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Retinoid Reversal of Squamous Metaplasia in Organ Cultures of Tracheas Derived from Hamsters Fed on Vitamin A-deficient Diet*

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Abstract—*Cytokinetic and ultrastructural studies were carried out to elucidate mechanisms involved in the reversal of squamous metaplasia (SM) by β-retinoic acid in organ cultures of tracheas derived from vitamin A-deficient hamsters. Tracheal cultures exhibiting focal areas of SM were treated with the retinoid for up to 7 days. The retinoid significantly inhibited [^{3}H]-thymidine labeling indices in the basal cells and stimulated the labeling indices in mucous cells. At the ultrastructural level the retinoid induced marked remodeling alterations in the metaplastic epithelium that included: (a) disruption of desmosomes and widening of intercellular spaces; (b) extensive vacuolation and degeneration of the metaplastic cells; (c) extrusion of the degenerated cells; (d) aggregation of keratin filaments; and (e) differentiation of certain basal cells into secretory cells. Consequently most degenerated metaplastic cells were extruded and the epithelium repopulated as a result of differentiation of basal cells into mucous cells and hyperplasia of the pre-existing mucous cells. The degenerative effects of the retinoid were limited to the metaplastic foci since the uninvolved epithelium adjoining metaplastic foci were not significantly altered. The results suggest that the restoration of normal tracheal epithelium following the retinoid treatment of explants exhibiting focal areas of squamous metaplasia is associated with the enhanced proliferation of the mucous cells. The inhibition of proliferation of basal cells further prevented hyperplasia and restored cell replication within the normal range.*

INTRODUCTION

THE REQUIREMENT of vitamin A in the regulation of normal growth and differentiation in epithelial tissues is well known. Under both *in vivo* [1–4] and *in vitro* [5, 6] vitamin A-deficient conditions epithelia of respiratory and urogenital tracts undergo squamous keratinizing metaplasia. Metaplasia in the respiratory tract epithelium involves stimulation of proliferation of basal cells and their subsequent transformation into squamous keratinizing cells instead of into mucous and ciliated cells [3, 7]. Other evidence indicates that retinoids also inhibit carcinogenesis in a number of experimental tumor models such as the respiratory tract [8, 9], mammary epithelium [10] and urinary bladder [11]. Vitamin A inhibited benzo(a)pyrene (BP)-induced lesions in the respiratory epithelium both *in vivo* [8] and *in vitro* [12]. Similarly, retinyl methyl ether, a synthetic retinoid, inhibited hyperplasia and squamous metaplasia in the organ culture of hamster trachea exposed to the amphibole types of asbestos, crocidolite and amosite [13].

Squamous metaplastic changes induced in the respiratory tract epithelium by carcinogens are histologically similar to that induced by vitamin A deficiency but they exhibit significant differences at the ultrastructural level. For instance, defects in the basement membrane, enlarged nuclei with cytoplasmic invaginations and pleomorphic nuclei occurred in lesions induced by BP [4]. Furthermore, the origin of lesions

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induced by the two manifestations may be different. The lesions induced by vitamin A deficiency reportedly originate in the basal cells while those induced by BP involve both the basal and mucous cells [4, 14].

The mechanism of action of vitamin A in the maintenance of normal differentiation is not completely understood. Further, its role against tumorigenesis is complicated by the variability in modes of tumor induction and tumor growth and the variable nature of carcinogenic insults. However, in respiratory carcinogenesis the most common preneoplastic response is characterized by squamous cell transformation. Studies in this laboratory are directed to elucidate cytokinetic and ultrastructural alterations associated with squamous metaplasia in the hamster trachea induced by various agents and its reversal by retinoids. Previously evidence has been presented to indicate that squamous metaplasia in organ culture of trachea derived from hamsters fed a vitamin A-deficient diet is caused by hyperplasia of the basal cells and subsequent differentiation of daughter cells into keratinizing cells. The surface layer containing the mucous and ciliated cells exfoliate as a result of population pressure from the underlying keratinizing cells [14]. In the present study cytokinetic and ultrastructural changes associated with the reversal by β-retinoic acid (RA) of squamous metaplasia in organ culture of trachea of hamsters fed a vitamin A-deficient diet were investigated. The results show that the RA inhibits basal cell proliferation, stimulates mucous cell proliferation and reverses squamous cell differentiation into mucous and ciliated cell differentiation, thereby restoring normal epithelial morphology. Following the retinoid treatment, focal areas of the metaplastic epithelium appear to undergo selective degeneration and are extruded out of the epithelium.

