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Pathogenesis of Lung Tumors Induced by N-Nitrosoheptamethyleneimine in F344 Rats

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Summary. Male F344 rats were given 3 mg N-nitrosoheptamethyleneimine (NHMI) per 100 g in olive oil by gavage twice a week for a maximum of 20 weeks. They were killed at predetermined intervals after the start of treatment and the sequential development of NHMI-induced squamous carcinomas in the lungs was followed by light and electron microscopy. Hypertrophy of the endoplasmic reticulum in mucous and Clara cells seen in an initial study suggested that these cells were involved in metabolic activation of the nitrosamine. Basal cells were identified as the cell type that later responded by proliferation, hyperplasia, and squamous metaplasia. Only in the bronchioles, where basal cells are not found in the healthy rat, did these lesions progress further to form squamous cell carcinomas.

Key words: NHMI – Pathogenesis – Lung tumors – Rat

Introduction

N-Nitrosoheptamethyleneimine (NHMI) is a powerful lung carcinogen in F344 and Sprague-Dawley rats producing an incidence of up to 100% squamous cell carcinomas when given in the feed (Lijinsky et al. 1969; Taylor and Lijinsky 1975; Taylor and Nettesheim 1975). All of the tumors were located in the bronchiolo-alveolar region. An electron microscopic examination revealed that the tumor cells had the characteristics of basal cells in the actively growing peripheral zones (Reznik-Schüller and Gregg 1981). The origin of the basal cells is not clear since, in the healthy rat, basal cells are restricted to the upper airways (trachea, stem, lobar, segmental bronchi). The bronchioles are lined with Clara cells and a few ciliated cells, and alveoles consist of alveolar cells.

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To clarify this problem, we followed the development of NHMI-induced lung tumors in rats sequentially.

Materials and Methods

Fifty male F344 rats (from the Frederick Cancer Research Center breeding colony) were housed in plastic cages in a barrier facility. Forty-five of these were given 3 mg/100 g body weight NHMI in 0.2 ml olive oil by gavage twice a week for a maximum of 20 weeks, when the treatment was discontinued. Pairs of animals were killed in the following sequence: at 24 h, weeks 1 to 10, then at two-weekly intervals until week 20. The remaining 13 rats were killed when clinical symptoms (loss of weight, abnormal breathing patterns) indicated the presence of lung tumors. The five control rats were given 0.2 ml/100 g body weight olive oil by gavage twice a week. Two were sacrificed at the start and the remaining three at the end of the experiment.

All animals were fixed in situ by vascular perfusion with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) under sodium pentobarbital anesthesia (Diabutal, Diamond labortories, Inc., Des Moines, IA). Five tissue samples per animal from each of the following areas were excised (Fig. 1): extrapulmonary stem bronchus, lobar bronchus, segmental bronchus, bronchioloalveolar region, and lung tumor (if macroscopically visible). Samples were cut into blocks of 1 mm^3 and immersed for an additional 2 h in fixative. They were then postfixed in 1% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.4) for 1 h, dehydrated in graded ethanols, infiltrated and embedded in Epon 812. The tissues were sectioned with an LKB Ultrotome III ultramicrotome. Semithin sections were stained with 1% toluidine blue in 1% sodium borate for light microscopy. Thin sections were stained with 2% uranyl acetate and lead citrate (Reynolds 1963) and examined in a Philips 201C electron microscope at 60 kv.

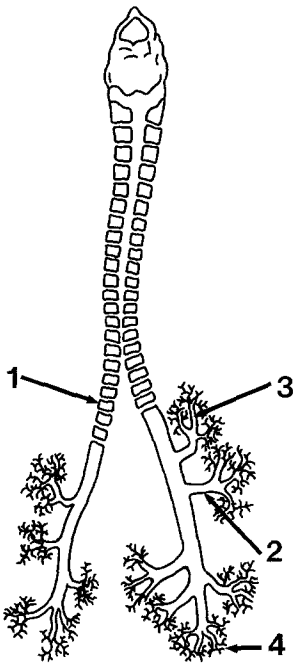

Fig. 1. Schematic drawing of the rat bronchial tree. The following samples were taken: *1*=extrapulmonary stem bronchus, *2*=lobar bronchus, *3*=segmental bronchus, *4*=bronchioloalveolar region

