Synergistic Effects of Diethylnitrosamine and Different Dusts on Respiratory Carcinogenesis in Hamsters

Frej G. Stenbäck, Alessandro Ferrero, and Philippe Shubik

The Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105 [F. G. S., P. S., and Downstate Medical Center, Brooklyn, New York 11203 [A. F.]

SUMMARY

A possible synergistic action between the carcinogen diethylnitrosamine injected s.c. and MgO, Al₂O₃, or carbon instilled intratracheally in the respiratory tract of Syrian golden hamsters was studied. Diethylnitrosamine alone induced tumors of the nasal cavity in 50% of the animals. Tumors were also produced in the larynx and trachea but not in the lungs. Groups receiving weekly intratracheal instillations of 0.9% NaCl solution suspensions of carbon or Al₂O₃ had no tumors of the respiratory system. MgO alone induced a significantly large number of lymphomas of the histiocytic type that were not induced by diethylnitrosamine and MgO. In the groups receiving diethylnitrosamine followed by intratracheal instillations of dust-0.9% NaCl solution suspensions, the tumor incidence in the upper respiratory tract was similar to that seen with diethylnitrosamine alone, but lung tumors were also observed. Intratracheal instillations of 0.9% NaCl solution after diethylnitrosamine also caused a number of tumors to occur in the lower respiratory tract.

It can be concluded that carbon and Al₂O₃ are not carcinogens for the respiratory system of hamsters. The increase in incidence of lower respiratory tract tumors previously seen with diethylnitrosamine followed by Fe₂O₃ and 0.9% NaCl solution was here obtained with diethylnitrosamine followed by 0.9% NaCl solution alone.

INTRODUCTION

A variety of chemicals administered by different routes induce tumors of the respiratory system in experimental animals (14). Recent observations suggest that synergism between systemically and topically administered carcinogens may occur in the respiratory tract of hamsters (4, 12, 13, 14, 18). Montesano et al. (12) reported a synergistic carcinogenic effect of diethylnitrosamine when it was given with benzo(a)pyrene and Fe₂O₃ in hamsters. The present studies investigated the possible synergistic effects of s.c. administered diethylnitrosamine and of intratracheally instilled dusts (Al₂O₃, MgO, and carbon) on the carcinogenicity of s.c. administered diethylnitrosamine in the respiratory system of hamsters.

MATERIALS AND METHODS

Male Syrian golden hamsters 6 to 7 weeks old randomly bred in our colony were housed in plastic cages, 5 or 6 per cage, containing sterilized San-i-cell bedding. They were fed Rockland rat diet in pellets and water ad libitum. Diethylnitrosamine, MgO, carbon, and Al₂O₃, all obtained from Fisher Scientific, Fairlawn, N. J., and 0.9% NaCl solution in water (sterile, nonpyrogenic) (Baxter Laboratories, Inc., Morton Grove, Ill.) were used. The distribution of the particle size of the dusts by microscopic examination was: (a) for MgO, >25 μm, 98.2%; >5 μm, 91.6%; >1 μm, 63.4%; (b) for carbon, >25 μm, 96.3%; >5 μm, 88.7%; >1 μm, 48.5%; and (c) for Al₂O₃, >25 μm, 96.4%; >5 μm, 86.4%; >1 μm, 53.3%

Animals were divided into 9 groups. Group 1 received weekly 1.0 mg of diethylnitrosamine s.c. for 12 weeks. Groups 2, 3, and 4 were treated weekly for 30 weeks with 2.0 mg of carbon, MgO, and Al₂O₃, respectively, administered intratracheally using the method of Saffiotti et al. (15). Groups 5 and 6 received the same diethylnitrosamine treatment as Group 1 followed 5 weeks later by 30 weekly intratracheal instillations of 0.25 ml 0.9% NaCl solution or 2.0 mg carbon. Groups 7 to 9 received the same diethylnitrosamine treatment as Group 1 followed 5 weeks later by 15 intratracheal instillations of 2.0 mg of carbon (Group 7), MgO (Group 8), and Al₂O₃ (Group 9) administered once every 2nd week. The dust was given intratracheally in 0.9% NaCl solution by the method of Saffiotti et al. (15).

Animals were weighed weekly and those in poor condition were isolated and left to die spontaneously or were killed when moribund. Autopsies were performed on all animals except for a few lost through cannibalism. At autopsy, the trachea was ligated and the lungs were removed en bloc while still fully expanded and fixed in 10% neutral buffered formalin. Histological sections were prepared from each lobe of the lungs, larynx, trachea, stem bronchi, liver, and other organs showing gross pathology. Nasal cavities were studied at 3 levels after decalcification. Sections were stained with hematoxylin and eosin. Special stains were used when required.
RESULTS

There were no differences in weight curves among the 9 groups. The survival rates of the animals are given in Table 1. Death in diethylnitrosamine-treated animals was most frequently attributed to suffocation produced by tumors in the larynx and trachea.

