the occurrence and severity of inflammatory and proliferative lesions not only in the trachea, but also in the larynx and lungs. In groups which received repeated BP-Fe$_2$O$_3$-saline treatments with or without the single MNU pretreatment (Groups 8, 11–13), only hamsters given ITC-induced tracheal wounds developed tracheal or intrapulmonary carcinomas.

Effect of Saline. Groups 4 and 9 each had a single MNU instillation and repeated ILC treatments, but only Group 9 received saline instillations. The survival curves were comparable. Group 9 had a significantly increased incidence of alveolar metaplasia (Table 5); no other respiratory lesions or tumors were increased in comparison with Group 4.

Groups 5 and 6 each had a single MNU instillation and repeated ITC treatments, but only Group 6 received saline instillations. No significant differences in the survival curves, nor in the frequency of nonneoplastic lesions of the larynx or trachea were seen; Group 6, however, had a significantly higher incidence of alveolar metaplasia. Tumor incidences were low in both groups, but only hamsters that received saline (Group 6) developed carcinomas (four laryngeal and one bronchial papilloma in Group 5; one laryngeal papilloma, three laryngeal carcinomas in situ, and one intrapulmonary bronchial epidermoid carcinoma in four hamsters in Group 6).

Alveolar metaplasia was distinct from BAH and appeared as poorly circumscribed discrete foci of increased cellularity in the distal alveolar ducts and proximal alveoli, with areas of interstitial thickening lined by hyperplastic epithelium that had undergone cuboidal, goblet cell, or epidermoid metaplastic change. The luminal spaces of these foci contained either clear eosinophilic secretions or foamy macrophages. Alveolar metaplasia occurred more frequently in groups that received saline as part of their treatment, but these small discrete lesions did not appear to be associated with an increased incidence of intrapulmonary carcinomas or other lesions.
Effect of Fe₂O₃ Particles. The effects of Fe₂O₃ particles were tested by comparisons of ILC Groups 9 with 10, and ITC Groups 6 with 7. Each of these four groups received a single MNU instillation. Group 9 had repeated ILC-saline and group 10 ILC-Fe₂O₃-saline instillations. Group 10 showed an earlier mortality (Table 2) and significant increases in the incidence and severity of laryngeal inflammation, hyperplasia, and metaplasia. Little difference was seen in the tracheas of these ILC-instilled groups, but the lungs of Group 10 had a significant increase in BAH and peripheral collections of macrophages. Group 10 (Fe₂O₃) had two papillomas and four carcinomas in situ in the larynx and one pulmonary adenoma in six hamsters, whereas no respiratory tumors were found in Group 9 (no Fe₂O₃).

Groups 6 (ITC-saline) and 7 (ITC-Fe₂O₃-saline) each received a single MNU instillation. These groups showed no difference in their survival curves. Group 7 had significantly increased incidence, severity and persistence of tracheal fibroplasia, and persistent intrapulmonary collections of Fe₂O₃ particle-laden macrophages in the peripheral lung with an increased incidence of BAH (Table 5). Tumor incidence in Groups 6 and 7 did not show any enhancing effects of Fe₂O₃. In Group 6 (no Fe₂O₃), there were three laryngeal carcinomas in situ, one laryngeal papilloma, and one bronchial epidermoid carcinoma in four hamsters. In Group 7 (Fe₂O₃), there was only one laryngeal carcinoma in situ. The laryngeal tumor response of Group 7 was unexpectedly low, as indicated by comparison with the laryngeal tumors in Group 10. Repeated instillations of Fe₂O₃-saline by ITC, but without MNU pretreatment, were previously reported to show no induction of respiratory tumors in hamsters (52, 53).

