American Journal of Pathology, Vol. 135, No. 6, December 1989

Diethylnitrosamine-Induced Pulmonary Endocrine Cell Hyperplasia and Its Association with Adenomatosis and Adenocarcinoma in Rabbits

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Pulmonary endocrine cells are suspected of being the precursors for small cell carcinoma of the lung (SCCL). The purpose of this study was to determine whether Diethylnitrosamine (DEN) can induce SCCL in rabbits. New Zealand white rabbits were injected subcutaneously with 20 mg/kg body weight of DEN, twice per week, starting when they were 1 week old. Controls received saline vehicle only. The animals were sacrificed 6 to 8.5 months after the first injection and lung tissues were processed for light microscopy. Using serotonin (5-HT) as a marker for the endocrine cells, tissue sections were stained immunohistochemically by the avidin-biotin complex method. In both control and DEN-treated animals, serotonin-immunoreactive cells organized into neuroepithelial bodies (NEBs). There was an apparent increase in the size, number, and stainability of NEB in DEN-injected animals. A majority of these NEBs were localized in the alveolar duct region. Small foci of adenomatosis and well-differentiated adenocarcinomas, which sometimes coexisted with hyperplastic pulmonary endocrine cells, were also found in the DEN-treated rabbits. (Am J Pathol 1989, 135:1119–1128)

Endocrine cells can be found in the lungs of normal humans and animals. They appear singly, or in clusters that are called neuroepithelial bodies (NEBs).[1] Because of the similarities in ultrastructural and immunohistochemical characteristics between normal pulmonary endocrine cells and small cell carcinoma of the lung (SCCL), the normal endocrine cells are suspected of being the progenitors of SCCL.[2-5] However, evidence from animal experiments suggests that during repair of tissue injury, pulmonary endocrine cells may be derived from secretory or Clara cells.[6,7]

In working toward establishing an animal model for SCCL, previous experiments have indicated that some nitrosamines can induce hyperplasia of pulmonary endocrine cells in hamsters[7-12] as well as in rats.[13] A recent report has shown further that diethylnitrosamine (DEN) treatment, coupled with simultaneous exposure to hyperoxia (70% O_2), produced neuroendocrine lung carcinoma in golden hamsters.[14] Because the rabbit is the only species in which lung endocrine cells have been quantitated at various pre- and postnatal ages,[15,16] this species was chosen for the current study on the effects of DEN on pulmonary endocrine cells. The results indicate that DEN not only induces endocrine cell hyperplasia but also adenomatosis and adenocarcinomas.

Material and Methods

New Zealand white pregnant rabbits were purchased from the supplier (White Hare, Stark City, MO), and were allowed to deliver in the University of Kansas Medical Center animal care unit. A total of seven newborns, from two litters, received DEN, 20 mg/kg body weight, twice per week beginning when they were 1 week old. Six control rabbits from two litters received equal amounts of saline vehicle only. Three of seven DEN-injected animals survived more than 6 months and were sacrificed along with three controls 6, 8, and 8.5 months after the first injection. At sacrifice, rabbits were premedicated with an intramuscular injection of ace-promazine maleate (0.1 mg/kg), and after a ten-minute interval they were anesthetized by a

Accepted for publication August 3, 1989.

Supported in part by BRSG S07 RR05373 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

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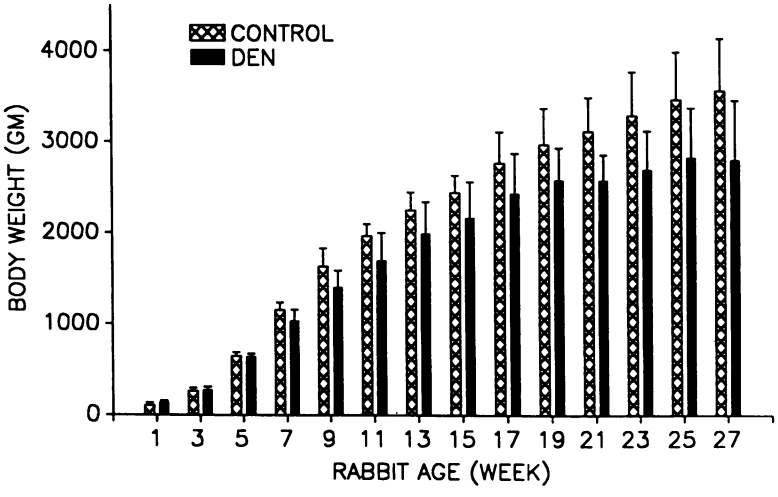

Figure 1. *Comparison of body weight. DEN-treated rabbits (N = 3) had consistently lower weights than that of the control animals (N = 3).*

combined intramuscular dose containing ketamine hydrochloride (25 mg/kg) and xylazine (3.0 mg/kg). The lung tissues were removed and fixed in 4% paraformaldehyde and processed for paraffin embedding.

An immunoperoxidase technique using avidin-biotin complex (ABC)[17] was used for localization of serotonin (5-HT), which is a marker for pulmonary endocrine cells.[15,18] Paraffin sections, 5 μm thick, were mounted on gelatin-chrome alum subbed glass slides and dried at 52 C for 2 hours. They were deparaffinized and hydrated through xylene and ethanol, pretreated with 0.3% hydrogen peroxide in absolute methanol for 30 minutes, and then incubated for 20 minutes with diluted normal goat serum. Sections were then incubated for 24 hours at 4 C with rabbit

