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Intratumorous distribution of catecholaminergic clone cells of human neuroblastoma

A catecholamine fluorescence study*

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Summary. The intratumorous distribution of catecholaminergic clone cells in 23 human neuroblastomas was studied using Falck-Hillarp's method, and the findings compared with the catecholamine (CA) content within the tumour. All the specimens contained elements with CA fluorescence, and the pattern of fluorescence was classified from the distribution of CA-positive cells and neurofibrils, as diffuse cellular (DC), diffuse fibrillary (DF), sporadic (S), clustered (C), island-shaped (I), and bundled (B). The strength of CA fluorescence of both cellular and fibrillary elements correlated well with the CA content within the tumour. In addition, all tumours of urinary VMA-negative cases also contained significantly larger amounts of CA than other, non-functioning, tumours in the paediatric age group. The results of this study suggest that firstly, the ratio of CA-positive cells to CA-positive neuronal processes is proportionately higher in the poorly-differentiated neuroblastomas and that secondly, even tumours negative for urinary VMA or HVA might be polyclonal and contain catecholaminergic elements.

Key words: Neuroblastoma – Catecholamine fluorescence – Catecholamine content – Catecholaminergic clone

Introduction

Catecholamine (CA) metabolism is one of the main characteristics of neuroblastoma, and interest has focused on its relationship to differentiation (Gitlow et al. 1973; LaBrosse et al. 1976; Graham-Pole et al. 1983). Clini-

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cally, urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) levels influence the prognosis, and a possible relation to the cellular differentiation was considered (Laug et al. 1978). Neuroblastoma is usually composed of heterogeneous clone cells (Amano et al. 1972). There has been little documentation of intratumorous catecholaminergic cells of neuroblastoma at the cellular or molecular levels in previous studies.

We studied the interrelationship of patterns of intratumorous distribution of catecholaminergic cells or fibrils, histological grades of differentiation of human neuroblastoma, and content of catecholamine within the tumours.

Materials and methods

Data on 24 patients with neuroblastoma from whom urine or tumour fragments were obtained are listed in Table 1. One neuroblastoma was not available for the CA fluorescence study. The average age of the patient was 3 years and 4 months (1 months ~11 years), and 9 were boys and 15 were girls. The primary sites were as follows; 16 adrenal region, 5 mediastinal area and 3 retroperitoneal space. Histologically, 13 were neuroblastoma (11 rosette-fibrillary types and 2 round cell types), 9 ganglioneuroblastoma (2 well differentiated types, 2 composite types and 5 poorly differentiated types), and 2 ganglioneuroma. Histological subclassification was according to the Histological Classification of Tumours in Infancy and Childhood by the Japanese Pathological Society (Ota and Shimizu 1980). The clinical stages of the disease were according to Evans et al. (1971). The numbers of patients were: stage I, 2; II, 2; III, 1; IV, 16; and IV-S, 4.

Table 1. Studied cases

No.	Sex	Age	Primary site	Stage	Histology[a]
1	F	1y 9m	Adrenal region	IV	NB
2	F	1y 0m	Mediastinum	IV	NB
3	F	5y 8m	Adrenal region	IV	NB
4	F	2m	Adrenal region	IV-S	NB
5	F	1m	Adrenal region	IV-S	NB
6	M	5m	Adrenal region	IV-S	NB
7	M	6y 0m	Adrenal region	IV	NB
8	F	4y 11m	Retroperitoneum	IV	NB
9	M	11y	Adrenal region	III	NB
10	M	2y 5m	Adrenal region	IV	NB
11	M	3y 4m	Adrenal region	IV	NB
12	M	1y 6m	Adrenal region	IV	NB
13	F	1y 7m	Adrenal region	IV	NB
14	F	1y 7m	Retroperitoneum	IV-S	GNB
15	M	2y 4m	Adrenal region	IV	GNB
16	F	3y 3m	Adrenal region	IV	GNB
17	M	3y 7m	Mediastinum	IV	GNB
18	F	5y 8m	Adrenal region	IV	GNB
19	F	3y 2m	Mediastinum	II	GNB
20	M	4y 0m	Retroperitoneum	IV	GNB
21	F	1y 5m	Adrenal region	II	GNB
22	F	1y 5m	Adrenal region	IV	GNB
23	F	6y 9m	Mediastinum	I	GN
24	F	6y 9m	Mediastinum	I	GN

