Tumor Induction in the Trachea of Hamsters with N-Nitroso-N-methylurea

Tsutomu Yarita, Paul Nettesheim, and Mary Lou Williams

ABSTRACT

Experiments were conducted to study the tumor response of hamster tracheas to N-nitroso-N-methylurea. Tracheas were exposed repeatedly with the use of a tracheal catheter. Ten to 30 exposures were given over a period of 5 to 20 weeks. The carcinoma incidence (including carcinoma in situ) was 0, 42, 67, 88, and 94% for 10, 15, 20, 25, and 30 twice-weekly exposures, respectively. With 10 exposures 2 of 12 hamsters developed benign tracheal tumors. Mean tumor induction time decreased when frequency of exposure was increased from 50 weeks with 10 to 15 exposures to 28 weeks with 25 to 30 exposures. The major histological types of invasive carcinomas observed were epidermoid carcinomas (54%), anaplastic large-cell and small-cell carcinomas (25%), adenocarcinomas (13%), and combined epidermoid-adenocarcinomas (7%). Sacrifice studies revealed that with 10 to 20 twice-weekly exposures only metaplastic lesions with varying degrees of cellular atypia are present at the time of the last exposure. Neoplastic lesions develop during the subsequent exposure-free interval. The data suggest that this tracheal tumor induction system may be well suited for studying problems related to development and progression of neoplastic disease.

INTRODUCTION

In recent years the development and fate of preneoplastic and early neoplastic lesions has been the subject of numerous investigations. Host and environmental factors enhancing or inhibiting the development of these lesions are being studied, since it is now realized that next to the prevention of exposure to carcinogenic agents the interruption of early stages of neoplastic development is probably the most effective means of cancer prevention.

Several laboratories (3) including our own (Ref. 1, 2, 5, and 9; for review see Ref. 4) have been involved in developing new experimental approaches for the study of neoplastic development in 1 major organ system that is a common target for carcinogenic substances in man, namely, the respiratory tract. For this purpose a method was devised in our laboratory by Schreiber et al. (9) to induce tumors in the tracheas of hamsters with the use of a tracheal catheter (Chart 1), which delivers fluid through a shorter outer cannula and aspirates the same fluid at a predetermined distance below through a longer inner cannula (8), thus exposing tissues only above the tracheal bifurcation. The advantages of this and other localized tumor induction systems, particularly in studies of neoplastic development, tumor promotion, and inhibition, are many and have been discussed in recent reviews of respiratory carcinogenesis (4, 6).

The purpose of this communication is to describe the qualitative and quantitative aspects of the tumor response induced in tracheal epithelium with repeated exposures to NMU.3

MATERIALS AND METHODS

Animals. Twelve- to 15-week-old male Syrian golden hamsters (ARS/Sprague-Dawley Division, The Mogul Corp., Madison, Wis.) were used throughout. The hamsters were housed in filter-top plastic cages and had free access to food and water.

Carcinogen Exposure. NMU was obtained from the National Cancer Institute chemical repository. Immediately before use the carcinogen was dissolved in 0.1 m sodium citrate buffer, pH 5.7. The NMU concentration was adjusted to 1% as measured by spectrophotometry at a wavelength of 392 nm. The NMU was stable at room temperature for at least 3 hr. The apparatus used for NMU exposure was principally the same as that described previously (8). Hamsters were anesthetized with a mixture of methoxyflurane (Metofane; Pitman-Moore, Fort Washington, Pa.) and oxygen and were then fastened to a slanted board (7) to facilitate insertion of the catheter through the open mouth into the larynx and trachea. Care was taken to obtain a constant depth of catheter insertion. The distance between the ends of the outer and the inner cannulas was 0.5 cm. The aim was to rinse the mucosal surface from the fourth to the tenth tracheal ring. The exposure conditions were as follows: washing time, 7 sec; volume released, 0.5 ml; vacuum for aspiration of washing fluid adjusted to ensure recovery of all washing fluid. Before the catheter was removed from the hamster trachea, the inner tubing was retracted into the outer tubing to prevent possible smearing of NMU upon withdrawal of the catheter. Animals were exposed to 1% NMU either once or twice weekly.