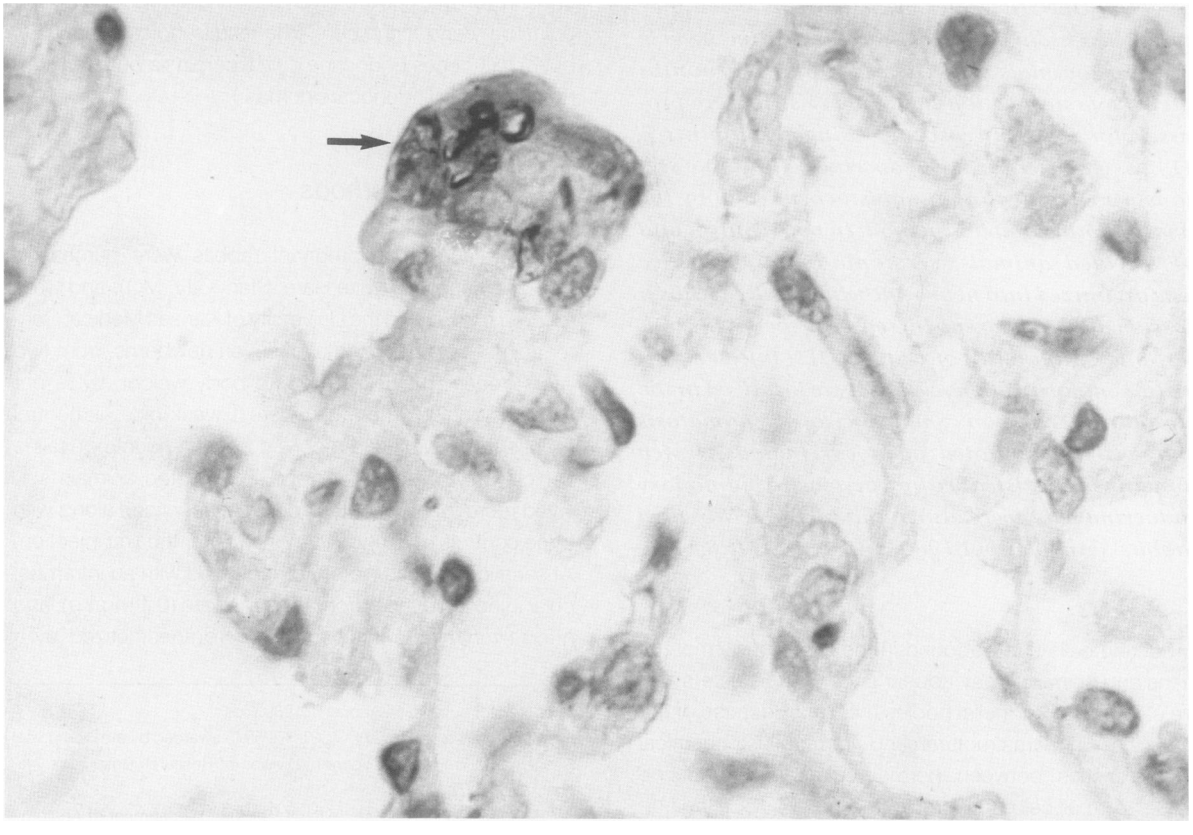

Figure 2. *A small NEB (arrow) in the alveolar duct region. The endocrine cells show weak immunoreactivity (Control, 5-HT immunoreaction, ×1660).*

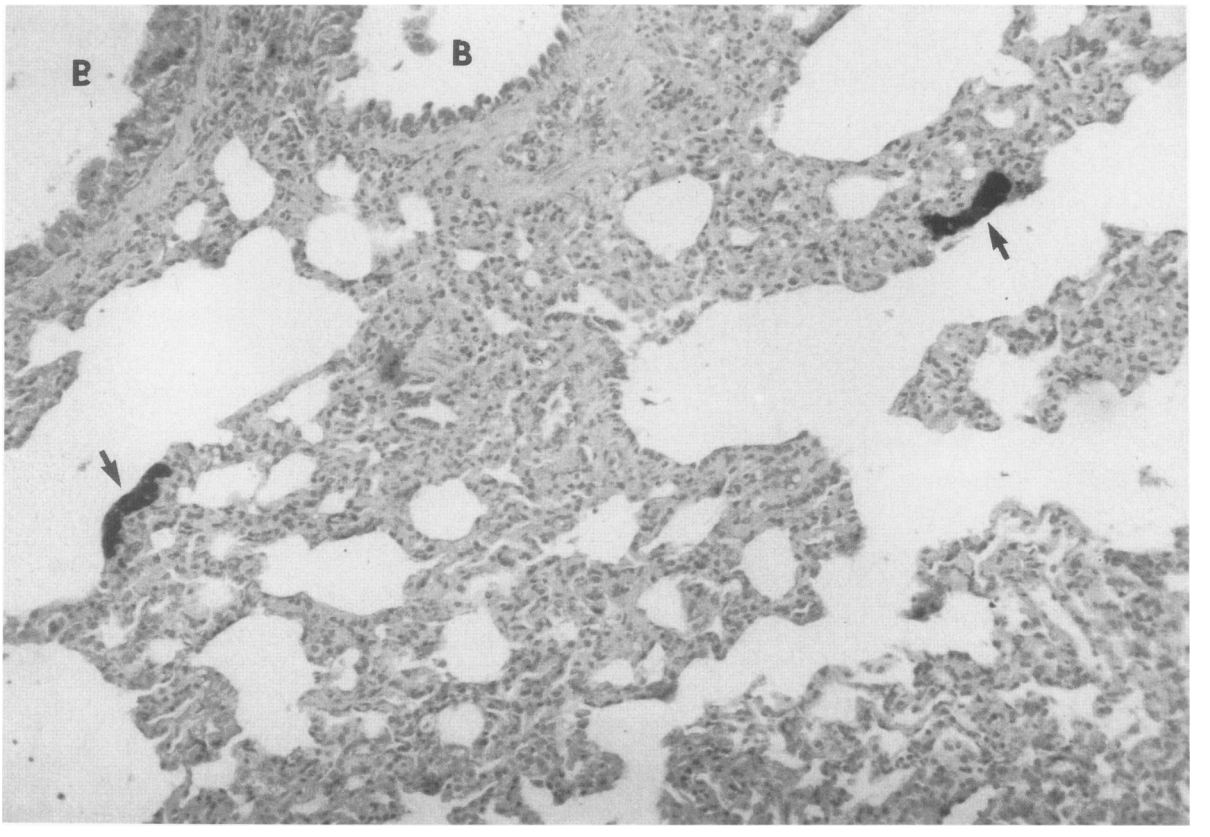

Figure 3. *Two NEBs (arrows) are shown in the alveolar duct region. Two sections of bronchioles* (B) *are near the upper left corner (DEN-treated, 5-HT immunoreaction, ×166).*

