Expression of a *neu/c-erbB-2*-like product in neuroendocrine cells of mammals

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Summary. The *neu/c-erbB-2* oncogen encodes a 185 kDa protein closely homologous to the epidermal growth factor receptor. The protein product (p185) is a glyco-protein with an external domain and an internal domain with tyrosine kinase activity. Amplification and/or overexpression of p185 is related to several human adenocarcinomas. Subsequent studies demonstrated its presence in certain neuroendocrine (NE) neoplasms, including phaeochromocytomas, insulinomas and medullary thyroid carcinomas. However, relatively little is known about its role in normal cell growth regulation and development. Therefore, our objetive was to determine whether *neu/c-erbB-2* was expressed in normal NE tissues of different mammals, specially in humans, as it was in their neoplasms. We have examined by immunohistochemistry different endocrine glands (thyroid, pancreas, suprarrenal and hypophysis) and the small intestine of human beings, rats and guinea pigs, using two polyclonal antibodies raised against the intracytoplasmic part of the protein, and specific antigen absorption controls. We have found that a *neu/c-erbB-2*-like product occurs in all normal NE tissues examined: C cells of the thyroid gland, chromaffin cells of the adrenal medulla, pancreatic islets, enteroendocrine cells of the small intestine and, finally, scattered cells of the adenohypophysis, according to a typical granular immunohistochemical pattern. Our results indicate that normal NE cells share a new common antigen in their cytoplasms, a *neu/c-erbB-2*-like product, with a similar immunostaining pattern to that presented by the neoplasms derived from them.

Key words: *neu/c-erbB-2* oncoprotein, Neuroendocrine cells, Immunohistochemistry, C cells, Chromaffin cells

Introduction

The *neu* oncogene was first described in rat neuro-blastomas, induced by treatment with ethylnitrosourea (Schechter et al., 1984). The human version of the *neu*

oncogene is also known as *c-erbB-2* or *HER-2*. This oncogene encodes a 185-kDa protein closely homo-logous to, but different from, the epidermal growth factor receptor (Bargmann et al., 1986; Dougall et al., 1994). The protein product, termed p185, is a glyco-protein with an extracellular ligand-binding domain, a transmembrane anchoring domain and an intracellular domain with tyrosine kinase activity (Coussens et al., 1985).

Much of our understanding of the physiological function of p185 is derived from observations in transformed cells. Thus, the expression of p185 is necessary for maintenance of the malignant phenotype of cell lines transformed by *neu* (Drebin et al., 1986). In addition, many studies have demonstrated amplification and/or overexpression of c-erbB-2 in several human adenocarcinomas, including those of breast and ovary (Slamon et al., 1989), pancreas (Hall et al., 1990) and urinary bladder (Coombs et al., 1991). However, relatively little is known about its role in normal cell growth regulation and tissue development. Several studies have been performed to determine specific expression of p185 in normal mammalian fetal and adult tissues. Kokai et al. (1987) have shown that the *neu* gene is expressed during normal development in the rat in a tissue- and stage-specific manner. p185 could be detected in neural and connective tissue during a relatively short embryonic period, but in several epithelial tissues expression of *neu* gene persisted into adulthood. In rats and humans, studies have reported expression of p185 in fetal and adult kidney (De Potter et al., 1987; Press et al., 1990), intestine (Quirke et al., 1989; Press et al., 1990) and female reproductive tract (De Potter et al., 1987; Quirke et al., 1989). The results of these studies imply that p185 has specialized functions in development and growth regulation of normal cells.

Nevertheless, none of the previous studies have analysed the expression of p185 in normal NE tissues, a subject of considerable interest taking into account that most of them are epithelial tissues with neural origin. Recently, overexpression of c-erbB-2 has been reported in several NE tumours, such as insulinomas, pulmonary atypical carcinoids and phaeochromocytomas (Roncalli et al., 1991; Castilla-Guerra et al., 1997). Our laboratory has also identified a significant p185 expression in all

medullary thyroid carcinomas (Utrilla et al., 1999). Surprisingly, the immunohistochemical pattern of *c-erbB-2* reactivity in all these NE tumours varied from the typical pattern found in adenocarcinomas -membrane and diffuse cytoplasmatic patterns- (Slamon et al., 1989) and in the normal mammalian tissues from which those tumours are derived -cytoplasmic pattern- (Kokai et al., 1987; Quirke et al., 1989; Press et al., 1990). NE tumours showed a granular cytoplasmic immunoreactivity with the antibody raised against the terminal part of the intracytoplasmic domain of the *c-erbB-2* oncoprotein, the same pattern presented by the central nervous system according to Stieber (1995) in adult rats.

