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Lung endocrine-like cells in hamsters treated with diethylnitrosamine: Alterations *in vivo* and in cell culture

(neuroepithelial bodies/carcinogen/small dense-cored vesicles/corticotropin immunoreactivity)

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ABSTRACT **Diethylnitrosamine is known to cause squamous cell carcinoma and adenocarcinoma of the lung in Syrian golden hamsters. Sections of lungs obtained from hamsters treated with the systemic carcinogen diethylnitrosamine showed a significant increase in the number of argyrophilic cells of neuroepithelial bodies. The hyperplastic response was retained at least 4 weeks after cessation of treatment. To examine whether these affected cells exhibited enhanced survival *in vitro*, lung cells were dissociated with Pronase and grown in culture. After 7 days, argyrophilia, dense-cored vesicles, and corticotropin-like immunoreactivity were observed in many of the cells derived from hamsters treated for 5 or 8 weeks. These findings suggest that the endocrine-like cells of neuroepithelial bodies are affected by diethylnitrosamine as evidenced by a numerical increase *in vivo* and by the properties exhibited by cells *in vitro*. The relationship of this diethylnitrosamine-induced reaction to bronchial carcinoid tumors or small-cell carcinoma of the lung remains to be established.**

There are experimental models for most human lung tumors but none for small-cell carcinoma or bronchial carcinoid tumors (1, 2). This is discordant with the fact that small-cell carcinoma constitutes 17–29% of human lung tumors (3). The cytoplasmic dense-cored vesicles observed in small-cell carcinomas and in bronchial carcinoid tumors have caused some investigators to postulate that these tumors originate from lung endocrine-like (Kultschitzky) cells (4–9). In addition, many patients who have these tumors develop "ectopic hormone syndromes," especially the corticotropin (ACTH) syndrome (10).

The finding that selected carcinogenic nitroso compounds such as diethylnitrosamine (Et_2NNO) can cause "proliferation" of endocrine-like cells in the hamster lung (11–13) may provide a model for studying the histogenesis of these tumors. Epithelial cells affected by carcinogen treatment of animals are known to have a greater proliferative capacity *in vitro* than cells of normal epithelium (14, 15). Therefore, we cultured cells dissociated from the lungs of Et_2NNO-treated hamsters in an attempt to obtain populations of endocrine-like cells. The observations *in vitro* were correlated with sequential histopathological changes occurring in the lung epithelium. We also examined the cell cultures and lungs of Et_2NNO-treated animals for the immunohistochemical localization of ACTH and growth hormone, reported to be synthesized by cultured cells of a lung carcinoma (16).

MATERIALS AND METHODS

Et_2NNO Treatment. Male Syrian golden hamsters (4–6 months old; Schmidt) were housed four per cage with free access to water and food (NIH 31 diet). Twice a week, 3 mg of Et_2NNO (Eastman) per hamster was injected subcutaneously. Hamsters, four per group, were treated for 5, 8, and 12 weeks with Et_2NNO for cell culture experiments. After 8 weeks of treatment, groups of four hamsters were allowed to recover for 4 and 8 weeks. Histochemical and immunocytochemical studies were performed on lung sections from hamsters, 6–8 per group, after 4, 8, 12, and 16 weeks of exposure to Et_2NNO. After the 8-, 12-, and 16-week treatment periods, groups of four hamsters were allowed to recover for 4 weeks. Control hamsters, two or three per group, received injections of saline and were sacrificed simultaneously with each group.