anti-serotonin serum (Accurate Co., Westbury, NY) (1:200 and 1:400 dilutions). Sections for negative controls were incubated for the same time period with nonimmune rabbit serum or PBS. Subsequent incubation procedures followed the recommendations of the supplier of the Vectastain ABC kit (Vector Labs, Burlingame, CA). Sections were rinsed with PBS (two times, ten minutes each) and then incubated with biotinylated secondary antibody (goat anti-rabbit) in the ABC kit for 30 minutes. The sections were washed again with PBS (two times, five minutes each) and incubated for 30 minutes with biotinylated horseradish peroxidase in the ABC kit. After washing with PBS (two times, five minutes each), the sections were incubated with a diaminobenzidine (DAB) mixture containing 100 mg DAB in 200 ml of Tris with 0.01% hydrogen peroxide. The slides were washed for 15 minutes in tap water and counterstained with hematoxylin for 15 seconds. Adjacent sections were used for routine H&E stain.

Sections from lung-tissue blocks containing bronchi and/or bronchioles were randomly selected for quantitation of NEBs,[15] adenomas, and adenocarcinomas. The tissue blocks were from different parts of the lung, and only one section derived from each block was used. The diameter of each NEB, the number of cells in each NEB, and the numbers of NEB, adenoma, and adenocarcinoma found in a section were recorded. The total areas of the lung sections were measured using a Bioquant Image Analysis System (R&M Biometrics, Inc., Nashville, TN). For each rabbit, the search for NEB, adenoma, and adenocarcinoma continued until the accumulated total areas of the lung sections reached at least 10 cm^2; this usually took from five to seven sections. The numbers of NEB, adenoma, and adenocarcinoma per cm^2 areas for each animal were calculated. For statistical analyses Student's *t*-tests ($P = 0.05$) were performed comparing the DEN-injected group with the controls in the size of NEBs, the number of cells per NEB, and the number of NEB, adenoma, and adenocarcinoma per unit area of the lung.

Results

Although the initial body weights (BW) of the DEN-injected rabbits (N = 3) were slightly higher than that of the controls (N = 3), BW of DEN-treated rabbits were consistently lower beginning at 7 weeks old (or 6 weeks after injection started) (Figure 1).

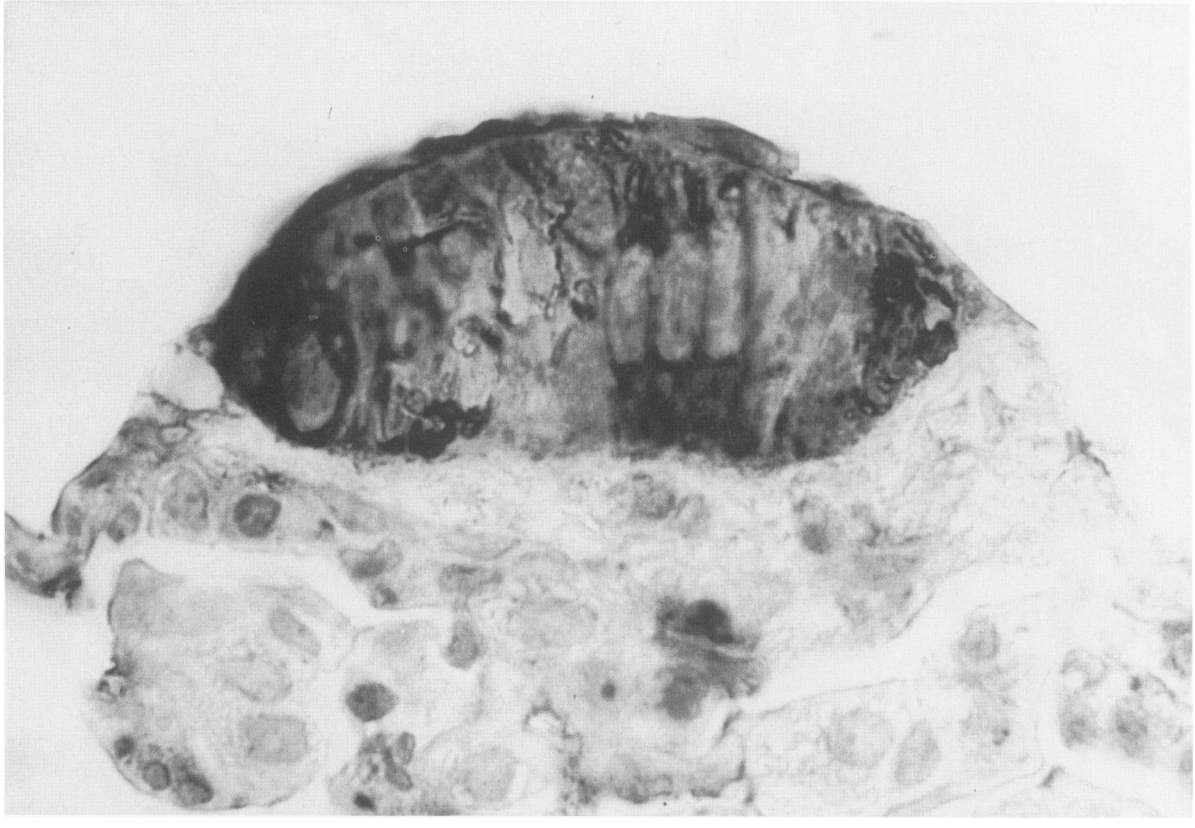

Figure 4. *Numerous endocrine cells in this NEB are strongly reactive for 5-HT, especially in apical and basal cytoplasm. Most cells are tall columnar (DEN-treated, 5-HT immunoreaction, ×1660).*

The light microscopic appearance of the control rabbit lungs was comparable to previous reports; notably the terminal bronchiole and alveolar duct regions were free of lumenal cell debris and the bronchial and bronchiolar epithelia were normal pseudostratified columnar, simple columnar, or cuboidal types. Occasional small NEB containing weakly immunoreactive cells were found in control rabbits (Figure 2). In DEN-injected animals, denuded epithelia in the bronchus and bronchiole were common and desquamated cells often filled the lumen; and regions of thickened alveolar walls were found. Strongly immunoreactive cell clusters resembling NEBs were mainly seen in the alveolar duct regions (Figure 3). The immunoreactive stain usually was accumulated in the apical and basal cytoplasm (Figure 4) of the rather regularly arranged columnar cells (Figure 5). In addition, grouped endocrine cells were seen to spread in thickened alveolar walls (Figure 6). Individual endocrine cells were seen in the bronchial and bronchiolar epithelia; and they appeared equally rare in both DEN-injected and control rabbits.

