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ARGYROPHIL, FLUORESCENT AND GRANULATED (PEPTIDE AND AMINE PRODUCING ?) APG CELLS IN HUMAN INFANT BRONCHIAL EPITHELIUM. LIGHT AND ELECTRON MICROSCOPIC STUDIES.

J.M. Lauweryns, J.C. Peuskens and M. Cokelaere

University of Leuven, Experimental Laboratory of Cardiopulmonary and Genital Pathology, 12, Minderbroedersstraat, B 3000 Leuven, Belgium

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We have recently reported (1) the occurrence in the bronchial and bronchiolar epithelium of newborn human infants of an impressive number of distinctly argyrophil and fluorescent cells, which were proposed to be involved at pulmonary level in the production of both amines and the kinin-generating system. These investigations were originally based on three lines of evidence : the common entodermal origin of the bronchi and the intestinal tract, the supposedly potent effects of kinins on the cardiopulmonary adaptation of the foetus to neonatal life (2), and the fine structural studies on the oncogenesis of bronchial carcinoid tumors and oat-cell carcinoma (3) which all suggested the possible occurrence of argyrophil or Kultschitzky-like cells in human lungs.

These earlier light optical observations have prompted us to carry out now combined light optical, histochemical and electron microscopical studies of the human infant tracheal and bronchial epithelium. As will be demonstrated next and while much speculation is still possible as to their exact rôle, we have observed the occurrence of argyrophil, fluorescent and ultrastructurally granulated cells, which do contain an impressive number of round and electron-opaque dense cored vesicles (800-1500 Å).

Material and Methods

Our studies include twenty-two newborns, on whom all general obstretical and pediatric data were available, the routine autopsy being performed usually within 1-2 days after death. The babies died from a wide variety of neonatal disorders as detailed (4).

In all cases, several large biopsies covering more or less the cut surface of a lung lobe (usually the right middle or lower lobe) were however

taken much earlier (i.e. within one hour) after death, immediately fixed in Bouin's fixative and formalin (neutral as well as acid) and embedded in paraffin. Serial sections were stained with hematoxylin-eosin, Masson's trichrome, Mayer's mucicarmine, Verhoeff-Van Gieson elastica stain (5) and Bielschowsky's method as modified by Foot for reticular fibers (5). In sixteen cases Jabonero's ammoniated silver carbonate technic as modified by Van Campenhout was carried out for argyrophilia (6). Fontana-Masson's technic (7) and Lison's azoreaction (7) for argentaffinity, Gomori's chromaffin stain (5) and Sevki's modified Giemsa method for chromaffinity (8), and Schmorl's method for lipofuchsin (8) was performed in ten cases.

In ten instances enough tissue was also available to be examined with the histochemical fluorescent amine technic according to Falck (9). Pulmonary tissue was quenched in liquid nitrogen and lyophilization performed for four days at temperatures decreasing from - 60°C to + 30°C. Afterwards the tissues were condensed for 1 hour with formaldehyde at 80°C. The paraformaldehyde used in this reaction was standardized in the atmosphere with diluted sulphuric acid (H_2SO_4/H_2O : 54,5/45,5) (10). The 6 μ thick sections of paraffin blocks were mounted in Entelan and examined with a fluorescence microscope Zeiss GFl (HBO 200 bulb, BG 12, UG 1 activating filters, 53/46, 65/47 barrier filters). During the photographic exposure in the fluorescence microscope, the sections were also studied with combined phase-contrast or ordinary light microscopy ; afterwards the sections were remounted and stained with hematoxylin-eosin in order to obtain an even better identification.

In all instances, control sections (including appendix, adrenal medulla ...) were run at the same time and through the same solutions.

For electron microscopy fresh tissue samples were taken in eight cases as soon as possible after death (within one hour). Using a dissecting microscope, sections were taken of the bronchi which did not exceed 1 mm. thickness. In four instances these were fixed in buffered osmium tetroxide alone (11) ; in three cases they were fixed in 2,5 % glutaraldehyde in 0,1 M phosphate buffer for 2 hours, repeatedly (6 times) washed in 0,1 M phosphate buffer and then postfixed in 1 % buffered osmium tetroxide. In one case enough tissue was available to allow a fixation of the tissues in buffered osmium tetroxide on the one hand, and in glutaraldehyde with postosmification on the other hand. The tissues were embedded in Epon and sections cut at 1 micron with a Porter-Blum MT-2 ultramicrotome, which were stained with toluidine blue. From the one micron sections appropriate areas were carefully selected for further trimming

of the tissue blocks and sectioning at 200 to 300 Angströms. These sections were stained with uranyl acetate and lead citrate (12) and examined with a Zeiss 9 A electron microscope.