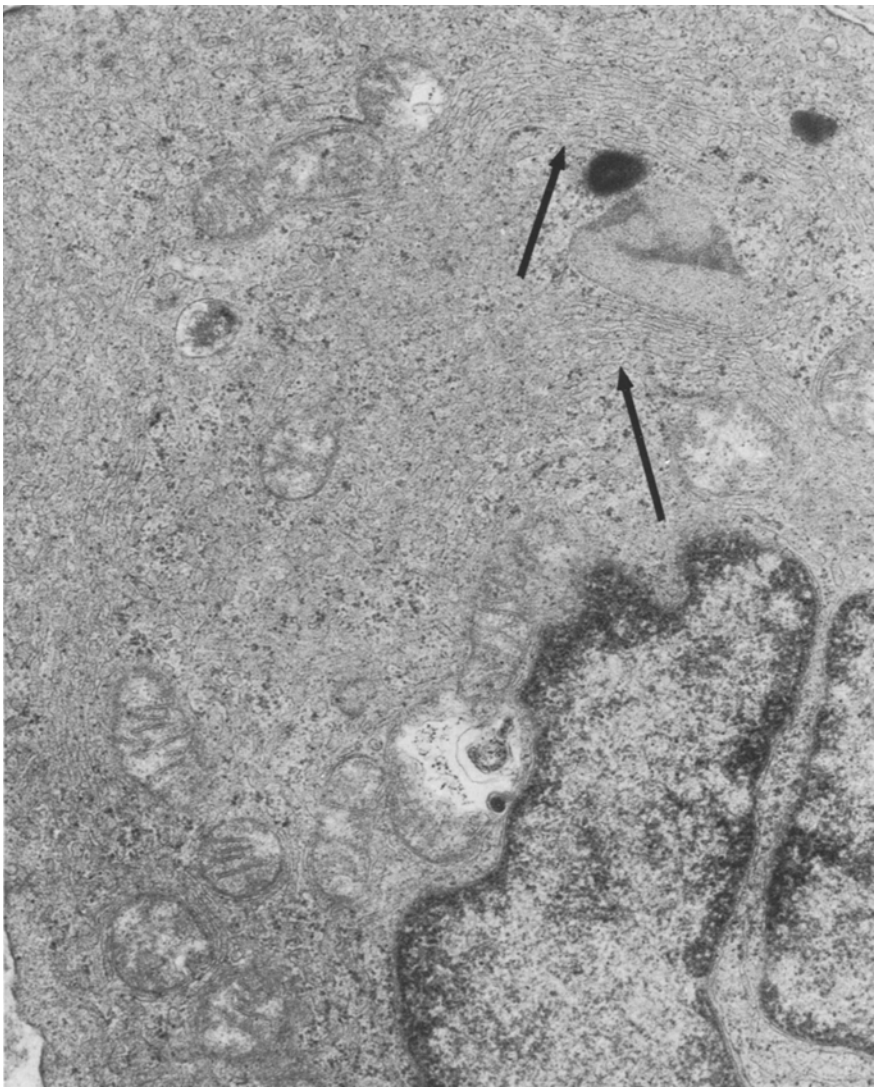

Fig. 2. Bronchiolar Clara cell 2 weeks after start of NHMI-treatment: hypertrophic smooth endoplasmic reticulum occupies the cytoplasm and forms arrays of parallel tubules (*arrowed*). Uranyl acetate, lead citrate; ×20,000

Results

Although many articles on the pathology of the lung in F344 rats have been published, there is no description of the normal respiratory epithelium in the literature, giving the anatomical location of the different cell types in the respiratory tree. The arrangement of cell types in the various segments of the airways in the healthy F344 rat is as follows. The trachea, extrapulmonary bronchi, lobar bronchi and segmental bronchi are coated by a pseudostratified respiratory

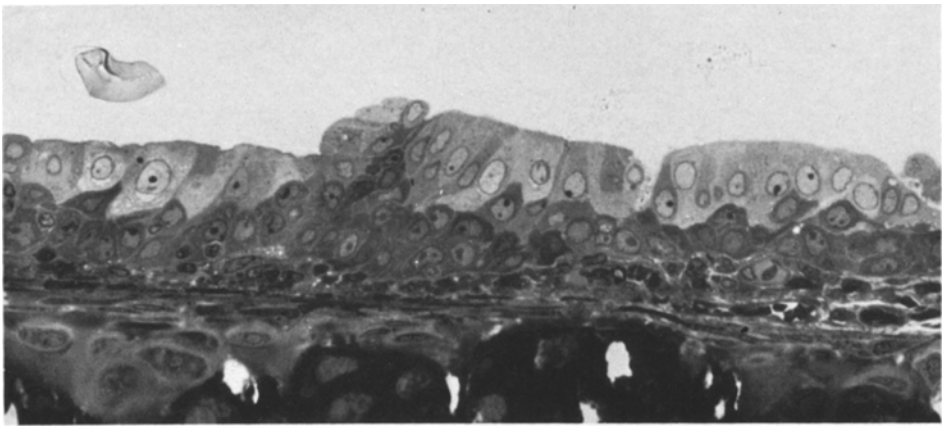

Fig. 3. Part of extrapulmonary stem bronchus after 4 weeks of NHMI-treatment; in the basal layer of the epithelium pronounced proliferation of basal cells. Semithin section, toluidine blue; ×400