In the DEN-treated rabbits peripheral adenomatosis was identified as minute lesions of alveoli lined by a mixture of simple columnar and cuboidal cells with glanduar appearance (Figure 7). In addition, there were rare, small well-demarcated aggregates of glandular structures lined by atypical stratified epithelium with some foci of glands within glands consistent with well-differentiated adenocarcinoma (Figures 8 and 9). Endocrine cells, singly or in groups, were occasionally found associated with clusters of adenomatoses (Figure 10) and adenocarcinoma (Figure 11). In general, the lung in DEN-treated rabbits showed focal thickening of alveolar septa composed of fibrosis and nonspecific chronic inflammation consistent with interstitial fibrosis.

Table 1 shows quantitative data for comparison of the control and DEN-injected animals. It was obvious that DEN-injected rabbits had statistically significant higher number of NEB (4.37/cm^2 *vs.* 0.08/cm^2 in controls), twice more cells per individual NEB (13.23 *vs.* 6.50 in controls), and NEBs with increased diameters (41.75 μm *vs.* 25.00 μm in controls). All NEBs in the controls were found in the alveolar ducts (100%), whereas in the DEN group, a majority of NEBs were associated with alveolar regions (96.47%), and those associated with bronchi (0.69%) and bronchioles (2.84%) were rare. In DEN rabbits, because of the often disruption of alveolar ducts and alveolar walls, NEBs found in these areas were combined. While no adenomatosis and adenocarcinoma were present in any

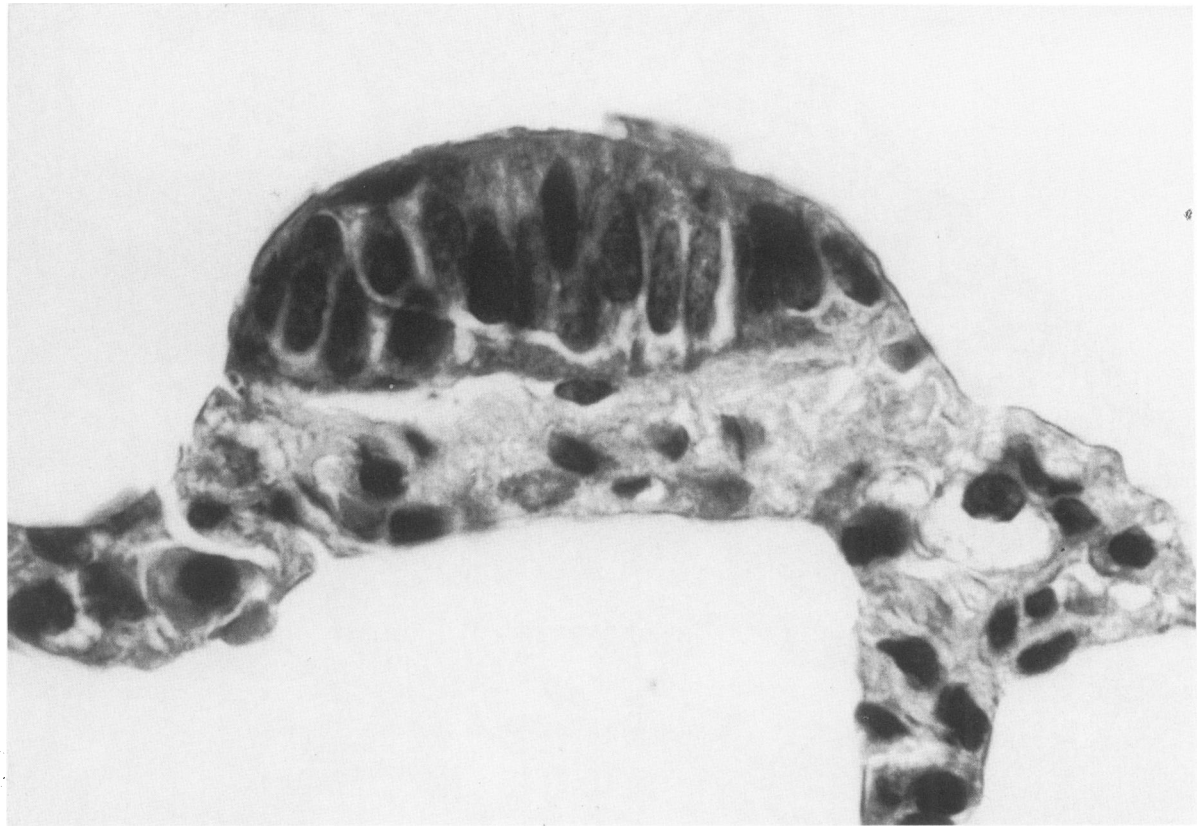

Figure 5. *An adjacent section of that of Figure 4 shows the tall columnar endocrine cells in the NEB (DEN-treated, H&E, ×1660).*

