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Neuroendocrine lung structures and tumours: immunohistochemical study by specific markers

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Summary. Out of 360 lungs or lobes surgically removed, 13 non neoplastic specimens and 16 neuroendocrine (NE) tumours are investigated with immunohistochemical methods, in order to evaluate the presence of NE structures in normal and pathological human lungs. The markers used are neuron specific enolase (NSE), chromogranin (CG) and the 80 kd antigen (80 kdAg) of NE secretory granules detected by the new monoclonal Phe-5 antibody. In non-neoplastic lung specimens, clearcut immunoreactivity for all three markers appears in NE cells, neuroepithelial bodies (NEB), NE cell-hyperplasias and dysplasias. In the same specimens 4 tumourlets with analogous clearcut immunoreactivities were also observed.

The NE tumours show distinct immunoreactivity for all three antisera in the 8 well differentiated cases. The 8 poorly differentiated tumours are variably immunoreactive for NSE and present low to nil staining with antisera to CG and 80 kdAg.

The immunohistochemical data are interpreted according to current views about a possible relationship between NE tumours and parent normal NE lung structures.

Key words: Neuroendocrine cells, Neuroepithelial bodies, Neuroendocrine tumours, Chromogranin, 80 kd neuroendorine antigen, Neuron specific enolase

Introduction

Lung neuroendocrine (NE) cells and neuroepithelial bodies (NEB) have been widely looked for by morphology, distribution and secretion (Di Augustine and Sonstegard, 1984). Similar immunohistochemical studies have dealt with NE lung tumours, believed to be histogenetically related to NE cells (Gould and Linnoila, 1982; Gould et al., 1983a,b; Gould, 1983; Mosca et al., 1986). The most commonly accepted immunohistochemical markers of the diffuse neuroendocrine system cells and tumours (Solcia et al.,1984) are neuron specific enolase (NSE), chromogranin (CG) and, recently, the 80 kd antigen of neuroendocrine (NE) granules, detected by the newly raised Phe-5 monoclonal antibody.

NSE is a cytoplasmic isozyme of the glycolytic enzyme enolase, mainly located in neurons, NE cells and related tumours (Schmechel et al., 1978; Tapia et al., 1981). Chromogranins, firstly described in adrenal chromaffin granules (Blaschko et al., 1967; Winkler, 1976) are a group of acidic polypeptides of various sizes associated with secretory granules. They have now been recognized in various NE cells and tumours (Rindi et al., 1986). Chromogranin A (CGA) is the largest of the CGs, with molecular weight of 78 Kd, and makes up about half of the total soluble granule proteins of the adrenal medulla. A recently developed monoclonal antibody (LK2H10) against CGA allowed an extensive study on various NE cells (Wilson and Lloyd, 1984) and to confirm the NE nature of argyrophilic cells and related tumours of the breast (Bussolati et al., 1985).

A new monoclonal antibody (Phe-5) has now been raised, which recognizes a polypeptide of 80 kd, present in cytoplasmic secretory granules of a variety of NE cells and tumours (80 KdAg).

NSE has been widely demonstrated in lung NE cells and tumours (Polak and Bloom, 1982; Wick et al, 1983; Linnoila et al., 1984; Springall et al., 1984; Sheppard et al., 1984; Blobel et al., 1985; Rode et al., 1985; Said et al., 1985; Warren et al., 1985; Wilson et al., 1985; Hamid et al., 1986; Nagle et al., 1986; Nomori et al., 1986), although other kinds of tumours can sometimes react, too (Leader et al., 1986). NSE can also be detected in serum of patients with NE lung tumours (Martínez et al., 1985; Pahlman et al., 1986).