Results

Due to the brief fixation interval after death, the ciliated pseudostratified columnar epithelium lining the trachea and the bronchi was beautifully preserved, directly adherent to the basement membrane and exhibiting no sloughing down or cellular necrosis as usually observed light optically on postmortem tissue sections. On the preparations stained for argyrophilia with the ammoniated silver carbonate technic (6), the occurrence of numerous argyrophil cells displaying the characteristics we have previously described (1), were again noted. These argyrophil cells (fig. 1A.) contain an oval to round nucleus and exhibit a triangular to pyramidal shape, the main cytoplasmic mass being ba-

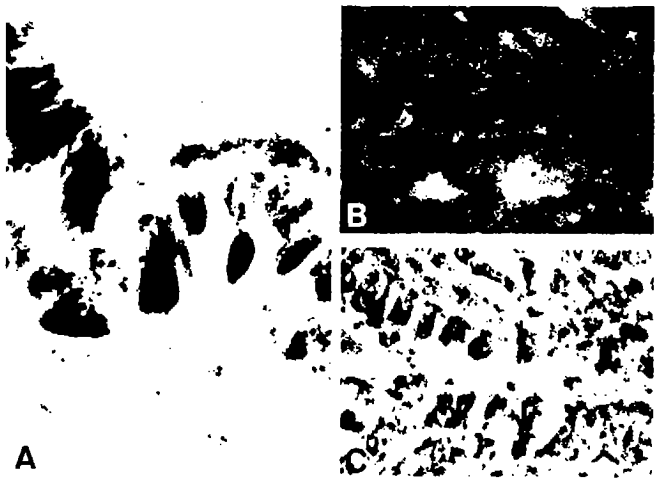

FIG. 1.

Fig. 1A. : Typical bronchial argyrophil AFG cells with their triangular shape and cytoplasmic argyrophilia, the deposit being mainly located in the basal cell cytoplasm (Jabonero's ammoniated silver carbonate technic as modified by Van Campenhout ; 800 X).

Fig. 1B. and C. : Occurrence of two bright green fluorescent AFG cells (arrow) in the bronchial epithelium of a newborn infant ; the main cytoplasmic mass is basally located (lyophilization and investigation with the histochemical fluorescent amine technique according to Falck, 1(B) : 600 X, 1(C) : same area, phase contrast microscopy ; 600 X).

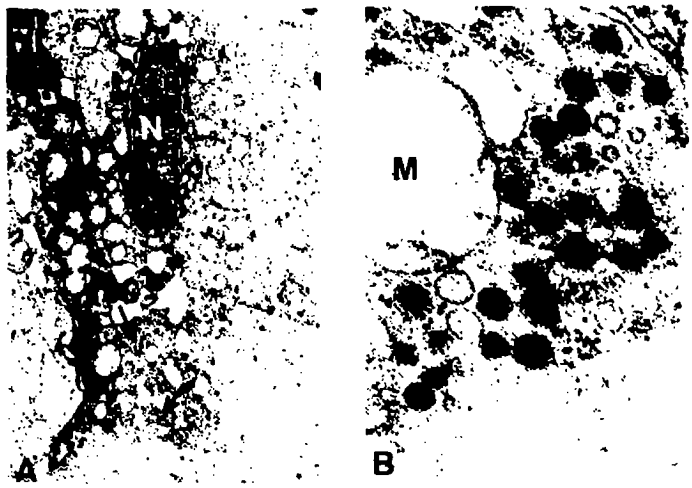

FIG. 2.

Fig. 2A. : Electron micrograph of typically granulated AFG cell, the basal portion of the dense cytoplasm lying immediately against the basement membrane ; occurrence of numerous intracytoplasmic electronopaque granulated vesicles (arrows).

