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In Vivo Autoradiography and Nitrosoheptamethyleneimine Carcinogenesis In Hamsters[1]

Hildegard Reznik-Schüller[2] and William Lijinsky

Chemical Carcinogenesis Program, NCI-Frederick Cancer Research Center, Frederick, Maryland 21701[3]

ABSTRACT

Quantitative autoradiograms were made, *in vivo,* in European hamsters with the use of [^{14}C]nitrosoheptamethyleneimine (260 μCi/animal; time between administration of nitrosamine and killing of animals, 6 hr). In this species, the lung is the principal target, and radioactivity was found in the Clara cells of the bronchial epithelium and in the nitrosoheptamethyleneimine-induced tumors which derive from these cells.

Tumors are not induced in the liver, which can metabolize this compound, and labeling is found principally in the cytoplasm, whereas in the target cells there is a high degree of labeling in both the cytoplasm and the nuclei.

INTRODUCTION

This study forms part of a series of morphological and biochemical investigations into the pronounced organotropic effects of some nitrosamines. NHMI,[4] when administered s.c. or i.g., induced up to 100% lung tumors in European hamsters (6). These neoplasms are adenomas, adenocarcinomas, squamous carcinomas, or mixed tumors (with areas of adenoid and squamous pattern) (6). On electron microscopic examination, areas with adenoid structure are seen to consist mainly of nonciliated Clara cells and of cells which have been shown to derive from the latter (7-9). Cells in squamous areas contain various amounts of cytoplasmic filaments and/or Apud-type granules (9). Although NHMI is probably metabolized in the liver, it causes no liver tumors in this organ. In this study, quantitative *in vivo* autoradiography was performed with [^{14}C]NHMI in European hamsters and was related to the results of chronic experiments.

MATERIALS AND METHODS

Four European hamsters were given s.c. injections once weekly of 0.20 of the dose lethal to 50% of animals (33 mg/kg body weight) unlabeled NHMI. Thereafter, these and 4 untreated control animals were given a single i.g. dose of [^{14}C]NHMI (260 μCi/animal) (12) (specific activity, 13.1 mCi/mmol). All animals were 1-year-old males and weighed approximately 500 g. Six hr after administration of the labeled compound, the animals were anesthetized with hexobarbital-NA (150 mg/100 g body weight) (Evipan-NA; Bayer, Leverkusen, Germany). They were then perfused with a plasma expander (Rheomakrodex; Knoll, AG, Ludwigshafen, Germany) via the portal vein to remove all blood from the vessels. Thereafter, they were fixed *in situ* by vascular perfusion with 2% cacodylate-buffered glutaraldehyde (pH 7.4). Samples of bronchi, peripheral lung tissues, lung tumors, heart, and liver were excised, immersed for an additional 2 hr in the fixative, and postfixed for 1 hr in 1% osmium tetroxide. After dehydration in a graded series of ethanols, they were embedded in Epon 812 (Ladd Research Industries, Burlington, Vt.). The dehydration steps were monitored by liquid scintillation counting to make sure that all unbound radioactivity was removed from the tissues. Semithin sections (1 μm thick) were cut on an LKB Ultrotome III (LKB, Bromma, Sweden) and placed on glass slides. They were dipped in Kodak NTB2 emulsion in a darkroom and stored in the dark for 30 days at 4°. Autoradiograms were then developed in D19, fixed in Tetanal rapid fixer, and washed in distilled water. They were stained with toluidine blue at room temperature. Light microscopic grain counts were made from 3 sections per sample (2 microscopic fields per section). Background counts were made from tissueless areas of the same size. In the case of bronchial epithelia which occupy only a narrow part of the microscopic field, the epithelium was oriented for grain counting so that it ran from one edge of the microscopic field to the opposite side (thus the respective epithelial area was always of the same length). With an ocular screw micrometer (Leitz, Wetzlar, Germany), the height of the epithelium was measured, and background counts were done on a tissueless strip of the same size. For the measurement of cellular and nuclear sizes, photomicrographs were taken using a 100 oil Planapochromat objective. They were printed at a 4-fold magnification. On the prints, the areas of cells and nuclei were measured with a computerized image analysis system (Digitizer Pen-X-31; Zscherpel Elektronik, Waiblingen, Germany), whereby the cytoplasmic area was calculated as the difference between total cellular area and nuclear area. In that way, 200 cells of each type were measured, and the calculated mean values were used to compare grain counts with area. All results were tested for significant differences by means of Student's *t* test and/or analysis of variance.

