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Neuronal and glial markers in tumours of neuroblastic origin

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Summary. The presence and distribution of different neural markers in 30 neuroblastic tumours (neuroblastomas, ganglioneuroblastomas) and 6 non-neuroblastic tumours were investigated by immunocytochemistry. Neuron-specific enolase (NSE), S-100 protein, tyrosine hydroxylase, neurofilaments and glial fibrillary acidic protein (GFAP) were localised in 3 undifferentiated neuroblastic tumours (group A), 12 poorly differentiated tumours (group B) and 15 well differentiated neuroblastic tumours (group C). Non-neuroblastic tumours (3 lymphomas and 3 Ewing sarcomas) showed no immunoreactivity.

Tyrosine hydroxylase and, in particular, NSE were found in mature ganglion cells and developing neuroblasts of poorly and well differentiated tumours (groups B and C). S-100 was localised in neuroblasts with slender cytoplasmic processes in the same groups. Neurofilaments were detected in ganglion cells and differentiated neuroblasts (groups B and C) while GFAP was localised in immature neuroblasts of undifferentiated and poorly differentiated tumours (groups A and B). Thus, there are differences in the neural proteins found in neuroblastic tumours and a wide panel of antibodies against neural markers may be a useful tool in the histological assessment of nervous system neoplasms.

Key words: Cytoskeleton components – Immunocytochemistry – Neuroblastomas – NSE – S-100 – Tyrosine hydroxylase

Neuroblastomas and their differentiated counterparts, ganglioneuroblastomas, are tumours frequently found in infancy.

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The histological diagnosis of neuroblastomas is sometimes difficult since their clinical and microscopical characteristics are extremely similar to those presented by other neoplasms such as undifferentiated sarcomas and lymphomas. Neuroblastomas may display different degrees of maturation (Makinen 1972) from undifferentiated through increasing differentiation to benign ganglioneuromas (Cushing and Wohlbach 1927; Fox et al. 1959). Signs of maturation are considered by various authors (Hughes et al. 1974; Ceccamea et al. 1981) to consist of enlarged vesicular nuclei, prominent nucleoli and an increase in the amount of cytoplasm with formation of cytoplasmic processes. The degree of tumour maturation is closely related to prognosis and survival, and an effective evaluation is thus necessary (Hughes et al. 1974).

Various proteins have been extracted from nervous tissue; some of them appear during maturation. They are often restricted to a single cell population (Bradshaw and Schneider 1980) and although some are components of the cytoskeleton, others are known to be involved in specific metabolic processes. These neural proteins include neuron-specific enolase, S-100, the enzyme tyrosine hydroxylase and two types of intermediate filaments forming part of the cytoskeleton – neurofilaments and glial fibrillary acidic protein.

Neuron-specific enolase, a $\gamma\gamma$ dimer of the glycolytic enzyme enolase, is present in neurons of the central and peripheral nervous systems (Marangos et al. 1978) as well as in paraneurons or APUD cells (Pearse 1968; Polak and Bloom 1979) throughout the entire body (Marangos et al. 1982). S-100 protein, a highly acidic water-soluble protein, first detected in astrocytes and Schwann cells (Moore 1965), has also been identified in the peripheral nervous system (Ferri et al. 1982). Tyrosine hydroxylase is localised in all sympathetic structures and can therefore be considered as a marker for this system (Thibault et al. 1981).

During the past few years the different components of the cytoskeleton have been characterised. Neurofilaments (Huneeus and Davison 1970), being about 10 nm in diameter, belong to the group known as intermediate filaments, since their diameter lies between those of the other two major cytoskeletal components, microfilaments (5 nm) and microtubules (about 25 nm). Neurofilaments are specifically restricted to neuronal cells in the central nervous system and in the periphery. On the other hand, astrocytes and glial cells of the central nervous system and peripheral ganglia, exhibit a different cytoskeletal intermediate filament, glial fibrillary acidic protein (GFAP) (Bignami et al. 1972; Jessen and Mirsky 1980).

Several attempts have been made to localize these substances in neoplastic tissues including neuroblastic tumours. NSE immunoreactivity has been localised in ganglioneuroblastomas (Tapia et al. 1981) and elevated levels of NSE have been found in the plasma of children with metastatic neuroblastoma (Zelter et al. 1983). The presence of S-100 has also been identified in neuroblastomas and ganglioneuroblastomas (Kahn et al. 1982; Nakajima et al. 1982). Osborn and Weber (1983) suggested that at least some neuroblastomas are characterised by the presence of neurofilaments.

