The Assessment of Tobacco Smoke Toxicity in Organ Culture. II. Ultrastructural Studies on the Immediate Response of Foetal Rabbit Tracheal Epithelium to Short-Term Exposures of Whole Smoke

It is generally assumed that the cytotoxic effects of cigarette smoke on the respiratory epithelium play a contributory role in the development of bronchogenic carcinoma[1]. An evaluation of these effects is therefore important, but can only be performed with difficulty in vivo, where both the conditions operating during smoking and the individual response are very variable. For these reasons, many workers have preferred to use tracheal or bronchial organ cultures for an assessment of the cytotoxicity of tobacco smoke or tobacco smoke condensate. Thus, CRESS et al.[2] found that in adult rabbit tracheal preparations exposed to a medium perfused with cigarette smoke, cilia ceased beating and were no longer positive for the enzyme ATPase. KENSLER and BATTISTA[3] studied ciliostasis in the rabbit and later[4] in chicken tracheal preparations exposed to fresh cigarette smoke, where filtration of the smoke through activated charcoal reduced its ciliotoxicity. DALHAMN[5] used rabbit tracheal preparations exposed to fresh cigarette smoke and observed that after diluting the smoke 10 times, the number of puffs preceeding ciliostasis increased by a factor of two. BLEIBERG[6] found that Alupent® (Orciprenaline) aerosol enhanced the recovery from ciliostasis of rabbit tracheae exposed to cigarette smoke. CROCKER[7] added cigarette smoke condensate to in vitro cultures of the bronchial mucosa of foetal monkey and hamster tracheae in order to test by light microscopy and autoradiography for the development of metaplastic and preinvasive neoplastic lesions.

Since none of these reports included any ultrastructural data, information of the morphology of the early response of the respiratory epithelium to smoke is still incomplete. We have therefore performed an electron microscope investigation on the effects in tracheal epithelium of acute in vitro exposure to fresh cigarette smoke under approximately physiological conditions. As previous exposure to atmospheric pollutants and/or infections could interfere with the effects of smoke per se, we have chosen the tracheal epithelium of foetal rabbits in order to minimize these factors. In this paper we report some preliminary results of the immediate effects of whole smoke on such a model system.

Materials and methods. Yellow silver strain rabbits[8] were sacrificed on the 28th–29th day of pregnancy by an i.v. injection of an overdose of sodium pentobarbital. After removal of the foetuses, the foetal tracheae were excised, cut into individual rings of approximately 2 mm thickness and washed in Parker's Medium 199 supplemented with 10% heat-inactivated rabbit serum. Immediately before exposure to smoke, the individual tracheal rings were touched on a moistened sterilized filter paper in order to remove most of the medium from the lumina. 3 to 5 rings were then placed on a stainless steel bridge within an open 35 mm glass petri dish, both of which had been sterilized at 160 °C for 2 h. The preparations were placed in a smoking machine, the details of which have been described elsewhere[9], and exposed to 2, 4, 8 or 12 puffs of whole smoke, respectively, from Kentucky standard research cigarettes. The lighting-up puff was not utilized for exposure and the cigarettes were replaced after the first 4 puffs. 35 ml (2 sec) puffs of whole smoke taken once every minute were diluted in a 1 to 10 ratio with a 95% air/5% CO_2 mixture within the exposure chamber. Temperature and relative humidity were maintained at 37 °C and 100%, respectively. 'Positive' control preparations were exposed to all the conditions prevailing in the smoking machine with the exception that the puff of smoke was replaced by a 2 sec intake of ambient air. 'Negative' control tracheal rings were kept in the medium at 37 °C for the duration of the individual experiments.

Immediately after the exposure was completed, test and control tracheal rings were washed in medium and fixed for 30 min in an ice-cold, freshly prepared mixture of equal parts of 2.0% glutaraldehyde and 1.0% osmium tetroxide in 0.1 *M* sodium cacodylate buffer at pH 7.4. Post-fixation was performed in 0.25% uranyl acetate in veronal acetate buffer (pH 6.4) for a further 30 min. Dehydration in a series of graded ethanols followed, and the material was embedded in Epon. The rings were trimmed so as to expose the central areas which had not been mechanically altered during their preparation and handling. Uranyl acetate and lead citrate solutions were used to enhance the contrast of the thin sections which were viewed in a Philips EM300 electron microscope.