Dissociation and Lung Cell Culture. Animals were anesthetized with methoxyflurane, the abdominal aorta was cut, and lungs were perfused through the pulmonary artery with Hepes/balanced salt solution (GIBCO) until all lobes were blanched. A polypropylene cannula was inserted into the trachea, and the lungs were dissected free. A solution of 0.1% Pronase P (Sigma) in Hepes/balanced salt solution was slowly infused (3.1 ml/hr) via the tracheal cannula through the lungs at 4°C for 11 hr by using a Sage pump. The lungs were then inflated with 6 ml of fresh Pronase P solution and left for 15 min at 37°C. The peripheral edge of each lobe was cut open, 100 ml of Hepes/balanced salt solution was infused into the cannula, and the effluent containing dissociated cells was collected. Lungs were minced and the pieces were stirred in 100 ml of Hepes/balanced salt solution/Ham's F-12 medium (GIBCO), 1:4 (vol/vol), at 4°C for 15 min. This suspension was filtered through two layers of cheesecloth and combined with the previously dissociated cells. The cells were centrifuged in the presence of 0.5% bovine serum albumin (Pentex, Kankakee, IL) and washed with Ham's F-12 medium, and viability was determined with trypan blue. After resuspending the cells in Ham's F-12 medium/10% fetal calf serum supplemented with penicillin at 30 mg/liter and streptomycin at 50 mg/liter, 7×10^5 viable epithelial cells were plated per 60-mm Falcon dish and incubated at 37°C in humidified 5% CO_2/95% air. The medium was changed after 24 hr and replaced after 48 hr with L-15 medium (GIBCO) containing the same supplements. Seven days after plating, cells were fixed for histological and ultrastructural examination.

Histological Techniques. Animals were anesthetized with 100 mg of Nembutal. After opening the thoracic cavity, fixative was infused for 1 hr into the tracheal cannula at a hydrostatic pressure of 20 cm. Whole lungs were then immersed in the

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Abbreviations: ACTH, corticotropin; Et_2NNO, diethylnitrosamine; FIF, formaldehyde-induced fluorescence; NEB, neuroepithelial body; APUD, amine precursor uptake and decarboxylation.

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same fixative for 24 hr and processed for 6-μm paraffin sections or ultrathin Epon sections. Fixed pellets of dissociated cells or cell cultures were also processed for light or electron microscopy. Bouin's fluid or 10% buffered formalin was used as a fixative for histochemical procedures and glutaraldehyde/formaldehyde (17) was used for ultrastructural studies.

Formaldehyde-Induced Fluorescence (FIF). Two to four hamsters at each time point received pargyline (100 mg/kg intraperitoneally), and after 1 hr, 5-hydroxytryptophan (100 mg/kg; Sigma) was injected. The animals were killed 1 hr later with Nembutal. The technique of Falck *et al.* (18) as modified by Eränkö (19) was used to demonstrate catecholamines by FIF.

Silver Staining. Grimelius's method (20) for argyrophilia, which requires an external reducing agent, and the modified Masson–Hamperl's stain (21) for argentaffinity were applied to deparaffinized sections or selected ultrathin Epon sections. Argentaffine cells contain chemical substance(s) that can reduce ammoniacal silver to metallic silver.

Immunocytochemistry. Specific antisera (A6 and A7) to the midregion of ACTH were obtained in rabbits after intermittent subcutaneous injections of synthetic ACTH 1-24 (Organon) coupled with carbodimide to bovine serum albumin. An antiserum to ACTH was also obtained from the National Pituitary Agency. Antiserum to bovine growth hormone was a gift from Peter Petrusz (Department of Anatomy, University of North Carolina, Chapel Hill). Primary antisera diluted 1:1000–1:2000 gave satisfactory staining on formalin-fixed or FIF sections for light microscopy (22).

RESULTS

Lung Endocrine-Like Cells. Throughout normal intrapulmonary airways, small clusters of cells known as neuroepithelial bodies (NEBs) (23) and occasional solitary cells were demonstrated by argyrophilia of FIF. We refer collectively to these as endocrine-like epithelial cells. Only solitary cells with these properties were observed in the tracheal epithelium. There was good correlation between the intensity of FIF and argyrophilia—i.e., cells with bright FIF also demonstrated strong argyrophilia (Fig. 1) The degree of argyrophilia, however, frequently varied among NEBs. Often a green fluorescent or silver-positive nerve fiber was observed beneath the basement membrane in the proximity of the endocrine-like cells. We could not demonstrate argentaffinity or immunostaining with antisera to ACTH or growth hormone in either the pulmonary epithelium or in selected NEBs previously identified in the same sections by FIF.