To elucidate the potential biological function of the *c-erbB-2* oncoprotein in normal NE cells and their neoplasias, we sought to determine whether the *neu/c-erbB-2* gene is expressed in different NE mammalian tissues. Using an immunohistochemical technique and different antibodies, we have demonstrated that all NE tissues that have been investigated presented an evident *neu/c-erbB-2*-like immunoreactivity with a typical granular cytoplasmic pattern, whose possible meaning is discussed in the present paper.

Materials and methods

Tissue samples

We have studied normal adult thyroid, pancreas, suprarrenal and pituitary glands and small intestine of human beings, rats and guinea pigs. Furthermore, we have also studied human fetal thyroid glands. The samples were fixed either in 10% formalin, in 4% paraformaldehyde or in Bouin's solution and embedded in paraffin.

Immunohistochemistry

Two antibodies were used: (1) Rabbit antibody raised against the intracytoplasmic part of the human *c-erbB-2* oncoprotein (A-485, DAKO, Denmark), at a dilution of 1/1000-1/2000; and (2) rabbit antibody raised against a peptide (residues 866-873) from the kinase domain of the human *c-neu* gene product (*c-neu* (Ab-1), Oncogene Science, MA, USA), at a dilution of 1/100.

The *c-erbB-2* oncoprotein was demonstrated by a three-stage streptavidin-biotin complex immuno-

peroxidase technique. Sections were cut at 5 micrometers thick, mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma, USA), and then dried at 60 °C for 1 h to promote adherence to the slide. After deparaffination and rehydration, a microwave oven pretreatment for antigen retrieval was carried out before immunostaining (Shi et al., 1991). The slides were transferred to a plastic Coplin jar filled with citrate buffer 0.1M, pH 6, and covered with a cap to minimise evaporation. Four Coplin jars were placed, evenly spaced, in a domestic microwave oven (Toshiba, ER 68608W) and irradiated for 4 min at 750 W power, and this cycle was repeated up to three times. After heating, the slides were allowed to cool to room temperature for 15 min. After a short rinse in PBS (0.01M phosphate-buffered saline, pH 7.2), the sections were treated with 3% hydrogen peroxide in distilled water for inhibition of endogenous peroxidase activity, washed in PBS, and then sequentially incubated with normal serum (Operon, Spain) diluted 1:10 in PBS (for 20 min at room temperature), specific antiserum (overnight at 4 °C), biotinylated antiserum to rabbit/mouse immunoglobulins (DAKO, LSAB 2 kit, CA, USA) for 30 min, and streptavidin-biotin-peroxidase complex (DAKO, LSAB 2 kit, CA, USA) for 30 min. Washing in PBS (three changes of 3 min each) was performed after antiserum incubations. Peroxidase activity was developed with diaminobenzidine tetrahydrochloride substrate (Sigma, USA). Sections were then counterstained with haematoxylin, dehydrated, cleared, and mounted. When immunopositive cells appeared, their NE character was confirmed in subsequent sections by using anti-calcitonin (only in the thyroid gland) or anti-chromogranin antibodies (DAKO, Denmark).

Positive control sections (breast carcinoma) were included in each bath of slides to ensure consistent immunostaining results. Specific controls included substitution of primary antibody with pre-immune serum from the same animal and prior absorption of the primary antibodies with the respective immunising peptides (courtesy of DAKO, Denmark; *c-neu* Peptide-1, Oncogene Science, MA, USA) at room temperature for 2 h.