Tumors found are listed in Table 1. The tumor incidence in the respiratory system in all diethylnitrosamine-treated groups was close to 100%. The trachea showed the highest tumor incidence (80 to 93%), followed by the nasal cavity (32 to 54%). Tumors observed in the larynx and trachea were papillomas, similar to those previously described in diethylnitrosamine-treated animals (12). They showed a papillary configuration with papillae lined by cuboidal mucus-secreting cells or squamous cells; in most cases both types of cells were found in the same tumor (Fig. 1). No evidence of invasive growth or metastasis was seen. One nodule of the lung was seen, similar to those seen in groups treated with diethylnitrosamine and 0.9% NaCl solution.

Animals that received repeated intratracheal instillations of carbon showed a black discoloration of the lungs. Carbon particles, phagocytized by macrophages, were seen in the alveolar spaces adjacent to the bronchi and bronchioles and around vessels, septa, and pleura. Aggregations of carbon dust in the lymph nodes were also conspicuous. No significant histological changes were seen in the trachea or bronchi nor were any tumors found.

Animals receiving dust alone (Groups 2 to 4) received 30 weekly instillations of the dust. In combination with diethylnitrosamine the dusts were given once in 2 weeks (Groups 7 to 9). The possibility that this affects the outcome is not very significant, as the tumor response in Groups 6 and 7 is basically the same, although the frequency of dust administrations is different.

Repeated instillations of Al₂O₃ produced lung fibrosis; macrophages and multinucleated giant cells containing yellow crystals were also observed (Fig. 2). The majority of the dust particles were in small aggregations. No tumors of the respiratory system were observed.

Repeated intratracheal instillations of MgO did not produce significant changes in the respiratory system. However, a significantly large number of animals developed lymphomas of the histiocytic type (Table 1), consisting of

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Initial animals</th>
<th>No. of animals autopsied</th>
<th>Survival rate (wk)</th>
<th>Total no. of tumor bearing animals</th>
<th>No. of Animals with tumors of</th>
<th>Other organs</th>
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<tr>
<td>1</td>
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<td>36</td>
<td>35</td>
<td>30</td>
<td>25</td>
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<td>29</td>
<td>25</td>
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<td>28</td>
<td>24</td>
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<td>6</td>
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<td>4</td>
<td>2 mg Al₂O₃ intratracheally weekly, 30 wk</td>
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<td>28</td>
<td>29</td>
<td>23</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
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<td>36</td>
<td>33</td>
<td>32</td>
<td>25</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1 mg diethylnitrosamine s.c., weekly, 12 wk; 2 mg carbon intratracheally, weekly, 30 wk</td>
<td>29</td>
<td>27</td>
<td>28</td>
<td>16</td>
<td>2</td>
<td>0</td>
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<td>7</td>
<td>1 mg diethylnitrosamine s.c., weekly, 12 wk; 2 mg carbon inetratracheally once/2 wk, 30 wk</td>
<td>36</td>
<td>33</td>
<td>31</td>
<td>24</td>
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<tr>
<td>8</td>
<td>1 mg diethylnitrosamine s.c., weekly, 12 wk; 2 mg MgO intratracheally, once/2 wk, 30 wk</td>
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<td>9</td>
<td>1 mg diethylnitrosamine s.c., weekly, 12 wk; 2 mg Al₂O₃ intratracheally, once/2 wk, 30 wk</td>
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<td>30</td>
<td>29</td>
<td>25</td>
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</tbody>
</table>

* One forestomach papilloma.
* Three forestomach papillomas, 2 adreno cortical adenomas.
* Two adreno cortical adenomas, 9 lymphomas.
* Three forestomach papillomas, 1 leiomyosarcoma of the small intestine.
* One forestomach papilloma.
* One adreno cortical adenoma.
* One forestomach papilloma, 1 adreno cortical adenoma.
* Two forestomach papillomas.
tightly arranged pleomorphic cells in a fine fibrillar network (Fig. 3). The neoplasms involved the lymph nodes, liver, and spleen.

