A comparative immunohistochemical study of phaeochromocytomas and paragangliomas

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Summary. There is no definite morphological distinction between phaeochromocytomas and paragangliomas. We, therefore, attempted to determine the universality and differential utility of a panel of tumour markers for diagnosis in formalin-fixed, paraffin-embedded specimens. Antibodies to neuron-specific enolase (NSE), chromogranin, synaptophysin, Leu-7, neurofilaments, cytokeratins, carcinoembryonic antigen (CEA), melanoma antigen HMB-45, S-100 protein and glial fibrillary acid protein (GFAP), were used on 11 phaeochromocytomas and 8 paragangliomas. NSE reactivity was detected in 10 phaeochromocytomas and in all paragangliomas. Chromogranin reactivity was found in all but two cases (one phaeochromocytoma and one paraganglioma). Synaptophysin reactivity was present in 10 phaeochromocytomas and in the 8 paragangliomas. Ten phaeochromocytomas stained for Leu-7, but none of the paragangliomas did. S-100-positive cells (sustentacular or type II cells) were found in 8 phaeochromocytomas and 7 paragangliomas. GFAP stained sustentacular cells of only one paraganglioma. Only in 5 phaeochromocytomas was there a focal reaction by neurofilaments. Cytokeratins, CEA and HMB-45 were never detected. We conclude that NSE, chromogranin, synaptophysin and S-100 protein are useful markers of both types of tumour, whereas GFAP staining is limited to a small number of these neoplasms. Leu-7 reactivity seems to favour diagnosis of phaeochromocytoma rather than paraganglioma, but further studies with larger series are needed to confirm this. Unlike previous reports, we did not find cytokeratin or HMB-45 immunostaining in any case.

Key words: Phaeochromocytomas, Paragangliomas, Immunohistochemistry, Tumour markers

Introduction

The paraganglion system is composed of widely dispersed collections of cells of two types (Kliewer et al., 1989). Chief (type I) cells contain neurosecretory granules and derive from the neural crest. Sustentacular (type II) cells are associated satellite elements that lack neurosecretory granules. Tumours arising from adrenal or extraadrenal chromaffin tissue, and which are usually related to overproduction of catecholamines, are termed phaeochromocytomas. Tumours arising from para-sympathetic-related (non-chromaffin) organs generally do not give rise to clinical evidence of neuroendocrine hyperfunction and are termed paragangliomas (Capella et al., 1988).

The purposes of this work were to study a) the usefulness of the neuroendocrine markers, neuron-specific enolase (NSE), chromogranin, synaptophysin and Leu-7, for recognition of phaeochromocytomas and paragangliomas; b) the prevalence of sustentacular cells, as identified with antibodies to S-100 protein and glial fibrillary acid protein; c) the cytoskeletal characteristics of these tumours (using antisera to neurofilaments and cytokeratins); d) the existence of signs of glandular differentiation (by detection of carcinoembryonic antigen (CEA)); e) the presence of HMB-45 (melanoma antigen) immunoreactivity, recently described in phaeochromocytomas (Unger et al., 1992); and f) whether on the basis of the foregoing it is possible to distinguish immunohistochemically between phaeo-chromocytomas and paragangliomas, which are often morphologically indistinguishable (Rosai, 1989).

Materials and methods

Eleven phaeochromocytomas and eight para-gangliomas were examined. All had been fixed in 10% buffered formalin and embedded in paraffin. 5 μm-thick sections were mounted on glass slides coated with chromealum-gelatin and dried overnight at 37 °C. After dewaxing and rehydration, the sections were surrounded