[a] NB: neuroblastoma, GNB: ganglioneuroblastoma, and GN: ganglioneuroma

Tumours other than neuroblastoma included in this study for comparison were as follows; malignant pheochromocytoma (14 years, female, right adrenal gland), rhabdomyosarcoma (11 years 5 months, male, right upper extremity), rhabdomyosarcoma (2 years 8 months, female, gluteal region), Hodgkin's disease (8 years, male left neck), rhabdomyosarcoma (6 months, female, right axillary region), Grawitz's tumour (7 years 2 months, male, left kidney), hepatic haemangioendothelioma (1 months, female), Wilms' tumour (1 years 2 months, male, left kidney) and a further Wilms' tumour (2 years, male, left kidney).

Urine used to measure VMA and HVA levels was collected for 24 h, in an acid condition, and food and drinks containing vanilla were restricted for the preceding 24 h.

Tumour specimens were obtained from operatively resected masses and all tissues with necrosis, haemorrhage or calcification were excluded. The samples were stored at −80° C until the study.

Urinary VMA and HVA were measured by methods using p-nitroaniline and high performance liquid chromatography, respectively, and the values were expressed as mg/day.

The intratumour adrenalin, noradrenalin and dopamine were quantified by high performance liquid chromatography (courtesy of Kitasato Biochemicals, Sagamihara, Japan).

The CA fluorescence study was carried out according to the method developed by Falck et al. (1962). Small solid pieces of tumour, usually 4 × 3 × 3 mm, were quickly frozen in isopentane cooled by solid carbon dioxide, and stored at −80° C until use. The frozen pieces were placed in the freeze-drying unit (Edwards High Vacuum, EPD-3, British) with a diffusion pump (Daia Vacuum, Model IT-L20P, Watanabe) for 4 days at −45° C at 10^{-4} atm. After being dried completely, the tissues were treated with paraformaldehyde vapor at 80° C for 60 min, and then embedded in paraffin. Sections of 7-μm thickness were cut and mounted in liquid paraffin, and photographed under a fluorescence microscope.

Serial sections were stained with haematoxylin and eosin.

Sections were examined in a Nikon fluorescence microscope equipped with an epi-illumination system (385–425 nm band-pass excitation filter and 470 nm long pass suppression filter). Under these conditions, blue-green fluorescence is specific for catecholamines, and yellow-green fluorescence non-specific. Most fluorescence observed in neuroblastoma specimens of this study was blue-green, but not yellow-green, and it could not be found when the specimen had not been treated with paraformaldehyde vapor. The concentrations of catecholamine within the tumour were enormously increased as stated in Results, while those of serotonin of 23 specimens were undetectable (less than 100 ng/g). Toluidine blue staining of serial sections excluded contamination by mast cells. This suggests that the fluorescence observed may result from the intratumorous catecholamines.

Results

Intratumorous catecholamine contents in human neuroblastoma

The CA contents within the tumour were measured in 13 of 14 neuroblastomas, 8 of 9 ganglioneuroblastomas and 2 ganglioneuromas (Tabl. 2, 3). The groups of neuroblastoma and ganglioneuroblastoma secreting increased amounts of urinary VMA and HVA showed higher amounts of intratumorous CAs than those secreting lower amounts (Table 3). The average contents of three CAs were highest in the former group, but the groups excreting a normal range of urinary VMA and HVA also showed higher amounts of intratumorous CAs than those in the non-neuroblastoma group except for malignant pheochromocytoma. Among the groups with negative urinary CA metabolites, the CA content, especially of noradrenalin and depamine, of neuroblastoma was significantly less than in cases of ganglioneuroblastoma and ganglioneuroma. Noradrenalin was the most highly concentrated of any CA within the tumours, while the concentration of adrenalin was