Effects of Et_2NNO Treatment. Paraffin sections from control animals showed 2–30 argyrophilic or fluorescent cells per NEB (6.9 ± 0.3, mean ± SEM). In the course of treatment with Et_2NNO, there was a geometric increase in argyrophilic cells per NEB (Figs. 2 and 3*a*). A significant increase in the number of argyrophilic cells per NEB was observed in hamsters treated for 4 weeks; a maximal 2-fold increase was observed in hamsters treated for 16 weeks (14.0 ± 0.9 argyrophilic cells per NEB). This effect occurred throughout different branches of the intrapulmonary airways. We could not find significant differences ($P > 0.3$) between the animals that were killed immediately after 8, 12, or 16 weeks of Et_2NNO treatment and those that were allowed to recover for 4 weeks after the last injection. ACTH or growth hormone-like immunoreactivity was not detected in NEBs after exposure to Et_2NNO.

The number of argyrophilic NEBs per cm^2 of sections remained unchanged during the first 4 weeks of Et_2NNO treatment; however, at 8 or 12 weeks, there was a significant increase in these structures (Table 1; Fig. 3*b*). The hamsters that were allowed to recover for 4 weeks after 8 or 12 weeks of treatment essentially retained the number of NEBs per cm^2 of lung section observed prior to recovery. The number (1–3 per cm^2) of solitary argyrophilic cells in hamster lungs did not change after Et_2NNO treatment. The proportion of stem bronchi that contained one or more NEBs was doubled; the proportion of medium size and terminal airways with NEBs was increased 4-fold after 8 weeks of Et_2NNO injections. An accurate classification of the small airways was hindered by hyperplastic changes in the epithelium after longer periods of exposure.

In addition to the changes in NEBs, the epithelium of conducting airways began to demonstrate focal hyperplasia after 8 weeks of Et_2NNO treatment. After 12–16 weeks of treatment, the hyperplastic foci often contained squamous metaplasia. At the same time, papillomas were found in larger airways, including the trachea. These affected areas seldom contained argyrophilic cells (Fig. 3*c*).

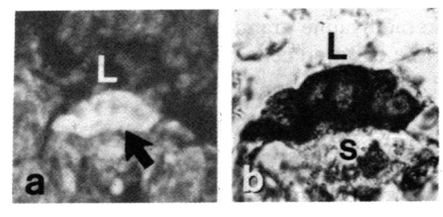

FIG. 1. Photomicrographs of a small NEB in a bronchiole of a control hamster. A paraffin section of lung which was fixed with paraformaldehyde vapor. L, lumen of a bronchiole. (*a*) Arrow indicates brightly fluorescing NEB (FIF). (×360.) (*b*) The same NEB after staining for argyrophilia. s, Submucosa. (×1000.)

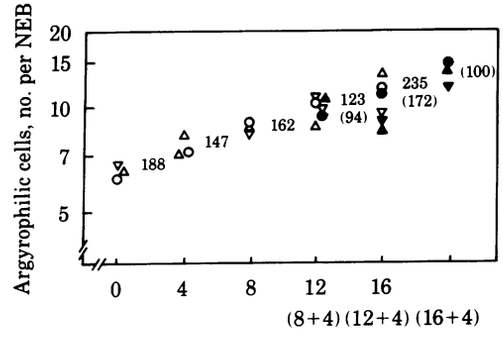

FIG. 2. Quantitation of argyrophilic cells of NEBs in various airways of Et_2NNO-treated hamsters. Deparaffinized sections of half lobes fixed in Bouin's fluid or hot paraformaldehyde vapor (10 sections per animal) were stained with silver. Argyrophilic cells per NEB were counted. An average of 36 NEBs per animal were examined. Data represent means from stem bronchi (△ and ▲), medium size bronchi (○ and ●), or preterminal and terminal bronchioles (▽ and ▼); △, ○, and ▽ represent values obtained immediately after Et_2NNO exposure, and ▲, ●, and ▼ represent values after recovery. Numbers represent total NEBs examined; those in parentheses represent NEBs examined after recovery. The number of argyrophilic cells per NEB exhibited increasing variance with increasing mean. Logarithmic transformation of the numbers achieved near homogeneity of variance. Statistical analysis was via an analysis of variance of the logarithms of the cell numbers in which between- and within-animal components of variability were estimated; the former was larger than the latter ($P < 0.001$) by a factor of ≈2. Other components of variability for bronchial type and exposure were computed and judged for statistical significance against the relevant components of within- and between-animal variability. Differences between airways were extremely small, albeit statistically significant ($P < 0.025$). The logarithm of argyrophilic cells per NEB was essentially linear in relationship to exposure ($P > 0.4$ for departure from linearity); the slope for this response was positive ($P < 0.0001$). A statistically significant elevation in response over control was detected as early as 4 weeks ($P < 0.05$).