Effect of BP. Groups 10 and 11 each received a single MNU instillation, followed by repeated instillations by ILC of BP-Fe₂O₃-saline (Group 11) or Fe₂O₃-saline (Group 10). No differences were seen in the survival curves or in the incidences of nonneoplastic lesions. Comparable tumor incidences were found in the larynges of both groups (an invasive laryngeal carcinoma was seen only in Group 11) but no cancers were observed in the trachea or lungs of either group (one pulmonary adenoma in Group 10, without BP). In contrast, Groups 7 and 8 received MNU pretreatment and repeated instillations of BP-Fe₂O₃-saline (Group 8) or Fe₂O₃-saline (Group 7) by ITC, with the consequent tracheal wounding. Group 8 (BP) compared to Group 7 (no BP), had a significantly lower survival rate (Fig. 1) and a highly significant increase in the onset, incidence, and severity of laryngeal, tracheal, and intrapulmonary inflammation, epithelial hyperplasia/metaplasia, papilloma, carcinoma in situ, epidermoid carcinoma, and overall respiratory carcinomas. Thus, considering these four MNU-pretreated groups, in the presence of the tracheal wounding (Groups 7 and 8), the administration of BP determined a major carcinogenic response in all segments of the respiratory tract, with a total of 38 tumors (34 carcinomas) in 17 hamsters of Group 8 (BP) versus a single tumor (a laryngeal carcinoma in situ) in Group 7 (no BP). The early mortality in Group 8 makes this difference even more striking (Fig. 2). However, in the absence of tracheal wounding (Groups 10 and 11), BP had no effect on respiratory tumor induction.

The effect of BP-Fe₂O₃ in the absence of MNU pretreatment was analyzed by comparing Groups 12 (BP-Fe₂O₃-saline by ITC) and 13 (BP-Fe₂O₃-saline by ILC) with Group 14 (saline by ITC). The total respiratory tumor yield was 26 tumors (18 carcinomas) in 15 hamsters of Group 12, only three tumors (one carcinoma in situ) in three hamsters of Group 13, and no tumors in Group 14. Of all the groups receiving multiple instillations of BP-Fe₂O₃-saline (Groups 8, 11-13) with or without MNU pretreatment, only Groups 8 and 12 given ITC-induced tracheal wounds during instillations, developed significant numbers of tracheal or intrapulmonary carcinomas.

Effect of MNU Once at 5 Weeks of Age. Group 3 (MNU only) compared with Group 2 (anaesthesia control) showed no significant differences in survival or in the incidence of respiratory lesions or tumors. The single epidermoid carcinoma of the larynx in Group 3 (MNU) is suggestive of a direct effect.

Groups 6 and 14 each received repeated saline instillations by ITC, but only Group 6 received MNU pretreatment. The survival curves were comparable. Group 6 had a significant increase in laryngitis and more frequent laryngeal hyperplasia/metaplasia and carcinoma in situ, but little difference in lower airway lesions compared to Group 14. Group 6 (MNU) had a total of five respiratory tumors (four carcinomas) in four hamsters, but Group 14 (no MNU) had none.

Groups 11 and 13 received repeated BP-Fe₂O₃-saline instillations by ILC but only Group 11 received MNU pretreatment. Group 11 had a significantly poorer survival than Group 13; however, few differences in their respiratory lesions or tumors were seen. Tumors occurred only in the larynx and the only invasive carcinoma was in Group 11 (MNU).

In contrast, significant differences were seen between Groups 8 and 12. Hamsters in both groups were given repeated instillations of BP-Fe₂O₃-saline by ITC but only those in Group 8 received MNU. Group 8 had a marked decrease in survival compared with Group 12 (Fig. 1). The total tumor yield was 38 tumors (34 carcinomas) in Group 8, and 26 tumors (18 carcinomas) in Group 12. Only Group 8 had tumors (six carcinomas) in the bronchi or lungs. Group 8 had a higher incidence and earlier onset of tracheal hyperplasia/metaplasia, and of carcinoma in situ, epidermoid carcinoma, and overall tracheal cancer. Moreover, these lesions and tumors were detected sooner in Group 8 than in Group 12. In Group 8, the first tracheal carcinoma in situ was seen at 21 weeks of age and the only tracheal carcinoma with metastasis seen in this study was detected at 28 weeks. In Group 12 the first tracheal carcinoma was not seen until 44 weeks of age. Group 8 had 1 in situ and five invasive carcinomas in the main bronchi, whereas Group 12 had no intrapulmonary tumors.