Table 2. Intratumorous catecholamine content of human neuroblastoma

Histology	Urinary VMA, HVA	No. of patients	Intratumorous catecholamines		
			Adrenalin	Noradrenalin	Dopamine
NB	+	8	140±62[a] (0–438)	22,421±10,562[a] (1,350–80,800)	4,015±1,419[a] (10–29,400)
	–	5	8±3 (0–18)	223±52 (74–359)	68±25 (30–153)
GNB	+	5	91±33 (1–185)	16,634±7,439 (1,470–23,900)	2,582±1,155 (1,530–4,450)
	–	3	37±23 (0–79)	4,060±1,553 (1,630–6,950)	1,155±1,035 (50–3,220)
GN	–	2	9, 0	2,240, 930	120, 130
Pheochromocytoma	+	1	58,200	48,400	670
Others	–	8	0.1±0.1 (0–0.5)	18±14 (0.2–114)	3.3±1.5 (0–10)

[a] ng/g wet weight, Mean±SEM.

small. On the other hand, a malignant pheochromocytoma contained large amounts of both adrenalin and noradrenalin (Table 2).

Catecholamine fluorescence of neuroblastoma

1. Neuroblastomas secreting higher amounts of urinary CA metabolites

As shown in Table 3, neuroblastomas secreting a large amount of urinary CA metabolites had a large number of CA-positive tumour cells with a moderately to highly positive CA fluorescence (diffuse cellular pattern, DC). The yellow-greenish fluorescence was diffuse in the cytoplasm, and the margin of most fluorescent cells was sometimes obscure. CA-fluorescent neurofibrils were rarely observed, especially in the rosette-fibrillary type neuroblastoma, and they did not form bundles; rather the process was random (Figs.1 A, 2). Neuroblastomas secreting a large amount of urinary CA metabolites all showed the diffuse cellular pattern of CA fluorescence. The pattern of CA fluorescence of ganglioneuroblastoma differed from that of neuroblastoma. The ganglioneuroblastoma contained large amounts of CA-positive neurofibrils which had a tendency to form fibrillary bundles (diffuse fibrillary pattern, DF, Figs. 3–5). Neuronal processes with varicosities were found in some tumour sections (Fig. 3). The ratio of CA-positive cells to CA-positive neuronal processes appeared to be higher in the poorly differentiated tumours and lower in the well differentiated ones. As shown in Fig. 6, the ganglion cells within the tumour showed no, or only weak, fluorescence. Four of six ganglioneuroblastomas with positive urinary CA metabolites showed a DF pattern. In case 15, however, most CA fluorescent

Table 3. Pattern of intratumour distribution of catecholamine fluorescence in human neuroblastoma

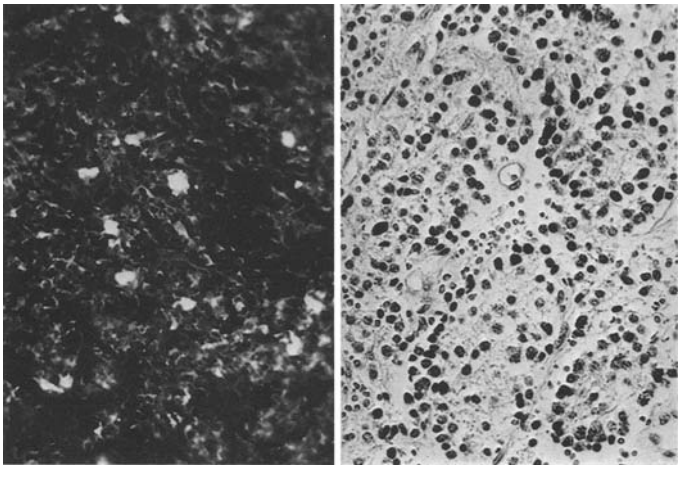
Group[a]	Case no.	Urinary VMA+HVA mg/day	Site of tumour	Histology[b]	Intratumorous CA (Ad.+Norad+Dopa.) ng/g	CA (+) cells		CA (+) Neurofibrils	Varicosity	Pattern of fluorescence[c]
						Number	Strength			
A	1	51.4	Primary	NB, round cell	2,399					
	2	37.5	Primary	NB, rosette	7,807	++	++	+	−	DC
	3	31.0	Iliac bone meta.	NB, rosette	18,175	++	+++	−	−	DC
	4	279.1	Liver meta.	NB, rosette	5,998	+++	+	−	−	DC
	5	33.2	Primary	NB, rosette	8,134	+++	+	−	−	DC
	6	38.0	Primary	NB, rosette	87,000	+++	++	++	−	DC
	7	51.1	Primary	NB, rosette	1,361	++	+	++	−	DC
	8	47.0	Primary	NB, rosette	81,738	+++	++	+	−	DC
	14	19.2	Primary	GNB, poorly	23,225	+++	++	+	+	DC
	15	58.0	Primary	GNB, poorly	22,879	++	+++	++	+	I
	16	71.4	Primary	GNB, composite	3,001	+	+++	+++	+	DF
	17	56.2	Primary	GNB, composite	ND[d]	+	+++	+++	+++	DF
	18	31.0	Primary	GNB, well	25,946	++	++	++	+	DF
	19	34.0	Primary	GNB, well	21,484	+	+++	+++	+	DF
B	9	15.7	Primary	NB, round cell	351	+	+	−	−	C
	10	13.0	Neck meta.	NB, rosette	287	+	+	−	−	C
	11	13.0	Testis meta.	NB, rosette	124	+	+	−	−	S
	12	5.7	Primary	NB, rosette	428	+	+	−	−	S
	13	4.0	Primary	NB, rosette	304	+	+++	−	−	C
	20	9.6	Primary	GNB, poorly	1,662	+	++	−	−	S
	21	8.7	Primary	GNB, poorly	3,923	++	++	++	+	DF
	22	8.1	Primary	GNB, poorly	10,170	++	+++	++	−	I
	23	11.7	Primary	GN	2,369	+	++	+++	−	B
	24	2.3	Primary	GN	1.060	+	++	+++	−	I