by Sigmacote (Sigma, St. Louis, USA) and rinsed with 0.01 M phosphate-buffered saline (PBS) pH 7.4. Sections assigned for cytokeratin detection were mounted on poly(L-lysine)-coated slides, dried, dewaxed, rehydrated and digested wth 0.1% trypsin for 30 min at 37 °C. The biotin-streptavidin-horseradish peroxidase technique was employed for immuno-histochemistry. Following blocking steps, the sections were consecutively incubated: 1) for 1h at room temperature in rabbit antisera to CEA (Dako Corp., 1:1000) or S-100 protein (Dako Corp., 1:500), or mouse antisera to NSE (Dako Corp., 1:1000), chromogranin (Biogenex, 1:200), Leu-7 (Becton-Dickinson, 1:100), synaptophysin (Biogenex, 1:200), GFAP (Dako Corp., 1:50; Biogenex, 1:500), neurofilaments (70-200 kd, Dako Corp., 1:100), cytokeratins (AE1-AE3, Boehringer, 1:400; CAM 5.2, Becton-Dickinson, prediluted) or HMB-45 (Enzo, 1:1000); 2) for 30 min at room temperature in biotinylated antibodies to rabbit or mouse (Biomakor, Rehovot, Israel) at a dilution of 1:30; 3) for 30 min at room temperature in streptavidin peroxidase (Biomakor, Rehovot, Israel) at a dilution of 1:30; 4) for 10 min at room temperature in 3,3'diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, USA) at a dilution of 0.06% with 0.003% H_2O_2. Between steps, the sections were rinsed with PBS (2x5 min). After step 4, they were rinsed with distilled water, dehydrated, cleared and mounted.

All antigen searches were performed in parallel with positive and negative controls; for negative controls, immunoreaction steps were omitted or the primary antiserum was substituted for normal rabbit or mouse serum.

The immunohistochemical results were graded as follows: a) for chief cells: -, negative; +/-, doubtful; +, less than 30% positive; ++, 30-60%; +++, more than 60%; b) for sustentacular cells, the number of immuno-reactive cells per high-power field (HPF) was counted for the whole available section and graded according to the following score: -, no reactive cells; +, 1-10 reactive cells; ++, 11-20 cells; +++, more than 20 sustentacular immunoreactive cells per HPF (Achilles et al., 1991).

Fresh tissue from seven tumours (five phaeochromo-cytomas and two paragangliomas) was fixed in cacodylate-buffered 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in Spurr resin, and ultrathin sections were cut, stained with uranyl acetate and lead citrate and observed in a Jeol 100B electron microscope.

Results

Phaeochromocytomas

The ten phaeochromocytoma patients were seven female and three males, and ranged from 6 to 66 years in age (mean 34.3 years). All had a clinical history of overproduction of cathecolamines. Eight patients had the tumours located in the adrenal gland (one bilaterally) and the other two had paraaortic neoplasms. Their maximum diameters were between 4 and 9.5 cm.

Tumour cells were generally polygonal, with a rather

Table 1. Immunohistochemistry of phaeochromocytomas and paragangliomas.

CASES	NSE	CG	SYN	LEU-7	NF	CK	CEA	HMB-45	S100*	GFAP*
Phaeochromocytomas										
1	+++	+++	+++	+++	-	-	-	-	+	-
2	+++	+++	+	+	+	-	-	-	++	-
3	+++	+++	+++	+	-	-	-	-	+	-
4	+++	+++	+++	+	-	-	-	-	+	-
5	+++	+++	-	-	-	-	-	-	+	-
6	+++	+++	+++	++	+	-	-	-	-	-
7	++	+++	+	++	-	-	-	-	+	-
8	+++	++	+++	++	+	-	-	-	++	-
9	+++	+++	+++	++	+	-	-	-	-	-
10	+++	+++	+++	+++	+	-	-	-	++	-
11	+/-	-	+++	++	-	-	-	-	-	-
Paragangliomas										
12	+++	-	+++	-	-	-	-	-	+	-
13	+++	+++	+++	-	-	-	-	-	+++	-
14	+++	++	+++	-	-	-	-	-	++	-
15	+++	+++	+++	-	-	-	-	-	+++	+++
16	+++	++	+++	-	-	-	-	-	+	-
17	+++	++	+++	-	-	-	-	-	++	-
18	++	+++	+++	-	-	-	-	-	+	-
19	++	++	+++	-	-	-	-	-	-	-

Abbreviations: CG, chromogranin; SYN, synaptophysin; NF, neurofilaments; CK, cytokeratins; *, only in sustentacular cells. Immunoreactivity of chief cells: -. negative; +/-, doubtful; +, less than 30% positive; ++, 30-60% positive; +++, more than 60% positive. Immunoreactivity of sustentacular cells (S-100 protein and GFAP): -, no reactive cells; +, 1-10% immunoreactive cells per high power field (HPF); ++, 11-20% reactive cells per HPF; +++, more than 20% immunoreactive cells per HPF.

abundant, granular, eosinophilic cytoplasm. Bizarre and multiple nuclei were common, but mitotic figures were found only in case 5. Sustentacular cells were usually difficult to identify in routine HE slides. Haemorrhagic foci were relatively frequent, but areas of necrosis were only seen in case 1.