Group 3 (a single MNU instillation by ILC at 5 weeks of age without additional treatments) only had two laryngeal tumors (one carcinoma). This single MNU instillation was subcarcinogenic for the hamster trachea and lungs. A few other tumors, mostly in the larynx, were seen in groups which had received MNU pretreatment and repeated ILC or ITC instillations without BP (Groups 3-7 and 10). Therefore, the effect of the MNU pretreatment was most marked as a determinant in combination with BP. In all groups given MNU, a carcinogenic effect was seen in other organs with the development of benign and malignant tumors in the oral/pharyngeal mucosa, esophagus, forestomach, pancreas, biliary tract and large intestine, vascular tumors, and internal sarcomas. These data will be reported separately.

DISCUSSION

The results of this long-term study provide experimental evidence of the interplay of multiple causative factors in the induction of respiratory cancers. Several factors act as major determinants of the carcinogenic response in this experimental model: a single pretreatment with MNU, repeated instillations
of BP-Fe₂O₃, and mucosal wounding with its sequence of injury, epithelial regeneration, and mesenchymal repair. Instillations of Fe₂O₃ and even saline alone contribute to the epithelial proliferative response (38). The role of mechanical mucosal injury, previously only surmised in this established experimental model for respiratory carcinogenesis (15, 39), has now been identified as one of major importance.

Mucosal Wounding. ILC and ITC, with the associated laryngeal and tracheal wounds, although not carcinogenic per se, were important determinants of the carcinogenic response. Mucosal wounding enhanced the incidence of airway tumors and shortened their latent period. We reported in a companion paper that these treatments induce a marked epithelial proliferative response and a subjacent mesenchymal reaction (38).

Repeated ITC-induced tracheal mucosal wounds had the most significant enhancing effect on respiratory carcinogenesis of all the noncarcinogen variables tested in this experiment. Tracheal wounding without carcinogens did not induce tumors, but it did influence the long-term survival and the incidence of airway inflammation, epithelial hyperplasia, metaplasia, atrophy, and submucosal fibroplasia. Of those groups treated with one or both carcinogens, only those receiving repeated, intratracheal wounds developed tracheal or intrapulmonary cancers. Most of these respiratory cancers occurred along the ITC-induced tracheal wounds or near the distal end of these wound sites in the main bronchi.

The distribution pattern of Fe₂O₃ or BP-Fe₂O₃ particles, the amounts of particulate material, and the incidence of particle-laden macrophage accumulations and BAH were similar in the distal lung irrespective of the level of instillation (ILC or ITC). There was no evidence to suggest that the instilled suspension is drawn into the lungs by the animal’s inspiratory movements. Particles of Fe₂O₃ and even saline alone contribute to the epithelial proliferative response and a subjacent mesenchymal reaction (38).

The enhancing effects of ITC-induced wounding on respiratory carcinogenesis may also be due to other mechanisms, in addition to increased cell proliferation. Wounding disrupts the normal epithelial-mesenchymal interactions, setting in motion a complex sequence of repair as the epithelium regenerates itself. In studies of mechanical injury to the hamster tracheal mucosa, the extent and severity of the wounding were important, with focal wounds returning to normal structure within 5–7 days but larger wounds having a delayed and extended period of cell proliferation and abnormal regeneration resulting in atrophic and/or metaplastic epithelial lesions and underlying submucosal fibroplasia (13, 14). Moreover, the frequency of carcinogen instillation by ITC, and thus of tracheal wounding, is also important in determining the carcinogenic response, since more tumors result from weekly treatments than from the same doses given every 2 weeks (15, 44, 45, 52).

These correlations suggest that incomplete resolution of the hyperplastic response at the end of each weekly treatment offered a higher number of target cells to the next dose. Also, more carcinogen is likely to be deposited at the incompletely regenerated wound site when repeatedly administered. The wound site provides an area for localization of BP-Fe₂O₃-laden macrophages. The acutely denuded wounds favor direct adherence of particles and infiltration of macrophages in the wound area. The persistent atrophy and epidermoid metaplasia that develop in severe and chronic wounds, resulting in extensive nonciliated areas, disrupts normal mucociliary clearance and bronchitis and bronchial carcinoma (61), and lung scarring and peripheral lung cancer (62, 63). Moreover, human lung cancers induced by cigarette smoking, radiation, asbestos, and crystalline silica develop in respiratory epithelial and mesenchymal tissues that are affected by marked inflammatory and fibrogenic reactions (64–68).