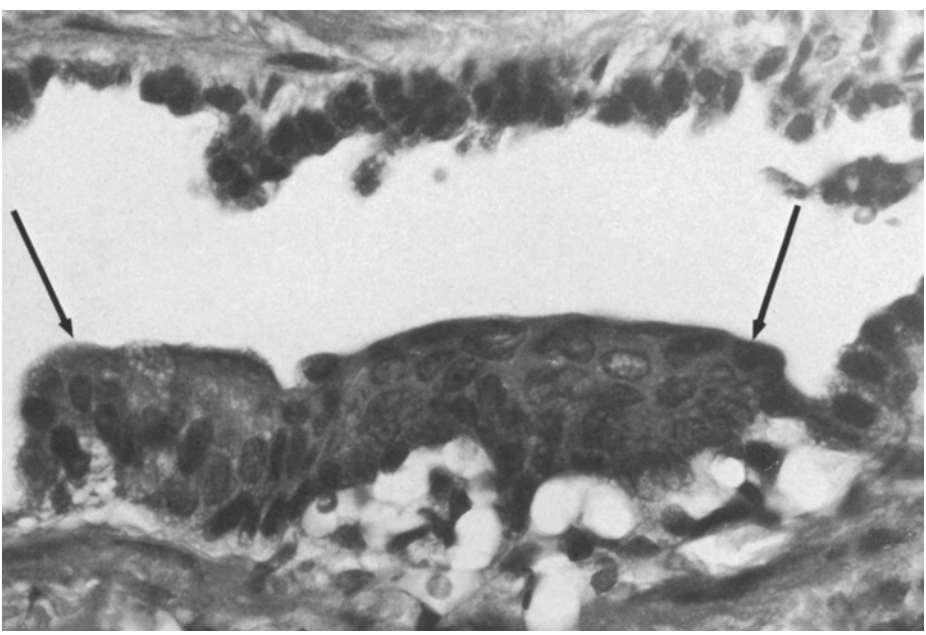

Fig. 4. Focal hyperplasia (*arrowed*) of basal cells in bronchiole after 5 weeks of NHMI-treatment. Semithin section, toluidine blue, ×1,000

epithelium composed of ciliated, mucous (goblet and small mucous granule cells) and basal cells. Occasionally, a single APUD-type cell or brush cell may be found. At the level of the subsegmental bronchi, basal cells and mucous cells disappear and in their place nonciliated Clara cells are found, as well as ciliated cells. In the bronchioles, ciliated cells are scanty with Clara cells being the predominate cell type.

