Sequential Morphologic Alterations in the Bronchial Epithelium of Syrian Golden Hamsters During *N*-Nitrosomorpholine-Induced Pulmonary Tumorigenesis

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***N*-nitrosomorpholine (NM)–induced pulmonary carcinogenesis was examined by light and electron microscopy in a 20-week serial sacrifice study using Syrian golden hamsters. First to be observed were a proliferation of endocrine APUD cells and a formation of lamellated inclusion bodies in the cytoplasm of Clara cells. After continued NM treatment, APUD cells underwent squamous metaplasia and Clara cells invaded the pulmonary tissues adjacent to the bronchi. Lung tumors consisted of cells possessing numerous lamellated inclusion bodies in their cytoplasm and a few squamous metaplastic and APUD cells. The observed pathologic alterations closely resembled those found after treatment with *N*-diethylnitrosamine (DEN) and *N*-dibutylnitrosamine (DBN) but were completely different from the cellular reactions induced by polycyclic aromatic hydrocarbons. It is concluded that the observed alterations of APUD cells and Clara cells are specific to nitrosamines. (Am J Pathol 89:59–66, 1977)**

LIGHT AND ELECTRON MICROSCOPIC examinations of the sequential alterations occurring in the bronchial epithelia of Syrian golden hamsters after treatment with benzo[a]pyrene,[1,2] *N*-diethylnitrosamine (DEN),[3-6] and *N*-dibutylnitrosamine (DBN)[4-6] have demonstrated profound differences between the early cellular reactions initiated by the hydrocarbon and those initiated by the nitrosamines. These differences corresponded with the different biologic properties of the carcinogens: that is, the local carcinogenicity of benzo[a]pyrene in contrast to the systemic carcinogenicity of the nitrosamines. The present investigations were performed to examine whether the cyclic nitrosamine, *N*-nitrosomorpholine (NM), induced intracellular reactions in the bronchial epithelia of the Syrian golden hamster similar to those induced by the two aliphatic compounds DEN and DBN.

Materials and Methods

Forty male Syrian golden hamsters (Centraal Proefdierenbedrijf, TNO, Zeist, The Netherlands) were subcutaneously injected once weekly for life with 1.5 LD_{50} *N*-nitrosomorpholine (98 mg/kg body weight). Ten control animals received once weekly

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subcutaneous injections of the solvent solution (physiologic saline, 0.1 ml/100 g body weight) only. All animals were kept 5 to a cage in Makrolon cages Type III (E. Becker & Co. GmbH, Castrop-Rauxel, Germany) under standard laboratory conditions (room temperature, 22 ± 2 C; relative humidity, 55 ± 5%; air exchange, 20 times/hour). They received a pelleted diet (RMH-TMB, RMH = rat mouse hamster; Hope Farms, Woerden, The Netherlands) and water *ad libitum*.

The sequential alterations of the bronchial epithelia were examined during the first 20 weeks of treatment. Starting with the second treatment week, 3 animals (2 experimental, 1 control) were fixed *in situ* by means of vascular perfusion with 2% cacodylate-buffered glutaraldehyde (pH, 7.4). Specimens were taken from lobar and segmental bronchi (20 pieces/animal) and peripheral lung tissues (10 pieces/animal) and immersed for a further 2 hours in the fixative. After being washed in cacodylate buffer, they were postfixed for an additional 2 hours in 1% osmium tetroxide, dehydrated through an ascending series of ethanols, and embedded in Epon 812 (Ladd Research Industries Inc., Burlington, Vt.). Sections were cut on an LKB Ultrotome III (LKB, Bromma, Sweden). Semithin sections (1 μ thick) were stained with toluidine blue. Ultrathin sections were mounted on uncoated copper grids and stained with uranyl acetate and lead citrate.

Exposures were taken with a Philips 201 electron microscope at an accelerating voltage of 40 kV.