We have therefore carried out a comparative and systematic search for

the presence of a variety of neural proteins in 30 tumours of neuroblastic origin and 6 of non-neuroblastic origin. Immunocytochemistry was undertaken using a panel of antibodies recognising the following neural antigens: neuron specific enolase, S-100, tyrosine hydroxylase, neurofilaments and GFAP. The purpose was to verify the existence and localization of these markers and their distribution pattern in relation to the degree of maturation of the tumours.

Materials and methods

All the material consisted of large surgical specimens collected during operations performed in the Policlinico Umberto I, Rome, from 1975 to 1980.

The series comprised 30 neuroblastic tumours, 3 small cell lymphomas and 3 Ewing sarcomas.

Table 1A

Antiserum to	Natural antigen	Optimal dilution
Neuron specific enolase (NSE) (Marangos et al. 1978)	Human	1/2000
S-100 protein (Cocchia and Michetti 1981)	Bovine	1/4000
Glial fibrillary acidic protein (GFAP) (Dahl and Bignami 1976)	Human	1/1000
Tyrosine hydroxylase (TH) (Thibault et al. 1981)	Feline (cat)	1/800
Neurofilament protein (68°, 150°, 200° K) (Dahl and Bignami 1977)	Avian (chicken)	1/500

Table 1B. Absorption controls

Antisera to	Antigen				
	NSE	S-100	GFAP	TH	NF
NSE	– (1)	+	+	+	+
S-100	+	– (1)	+	+	+
GFAP	+	+	– (1)		+
TH	+	+	+	– (0.5)	+
NF	+	+	+	+	– (1)

The minimum concentration (nmol/ml diluted antiserum) of antigen required to successfully quench immunostaining is shown in parentheses. (– = negative staining; + = positive staining) Cross-absorption controls were carried out using up to 10 nmol of antigen per ml of diluted antiserum

Table 2A.

Case	Sex	Age (months)	Location	Signs of maturation	Detectable markers				
					NF	NSE	S-100	GFAP	TH
Group A (undifferentiated)									
1	M	29	Neck	0	−	−	−	−	−
2	M	24	Adrenal	0	−	−	−	+	−
3	F	7	Adrenal	0	−	−	−	−	−
Group B (partly differentiated)									
4	F	34	Adrenal	1	+	−	+	−	−
5	F	0	Adrenal	1	+	−	+	−	−
6	M	36	Adrenal	1	−	−	+	+	−
7	F	22	Adrenal	1	−	+	+	−	−
8	M	19	Adrenal	2	+	+	+	−	+
9	M	31	Adrenal	2	−	+	+	−	−
10	F	20	Adrenal	2	−	+	+	+	−
11	M	25	Adrenal	2	+	+	+	−	−
12	F	37	Adrenal	3	+	−	+	−	−
13	F	25	Paraortic	3	+	+	+	−	+
14	M	24	Adrenal	3	+	+	−	−	+
15	M	27	Adrenal	3	+	+	+	−	+
16	F	34	Adrenal	3	−	+	+	−	+
17	M	29	Adrenal	3	+	−	+	−	+
Group C (well differentiated)									
18	M	29	Mediastinum	4	+	+	+	−	−
19	F	11	Adrenal	4	+	+	−	−	+
20	F	22	Adrenal	4	+	+	+	−	+
21	M	25	Adrenal	4	+	+	−	−	+
22	M	20	Adrenal	4	+	−	+	−	−
23	F	26	Adrenal	4	+	+	−	−	+
24	F	29	Adrenal	4	−	+	+	−	−
25	F	29	Adrenal	5	+	+	−	−	+
26	F	8	Adrenal	5	+	+	−	−	+
27	M	34	Adrenal	5	+	+	−	−	+
28	M	29	Neck	5	+	+	+	−	+
29	F	34	Adrenal	5	+	+	−	−	+
30	F	35	Adrenal	5	+	+	+	−	+

Table 2B

Case	Sex	Age (years)	Primary location	Histology	Detectable markers
31	F	4	Left kidney	Lymphoblastic lymphoma	None (within tumour cells)
32	F	3	Paraortic	Lymphoblastic lymphoma	None (within tumour cells)
33	M	3	Pelvic	Lymphoblastic lymphoma	None (within tumour cells)
34	F	5	Femur	Ewing sarcoma	None (within tumour cells)
35	F	10	Knee joint	Ewing sarcoma	None (within tumour cells)
36	M	13	Femur	Ewing sarcoma	None (within tumour cells)

The ages of patients with neuroblastic tumours (14 male, 16 female) varied from 0 to 4 years (2.1 mean) (Table 2A), while patients with non-neuroblastic tumours (2 male, 4 female) ranged from 3 to 13 years (6.3 years mean) (Table 2B).