Hamsters were killed either at predetermined time intervals or when moribund and showing signs of respiratory distress. The larynx, trachea and bifurcation, and, in some cases, the lungs were fixed in Bouin's fixative for 24 hr. The tissues were then cut transversely into 16 tracheal rings, and the
when moribund or when signs of severe dyspnea became apparent. Tumor incidence, tumor type, and tumor "la-

dency" (i.e., time to development of severe respiratory distress or death) were the major end points studied. The extent of spreading of the tumors and other epithelial lesions within the trachea was also determined.

RESULTS

Sacrifice Studies. Eight hamsters were killed within 1 week after 1 exposure to 1% NMU. No marked mucosal changes could be recognized. Four to 8 hamsters were killed within 1 week after 2 exposures given in 1 or 2 weeks. Mild hyperplastic changes were seen in the midportion of the trachea. These were not detected 1 week later. After 5 NMU exposures, 2 exposures/week, marked epithelial changes were noticed. The epithelium was flattened. Cells with irregularly shaped, hyperchromatic, large nuclei were seen. In this group 2 to 3 animals were also killed 2, 4, and 8 weeks after the last exposure. At that time most of the epithelium appeared normal except for small metaplastic patches showing mild cellular atypia. When NMU was given 5 times only once weekly, the epithelial changes were much milder and did not persist beyond a few weeks. With 10 exposures given at weekly intervals, the epithelial abnormalities were only slightly greater, but small focal metaplasias developed 8 weeks after the last exposure.

After 10 to 20 exposures, groups of 10 or more animals were killed. Since these exposure regimens are probably the most important ones for purposes of tumor induction, the histological findings are presented in some detail. The distribution of lesions within each trachea was "mapped," as illustrated in Chart 2. After 10 washings with NMU (2/week), 11 of 13 hamsters showed squamous metaplastic lesions with and without mild cellular atypia (Chart 2A). Some of these were circumscribed lesions located between the fifth and tenth tracheal rings. Areas adjacent to these metaplasias showed either flattening and attenuation of the epithelium with bizarre-shaped cells or various types of hyperplasias. After 15 exposures (Chart 2B), 10 of 10 hamsters showed widespread squamous metaplasias with mild to moderate atypia (Fig. 1a), in some cases covering much of the surface of the affected tracheal rings. Hyperplastic changes were only occasionally seen. Instead, large areas of mucosa were covered with an almost completely undifferentiated epithelium with either extremely flattened cells or large "bizarre-shaped" cells (Fig. 1, b and c). Most of the metaplasias occurred between the third and tenth tracheal rings (Chart 2b). No neoplasias were present in either of the 2 groups.

Two groups of animals were killed after 20 washings with NMU: 21 animals receiving 2 exposures/week and 18 hamsters receiving 1 exposure/week (total exposure period, 20 instead of 10 weeks). All animals of the former group showed widespread squamous metaplasias with moderate to marked cellular atypia (Fig. 1d); some of these were thin, composed of flattened cells. In many cases the entire surface area of the affected tracheal ring was occupied by the metaplastic lesion. Undifferentiated epithelium with bizarre hyperchromatic cells was found in areas adjacent to the metaplasias. In most animals killed 1 week after the last exposure, much of the epithelial surface between the 2nd and 12th tracheal rings was severely altered. No tumors
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Table 1

Respiratory tract tumor response in hamsters multiply exposed to intratracheal 1% NMU

<table>
<thead>
<tr>
<th>Time to first tumor (wk)</th>
<th>From start of exposure</th>
<th>From end of exposure</th>
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<table>
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<tr>
<th>Treatment</th>
<th>No. of exposures</th>
<th>Effective no. of hamsters</th>
<th>No. of hamsters with carcinoma (all types)</th>
<th>No. of hamsters with carcinoma (invasive type)</th>
<th>Mean tumor induction time (wk)</th>
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<td>12</td>
<td>2 (17)*</td>
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<td>50</td>
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<td>Citrate buffer</td>
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<td>25</td>
<td>17</td>
<td>15 (88)</td>
<td>12 (71)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>17</td>
<td>16 (94)</td>
<td>16 (94)</td>
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</table>

Numbers in parentheses, percentages.

* This group received 1 exposure/week instead of 2, as did all other groups. Two animals died after 19 exposures.