Immunohistochemistry

The immunohistochemical results for phaeochromocytomas are summarized in Table 1. Reactivity for NSE, chromogranin, synaptophysin, Leu-7 and neurofilaments appeared only in chief cells. NSE reactivity was found in 10 of the 11 tumours. Chromogranin positivity was found in the same number of cases, usually in almost all chief cells (Fig. 1). Synaptophysin reactivity was widespread in eight tumours, focal in two, and was not detected in case 5. Leu-7 reactivity was widespread in three cases, moderate in four and focal in three (Fig. 2). Neurofilaments immunostained scattered cells in five tumours (Fig. 3). No cytokeratin, CEA or HMB-45 reactivities were detected. Staining for chromogranin was polar in one case, staining for Leu-7 was peripheral

and diffuse cytoplasmic in one case, and reactivity for neurofilaments was paranuclear also in one case; diffuse cytoplasmic positivity was observed in all other stained chief cells.

Antibody to S-100 protein reacted strongly in the nuclei and cytoplasm of a small or moderate number of sustentacular cells of eight tumours. One tumour (no. 9) also showed some degree of staining in the nuclei and cytoplasm of chief cells, but the results obtained after titulation of the antibody lead us to pressume that this staining was nonspecific. No GFAP positivity was detected.

Ultrastructure

Ultrastructural study was performed in five cases (nos. 1, 6, 7, 8, 9). The chief cells contained abundant catecholamine granules. These granules usually had a clear space or halo between the limiting membrane and the electron-dense, eccentric, core (norepinephrine-containing granules). There were also increased numbers of mitochondria, prominent rough surfaced endoplasmic reticulum and scattered lysosomal dense bodies. Some
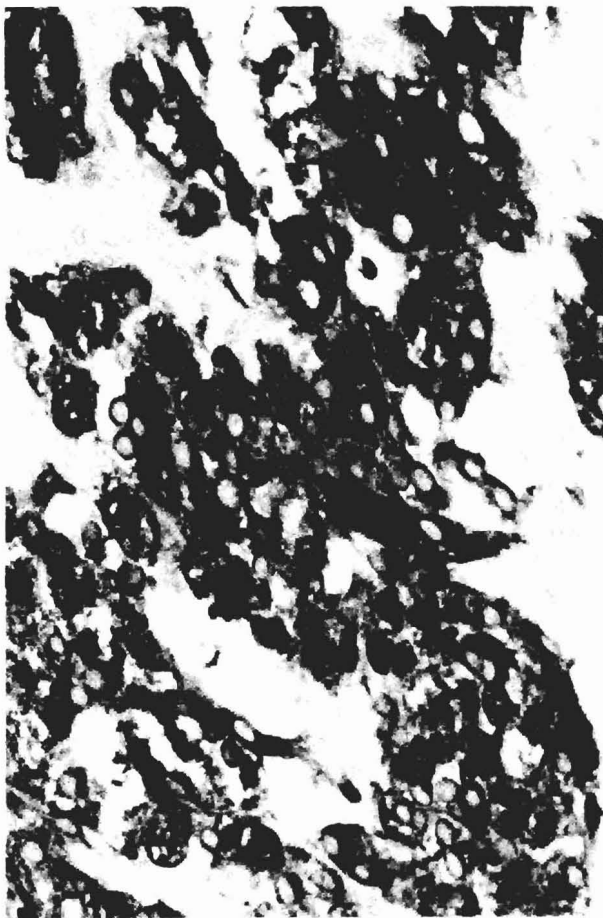
Fig. 1. Phaeochromocytoma (case 3): diffuse cytoplasmic staining for chromogranin (light haematoxylin counterstain). x 330
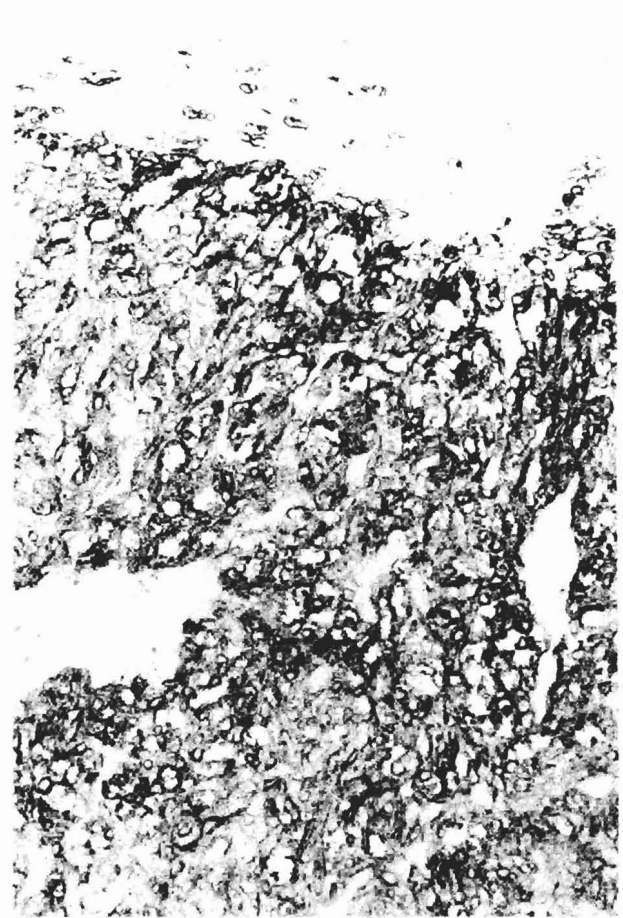
Fig. 2. Phaeochromocytoma (case 1): peripheral and cytoplasmic staining for Leu-7 (not counterstained). x 165