Results. The tracheal epithelium of the 28–29 day rabbit foetus has been briefly described in light and electron microscope studies by LEESON[10]. In 'negative', i.e. non-exposed, light and EM control preparations, this epithelium is pseudostratified and consists of ciliated, non-ciliated and basal cells (Figure 1). The ciliated and non-ciliated cells are columnar in shape. The nucleus in the ciliated cells possesses a finely dispersed heterochromatin. In the cytoplasm, the rough-surfaced endoplasmic reticulum (rER) is often present as whorls, and the mitochondria are concentrated at the apical end, beneath the basal bodies of the cilia. Long, slender microvilli occur between the individual cilia. Golgi profiles are common in the supranuclear region, as are secondary lysosomes and multivesicular bodies. In the non-ciliated cells, the heterochromatin is concentrated at the periphery of the nucleus. The cytoplasm is more electron dense than that of the ciliated cells and contains a highly developed system of rER in the supranuclear region together with smooth surfaced ER near the apex of the cell. Golgi profiles are abundant, and beneath the apical membrane lie a few electron dense granules approximately 400 nm in diameter. In some of the non-ciliated cells at this stage, there is evidence of the assembly of secretory granules near the Golgi region. These granules are lined by a single unit membrane and are of medium electron density. They may represent the early stages of mucin production. Otherwise there are no mature goblet cells in the epithelium at this stage. The microvilli at the apical surface are shorter and thicker than those present at the apex of the ciliated cells. Junctional complexes are identifiable between the contiguous cells of the epithelium. They are tripartite, consisting of a tight and an intermediate junction and a desmosome.

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The basal cells are polygonal in shape with a higher nuclear/cytoplasmic ratio than in the other two types of cell. The heterochromatin is concentrated at the periphery of the nucleus. rER is only present as few short strands. Bundles of microfilaments are common and are often inserted into desmosomes which occur between individual basal and between the basal and the ciliated or non-ciliated cells. No enterochromaffin-like cells have been observed in the epithelium at this stage. Separating the epithelium from the underlying lamina propria is a basal lamina of about 40 nm thickness. The lamina propria contains fibroblasts, embedded in collagen fibrils, and plasma cells characterized by their extensively developed and dilated rER. 'Positive' control organ cultures were indistinguishable from non-exposed 'negative' controls.

Test cultures: After exposure to 2 puffs of whole smoke from the Kentucky cigarette, there were no identifiable changes in the ultrastructure of the epithelium as compared with the controls. After exposure to 4 or 8 puffs of whole smoke, the intercellular spaces between adjacent cells were very much larger than in the controls (Figure 2). However, no changes in the ultrastructure of the junctional complex could be observed. Both ciliated and nonciliated epithelial cells showed protrusions of the apical membrane. In most cultures exposed to 4 or 8 puffs, these protrusions were long and thin (Figure 3),