This control group has been under study for 50 weeks.

were present. A few hamsters were killed 2 to 8 weeks after the last exposure to determine whether some of the mucosal alterations would regress. Some recovery seemed to occur since the lesions were less extensive; however, 2 of 3 animals killed 8 weeks after the last exposure showed small invasive carcinomas in the midportion of the trachea.

The animals receiving 20 NMU washings over 20 weeks showed much the same epithelial pathology as did the previous group. However, undifferentiated lesions with bizarre and often atrophic cells were less frequent. Hyperplastic lesions were more common; 5 of 12 animals killed at 1 week after exposure had carcinoma in situ, and 1 animal had an early invasive carcinoma. Six animals were killed between 2 and 8 weeks after the last exposure. Three of these had carcinoma in situ, 1 had a squamous papilloma, and 4 had early invasive carcinoma (Fig. 2). The distribution of lesions in individual hamsters is illustrated in Chart 2C.

Control animals were washed 1 to 20 times with buffer solution without carcinogen. In half of the animals (9 of 18), small patches of regular squamous metaplasia were found. Small areas of mild hyperplasia were also seen; however, most of the tracheal mucosa was normal.

Tumor Induction Studies. The mortality occurring during repeated NMU exposures is summarized in Chart 3. The findings of 5 different studies representing over 450 hamsters carried out for various purposes during an 18-month period are summarized. The incidence of mortality was somewhat variable from experiment to experiment but did not exceed 10% with up to 15 exposures, and it was between 6 and 14% with 20 exposures regardless of whether the doses were delivered once or twice/week. With 25 and 30 exposures, the cumulative mortality increased to 22 and 35%, respectively. It is obvious that the deaths are not caused by the anesthesia and/or the washing procedure but are due solely to the effects of NMU, since exposure to the citrate buffer alone, without carcinogen, caused no mortality. The cause of death in almost all of the NMU-exposed animals was laryngitis, tracheitis (Fig. 3), and/or pneumonia.

Mortality data from animals killed when moribund or dying spontaneously after various NMU exposure regimens had been completed are presented in Chart 4. The median
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Chart 3. Mortality of hamsters during repeated exposure to 1% NMU by intratracheal washing. Three groups of hamsters were exposed twice a week to NMU (O, n = 93; D, n = 179; A, n = 182). One group was exposed to NMU once a week (O, n = 79); 1 control group (●) was exposed to citrate buffer without carcinogen.

Graph 3. Mortality of hamsters exposed to 1% NMU by intratracheal washing. The survival time of the groups receiving 20 or 30 exposures was approximately 20 to 30 weeks; that of the groups receiving 10 or 15 exposures was 50 to 60 weeks. In contrast, no mortality has been encountered in the control group receiving 30 exposures to the washing fluid without carcinogen during the 50 weeks that have elapsed since the start of the experiment. One control group receiving 20 exposures developed "wet-tail" disease at 12 weeks after the last exposure, and 80% of the animals died within 4 weeks.

The tumor incidence data are summarized in Table 1 (see also Chart 4). With 10 NMU exposures only 2 of 12 hamsters developed tumors. Both of these occurred rather late, at 33 and 62 weeks after the start of exposure. One was classified as polyp, the other as papilloma. The other animals died with hyperplastic, metaplastic, or atrophic changes of the tracheal epithelium or, in most cases, with normal tracheas. These animals died from wet-tail disease, renal sclerosis, amyloidosis, or tumors of other organs.

With 15 exposures 9 of 12 hamsters (i.e., 75%) developed tracheal and laryngeal neoplasms. Only 2 hamsters, however, carried invasive carcinomas, while the other lesions were papillomas and noninvasive carcinomas (carcinoma in situ). The mean "tumor induction time" (more precisely, time to death with tumor) in these 2 low-dose groups is approximately 50 weeks (see Table 1). The time to first tumor, counting from the beginning of exposure, is 33 and 25 weeks for the groups receiving 10 and 15 exposures, respectively.

With 20 NMU exposures in 10 weeks, 10 of 15 animals developed tumors. All of these occurred in the trachea and were invasive carcinomas. Thus, the percentage of animals with invasive tumors was close to 70%. The mean tumor induction time in this and all subsequent NMU-exposed groups was between 28 and 35 weeks (from the start of exposure) and was thus 4 to 5 months shorter than that in the 2 previous groups receiving only 10 and 15 NMU doses, respectively. The time to first tumor was between 13 and 19 weeks.