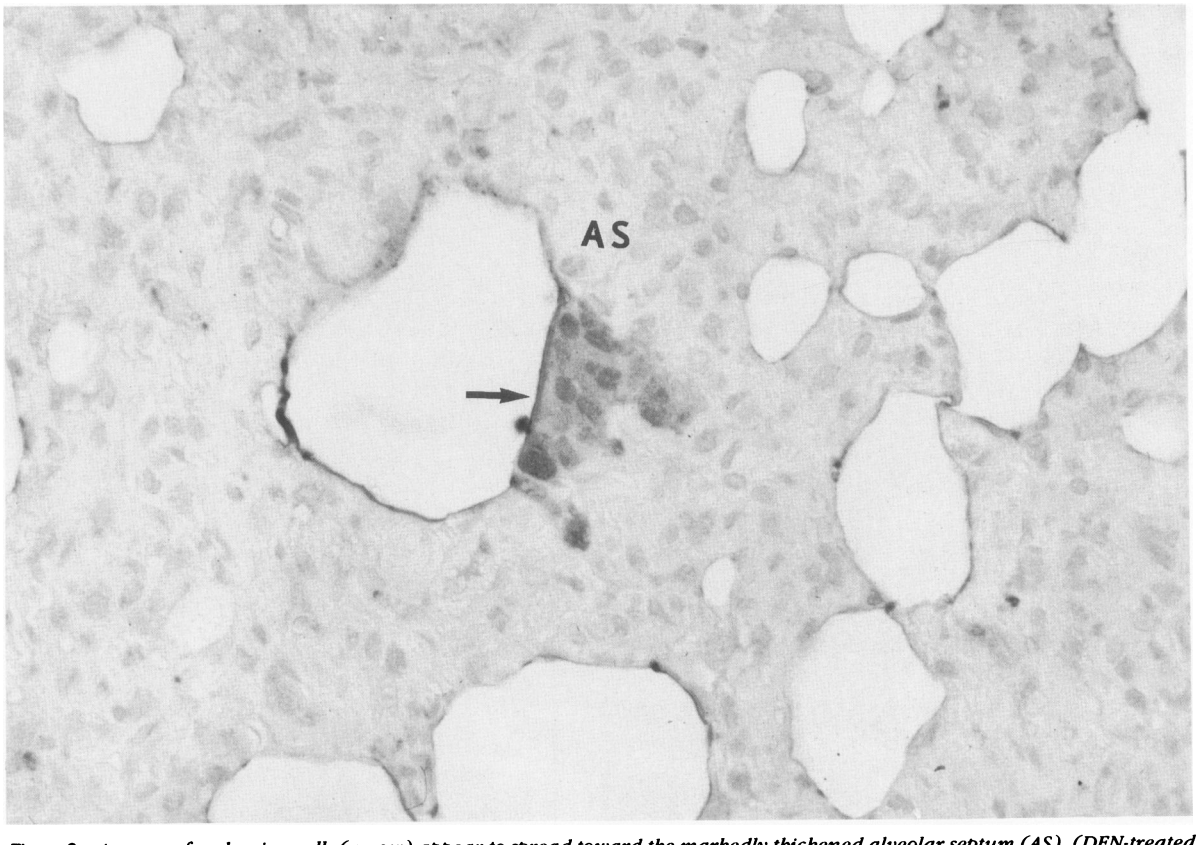

Figure 6. *A group of endocrine cells (arrow) appear to spread toward the markedly thickened alveolar septum (AS). (DEN-treated, 5-HT immunoreaction, ×660).*

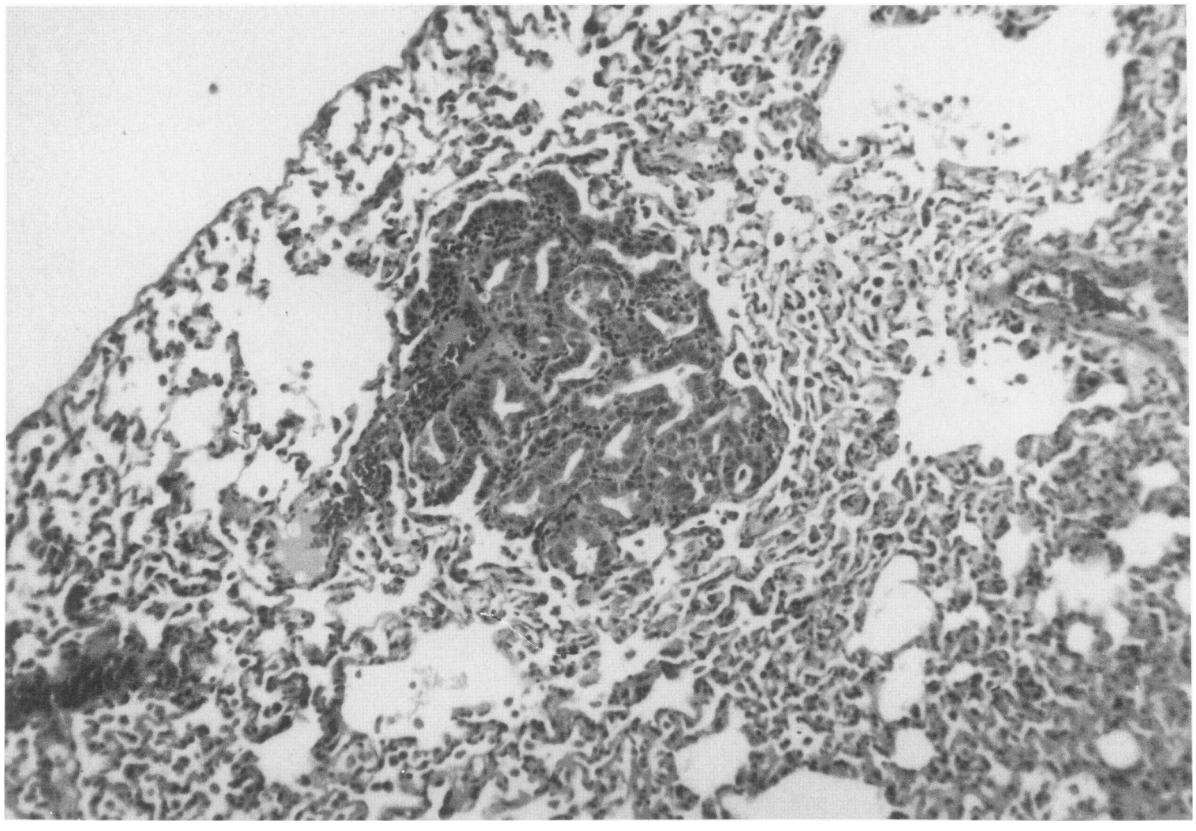

Figure 7. *Peripheral adenomatosis consisted of glandular structures lined by benign-looking simple epithelium. (DEN-treated, H&E, ×166).*