cells and their cytoplasmic processes were joined by desmosome-like junctions.

Paragangliomas

The seven paraganglioma patients (six females and one male) had ages ranging from 21 to 65 years (mean 40.8 years); six had carotid body tumours (one bilaterally) and one a retroperitoneal tumour. The maximum diameters of carotid body tumours ranged from 2.5 to 6 cm and the retroperitoneal tumour was 20 cm across.

Microscopically, all these tumours were composed predominantly of round to polygonal chief cells with moderate to abundant eosinophilic granular or clear cytoplasm and a round or oval nucleus. These cells were arranged in solid nests separated by a rich vascular network. Less commonly, a trabecular pattern was also seen. Pseudoacini were rarely encountered. Occasionally, spindle or putative sustentacular cells were seen at the periphery of the chief cell nests. In some tumours, nuclear pleomorphism and multinuclearity were often observed. Necrosis was prominent in case 6.

Immunohistochemistry

The immunohistochemical findings for paragangliomas are also summarized in Table 1. Again, NSE, chromogranin and synaptophysin were stained for only in chief cells; Leu-7, neurofilaments, cytokeratins, CEA and HMB-45 were not detected in any tumour. NSE reactivity was always present in over 60% of chief cells (Fig. 4). Chromogranin reactivity was present in seven of the eight tumours usually in under 60% of the chief cells. Synaptophysin reactivity was detected in all the paragangliomas, with a strong immunoreaction in the great majority of cells (Fig. 5). Diffuse cytoplasmic staining was observed for all the antigens detected, and NSE reactivity also appeared in nuclei.