[a] A: group with elevated urinary VMA (≧8.0 mg/day) and HVA (≧12.0 mg/day). B: group with normal urinary VMA and HVA

[b] NB, rosette: rosette-fibrillary type of neuroblastoma (neuroblastoma grade IIIr, Fortner et al. 1968). NB, round cell: round cell type of neuroblastoma (neuroblastoma grade III, Fortner et al. 1968). GNB, well: well differentiated ganglioneuroblastoma (neuroblastoma group I, Horn et al. 1956). GNB, composite: composite type ganglioneuroblastoma (composite ganglioneuroblastoma, Evans 1966). GNB, poorly: poorly differentiated ganglioneuroblastoma (complex ganglioneuroblastoma, Evans 1966)

[c] DC: diffuse cellular pattern. I: island-shaped pattern. DF: diffuse fibrillary pattern. C: clustered pattern. S: sporadic pattern. B: bundled pattern

[d] ND: not determined



A B

Fig. 1 A and B. Neuroblastoma, rosette-fibrillary type, case 3. Catecholamine fluorescence. **A** Small round cells with either strong or weak CA fluorescence are diffusely distributed together with some CA-positive neuronal processes ×150. **B** Photomicrograph of a section near to **A**. H & E, ×220

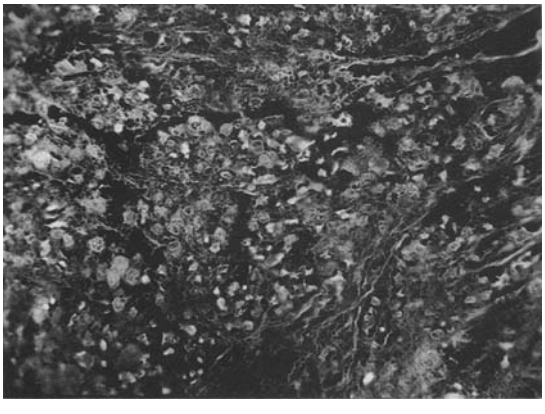


Fig. 2. Neuroblastoma, rosette-fibrillary type, case 6. Diffuse cellular pattern of CA fluorescence. The section is occupied mainly by CA-positive tumour cells with a minority of CA-positive neuronal processes ×128

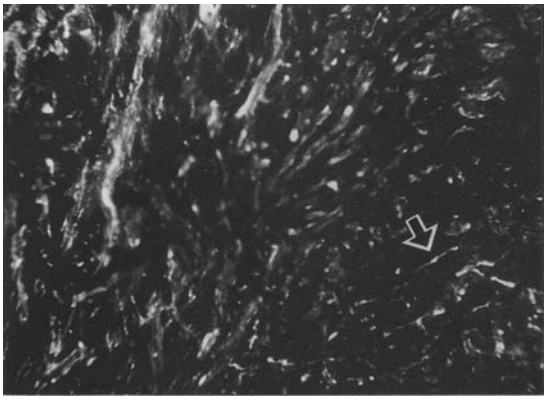


Fig. 3. Ganglioneuroblastoma, composite type, case 17. Diffuse fibrillary pattern of CA fluorescence. There are numerous varicosities (*arrow*) ×65