Another group of animals received 20 NMU exposures spread over 20 instead of 10 weeks. The tumor response was distinctly greater than that of the group receiving 20 exposures within 10 weeks. Ninety-six % of the animals developed tumors. Several animals had more than 1 neoplastic lesion, 5 of which involved the larynx. Seventy-four % of the animals in this group had 1 or more invasive carcinomas. The average tumor induction time was 35 weeks, as compared to 30 weeks in the group receiving 20 exposures within 10 weeks.

With 25 and 30 NMU exposures, 15 of 17 and 16 of 17 hamsters, respectively, had noninvasive or invasive carcinomas. The percentages of animals with invasive carcinomas were 71 and 94%, respectively. Three hamsters died within 1 to 3 weeks after the last exposure without neoplasia from an obstructive tracheitis. Both exposure groups had several animals with more than 1 neoplastic lesion. The group receiving 30 NMU exposures had a particularly high incidence of multiple carcinomas of distinct histological types. No animals from the 2 control groups developed any neoplasms. The animals that died in these groups showed no significant epithelial abnormalities in larynx and trachea.

A minimum of 2 histological sections/tracheal ring and 8 sections/larynx was prepared from each animal. This allowed us to map the distribution and extent of lesions. Chart 5 depicts the distribution of "toxic" lesions, metaplasias with and without cellular atypia, polyps and papillomas, noninvasive carcinomas, and invasive carcinomas in the tracheas of the hamsters from the largest group, which received 20 weekly NMU exposures, and, for comparison, from the group receiving 10 NMU exposures. It can be seen (Chart 5B) that the majority of the neoplastic lesions (30 of 36) were somewhere between the 4th and 12th tracheal rings. In some cases the neoplasms had spread over this entire surface. Approximately half (4th to 12th rings) of the entire trachea seemed to have been exposed extensively to carcinogen. While the majority of the lesions were concentrated in this region (we intended to expose the 4th to 10th tracheal rings), metaplastic and neoplastic lesions did oc-
cur above (including in some cases the larynx) as well as below. The malignant lesions tended to occupy the entire tracheal ring (or rings) indicated in the chart, while the nonmalignant abnormalities were in many cases only focal. The other exposure groups (not shown here) were evaluated in the same manner. They presented a similar picture except for the group receiving 30 exposures. In this group many tracheas seemed to have been heavily exposed from the larynx down to the 12th tracheal ring, as was indicated by the presence of severe preneoplastic and neoplastic lesions over much of this area.

Table 2 summarizes the histological types of tumors appearing in groups of hamsters receiving different numbers of NMU exposures. Of the 141 tumors observed in 96 animals (not counting the controls), 54% were diagnosed as invasive carcinomas, 32% as noninvasive carcinomas (the term is used as synonymous with carcinoma in situ or intraepithelial carcinoma), and 13% as polyps and papillomas. Only 1 sarcoma was found. A group of “growths” not listed in Table 2 resembled hemangiomas (Fig. 4). These were sometimes large enough to obstruct the tracheal lumen and cause suffocation. The frequency of these tumor-like lesions was 1, 0, 5, 3, and 1 in groups exposed 15, 20 (twice/week), 20 (once/week), 25, and 30 times, respectively. Whether these growths were true hemangiomas, i.e., of neoplastic nature (or venous ectasias associated with inflammation), was doubtful; they were therefore not included in Table 2. As indicated by the data given in Table 2, the incidence of neoplasms was clearly dose related. In the context of this study, this means related to the cumulative number of exposures. An average of 0.17 tumor/animal was present in the lowest dose group, while 2.53 tumors/animal were observed in the highest dose group. However, the dose rate was apparently also of significance since the tumor incidence in the group receiving 20 weekly injections was 1.87 tumors/animal, compared to 0.93 tumor/animal in the group receiving 20 exposures with 2 exposures/week. The proportion of benign tumors clearly diminished with increasing dose: 100% of the tumors were benign in the lowest exposure group as compared to only 2% in the highest exposure group. The relative carcinoma frequency did not show any recognizable trend. A total of 45 noninvasive carcinomas was found as compared with 76 invasive carcinomas. Of the invasive carcinomas 54% were epidermoid carcinomas. Anaplastic carcinomas were the next most common type (26%). These were subdivided into a

