Thorax 1985;**40**:862–865

Pulmonary endocrine cells immunoreactive for calcitonin in the lungs of fetal and neonatal rats

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ABSTRACT Previous studies have shown that the size of the population of bronchopulmonary endocrine (Feyrter) cells appears to be greatest in fetal and neonatal life. This has led to the logical assumption that these cells are important during this period and undergo a subsequent decline in number thereafter. In this study endocrine cells containing calcitonin or a closely related cross reacting peptide have been demonstrated in the lungs of fetal and neonatal rats by immunoenzyme histochemistry. They appear first about three days before birth and their number, expressed as immunoreactive cells per cm^2 of histological section, remains relatively constant for up to three weeks after birth. It has been shown previously in this department that endocrine cells immunoreactive for calcitonin are present in the lungs of the normal adult rat. Their number in these adult animals is closely similar to the numbers of cells in the lungs of the developing animals of the present study. It is suggested that, at least in the rat, bronchopulmonary endocrine cells immunoreactive for calcitonin have a role that is not confined merely to the period of transition from fetal to neonatal life.

The number of bronchopulmonary endocrine cells, both solitary and clustered (neuroepithelial bodies), is generally considered to be greater in the fetus and newborn animal than at any other time during life. It has been suggested therefore that these cells may have an important function during intrauterine and early extrauterine existence,[1] with a subsequent decrease in their number after this period.[2 3] Having recently shown that pulmonary endocrine cells immunoreactive for calcitonin are widespread in normal adult rats,[4] we have made a qualitative and quantitative study of such cells in the lungs of fetal and neonatal animals of the same species. We hoped that by this means any alteration in the morphological characteristics or the number of these cells during this period would be detected.

Methods

Ten healthy young adult female Wistar albino rats were mated with four young adult males, and the former observed for signs of pregnancy. Some of those in which mating had been successful were killed at regular intervals during gestation. Others were allowed to deliver, and their offspring were then killed at regular intervals. By this means, 11 groups of fetal and neonatal rats were obtained from two weeks before birth to three weeks after birth at intervals of about three days. No group contained less than six animals. Owing to the small size of the fetuses, each was processed whole. In the case of those obtained at birth and thereafter, the lungs were removed by careful dissection and processed alone. All lungs were in the collapsed state, fixation in all cases was in Bouin's solution for four hours, and tissues were embedded in paraffin wax. Three groups of serial sections were cut from each block at a thickness of 4 μm.

Adjacent sections were studied after staining with haematoxylin and eosin, by the Grimelius method for argyrophilia,[5] and after labelling for calcitonin by the peroxidase antiperoxidase (PAP) method of Sternberger.[6] The antisera used and the precise details of the method have been described previously.[4] In this case two different primary antisera were used. These were purchased from Steranti Research Ltd and from Dako Corporation of Denmark. Both were polyclonal. The working dilution was 1:8000 in both cases with incubation at constant humidity and at 4°C for 18 hours. Both

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Accepted 10 May 1985

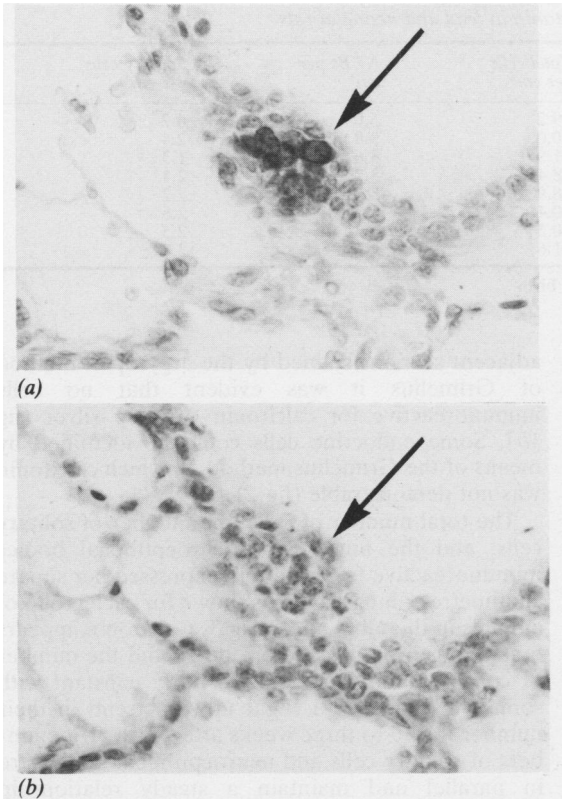

Fig 1 *Adjacent sections showing a neuroepithelial body immunoreactive for calcitonin (arrows) in a terminal bronchiole of a rat aged 21 days: (above) peroxidase antiperoxidase anticalcitonin preparation; (below) stained by the silver impregnation method of Grimelius. The calcitonin containing cells of the neuroepithelial body do not take up silver. (× 1250.)*

showed 100% cross reactivity with purified antigen, but less than 0.1% with other purified peptide hormones as determined by radioimmunoassay. Liquid phase absorption studies were performed in which increasing amounts of calcitonin, katacalcin, and calcitonin gene related peptide (CGRP) were incubated with 1 ml of antiserum at working dilution for 18 hours at 4°C. Complete quenching of immunostaining was obtained with less than 100 ng of calcitonin, but the addition of up to 100 μg of katacalcin and CGRP had no effect.