MATERIALS AND METHODS

Organ culture

The method for rearing hamsters fed a vitamin A-deficient diet has been previously described [14]. For tracheal organ culture hamsters weighing between 48 and 55 g (28–32 days old) were used. At this time most animals were gaining weight and showed no external sign of vitamin A deficiency. Animals were killed by i.p. injection of 0.5 ml of a 1% solution of Brevital Sodium (Eli Lilly & Co., Indianapolis, IN), their tracheas were excised aseptically and placed in serum-free cold L-15 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Grand Island Biological Co., Grand Island, NY). Each trachea was opened longitudinally along the membranous wall and cultured individually in 60-mm culture dishes, each containing 2 ml of CMRL 1066 medium (serum-free) supplemented with insulin (1 μg/ml, Sigma Chemical Co., St. Louis, MO), hydrocortisone hemisuccinate (0.1 μg/ml, Upjohn Co., Kalamazoo, Mich.), glutamine (2 mM) and antibiotics [6]. The cultures were maintained in a rocker chamber (Bellco Glass Co., Vineland, NJ) at 35–36°C in an atmosphere of 50% O_2 + 45% N_2 + 5% CO_2. The medium was changed three times every week.

Cell kinetic studies

Sporn *et al.* [15] have reported that when tracheas of hamsters fed on vitamin A-deficient diets are cultured in serum-free medium approximately 50% of the explants develop squamous metaplasia at 3 days after incubation. After 10 days approximately 90% of the explants exhibit lesions. In this study the retinoid treatment was started at 3 days after culture when approximately 50% of the explants exhibited focal areas of squamous metaplasia.

After three days of incubation in the control medium cultures were divided into 4 groups (5 cultures per group). One group was kept as the control and the remaining 3 groups were treated with RA at 10^{-8}, 10^{-9} and 10^{-10} M concentrations. The RA was dissolved in dimethylsulfoxide (DMSO) and added to the culture medium at the time of changing the medium. The control group received an equivalent amount of DMSO. Three days after treatment all cultures were terminated. Before termination cultures were pulse-labeled with tritiated thymidine ([^{3}H]-TdR, 2 μCi/ml, 6.7 Ci/mmol) for 1 hr. They were embedded in paraffin, sectioned at 1–2 μm thickness using a Porter-Blum MT-2B ultramicrotome and glass knives and processed for autoradiography. Prior to autoradiography tissue sections were stained by the Periodic Acid Schiff method to identify mucous cells [14]. The total number of basal and mucous cells and the number of labeled cells of each type were counted in 2–3 sections from each tracheal culture. The labeling index was expressed as per 1000 cells and the mean labeling index and standard deviation calculated for each group (LI ± S.D.). The significance of the difference between two groups was determined by Student's *t* test.

Ultrastructural studies

These studies were designed to elucidate the sequence of morphological events involved in the reversal of metaplastic lesions by RA. Three days after incubation in the control medium cultures were treated with 10^{-9} M RA. A control group received only DMSO. The 10^{-9} M RA concentra-

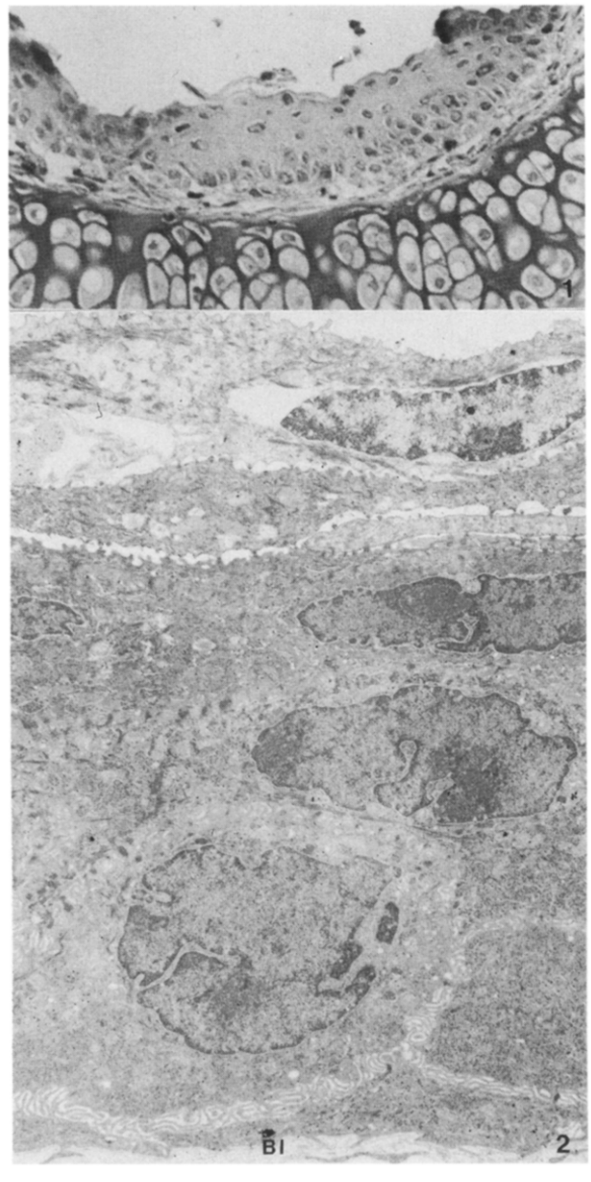


Fig. 1. Cornified epithelium. The pre-existing mucous and ciliated cells are defoliating ensuing keratinization. ×400.