<table>
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<th>Total no. of tumors</th>
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<th>Papilloma and polyp</th>
<th>Noninvasive carcinoma</th>
<th>Invasive carcinoma</th>
<th>Epidermoid</th>
<th>Adeno</th>
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<td>5</td>
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<td>3</td>
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a Not included are 10 hemangioma-like growths occurring in the tracheas of hamsters of 4 of the 6 NMU-exposed groups (see text).
b For details of exposure see Table 1.
c This group has not yet been terminated. It has been carried for 50 weeks.
large- and a small-cell type, the large-cell type being the more common. Adenocarcinomas made up 13% of the carcinomas, and a few tumors were of the combined epidermoid-adenocarcinoma type (7%). Only 1 sarcoma was observed. Examples of the various tumor types are illustrated in Fig. 5.

DISCUSSION

In preliminary experiments it had been established that, at a concentration of 1% NMU, hamsters would not tolerate more than 2 exposures/week. We therefore limited our exposures to 1 or 2/week. Our experiments showed that the tumor response in this experimental system was indeed ‘dose’ dependent, which means, in this case, dependent upon the number of exposures. This was most clearly reflected in the number of animals with noninvasive and invasive carcinomas or the total number of tumors per group (see Tables 1 and 2). With 10 and 15 exposures, 12 of 14 tumors were noninvasive, suggesting that less frequent exposures to NMU caused development predominantly of benign or slowly growing lesions.

The mean tumor induction time and the time to first tumor (i.e., the average time required for animals to die with tracheal tumors and the time required for the first animal to die with tumor) were inversely related to the number of exposures (see Table 2). The interval between the last carcinogen exposure and the appearance of the first tumor was over 4 months for the 2 low-dose groups and 2 months for the group receiving 20 exposures (at the rate of 2/week), and for all other groups it was 0 to 3 weeks.

Of particular interest is a comparison of the tumor responses in the 2 groups receiving 20 exposures at different rates, namely, 2 and 1 exposure/week, respectively. In terms of tumor incidence, 20 NMU exposures given over 20 rather than 10 weeks were clearly more effective: 96% tumor-bearing animals compared to 67% (see Table 1). The average number of tumors per animal was twice as high (1.87 compared with 0.93 tumor/hamster) in the group receiving 1 exposure/week as it was in the group receiving exposures twice weekly (see Table 2). This suggests that not only total dose but also duration of exposure (or, more accurately, the time span during which exposures are given) may be a crucial determinant of the magnitude of the tumor response, since the same cumulative number of exposures caused a substantially stronger response when spread out over a longer time. The mean tumor induction time was virtually the same in both groups (see Table 2).

A comparison of the frequency and extent of epithelial abnormalities at the time of the last exposure (e.g., Chart 2A) and at the time of death several months later (e.g., Chart 5A) shows that fewer and less extensive metaplasias were found at death. This indicates that many of these early, recognizable changes were of a toxic nature and were reversible.

The sacrifice studies yielded another important piece of information. Of the 12 animals killed 1 week after 20 exposures given within 10 weeks, none showed carcinomas (noninvasive or invasive); all showed metaplastic lesions with moderate to marked cellular atypia. However, 2 of 3 hamsters killed 8 weeks later had invasive carcinomas, suggesting that at least some of the neoplasms developed rather rapidly. With 20 exposures given over a period of 20 weeks (Chart 2C), noninvasive carcinomas were already present in several animals 1 week after the last exposure, and invasive carcinomas were present as well in hamsters killed 1 to 8 weeks later. These findings are consistent with the data obtained in the survival study (see Chart 4A).

Our experiments showed that, with an increasing number of exposures, increasingly larger areas of the tracheal epithelium were visibly altered and presumably transformed. The extent to which various levels of the trachea (and larynx) showed pathological changes was greater than anticipated. Although in general the most severe damage seemed to be limited to the midportion of the trachea, the carcinogen exposure of tracheal mucosa was not as sharply circumscribed as was originally expected. This suggests that one might be able to deliver a more uniform dose to the trachea by increasing the distance between the ends of the inner and outer tubing (Chart 1). The occasional induction of tumors in the larynx is clearly due to technical imperfections that can probably be avoided with further refinement of the techniques.