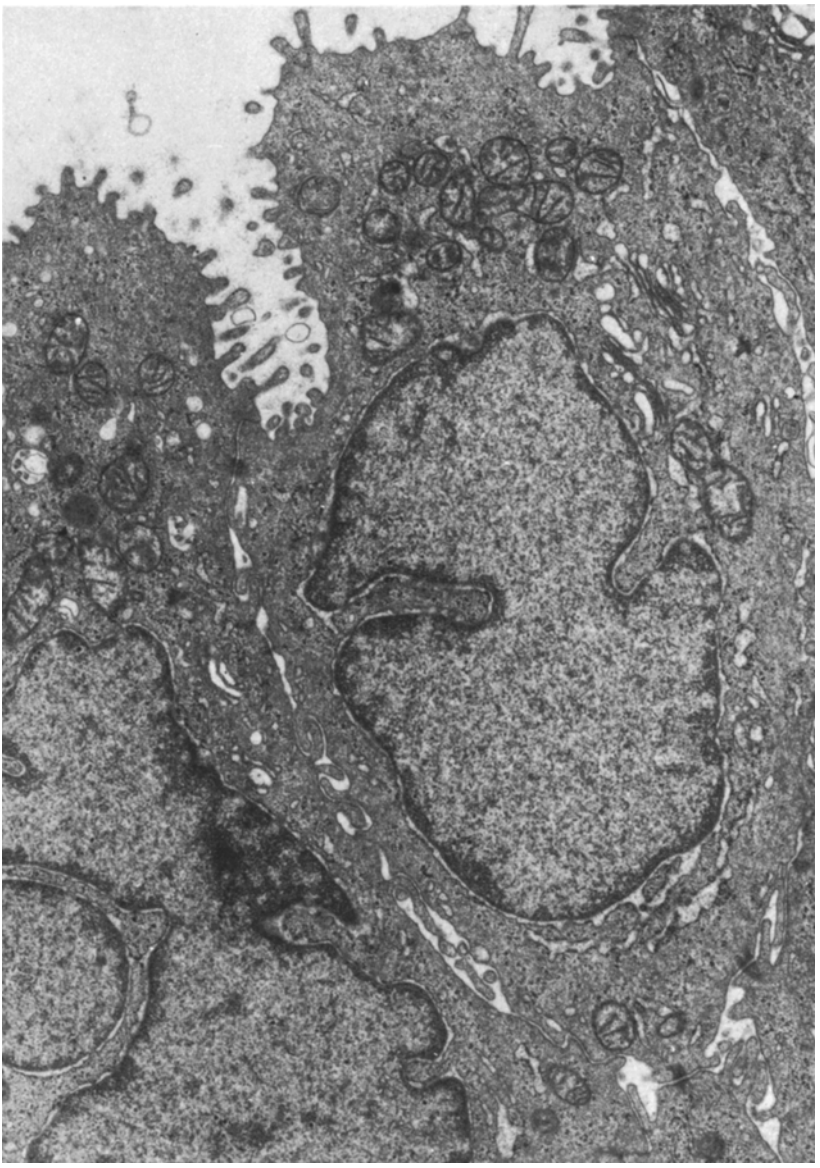

Fig. 5. Electron micrograph of transitional cells which form the surface layer of a hyperplastic area in a segmental bronchus after 6 weeks of NHMI-treatment: These cells have features identifying them as transitional in their differentiation between basal cells and normal surface cells of the respiratory epithelium. Uranyl acetate, lead citrate; ×14,000

In the NHMI-treated rats, the first morphological changes, 2 weeks after the start of the treatment, were hypertrophy (formation of concentric parallel lamellae) of rough endoplasmic reticulum in the mucous cells of the upper airways. In the subsegmental bronchi and bronchioles Clara cells demonstrated hypertrophy of smooth (Fig. 2) or rough endoplasmic reticulum. At this time,

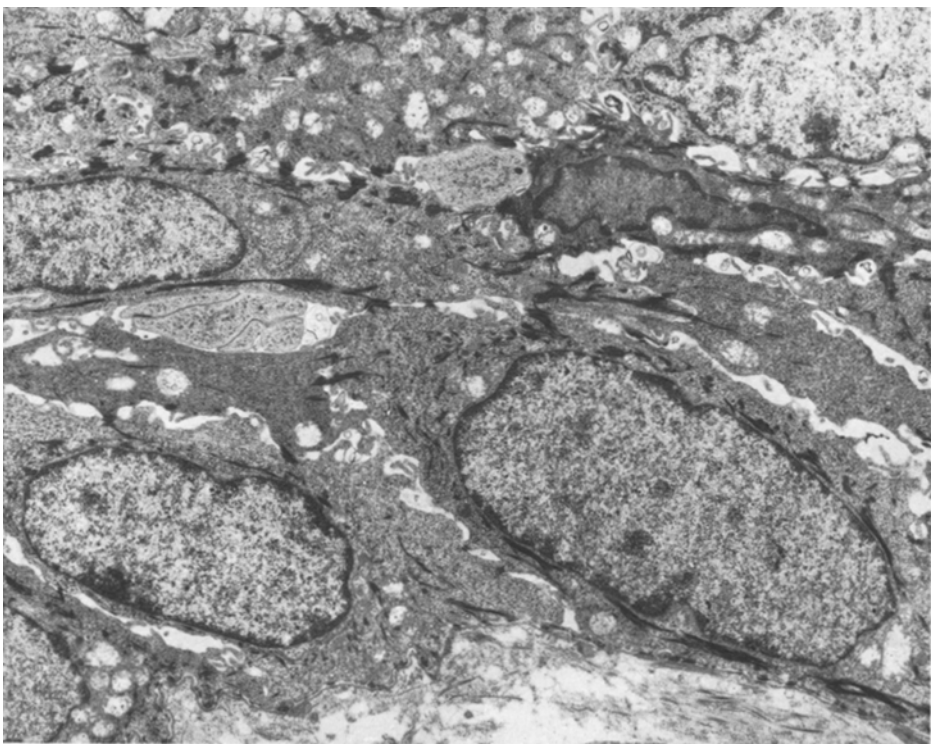

Fig. 6. Basal portion of bronchiolar hyperplasia after 6 weeks of NHMI-treatment; prominent bundles of cytoplasmic tonofilaments are characteristic of early squamous metaplasia. Uranyl acetate, lead citrate; ×6,100