Fig. 4. Ganglioneuroblastoma, well differentiated type, case 19. Diffuse fibrillary pattern of CA fluorescence ×128

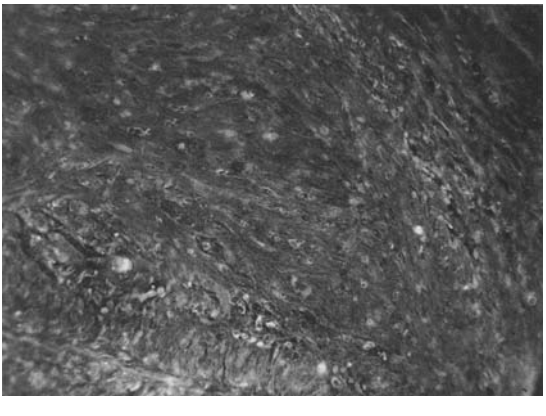
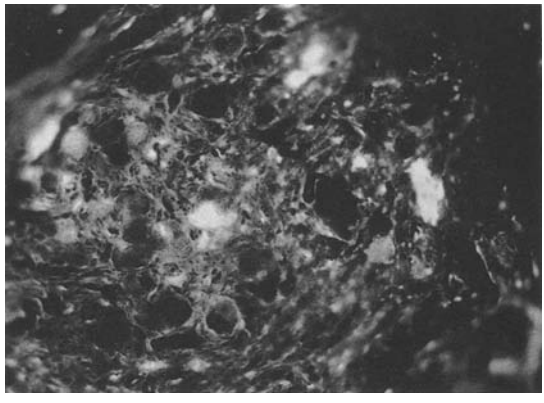


Fig. 5. Ganglioneuroblastoma, poorly differentiated type, case 21. Diffuse fibrillary pattern of CA fluorescence. ×128. The urinary excretions of VMA and HVA of this case were within normal limits

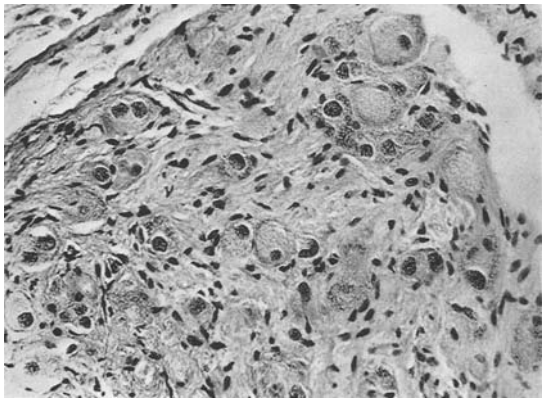
cells and neuronal processes were present around the ganglion cells, and looked like a fluorescent island (island-shaped pattern, I, Fig. 7).

2. Neuroblastomas secreting normal amounts of urinary CA metabolites

All the tumours with normal urinary CA metabolites contained CA fluorescent cells or neuronal proceses. The strength of the fluorscence was comparable with the level of intratumorous CA contents. In case of neuroblastoma, CA-positive tumour cells were rarely observed in the main part of non-fluorescent dark area, and the pattern of distribution was sporadic (S, Fig. 8) or clustered (C, Fig. 9). However, 3 ganglioneuroblastomas with poorly differentiated histological type showed sporadic, diffuse fibrillary and island-like patterns, with or without CA-fluorescent fibrillary elements. In cases of ganglioneuroma, the fluorescent component was mainly composed of neuronal processes or bundles (B, Fig. 10). Varicosities were nil. In case 24, ganglion cells showed no or weak fluorescence, and CA-positive areas were island-shaped.