All diethylnitrosamine-treated animals (with and without tracheal instillations) also had tumors of the nasal cavity. They were adenomas, adenocarcinomas (Fig. 4), papillomas, squamous cell carcinomas, and olfactory neuroepithelial tumors. The olfactory neuroepithelial tumors were composed of small, round, or oval cells with hyperchromatic nuclei and a scanty cytoplasm. The blood vessels were thin walled and there were marked clumping and perpendicular palisading of tumor cells around these vessels. In some areas the tumor cells formed islands and sheets of various sizes and shapes. “Pseudorosettes,” consisting of a ring of poorly differentiated cells with a central space containing fine eosinophilic fibrils and debris, were common. Another tumor pattern consisting of organized structures and undifferentiated cells, a true rosette, was also seen that resembled the type seen in retinoblastomas and medulloblastomas. These tubular structures consisted of groups of elongated cells arranged radially around a central circular or oval cavity. Squamous metaplasia of the respiratory epithelium lining the nasal cavity was not uncommon. In some animals papillomatous hyperplasia, with mixed glandular and squamous structures, was seen. The squamous carcinomas were solid epithelial structures with scant keratin formation; in some animals the difference between a glandular tumor with squamous metaplasia and a squamous cell carcinoma with glandular areas was not distinct. The glandular tumors derived either from the surface epithelium or from the submucosal glands were of different types, tubuloalveolar, adenoacinar, and papillary. [These are described in detail in a separate paper (17).]

In experiments combining s.c. injected diethylnitrosamine and intratracheally instilled 0.9% NaCl solution a large number of tumors were found in the larynx and trachea as well as in the lung. Grossly, the lung tumors appeared as firm nodules, grayish white in color, 0.1 to 0.5 cm in diameter, sometimes reaching a size of up to 1.5 cm. Histologically, they were composed of squamoid cells with an adenoid pattern (Fig. 5). The lesions started out as aggregations of small monomorphic polygonal cells arranged in concentric layers, occasionally with a pseudorosette-like appearance. Kreyberg-stained sections were weakly positive for keratin; no horn pearls or evidence of mucoid secretion occurred. Later the lesions attained a larger size, some replacing the entire lung lobe, others remaining small (Fig. 6). Some of the tumors were located closely to a bronchus, although the connection was not always obvious. The bronchial epithelium was mostly regular sometimes the epithelium showed a papillary configuration.

The fully developed lesions showed 2 main patterns of differentiation. Mostly, the tumors were solid, composed of cords or islets of tightly arranged small cells with rounded basophilic nuclei, faintly staining cytoplasm, and distinct cell borders (Fig. 7). The superficial cell layers were occasionally flattened; horn pearls or keratinization did not occur. Intercellular bridges could not be unequivocally demonstrated. Another pattern of differentiation occurred less frequently and only in large, more fully developed lesions (Fig. 8). These showed papillary or acinar structures, with a scanty fibrous stroma. The lumen contained substances staining weakly for mucin; the epithelium surrounding these structures consisted of cuboidal basophilic cells rarely showing secretory activity. The lesions were always clearly delineated with no signs of infiltration into surrounding lung tissue or formation of metastasis.

When dusts were added to 0.9% NaCl solution in diethylnitrosamine-treated hamsters, the number of lung tumors was significant. The slightly lower number may depend on the shorter life-span of the animals. The histological structure was not significantly altered (Table 1), the main difference being the presence of fibrosis and dust particles in the tumors.

Tumors in other organs were not common; papillomas of the forestomach, adenomas of the adrenal cortex, as well as a leiomyosarcoma of the small intestine were seen (Table 1).

**DISCUSSION**

The known carcinogenic effect of diethylnitrosamine for the upper respiratory tract of hamsters (5, 10-12) was reaffirmed by the results of this study, which showed a large number of papillary tumors in the larynx and trachea. Hyperplasia and dysplasia, as well as nonspecific metaplastic changes, were common features in the respiratory epithelium. Most tumors showed both glandular components and squamous keratin-containing epithelium, similar to tumors previously described (10). Infiltrative growth and marked cytological irregularities did not occur. The neoplastic response in the nasal cavity, with tumors deriving both from the respiratory lining and olfactory epithelium as well as from the submucosal glands, indicated an effect of diethylnitrosamine on both these tissue components.

Al₂O₃ has been shown to produce severe lung fibrosis in humans. So far the incidence of lung tumors reported among workers in the aluminum industry (9) has been the same as in the general population. In experimental animals aluminum dextran and toxoid produced granulomas and sarcomas at the injection site (3, 7).

Fibrosis and vascular lesions in the lungs caused by carbon particles are well known in man (16). Blacklock et al. (2) have studied the relationship between particle size in soil and coal and lung cancer. Lesions seen in the lungs in hamsters in this experiment were similar to those occurring in humans after exposure to excessive quantities of dust-containing coal particles.

MgO alone produced histiocytic type lymphomas in 30% of the animals but surprisingly the combination of s.c. administered diethylnitrosamine and intratracheal instillations of MgO did not induce lymphomas. The significance of the result is difficult to assess at the present moment. Magnesium has not been previously reported as potentially carcinogenic. Spontaneous histiocytic lymphomas have been reported in Syrian golden hamsters but at a much lower percentage, 2%, and at a later age (11).