ciliated cells in all parts of the airways had swollen mitochondria with partial loss of their cristae, and an increase in the amount of lysosomal derivatives (primary, secondary lysosomes). At four weeks we found multiple proliferation of small cells in the basal epithelial layer (Fig. 3) in all segments (including bronchioles) of the respiratory tree. These cells were identified as basal cells by electron microscopy. The cells (ciliated, mucous, Clara cells) located above such proliferating basal cells appeared often disoriented. At later stages in the experiment such foci with proliferated basal cells had become more numerous and enlarged to form focal hyperplasias. (Fig. 4). The normal surface cells of the respiratory epithelia (ciliated, mucous, Clara) disappeared from the surfaces of such hyperplasias, to be replaced by a new kind of cell (Fig. 5). This cell type had some features (microvilli, RER, SER) in common with the mucous and Clara cells of untreated animals but resembled the basal cells in having a large nucleus with little heterochromatin and a relatively large number of polyribosomes. We have chosen to call them "transitional cells". The microvilli of these transitional cells exhibit a prominent glycocalyx.

At 6 weeks, basal cells in hyperplastic areas exhibited bundles of tonofilaments (Fig. 6), a phenomenon characteristic of early squamous metaplasia. As the treatment was continued, squamous metaplasia advanced further leading

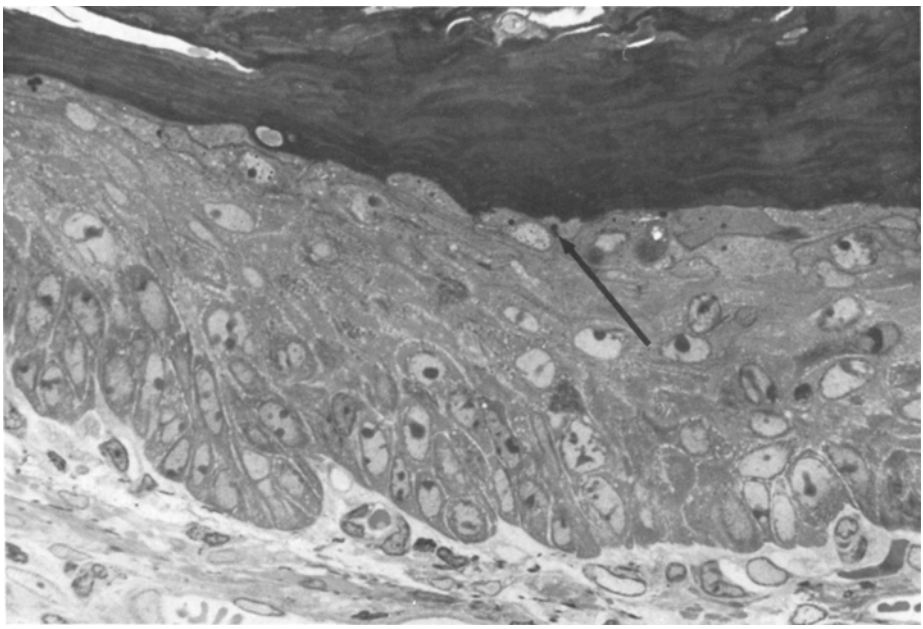

Fig. 7. Part of bronchiole after 12 weeks of NHMI-treatment; the epithelium is highly hyperplastic and has undergone squamous metaplasia. A thick layer of keratin covers the luminal surface and some keratohyalin granules (*arrowed*) are visible in the adjacent layer of cells. Semithin section, toluidine blue; ×1,000