In all cases, a strong S-100 protein reaction appeared in the nucleus and cytoplasm of spindle-shaped or stellate cells which ranged in density from sparse to abundant (Fig. 6). As in phaeochromocytomas, we observed staining of chief cells in one case (no. 16),
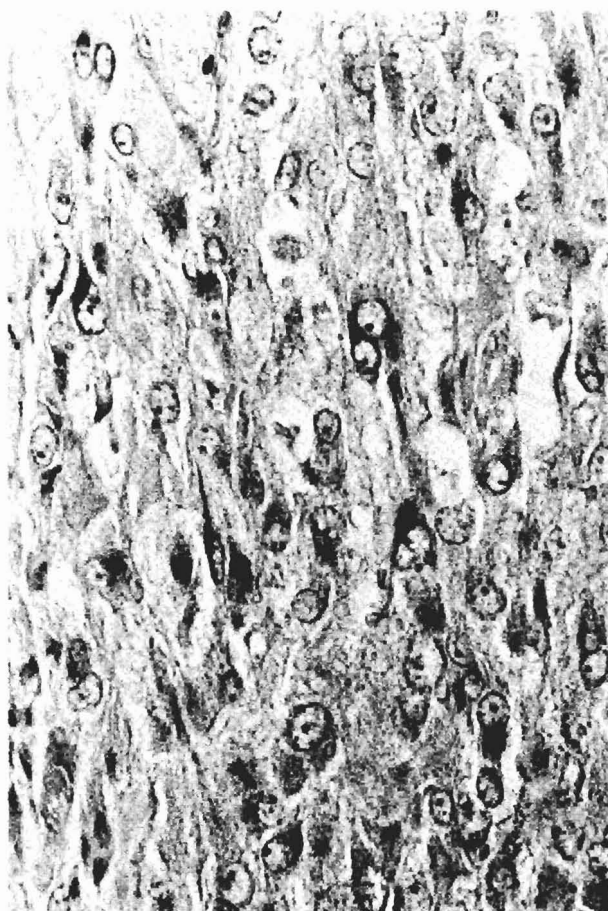
Fig. 3. Phaeochromocytoma (case 10): paranuclear cytoplasmic staining for neurofilaments (haematoxylin counterstain). x 400
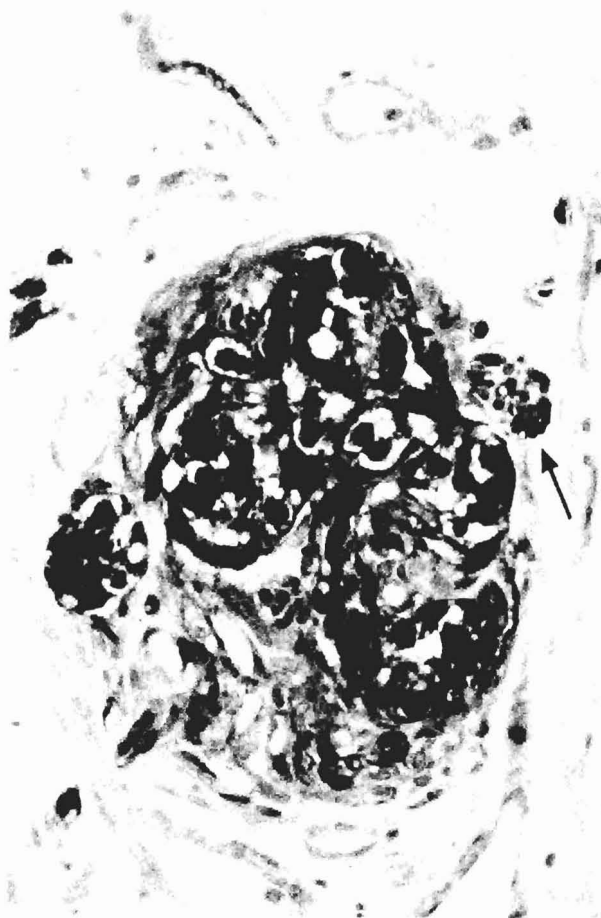
Fig. 4. Paraganglioma (case 14): diffuse cytoplasmic and nuclear staining for NSE. Nerve fibre also positive (arrow) (haematoxylin counterstain). x 330

more intense in cytoplasm, that we interpreted as nonspecific.

GFAP positivity in cytoplasm of sustentacular cells was noted only in one case (Fig. 7).

Ultrastructure

Case 16 was examined at ultrastructural level and consisted of closely packed cells which were intimately related to the blood vessels. There were numerous neurosecretory granules, heterogeneous in size and electron density, as well as mitochondria, an abundant rough surfaced endoplasmic reticulum and occasional lysosomal dense bodies. Neuritic or dendritic processes with microtubules were also present (Fig. 8).

Discussion

Our immunohistochemical results support the current view that phaeochromocytomas and paragangliomas are composed of two cell populations (Schroder and Johannsen, 1986; Korat et al., 1988; Kliewer et al., 1989). Chief cells exhibited reactivity for neuro-

endocrine markers (NSE, chromogranin, synaptophysin, Leu-7, bombesin) and sustentacular cells for S-100 protein and occasionally for GFAP. Sustentacular cells did not exhibit reactivity for neuroendocrine markers. Though we observed some staining of chief cells with S-100 protein in two cases, which could be viewed as in agreement with the results of Johnson et al. (1988), we think -after titulation tests- that in our cases this reactivity was not specific. GFAP positivity in sustentacular cells was detected only in one para-ganglioma. This is in agreement with the results of Schroder and Johannsen (1986), Johnson et al. (1988) and Korat et al. (1988). Nevertheless, other authors have obtained more frequent immunostaining for GFAP by using a polyclonal antisera, specially in paragangliomas (Kliewer et al., 1989; Achilles et al., 1991).

