# Ultrastructural Alterations of Nonciliated Cells After Nitrosamine Treatment and Their Significance for Pulmonary Carcinogenesis

Hildegard Reznik-Schüller, DVM

**Syrian golden hamsters received multiple subcutaneous injections of *N*-diethylnitrosamine (DEN) or *N*-dibutylnitrosamine (DBN). Ultrastructural examination of the sequential alterations of bronchial epithelia was performed. Starting from the third to fourth treatment, the formation of lamellated inclusion bodies (LBs) was observed in nonciliated cells of segmental bronchi and peripheral bronchioles. These organelles closely resembled those LBs normally found in alveolar epithelial cells Type 2, which are a characteristic feature of alveolar cell carcinomas. (Am J Pathol 85:549–554, 1976)**

THE CARCINOGENICITY of *N*-diethylnitrosamine (DEN) and *N*-dibutylnitrosamine (DBN) for pulmonary and tracheal tissues in hamsters has been frequently reported.[1-5] Electron microscopy revealed DEN-induced pulmonary neoplasms in Syrian golden hamsters to consist of several cell types. Two of these were different types of poorly differentiated cells, both of which were regarded as originating from basal cells. A few mucus-producing cells, as well as a fourth type closely resembling a Type 2 alveolar epithelial cell in its contents of lamellated inclusion bodies, were also found.[6,7] These latter cells occurred mainly in the periphery of lung tumors and, therefore, were thought to represent a proliferative reaction of lung tissue upon the tumor growth rather than actually being neoplastic themselves.[6,7] Moreover, because of certain structural similarities between pulmonary and coincidentally occurring tracheal neoplasms, it has been suggested that during the procedure of intratracheal instillation, there takes place an accidental transfer of parts of tracheal neoplasms to the lungs.[7] Recently, DBN-induced pulmonary adenocarcinomas of the European hamster were shown to consist predominantly of cells demonstrating lamellated inclusion bodies.[8] Nevertheless, due to the occasional occurrence of the same cell type within the linings of the tumor-adjacent bronchi, a bronchogenic origin of these tumors was discussed.[8] The present investigations were performed to gather further information about the histogenesis of nitrosamine-induced pulmonary neoplasms and hence to reply to the various speculations concerning which is their cell of origin.

From the Abteilung für Experimentelle Pathologie, Medizinische Hochschule Hannover, Hannover, Germany.

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Address reprint requests to Dr. Hildegard Reznik-Schüller, Medizinische Hochschule Hannover, Abteilung für Experimentelle Pathologie, Karl-Wiecher-Allee 9, 3 Hannover 61, Germany.

## Materials and Methods

Eighty male Syrian golden hamsters (Centraal Proefdierenbedrijf, TNO, Zeist, The Netherlands) were housed 5 to a cage in Makrolon cages Type II (E. Becker & Co., GmbH, Castrop-Rauxel, West Germany) under standard laboratory conditions (room temperature, 22 ± 2 C; relative humidity, 55 ± 5%; air exchange, 20 times/hour). They received a pelleted diet of RMH-TMB (RMH = Rat Mouse Hamster, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. Forty of the animals were subcutaneously injected twice weekly for life with 1/10 $LD_{50}$ DEN (178 mg/kg body weight). The remaining 40 hamsters were subcutaneously injected once a week for life with 1/5 $LD_{50}$ DBN (351 mg/kg body weight). Twenty animals, kept under the same conditions, were treated with 0.1 ml/100 g body weight physiologic saline (the solvent for DEN) or 0.1 ml/100 g body weight Livio oil (the solvent for DBN). Starting with the second treatment week, 2 DEN-injected animals were fixed *in situ* every week by means of perfusion and 2 DBN-treated animals every 2 weeks. One animal per group of the respective controls was sacrificed in the same manner every 2 (DEN controls) or every 4 (DBN controls) weeks. Perfusion was performed under anesthesia with 100 mg/kg body weight Evipan-Na (Bayer, Leverkusen, West Germany) using 2% cacodylate-buffered glutaraldehyde (pH = 7.4) as fixative, following preperfusion with Rheomakrodex (Knoll A. G., Ludwigshafen, West Germany). Samples of lobar and segmental bronchi were excised from each lung, cut into small pieces, 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 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 or 60 kV.