Results

All NE human tissues investigated in the present

Fig. 1. Immunohistochemical demonstration of a *neu/c-erbB-2*-like product in a human thyroid gland of an adult. Note uniform, granular immunostaining of the entire C-cell population and negativity of thyroid follicles and stroma. The tissue was fixed in formalin and immunostained with the DAKO antibody. A, x 200; B, x 1,000.

Fig. 2. High power view of a fetal human thyroid gland. C cells are prominent and uniformly granular immunostained. The tissue was fixed in formalin and immunostained with the *c-erbB-2* DAKO antibody. x 1,000

Fig. 3. Section of the thyroid gland of a guinea pig. C cells are delicately immunostained showing a diffuse granular pattern. The tissue was fixed in paraformaldehyde and immunostained with the *c-erbB-2* Oncogene antibody. x 400

Fig. 4. Section of the suprarrenal gland of a human being. All chromaffin cells show a conspicuous immunoreactivity in contrast with the negativity of the adrenal cortex. Higher magnification of the adrenal medulla reveals a diffuse granular pattern. The tissue was fixed in formalin and immunostained with the *c-erbB-2* DAKO antibody. A, x 200; B, x 400
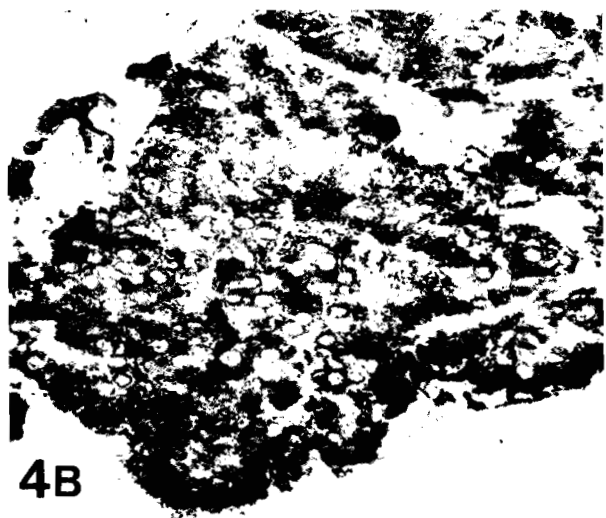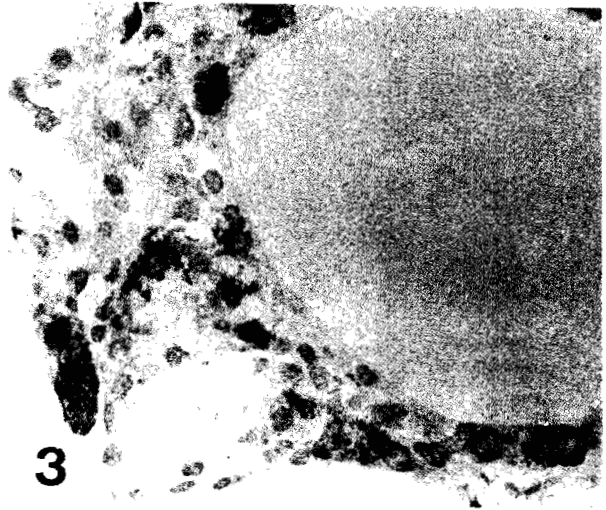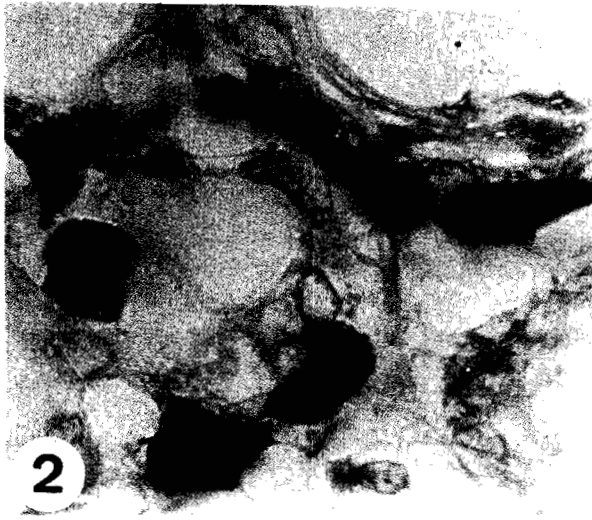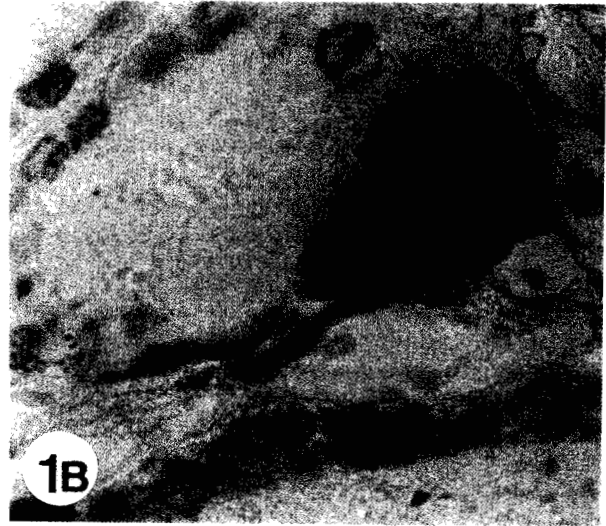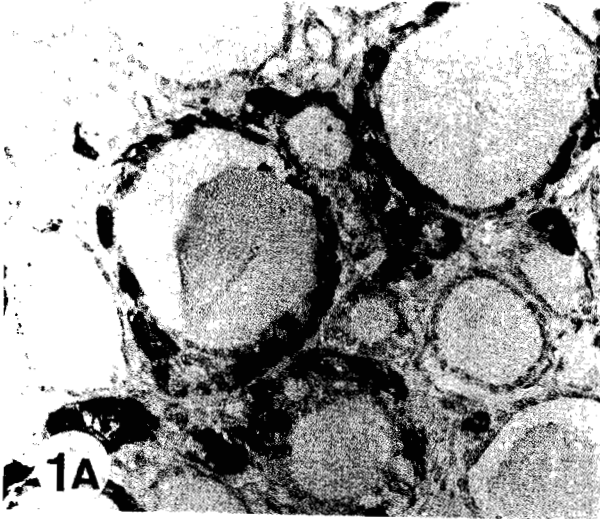
study showed a conspicuous immunostaining for the polyclonal *c-erbB-2* antibody manufactered by DAKO (Table 1). The immunoreactive cells presented a prominent granular cytoplasmatic pattern. C cells were immunostained both in adult and fetal thyroid glands and showed a dark *c-erbB-2*-like product distributed as a punctate material diffusely in the cytoplasm (Figs. 1, 2). A similar immunohistochemical pattern was presented by apparently all pancreatic endocrine cells (Fig. 9), enteroendocrine cells of the small intestinal epithelium (Figs. 7, 8), scattered cells on the adenohypophysis (Fig. 5) and, finally, the chromaffin cells of the adrenal medula (Fig. 4). The nervous myenteric plexus located in the small intestinal wall also presented a granular immunostaining pattern coinciding with the soma of ganglion cells or diffusely around them, which may be suggestive of synaptic localizations. A similar immunohistochemical pattern was found in thyroid, pancreas and small intestine after applying the polyclonal antibody raised against the peptide (residues 866-873) of the internal domain of the *c-neu* product (Oncogene). However, the material showed a lower intensity.