A



B

Fig. 6 A and B.
Ganglioneuroblastoma, composite type, case 17. **A** CA fluorescence ×128. Large-sized ganglion cells are non-fluorescent.
B Light micrograph of the near section of **A**. H & E, ×153

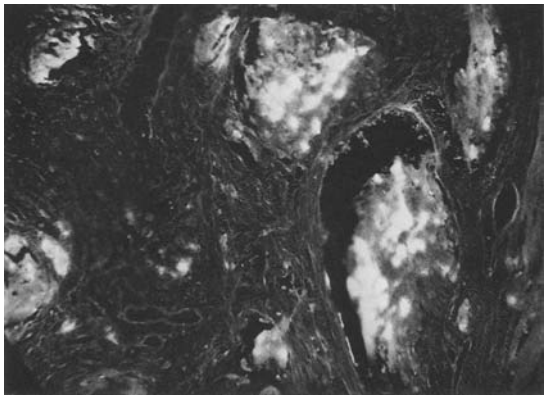


Fig. 7. Ganglioneuroblastoma, poorly differentiated type, case 15. Island-shaped pattern of CA fluorescence ×65

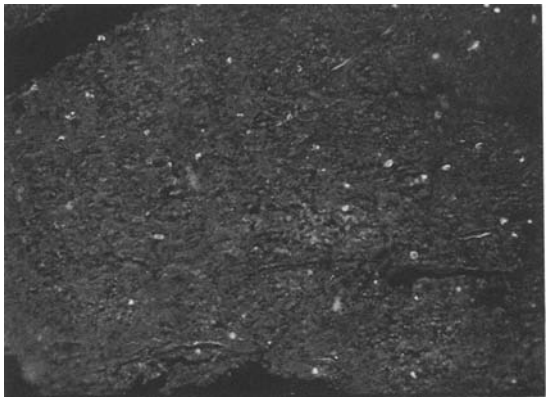


Fig. 8. The typical sporadic pattern of CA fluorescent cells. Most of the neuroblastoma cells are nonfluorescent ×65

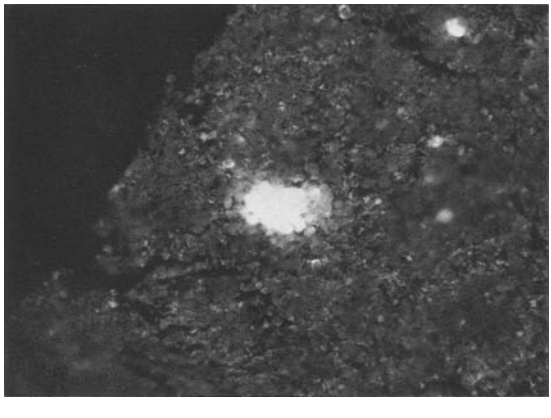


Fig. 9. Neuroblastoma, rosette-fibrillary type, case 13. Clustered pattern of CA fluorescence. The strongly fluorescent tumour cells form a cluster among the nonfluorescent tumour tissue. The urinary VMA was negative ×128

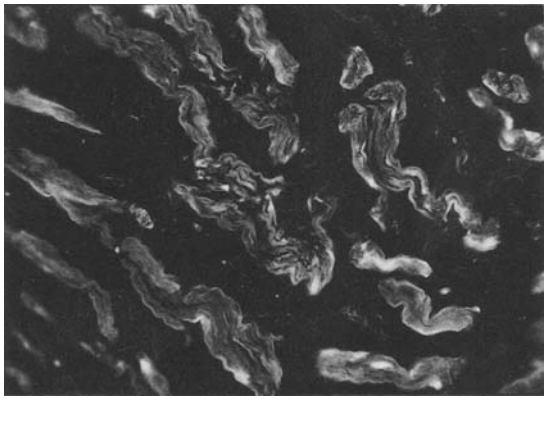


Fig. 10. Ganglioneuroma, case 23. Bundled pattern of CA fluorescence ×65

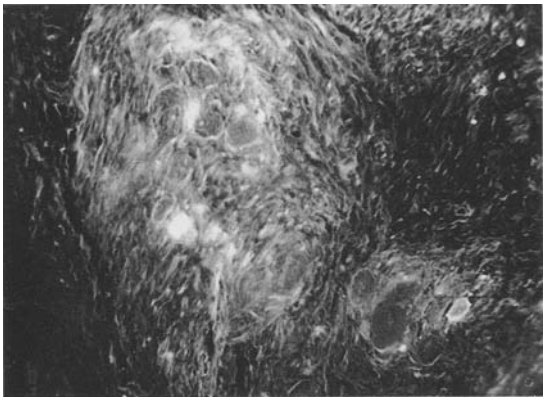


Fig. 11. Ganglioneuroma, case 24. Island-shaped pattern of CA fluorescence ×65. CA fluorescent elements distributed around ganglion cells.