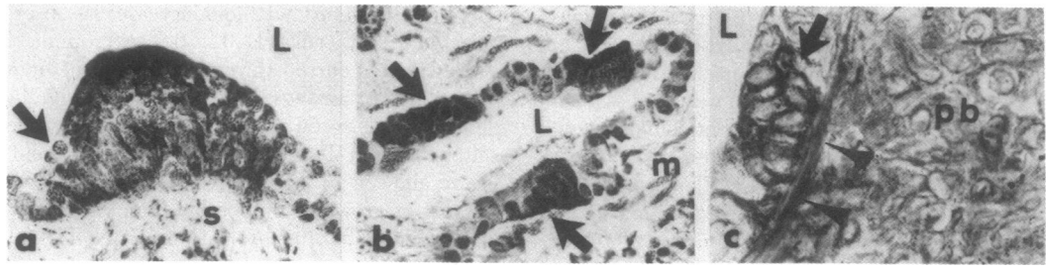

FIG. 3. Photomicrographs of NEBs in airways of Et_2NNO-treated hamsters. Paraffin sections of lungs were stained for argyrophilia. L, lumen of an airway. (*a*) Large NEB of a hamster treated with Et_2NNO for 16 weeks; paraformaldehyde vapor fixation. Arrow indicates nonargyrophilic cells closely associated with dark argyrophilic cells of the NEB. s, Submucosa. (×240.) (*b*) Medium-sized bronchus of a hamster treated with Et_2NNO for 12 weeks; paraformaldehyde vapor fixation. Arrows indicate NEBs with dark argyrophilic cells in the epithelium. m, Muscle layer. (×240.) (*c*) NEB (arrow) of a hamster treated with Et_2NNO for 12 weeks; Bouin's fixation. Airway is surrounded by peribronchiolar adenomatous growth (pb) of parenchyma. Arrows indicate basement membrane that was preserved. Note that there are no argyrophilic cells in pb area. (×640.)

Confirming previous reports (24), the major ultrastructural features in component cells of NEBs were cytoplasmic dense-cored vesicles with a diameter of 160 ± 6 nm (mean ± SEM; $n = 102$) and abundant free ribosomes. In addition to cells with small dense-cored vesicles, cells with prominent bundles of tonofilaments were also observed in NEBs after 12 weeks of exposure; we did not observe dense-cored vesicles in these cells.

Dissociated Cells. An estimated 7.3×10^6 viable nucleated cells were obtained from each lung by digestion with Pronase. Ciliated, mucus-producing or globlet, and type 2 cells, as well as macrophages, were identified by electron microscopy among dissociated cells. Of the total yield, 75–87% consisted of erythrocytes; 4–7% of the nucleated cells were type 2 cells. This is in agreement with previous studies on the proteolytic separation lung cells (25). In both control animals and those treated with Et_2NNO, 2–7% of the nucleated cells were argyrophilic, with macrophages accounting for at least one-half the cells that gave a positive reaction. Cells with small dense-cored vesicles, identified by electron microscopy, comprised 0.3–1.0% of the nucleated cells. Approximately 0.5% of the nucleated cells derived from hamsters treated for 8 weeks demonstrated immunoreactivity with antisera to ACTH; samples from other hamsters, treated or control, were negative.

Table 1. Quantitation of NEBs and their component cells in lungs of Et_2NNO-treated hamsters

Treatment period, weeks	NEBs per cm^2*	Argyrophilic cells per cm^2†
	No recovery period	
0 (control)	0.4 ± 0.1 (6)	100
4	0.3 ± 0.1 (4)	120
8	1.4 ± 0.1 (4)‡	590
12	1.0 ± 0.2 (4)‡	420
	Plus 4 weeks recovery	
8	1.3 ± 0.1 (4)‡	620
12	1.0 ± 0.1 (4)‡	440

* NEBs consisting of two or more argyrophilic cells were counted on sections of half lobes prepared after fixation with Bouin's fluid under controlled hydrostatic pressure. All lobes were examined (10 sections per animal), and the area of sections was determined with a planimeter on the image obtained with a Zeiss Ultraphot microscope. On average, 19 cm^2 per animal was examined. Data are mean ± SEM; numbers of animals are given in parentheses. The overall significance of differences among the groups was tested with the Krushal–Wallis one-way analysis of variance ($P < 0.001$). The differences between the individual means over the control group were tested with the Mann–Whitney *U* test.