NE cells and some small cell carcinomas of the lung have also been immunostained for CGA, (Wilson et al., 1984; Said et al., 1985). Moreover, NE cells, NEBs and various lung tumours have proved to be immunoreactive with antisera against 68 K, 150 K and 200 K dalton components of the neurofilaments (NF) (Lehto et al., 1983; Bergh et al.,

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1984; Lehto et al., 1985; Torikata et al., 1986). Some bronchial carcinoids give a double expression of NF and keratin (Lehto et al., 1985). An additional marker for human neuroendocrine lung tumours is PGP 9.5 (Rode et al., 1985; Hamid et al., 1986).

The aims of the present work are: to investigate the possible evolution from the NE cells and NEBs to hyperplasia, dysplasia, tumourlets and various types of neuroendocrine carcinomas; to check the immunoreactivity of the NE tumours with the available markers; to find out the most reliable markers for diagnostic purposes; to look for possible detection of paraneoplastic syndromes in patients bearing a lung neoplasia histologically ascertained as neuroendocrine in type.

Materials and methods

Out of our surgical material (360 lungs or lobar excisions) 29 cases were selected. Nine specimens were lungs removed for non-NE tumours, four lungs bore chronic bronchiectatic inflammatory disease and sixteen were lungs with NE tumours (Table 1). Lung NE tumours were classified, according to Gould et al., (1983a), Paladugu et al., (1985) and Mosca et al., (1986), in the following groups, as detailed in Table 1:

- neuroendocrine tumours of carcinoid type, or typical carcinoids
- NEC of well differentiated type: NECWED (or atypical, peripheral carcinoids).
- NEC of intermediate cell type: NECINT (poorly differentiated carcinomas).
- NEC of microcytoma type: NECMIC (or small cell carcinomas).

The specimens, fixed in 10% formalin and/or Bouin's fluid, were routinely paraffin embedded and serial sections of each specimen were cut. A section of each series was stained with hematoxylin and eosin, another section with Alcian-blue-PAS and the next one with a modified Grimelius silver impregnation (Lack and Mercer, 1977). The remaining sections, were mounted on glue-coated slides and immunostained (Sternberger, 1970) for NSE, CG and 80 kdAg, using the indirect PAP method or the indirect conjugated method.

Primary antisera were commercially available rabbit polyclonal anti-NSE (Immunonuclear Corp. Cod. 3A048 lot. 8544050), rabbit polyclonal anti-porcine CG (Immunonuclear Corp. Cod 63H2TP Lot. 8541012) and the mouse monoclonal Phe-5 anti-80 KdAg antibody (Ortho Diagnostic System S.P.A. Cod. ENDO, ENZO BIOCHEM., Cat. n. EAB-932 Lot. 51FC2). Anti-NSE was used at kit dilution, anti-CG was diluted 1:200 and the Phe-5 was diluted 1:500. All primary antisera were incubated overnight at 4°C. Swine antirabbit immunoglobulins (Dako Corp. Cod. Z196) and PAP immunocomplex (Dako Corp. Cod. Z113) were used at 1:50 and 1:100 dilutions respectively for 30 min. Rabbit anti-mouse immunoglobulins peroxidase conjugated (Dako Corp. Cod. P161) was used at 1:75 dilution, for 30 min. Peroxidase was evidenced with diaminobenzidine, occasionally with osmium tetroxyde enhancement.

Negative controls were obtained by omitting primary antiserum on a section of each series. As a positive control slides of normal human adrenal medulla were used.

Results

In non neoplastic specimens the Grimelius impregnation allowed the detection of NE cells, NEBs and some other NE structures, interpreted as «linear hyperplasias» or «ne cells dysplasias». Four tumourlets were detected among the specimens; three were argyrophilic and one was not.

«Linear hyperplasias» essentially consisted of a localized increase of NE cells along the basement membrane of bronchial epithelia; «NE cells dysplasias» were larger collections of NE cells, which piled up in the context of bronchial epithelium to give irregular nests extending for variable length and thickness along the bronchial wall. These cells presented large nuclei with coarse chromatin and sometimes clearcut nucleoli (Figs. 2a, 3a). All these NE structures showed prominent immunoreactivity for NSE, CG and 80 KdAg (Figs. 1, 2b, 2c, 3b, 3c, 3d).