The tissues were identically fixed in aqueous 10% formalin for 24 h, dehydrated and embedded in paraffin (56° M.P.). Five μm serial sections were mounted on slides pre-coated with poly-L-lysine (Huang et al. 1983). Haematoxylin-eosin staining was carried out for histological examination.

Tumour typing

Various criteria, previously established by others (Makinen 1972) were adopted in order to assess the degree of differentiation among our cases.

The following parameters were considered: 1) presence of large vesicular nuclei 2) development of abundant cytoplasm 3) formation of cytoplasmic processes 4) rosette-like formation of neuroblasts 5) presence of ganglion cells. Depending on the presence of one or more of these our cases were divided into: group A) no parameters of differentiation (undifferentiated), group B) one to three parameters of differentiation (poorly differentiated), group C) four or five parameters of differentiation (well differentiated).

Since our attention was focused on the degree of maturation of tumour cells, other parameters such as lymphocytic infiltration, calcification, extent of necrosis, etc., were not considered, although they may represent important factors with clinical and prognostic implications (Ceccamea et al. 1981).

Immunocytochemistry

The peroxidase anti-peroxidase technique (Sternberger 1979) was applied as follows: The sections were brought to water through xylene and graded alcohols. Endogenous peroxidase was then inactivated by immersion in 0.3% H_2O_2 in phosphate buffer (phosphate-buffered saline 0.2 M, pH 7.0) for 30 min at room temperature. Normal goat serum diluted 1/30 in phosphate-buffered saline was then applied for 30 min at room temperature, followed by the primary antiserum. The primary antisera, all raised in rabbits, were applied at pre-determined optimal dilutions (see Table 1A) in phosphate-buffered saline for 16 h at 4° C in a damp chamber. For characterisation of antisera see Table 1A and B. After rinsing, the second layer antibody (goat anti-rabbit IgG, Miles, 1/100) was applied for 30 min at room temperature. Finally, after washing in phosphate-buffered saline, rabbit peroxidase anti-peroxidase (UCB) diluted 1/300 was placed on the sections. The specific sites of immunoreaction were developed in a solution of phosphate-buffered saline containing 0.0006% H_2O_2 and 0.025% diaminobenzidine (DAB) (Sigma St. Louis, MO, USA). The sections were then dehydrated through graded alcohols and xylene and mounted in a permanent medium (DPX).

Controls

The controls that were carried out comprised dilution of the primary antisera, their substitution with non-immune serum and their use after absorption with an excess of antigen against which they were thought to be specific. In view of the reported similarities among some of the antigens investigated (Bradshaw and Schneider 1980) the immunostaining was also verified using antisera preabsorbed with an excess of structurally related and unrelated antigens included in this study (Table 1B).

Results

A. Histological findings

Neuroblastic tumours. According to their histological appearance and signs of differentiation, cases were classified as follows: 3 cases as group A (undif-

