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Localization of serotonin, cholecystokinin and somatostatin immunoreactivity in the lower respiratory tract of embryonic, foetal and postnatal sheep

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Summary. The lower respiratory tract of the sheep was studied by light-microscopical immunocytochemistry for serotonin, cholecystokinin, somatostatin, bombesin and calcitonin during different periods of lung development; embryonic, foetal and postnatal. At embryonic period only intraepithelial serotonin-containing cells as solitary neuroendocrine cells (NEC) and neuroepithelial bodies (NEB) were found. At foetal stages, immunoreactive cells to serotonin, cholecystokinin and somatostatin were observed in airway epithelium, as solitary NEC and NEBs, and in autonomic intrapulmonary ganglia as single or clusters of small intensely-fluorescent (SIF) cells. In postnatal sheep, serotonin- and cholecystokinin-containing cells were found within airway mucosa as solitary NECs and NEBs. No immunoreactive cells were observed with antiserum to bombesin and calcitonin. Quantitative studies showed that serotonin was the predominant substance, and that solitary neuroendocrine cells were more numerous in distal conducting airways and at foetal stages.

Key words: Sheep, Respiratory, Neuroendocrine, Paraneuron, Serotonin, Cholecystokinin, Somatostatin

Introduction

Components of the Diffuse Neuroendocrine System (Pearse and Takor, 1979) and the paraneuron group (Scheuermann, 1987; Fujita et al., 1988) have been described in airway mucosa and in autonomic intrapulmonary ganglia of many species.

These cells are found in the airway epithelium as single cells (solitary neuroendocrine cells), from the trachea to the alveoli, or in groups (neuroepithelial bodies) in intrapulmonary airways. They are more numerous during foetal and neonatal periods and they are assumed to be able to provide a paracrine and haemocrine, as well as neurocrine regulation of the airway function (Becker, 1984).

Using immunohistochemical techniques, serotonin and several regulatory peptides including bombesin/gastrin-releasing peptide (bombesin/GRP), calcitonin-, somatostatin-, cholecystokinin- and leu-enkephalin-containing cells have been identified in intraepithelial neuroendocrine cells (for references see Scheuerman, 1987). More recently, calcitonin gene-related peptide (Cadieux et al., 1986), substance P (Gallego et al., 1990), PYY (Keith and Ekman, 1990) and helodermin (Luts et al., 1991), have also been demonstrated in solitary neuroendocrine cells and neuroepithelial bodies.

Ganglionic cells with APUD and paraneuronal features are found within autonomic ganglia of several species including foetal sheep. They are known as paraganglionic cells, granule-containing cells or small intensely-fluorescent (SIF) cells (Mann, 1971; Knight, 1980; Böck, 1982; Scheuermann, 1987).

In previous studies we reported the presence of serotonin-immunoreactive SIF cells within autonomic intrapulmonary ganglia of foetal sheep (Balaguer et al., 1991). Recently, we identified intraepithelial neuroendocrine or paraneuron cells in embryonic, foetal and postnatal sheep by immunostaining to neuron-specific enolase, Grimelius's method and conventional ultrastructural methods (Balaguer and Romano, 1991). The aim of this paper is to investigate the serotonin and neuropeptide immunoreactive content of the sheep pulmonary paraneurons.

Materials and methods

Rasa Aragonesa sheep embryos, foetuses and young lambs used in our study were obtained at a homologated slaughterhouse (Mercazaragoza). Pregnant ewes and young lambs were examined before and after slaughter by Official Veterinary Meat Inspectors and considered free of respiratory and/or systemic disease.

Animals were distributed in five groups as follows:

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group A, pseudoglandular stage (gestational days 55-65, crown-rump 8-13 cm, three foetuses); group B, canalicular stage (gestational days 95-105, crown-rump 25-30 cm, three foetuses); group C, alveolar stage (gestational days 125-130, crown-rump 39.5-42 cm, three foetuses); group D, postnatal period (three 3 month-old lambs); and group E, embryonic period (gestational days 25-35, crown-rump 14-30 mm, three embryos) (Green and Winters, 1945; Evans and Sack, 1973).