The three argyrophilic tumourlets were mainly composed of nests of elongated cells, sometimes spindle-shaped and palisaded (Fig. 4), resembling those of peripheral carcinoids (NECWEDs). The non argyrophilic tumourlet could be classified as a small NECNID. Histologically it showed a mosaic pattern of polygonal cells, with granular eosinophilic cytoplasm and regular oval nuclei.

Some acinar structures were here detectable, too. In a section the tumourlet appeared to fill the bronchial lumen and to extend into adjacent lung parenchyma (Fig. 5a). Its following serial section showed that there was a portion of the tumourlet with exclusive intraluminal growth (Fig. 5b), which merges with an area of NE cell dysplasia (Fig. 5c).

Serial sections of the cited non tumoural specimens were immunostained with polyclonal antisera against NSE and CG and with the newly raised Phe-5 monoclonal antibody which recognizes the 80 KdAg of NE granules. Identical staining patterns were obtained in NE cells, NEBs (Fig. 1), NE linear hyperplasias and dysplasias (Figs 2, 3, 5) and tumourlets (Fig. 4). Slight variations in staining intensity were probably due to different antigenic preservation during fixation and embedding procedures. However, the best results were obtained with the Phe-5 monoclonal antibody (Fig. 5). Interestingly, the non-argyrophilic tumourlet and related dysplasia were clearly immunostained with all three antisera (Fig. 5).

The 16 lung neuroendocrine neoplasms were tested with all above mentioned markers. Argyrophilia was shown by typical carcinoids, by 1 out of 2 NECWEDs, by 1 of 3 NECINTs and by 1 of 5 NECMICs. Staining patterns and intensity displayed variations from diffuse positive staining of all tumour cells to scattered positive cells.

The 6 carcinoids and the 2 NECWEDs revealed

Table 1. Studied cases and immunohistochemical data.

	No. of CASES	ARGYROPHILIA	IMMUNOHISTOCHEMICAL DATA °°												
			N S E				C G				80 KdAg				OTHER
			S	C	D	A	S	C	D	A	S	C	D	A	
NE cells	13 ◇	all +	13				13				13				
NEB	13 ◇	all +	13				13				13				
LINEAR HYPERPLASIA	2 °	all +		2				1	1			2			
NE cell DYSPLASIA	4 °	3 + 1 −	2	2			1	3			1	3			
TUMOURLET (NECNID type)	1 °	−		1				1			1				S-100 − **
TUMOURLET (NECWED type)	3 °	all +		3				3			1	2			S-100 − **
NECNID	6	all +	1	5			2	4			3	3			S-100 − **
NECWED	2	1 + 1 −		1	1		1	1				2			S-100 − **
NECINT	3	1 + 2 −		2		1	1		1	1		1	2		S-100 − **
NECMIC	5 *	1 + 4 −	1	3	1				2	3		1	2	2	S-100 − **

(Notes to Table 1)

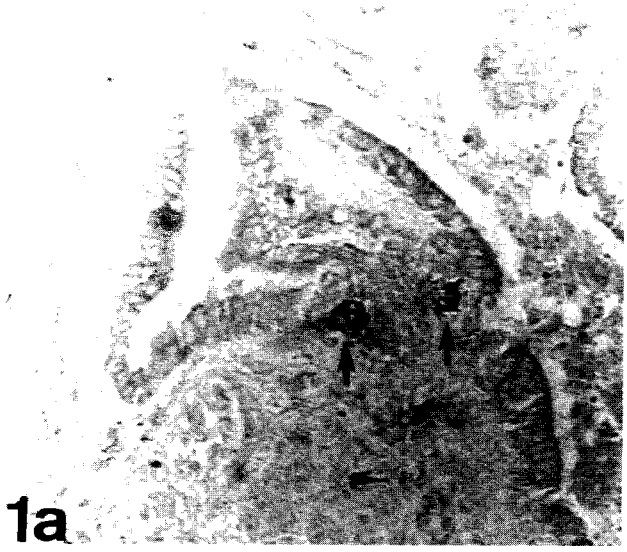
◇: N° of cases from non tumoural specimens examined for detection of NE cells and NEBs. Out of the 13 cases, 4 are bronchiectatic lungs and 9 consist of normal lung parenchyma, adjacent to non-neuroendocrine tumours.