Fig. 2. Electron micrograph of cornifying epithelium in a tracheal organ culture. Basal lamina (Bl). ×3840.

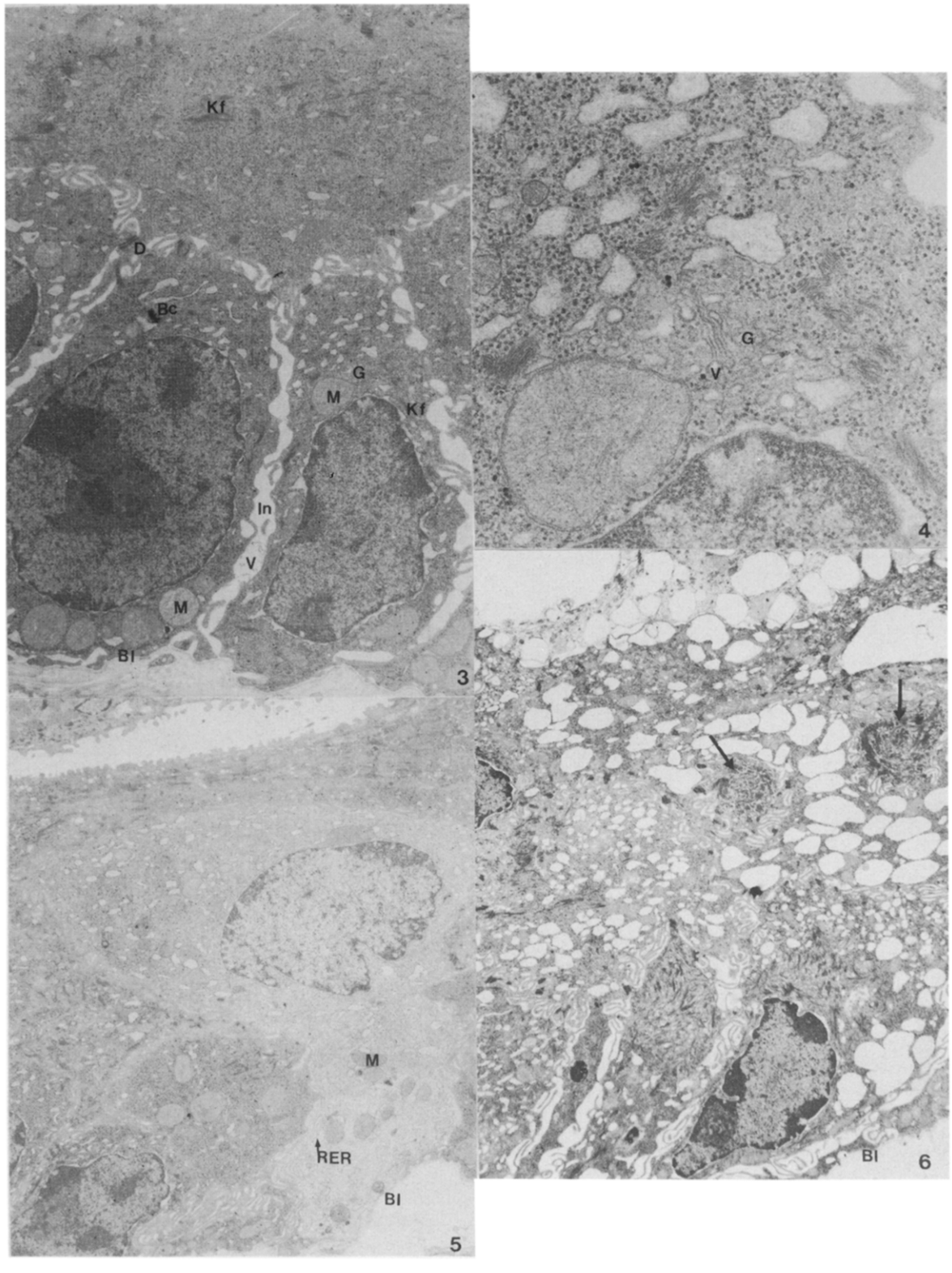
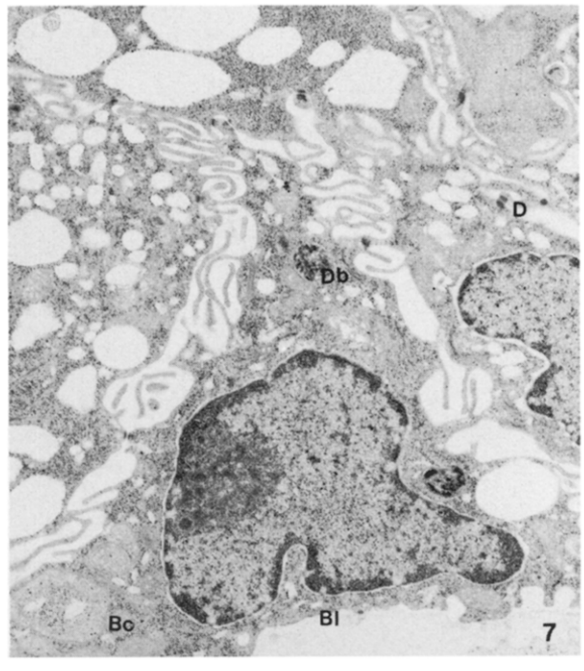