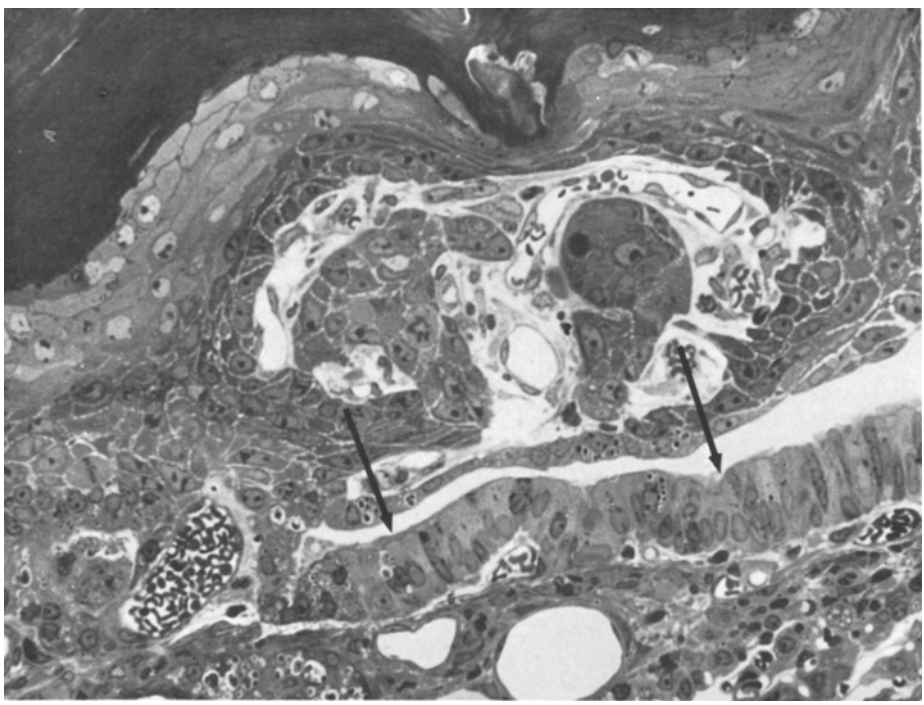

Fig. 8. Bronchiole at 14 weeks of NHMI-treatment: proliferating squamous cells overlay the adjacent normal epithelium (*arrowed*) while they are growing along the bronchiolar lumen into the alveole. Semithin section, toluidine blue; ×500

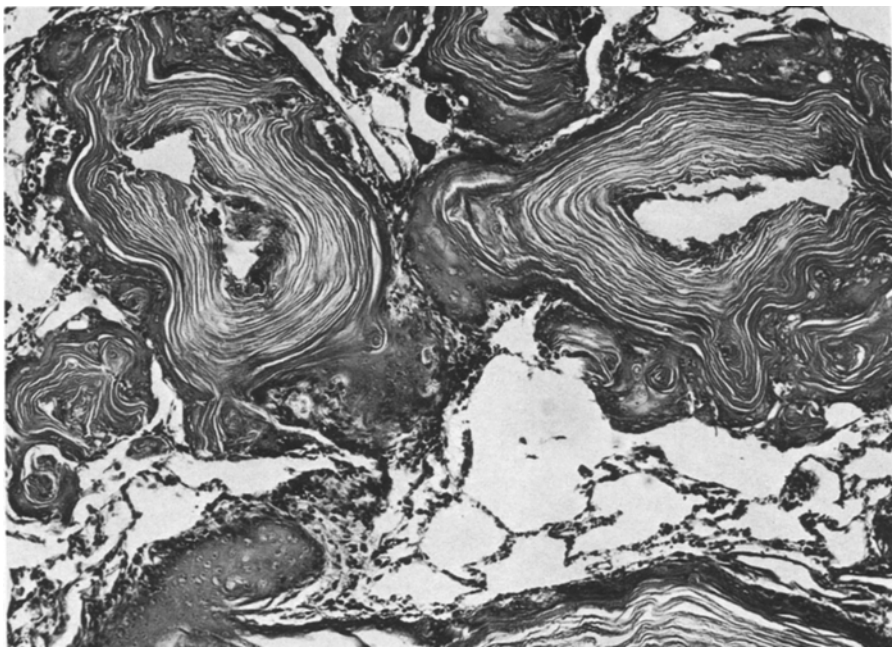

Fig. 9. Cross section of keratinizing squamous metaplasia in bronchioloalveolar region after 15 weeks of NHMI-treatment: at this plane of section, the bronchiolar origin of the squamous cells cannot be identified. Semithin section, toluidine blue; ×400