## Results

As early as 3 to 4 weeks after commencement of DEN or DBN treatment, segmental bronchi or peripheral bronchioles demonstrated numerous nonciliated cells containing dark granules in their cytoplasm. This phenomenon was observed throughout the whole experimental period. When examined light microscopically, these inclusion bodies resembled mucus droplets (Figure 1, inset). However, electron microscopy established these organelles as possessing a specific substructure, which clearly excluded them from containing any mucus substance (Figures 1–3). They exhibited numerous parallel lamellae of the cross-barred or concentric types (Figures 1 and 2). At higher magnification these lamellated inclusion bodies were observed to be enclosed by a membrane and to possess a narrow marginal rim composed of a lysosome-like fine-granular dense substance (Figure 3). Nonciliated cells in the control animals did not contain such described inclusion bodies. However, as normal, the animals did possess organelles of the same fine structure in alveolar epithelial cells Type 2 (Figure 4).

## Discussion

The ultrastructure of the described lamellated inclusion bodies (LBs) found in the nonciliated cells of segmental bronchi and peripheral bronchioles of DEN- and DBN-treated hamsters was typical of those LBs normally only occurring in alveolar cells Type 2. These organelles, which up until now have not been described within the bronchial linings, are believed to be the source of lung surfactant.[9-12] They are a typical feature of human alveolar cell carcinomas, leading to the conclusion that such tumors derive from alveolar epithelial cells Type 2.[13,14] Diethylnitrosamine treatment in Syrian golden hamsters led to the development of pulmonary neoplasms composed of both respiratory epithelial and alveolar epithelial cells Type 2.[6,7] This was thought to indicate that the tumors had originated from simultaneously occurring tracheal neoplasms, parts of which had been transferred to the lungs during the intratracheal instillation procedure.[7] The presence of LB-containing cells in the pulmonary tumors was explained as a proliferative reaction of alveolar cells upon the tumor growth.[7] However, the present findings have shown that DEN or DBN treatment results in the formation of LBs in the nonciliated cells of segmental bronchi and peripheral bronchioles. This allows for the conclusion that LB-containing cells occurring in pulmonary neoplasms need not necessarily derive exclusively from alveolar epithelial cells but could also originate from the bronchial linings. If, in addition, respiratory epithelial cells are found in such tumors—a phenomenon reported by several authors[6,7,14]—then a bronchogenic origin becomes even more probable. Nevertheless, the possible implication of transplanted parts of tracheal neoplasms in the development of pulmonary neoplasms cannot be excluded. This is especially the case with the Syrian golden hamster, which develops a particularly high rate of tracheal neoplasms. The possible falsification of results through this "transfer danger" could be excluded by either administering the carcinogen subcutaneously or by using another animal model, such as the European hamster, which only seldomly develops tracheal neoplasms.

The here-reported early alterations of nonciliated cells after nitrosamine treatment suggest a bronchogenic origin of the so-called alveolar cell carcinoma. They demonstrate that after DEN or DBN treatment the nonciliated bronchial cells developed ultrastructural features normally only found in alveolar epithelial cells Type 2. Human alveolar cell carcinomas have been shown to be composed of this cell type,[13,14] which has recently also been found to be a characteristic feature of DBN-induced pulmonary adenocarcinomas in the European hamster.[8] Upon

consideration of the fact that both bronchial and alveolar epithelial cells derive from the same embryologic columnar epithelium,[15-17] it would seem possible that the nitrosamines cause the bronchial cells to display developmental steps which are normally only performed during embryonic development.

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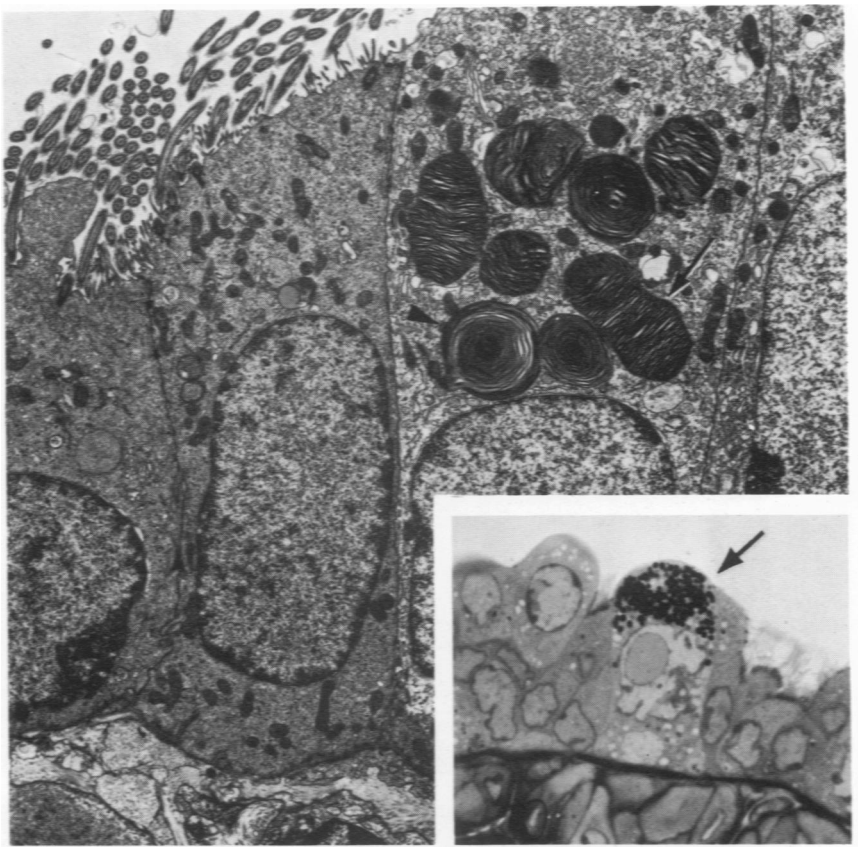