The primary antisera were linked to rabbit immunoglobulin peroxidase antiperoxidase complex by swine antirabbit serum. The chromogen was 3-amino 9-ethylcarbazole. Endogenous peroxidase was removed by prior incubation with 1% hydrogen peroxide in methanol, and non-specific binding of immunoglobulin was prevented by the application of normal swine serum. Negative control procedures depended on omission of each stage of the procedure in turn and on replacement of the primary antisera with antisera to hormones other than calcitonin. Positive tissue controls comprised sections of human and non-human thyroid glands and of tissue from cases of human medullary carcinoma of the thyroid.

Serial sections were examined and compared with a Leitz Wetzlar Dialux microscope. After subjective examination the number of cells immunoreactive for calcitonin was expressed per square centimetre of lung section,[7] the area in question being measured by planimetry. Other methods have been used to quantify endocrine cells in the airways,[2 8] but in the present study we considered that since positively labelled cells were present in both airways and parenchyma the method used by Taylor[7] would be most readily applicable and accurate. Although the lungs were all in the collapsed state when examined, there was a slight decrease in the amount of tissue per unit area in those from the neonatal animals in comparison with those from the fetuses since previ-

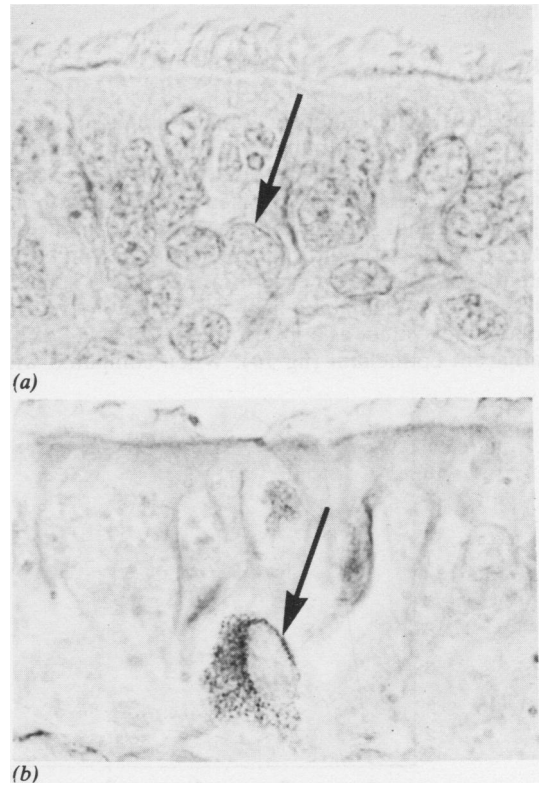

Fig 2 *Adjacent sections showing a single endocrine cell (arrows) situated basally in the epithelium of a bronchus of a rat aged 18 days: (above) peroxidase antiperoxidase anticalcitonin preparation; (below) stained by the silver impregnation method of Grimelius. The cell is argyrophilic but does not contain calcitonin. (× 1600.)*

Bronchopulmonary endocrine cells immunoreactive for calcitonin in fetal and neonatal rats

Time (days after birth)*	*No of rats*	*Total section area (cm^2)*	*Total cells per cm^2*	*NEBs per cm^2*	*Solitary cells per cm^2*
−3	7	1.20	19.2	5.0	4.2
0	8	2.90	20.0	3.8	2.4
+3	7	4.75	21.9	3.6	1.3
+7	10	3.76	18.4	3.2	2.1
+10	6	3.71	18.9	3.0	2.2
+14	6	3.98	20.4	3.5	2.8
+18	6	4.48	20.5	3.3	2.5
+21	6	3.71	21.6	4.9	3.8

*No immunoreactive cells were seen earlier than three days before birth.
NEBs—neuroepithelial bodies.

ously aerated lungs never collapse completely, unlike lungs in utero before expansion occurs. This would tend to lead to some underestimation of the numbers of endocrine cells in the neonatal period, but we did not consider that this would affect the results significantly. Three counts were performed: the total number of positive cells, the number of solitary cells, and the number of neuroepithelial bodies.