Administration of diethylnitrosamine s.c. followed by
intratracheal instillation of 0.9% NaCl solution induced a striking tumor response in the lower respiratory tract.

The slightly lower number of lung tumors seen in the groups treated with diethylnitrosamine and different dusts as compared to diethylnitrosamine and 0.9% NaCl solution may depend on a number of factors. One explanation is the shorter life-span of the animals, as these lesions occurred with increasing frequency at an older age. It is also possible that the regenerative and reparative effects caused by the dust-induced injury made the lung tissue less responsive to the tumor-inducing effect of the treatment. When comparing the results from the different groups in these studies one must take into account the fact that diethylnitrosamine was followed by intratracheal instillations of 0.9% NaCl solution weekly for 30 weeks, while the dust treatments in the 3 groups were once every 2 weeks for 30 weeks after initial diethylnitrosamine treatment. This does not appear to affect the results, as diethylnitrosamine followed by carbon once every 2 weeks or followed by carbon once weekly induced the same number and types of tumors.

The tumor response seen here after s.c. injected diethylnitrosamine and intratracheally instilled 0.9% NaCl solution is similar to that seen when diethylnitrosamine was followed by Fe₂O₃ suspended in 0.9% NaCl solution (12, 14). Fe₂O₃ was originally introduced as a carrier for particles of carcinogenic chemicals, the essential property being its capacity to facilitate the penetration and retention of the carcinogen in the lung tissue (15). Montesano et al. (12), however, observed that s.c. injections of diethylnitrosamine followed by intratracheally instilled Fe₂O₃ resulted in a higher incidence of bronchoalveolar tumors than when using diethylnitrosamine alone. Harris et al. (8) suggested that Fe₂O₃ may act as a cofactor in respiratory carcinogenesis, as they observed a marked basal cell hyperplasia in Fe₂O₃-treated hamsters. Feron et al. (6), on the basis of studies of the effect of diethylnitrosamine and Fe₂O₃, concluded that the latter might be considered as a cocarcinogen.

As the tracheal papillomas in diethylnitrosamine-treated hamsters developed relatively early, Feron et al. (6) also suggested the possibility that the high incidence of pulmonary tumors in these animals could be due to transplantation of the tracheal papillomas into the lower respiratory tract as a consequence of the instillation procedure. Another possibility, that the metabolism of diethylnitrosamine in the lung could be altered by the administration of foreign material in the alveolar cavity, is unlikely as the diethylnitrosamine is probably metabolized at the time that the intratracheal injections of diethylnitrosamine are done.

Morphologically, the peripheral tumors observed in the lung were markedly similar to the papillomas observed in the trachea. This type of diethylnitrosamine-induced tracheal neoplasms has often been described as squamous cell tumors (4, 11). The morphological picture of peripheral tumors has been considered indicative of alveolar cell origin (6). However, studies on these tracheal papillomas using semithin techniques revealed no metaplastic alterations to cornified or uncornified squamous cells (1).

Tumors produced by benzo(a)pyrene and Fe₂O₃ are morphologically completely different as shown by Saffiotti et al. (15). The peripheral tumors seen here are monomorphic and well circumscribed, with no tendency to keratinization. Those produced by benzo(a)pyrene and Fe₂O₃ are malignant, infiltrating with extensive keratinization, markedly similar to human respiratory tumors.

REFERENCES

Fig. 1. Diethylnitrosamine-treated hamster showing papillary tumor of the trachea composed almost entirely of squamous cells. H & E, x 60.
Fig. 2. Fibrosis and dust accumulations in lung produced by aluminum. H & E, x 60.
Fig. 3. Malignant lymphoma in hamster treated with MgO. H & E, x 85.
Fig. 4. Poorly differentiated adenocarcinoma of the nasal cavity in hamster treated with diethylnitrosamine and carbon. H & E, x 150.
Fig. 5. Aggregates of squamoid cells in hamster lung in animal treated with diethylnitrosamine and 0.9% NaCl solution. H & E, x 185.

Fig. 6. Lung of hamster treated with diethylnitrosamine and 0.9% NaCl solution almost completely filled with tumor tissue, a large one in the top part and a smaller one in the lower part of the picture. H & E, x 6.5.

Fig. 7. Lung tumor composed of cuboidal tightly arranged cells in hamsters treated with diethylnitrosamine and 0.9% NaCl solution. H & E, x 270.

Fig. 8. Lung tumor, partly solid, partly showing a lumen containing weakly alcianophilic material. Kreyberg's stain, x 65.
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