°: Hyperplasias, dysplasias and tumourlets detected in the 13 non-neoplastic specimens.

*: One of these cases is a nodal metastasis of an unresected NECMIC.

°°: Immunostaining is classified as S = strong; C = Clear; D = doubtful and A = absent.

**: Only isolated positive cells are S-100 positive and are interpreted as dendritic reticulum cells; one NECNID, however, shows abundant stellate-shaped immunoreactive cells, as a sort of satellite cell (Barbareschi et al., 1986).



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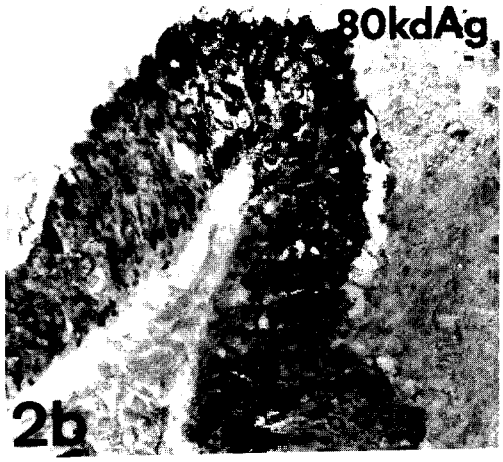
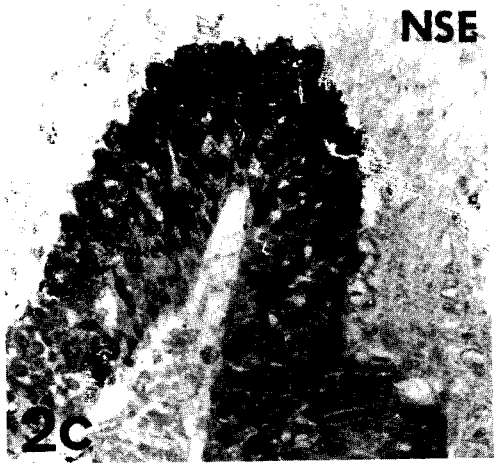


Fig. 1a. Two neuroepithelial bodies immunostained for 80 kdAg (arrows). **b.** Suprarenal medulla used as positive control for the same antigen. Immunoperoxidase without counterstain. × 250