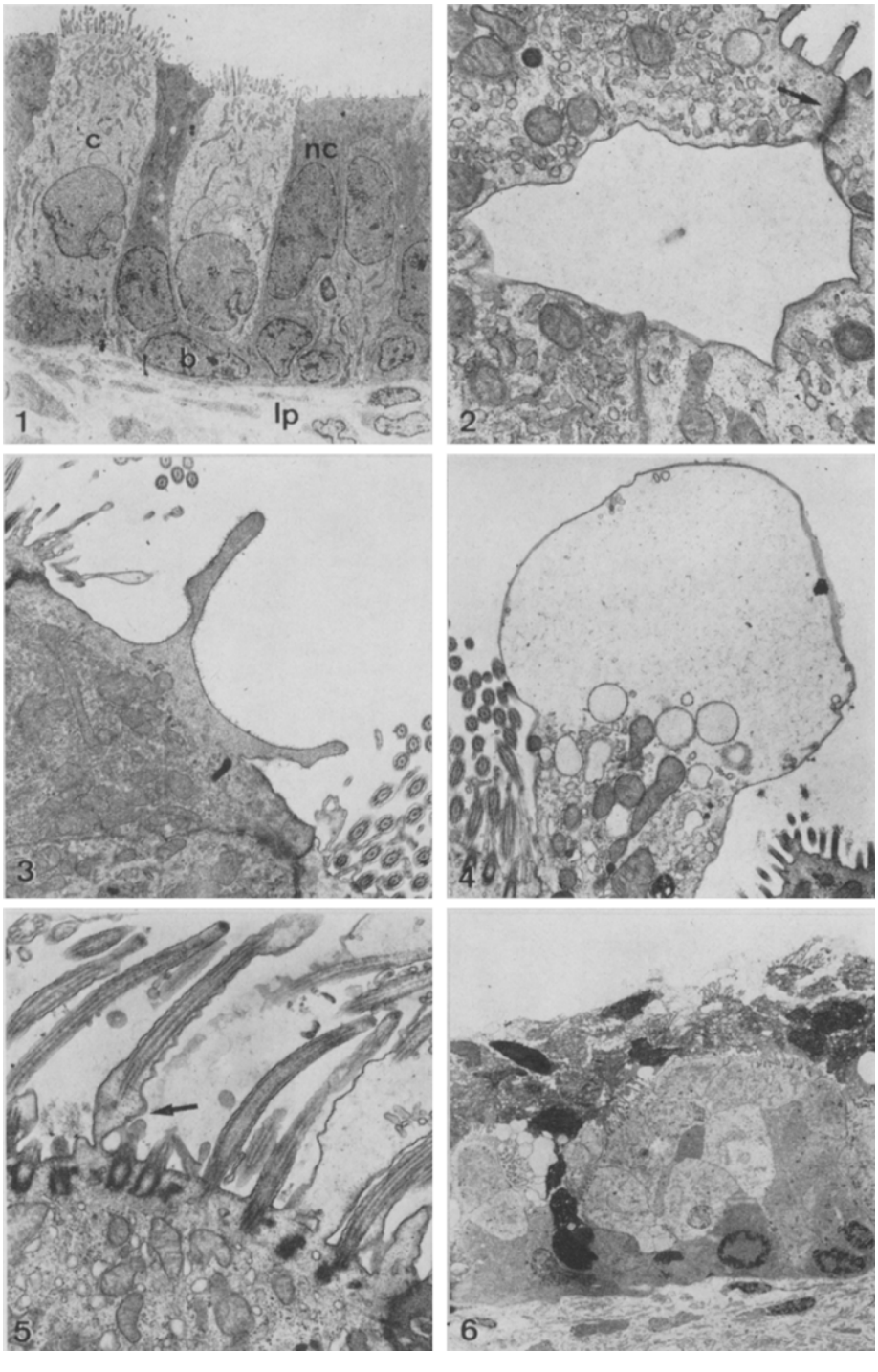

Fig. 1. Non-exposed, 'negative' control foetal rabbit tracheal epithelium. b, basal cell; c, ciliated cell; lp, lamina propria; nc, non-ciliated cell. ×1,500.

Fig. 2. Large intercellular space between 2 non-ciliated epithelial cells in a foetal trachea exposed to 4 puffs of whole smoke. Note morphologically intact junctional complex (arrow). ×12,500.

Fig. 3. Apical protrusions of a non-ciliated epithelial cell from a trachea exposed to 8 puffs of whole smoke. ×6,500.

Fig. 4. Ballooning of the apical cell area in a non-ciliated epithelial cell from a trachea exposed to 4 puffs of whole smoke. ×6,500.

Fig. 5. Swelling (arrow) of the basal portion of a cilium in an epithelial cell from a trachea exposed to 12 puffs of whole smoke. ×10,500.

Fig. 6. Epithelium from a trachea exposed to 12 puffs of whole smoke. Note the layer of cellular debris in the lumen. × 900.

but in some epithelial cells from cultures exposed to 8 puffs, the whole of the apical cell area bulged out into the lumen (Figure 4). The cilia were also observed to have swollen in the same way, but the internal microtubular structure of the individual cilia was not affected (Figure 5). After exposure to 12 puffs of whole smoke, these effects were present in more of the epithelial cells and many of the columnar cells were found to have undergone lysis, leaving a layer of cellular debris and exposing the less differentiated basal cells (Figure 6). An increase in the number of secondary lysosomes, especially of the autophagic vacuole type, was noted in all types of cells including the basal cells.