Basal cells are so scanty in the bronchial epithelium of this species that their values were excluded from the statistical analysis.

RESULTS

Autoradiograms from heart and peripheral lung tissues (alveolar epithelial cells, connective tissue cells, and blood

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[2] To whom requests for reprints should be addressed.

[3] The animal experiments were done at the section of experimental pathology, Medical School, Hannover, Federal Republic of Germany.

[4] The abbreviations used are: NHMI, nitrosoheptamethyleneimine; i.g., intragastrically.

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vessels) were negative in all animals. In the lungs, grains were concentrated over the bronchial epithelia (Figs. 1 and 2). Thereby, the majority of grains was found over the nonciliated Clara cells (Figs. 1 and 2), which make up 38% of the bronchial epithelial cells in this species. This cell type demonstrated significantly ($p < 0.01$) higher amounts of grains per area cytoplasm and per area nucleus than did ciliated cells (Chart 1). Basal cells, which due to their scanty occurrence were excluded from statistical comparisons, demonstrated only occasionally 1 or 2 grains/cell. No statistically significant differences were found between the distribution of grains over morphologically normal-appearing bronchial epithelia in NHMI-pretreated and control animals. In the lung tumors, which all were adenomas and adenocarcinomas (all NHMI-pretreated hamsters had multiple lung tumors) which consisted mostly of nonciliated cells, grains/area cytoplasm (Chart 1) were similar as in the Clara cells of normal bronchi. However, the number of grains [16.9 ± 2.2 (S.D.) grains/100 sq μm] over the nuclei of tumor cells was higher ($p < 0.1$) than in the normal bronchial epithelia. In the liver, the amount of grains per area cytoplasm equaled that found in unaltered Clara cells and in lung tumor cells (Chart 1). In contrast, the amount of grains per area nucleus was extremely low (2.47 grains/100 sq μm) and thus differed significantly ($p < 0.01$) from the respective findings in bronchial epithelia and lung tumor cells.

DISCUSSION

In autoradiograms made under these conditions, grains are generally regarded as indicators of labeled material bound to cellular constituents (1). Although it is not possible to determine whether the activity is directly bound nitrosamine or incorporated after metabolism to 1- or 2-carbon units, the amount of incorporation is expected to be very low in this experiment because of the short time interval between administration of the labeled compound and the death of the animals. In view of the widely accepted concept that nitrosamines are metabolically activated within the cells (4, 5), the distribution of grains after administration of NHMI in the organs of European hamsters indicates that the chemical is metabolized and bound in the target organ (the lungs) as well as in the nontarget organ (the liver). The question arises as to why tumors develop only in the lungs and not in the liver. Since the number of grains per area of cytoplasm is almost the same in target and nontarget cells, this cannot reflect a quantitative difference in the production of metabolites in liver and lungs but suggests that qualitatively different metabolites are formed. This hypothesis gains support from the fact that the nuclei of target bronchial and tumor cells contain significantly more radioactivity than those of liver cells. Thus, these results agree with the recent findings at the Frederick Cancer Research Center, in rats (J. Farrelly and W. Lijinsky, personal communication). After NHMI was administered to rats, the metabolites isolated from lung (target organ) and liver (nontarget organ) homogenates were different. They also found, however, that binding of NHMI and its metabolites to nuclear components of lung cells was very low and that binding of NHMI overall in the lung is not very great. This might be due to the fact that the bronchial epithelial cells comprise only about 20% of lung tissue. We find that the bronchial epithelial tissue is the only one which binds NHMI, and most of this binding takes place in the nonciliated Clara cells, which make up only about one-half of the total bronchial epithelial tissue. Biochemical investigations on whole-tissue homogenates are somewhat unsatisfactory because of the mixture of types of cells present. In this respect, our results will be a basis for future biochemical studies on better defined epithelial cell populations. The results also suggest that the nonciliated Clara cells of the bronchial epithelium play a key role in pulmonary nitrosamine carcinogenesis in hamsters. These cells not only bind significantly more of the activity administered than do all other cell types of the lungs but, since they were found to contain more grains per unit area of cytoplasm, they, like the liver, can obviously metabolize the nitrosamine. This assumption is supported by histochemical studies (2) which demonstrate that the Clara cells possess the highest amount of oxidative enzymes of all cell types in the lungs. Boyd (3) recently found that Clara cells are the main site of metabolism of 4-ipomeanol, a toxic lung agent that requires metabolic activation. The important role that Clara cells play in the mechanism of nitrosamine-induced lung carcinogenesis was already suspected from the results of several serial sacrifice studies in Syrian hamsters (7, 8). These electron microscopic investigations show that diethylnitrosamine, dibutyl-nitrosamine, and nitrosomorpholine all cause the same specific early alterations in Clara cells which, in contrast, do not occur during polycyclic hydrocarbon-induced lung carcinogenesis (10, 11). The present findings thus support the view that the first ultrastructural changes are most probably found at the site of metabolism (which leads to the ultimate carcinogen) of the nitrosamine. It would be helpful to isolate the different cell types identified by electron microscopy for use in biochemical investigations aimed at elucidating the intracellular mechanism of nitrosamine carcinogenesis.