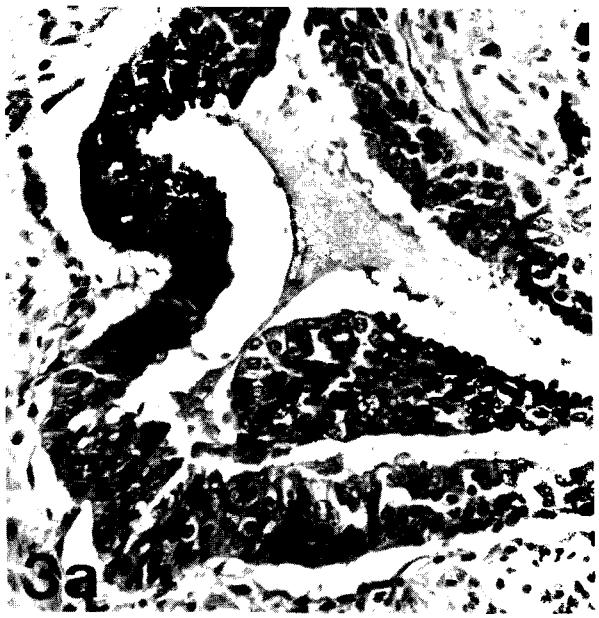
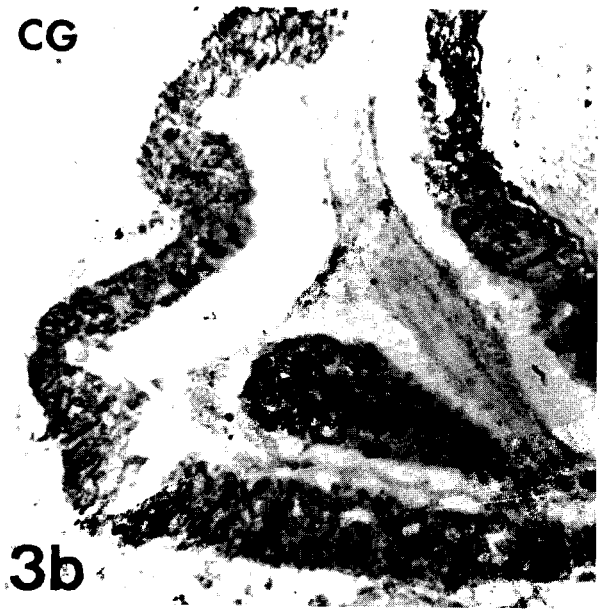
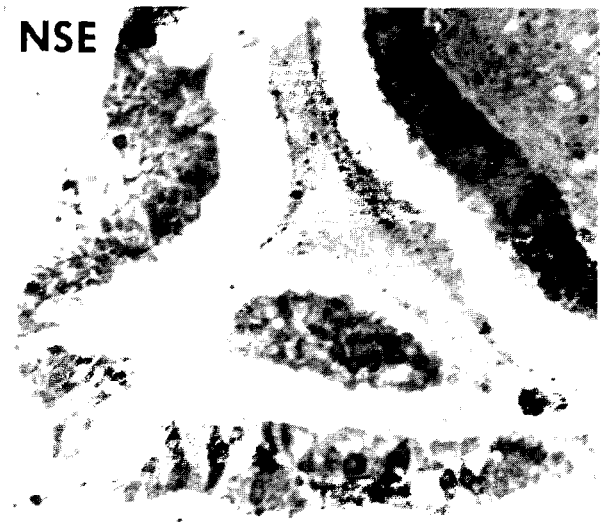


Fig. 2. NE cell dysplasia. **a.** NE cells collect along basement membrane of a small bronchiolus and pile up to form an irregular nest. Haematoxylin and eosin. × 250 **b.** Next section stained for 80 kdAg. Immunoperoxidase without counterstain × 250 **c.** Next section stained for NSE. Immunoperoxidase without counterstain × 250





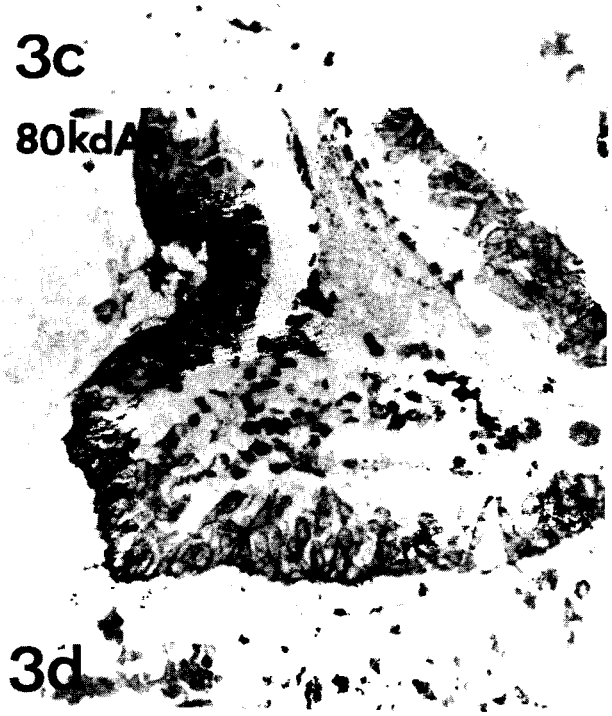


Fig. 3. NE cell dysplasia. **a.** NE Cells are irregularly distributed and show nuclear atypia. Haematoxylin and eosin. × 250. **b,c,d.** Serial sections immunostained for CG, NSE and 80 KdAg respectively. Immunoperoxidase without counterstain. × 250