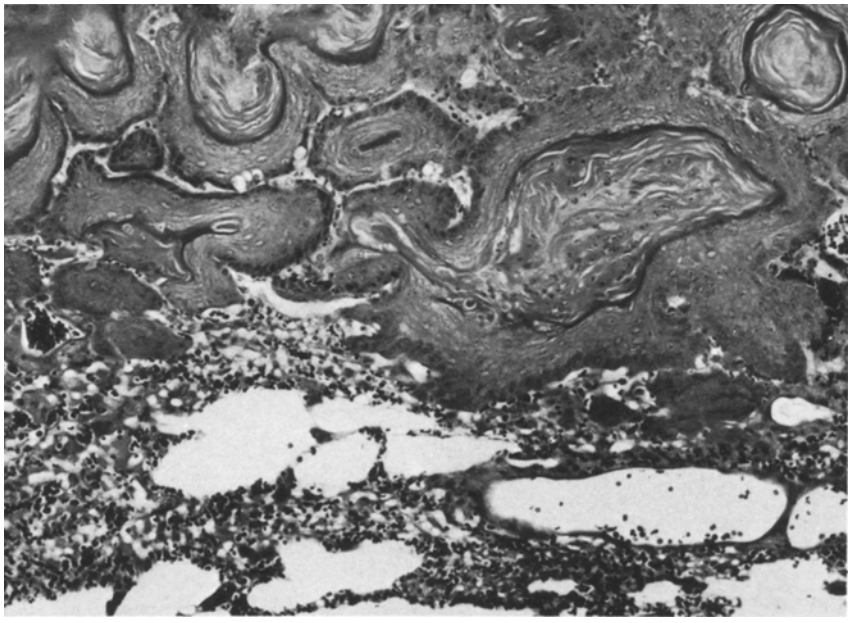

Fig. 10. Example of squamous cell carcinoma in the lung periphery after 10 weeks of NHMI-treatment. Note pronounced keratinization. Semithin section, toluidine blue; ×130

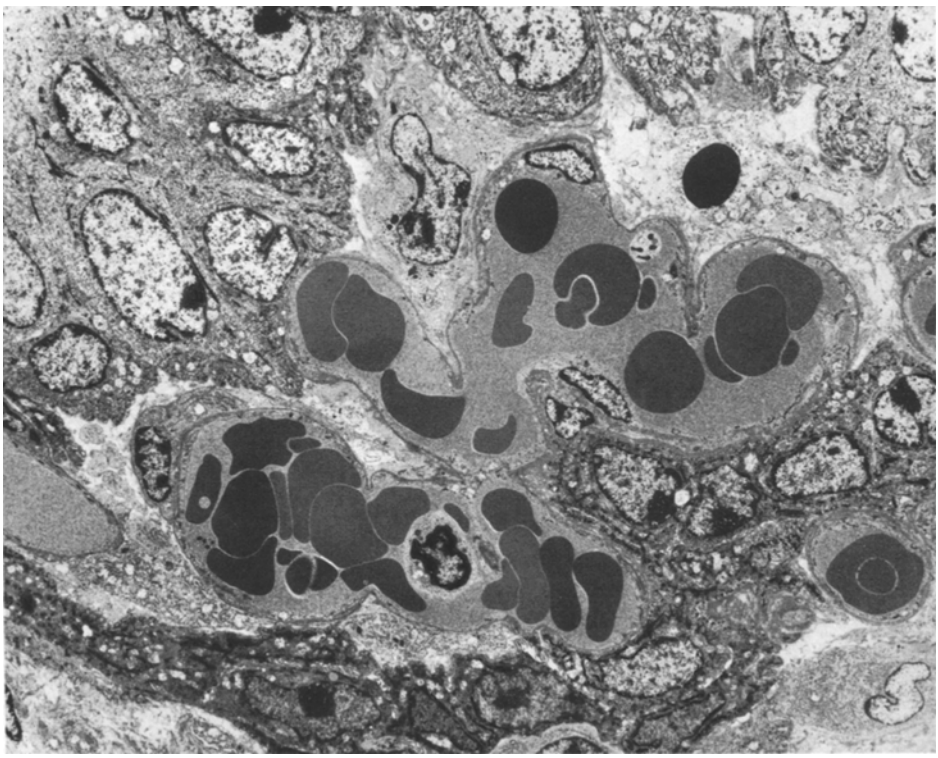

Fig. 11. Electron micrograph exemplifying peripheral area of squamous cell carcinoma: the main tumor mass is located above the upper margin of the picture. In the lower portion, strands of tumor cells infiltrating lung parenchyma. Uranyl acetate, lead citrate; ×3,000