Fig. 2B. : Cytoplasm of AFG cell containing numerous and typical electronopaque dense-cored vesicles (800-1500 Ångström), limited by a unit membrane ; the central dense core is surrounded by a small peripheral halo ; Nucleus (N) ; Dilated mitochondrium (M) ; Fig. 2A. : 6300 X ; Fig. 2B. : 52100 X).

sally located in the bronchial epithelium, i.e. immediately adjacent to the epithelial basement membrane. The cytoplasmic precipitate appears as a massive black, more or less amorphous to more granular diffuse deposit which is mainly and preferentially present in the basal portions of the cell cytoplasm (i.e. beneath the nucleus). On the tissue sections sometimes only the basal portions of the argyrophil cells are present, which appear then still more triangular to oval, "tadpole" shaped. In other (more unusual) instances they exhibit even ramifying or branching basal cytoplasmic processes, suggestive of the complex interdigitations observed on electron micrographs in cases of peripheral pulmonary carcinoid tumor (3). These cells failed to demonstrate a positive argentaffin and chromaffin reaction. The lipofuchsin stain was also negative. On the other hand cells with analogous morphology and cellular localization were observed on the freeze-dried specimens and did reveal an intensive bright green

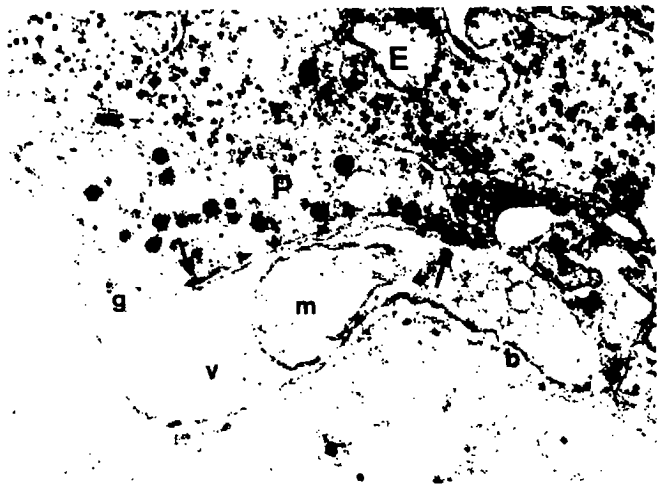

FIG. 3.

Electron micrograph of basal cytoplasmic process (P) of an AFG cell with its dense cytoplasm and typical membrane bound granulated vesicles ; Nerve ending close to the basement membrane (b) and separated from the AFG cell by a gap of only about 200 Ångström and containing many agranular vesicles (v) and an occasional granulated vesicle (g), suggesting a "direct contact" ; Bronchial epithelial cell (E) ; membrane thickenings (arrows) ; mitochondrium (m) ; collagen fibers (c) (34200 X).

cytoplasmic fluorescence (fig. 1,B,C).

Studied with the electron microscope on tissue sections fixed with glutaraldehyde, granulated cells (fig. 2,A) were noted which revealed a profile comparable in its morphology and topography, to the argyrophil and fluorescent cells. Paralleling the light optical morphology, they appear indeed on the electron micrographs to be intercalated amoung the other and classicaly known mucosal cells, their basal cell cytoplasm lying above as well as immediately adjacent to the basement membrane. The cell membrane is of the classic unit membrane type ; typical desmosomes are present, supporting furthermore the epithelial character of the granulated cells and excluding at the same time and besides other arguments an eventual migrated intra-epithelial mesenchymal or blood cell. The cytoplasmic matrix is denser and more electronopaque than the surrounding mucosal cells. It is characterized by the occurrence of numerous round granulated vesicles (800 to 1500 Å) which are limited by a single unit membrane and do contain a distinct and centrally located dense core, which is

surrounded by a small and translucent peripheral halo (100 to 150 Å). In this halo some barely discernible granular material can be traced. The prominent central core is usually highly electronopaque, the electron density being somewhat variable in individual instances and sometimes less pronounced ; a faint granular substructure can sometimes be traced in the electronopaque core itself (fig. 2,B).

Besides these typical electronopaque dense-cored vesicles, the cell cytoplasm contains numerous mitochondria. Due to terminal agony, hypoxia and the unavoidable postmortem fixation interval, they usually appear dilated on the electron micrographs. (In this respect it may be noted that hypoxia does not affect the ultrastructure of the granulated vesicles (13). A moderately well developed endoplasmic reticulum (mainly smooth), some glycogen (in its granular form) and a rather small Golgi-complex can also be seen. In the vicinity of the Golgi-complex- and as had been noted by de Robertis and Sabatini in the adrenal medulla (14) and by Ekholm and Ericson in the parafollicular cells of the thyroid gland (15) - some small vesicles are sometimes observed which do contain a dense material. This dense deposit is separated from the membrane of the vesicle by a clear space.