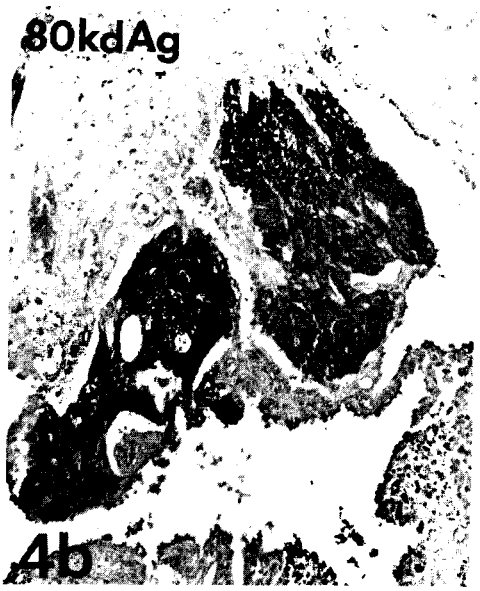


Fig. 4. Tumourlet of the NECWED type. Serial sections show similar CG (**a**) and 80 KdAg-immunoreactivity (**b**). Immunoperoxidase without counterstain. × 100

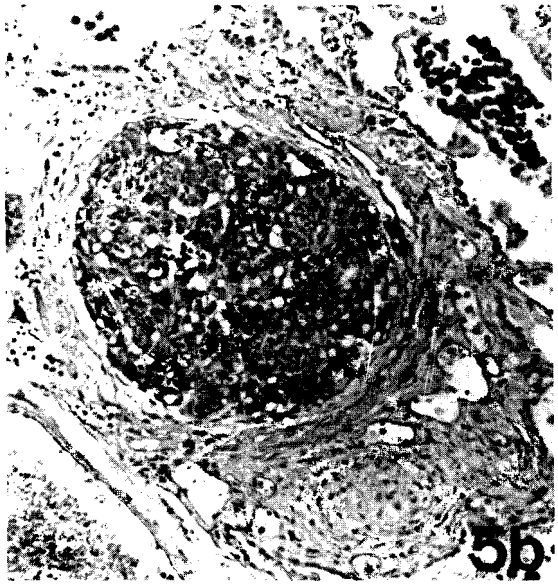
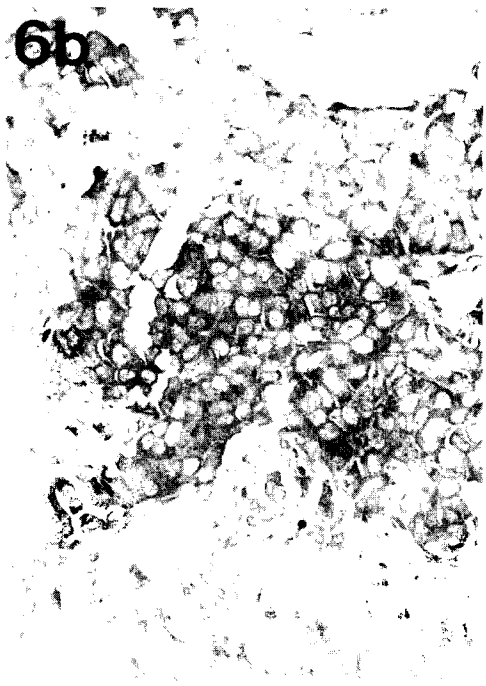