In regard to adult NE tissues of the rat, an evident immunoreactivity was only found in scattered cells of the adenohypophysis, when the gland was fixed in Bouin's solution and immunostained with the DAKO antibody (Fig. 6). Furthermore, a faint immunopositivity was also observed in C cells of the thyroid gland fixed in paraformaldehyde and immunostained with the polyclonal antiserum produced by Oncogene.

Finally, all NE tissues of the guinea pigs showed a certain degree of immnunopositivity, either using the DAKO antibody (pancreas, small intestine and adrenal medula) or Oncogene antibody (thyroid, pancreas and small intestine), but the second ones were always less intensive (Fig. 3).

All the granular immunostainings disappeared completely after adding the free peptides against which both antibodies, DAKO and Oncogene, were raised, respectively.

To exclude the possibility that the immunization peptides could exhibit certain homology to other known peptides or proteins, we have introduced their sequences in a protein database (SWISS-PROT/TrEMBL). As a result of this examination only the expected coincidences with proteins belonging to the EGF-Receptor family appeared, as *c-erbB-2* is a member of this family. No sequence homology with synaptophysin or chromo-granin was found. Nevertheless, we cannot completely exclude that the antibodies used in the present study do not crossreact with those proteins that are highly expressed in many NE cells. However, this possibility is inherent to the immunohistochemical techniques in general.

Discussion

In the present study the existence of *neu* product in different NE tissues from diverse mammals has been studied, finding a variable immunoreactivity with a granular cytoplasmic pattern in the thyroid C cells, the pancreatic endocrine islets, the enteroendocrine intestinal cells, scattered cells in the adenohypophysis and, finally, the chromaffin cells of the suprarenal gland. The immunostaining was mainly obtained with the rabbit polyclonal antiserum raised against the terminal part of the internal domain of *c-erbB-2* human oncoprotein (DAKO), being completely abolished after absorption with the immunising peptide (courtesy of DAKO). It is the first time that an exhaustive study of *neu* product in normal adult and fetal NE tissues has been carried out and that the existence of *neu/c-erbB-2*-like immunoreactivity in these has been confirmed.

The *neu* oncogene was first identified as a tranforming gene in chemically-induced neuroglio-

Table 1. *neu/c-erbB-2*-like expression in mammalian adult NE tissues using DAKO antibody.

| NE TISUES	HUMANS	RATS	GUINEA PIGS
Thyroid: C cells	++++	-	-
Pancreas: islets	++++	-	+
Small intestine: enteroendocrine cells	++++	-	+++
Adrenal medulla	++++	-	++
Pituitary gland	++++	++	N.D.*

N.D.: non determined.

Fig. 5. Immunohistochemical demonstration of a *neu/c-erbB-2*-like product in a human pituitary gland of an adult. Note the presence of numerous scattered cells showing an evident granular immunoreactivity. The tissue was fixed in formalin and immunostained with DAKO antibody. x 400

Fig. 6. Section of a pituitary gland of the rat. There are scarce cells immunostained with the antibody. The tissue was fixed in Bouin's solution and immunostained with the *c-erbB-2* DAKO antibody. x 400

Fig. 7. Immunohistochemical demonstration of a *neu/c-erbB-2*-like product in the Brünner's glands of the human duodenum. The glands present abundant enteroendocrine cells prominently immunostained. The tissue was fixed in formalin and immunostained with DAKO antibody. x 200

Fig. 8. Immunohistochemical demonstration of a *neu/c-erbB-2*-like product in the intestinal villi of the human duodenum. Enteroendocrine cells show a prominent granular immunoreactivity located at the basal cytoplasm. The tissue was fixed in formalin and immunostained with DAKO antibody. x 400

Fig. 9. Photomicrography of human pancreatic islets from an adult. Note uniform, granular immunopositivity of the entire islets population and negativity of pancreatic acini and stroma. The tissue was fixed in formalin and immunostained with the *c-erbB-2* DAKO antibody. A, x 200; B, x 400