Results

The bronchial linings of the control animals remained unaltered throughout the observation period. As early as 4 weeks after the start of the experiment, the segmental bronchi of NM-treated animals demonstrated focal proliferations of small, round to oval cells (Figure 1, inset); these were identified by their contents of dense-cored, cytoplasmic granules as endocrine APUD-type cells (Figure 1). This phenomenon was found consistently throughout the observation period. However, the granules tended to decrease in number during prolonged NM treatment (Figure 2), while cytoplasmic filaments and filament bundles increased in number (Figure 2). From the third treatment week onwards, the nonciliated cells (Clara cells) occasionally demonstrated intracytoplasmic formation of lamellar bodies (Figure 3 and inset). These latter organelles closely resembled the lamellar bodies that are a typical feature of great alveolar cells. They consisted of parallel lamellae surrounded by a narrow rim of lysosome-like material and were enclosed by a membrane. The number of Clara cells containing lamellar bodies increased in number under continued NM treatment. Moreover, it was occasionally noted that mature lamellar bodies were extruded into the bronchial lumen by means of a merocrine secretion (Figure 3). Around the fifteenth treatment week, hyperplasia of nonciliated cells was frequently observed in lobar and segmental bronchi. After 17 weeks of NM treatment, nonciliated cells were found invading the lung tissue through defects in the basement membrane. One of the 2 animals sacrificed after 20 weeks of NM treatment exhibited multiple pulmonary tumors. The majority of these were composed of cells containing lamellar bodies (Figure 4); occasionally a

few cells were interposed that were characterized by the presence of cytoplasmic filaments (Figure 5) and/or dense-cored granules.

Discussion

The present findings demonstrate that the cyclic nitrosamine *N*-nitrosomorpholine causes the same specific alterations of APUD cells and Clara cells as the aliphatic DEN and DBN.[3-6] Moreover, the morphology of the resultant tumors closely resembled that of pulmonary neoplasms found in Syrian golden hamsters after DEN and DBN administration [3-6] and in European hamsters after DBN treatment.[7] The fact that the described cellular responses occurred consistently with all three examined nitrosocompounds, but not after treatment with benzo[a]pyrene [2] or chronic intratracheal instillation of automobile exhaust condensate [8] strongly suggests that the described reactions are specific to nitrosamines. These findings also indicate that Clara and APUD cells could be the main sites of metabolism of these carcinogens in the lungs. This hypothesis is partially supported by recent findings with radioactively labeled nitrosoheptamethyleneimine (NHMI) [9] and labeled DEN.[10] These two studies showed that NHMI and DEN were primarily bound to cellular macromolecules of Clara cells. However, it was difficult to draw any such conclusion for the APUD cells, since only a small number of these cells were observed in the investigated animals. The present findings prove that nitrosamine-induced pulmonary tumors in hamsters, which due to their contents of lamellar bodies in the neoplastic cells closely resemble alveolar cell carcinomas, derive from the Clara cells of the bronchial linings.

Moreover, these results demonstrate that Clara cells are able to produce mature lamellar bodies and to extrude them into the bronchial lining by means of a merocrine secretion. This phenomenon illustrates that under nitrosamine treatment Clara cells have a capacity for the formation of surfactant, the production of which is restricted to alveolar epithelial cells Type 2 in healthy individuals.[11-14] Both Clara cells and alveolar epithelial cells derive from the same embryonal columnar epithelium of fetal airways.[15-17] The present findings suggest, therefore, that the nitrosamines deactivate an "inhibitory system" which normally prevents surfactant production; this would then mediate the development of the embryonal cells into alveolar cells Type 2. Conversely, they could produce this same effect by the activation of a "release system" for surfactant production. The proliferation and ultrastructural changes of APUD cells coincides with the above mentioned findings after DEN and DBN treatment. These results demonstrate that cells of this type can undergo squamous metaplasia, since the phenomenon of cytoplasic bundle formation in respi-

ratory epithelia is regarded as an early stage of squamous metaplasia.[18,19] APUD cells have been shown to be the source of carcinoid tumors and oat cell carcinomas in human lungs,[20,21] while human alveolar cell carcinomas are characterized by cells containing lamellar bodies.[22,23] The present results demonstrate that nitrosamine-induced pulmonary tumors in the Syrian golden hamster can serve as a model for further studies concerning the biochemical characteristics of such human neoplasms.