Experimentally, cell proliferation and hyperplasia are essential features of tumor development in many tissues, such as liver (17, 18, 56) and skin (69–72). In the mouse skin, mechanical wounding has been shown to be as effective as phorbol esters in promoting skin carcinogenesis (70–72). Colon injury (57), hepatic injury or resection (17, 18), bladder injury (73), foreign body tumorigenesis (59, 60) and even wounding related to the development of plant tumors (74) are examples of chronic injury, wounding, and cell proliferation associated with carcinogenesis. Moreover, similar phenomena involving inflammation and proliferation have been shown to be key features of the enhancing effects of “inert” particles, fibrogenic fibers and dusts, cigarette smoke, aldehyde smoke constituents, sulfur dioxide, antioxidants, and vitamin A deficiency in respiratory carcinogenesis studies (15, 16, 53, 64–68, 75). Chronic infection can also be a major determinant of the carcinogenic response in the respiratory tract, as suggested by a study in male rats treated with the systemic carcinogen N-nitrosopentamethylenamine (76), which resulted in progressively higher incidences of peripheral lung carcinomas in germ-free (17%), specific-pathogen-free (37%), and conventional infected rats with chronic pneumonia (83%).

In tissues that usually have low proliferation rates, such as the epithelium of the lung, liver, and pancreas, an important limiting step in carcinogenesis may be the rate of cell proliferation. A round of epithelial cell division is required in adult tissues for initiation and is an essential feature of promotion and progression (17, 18, 69). Toxicological injuries to the respiratory epithelium that produce cell death, proliferation, and regeneration are considered as enhancing factors in respiratory carcinogenesis.

The persistent atrophy and epidermoid metaplasia that develop in severe and chronic wounds, resulting in extensive nonciliated areas, disrupts normal mucociliary clearance and bronchitis and bronchial carcinoma (61), and lung scarring and peripheral lung cancer (62, 63). Moreover, human lung cancers induced by cigarette smoking, radiation, asbestos, and crystalline silica develop in respiratory epithelial and mesenchymal tissues that are affected by marked inflammatory and fibrogenic reactions (64–68).

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collects migrating macrophages.

Loss of normal epithelial cells from the cannula-induced wounds and/or depression of normal epithelial cell growth adjacent to the wounds might also contribute to the enhancement of tumorigenesis following wounding since paracrine secretions from normal rat tracheal epithelial cells were shown to inhibit growth of carcinogen-altered cells in vitro (77), and normal epithelial cells in tracheal grafts to inhibit expression of the neoplastic phenotype of carcinogen-altered cells (78). Following wounding, neutrophils, macrophages, platelets, and lymphocytes are recruited into the wound site in large numbers. An array of growth factors (including TGF-\(\beta\)) is secreted by inflammatory cells (23, 24, 35). In humans, TGF-\(\beta\) inhibited proliferation of normal bronchial epithelial cells and induced terminal differentiation (36, 37). Therefore, loss of normal epithelial cells and/or suppression of their growth by TGF-\(\beta\) (from inflammatory cells) might give the carcinogen-altered cells a growth advantage at the wound site, following repeated cannula-induced tracheal wounding in hamsters.

The inflammatory response likely contributes further in that neutrophils and macrophages produce proteolytic enzymes and reactive oxygen species that enhance cell damage and proliferation (79–85). Oxygen free radicals produce genotoxic, mitogenic, and promoting effects in several carcinogenesis systems (86–91). Little is known of the effects of inflammatory cell mediators and cytokines on the respiratory epithelium. Inflammation at the wound may also enhance delivery of the active carcinogen to the proliferating target epithelium, as alveolar macrophages can metabolize polycyclic hydrocarbons such as BP (92).

**MNU Pretreatment and BP Instillations.** The single MNU treatment by ILC at 5 weeks of age was, by itself, subcarcinogenic for the trachea and lungs; however, it markedly enhanced the carcinogenic response in these organs when followed by ITC instillations of BP-Fe\(_2\)O\(_3\)-saline. MNU is a direct-acting carcinogen with a short biological half-life (10, 93). A synergistic effect of repeated MNU and BP treatments on hamster respiratory carcinogenesis has been reported (10), but the present findings show that even a single exposure to this agent can be a major determinant of the subsequent carcinogenic response to the BP-Fe\(_2\)O\(_3\) instillations. The cytotoxicity of MNU has been cited as a factor in experiments with multiple MNU treatments (43–47); however, no acute cytotoxicity was seen in the respiratory tracts of young hamsters in our preliminary acute toxicity study. The most likely mechanism for the carcinogenic enhancement by MNU in our long-term study is the normally high rate of cell divisions in the trachea of 5-week-old hamsters (48), which provided a pool of proliferating cells for initiation by the single, brief MNU exposure (17). The single MNU exposure by ILC was also found to have a marked carcinogenic effect on distant organs (to be reported separately); therefore, a systemic effect on the respiratory tract epithelium cannot be ruled out.