5

6

7

8

9A

9B
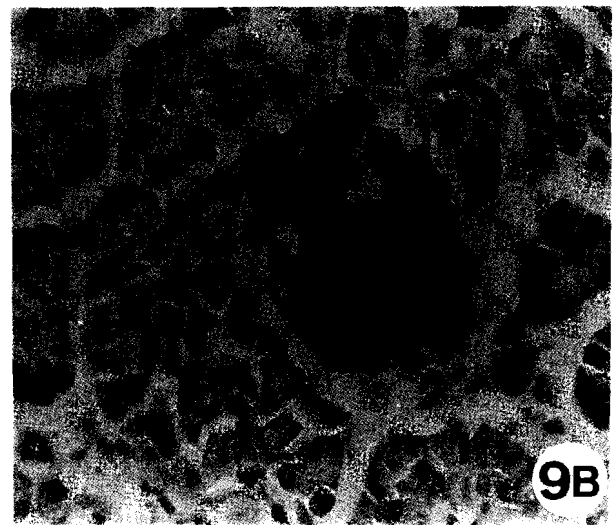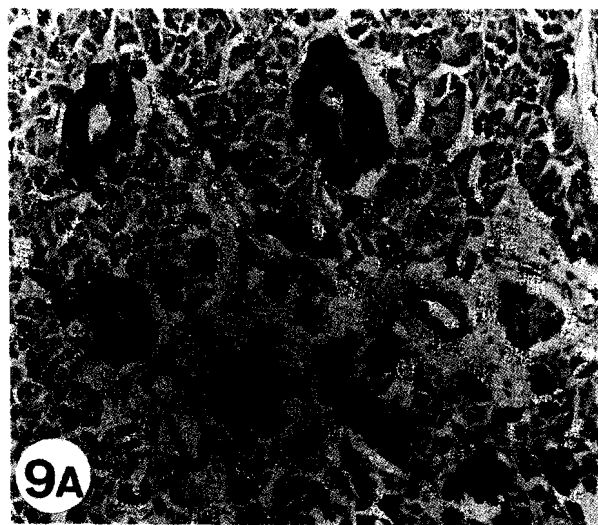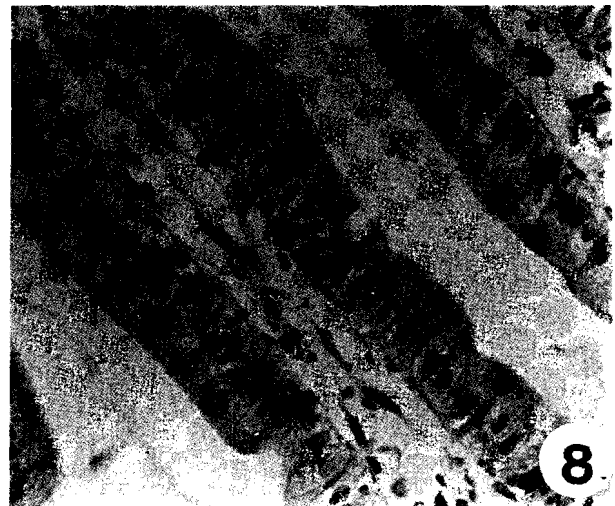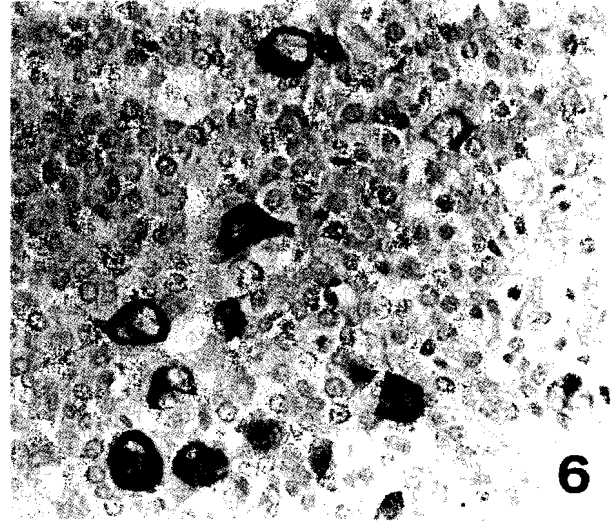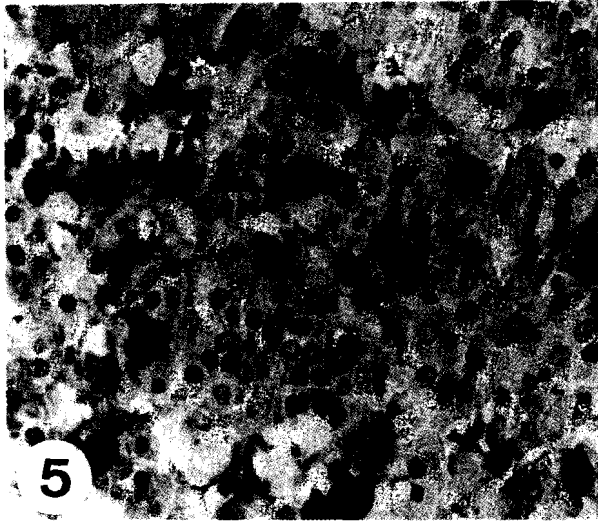
blastomas (Schechter et al., 1984). Subsequently, amplification and/or overexpression of the human form of *neu* in several human adenocarcinomas was demonstrated (Slamon et al., 1989; Hall et al., 1990; Coombs et al., 1991). Initially, an immunohistochemical pattern characteristically membranous of *c-erbB-2* positivity, exclusive to the tumoral cells, was described in those tumours, and which was correlated with the presence of a 185 kDa protein by means of Western blot analysis (De Potter et al., 1987). However, subsequent reports described the existence of diverse tumours (bladder, thyroid gland, pancreas) showing a different cytoplasmic immunostaining pattern, whether they coincided or not with the membranous pattern, which corresponded with a 155 kDa protein by Western blot analysis (Hall et al., 1990; Coombs et al., 1991; Soares et al., 1994). According to De Potter et al. (1987) this could be a *neu*-like cross-reacting protein or a different *neu* oncogene product derived by alternative splicing from the same gene and having a different destination in the cell. Other authors reported that p155 may represent precursor forms of the *c-erbB-2* molecule (Corbett et al., 1990; Coombs et al., 1991; Soares et al., 1994), since inhibition of N-linked glycosylation by tunicamycin has been shown to produce an immunoprecipitable protein of 155-160 kDa in human cells which express *c-erbB-2* (Akijama et al., 1986). Nevertheless, the real meaning of this protein has still not been made definitely clear.