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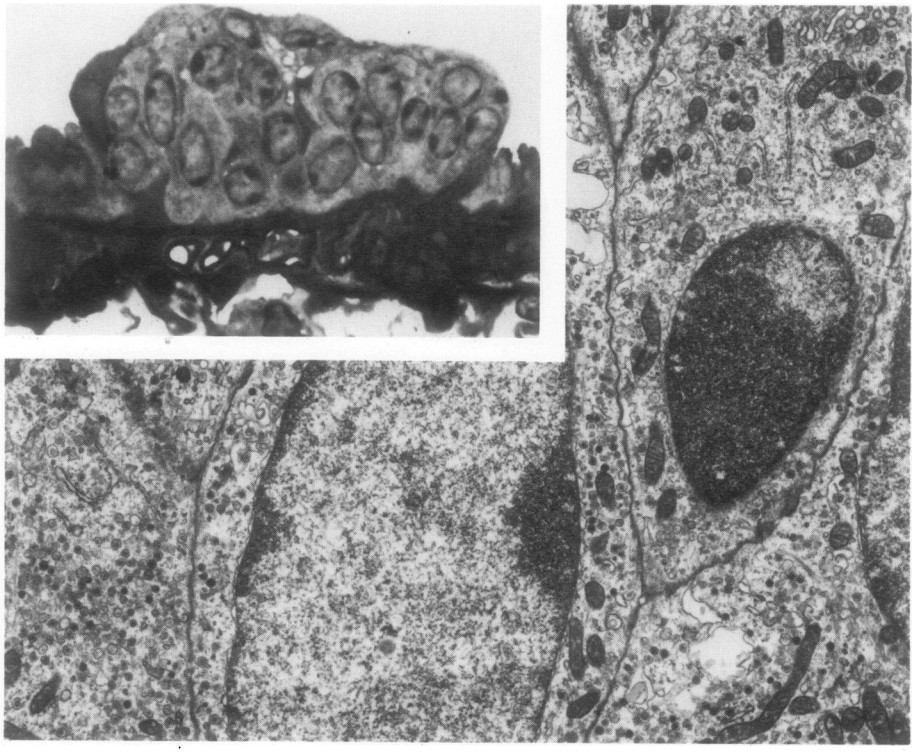

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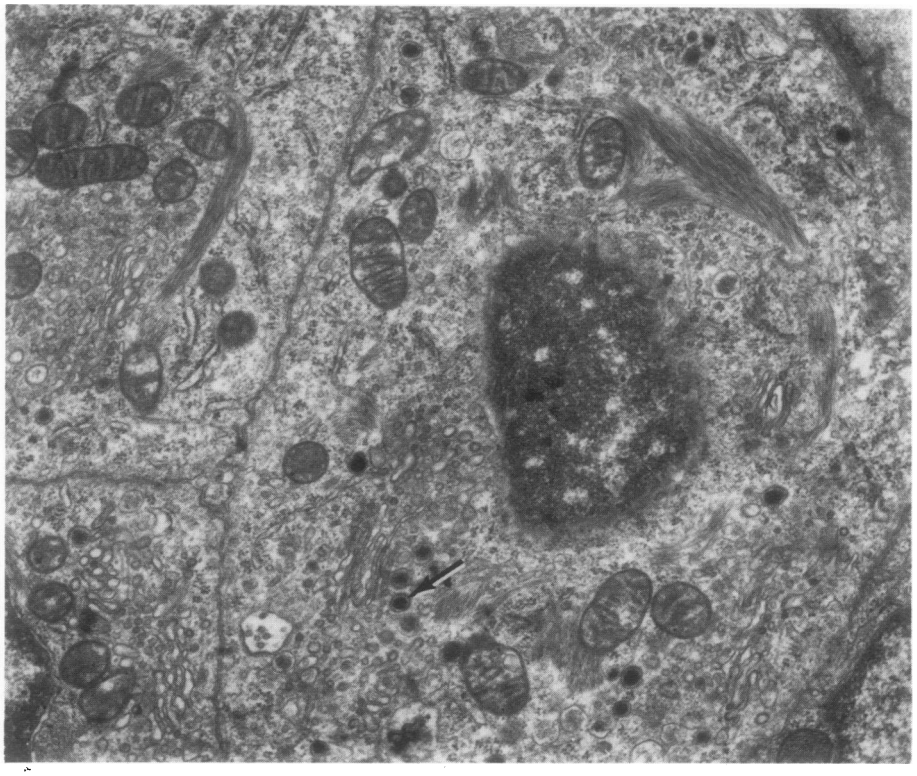

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Figure 1—Fine structure of cells shown in **inset**; the cytoplasm of the proliferated cells contains numerous dense-cored granules, typical features of APUD cells (× 14,000). **Inset**—Area with proliferated small, round to oval cells in segmental bronchus of hamster after 4 weeks of NM treatment (× 860). **Figure 2**—APUD cell in bronchial epithelium after 12 weeks of NM treatment; dense-cored granules (*arrow*) are scanty, while filaments and filament bundles have increased in number. (× 20,000).