The types of tumors induced were similar to those described previously by other investigators in hamsters given intratracheal injections of polycyclic hydrocarbons (e.g., Ref. 7). The large number of noninvasive carcinomas was surprising. The majority were topographically unrelated to other tumors (28 of 45), while 17 occurred in close proximity to invasive carcinoma. Most were small and confined to only a small portion of a single tracheal ring. In this respect they were clearly distinct from both the metaplasias and the invasive carcinomas, which often spread over large areas of mucosa. The earliest carcinoma in situ was observed at 13 weeks after start of exposure. Most, however, occurred much later. In contrast to the metaplasias with and without cellular atypia, this lesion was clearly a late development.

In the group receiving 15 exposures, noninvasive carcinomas were observed at 50 to 65 weeks after start of exposure. By far the most common type of invasive carcinoma was the epidermoid carcinoma, followed by the anaplastic carcinoma. In this latter class the majority were of the large-cell type and 3 were of the small-cell type (fusiform and polygonal). Whether these latter tumors are equivalent to human small-cell carcinomas remains to be determined.

Adeno- and combined epidermoid-adenocarcinomas were the least frequent classes of invasive carcinomas. There seemed to be no predilection for tumor types in the various tracheal segments. No distant metastases were observed; however, local invasion of lymph nodes, mediastinum, and thoracic cavity was frequently seen. The animals probably died from tracheal obstruction before metastases could develop.

Significant mortality occurred in all NMU exposure groups during the exposure period. With 10 to 20 exposures, the number of deaths remained near or below 10% in most cases. With higher exposure numbers the loss of animals became, however, quite considerable. We found that in almost all cases death was due to necrosis of
tracheal epithelium followed by the development of an obstructive granulomatous tracheitis. Studies with 1.5% NMU have shown that the tracheal necrosis and subsequent obstruction occur even earlier at higher NMU concentrations. The evidence suggests that the primary cause of this tracheitis is NMU toxicity rather than infectious agents that might have been introduced with the repeated intratracheal washing. This suspicion is confirmed by ongoing studies with lower NMU concentrations. The low incidence of mortality in the earlier study (with 30 exposures to 1% NMU) (9) is probably due to a lower concentration of NMU than that assumed by the investigators.

In conclusion, we found that the tumor development in this tracheal tumor system follows a dose-response pattern in terms of number of tumor-bearing animals, number of tumors per animal, and tumor induction time. The main histological types of respiratory tract cancers are induced. Most of them are confined to the tracheal segment between the 3rd and 12th tracheal rings. Depending on the frequency of NMU exposures, several months may elapse between the last exposure and death from cancer. Exposure-free intervals of several months can be obtained before the appearance of tumors with 20 exposures or less, making the system useful for investigations of neoplastic evolution.

REFERENCES
Fig. 1. Early mucosal changes in hamsters exposed to NMU 15 to 20 times. 

a, focal squamous metaplasia with mild atypia. H & E, × 400. 
b, "flattened" epitheleum with very elongated cells and thin epithelial lining. H & E, × 400. 
c, bizarre cells with large irregular nuclei. Note inflammatory exudate. H & E, × 400. 
d, focal squamous metaplasia with marked atypia. H & E, × 312.
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Fig. 2. Early neoplastic lesions in tracheas of hamsters exposed intratracheally 20 times. a, carcinoma in situ. H & E, × 312. b, early invasive squamous cell carcinoma. H & E, × 312.

Fig. 3. Obstructive tracheitis in hamster exposed 30 times to NMU. Only a small central channel is open; no epithelial lining is present. Note granulomatous reaction with giant cells. H & E, × 125.

Fig. 4. Hemangioma-like growth protruding into the tracheal lumen in a hamster given 15 NMU exposures. Dilated spaces are lined with what appears to be endothelium and are filled with blood. H & E, × 200.
Fig. 5. Major types of invasive carcinomas in hamsters following 15 to 20 NMU exposures. a, squamous carcinoma. H & E, × 312. b, adenocarcinoma. H & E, × 200. c, anaplastic large-cell carcinoma. H & E, × 312. d, anaplastic small-cell carcinoma. H & E, × 400.
Tumor Induction in the Trachea of Hamsters with \textit{N}\text{-}Nitroso\textit{N}\text{-}methy lurea

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