† The total number of argyrophilic cells per cm^2 was determined by multiplying the mean number of NEBs per cm^2 by the mean number of argyrophilic cells per NEB. Values are expressed as percent of control.

‡ $P < 0.005$ by the one-tailed test.

Cell Culture. Small round cell colonies were observed by phase-contrast microscopy 1 day after plating the dissociated lung cells from treated or control animals. After 7 days, cell cultures were not confluent, but larger epithelial-like colonies were observed that were surrounded by fibroblasts. At that time, ≈40% of the cells in culture from animals treated with Et_2NNO for 5 weeks appeared argyrophilic with Grimelius's silver stain (Table 2). These cells also demonstrated argentaffinity. Sixteen percent of the cells obtained after 5 weeks of Et_2NNO treatment had ACTH-like immunoreactivity (Fig. 4); this percentage decreased with cells from animals treated for longer periods. Serial sections of the cells showed no immunostaining when incubated with antigen-inactivated (150 μg of hormone per diluted antiserum) or nonimmune serum. We rarely observed cells in culture with ACTH-like immunoreactivity in specimens from animals allowed to recover after 8 weeks of exposure (Table 2). Growth hormone-like immunoreactivity was not detected in cells in culture. Samples from unexposed animals did not show immunoreactivity for either hormone.

Electron microscopy of cultured cells from Et_2NNO-treated animals showed many cells with small dense-cored vesicles and other organelles (Fig. 5) that resembled the component cells of NEBs in airways. These cells were most numerous (≈30%) in cultures from animals treated with Et_2NNO for 5 weeks (Table 2).† They contained either small dense-cored vesicles with large pale halos [diameter 400 ± 15 nm (mean ± SEM), $n = 100$] or vesicles of a smaller diameter (240 ± 10 nm, $n = 100$) with a less-electron-dense core. Cells with small vesicles (diameter 180 ± 5 nm, $n = 100$) having moderately dense cores were also found after 8–12 weeks of exposure. Large highly polymorphic dense bodies (700- to 1500-nm diameter) were also frequently observed. In the cultures from control animals 0–7% of cells contained small dense-cored vesicles. Cells with numerous electron-lucent vesicles and abundant smooth endoplasmic reticulum were occasionally observed in cultures from treated or control animals. After 8 or 12 weeks of exposure, we also observed an increasing number, 8% and 23%, respectively, of squamous (epidermoid) cells with thick bundles of tonofilaments or keratohyaline granules. These cells constituted only 0–0.5% of the cultured cells from control animals.

DISCUSSION

Rare endocrine-like cells in hamster lungs were identified by the following properties: (*i*) the presence of small cytoplasmic

† Different fixatives were required for optimal demonstration of ACTH, argyrophilia, and small dense-cored vesicles in cells in culture. This precluded showing coincidental expression of these characteristics in the same cell, as might have been accomplished with serial sections. However, the small dense-cored vesicles in ultrathin sections of the cells were argentaffine as viewed by electron microscopy.

Table 2. Quantitation of endocrine-like cells after 7 days in culture, in comparison with dissociated cells, obtained from lungs of hamsters treated with Et_2NNO

Treatment period, weeks	Argyrophilic cells*	Cells with dense-cored vesicles†	Cells with ACTH-like immunoreactivity‡
	No recovery period		
0 (control)	ND	6	ND
5	40	30	16
8	20	18	9
12	ND	24	4
	Plus recovery period		
8§	ND	16	0.3
8¶	ND	9	ND
	Dissociated cells		
All time points	4	0.6	ND‖

Values are expressed as % of nucleated cells and were obtained by examining cultures from three separate trials. Less than 20% variation was observed between trials. ND, not detected.