The carcinogenic effects of BP were conspicuous, but definitely conditioned by the presence of the tracheal wounding and by the MNU pretreatment. In the absence of these two cofactors (Group 13), only two papillomas and one carcinoma in situ of the larynx were produced by a total instilled dose of 45 mg BP per hamster. The effects of BP and other polycyclic aromatic hydrocarbons in this experimental model have been studied in many previous experiments always by means of ITC (2–4, 6, 8, 10, 15, 40, 53, 55, 94). The present protocol made it possible to identify the separate and interdependent role of mucosal wounding in determining the response to BP. Other carcinogens, having different toxic properties, may contribute differently to this combined response mechanism. The role of other agents, capable of producing epithelial damage and regeneration per se, could be studied using instillation by ILC or inhalation exposure in combination with BP or other carcinogens given by ILC.

Saline and Fe\(_2\)O\(_3\). The only significant effects of saline per se were to increase the incidence of alveolar metaplasia, as seen in MNU-pretreated groups with either ILC or ITC. Even Group 14, which had received saline by ITC but no MNU pretreatment, had a higher incidence of alveolar metaplasia and BAH than Group 5 which had received ITC without saline, although Group 5 had also received MNU pretreatment. This response suggests that the repeated ITC-tracheal wounding induced an altered mucociliary clearance of the lower respiratory tract (95, 96). Altered clearance would increase retention of inflammatory cells in the peripheral lung with release of their mediators on the epithelium of the distal airways. These considerations suggest that the reported enhancement of carcinogenesis by ITC-instilled saline, following treatment with \(^{210}\)Po (97, 98) or with systemic DEN (99), may be largely the result of ITC-induced tracheal wounds, possibly through interference with airway clearance mechanisms, although saline may have had an additional separate role, which would be testable by the present protocols. The fact that, of the groups receiving MNU and repeated ITC, only Group 6 (saline) had carcinomas, whereas Group 5 (no saline) had only papillomas, is merely suggestive of an effect on tumor progression.

The role of Fe\(_2\)O\(_3\) particles was extensively documented in previous studies by ITC: whereas no tumors were induced by Fe\(_2\)O\(_3\) without carcinogens, Fe\(_2\)O\(_3\) particles were a major determinant of the carcinogenic response to BP, when both were given by ITC (2–4, 15, 40, 52, 53, 94). The effects of Fe\(_2\)O\(_3\) observed in the present study, namely an increased incidence of tracheal fibroplasia in ITC-treated animals and an increased incidence of BAH with alveolar macrophage accumulation in the lungs of both ILC and ITC groups, suggest that in addition to the role of Fe\(_2\)O\(_3\) as a carrier particle, the effects of Fe\(_2\)O\(_3\) may be mediated by the recruitment of alveolar macrophages. These cells would contribute to the production of toxic and carcinogenic BP metabolites, and would stimulate other cellular reactions at the wound sites and the development of tumors by producing cellular mediators of inflammation and peptide growth factors capable of inducing cell damage and/or stimulating cell proliferation. The mechanisms involved in the determinant role of particulates in the induction of respiratory tumors in this remarkably effective experimental carcinogenesis model still need to be further clarified. For example, we do not know why BP, ground with gelatin and then simply mixed with Fe\(_2\)O\(_3\) particles, without surface adsorption and given repeatedly by ITC, is almost ineffective in spite of the concomitant reaction to Fe\(_2\)O\(_3\) particles and to ITC-induced mucosal wounding (40). These mechanisms could be further clarified by using the present protocol, comparing the ILC and ITC routes, to distinguish the effects due to particulates such as Fe\(_2\)O\(_3\) per se from those due to the repeated tracheal wounding, which, in the present study, appears to overwhelm the reactions to the particulates.