Fig. 3. Basal cell layer in squamous epithelium at 1 day *after treatment. Notice disruption of intercellular cytoplasmic processes, widening of intercellular spaces and intercellular vacuoles containing degenerating cytoplasmic processes (V), mitochondria (M), basal lamina (Bl), basal cell (Bc), intercellular space (In), Golgi complex (G), desmosome (D) and keratin filaments (Kf).* ×10,240.

Fig. 4. A basal cell (from Fig.3) apparently undergoing secretory cell differentiation as indicated by Golgi complexes (G) and secretory vesicles (V). ×41,600.

Fig. 5. A large cell extruding out of the basal cell layer. Notice the undifferentiated morphology of the cell. Basal lamina (Bl), mitochondria (M) and rough endoplasmic reticulum (RER). ×4160.

Fig. 6. Squamous metaplastic epithelium undergoing widespread degeneration (5 days *after treatment). Notice large vacuoles with no internal structure and aggregating whorls of keratin filaments (arrows). Most vacuoles are lined with strings of ribosomes. Basal lamina (Bl).* ×60,000.



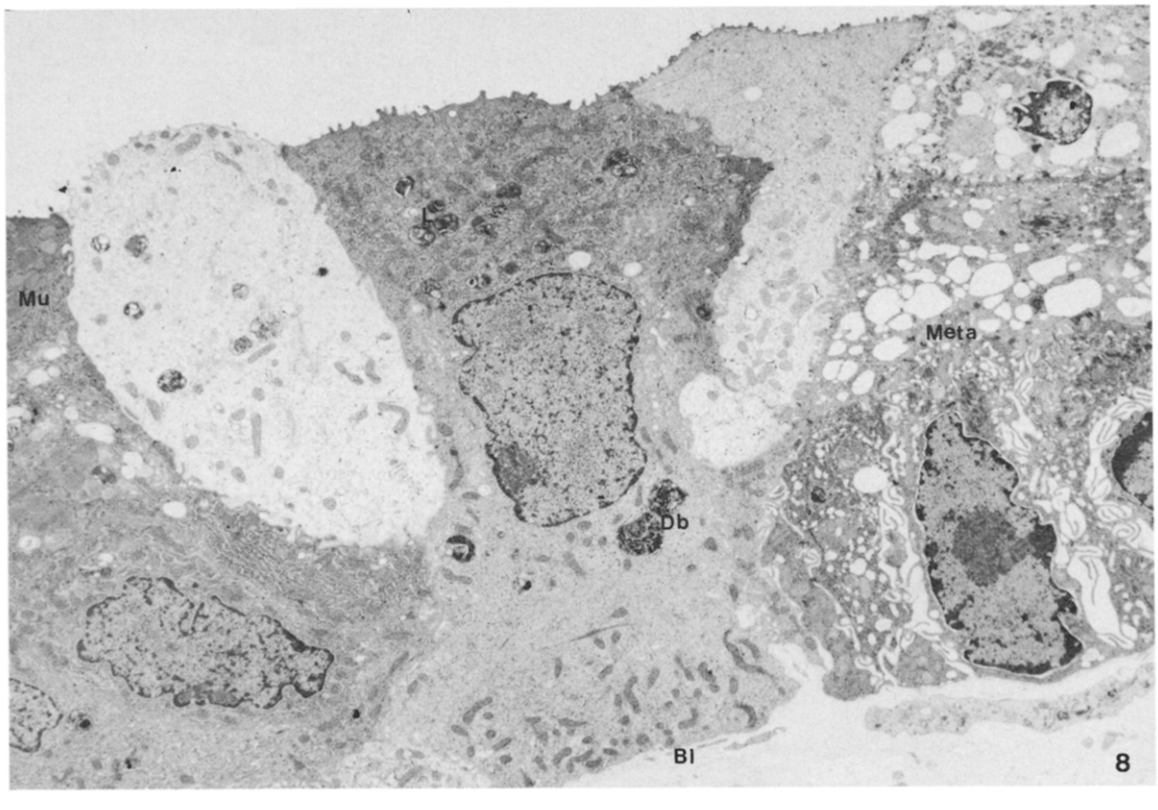


Fig. 7. Same material as in Fig. 6. A basal cell containing dense bodies (Db) which apparently are formed by aggregation of keratin filaments. Basal lamina (Bl), basal cell (Bc) and dense body (Db). ×10,240.

Fig. 8. Tracheal epithelium showing focal area of squamous metaplasia (Meta) and adjoining normal-looking epithelium. The degenerative changes are limited to the metaplastic area. The normal-appearing epithelium appears relatively unaltered (5 days *after treatment). Dense bodies (Db), lysosomes (L), basal lamina (Bl) and mucous droplets (Mu).* ×3840.

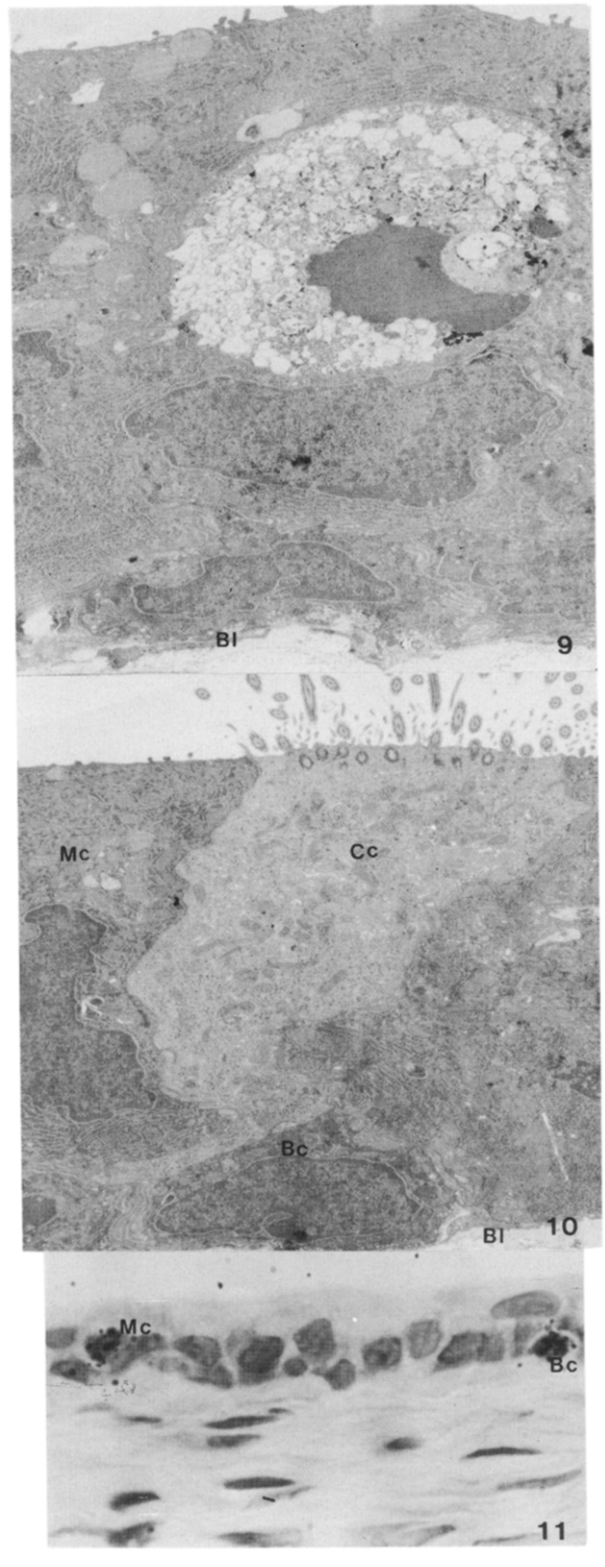


Fig. 9. Tracheal epithelium at 7 days after treatment. A giant vacuole containing degenerated metaplastic cells intermingled with superficial mucous and/or ciliated cells. Basal lamina (Bl). ×7360.

Fig. 10. Normal-appearing epithelium at 7 days after treatment. Basal lamina (Bl), basal cell (Bc), mucous cell (Mc) and ciliated cell (Cc). ×5840.