The more sensitive markers of the chief cells of phaeochromocytomas and paragangliomas were NSE and synaptophysin reactivity (both detected in 18 of 19 tumours), followed by reactivity for chromogranin (detected in 17 of 19 tumours). Because of its sensitivity, NSE reactivity is widely used as a marker of neuro-endocrine neoplasms, but since it occurs in many non-
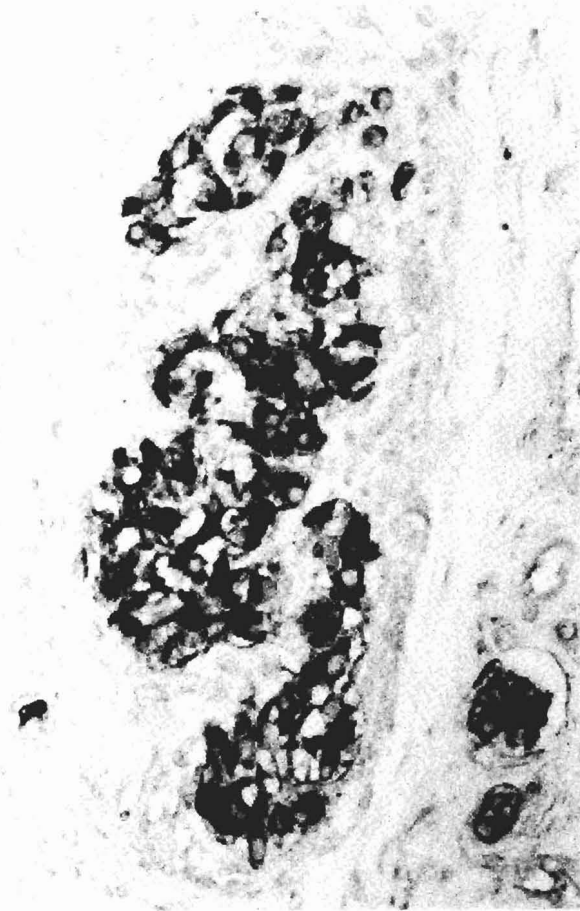
Fig. 5. Paraganglioma (case 14): diffuse cytoplasmic staining for synaptophysin (haematoxylin counterstain). x 330

Fig. 6. Paraganglioma (case 13): nuclear and cytoplasmic staining for S-100 protein in sustentacular cells (haematoxylin counterstain). x 330

neuroendocrine normal tissues and tumours (Haimoto et al., 1985; Leader et al., 1986; Pahlman et al., 1986), it must be used in combination with other neuroendocrine markers (Schmechel, 1985; Gould and DeLellis, 1990).

Immunoreactivity for chromogranin was more widespread in phaeochromocytomas than in paragangliomas, reflecting the less frequent secretion of catecholamines by paragangliomas (Kliewer et al., 1989). It is well known that intense immunostaining for chromogranin is associated with the presence of epinephrine storage granules (Lloyd et al., 1985).

Recent studies suggest that immunohistochemical identification of synaptophysin is affected negatively by prolonged tissue fixation in formalin (Hoog et al., 1988), but we found synaptophysin reactivity even slightly more frequent than staining for chromogranin, which in formalin-fixed material is of proven immunohistochemical stability (Wilson and Lloyd, 1984; Kimura et al., 1988). This is not surprising if we remember that the immunogen employed for obtaining the commercial antibody are cells of a human phaeochromocytoma.

Leu-7 antigen has been detected in a variety of neuroendocrine neoplasms, including phaeochromo-cytomas (Tsutsumi, 1984) and paragangliomas (Caillaud et al., 1984; Bunn et al., 1985). In our study phaeo-chromocytomas exhibited Leu-7 immunoreactivity, but none of the paragangliomas did. Our results may support the usefulness of anti-Leu-7 for identification of specific subsets of secretory granules (Tischler et al., 1986), and suggest that Leu-7 can aid differential diagnosis between phaeochromocytomas and paragangliomas. There are two previous reports of Leu-7 immunoreactivity in paragangliomas (Caillaud et al., 1984; Bunn et al., 1985). However, Bunn et al. (1985) studied only one case, and Caillaud et al. (1984) found in paragangliomas only scattered Leu-7 reactive cells throughout the bulk of Leu-7 negative tumour cells and, by contrast, they reported that virtually all cells of phaeochromocytoma were stained for Leu-7 antiserum. Further studies with larger series are needed to elucidate this question.