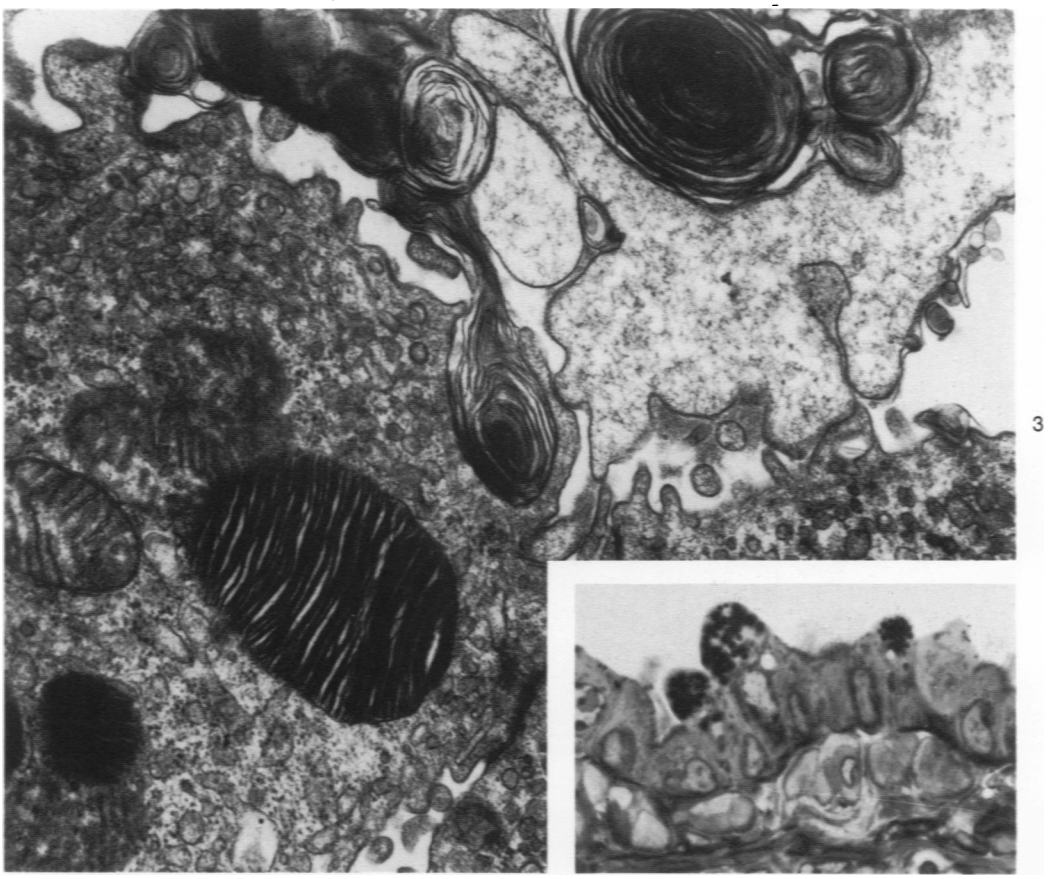

Figure 3—Merocrine secretion of mature lamellar body from a Clara cell in the bronchial lumen (× 27,400). **Inset**—Clara cells in segmental bronchus containing numerous dense intracytoplasmic inclusions (*arrow*) after 3 weeks of NM-treatment (× 860).