**Figure 1**—Segmental bronchus of a DEN-treated hamster; a nonciliated cell demonstrates lamellated inclusion bodies with crossbarred (*arrow*) and concentric (*arrowhead*) lamellae. (× 6100) **Inset**—Segmental bronchus of a DEN-treated hamster; a nonciliated cell (*arrow*) contains numerous dark granules and a large nuclear inclusion body (Semithin section, toluidine blue, ×850).

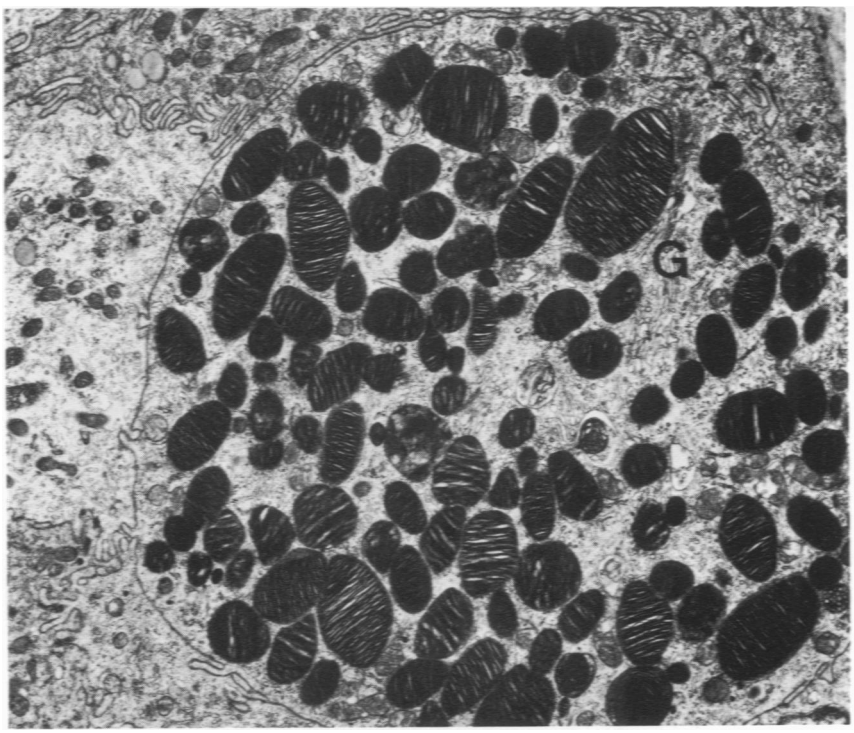
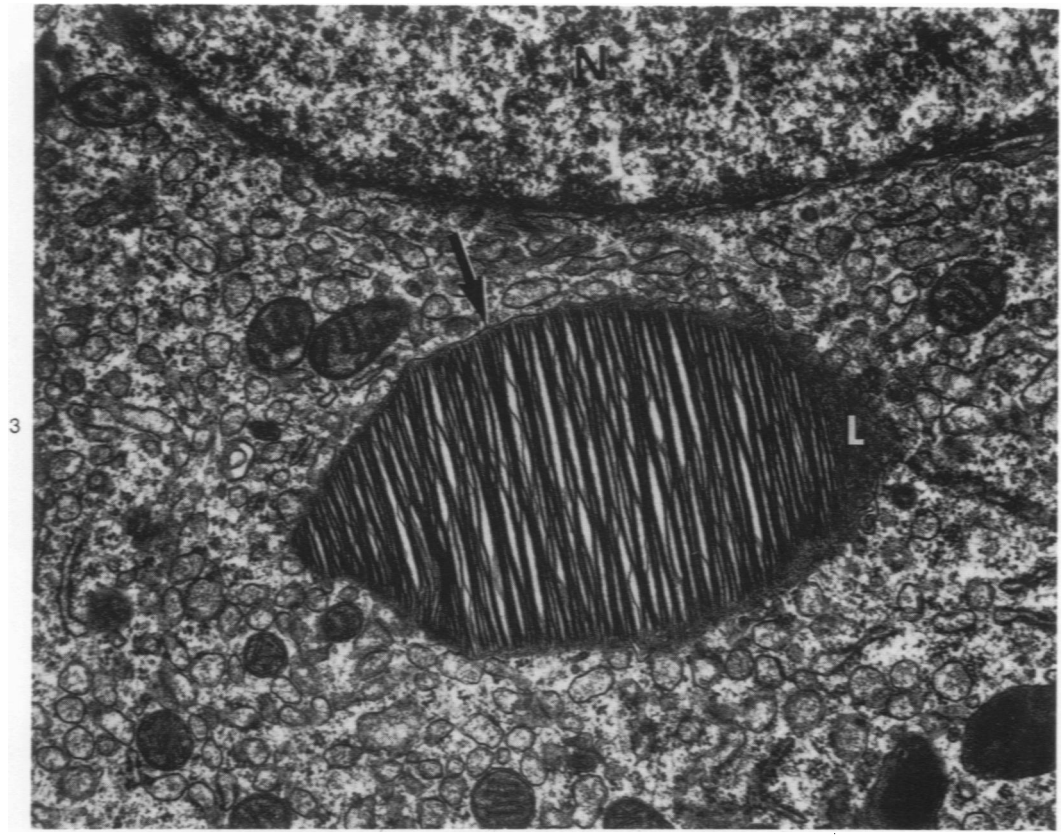