Foetuses and embryos were removed from pregnant ewes immediately after slaughter and processed without being allowed to breath. Embryos were fixed *in toto* by immersion in cold (4 °C) Bouin's solution for 72 h and later in cold (4 °C) 70% ethanol for three days. The tracheas and lungs were removed from foetuses and lambs and 3-5 mm-thick samples were excised from cervical thoracic trachea, primary and lobar bronchi, apical and cardiac segments of the apical-cardiac lobe and additional segments of the left diaphragmatic lobe in pseudoglandular foetuses. Samples were fixed rapidly after excision in cold (4 °C) Bouin's solution overnight and immersed in cold (4 °C) 70% ethanol for three days. All tissues were embedded in paraffin and 4 µm sections were cut.

For immunoperoxidase staining, Bouin's solution-fixed sections were labelled with rabbit polyclonal antiserum to serotonin, bombesin, calcitonin (Incstar Co.), somatostatin and cholecystokinin (Immunonuclear Co.), using a commercially available avidin-biotin-peroxidase complex (ABC) immunostaining kit (Lipshaw Co.). After blocking of endogenous peroxidase, primary antiserum were used at a dilution of 1/1000 in phosphate-buffered saline (pH 7.2) and incubation time was 18-22 hours at 4 °C in a moist chamber. Peroxidase activity in some sections was revealed by incubation 0.05 M tris buffer (pH 7.6) containing 50 mg of 3,3'-diaminobenzidine tetra-hydrochloride (DAB) (Sigma) and 75 µl of 30 vol. hydrogen peroxide in 100 cm^3. After rinsing, sections were lightly counterstained with Carazzi's haematoxylin, dehydrated and mounted. Other sections were revealed by 3-amino-9-ethylcarbazol (AEC), included in the staining kit.

Negative staining controls were performed by replacing the primary antisera with non-immune rabbit serum or phosphate-buffered saline. As positive controls for serotonin and CCK, sections of sheep duodenum were used; sections of sheep pancreas were used as positive controls for somatostatin; thyroid gland for calcitonin and child lung as positive control for bombesin. Additional positive controls included in the kit, Anti Kappal/Lambda, were also used.

For quantitative studies, single immunoreactive epithelial cells were counted with a reticle at x 1000 from 15 randomly-selected fields from sections of cervical and thoracic trachea, primary, lobar-segmental and subsegmental bronchi, and bronchioli of all foetuses and postnatal sheep (total of 90 fields per animal). Mean number of single immunoreactive neuroendocrine cells/100 epithelial cells ± SE was calculated.

Results

During the embryonic period, only serotonin-immunoreactive cells distributed throughout better-differentiated airways were identified in the embryo at gestational day 35 (Fig. 1).

At foetal stages, serotonin-, cholecystokinin- and somatostatin-immunoreactive cells were found. In postnatal period, only serotonin and cholecystokinin immunoreactivity was observed.

Serotonin-immunoreactive cells were found in the airway epithelium of all groups of foetal and postnatal sheep and within autonomic intrapulmonary ganglia at pseudoglandular and canalicular stage. In airway epithelium, they appeared individually (Fig. 2) or in clusters (Fig. 3). Single neuroendocrine cells were

Fig. 1. Serotonin-immunoreactive cells in the undifferentiated bronchial tube of an embryo at gestational day 35. ABC/DAB immunoperoxidase stain. x 400

Fig. 2. Single serotonin-containing cells in the airway epithelium of a foetus at alveolar stage. Note the bifurcation of the cell prolongation (arrow). ABC/DAB immunoperoxidase stain. x 650

Fig. 3. Serotonin-immunoreactive neuroepithelial body in distal airway of a foetus at alveolar stage. ABC/DAB immunoperoxidase stain. x 650