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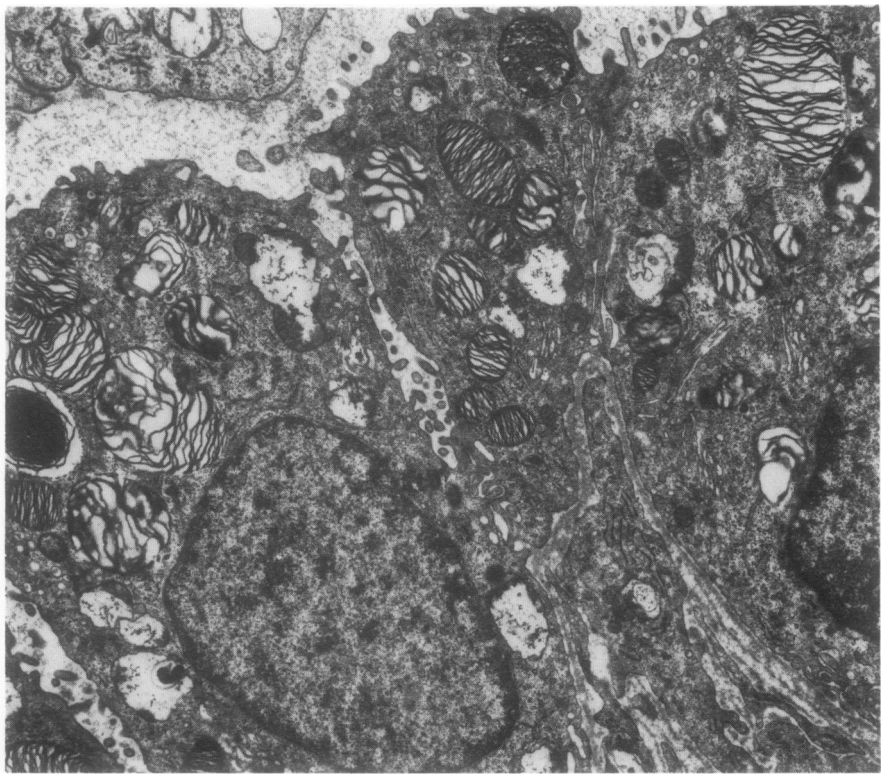

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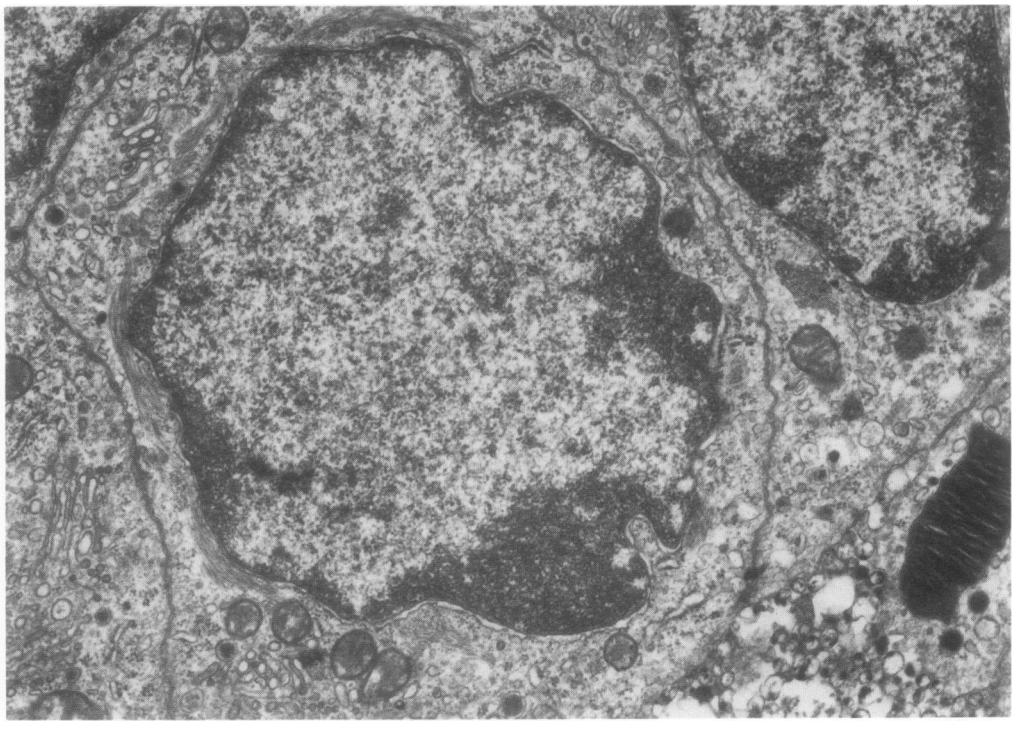

Figure 4—Fine structure of tumor cells; the secretory granules are identifiable as lamellated inclusion bodies (× 14,000). **Figure 5**—Fine structure of another part of the tumor shown in Figure 4; dense-cored granules and/or prominent cytoplasmic filaments are visible (× 20,000).