**Figure 2**—Basal part of a hyperplastic segmental bronchus after DBN treatment; a nonciliated cell demonstrates excessive formation of LBs. Note also the well-developed Golgi apparatus (G). (× 9300)

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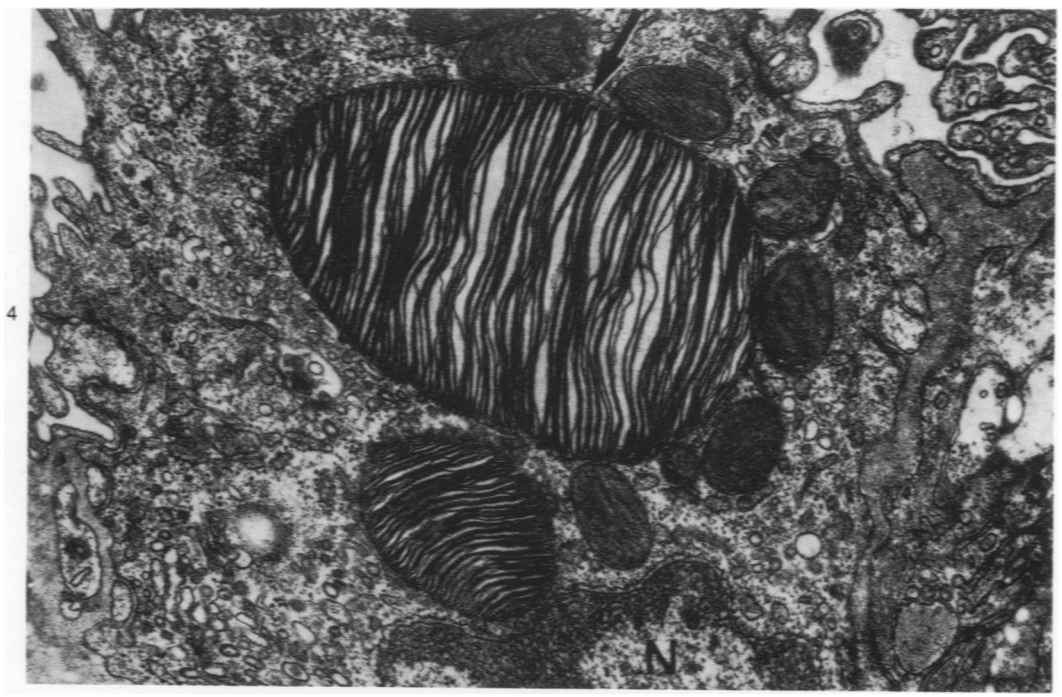


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**Figure 3**—Lamellated inclusion body in a nonciliated cell at higher magnification; the organelle is surrounded by a membrane (*arrow*). Its parallel lamellae seem to originate from a marginally situated lysosome-like substance (*L*). *N* = nucleus. (× 27,400) **Figure 4**—Lamellated inclusion bodies in an alveolar epithelial cell Type 2 from a control animal. Note close resemblance with the organelle of Figure 3. The limiting membrane of the LB is indicated by an *arrow*. *N* = nucleus. (× 27,400)