Present also are bundles of intracytoplasmic filaments, as well as occasional small and empty appearing vesicles, dense granules, dense bodies and microtubules.

Another striking finding was the occurrence of several unmyelinated nerve fibers which were seen as well in the submucosal connective tissue as above the basement membrane or intramucosal. These intramucosal nerve fibers which have no Schwann cell envelopment, are characterized by an electron-lucent axoplasm and several beaded enlargments or varicosities which contain many characteristic small vesicles and mitochondria. While there are several such varicosities along the course of a single axon, resembling terminations "en passage" throughout the various cells of the bronchial mucosa, nerve endings suggesting a "direct contact" with the granulated cells have been observed. They are localized within the mucosa, deep to the basement membrane and in intimate relationship to the granulated cells, being separated from the latter by a gap of only about 200 Ångström and devoid of any other cellular processes. At this site of presumed "contact", the granular cells and the axon membrane do exhibit thickenings ; the axoplasm contains mitochondria and large accumulations of vesicles. The axoplasmic agranular and granular vesicles are of the classic types (16,17), with a distinct predominance of the agranular vesicular type (fig.3).

Finally and as we anticipated, no granulated cells were observed after osmiumtetroxide fixation alone.

Discussion

To our earlier studies (1) revealing the occurrence of argyrophil and fluorescent cells in the normal bronchial epithelium of human infants, we may now add the results obtained on a much larger series of infants and after a combined electronmicroscopical investigation with various fixation technics. This electron microscopical study has confirmed the existence of a specific cell and has revealed its characteristic ultrastructure, these unique cells being granulated and possibly innervated. To the best of our knowledge the occurrence of such argyrophil, fluorescent and granulated cells, which will hereafter and tentatively be called "AFG cells", has never been systematically explored nor reported in the normal tracheal and bronchial epithelium by combined light optics, fluorescence microscopy and with the electron microscope.

Fröhlich (18) has observed the existence of so-called bronchial "Helle Zelle" (clear cells) which were argyrophilic in several animal species (but he did not study this in the human), while Feyrter (19) considered all "clear cells" (most of them being argyrophilic according to his experience) as part of an endocrine ("paracrine") gland with widespread distribution throughout many body organs, including the lungs. A possible relationship of the cells we have observed to these so-called "clear" cells, remains however entirely speculative and unproven, as there are evidently much more numerous "clear", empty appearing and basally located cells in the human bronchi and bronchioles than the cellular type reported by us.

Following the classical contributions on "argyrophil" and "argentaffin" cells (20) and the more recent cytochemical studies of Carvalheira e.a. (21), the bronchial cells observed by us might be defined as "argyrophil", i.e. cells having an affinity for silver (the reduction of the silver solution being aided by an extraneous reducing agent) and revealing ultrastructurally a comparable appearance, the common characteristics of these cells being the intracytoplasmatic occurrence of a secretory product which is stored in membrane bound granules. They are however not entirely identical to the "argyrophil" or so-called polypeptide hormone-producing APUD cell series of Carvalheira e.a. (21) as they do disclose a distinct green fluorescence after freeze-drying and formaldehyde vapour treatment. Also the possible "direct contact" innervation-revealed by the electron microscopical investigation-appears a rather unique finding.

Hence the AFG cells of the human infant lung appear to be related to the growing list of presumably peptide and amine secreting cells which have been observed in different tissues and organs (22). The list of APUD cells includes pituitary corticothrophs (ACTH) and melanotrophs (MSH), pancreatic islet betha-(insulin) and alpha 2-(Glucagon) cells, thyroid C cells (Calcitonin), ultimobranchial C cells (Calcitonin) and the G cells of the antro-pyloric mucosa. Another category of related cells which possess a sufficient number of common characteristics, though not identical to the APUD cells and to be distinguished from them in the current status of our knowledge, include the enterochromaffin cells, the adrenal medullary (noradrenalin) cells and carotid body (Type I) cells (23,24).

The possible innervation of the AFG cells which seems in our studies to be of the "direct contact type" according to the classic criteria (17), may be related to comparable ultrastructural observations of the secretory parenchyma of the ultimobranchial body (25), the carotid body (24,26,27) and even the human salivary glands (28) which are also involved in the kinin generating system (29). To determine whether the terminal "direct contact" is adrenergic or cholinergic and even if the small vesicle population predominates in the nerve endings, is impossible for technical reasons as this would require fixation with permanganate (30). Even the challenging studies of Biscoe and Stehbens (26) and of Kobayashi (27) on a comparable controversy in the carotid body, have revealed that also theoretically the solution cannot be given readily.