**CONCLUSIONS**

The present study demonstrated the highly significant and previously undocumented role of a noncarcinogenic variable in the hamster respiratory carcinogenesis model, namely repeated
laryngeal and tracheal mucosal wounding. The respiratory tract tumors occurred at the wound sites (larynx or trachea) or near them (main bronchi), although the entire respiratory tract was exposed to the BP-Fe$_2$O$_3$ particles, which were mostly deposited at the lung periphery. These findings help explain why ITC-instillations frequently result in a greater tumor incidence than inhalation of similar carcinogens by the same species (4, 15, 100).

The localized mucosal injury and the resulting reactions caused by tracheal wounds, described here and in our companion paper (38), are important in determining the final phenotypic expression of carcinogen-exposed cells. The critical mechanisms in this model are most likely changes in proliferation and differentiation of normal and preneoplastic cells in the wound areas influenced by locally released paracrine peptide growth factors (23, 36, 37).

Respiratory carcinogenesis, especially the induction of carcinomas closely comparable with human bronchogenic carcinomas, is clearly dependent on a multifactorial etiology. Various carcinogens are known to be effective in the hamster model, including polycyclic aromatic hydrocarbons, nitrosamines, nitrosamides, and asbestos (15, 43, 53, 64, 66, 100). Radiation from a variety of sources is an effective respiratory carcinogen (64, 66, 97, 98). Various inhaled carcinogens are effective in the hamster respiratory system (100) and inhaled toxic agents, such as ozone or oxygen, can be important factors in appropriate circumstances (101-104). Inorganic particulates with different chemical and physical characteristics act as critical determinants of the response (2-4, 15, 40, 66, 67). Genetic factors also determine the carcinogenic response, as shown by marked species differences in respiratory carcinogenesis (100). Nutritional factors are of importance, as shown by the inhibitory role of retinoids in the induction of experimental respiratory cancer (105, 106). Infectious agents are also important contributing factors (76). Finally, mechanical mucosal injury, as documented in the present report, is clearly a major determinant of the carcinogenesis response, as it must have been in some of the early animal models of respiratory carcinogenesis involving pellet implantation, thread transfixion or intrapulmonary implantation (100).

Our study shows that interplay and synergism of factors are typical of the highest carcinogenic responses. Remarkably similar considerations can be applied to the human situation, not only in the analogy of the cellular responses and in the characteristics of the induced tumors, but also in the interplay of multifactorial exposures. Cigarette smoking, the most widely recognized factor in human respiratory carcinogenesis, represents a complex multifactorial exposure to toxic, carcinogenic, and enhancing factors, and it is known to interact synergistically with other exposures, such as radiation and asbestos (64-68). In addition, many other human exposures to carcinogens, possibly of brief duration and at young ages, such as the single MNU exposure described here, may have a "predisposing" effect for cancer development.

In these respects, although the exposure methods are experimentally "exaggerated" (4), our hamster model can be viewed as a model of multifactorial human respiratory carcinogenesis. Can risk be apportioned to the many interdependent factors mentioned above? If we consider the total of 94 respiratory tract tumors reported here, we find no satisfactory way of attributing any one of them to a single causative factor, but rather we must consider them all as derived from complex etiological and pathogenic interactions. If all the 94 tumors were considered for each of the factors, we could attribute 64 tumors to MNU, 71 to BP, 16 to laryngeal wounding without tracheal wounding, and 75 to combined laryngeal and tracheal wounding, obviously a wide overlap. A further contributing role should be attributed to Fe$_2$O$_3$ for 79 tumors and to saline for 87 tumors, in addition to the roles of genetic, nutritional, and infectious factors. Clearly a quantitative risk estimate based on any single factor would provide an unrealistic assessment.

Prevention, by removal or reduction of each one of the interplaying factors, can be seen by appropriate group comparisons to result in drastic reductions of the total carcinogenic response.

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References


Multifactorial Hamster Respiratory Carcinogenesis with Interdependent Effects of Cannula-induced Mucosal Wounding, Saline, Ferric Oxide, Benzo[a]pyrene and N-Methyl-N\text{-}nitrosourea

Kevin P. Keenan, Umberto Saffiotti, Sherman F. Stinson, et al.