Fig. 11. Autoradiogram of tracheal epithelium showing a labeled basal cell (Bc) and a labeled mucous cell (Mc). PAS and hematoxylin. ×2000.

tion was selected because it has previously been shown to reverse squamous metaplasia in the tracheal explants [15]. At 1, 3, 5 and 7 days after treatment groups (3 explants each) of treated and control cultures were fixed in 3% glutaraldehyde in phosphate buffer. They were cut into 1 × 2-mm pieces, post-fixed in osmium, dehydrated in acetone and embedded in polybed 812 (Polysciences Inc., Warrington, PA). Thin sections were cut with a diamond knife in a Porter-Blum ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Philip-300 or a Hitachi HU12 electron microscope.

RESULTS

Effects of RA on tracheal organ cultures; morphological observations

Tracheal cultures in medium without RA. At the initiation of cultures the tracheal epithelium of vitamin A-deficient hamsters (28–32 days old) exhibited typical morphology. The epithelium consists of three major types of cells: basal cells, mucous cells and ciliated cells. Incubation of tracheas in the control medium was followed by the development of keratinizing squamous metaplasia (Figs 1 and 2). After three days of culture cornifying squamous metaplastic areas were present in approximately 50% of the explants. The degree and severity of the lesions progressed with the length of culture period and keratinized lesions were present in approximately 90% of the explants after 10 days [14].

Tracheal cultures in medium supplemented with RA. Tracheas were first cultured in the control medium for 3 days, followed by treatment with 10^{-9} M RA. Groups of control and treated cultures were terminated at 1, 3, 5 and 7 days thereafter for ultrastructural studies. The effect of RA was examined in the metaplastic lesions and in the uninvolved epithelium. Two to three metaplastic foci were studied from each treated explant.

The RA caused a marked remodeling of the squamous metaplastic epithelium. The effect was specific for the metaplastic lesions since the uninvolved epithelium appeared unaltered (see below). Within 1 day the intercellular cytoplasmic processes, particularly between the basal cells, were disrupted and the intercellular spaces had widened (Fig. 3). This was accompanied by swelling and disruption of desmosomes. Frequently halves of disrupted desmosomes were seen. A large number of broken cytoplasmic processes were seen in the widened intercellular spaces. Frequently autophagic vacuoles containing fragments of the cytoplasmic filaments were formed. The superficial squamous layers were loosening and exfoliating.

Some of the basal cells appeared to be differentiating into columnar cells (Figs 3 and 4). The nuclei of these cells were oval and appeared to occupy the basal position. The mitochondria were large, oval or round in shape, with moderately dense matrix. The supranuclear cytoplasm contained Golgi complexes that were producing spherical secretory vesicles (Fig. 4). There were many vacuoles sometimes throughout the cells but mostly in the supranuclear region. The vacuoles varied considerably in size and shape and appeared to have been formed from dilated rough endoplasmic reticulum (RER). The smaller membrane-coated vesicles in the supranuclear region may have originated from the Golgi vesicles. Numerous free ribosomes and bundles of keratin filament were still present in the basal cells. Occasionally a large cell with undifferentiated morphology extruded out of the basal layer (Fig. 5). It had pale cytoplasm with scant organelles. The RER was mainly dilated and frequently bordered the mitochondria. The keratin filaments were almost completely absent from these cells.

At later intervals (3–5 days after treatment) most cornified layers had exfoliated. The cells in the metaplastic lesion were undergoing widespread degenerative changes. They had pyknotic nucleic, vacuolated cytoplasm and remnants of cytoplasmic organelles (Fig. 6). However, there was little evidence of greater numbers of lysosomes. The vacuolation was maximum in the most superficial cell layer, but it also occurred extensively in cells situated deeper in the metaplastic lesions. The keratin filaments appeared aggregating to form whorl-shaped structures. Some basal cells contained dense bodies that appeared to be formed by aggregating keratin filaments (Fig. 7). Strangely, the basal cells that contained dense bodies had relatively few filaments, as if these were aggregated selectively to form dense autophagic bodies. Furthermore, the dense bodies had limiting membranes and their internal structure was that of extremely dense fibrillar material. These basal cells exhibited morphology of relatively undifferentiated cells. Their nuclei were large and the cytoplasm contained scant RER and numerous ribosomes.

The degenerative changes caused by RA were limited to the metaplastic lesions. In the epithelium where lesions were small the degenerative changes occurred largely in the metaplastic cells (Fig. 8). With the exception of a few lysosomes and dense bodies, the adjoining uninvolved epithelial cells appeared unaltered. Even in areas where the epithelium consisted of basal cells with metaplastic alterations (i.e. contained keratin filaments) and overlying

mucous and ciliated cells, the degenerative changes described above occurred in the basal metaplastic cells. Occasionally mucous cells also contained vacuoles and some lysosomes but their numbers were not extensive enough to represent a significant adverse effect.