With regard to the immunohistochemical staining pattern for *c-erbB-2* found in the present study, the granular cytoplasmic pattern had not been described until now in any kind of tumour, except diverse NE neoplasias, such as phaeochromocytomas, insulinomas, pulmonary atypical carcinomas and, according to our own experience, in medullary thyroid carcinoma (Roncalli et al., 1991; Castilla-Guerra et al., 1997; Utrilla et al., 1999). Considering all results together, we can assert that both normal and tumoral NE cells share the same pattern for *c-erbB-2* immunoreactivity. The meaning of the granular cytoplasmic pattern may be the same as for the diffuse cytoplasmic pattern described by De Potter et al. (1987), a *neu*-like cross-reacting protein. Alternatively, it may represent membrane traffic of the newly synthesized protein, since immunoelectron microscopic studies have shown the presence of *c-erbB-2* immunoreactivity in cytoplasmic elements such as endoplasmic reticulum (Mori et al., 1987) or in endosomal compartments (Miller et al., 1986).

Nevertheless, we are tempted to suggest an alternative explanation for this. Recently, Stieber et al. (1995) described the presence of a p140 kDa protein in membrane synaptosomes of rat central nervous system using a polyclonal antiserum raised against the carboxy-terminal part of the p185 *neu* rat protein. The same antiserum reacted with a 185 kDa protein in microsome membranes from rat phaeochromocytoma cells. In both cases, the immunohistochemical pattern was finely granular and coincided in the central nervous system with synaptic localizations. The identity of the 140 kDa

protein was unknown, but the authors suggested that it could be related to p185 *neu* protein or to other kinases. Considering that neural cells and NE cells share a common origin in the neuroectoderm, they may also share many differente features, as is generally accepted. One of the best known common features is the presence of synaptophysin as an immunohistochemical marker for both neural and NE cellular systems. Synaptophysin is found in synaptic vesicles (Jahn et al., 1985), in adrenal chromaffin granules and in secretory granules of all NE cells (Gould et al., 1986; Buffa et al., 1987). In fact, parafollicular cell granules have been considered analogous to the synaptic vesicles of serotonergic neurons (Barash et al., 1987). Weiler et al. (1989) have also described other common membrane antigens in vesicles obtained from neural crest-derived endocrine cells, like cytochrome b-561 and carboxypeptidase H, enzyme involved in the processing of peptides. As happens with synaptophysin and these other antigens, a *neu/c-erbB-2*-like product may be shared by synaptic vesicles, chromaffin granules and secretory granules of NE cells.

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