Neurofilament immunoreactivity has been detected in paragangliomas and phaeochromocytomas (Lehto et al., 1983; Perentes and Rubinstein, 1987; Korat et al., 1988).

Fig. 7. Paraganglioma (case 15): GFAP reactivity in cytoplasm of sustentacular cells (haematoxylin counterstain). x 330
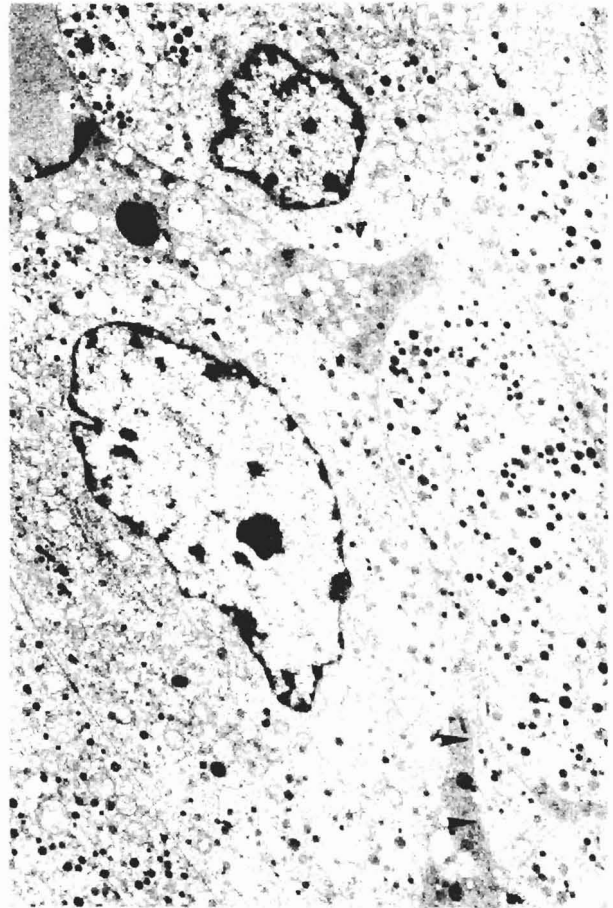
Fig. 8. Paraganglioma (case 16): ultramicrograph showing chief cells, rich in cytoplasmic organelles, and their relation to a blood vessel. Numerous electron-dense core granules can be seen, as well as mitochondria, an abundant rough surfaced endoplasmic reticulum and occasional lysosomal dense bodies. Note a neuritic (dendritic) process (arrowheads). x 3,900

We observed only a focal reaction in five phaeochromocytomas. Our poor results, like these of other authors (Johnson et al., 1988), may have been due to formalin fixation (or overfixation) (Gould and DeLellis, 1990). Since we used a monoclonal antibody for the 70 and 200 kd polypeptide subunits of neurofilaments (the former being the most prevalent in cells of these tumours (Miettinen, 1987), we do not believe our negative results have been due to any problem of antibody specificity.

Signs of epithelial differentiation such as true acini formation, the presence of intracytoplasmic lumina with PAS-positive material and, rarely, immunoreactivity for cytokeratins or CEA have been reported in some paragangliomas (Sneige et al., 1983; Spagnolo and Paradinas, 1985; Johnson et al., 1988). We did not observe cytokeratins or CEA reactivity.

Recently, Unger et al. (1992) described HMB-45 (melanoma antigen) reactivity in chief cells of some phaeochromocytomas (4 of 12 cases). They suggested that this fact could be due to cross-reactivity between neurosecretory granules and premelanosomes, perhaps reflecting the common embryologic origin of the respective cells. However, we did not find immunoreactivity for HMB-45 in any of the 11 phaeochromocytomas and 8 paragangliomas studied.

In conclusion, NSE, synaptophysin and chromogranin are good markers of phaeochromocytomas and paragangliomas. S-100 protein reactivity identifies the associated component of these tumours, the sustentacular cells. Leu-7 immunoreactivity seems to favour diagnosis of phaeochromocytoma rather than paraganglioma, but further studies with larger series are needed to confirm this.

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