* Deparaffinized sections of cell pellets fixed in Bouin's fluid were stained (20), and 500 nucleated cells were examined (×250) for argyrophilia.

† Ultrathin sections of cells fixed with 2.6% glutaraldehyde/2% formaldehyde (18) were examined with an electron microscope, and 200 cells with small dense-cored vesicles were counted.

‡ Deparaffinized sections of formalin-fixed cells were stained by the immunoperoxidase-bridge method (22) using specific antisera to ACTH, and immunopositive cells were counted as described above.

§ Recovery period = 4 weeks.

¶ Recovery period = 8 weeks.

‖ Some positive cells (0.5%) were found at 8 weeks of treatment.

dense-cored vesicles; (*ii*) FIF of catecholamines after treatment with 5-hydroxytryptophan, a serotonin precursor; (*iii*) argyrophilia (or argentaffinity); and (*iv*) the presence of a polypeptide hormone. Pearse has postulated that nearly all endocrine cells have these properties and can be classified as members of an amine precursor uptake and decarboxylation (APUD) endocrine-cell system (26, 27). Argyrophilia itself by Grimelius's method is not dependent on the amine content (28), nor does the reaction seem to depend on the occurrence of polypeptide hormones. Despite the uncertainty of the substance(s) responsible for the reaction, the empirically developed argyrophilic staining is useful for identifying endocrine cells with small dense-cored vesicles (29). Similar to other species, including man, adult hamsters have two principal types of APUD cells in their lungs—i.e., solitary endocrine-like cells and NEB cells (2, 24, 30–37).

Our findings confirm the report of Reznik-Schüller (11) that Et_2NNO can cause an increase in lung endocrine-like cells. In her study, however, only electron microscopy was used and a systematic quantitation of endocrine-like cells was not performed. This probably accounts for the fact that she did not

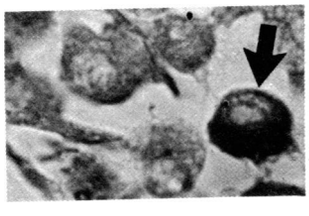

FIG. 4. Photomicrograph of a cultured cell with ACTH-like immunoreactivity. Cells were obtained by Pronase treatment of airways of a hamster treated with Et_2NNO for 5 weeks and cultured for 7 days. Cells were fixed, pelleted, and immunostained with antiserum against ACTH (A-6). Arrow indicates a cell with dark cytoplasmic precipitate; immunoperoxidase-bridge method. The nucleus did not stain. (×900.)

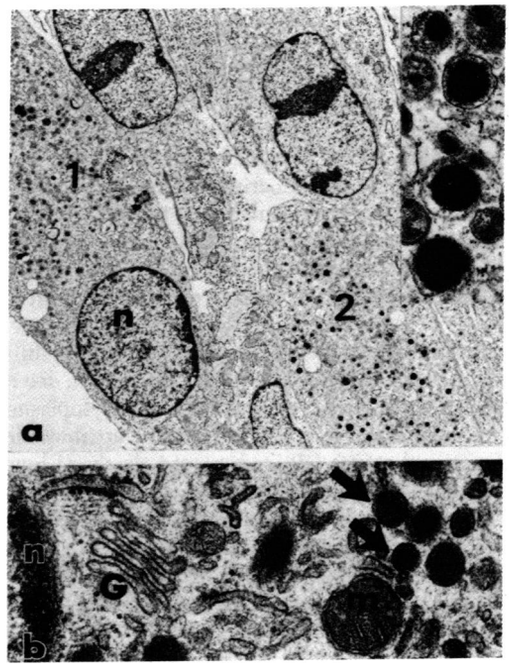

FIG. 5. Electron micrographs of cultured cells. Cells were obtained by Pronase treatment of airways of a hamster treated with Et_2NNO for 5 weeks and cultured for 7 days. n, Nucleus. (*a*) Two cells (1 and 2) with small dense-cored vesicles. (×2600.) (*Inset*) Details of the cytoplasmic dense-cored vesicles in cell 2. (×17,000.) (*b*) Typical organelles in a cultured cell that contains numerous small dense-cored vesicles (arrows). Note also a piece of a rough endoplasmic reticulum in the upper left hand corner close to the nucleus and some smooth endoplasmic reticulum in the center. G, Golgi apparatus; m, mitochondria. (×34,000.)