As regards the possible secretory function(s) of the bronchial AFG cells, we assume that these cells play a paramount rôle in the production of amines (histamine, catecholamines, 5-hydroxytryptamine and related substances) and the peptide-generating system ? Indeed, as recently suggested by pharmacological studies of Melmon e.a. (2) an intimate relationship exists between catecholamines and the kinin system, which could hence be shared and explained at pulmonary level by a common cell : the AFG cell. This unique cell could indeed interfere with a wide variety of morphologically partially unexplained pulmonary functions, adaptations and disorders, i.e. bronchial smooth muscle tone (31), pumonary vasomotion (32), the circulatory adaptation of the fetus to neonatal life, the oncology of bronchial carcinoid and oat cell tumors, and the carcinoid syndrome sometimes associated with these tumors.

Light and especially electron microscopical studies have indeed indicated a distinctive analogy between bronchial carcinoid tumors and Kultschitzky type-cells, the cell of origin claimed to be a normal but scant component of

bronchial glands and tumorous bronchial epithelium (3). The carcinoid syndrome has now also been described in association with primary bronchial carcinoids (33) and even with apparently more typical carcinomata originating in the bronchus, especially oat-cell carcinomata (34), whose cells contain serotonin granules (3).

The biological hallmark of the carcinoid syndrome was originally considered to be an excessive production of serotonin and its metabolites ; recently however, the rôle of serotonin as the unique mediator in some carcinoid manifestations has been challenged (35). Kinins were also present in some carcinoid tumors (36) and oat-cell carcinomas of the lung (37).

As kinins have been held possibly responsible for the circulatory adjustments associated with birth causing i.e. a dilatation of the pulmonary artery musculature in the lamb fetus in vivo (2) and at an oxygen partial pressure within the physiologic range, we assume that this effect could be mediated through the AFG cells.

On the other hand, a possible chemoreceptor-like function of the AFG cells, which may be "tasting" the PO_2, the PCO_2 and (or) other constituents of the intratracheal and intrabronchial gas cannot entirely be excluded and may be possible also. Indeed - and as already detailed - these cells share some common characteristics whith the type I cells of the carotid body, such as their granulated cytoplasmic appearance and the possible presence of "direct contact" nerve endings (24,26,27). Additional pharmacologic and physiologic data are however needed to substantiate this hypothesis.

Finally, it may be suggested that the whole or partial failure of the AFG cells in the cardiopulmonary adaptation at birth might be held responsible to an unknown extent for the pulmonary arterial vasoconstriction and hypoperfusion which has been claimed to be fundamental in the idiopathic respiratory distress syndrome (38).

Summary

Light optical, histochemical and electron microscopical studies have revealed that the epithelium of the bronchi of newborn human infants contains an impressive number of distinctly argyrophil, fluorescent (after freeze-drying and formaldehyde vapour treatment) and ultrastructurally granulated cells. These AFG cells do indeed contain numerous round electronopaque dense-cored vesicles. Intramucosal nerve endings are also present, suggesting a "direct contact" with the AFG cells.

It is proposed that these cells in the human lung are related to the

ty and pulmonary hemorrhage ; Case 11 : 560 gm., sex unknown, neonatal death due to severe immaturity ; Case 12 : 1120 gm., f, 4 days, IRDS and pulmonary hemorrhage ; Case 13 : 1720 gm., f, 2,5 days, prematurity and IRDS ; Case 14 : 1025 gm., m, 70 hrs, viral pneumonia ; Case 15 : 825 gm., f, neonatal death due to prematurity and IRDS ; Case 16 : 1680 gm., f, 11 hrs, right cerebral intraventricular hemorrhage and IRDS ; Case 17 : 1575 gm., m, 4 hrs, interstitial pulmonary emphysema ; Case 18 : 1600 gm., f, 15 hrs, congenital hydronefros and pneumonia ; Case 19 : 600 gm., f, neonatal death due to extreme immaturity ; Case 20 : 860 gm., neonatal death due to hydrocephaly ; Case 21 : 2800 gm., m, 1,5 days, bilateral intraventricular cerebral hemorrhage and pneumonia ; Case 22 : 2950 gm., f, 11 hrs, first arch syndrome.

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