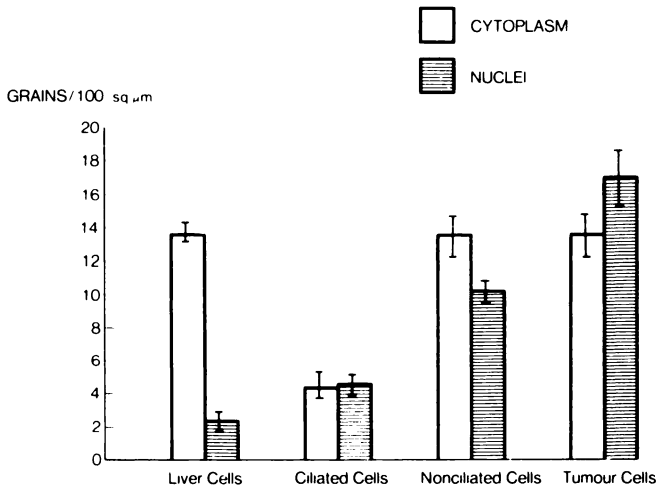

Chart 1. Grains per area of cytoplasm and nuclei in cells of bronchial epithelium, lung tumors, and livers. The distribution of grains per unit area demonstrates that equal amounts of radioactivity are found in the cytoplasm of liver cells, nonciliated bronchial cells, and lung tumor cells. The nuclei of liver cells contain extremely few grains, whereas the number of grains is high in the nuclei of target (nonciliated bronchial and lung tumor) cells.

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REFERENCES

1. Amlacher, E. Autoradiographie in Histologie und Cytologie. Stuttgart: Gustav Fischer Verlag, 1974.
2. Azzopardi, A., and Thurlbeck, W. M. The Histochemistry of the Nonciliated Bronchiolar Epithelial Cell. Am. Rev. Resp. Diseases. *99:* 516–525, 1969.
3. Boyd, M. R. Specific Alkylation and Necrosis of Pulmonary Nonciliated Bronchiolar Cells by the Lung-Toxic Furan, 4-Ipomeanol. Proc. Am. Assoc. Cancer Res., *18:* 246, 1977.
4. Magee, P. N. Activation and Inactivation of Chemical Carcinogens and Mutagens in the Mammal. Essays Biochem., *10:* 105–134, 1974.
5. Miller, E. C., and Miller, J. A. The Metabolism of Chemical Carcinogens to Reactive Electrophiles and Their Possible Mechanisms of Action in Carcinogenesis. ACS Monograph Series No. 173, Chemical Carcinogens, pp. 737–762, 1976.
6. Reznik, G., Mohr, U., and Lijinsky, W. Carcinogenicity of Subcutaneously Injected *N*-nitrosoheptamethyleneimine in European Hamsters. J. Natl. Cancer Inst., *61:* 239–243, 1978.
7. Reznik-Schüller, H. Ultrastructural Alterations of Nonciliated Cells after Nitrosamine Treatment and Their Significance for Pulmonary Carcinogenesis. Am. J. Pathol., *85:* 549–554, 1976.
8. Reznik-Schüller, H. Sequential Morphological Alterations in the Bronchial Epithelium of Syrian Golden Hamsters during *N*-Nitrosomorpholine-induced Pulmonary Tumorigenesis. Am. J. Pathol., *89:* 59–66, 1977.
9. Reznik-Schüller, H. Ultrastructure of Lung Tumors in European Hamsters Treated with Nitrosoheptamethyleneimine. Am. J. Pathol., *93:* 45–51, 1978.
10. Reznik-Schüller, H., and Mohr, U. Investigations on the Carcinogenic Burden by Air Pollution in Man. IX. Early Pathological Alterations of the Bronchial Epithelium in Syrian Golden Hamsters after Studies from Semithin Sections. Zentr. Bakteriol. Parasitenk. Abt. I. Orig. B, *159:* 493–502, 1974.
11. Reznik-Schüller, H., and Mohr, U. Investigations on the Carcinogenic Burden by Air Pollution in Man. XII. Early Pathological Alterations of the Bronchial Epithelium in Syrian Golden Hamsters after Intratracheal Instillation of Benzo[*a*]pyrene. 2. Further Ultrastructural Studies. Zentr. Bakteriol. Parasitenk. Abt. I. Orig. B, *160:* 108–129, 1975.
12. Snyder, C. M., Farrelly, J. G., Lijinsky, W. Metabolism of Three Cyclic Nitrosamines in Sprague-Dawley Rats. Cancer Res., *37:* 3530–3532, 1977.