Discussion

Since the report of Mason et al. (1957), neuroblastoma has been recognized as one of the functioning tumours belonged to the APUD system which originates from the embryonal neural crest. The measurement of urinary CA metabolites such as VMA or HVA is useful for the diagnosis of this tumour and for determining therapy. Laug et al. (1978) suggested that the ratio of urinary VMA and HVA levels may relate to the prognosis. The enzymatic or molecular basis of this proposal is poorly understood.

The electron microscope has facilitated visualization of the presence of cored vesicles containing CA and the metabolites (Greenberg et al. 1969; Yokoyama et al. 1971). Hata et al. (1984) suggested that the size, numbers and ultrastructure of secretory granules might vary depending on the stage of maturation and functional activity of the tumour. However, little attention was directed to the fact that neuroblastoma consists of polyclonal cells. The detection of CA fluorescence from neuroblastic cells has apparently only been used for differential diagnosis of neuroendocrine tumours such as neuroblastoma and pheochromocytoma (Reynolds et al. 1981). In the present study, we used the CA fluorescence method for observation of tumour specimens, and patterns of distribution of catecholaminergic cells or fibrils in the neuroblastoma became evident.

The pattern of CA fluorescence could be classified as 1) diffuse cellular, 2) diffuse fibrillary, 3) sporadic, 4) clustered, 5) island-shaped, and 6) bundled. In case of neuroblastoma of the poorly differentiated histological type, all 7 VMA-positive cases showed a diffuse cellular pattern of distribution of the catecholaminergic cells, as the tumour was mainly composed of small round cells. In cases of urinary VMA-negative neuroblastoma, the patterns were sporadic or clustered, and contained relatively higher amounts of CA within the tumour. These findings indicate that even in cases of normal urinary VMA or HVA, there are catecholaminergic elements within the tumour.

Ganglioneuroblastoma showed a wide variety of patterns of catecholaminergic cells. CA-positive neurofibrillary elements were more numerous than in neuroblastoma. Among 9, 5 were of the diffuse fibrillary pattern and the island-shaped pattern was observed in two, one of whom was a VMA-negative patient. The intratumorous CA content of the VMA-negative ganglioneuroblastoma was fairly high and correlated with the findings of CA fluorescence. Possible explanations are the presence of unsecretable CA and/or the rapid turnover within the tumour or other organs such as the liver. Another important finding was the intratumorous formation of varicosities similar to those found in normal adrenergic neurofibrils (Furness and Costa 1973).

In case of ganglioneuroma, CA fluorescence was observed mostly in the neurofibrils or its bundles. The presence of measurable CA in the ganglioneuroma was also noted by Hörtnagl et al. (1972). These CAs may not be secreted because urinary CA metabolites were within normal range in the present two cases.

In both ganglioneuroblastoma and ganglioneuroma, ganglion cells had no, or only weak, fluorescence, but there was a tendency to form a fluorescent network around these cells. All 4 sections obtained from Stage IV-S patients showed a diffuse cellular pattern of CA fluorescence, and no pattern specific for this stage was observed.

The phenotypic diversity of neuroblastoma has recently been studied biochemically from the aspect of neurotransmitter syntheis. However, the biochemical measurement of tyrosine hydroxylase and choline acetyltransferase activities in human neuroblastoma does not give a clear explanation of the clinical aspect of the diversity (Imashuku et al. 1975; Yokomori et al. 1983). It has also been suggested that even the cloned murine or human neuroblastomas were not always simply classified into adrenergic or cholinergic, because some had multiple neurochemical characteristics (Biedler et al. 1978). The CA fluorescent cell components may also be composed of such multifunctional CA producing tumour cells or fibers.

Thus, the present observations revealed that even in cases of neuroblastoma not secreting significantly higher amounts of CAs into the urine, the tumour still contains catecholaminergic tumour cells or neurofibrils and has polyclonal characteristics. The pattern of distribution of catecholaminergic clone cells within the tumour may correlate with prognosis or sensitivity to therapy.

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