Fig. 5. Tumourlet of the carcinoid type. **a.** The tumourlet cells are arranged in nests extending through the bronchial wall in adjacent lung parenchyma. Haematoxylin and eosin. × 100 **b.** A deeper section shows a completely intraluminal growth of the tumourlet, which is devoid of any argryophylia. Grimelius silver impregnation. × 100 **c.** At a deeper level the tumourlet merges with an area of NE cell dysplasia, which is clearly immunoreactive for NSE, CG and 80 KdAg. Immunoperoxidase, without counterstain × 100



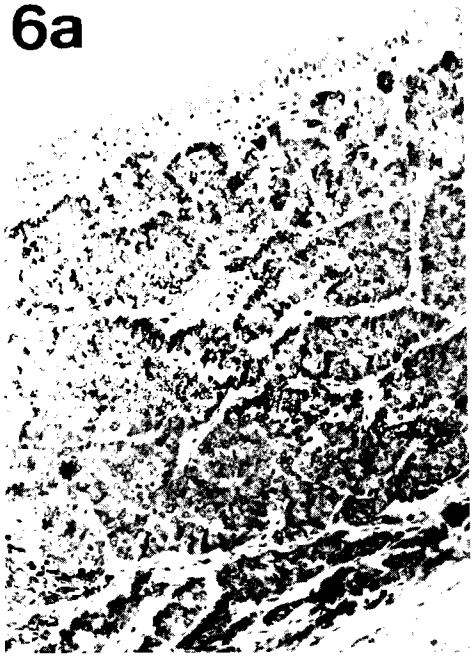


Fig. 6. NE Lung tumours. **a.** Bronchial carcinoid immunostained for the 80 Kd Ag; displaying «strong staining». Immunoperoxidase with haematoxylin counterstain × 100. **b.** Lung «intermediate type» carcinoma (NECINT) immunostained for the 80 KdAg; «clear staining». Immunoperoxidase without haematoxylin counterstain. × 250

strong immunoreactivity for CG, NSE and the 80 kd (Fig. 6). The other less differentiated neuroendocrine carcinomas (3 NECINTs and 5 NECMICs) showed a variable and less intense staining with antisera against NSE and presented very low to absent staining for CG and the 80 KdAg.

In our present series no patients presented clinically detectable paraneoplastic syndrome.

Discussion

Lung neuroendocrine cells (NE cells) were first mentioned by Froelich (1949) and Feyrter (1954), as «Helle Zellen». The local distribution of NEC in human bronchial tree was firstly tentatively suggested by Tateishi (1973). Froelich (1949) not only described the existence of solitary «Helle Zellen», he also recognized that these cells occurred in small clusters («Knotchen»), which were successively called neuroepithelial bodies (NEB) by Lauweryns et al. (1972) and were then described in juvenile and adult human lung (Lauweryns and Goddeeris, 1975).

Although there are some differences between NE cells and NEBs, a great deal of data point out to their common relationships to the so-called APUD system, or diffuse neuroendocrine system (Capella et al., 1978, 1981; Gould , 1983; Gould et al., 1983a,b; Di Augustine et al., 1984 Stahlman and Gray, 1984; Stahlman et al., 1985). Immunohistochemical data support such relationships.

Indeed, in addition to a variety of endocrine polypeptides (Mosca et al., 1986), NE cells and NEBs have proven to contain NSE (Polak et al., 1982; Linnoila

et al., 1984), CGA (Wilson et al., 1984) and the 68 Kd, 150 Kd and 200 Kd components of the neurofilaments (Torikata et al., 1986).

In the present study we find distinct immunoreactivity in human lung NE Cells, NEBs and their hyperplasias using polyclonal antisera against NSE and native porcine chromogranin. Moreover, strong immunostaining is obtained with the newly raised monoclonal Phe-5 antibody. Immunohistochemical data obtained with anti-CG antiserum and with the Phe-5 are similar in our hands.