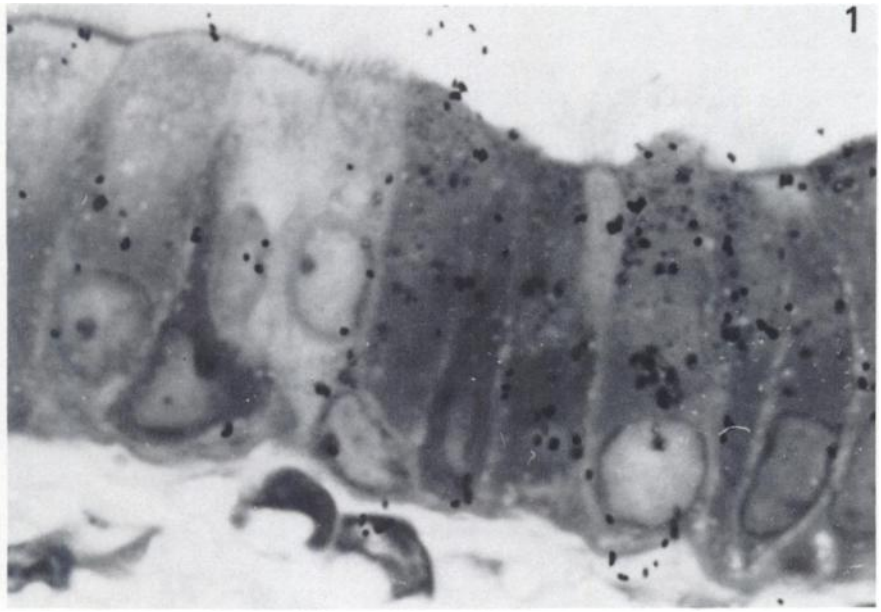

Fig. 1. Epithelium of segmental bronchus in European hamster after 1 i.g. dose of 260 μCi [^{14}C]NHMI. Autoradiographic grains are numerous over the nonciliated Clara cells, whereas only a few grains are seen over the ciliated cells. Semithin section, 1 μm thick. Toluidine blue, × 1400.

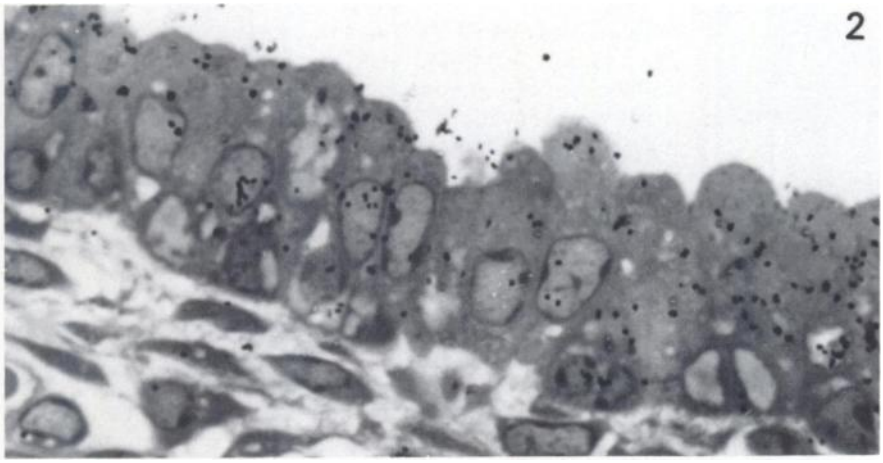

Fig. 2. Epithelium of bronchiole in European hamster after 1 i.g. dose of 260 μCi [^{14}C]NHMI. In this area Clara cells predominate, and they show numerous autoradiographic grains. Semithin section, 1 μm thick. Toluidine blue, × 860.