Results

The cells that contained granules immunoreactive for calcitonin were found in developing airways and parenchyma as either solitary cells or clusters (neuroepithelial bodies). When compared with adjacent sections stained with haematoxylin and eosin these cells were difficult to separate from their epithelial neighbours except in the case of some of the neuroepithelial bodies, when they were frequently elevated, as a group, above the level of the adjacent epithelium (fig 1*a*). When compared with adjacent sections stained by the argyrophil method of Grimelius it was evident that no cells immunoreactive for calcitonin took up silver (fig 1*b*). Some endocrine cells could be identified by means of the Grimelius method, in which calcitonin was not demonstrable (fig 2).

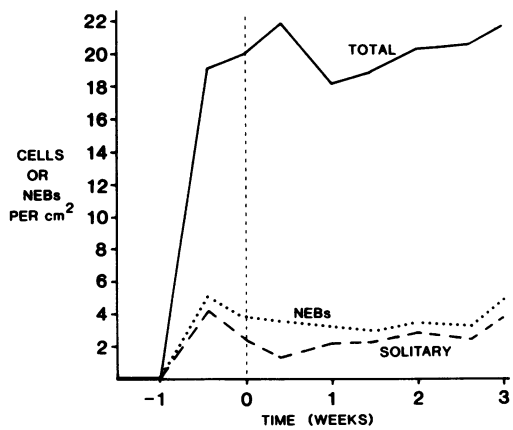

Fig 3 *Numbers of calcitonin containing cells per cm^2 of section in lungs from fetal and neonatal rats. The vertical broken line indicates the time of birth.*
NEBs—neuroepithelial bodies.

The total number of cells, the number of solitary cells, and the number of neuroepithelial bodies immunoreactive for calcitonin expressed per square centimetre of lung section is shown for each group of animals in the table and in fig 3. Calcitonin appears first about three days before birth, and the number of cells per unit area remains fairly constant with some suggestion of a slight upward trend in their number for up to three weeks after birth. The numbers of solitary cells and neuroepithelial bodies are in parallel and maintain a steady relationship throughout the period of the experiment (fig 3).

Discussion

Although pulmonary endocrine cells are of uncertain function, in the case of man they have been shown to contain at least one amine—5-hydroxytryptamine (serotonin)[9]—and at least three peptides (calcitonin, gastrin releasing peptide, and leu-enkephalin).[10] Since these cells appear to be less prevalent during adult life than during the fetal and neonatal period, it has been proposed that they are of greatest importance around the time of birth.[2] It has, for example, been suggested that they may play a part in maintaining pulmonary vasoconstriction in the hypoxic lung of the fetus, and in promoting vasodilatation when the newborn animal takes its first breaths.[1]

As with studies of human lungs, most previous investigations in the rat have relied on the use of silver impregnation methods as a means of labelling these endocrine cells.[2 11] Such methods, however, may not reveal all of the endocrine cells present[8] and they provide no information on the extent and distribution of the subpopulations that almost certainly exist. We have, for example, recently found a wide-

spread distribution of endocrine cells immunoreactive for calcitonin in the airways and alveoli of normal adult rats.[4] This was an unexpected finding in the light of current beliefs about the numbers and distribution of endocrine cells in this species. The present study provides equally interesting information about endocrine cells immunoreactive for calcitonin in late fetal and early postnatal life. It is clear that argyrophilia and the presence of calcitonin do not correspond. This is not necessarily surprising, since the mechanism of the former is still uncertain,[12] and it may not be related to the content of peptide. The fact that endocrine cells may be identified before calcitonin appears within some of their number is consistent with previous investigations showing development of endocrine cells in early fetal life.[13–15] Presumably those destined to produce calcitonin do not do so until later. It could be argued that the appearance of granules of calcitonin in these cells in the late fetal period reflects increased storage after a period of secretory activity during early pregnancy. In this case, however, one would expect a subsequent gradual decline in the number of cells, their function having been fulfilled, but this does not seem to occur. These findings suggest that they do not have a particular role in the transition from the fetal to the neonatal state, but become evident in the late fetal period and persist at an almost constant level as long as three weeks after birth, when the animals are well on the way to maturity.

In the neonatal rats of the present study, the total number of calcitonin containing endocrine cells per cm^2 of lung was about 20 from three days before birth to three weeks after birth (table). In the adult rats of our previous study[4] it was 18.16 cells per cm^2 of lung. These figures are in close agreement, and it is likely that these cells maintain their number, in health, from the neonatal period through to adulthood.

One may only speculate on the function of these cells. Local (paracrine) effects on cell differentiation, growth, or secretory activity of other epithelial cells of the lung, or on the tone of the smooth muscle of the airways or pulmonary vessels, cannot be discounted and may even be of fundamental importance.

We would like to thank Miss C Youd for typing the manuscript and Mr AR Williams for taking the photographs. This study was part of a larger body of work supported by a grant from the Mersey Region Health Authority.

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