Fig. 4. Serotonin-containing cell in a bronchial gland of a foetus at alveolar stage. ABC/DAB immunoperoxidase stain. x 650

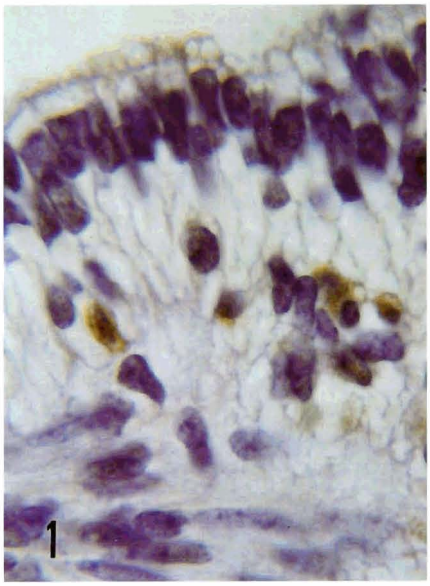
Fig. 5. Solitary cholecystokinin immunoreactive cells in bronchioli of a foetus at alveolar stage. ABC/DAB immunoperoxidase stain. x 650

Fig. 6. Cholecystokinin-containing neuroepithelial body in more distal airway of a foetus at canalicular stage. ABC/DAB immunoperoxidase stain. x 650

Fig. 7. Cholecystokinin-containing cells within an intrapulmonary autonomic ganglia of a foetus at pseudoglandular stage. ABC/DAB immunoperoxidase stain. x 250

Fig. 8. Cholecystokinin-containing cells in close contact with a small blood vessel (*) within an intrapulmonary autonomic ganglia of a foetus at pseudoglandular stage. ABC/AEC immunoperoxidase stain. x 400