detect endocrine-like cells—i.e., cells with small dense cored vesicles—in the bronchial epithelium of normal hamsters. Our studies show that Et_2NNO increases the number of NEBs per cm^2 and the number of cells per NEB. It should be noted that hyperplasia in NEBs would enhance the probability of the detection of these structures. Thus, the observed increase in number of NEBs per cm^2 of lung section in treated animals may not represent an absolute increase of NEBs per lung. The number of argyrophilic cells in NEBs appeared to be dependent on the cumulative dose of Et_2NNO; when animals were allowed to recover, the number of such cells remained constant between the time of the last treatment and the time of sacrifice, 4 weeks later. Since component cells of NEBs neither take up [^{3}H]thymidine (24 hr after injection) nor undergo mitosis (38), it is probable that these cells originate from a separate stem cell population. Possibly nonciliated epithelial cells adjacent to the NEBs (Clara cells?) proliferate and differentiate to form hyperplastic NEBs as an early response to Et_2NNO.

The short-term cultures from the lungs of Et_2NA-treated hamsters yielded cells histochemically and ultrastructurally similar to the endocrine-like cells of hamster lungs observed *in vivo*. It is likely that the enlarged NEBs were dissociated by the Pronase treatment and served as the source of the endocrine-like cells *in vitro*. The relatively large proportion of endocrine-like cells in tissue culture from animals exposed for 5 weeks suggests either selective survival or selective proliferation of these cells *in vitro*. Expression of ACTH-like immunoreactivity occurred *in vitro* in cells from Et_2NNO-treated animals but was not observed *in vivo*. Culture conditions may favor the synthesis or intracellular accumulation of ACTH or may select for endocrine-like cells capable of synthesizing the hormone. This may explain why immunoreactivity to ACTH was observed *in*

vitro but not *in vivo* in the NEB cells of Et_2NNO-treated hamsters. It is unlikely that the culture medium would have served as a source for the hormone; ACTH-like immunoreactivity in fetal calf serum was not detected by a sensitive radioimmunoassay. Based on the temporal relationship between the appearance of cells with ACTH-like immunoreactivity in culture and the first increase of endocrine-like cells *in vivo* following injection of Et_2NNO, we speculate that the cultured hormone-containing cells are derived from the cells responding to Et_2NNO *in vivo*. Likewise, the appearance of cells with epidermoid characteristics in cultures established from hamster lungs after 8 or more weeks of Et_2NNO treatment may reflect the squamous metaplastic changes known to occur in the airways of hamsters after such treatment (for review, see ref. 39).

The variations in ultrastructural features of cytoplasmic granules in endocrine-like cells *in vitro* may be attributable to different functional stages (40) or separate classes of lung endocrine-like cells (41). Similar heterogeneity is also characteristic of cells in bronchial carcinoid tumors (42). A positive argentaffine reaction by the cultured endocrine-like cells is typical of classical carcinoid tumors but was not observed in endocrine-like cells of the hamster lung. This may reflect a change in the composition of small dense-cored vesicles in the endocrine-like cells and may correlate with the appearance of ACTH-like immunoreactivity. Also, an earlier report showed that the lysozome-like structures (large electron-dense bodies) of bronchial carcinoid tumors become more prominent when this tissue is maintained in culture (43), as was observed in the present study for the endocrine-like cells *in vitro*. Although various histological types of lung tumors have been produced experimentally with Et_2NNO (1, 44), lung endocrine-like cells or their progenitors may be particularly sensitive to its carcinogenic effects (45). Taken together, the hyperplastic and progressive changes observed in NEBs in the present study may recapitulate the histopathological events occurring in the formation of peripheral pulmonary carcinoids (46) and bronchial carcinoid tumors and the progression to less differentiated lung carcinomas, such as small-cell carcinomas, which exhibit few cells with APUD characteristics. However, the relationship between the cellular alterations of the NEB cell population and tumor induction with Et_2NNO in various segments of the airways of hamsters is as yet uncertain.

The recovery of endocrine-like cells *in vitro*, apparently derived from endocrine populations of the lung, should be helpful toward identifying any bioactive peptides and, ultimately, in determining the physiological functions of these cells.

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