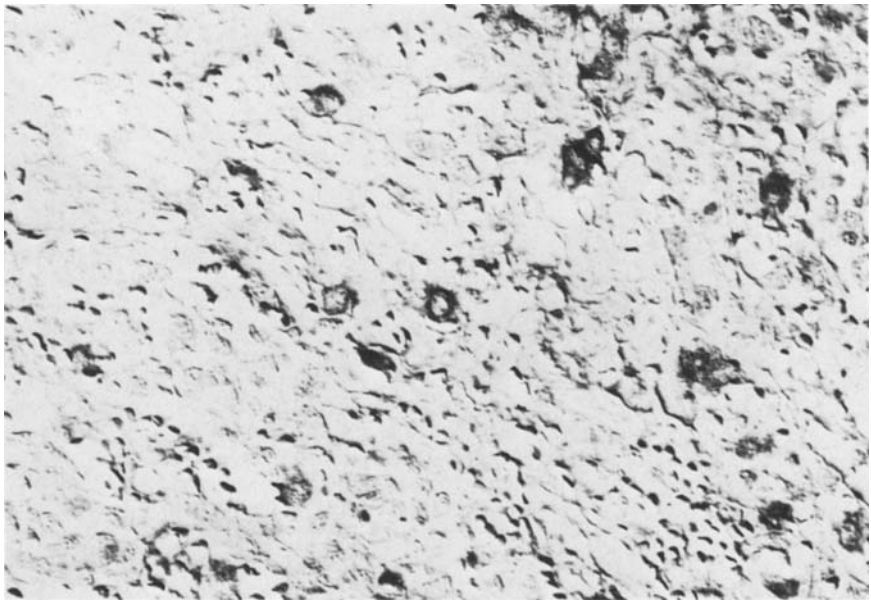

Fig. 1. Undifferentiated neuroblastoma. Scattered immature neoplastic cells stained with antibodies to GFAP. Nomarski optics (mag. × 540)

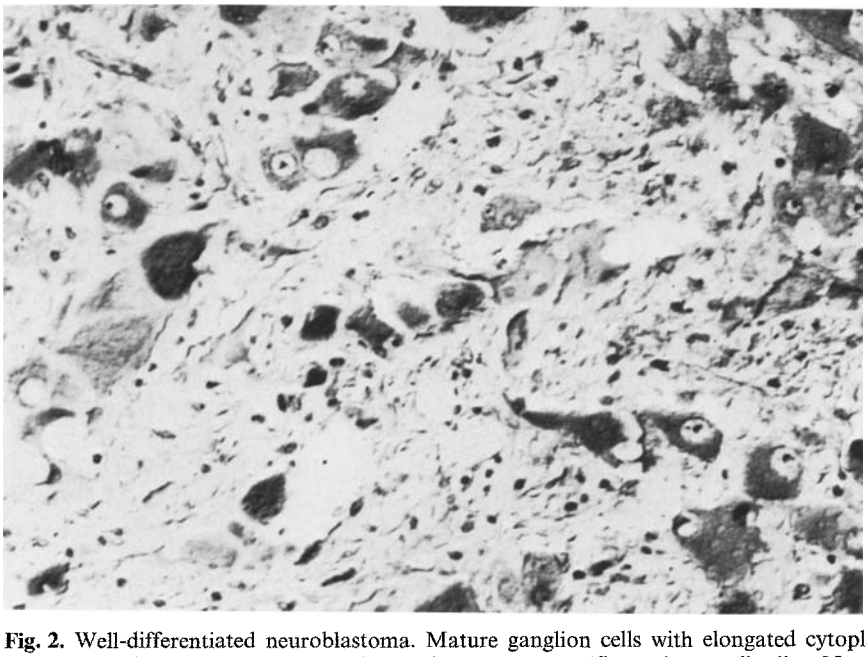

Fig. 2. Well-differentiated neuroblastoma. Mature ganglion cells with elongated cytoplasmic processes and a few neuroblasts stained using neuron specific enolase antibodies. Nomarski optics. Nuclear counterstain with diluted haematoxylin (mag. × 540)

ferentiated), 15 cases as group B (poorly differentiated) and 12 cases as group C (well differentiated). Ganglion cells were detected in all cases from group C, while in group B they were observed in 7 cases out of 15.

Lymphomas. All 3 cases were classified as lymphoblastic lymphomas of diffuse pattern (Lennert 1978).

Ewing sarcomas. In these three cases small, uniform cells with scanty cytoplasm were observed. In two cases a rosette-like pattern was also detected.

B. Immunocytochemistry

All antibodies provided positive immunostaining. Suppression of the staining was obtained either using non-immune primary antisera or by preadsorption with their specific antigens but not by structurally related or unrelated ones (Table 1 B).

Undifferentiated neuroblastic tumours (group A). The 3 cases examined showed no positive immunoreactions for most of the antibodies tested.

Nevertheless, glial fibrillary acidic protein immunoreactivity was observed in one case. Scattered neoplastic cells were positively stained, although the majority of neuroblasts remained unreactive (Fig. 1).

No immunoreactivity for NSE, neurofilaments, S-100 or tyrosine hydroxylase was found in these 3 cases.

Poorly differentiated neuroblastic tumours (group B). Neuron-specific enolase and tyrosine hydroxylase were clearly demonstrated in the majority of mature ganglion cells and in numerous neuroblasts showing signs of differentiation such as increased cytoplasm and axon-like processes.