Fig. 9. Small somatostatin-containing neuroepithelial body in distal airway of a foetus at pseudoglandular stage. ABC/DAB immunoperoxidase stain. x 650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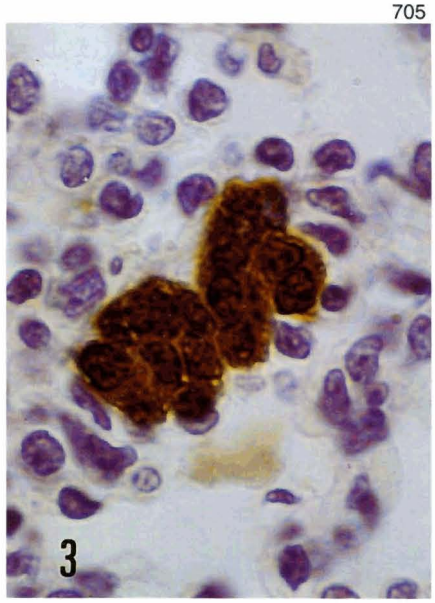
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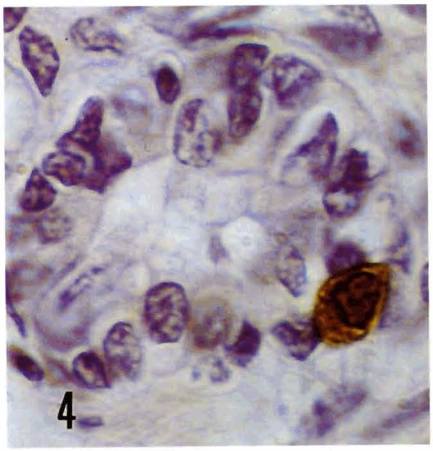
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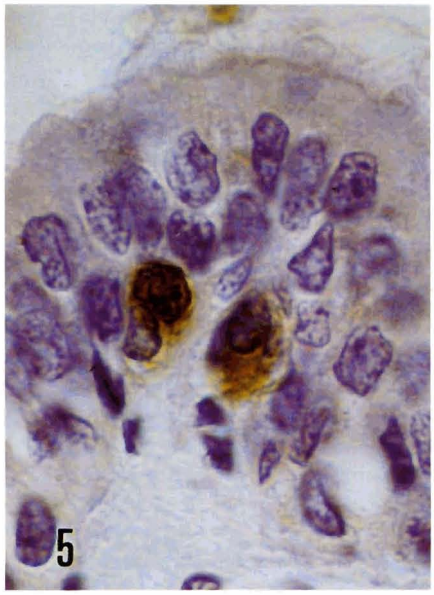
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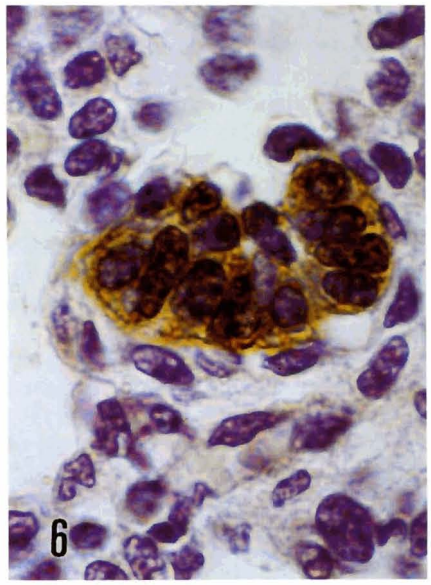
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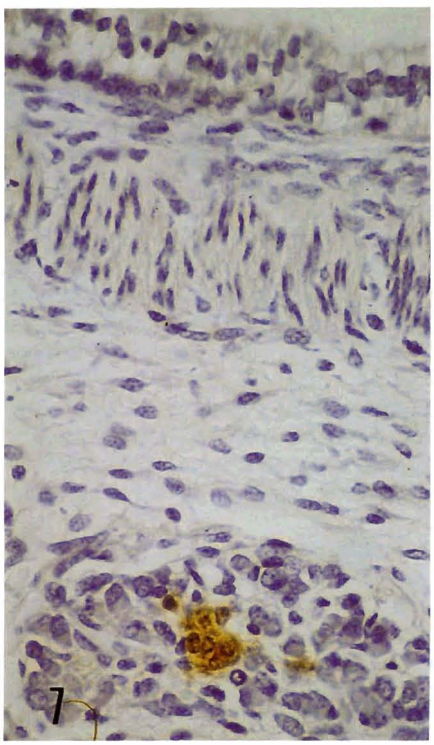
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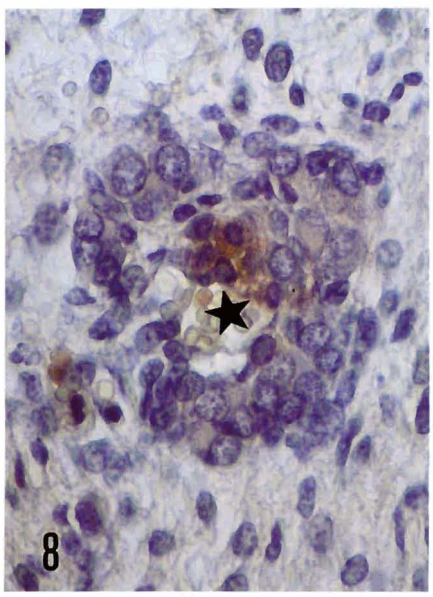
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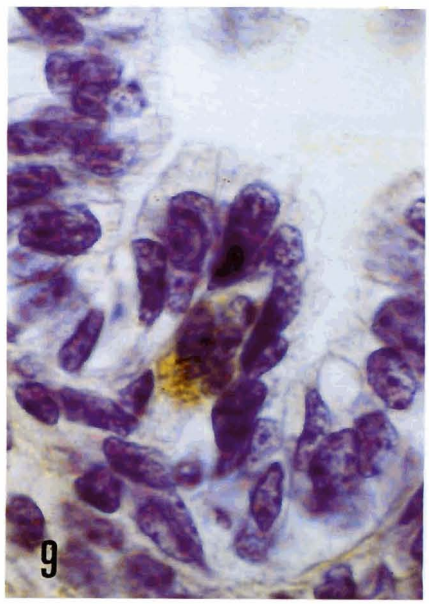
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Table 1. Mean number of solitary neuroendocrine cells/100 epithelial cells ± SE at different stages of lung development.