At seven days after treatment the explant epithelium at the light microscopic level appeared to have regained normal morphology. The pseudostratified structure consisted of a superficial layer of mucous and ciliated cells and a discontinuous row of basal cells, although the proportion of basal cells still appeared greater than in the epithelium prior to organ culture. However, at the ultrastructural level the epithelium apparently was still undergoing remodeling changes. Frequently giant vacuoles presumably containing degenerative cells were found intermingled with mucous and ciliated cells (Fig. 9). The necrotic cells exhibited highly vacuolated cytoplasm and pycnotic nuclei that had electron-dense nucleoplasm. Except for these necrotic cells the epithelium was well organized and contained highly differentiated mucous goblet and ciliated cells (Fig. 10). The basal cells were flat, situated along the basal lamina and contained relatively scanty cytoplasmic organelles, but their cytoplasm still contained bundles of keratin filaments.

The retinoid effect, as indicated above, was limited to the metaplastic cells since the uninvolved epithelium adjoining the metaplastic areas remained relatively unaltered by the treatment. Even at 7 days after treatment the uninvolved epithelium exhibited relatively typical ultrastructural morphology.

Effect of RA on labeling indices

After 3 days of incubation in the control medium and when approximately 50% of the explants developed squamous metaplasia they were treated with different concentrations of RA for an additional 3 days. The number of total and labeled basal and mucous cells (Fig. 11) were counted and the LI ± S.D. determined for each group. The results show that RA inhibited replication of the basal cells and stimulated replication of mucous cells (Fig. 12). For example, at 10^{-8} M RA inhibited the LI of basal cells by approximately 77% ($P < 0.05$). At 10^{-9} and 10^{-10} M concentrations, however, an inhibition of approximately 36% of basal cell LI was not statistically significant. The stimulation of mucous cell replication was significant ($P < 0.01$) at all concentrations tested. The data presented in Fig. 12 represent one experiment, but it was repeated and similar results obtained.

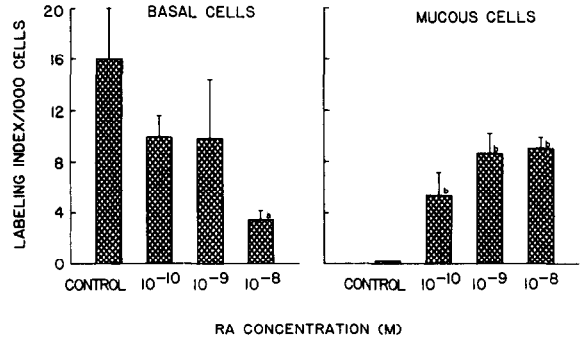


Fig. 12. Effect of different concentrations of RA on LI ± S.D. of mucous and basal cells. Tracheal explants were first cultured in the control medium for 3 days *and then treated with RA for an additional* 3 days. *(a) Inhibition of labeling index significant* ($P < 0.05$) *as compared to the corresponding control group; (b) stimulation of labeling index significant* ($P < 0.01$) *as compared to the corresponding control group.*

DISCUSSION

In agreement with previous studies [6, 15], RA reversed squamous metaplastic lesions in organ cultures of tracheas derived from hamsters fed a vitamin A-deficient diet. The remodeling of the epithelium was rapid since almost complete reversal of squamous metaplasia occurred within 7 days after the treatment. The purpose of this study was to examine cytokinetic and ultrastructural alterations involved in the reversal of squamous metaplasia in an effort to elucidate the mechanism of action of retinoids.

At the inception of organ cultures the tracheal morphology of hamsters fed a vitamin A-deficient diet was similar to that of the normal hamster trachea. The hyperplasia of basal cells and subsequent squamous metaplasia in tracheal cultures derived from the deficient animals appear to be related to the vitamin A status of animals prior to organ culture.