S-100 protein was often demonstrated in differentiating neuroblasts and in the tangled network of cell processes emanating from them (Fig. 4).

Positive staining for neurofilaments was observed in the perikarya of ganglion cells and in nerve bundles originating from them (Fig. 5). Neurofilaments were also detected in the cytoplasm of neuroblasts with large vesicular nuclei and in their cytoplasmic processes (Fig. 6) while immature neuroblasts were almost entirely unreactive.

Glial fibrillary acidic protein was found in some neoplastic cells with few or no signs of differentiation.

Well differentiated neuroblastic tumours. In this group of neoplasms NSE, tyrosine hydroxylase and neurofilaments were the antigens most frequently observed in the neoplastic cells.

NSE and tyrosine hydroxylase were detected in mature ganglion cells as well as in differentiating neuroblasts or "intermediate cells" (Figs. 2 and 3). Neurofilaments were localised in the perikarya of ganglion cell bodies and in their axons. Most of the differentiating neuroblasts were also positive.

S-100 protein was detected in the cytoplasm of differentiating neuro-

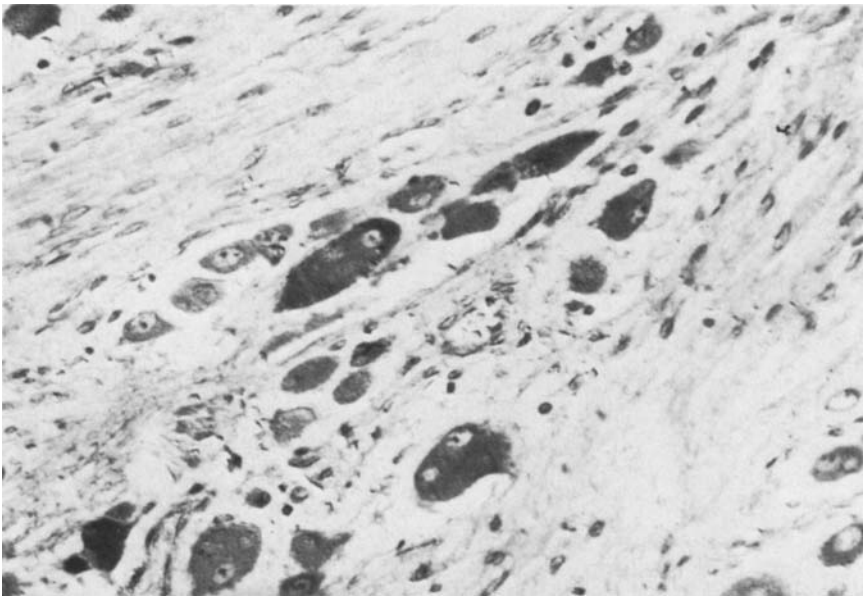

Fig. 3. Well-differentiated neuroblastoma. Numerous ganglion cells, often binucleate, showing strong immunoreactivity to tyrosine hydroxylase antibodies. Nomarski optics (mag. × 432)

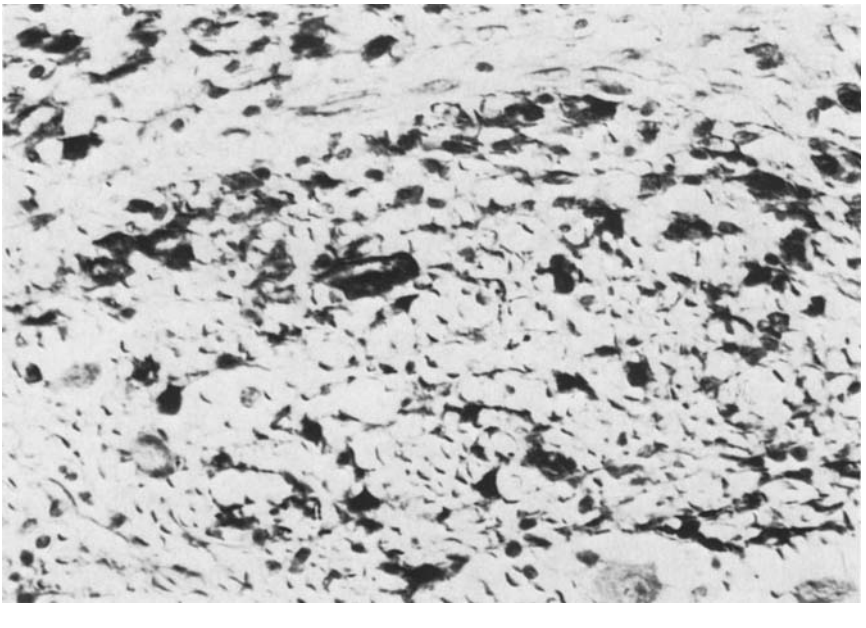

Fig. 4. Poorly differentiated neuroblastoma. A cluster of neuroblasts demonstrated using S-100 antibodies. Nomarski optics, nuclear counterstain with diluted haematoxylin (mag. × 675)