GROUP		LOCALIZATION					
		C.T	T.T	P.B	Ls.B	Ss.B	br.
A	Ser.	0.14±0.04	0.193±0.091	0.53±0.02	1.52±0.18	1.72±0.68	1.46±0.4
	CCK	*	0.147±0.073	0.28±0.09	0.4±0.0	0.473±0.065	0.21±0.075
B	Ser.	0.033±0.02	0.53±0.027	0.047±0.023	0.14±0.04	0.337±0.09	0.187±0.05
	CCK	0	0	0	*	*	*
C	Ser.	0	*	*	*	0.283±0.057	0.133±0.027
	CCK	0	0	0	*	0.223±0.140	0.067±0.027
D	Ser.	*	*	*	*	*	*
	CCK	0	0	0	0	*	*

C.T, cervical trachea; T.t, thoracic trachea; P.B, primary bronchi; Ls.B, lobar-segmental bronchi; Ss.B, subsegment bronchi; Br., bronchioli.

distributed throughout all levels of the lower respiratory tract, from trachea to alveolar ducts, including the bronchial glands (Fig. 4), while neuroepithelial bodies were only found in intrapulmonary airways.

Cholecystokinin-immunoreactive cells similar to those described above (Figs. 5, 6) were found in airway epithelium in all groups of foetal and postnatal sheep, and in autonomic ganglia at pseudoglandular stages as solitary cells or in clusters (Fig. 7). Occasionally they were seen around or in close contact with wide, thin-walled vessels (Fig. 8).

Occasionally, small clusters of somatostatin-immunoreactive cells were detected in intrapulmonary airways at pseudoglandular stage (Fig. 9).

No immunoreactive cells were observed when using primary antisera antigen against bombesin and calcitonin, although immunoreactivity was observed in appropriate positive-control tissues.

Quantitative results are shown in Table 1. Only serotonin- and CCK-containing solitary neuroendocrine cells were counted and somatostatin-containing cells were only observed in sparse clusters at pseudoglandular stage.

Discussion

With our study we have added new information on the neuropeptide content of the sheep pulmonary paraneurons during lung development. Intraepithelial neuroendocrine cells in the airway of many vertebrate species are known to contain serotonin and/or regulatory peptides (Cadieux et al., 1986; Scheuermann, 1987; Gallego et al., 1990; Keith and Ekman, 1990; Luts et al., 1991), although in sheep, only intraepithelial neuroendocrine cells containing serotonin have been reported (Ceccarelli et al., 1990; Luts et al., 1991). Besides serotonin, we have also identified cholecystokinin- and somatostatin-containing paraneurons.

Sheep intraepithelial neuroendocrine cells begin to appear very early in development, towards the end of the embryonic period (35-day gestation) (Balaguer and Romano, 1991), as solitary cells or as neuroepithelial bodies, and contain serotonin, as we have now demonstrated. This agrees with studies performed on human lungs in which neuroendocrine cells appear in the respiratory epithelium at 8 weeks gestation (early pseudoglandular stage), showing immunoreactivity to serotonin (Cutz et al., 1984).

During foetal and neonatal stages we have revealed the presence of serotonin, cholecystokinin and somatostatin within intraepithelial paraneurons. The quantitative study shows that, as in most species (Scheuerman, 1987), serotonin is the predominant substance, and that single neuroendocrine cells are more numerous in distal conducting airways and at foetal stages (see Table 1). Although there is a general agreement as to the decrease of the number of intraepithelial neuroendocrine cells with age, some studies explain this on the basis of a dilution effect related to lung growth (Redik and Hung, 1984), which could account for the higher number of solitary neuroendocrine cells we have observed at pseudoglandular stage.