to the formation of keratohyalin granules and mature keratin in the surface layers of such lesions (Fig. 7). In the bronchioles, the squamous cells appeared to proliferate more rapidly than at other levels of the airway. This resulted in the growth of squamous cells which then overlaid the adjacent normal epithelia (Fig. 8), thereby extending through the luminal spaces of the bronchioles into the alveoles. In cross section, such alveoles appeared to be filled with central keratin enclosed by squamous and basal cells (Fig. 9), while the adjacent alveolar cells were compressed. The development of peripheral squamous carcinomas (Fig. 10) from such areas was characterized by enlargment of the lesions, central hyperkeratinization, and infiltrative growth (Fig. 11) of squamous cells into adjacent blood vessels and alveoles. Thus, all the animals killed at week 20, and later, had developed multiple squamous tumors in their bronchiolo-alveolar region. In one rat, an adenoma composed of cells with the morphological features of alveolar type II cells was also found. It is noteworthy that the squamous metaplasia of the upper airways (extra-pulmonary, lobar, segmental bronchi) only increased in extent and in contrast to the lesions in the bronchioles did not grow into the adjacent parenchyma to form tumors.

Discussion

Our study shows that NHMI-induced pulmonary carcinogenesis in F344 rats develop through four major stages. The first stage is characterized by quantitative changes of the cytoplasmic organelles (increase in endoplasmic reticulum of mucous and Clara cells) which are the most likely to be involved in the metabolic activation of the nitrosoamine. This interpretation is supported by recent findings in Syrian golden and European hamsters in which the endoplasmic reticulum of mucous and Clara cells has been identified as the principle site of binding after the in vivo administration of labeled N-diethylnitrosamine and NHMI (Reznik-Schüller and Lijinsky 1979; Reznik-Schüller and Hague 1980). The concomitant degenerative changes (swelling of mitochondria, increased amount of lysosomes) in ciliated cells suggest that toxic metabolites may also be produced during the activation of NHMI.

The second stage reflects the response of the respiratory epithelia to the presence of carcinogenic metabolites: basal cells start to proliferate. This reaction is to be expected in the upper airways (trachea, extrapulmonary stem, lobar, and segmental bronchi) since, in this region, basal cells function as stem cells, and are responsible for cell renewal (Kuhn 1976). In the subsegmental bronchi and bronchioles, however, basal cells are not found in healthy rats. At these levels, Clara cells have been shown to provide for cell renewal after toxic injury in place of the stem cells (Evans et al. 1976). Thus, the formation, and subsequent proliferation, of basal cells in this peripheral area represents the first specific pre-cancerous change which can be seen in the rat lung.

During the third stage, the proliferation of basal cells is more advanced, and basal cells are transformed into squamous cells (formation of tonofilaments, keratohyalin and finally keratin). The final stage is characterized by invasive growth of squamous cells into adjacent lung parenchyma, a phenomenon generally accepted as a definite indicator of malignancy.

The development of NHMI-induced lung tumors in the rat model appears to be in sharp contrast with the pathogenesis of lung tumors induced by this compound in the European hamster (Reznik-Schüller 1978; Reznik-Schüller and Lijinsky 1979; Reznik-Schüller and Hague 1981). It is also different from the pathogenesis of lung tumors induced by a variety of other nitrosamines in Syrian golden hamsters (see review by Reznik-Schüller and Reznik 1979). In both hamster species, the nitrosamine-induced lung tumors originated from bronchial Clara cells and APUD-type cells, with the Clara cells forming adenomas and adenocarcinomas, while APUD-cells were the source of neoplastic squamous cells. The hamster tumors unlike those in the rat, originated from all types of intrapulmonary bronchi and were not restricted to the bronchiolo-alveolar region. It seems unlikely that these differences in localization of the tumors reflect differences in the distribution of nitrosamines in the lungs of rats and hamsters. Although the distribution of NHMI has not yet been studied in the rat and hamster lungs, it has been shown with other nitrosomines that the non-metabolized compounds freely pass the biological membranes and distribute evenly in the intra- and extra-cellular tissue water (Brittebo et al. 1981). Moreover, it was found that rat lung microsomes metabolize NHMI an order of

magnitude better than microsomes of non-target organs (liver, kidneys) (Hecker et al. 1981). The early quantitative changes (increase in smooth and rough endoplasmic reticulum) observed in the rat mucous and Clara cells indicate that, as in the hamster (Reznik-Schüller and Lijinsky 1979; Reznik-Schüller and Hague 1981), it is most probable that these are the same cells types which metabolically activate the parent nitrosamine. The neoplastic response of basal cells in the rat, and Clara and APUD-cells in the hamster, may then reflect the production of different metabolites in these species or some inherent differences in the capacity of the cells to proliferate. These questions cannot be answered by data obtained in this type of study, but require a more detailed understanding of the pathways by which the different cell types metabolize nitroso compounds.

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