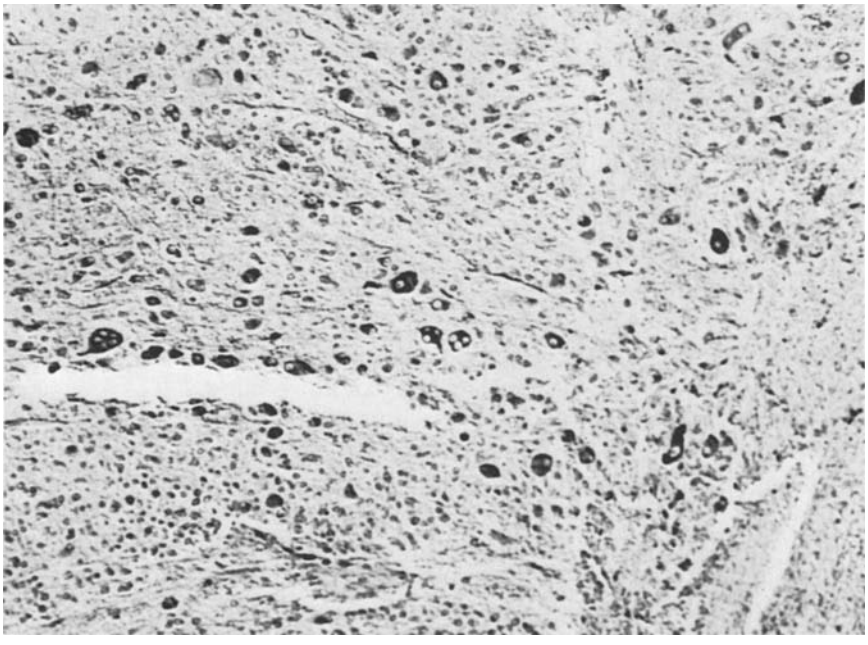

Fig. 5. Poorly differentiated neuroblastoma. Numerous mature ganglion cells and maturating neuroblasts immunoreactive to antibodies to neurofilaments. Nomarski optics. Nuclear counterstain with diluted haematoxylin (mag. × 230)

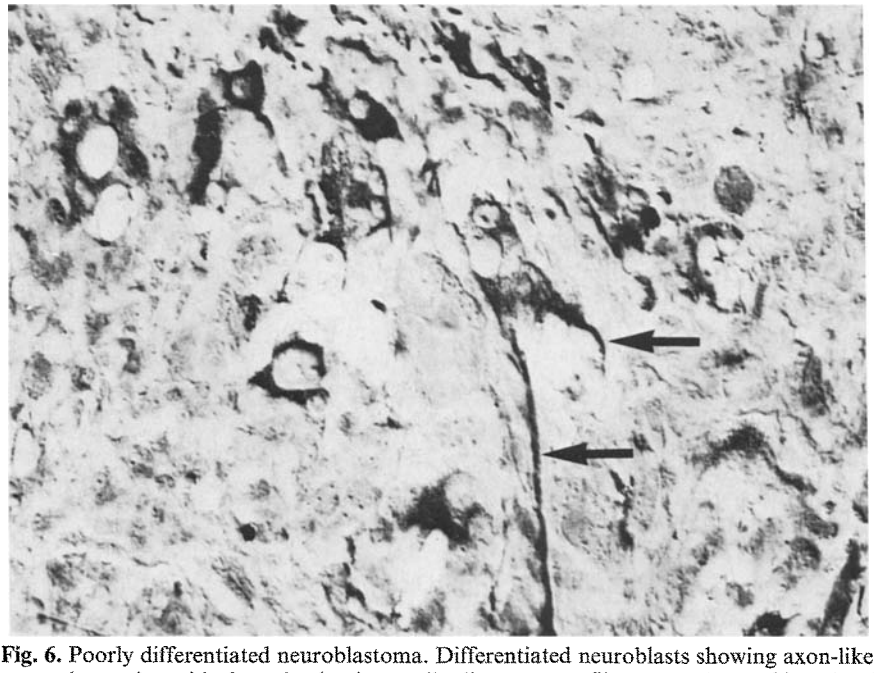

Fig. 6. Poorly differentiated neuroblastoma. Differentiated neuroblasts showing axon-like processes (*arrows*) positively stained using antibodies to neurofilaments. Nomarski optics (mag. × 540)

blasts, while mature ganglion cells were negative. Glial fibrillary acidic protein was never found in this group of neoplasms.