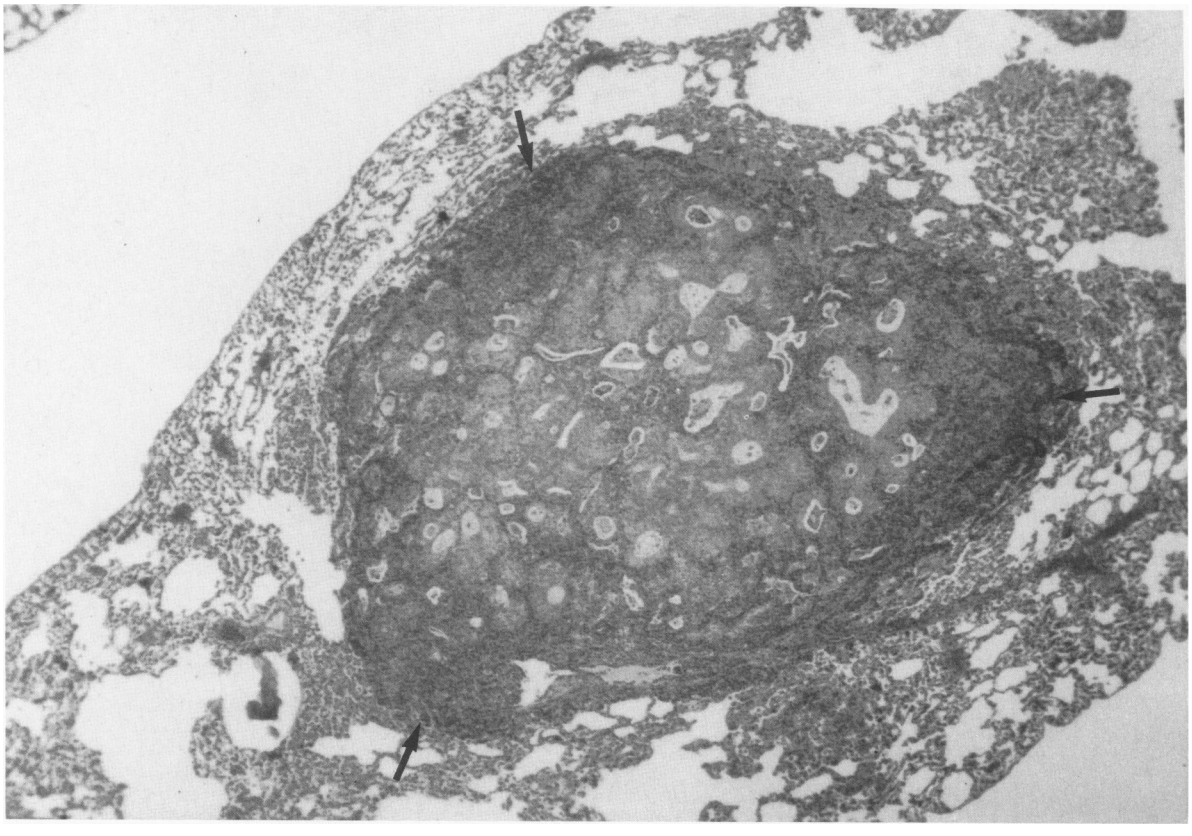

Figure 8. *Peripheral adenocarcinoma consisted of glandular structures surrounded by aggregates of lymphocytes (arrows). (DEN-treated, H&E, ×66).*

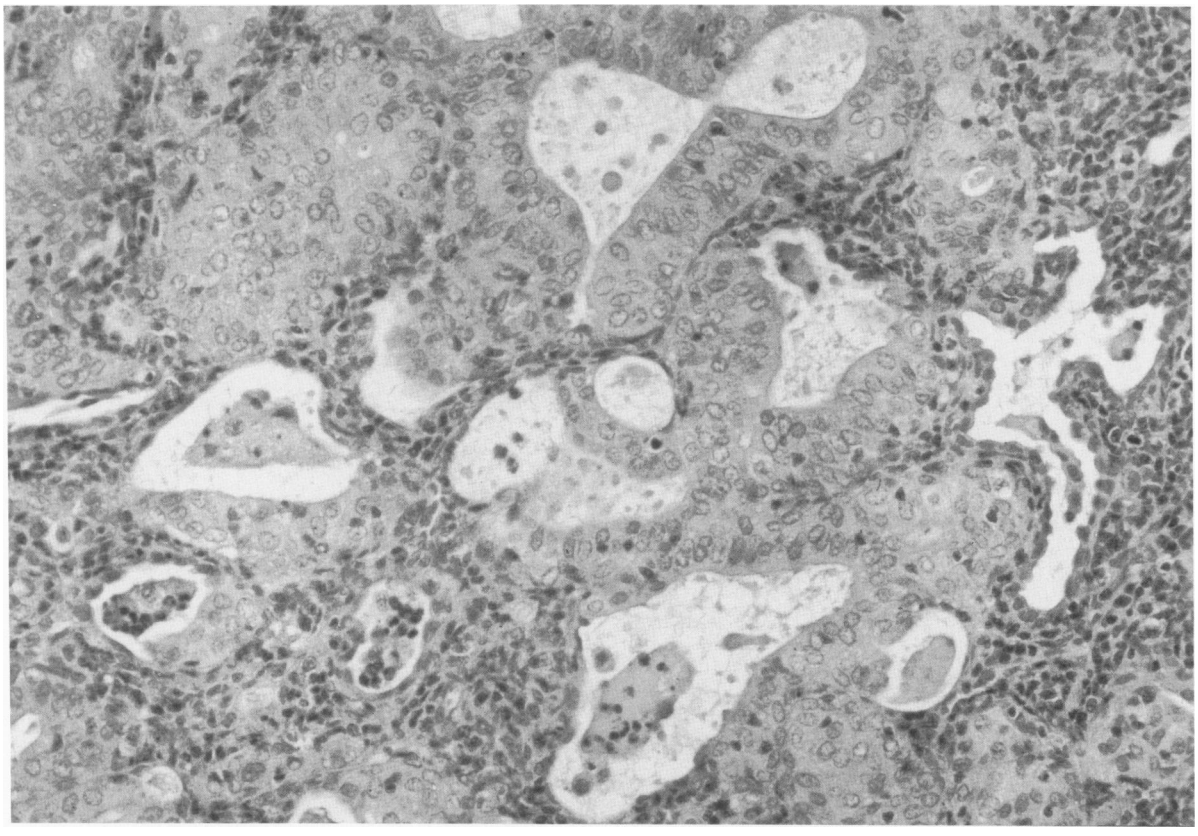

Figure 9. *Higher magnification of an area of Figure 8 showing the glandular nature of adenocarcinoma lined by stratified epithelium (DEN-treated, H&E, ×330).*