Discussion. The maintenance of the basic morphology of 'positive' control preparations under the experimental conditions described demonstrates that this model system is potentially useful for studying the effects of in vitro exposure of a respiratory epithelium to cigarette smoke. The use of a standardized fixation following immediately upon exposure may visualize cellular changes which would subsequently be lost in a more extensive cell and tissue breakdown or, alternatively, in recovery and repair.

FRASCA et al.[11] described ballooning of the apical membranes of cells in the bronchial epithelium of dogs which had been exposed daily to up to 12 cigarettes for a period of 44 days. Our similar findings were produced after only 8 or 12 puffs, suggesting that the results observed in the dogs may have been due to the acute cytotoxicity of the smoke exposure preceeding fixation rather than to any long term cumulative effects. It is interesting to note that NIDEN[12] observed ballooning of the apex of bronchiolar Clara cells in adult mice exposed to ammonia. Exposure to smoke may inhibit the energy-dependent maintenance of osmotic pressure within the cells such that solutes will pass across the cell membrane along a concentration gradient. As the apical surface of the cell is the most exposed to the smoke, this might explain the development of ballooning at this site. Swelling of cilia could be an explanation for ciliostasis.

An effect of tobacco smoke on cell junctions in the respiratory epithelium has been noted by SIMANI et al.[13] in guinea-pigs exposed to more than 50 cigarettes. These workers used horseradish peroxidase to demonstrate the failure of the junctions to maintain an effective seal against the passage of larger molecules. Although in the present study there were no changes in the ultrastructure of the junctions, the development of widened intercellular spaces immediately adjacent to these structures may have been due to an impairment of their highly specific function.

In the foetal tracheae used in this study, no mature goblet cells could be observed. Thus the lack of a mucin layer overlying the epithelium might exacerbate the cytotoxic effects of the smoke. In the post-natal trachea, where goblet cells are present, such a layer would act, in short-term exposures, as a barrier to both the particulate and gas vapour phases of smoke. Work is continuing to determine if there are any differences in the response to smoke exposure between the foetal and the adult rabbit tracheal epithelium[14].

Summary. Foetal rabbit tracheal organ cultures were exposed under defined conditions to whole cigarette smoke and fixed immediately for electron microscopy. After an exposure to 4 or 8 puffs, epithelial intercellular spaces were enlarged, the apical portion of many cells bulged out into the lumen and many cilia were swollen. An exposure to 12 puffs produced a breakdown of the epithelium.

P. DAVIES and G. S. KISTLER

Department of Anatomy, Division of Electron Microscopy, University of Zürich, Gloriastrasse 19, CH–8006 Zürich (Switzerland), 17 December 1974.

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The Sperm Bundles of Honeybee *Apis cerana indica* Fabr.

The sperm bundles are characteristics of the insect testes. They have been reported in Coleoptera, Orthoptera, Homoptera and Odonata[1–5]. The present work was undertaken to study the sperm bundles of the honeybee, *Apis cerana indica* Fabr.

The honeybee brood was reared at 34 °C in the laboratory. The testes, seminal vesicles, and accessory glands from living pink head pupae and newly emerged adults were used. For routine microanatomy, Zenker and Carnoy fixed material was sectioned and stained in haematoxylin/eosin.

Cytochemical localization of lipids was made on neutral formalin and gelatin embedded material postchromed according to BAKER[6]. Frozen 10 µm thick sections were cut and stained for lipids with Sudan black B in propylene glycol after the method of CHIFFELLE and PUTT[6]. Phospholipids were stained with acid haematein along with pyridine extracted controls according to BAKER[6], and neutral and acidic lipids with Nile blue after Cain[6]. Carbohydrates were localized in material fixed for 3–6 h after Zenker, or 1–4 h at 0–4 °C after Carnoy or Gendre's. The PAS reaction and Best's carmine along with acetylation and KOH reversal after MCMANUS[6] and diastase digestion were used as controls. Acid mucopolysaccharides were stained with alcian blue.

The sperm bundle of honeybee *A. cerana indica* Fabr. is comprized on an average of 72 sperms arranged in a definite hexagonal geometric array (Figure 2b). Each sperm bundle has a hyaline cap at its anterior region (Figure 1). This is formed by a double walled nutritive sac, covering most of the anterior portion of the bundle

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