Effects of Route of Administration and Dose on the Carcinogenicity of N-Nitrosodiethanolamine in the Syrian Golden Hamster

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ABSTRACT

N-Nitrosodiethanolamine was assayed for carcinogenicity in Syrian golden hamsters by s.c. injection, topical application, and oral cavity swabbing. Three groups of 30 hamsters each received 27 weekly s.c. injections of either 500, 170, or 58 mg of N-nitrosodiethanolamine per kg in 0.9% NaCl solution. In the group treated with 500 mg/kg, 19 of 30 animals developed nasal cavity tumors, 7 of 30 had tracheal tumors, and 2 of 30 had tumors of the larynx. Among the animals treated with 170 mg/kg, 7 of 29 presented with nasal cavity tumors and 4 of 29 presented with tracheal tumors. In the group treated with 58 mg/kg, only two tracheal tumors were observed. Acetone solutions of N-nitrosodiethanolamine were applied to the shaved backs of three groups of 30 hamsters, each three times weekly for 36 weeks, at doses of 25, 8, or 2.5 mg; the total doses were the same as in the groups treated by s.c. injection. At the 25-mg dose level, 5 of 30 animals developed nasal cavity tumors and 4 of 30 animals had tumors of the trachea. No skin tumors were observed. The incidence of respiratory tract tumors in the groups treated with 8 or 2.5 mg was not significant compared to controls. The oral cavities of 40 hamsters were swabbed three times weekly for 45 weeks with 20 mg of N-nitrosodiethanolamine; the total dose was the same as the highest doses given by s.c. or topical administration. Seventeen of 38 hamsters had nasal cavity tumors, 6 of 38 developed tracheal tumors, and 1 of 38 presented with a tumor of the larynx. No tumors were observed in the oral cavity. The results of this study demonstrate that N-nitrosodiethanolamine is organospecific for the Syrian golden hamster nasal cavity and trachea and that it induces tumors in these sites at doses lower than previously reported.

INTRODUCTION

NDELA, an environmentally prevalent nitrosamine, is found in relatively high concentrations in tobacco, tobacco smoke, cosmetics, and industrial cutting fluids. It induces tumors of the liver and nasal cavity in rats and tumors of the respiratory tract in Syrian golden hamsters. Dose-response studies in rats have demonstrated that it is a relatively potent carcinogen than previously assumed. However, little is known about the effect of route of administration on NDELA carcinogenesis. Two of the major routes of human exposure to NDELA are through the skin, from cosmetics, and through the oral cavity from chewing tobacco. Therefore, we have compared the carcinogenicity of NDELA administered s.c., topically, and to the oral cavity of Syrian golden hamsters and have investigated its effects at doses lower than those previously examined.

MATERIALS AND METHODS

Apparatus

High-performance liquid chromatography was performed on a system consisting of 2 Model 6000A solvent delivery systems, a Model 660 solvent programmer, a Model U6K injector, a Model 440 UV-visible detector (Waters Associates, Milford, Mass.) and a 250- x 4.6-mm Lichrosorb SI-60 10-µm column (EM Reagents, Cincinnati, Ohio). Compounds were eluted with 4% ethanol in CHCl₃ at a flow rate of 2.5 ml/min. Preparative high-performance liquid chromatography was carried out with a Waters Associates Prep LC/system 500 with Prep-pak-500/silica cartridges (Waters Associates). Thin-layer radiochromatography was performed with a Packard Model 7201 radiochromatogram-scanner. Scintillation counting was done with a Nuclear Chicago Isocap 300 system.

Chemicals

Synthesis of NDELA. Diethanolamine (50 g, 0.48 mol; Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in 35 ml of H₂O and 5 N HCl was added dropwise until pH 3.5 was reached. A solution of NaNO₂ (38 g, 0.55 mol) in 45 ml of H₂O was added dropwise over a 30-min period while the pH was maintained between 3 and 4. After 4 hr of stirring at room temperature, absolute ethanol was added, and the mixture was concentrated at reduced pressure. NaCl was removed by filtration and was washed with ethanol and acetone. The filtrate and washings were concentrated to give crude NDELA which contained some diethanolamine. This residue was dissolved in chloroform:ethanol (6:1) and passed 3 times through 400 g of activity III acidic alumina (Woelm) to remove the diethanolamine. The NDELA was purified by preparative high-performance liquid chromatography on 2 silica cartridges with elution by chloroform:ethanol (10:1 at 0.1 liter/min, yielding 25 g (39%), which was pure by thin-layer chromatography [silica, chloroform:ethanol (5:1); Rf 0.43] and high-performance liquid chromatography. Its mass spectrum was identical to the published spectra (1, 2). This procedure was carried out repeatedly to provide 350 g NDELA for the bioassay.