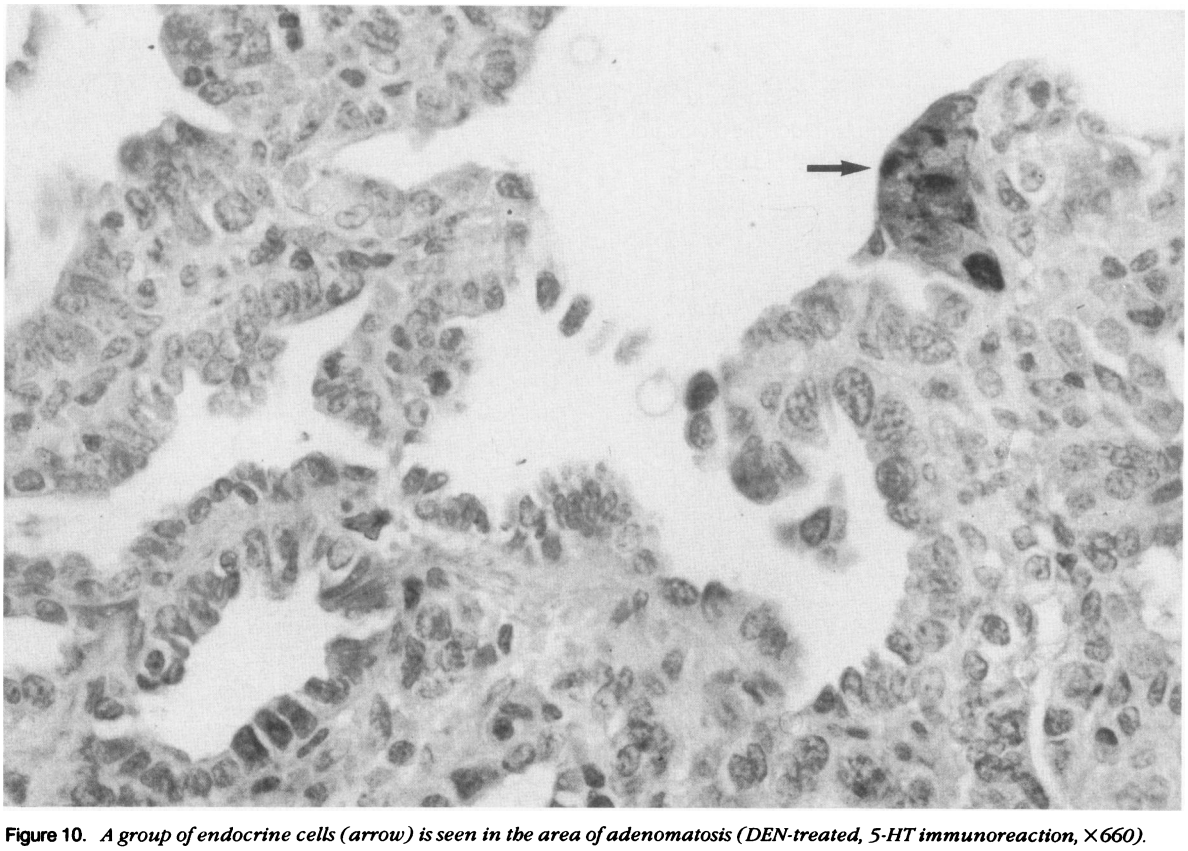

Figure 10. *A group of endocrine cells (arrow) is seen in the area of adenomatosis (DEN-treated, 5-HT immunoreaction, ×660).*