The RA induced complex remodeling changes in the metaplastic epithelium that accompanied vacuolation and exfoliation of the lesions, subsequently re-establishing epithelium with an almost normal morphology. At 3 days after treatment most squamous cell layers desquamated and the intercellular cytoplasmic processes and desmosomes which are numerous in the untreated cultures were disrupted, causing widened intercellular spaces and extrusion of cells from the basal layer. Degenerating cells and vacuoles containing fragments of cytoplasmic organelles were present at different levels in the epithelium. The degeneration caused by RA, however, was limited to the metaplastic cells since the uninvolved epithelium adjoining squamous metaplasia was not altered. At 7 days after treatment the epithelium regained almost normal morphology. Although several different mech-

anisms are probably involved in these rapid remodeling alterations, the exfoliation of squamous cells and disintegration of cytoplasmic processes and desmosomes may be related to the surfactant action of RA on the plasma membrane [16]. However, the selectivity of the retinoid effects in the tracheal organ cultures to the metaplastic foci and a lack of widespread degeneration in the uninvolved epithelium in the same culture suggest that the surfactant property of the retinoid is not the major factor involved in the remodeling of the tracheobronchial epithelium. Recently retinol was shown to cause degenerative changes specifically in phytohemagglutinin (PHA)-stimulated lymphocytes but not in the resting cells [17]. Since vitamin A action may be associated with cells containing binding proteins [18, 19], it was postulated that a majority of the stimulated cells contained high levels of the binding protein, making them susceptible to its action, while the resting cells, which contained low levels of the receptor, were not affected [17]. In fact, concentrations of retinol and retinoic acid-binding proteins are reportedly higher in rapidly proliferating embryonic and tumor cells than in the adult and normal cells [20]. Thus it is possible that the rapidly proliferating cells in the metaplastic lesions of the tracheal explants contain high concentrations of retinoic acid-binding protein, making them susceptible to its effect, while the resting mucous and ciliated cells contain lower concentrations of the binding protein and therefore are not altered by RA. It should be noted, however, that the retinoid effects are not necessarily mediated by cell-binding proteins. Recently Douer and Koeffler [21] reported inhibition of clonal growth by retinoids of human leukemic cell lines which lacked detectable quantities of retinoid-binding proteins. Similarly, Lotan *et al.* [22] found no correlation between the level of cell-binding proteins and growth inhibition by retinoids of several neoplastic cell lines.

Alternatively, the retinoid may cause an increase in the number of lysosomes, and the subsequent release of lysosomal enzymes into the cytoplasm may be responsible for the degenerative changes [23, 24]. This did not seem to occur in the tracheal explants since the increase in the number of lysosomes after the retinoid treatment was not striking. Previously Matter and Bollag [25] have reported regressions of 7,12-dimethylbenz(a) anthracene-induced skin papillomas of the mouse without significant increase in lysosomes. Vitamin A influences cellular differentiation and increases production and secretion of mucopolysaccharides, causing widening of extracellular spaces [26–28]. Although RA also causes mucous cell differentiation in the tracheal epithelium, it was not possible to demonstrate increased secretion into extracellular space. Nevertheless, a relationship between mucous cell differentiation and tumor regression has been reported for rabbit keratocanthoma and mouse papilloma treated with retinoids [27, 28]. In the tracheal epithelium hyperplasia of mucous cells caused by RA is probably partially responsible for re-establishing the normal epithelial morphology.

Retinoids have been shown to inhibit [29–31] or stimulate [32–34] proliferation in different tissues. However, with certain exceptions [25], their anticarcinogenic activity is generally equated with their capacity to inhibit cell proliferation. In most studies the proliferative activity is estimated without any regard to the different types of cells that constitute a tissue. This study for the first time shows that RA can have different effects on the mucous and basal cells in the tracheobronchial epithelium. It stimulated [^{3}H]-TdR incorporation in the mucous cells and inhibited the incorporation in the basal cells. After treatment of the metaplastic explants with RA the squamous element was sloughed off and a certain proportion of the basal cells appeared to differentiate into columnar cells exhibiting characteristics of the mucous cells. Similarly, in the mouse prostate explants in which RA also inhibited proliferation and reversed squamous metaplasia induced by 3-methylcholanthrene, Muller-Salamin *et al.* [31] have suggested that the switch from squamous to mucous differentiation occurs in the uncommitted basal cells. Seemingly, this also occurs to a certain degree in the tacheobronchial epithelium after treatment of the metaplastic explants with RA. However, it is important to indicate that a large proportion of the basal cells in the metaplastic area of tracheobronchial epithelium appear to undergo degenerative changes after RA treatment. It is possible that these cells have already been committed to differentiate into keratinizing cells and are unable to revert to mucous cell differentiation. However, the results that RA stimulates proliferation of the pre-existing mucous cells in the tracheal explants indicates that, in addition to the differentiation of basal cells into mucous cells, hyperplasia of the mucous cells contributes to re-establishing the normal morphology of the tracheal epithelium.

This study demonstrates the complexity of the effect of RA on the metaplastic tracheal epithelium. The results indicate that all of the effects of RA discussed above occur simultaneously and probably synergistically to reverse the metaplasia and induce mucous cell differentiation typical of the normal tracheal epithelium.

The observation that the effect of RA is relatively specific for the metaplastic lesions is important from the viewpoint of potential usefulness of retinoids in chemoprevention.

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