[U-¹⁹C]NDELA (10 mCi/mmol) was obtained from Rosechem Products, Los Angeles, Calif. Its purity was established by thin-layer radiochromatography under the conditions described above for NDELA. NCS solubilizer was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.).

Absorption of [U-¹⁹C]NDELA. Six male Syrian golden hamsters, 8 weeks old, were housed individually in glass metabolism cages (Eck and Krebs, Long Island City, N. Y.). Two animals were given s.c. injections of [U-¹⁹C]NDELA (500 mg/kg body weight, 7 x 10⁶ dpm), 2 animals were treated by topical administration to their shaved backs of [U-¹⁹C]NDELA (25 mg, 8 x 10⁶ dpm) in 0.5 ml of acetone, and the oral cavities of 2 animals were swabbed with [U-¹⁹C]NDELA (25 mg, 2 x 10⁶ dpm) in 0.5 ml of 0.9% NaCl solution. The remaining radioactivity in the swabs was determined. Urine, feces, and expired air were collected, the latter in gas wash bottles containing 2 n NaOH. Aliquots of the urine and NaOH

1 This study was supported by USPHS Contract NO1-CM-55666 and National Cancer Institute Grants CA 23901 and CA 29580. This is paper 24 in the series, "A Study of Tobacco Carcinogenesis."

2 The abbreviation used is: NDELA, N-nitrosodiethanolamine.

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solutions were subjected to scintillation counting. Feces were extracted with acetone in a Soxhlet extractor, and aliquots were counted. The topically treated animals were sacrificed after the collections, and the treated areas (3 x 3 cm) were excised, solubilized in NCS, and counted. Animals treated by oral swabbing were sacrificed, and their oral tissues were removed, solubilized in NCS, and counted.

Bioassays for Carcinogenicity. Outbred Syrian golden hamsters (8 to 10 weeks old) from Simonsen Laboratories (Gilroy, Calif.) were housed in groups of 5 in solid-bottomed polycarbonate cages with hardwood bedding and kept at 20 ± 10% (S.D.) relative humidity with a 12-hr light-dark cycle. The hamsters were given Purina laboratory chow and tap water ad libitum.

Division into experimental groups is shown in Table 1. Animals in Groups 1 to 8 received s.c. injections of NDELA in 0.9% NaCl solution or 0.9% NaCl solution alone once weekly for 27 weeks. Average weights of the animals during this period were 190 g (females) and 165 g (males). The backs of the hamsters in Groups 9 to 16 were shaved at the beginning of the experiment and as necessary during the course of treatment. NDELA in acetone (0.5 ml) or acetone alone was applied with a biopipet 3 times weekly for 36 weeks. The oral cavities including the

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Sex of animals</th>
<th>Dose/application</th>
<th>Total dose/animal</th>
<th>Mean wt after 1 yr (g)</th>
<th>Early</th>
<th>Advanced</th>
<th>With brain invasion</th>
<th>Tracheal tumors</th>
<th>Larynx tumors</th>
<th>Other tumors</th>
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<td>1</td>
<td>15 (15)</td>
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<td>2</td>
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<td>500 mg/kg s.c.</td>
<td>2570</td>
<td>19.2</td>
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<td>1</td>
<td>4</td>
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<tr>
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<td>M</td>
<td>170 mg/kg s.c.</td>
<td>760</td>
<td>5.7</td>
<td>156</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>4</td>
<td>15 (14)</td>
<td>F</td>
<td>170 mg/kg s.c.</td>
<td>860</td>
<td>6.4</td>
<td>165</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
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<td>2.2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>175</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>10 (8)</td>
<td>F</td>
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</tr>
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<td>2700</td>
<td>20</td>
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<td>2.5 mg topical</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>F</td>
<td>Acetone topical</td>
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<td>0</td>
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<td>4</td>
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<td>F</td>
<td>20 mg oral swabbing</td>
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<td>146</td>
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<tr>
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<td>163</td>
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<td>0</td>
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<td>1</td>
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<td>170</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* Injections were given s.c. in 0.9% NaCl solution once weekly for 27 weeks. Topical applications were given in acetone, 3 times weekly for 36 weeks. Oral swabbing was done with 0.9% NaCl solution, 3 times weekly for 45 weeks.