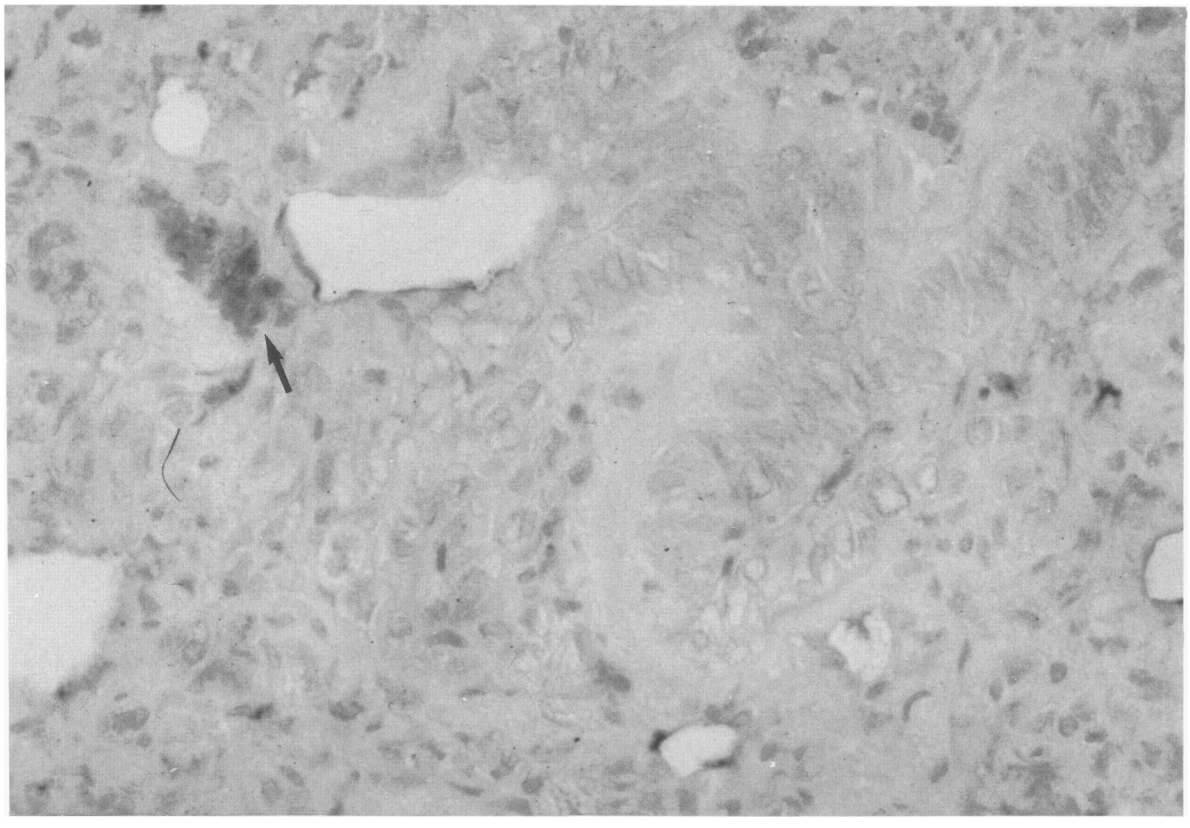

Figure 11. *A group of endocrine cells (arrow) is seen in the area of adenocarcinoma (DEN-treated, 5-HT immunoreaction, ×660).*

control rabbits, lesions of adenoma (1.13/cm^2) were common in DEN-injected rabbits, and two foci of adenocarcinoma (0.06/cm^2) were seen in DEN-injected rabbits.

Table 1. *Neuroepithelial Bodies (NEBs), Adenomas, and Adenocarcinomas Found in the Control and DEN-Injected Rabbits.*

	Controls (N = 3)	DEN (N = 3)
Number of NEBs/cm^2	0.08 ± 0.08	*4.37 ± 0.19
Number of cells/NEB	6.50 ± 1.50	*13.23 ± 1.78
Diameters of NEB (μm)	25.00 ± 5.00	*41.75 ± 1.28
Number of adenomas/cm^2	0	*1.13 ± 0.94
Number of adenocarcinoma/cm^2	0	0.06 ± 0.03
Relative number of NEBs in bronchi (%)	0	0.69
Relative number of NEBs in bronchioles (%)	0	2.84
Relative number of NEBs in alveolar region (%)	100	96.47

* indicates statistically significant difference between control and DEN groups ($P = 0.05$).

Means and standard errors are shown for all parameters except for the distribution for NEBs which is shown in percentages.

Discussion

Our study is the first to demonstrate that DEN can induce lung endocrine cell hyperplasia in the rabbit. In this species, hyperplastic lesions resembling enlarged NEBs occur mostly in the alveolar duct regions. In contrast, in the hamster endocrine cell hyperplasia was seen in proximal airways such as bronchioles and small bronchi,[12] or at various levels from lobar bronchus to the bronchiolo-alveolar junctional areas.[7,19] Our findings support the contention that selected carcinogenic nitroso-compounds such as DEN can cause proliferation of endocrine cells in the animal lungs. Although our present data indicate no evidence of atypical cells or dysplastic cells that might suggest a malignant change toward SCCL, further prolonged experiments may lead to the development of an ideal animal model for studying the histogenesis of SCCL.

The induction of adenoma and adenocarcinoma by nitrosamines has been reported in the past.[11,20,21] Adenocarcinoma developed in 42% of nitrosamine-treated hamsters, but endocrine cells appeared not to participate in the development of these adenocarcinomas.[12] However, we have noticed in the DEN-treated rabbits the occurrence of NEB or single endocrine cells in the vicinity of

adenomatosis and adenocarcinoma, which occupied much wider areas than that of the NEB. Previously, cells with dense core granules, which are usually considered endocrine in nature, have also been seen in areas with squamous differentiation in the hamster.[21] Although the significance of the coexistence of endocrine cells with pulmonary neoplastic lesions is not known, it is possible that the minute aggregates of endocrine cells in the periphery of adenomatosis or adenocarcinoma may develop into neuroendocrine carcinoma, resulting in a mixture of SCCL with squamous carcinoma and adenocarcinoma.[22]

Several investigators have postulated that pulmonary endocrine cells are the cells of origin for SCCL and bronchial carcinoid tumors.[2-5] The basis for this concept lies in the fact that the tumor cells contain dense core vesicles and other cytochemical properties similar to pulmonary endocrine cells. However, other investigators consider secretory cells[6] or Clara cells[7] to be the likely precursors for the endocrine cells. Previously we have shown that 1-week-old rabbits have relatively larger and more numerous NEBs than adult rabbits. These NEB are localized in the developing bronchi and bronchioles.[15] Because in the present studies DEN treatments began when the rabbits were 1 week old, it is possible that the size and number of NEBs were maintained by DEN. However, the fact that NEBs in the DEN-treated animals were mostly localized to the alveolar duct regions, rather than in the bronchial or bronchiolar locations seen in newborns, suggests that at least some of these NEBs are newly formed.

The effects of nitrosamines on the lung may be initiated by binding of nitrosamines and/or their metabolites to pulmonary epithelial cells, as demonstrated by autoradiography.[23] Because it is well known that some endocrine cells have the ability to take up, store, and metabolize simple amines and their precursors, it is reasonable to speculate that nitrosamines may act generally as endocrine cell proliferators in the lungs of many species, including humans.[11] This may explain how nitrosamines induce SCCL.[11]

Our results indicate that DEN can cause an increase in the number and size of NEB in the rabbit. In addition, the coexistence of pulmonary endocrine cells with adenomatosis and adenocarcinoma is unique and may be significant in the understanding of the development of SCCL and its association with other types of lung carcinoma. Further experiments in this species and in other animals should provide important models for studying the histogenesis of various forms of SCCL.

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Acknowledgment

The authors thank H. C. Anderson, M.D., for kind support and encouragement, and Deborah Borek, M.D., for editorial assistance. Excellent assistance in histologic techniques was provided by Rosetta Barkley and Airlean Bowls.