In our study we failed to detect immunoreactivity to bombesin/GRP and calcitonin in embryonic, foetal and neonatal sheep. Previous reports in neonatal lambs have detected a bombesin/GRP content, although it was determined by RIA and not by immunocytochemistry (Kulik et al., 1983). The low concentration of this peptide, as determined by RIA, probably accounts for failure to locate it by immunocytochemistry, in which a concentration thereshold must be exceeded in order to reliable differentiate specific staining from background (Ghatei et al., 1982).

Bombesin/GRP-like immunoreactivity has thus far been demonstrated in human, monkey, and occasionally in cat lungs (Cutz et al., 1984; Dayer et al., 1985; Ghatei et al., 1982), while calcitonin has been reported in laboratory animals and man (Scheuerman, 1987). Nevertheless, immunostaining of lung sections from other mammals, including adult sheep, with the same

antibodies has yielded negative results (Sonstegard et al., 1982; Luts et al., 1991). Species variations, presumably due to restricted specificities of antibodies or species differences in the chemical structure of respective substances (Cutz et al., 1986), could be other factors influencing our negative results.

In foetal and postnatal sheep lung, the number of single neuroendocrine cells immunoreactive to neuron-specific enolase, considered a universal marker of the Diffuse Neuroendocrine System (Wharton et al., 1981), was higher than the sum of cells that displayed serotonin and/or cholecystokinin immunoreactivity (Balaguer and Romano, 1991). This fact suggests the possibility of the presence of additional cells which we have failed to detect, as already discussed, or the presence of other substances not used in this study.

In this way, CGRP in several species, PYY in hamster, substance P and leu-enkephalin in man, have been identified in single neuroendocrine cells (Cutz et al., 1981; Stahlman et al., 1985; Cadieux et al., 1986; Scheuermann, 1987; Johnson and Wobken, 1987; Keith and Ekman, 1988, 1990; Gallego et al., 1990).

Our investigation has also revealed the presence of paraneurons within intramural ganglia. Paraneurons or SIF cells of the autonomic ganglia are known to contain biogenic amines and several regulatory peptides (Fujita et al., 1988; Chiba and Masuko, 1989). In intramural ganglia of the lung, the substance responsible for the formaldehyde-induced fluorescence of SIF cells has been identified as dopamine (Scheuermann et al., 1984). Recently, we reported the presence of serotonin-containing SIF cells within autonomic ganglia of the sheep (Balaguer et al., 1991) and to our knowledge this is the first report of cholecystokinin-containing paraneurons in intramural ganglia of the lung.

The morphological features of SIF cells observed are similar to those described previously in other ganglia (Matthews, 1989) in which two types of SIF cells are proposed. One type is solitary, displaying elongated cell processes and is assumed to act as an interneuron. The other type, with few or no processes, forms clusters in close proximity to fenestrated capillaries and possibly functions as a local endocrine-chemoreceptor. In intrapulmonary ganglia, Scheuermann et al. (1984) suggest that these cells, under neural control, may operate in different ways as endocrine or paracrine cells, modulating the activity of the ganglionic neurons, or that they could influence nearby trabecular smooth muscles and could result in general neuroendocrine effects on the pulmonary circulation in the same way as intraepithelial paraneurons.

In conclusion, sheep lung members of the Diffuse Neuroendocrine System and Paraneuronic System are present in two localizations; in the airway mucosa, as solitary neuroendocrine cells and neuroepithelial bodies, and in intramural autonomic ganglia, as single and clusters of SIF cells. They contain serotonin, cholecystokinin and somatostatin. The fact that all these cells share structural features, as well as immunoreactivity to similar neuroactive amines and peptides, strongly suggests the possibility of a morphofunctional relation between them. Nevertheless, further investigations to clarify this point are necessary.

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