| Numbers in parentheses, number of animals autopsied.
| Two animals with nasal and tracheal tumors.
| Pancreas islet tumor.
| One pancreas islet tumor; one lymphoma; one (pleomorphic) sarcoma; one lung adenoma.
| Lung metastases of a giant cell tumor.
| Thyroid papillary adenoma.
| Pleomorphic sarcoma; squamous cell carcinoma stomach; hemangiomia (mixoid) of spleen; liver hemangiomia; spindle cell sarcoma (low grade) of spleen; one large adrenal adenoma.
| Parathyroid adenoma; thyroid clear-cell adenoma; liver adenoma; low-grade spindle cell sarcoma of spleen; one large adrenal adenoma.
| Lymphoma.
| One animal with nasal tumor plus tracheal tumor.
| Liver adenoma.
| Anaplastic carcinoma of salivary gland with liver metastases.
| Skin acanthosis plus hyperkeratosis; adenocarcinoma in a lymph node (primary unknown).
| Bladder papilloma; one large adrenal adenoma.
| Lymphoma; sebaceous gland hyperplasia, one large adrenal adenoma.
| Three animals with nasal tumors and tracheal tumors.
| Two animals with lung adenomas; one liver adenoma.
| Possible lung metastases in one case.
| Lip papilloma.
| One large adrenal adenoma.
| Three animals with spleen angiomas, one large adrenal adenoma.

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RESULTS

To assess the absorption of NDELA by the 3 routes to be used in the bioassays, we administered [14C]NDELA to Syrian golden hamsters by the s.c., topical, and p.o. routes. Sixteen hr after s.c. injection of [U-14C]NDELA, 49% of the dose appeared in the urine and 11% appeared in the feces; no radioactivity was detected in the expired air. Sixteen hr after oral swabbing with [U-14C]NDELA, 34% of the dose was excreted in the urine and 6% in the feces. No radioactivity was detected in the expired air, and only 2% of the dose remained in the oral cavity. In contrast, 16 hr after application of [U-14C]NDELA to the skin, 41% of the dose was still present in the skin, and the levels of excretion in the urine and feces were 21 and 4%, respectively.

Except in Group 9, all animals gained weight steadily for the first 6 months of the bioassay, reaching a mean of 181 g for males and 204 g for females. Subsequently, weight declined somewhat, as indicated in Table 1. The animals in Group 9 reached a maximum mean weight of 165 g 14 weeks after treatment. With the exception of Groups 1, 17, and 18, survival was 80% or greater in all groups 10 months after treatment. The percentages of survivors in these groups 10 months were: Group 1, 73%; Group 17, 75%; and Group 18, 65%. Survival decreased steadily in all groups beginning between 10 and 20 months after treatment.

Regardless of the route of administration, the main target organs for NDELA were the nasal cavity and trachea (see Table 1). Within the groups treated by s.c. injection, the incidence of nasal cavity, tracheal, and laryngeal tumors was significantly greater in Groups 1, 2, and 4 (p < 0.01) and in Group 3 (p < 0.05) than in controls. Since tracheal tumors are rarely observed as spontaneous tumors in untreated hamsters, the 2 tracheal tumors in Group 5 are likely to be treatment related. Among the animals treated by topical administration, the tumor incidence in Group 9 was significant (p < 0.05) compared to controls. The 3 nasal cavity tumors in Group 10 were almost certainly treatment related, although the incidence was not statistically significant at p < 0.05. Significant incidences of tumors were also observed in the animals in Groups 17 (p < 0.01) and 18 (p < 0.05) treated by oral swabbing.

The nasal cavity tumors affected both respiratory and olfactory areas and presented a large sequence of aspects, from squamous papillomas to olfactory esthesioneuroepitheliomas with brain invasion. One animal, carrying a large olfactory tumor, had also lung metastases. The morphological structure of the metastases was not identical with the primary nasal tumor but was sufficiently similar to suggest that the olfactory tumor was the primary focus, especially since no other cancer was found elsewhere in the body. All animals, untreated as well as treated, had adrenal tumors, mostly of small size, probably cortical adenomas sometimes with an irregular, bizarre cellular arrangement. Several hamsters developed very large adrenal tumors (2 cm in diameter) with extensive hemorrhagic necrotic areas. Two of these tumors had a histological structure suggesting neuroblastomas. The kidneys and livers of all animals showed marked chronic degenerative and inflammatory changes ("old hamster kidney").

DISCUSSION

This study demonstrates that NDELA is an organospecific carcinogen for the Syrian golden hamster trachea, larynx, and nasal cavity. While previous studies have shown that NDELA administered in the drinking water or by injection is carcinogenic in the rat and Syrian golden hamster, application of NDELA to the skin, which is a major route of human exposure, obviously also results in tumors of the respiratory tract in the Syrian golden hamster (3, 6, 10, 15, 17). Since the animals were not caged individually, it is possible that some of the tumors may have resulted from ingestion of NDELA during grooming. While the results of the bioassays are in agreement with the generally observed organospecificity of nitrosamines, topical administration to Syrian golden hamsters or oral swabbing of some related nitrosamines did induce local as well as systemic tumors. Thus, N-nitrosobis(2-hydroxypropyl)amine induced local epithelial neoplasms upon administration to the hamster lip, and N-nitroso-bis(2-oxopropyl)amine gave a high incidence of skin tumors upon application to hamster skin (14, 16). This suggests that the latter 2 nitrosamines, but not NDELA, can be activated in the hamster oral cavity and skin.