Since lung neuroendocrine structures are subject to physiopathological hyperplasia and dysplasia (Gould et al., 1983b; Mosca et al., 1986), a great deal of work has been done to relate them to lung tumours of supposed neuroendocrine origin (Gould et al., 1983a,b). NE cells and tumours were investigated for a variety of neuroendocrine markers. NE tumours proved to be immunoreactive for NSE (Wick et al., 1983; Sheppard et al., 1984; Springall et al., 1984; Blobel et al., 1985; Martínez et al., 1985; Rode et al., 1985; Said et al., 1985; Warren et al., 1985; Wilson et al., 1985; Nagle et al., 1986) and for the 68 Kd, 150 Kd, 200 Kd components of neurofilaments (Lehto et al., 1983; Bergh et al., 1984; Lehto et al., 1985). Somewhat different is the immunostaining of NE lung tumours for CG: carcinoids are reported to stain intensely (Said et al., 1985), whereas small cell carcinomas are reported to be either positive (Wilson et al., 1984) or negative (Said et al., 1985). Moreover a great variety of hormones have been immunohistochemically shown in the NE lung tumours, as summarized by Yang et al., (1983), Linnoila and Petrusz (1984), Solcia et al., (1984b), Stahlman et al., (1985) and Mosca et al., (1986).

We must also mention that one of our typical carcinoids displays abundant stellate-shaped S-100 immunoreactive cells (see literature data summarized by Barbareschi et al., 1986). These cells can be interpreted as a sort of satellite cell analogous to those of paragangliomas and pheochromocytomas, in support of the hypothesis of a paraganglioid type of some carcinoids (Capella et al., 1979).

In the present study our attention was particularly pointed to hyperplasias, dysplasias and so-called tumourlets, which can in fact be considered the very first stage of neoplastic growth of all neuroendocrine structures. Indeed, the tumourlets can even metastasize (D'Agati and Perzin, 1985). Interestingly the examined tumourlets, of either the carcinoid or NECWED type, are all immunoreactive for NSE, CG and 80 KdAg, independently from their positive or negative Grimelius reactivity.

All these data suggest that, in fact, the tumourlets eventually appear to be an early stage of neoplastic growth and to be possibly heralded by dysplastic mucosal areas (Figs. 5b, c). These data are in keeping with previous studies and furtherly confirm the relationships of NE cells and NEBs and their derivatives with the diffuse neuroendocrine system. Immunoreactivity of NE cells and SCCs for the Leu-7 antigenic determinant present in natural killer cells (Bunn et al., 1985), and detection in SCCs of an antigen derived from a SCC line, present in a subset of normal and malignant neuroendocrine tissue (Postmus et al., 1986) give further support to such a conviction.

The diagnostic usefulness of combining the Grimelius silver impregnations with the three neuroendocrine markers (NSE, CG and 80 KdAg) can be suggested. Of course, a possible combination of light microscopy with immunohistochemistry and electron microscopy can sometimes give more precise definition of controversial cases (Hammond and Sause, 1985; Warren et al., 1985). In fact, the distinction among the different lung carcinomas is not always easily achieved with conventional histological methods (Mooi et al., 1986), while it is of paramount prognostic and therapeutic importance (Dhillon et al., 1985; Vollmer et al., 1985; Mooi et al., 1986).

Two final considerations can here be added. If we look at the total amount of surgical specimens we studied, the mean frequency of NE derangements we find is about 8% and this figure practically parallels the statement of Carter and Eggleston (1980), who say that about 10% of all lung tumours are hormonically active.

On the other hand, a possible paraneoplastic syndrome is very seldom detectable in the patients before surgery. This does not imply that at a subclinical level an endocrine disturbance due to ectopic hormone secretion can be present. Indeed, we already suggested to the clinicians to withdraw some serum from each lung-Patient to be operated upon, to be able a posteriori to research a hormonal pathology when morphology shows a NE abnormal picture. These investigations are insufficient at the present time.

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