Non-neuroblastic tumours. None of the neural antigens investigated was observed in the neoplastic cells of non-neuroblastic tumours (lymphomas and Ewing sarcomas), although positive staining for some of these antigens was sometimes found in non-neoplastic elements surrounded by the neoplastic growth. In fact, S-100 was localised in nerves and neurofilaments as well as NSE in nerves and ganglion cells.

Discussion

We have demonstrated the presence of neural antigens in 28 of 30 cases of neuroblastic tumours using a wide variety of antibodies.

Neuron-specific enolase was detected in partially differentiated and well differentiated tumours, localised in all mature ganglion cells as well as in differentiating neuroblasts. Its localisation in mature elements lends support to the hypothesis that the enzyme is not present in its specific form during the earlier stages of neuroblastic differentiation (Schmechel et al. 1980). NSE may therefore be considered as a reliable marker for partially differentiated neuroblastic tumours.

S-100 protein was found in neuroblasts with signs of maturation, particularly in poorly differentiated neuroblastic tumours. This observation suggests that S-100 may be present in neuroblasts during certain stages of maturation while progressively disappearing in more differentiated tumours. The occurrence of S-100 in the network of cytoplasmic processes arising from neuroblasts indicates that S-100 immunocytochemistry may be a useful tool for demonstrating these formations, so far difficult to stain with histochemical methods.

Neurofilaments were present in ganglion cells and maturing neuroblasts of more differentiated tumours. They were detected in cell bodies as well as in cytoplasmic processes.

These findings confirm the observation that at least some neuroblastomas may contain neurofilament immunoreactivity (Osborn and Weber 1983). Thus, neurofilaments could be another useful marker for neuroblastic neoplasms, particularly those undergoing initial differentiation.

The demonstration of NSE and NF in these cases supports the hypothesis that immature neoplastic cells tend to differentiate toward neuronal cell lines exhibiting two specific markers of neurons, both localised in the cytoplasm of the same cell types (maturating neuroblasts and ganglion cells).

Abnormal tangles of neurofilaments have been observed in some pathological non-neoplastic conditions (Dahl et al. 1982; Kluck et al. 1984). Further biochemical and ultrastructural analyses are needed to clarify the chemical composition and the spatial arrangement of neurofilament immunoreactivity in neuroblastoma cells.

In our study we have also observed the presence of glial antigens in neuroblastic tumours. Since S-100 as well as glial fibrillary acidic protein was shown in some neoplastic cells in cases with few or no parameters of maturation.

Evidence has been published suggesting that the GFAP molecule shares important similarities with neurofilaments (Goldman et al. 1978). The demonstration of GFAP immunoreactivity in some cases with no signs of maturation appears not to be due to cross reactivity with NF-like material, since preabsorption of GFAP antisera with a great excess of NF did not interfere with the immunostaining (see Table 1 B). Moreover, NF immunoreactivity is present in those cases where GFAP immunoreactivity is virtually absent. These findings suggest that a minority of pluripotential precursor cells are present in most immature cases and that they may display, at least in these stages, specific glial markers such as GFAP.

Tyrosine hydroxylase has also been demonstrated in the majority of ganglion cells and in the so-called "intermediate cells" present in cases with signs of differentiation. Therefore our findings support the hypothesis that these tumours often contain adrenergic substances (Sunderman 1974) that are produced in ganglion cells. In every case each neural protein showed a specific pattern of distribution in a particular cellular element (neuroblast, ganglion cell, etc.). The predominant localisation of each neural protein depended on the degree of maturation of the tumour (Table 2 A).

We conclude that a panel of antibodies to neuronal and glial markers may be extremely useful in the histological assessment of neuroblastic tumours and that comparison of the immunocytochemical pattern with the prognosis and the clinical stage of the disease is warranted.

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