The incidences of respiratory tract tumors after s.c. injection (Groups 1 and 2) and oral swabbing of NDELA (Groups 17 and 18) were comparable. An approximately equivalent dose of NDELA applied to the skin (Groups 9 and 10) gave a somewhat lower incidence of respiratory tract tumors. This may be partially due to differences in absorption; our studies with [14C]NDELA suggest that its absorption from the skin is fairly slow. The lower carcinogenicity of NDELA applied to the skin compared to that given s.c. was also evident by the lack of response in Groups 11 and 12 compared to the results of an equivalent dose in Groups 3 and 4.

The carcinogenicity data from Groups 1 and 2 are essentially identical to our earlier observations in a bioassay in which we used a similar dose and protocol (6). Similar results were also reported in a dose-response study of NDELA in Syrian golden hamsters (15). The lowest dose in that study was approximately 10.5 g/kg, which gave a significant incidence of nasal cavity and tracheal tumors (15). Animals in our Groups 3 and 4 received total doses of approximately 4.6 g/kg, and the incidence of respiratory tract tumors was significant. A total NDELA dose of approximately 1.6 g/kg (Groups 5 and 6), however, was practically ineffective in producing tumors of the nasal cavity and trachea. Two recent dose-response studies in rats have indicated that NDELA is a more potent carcinogen than previously assumed based on the original bioassay by Druckrey et al. (3, 10, 17). The lowest dose in the recent experiments was 1.5 mg/kg/day, administered in the drinking water. This regimen resulted in the induction of liver tumors after a total dose of approximately 0.86 g/kg (17).

The morphology and distribution of tumors in the nasal cavity and trachea in this experiment are similar to the nasal and...
tracheal tumors induced in hamsters by other nitrosamines (20). The laryngotracheal papillomas never became squamous carcinomas probably due to the particular location of these tumors. The hamsters die by airway obstruction long before the malignant conversion of papillomas can occur.

To the contrary, nasal tumors always become malignant. Several types of tumors may develop simultaneously in the same animal. In general, the olfactory tumors grow faster and more aggressively, invading and destroying the nasal cavity and other benign or malignant tumors present there. Islands of epidermoid tumors can remain encircled in the esthesioneuroepithelium. In our experience, the olfactory tumors are the only nasal cavity tumors invading the brain. That is explicable, considering the proximity of the brain and the aggressiveness of the olfactory neurogenic tumor.

The results of this bioassay allow us to compare the carcinogenicity of NDELA to assays in Syrian golden hamsters of other structurally related or environmentally prevalent nitrosamines. N-Nitrosodiethylethylamine appears to be at least 250 times as active as NDELA, since a total dose of 0.06 mmol induced respiratory tract tumors in 29 of 35 hamsters (12). N-Nitrosomorpholine and 4-(methylamino)-1-(3-pyridyl)-1-butanone are both clearly more carcinogenic than is NDELA because a total dose of approximately 1.5 mmol of the former induced tracheal tumors in 23 of 33 hamsters and a total dose of 0.9 mmol of the latter caused respiratory tract tumors in 18 of 20 hamsters (5, 7). A total dose of 2 mmol of N-nitrosopyrrolidine induced nasal cavity and tracheal tumors in 15 of 21 hamsters, whereas the same dose of NDELA administered in Groups 5 and 6 was inactive (11). N'-Nitrosonornicotine, which gave nasal cavity and tracheal tumors in 13 of 21 hamsters after a dose of 2 mmol, also seems to be more active than in NDELA (11). Thus, while the results of the present study demonstrate that NDELA is somewhat more carcinogenic in Syrian golden hamsters than previously assumed, it is a weaker carcinogen in the Syrian golden hamster than are these other nitrosamines. This may be due partially to its low rate of metabolism which is indicated by our failure to detect $^{14}$CO$_2$ and by previous studies in which relatively high percentages of unchanged NDELA were detected in urine (8, 9, 18, 19). However, human exposure to NDELA through tobacco and cosmetics, and in certain occupational settings in which cutting fluids are used, is often greater than exposure to N-nitrosodimethylamine, N-nitrosodiethylethylamine, N-nitrosomorpholine, or N-nitrosopyrrolidine (13). Thus, NDELA should be considered as a significant environmental hazard, and its occurrence should be minimized.

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