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Distribution of neurofilament protein and neuron-specific enolase in peripheral neuronal tumours

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Summary. Peripheral neuronal tumours were studied by the peroxidase-antiperoxidase (PAP) method for the presence of the neurofilament protein (NFP) and neuron-specific enolase (NSE). All cases of ganglioneuromas and ganglioneuroblastomas were positive for NFP and NSE. Both markers were observed only in tumour cells showing differentiation towards ganglion cells. Of the 14 cases of neuroblastoma, 8 were positive for NFP and 12 were positive for NSE. NSE was detected in most neuroblastic tumour cells. However, NFP was found in neuroblasts with signs of differentiation, such as nuclear enlargement, but not in immature, small round cells. NFP was present in cell bodies as well as in cytoplasmic processes of partially differentiated neuroblasts. The majority of pseudorosettes showed no NFP stain. Thus, antibodies against both NFP and NSE are useful in the diagnosis of peripheral neuronal tumours. Moreover, the presence of NFP seemed to be related to the degree of tumour cell differentiation.

Key words: Peripheral neuronal tumours – Immunohistochemistry – Neurofilament protein – Neuron-specific enolase – Pseudorosette

Neurofilaments are intermediate filaments (10 nm) that play a role in the structural integrity of the neurons. Neurofilament protein (NFP) is composed of three major polypeptides with molecular weights of 68,000 (68Kd), 160,000 (160Kd) and 200,000 (200Kd). Neuron-specific enolase (NSE) is one of a group of isoenzymes in the glycolytic pathway and is a dimer comprised of two γ subunits with a molecular weight of about 80,000. It has been found by the use of immunohistochemical methods, that both proteins are confined to neurons and neuroendocrine cells in normal adult tissues (Trojanowski and Lee 1983). Both proteins have the advantage that

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their antigenicity is preserved in formalin-fixed and paraffin-embedded tissues; thus, they may provide important information on the histogenesis or the degree of differentiation of a given tumour. The peripheral neuronal tumours are considered to be derived from neural crest cells and are classified into neuroblastoma (NBL), ganglioneuroblastoma (GNBL), and ganglioneuroma (GN) by tumour cell differentiation towards mature ganglion cells. NBL is one of the most common, malignant solid neoplasms developing in childhood, however, the diagnosis of NBL by conventional histological procedure is sometimes difficult, because NBL and small round cell sarcomas, such as embryonal rhabdomyosarcoma, Ewing's sarcoma, and malignant lymphoma, have almost identical morphological features.

In this report, we show immunohistochemically the localization of NFP and NSE in neuronal tumours and discuss the correlation between both proteins and neuronal differentiation of the tumours.

Material and methods

A total of 24 peripheral neuronal tumours, including 5 GN (5 mediastinum), 5 GNBL (5 adrenal gland) and 14 NBL (8 adrenal gland, 3 retroperitoneum, 1 pelvis, 1 neck, 1 unknown), was studied. Seven specimens (2 GNBL and 5 NBL) were obtained from autopsy cases. Classification of these tumours was based on conventional histology, ultrastructural morphology and clinical observations (the patient age, primary site, urinary catecholamine metabolites etc.). Although the differential diagnosis between GNBL and NBL was sometimes difficult because of limited numbers of surgically resected specimens, GNBL was defined as a tumour which contained areas similar to the NBL together with collections of ganglion cells.

Human NFP was extracted and purified by a modified version of the method of Mori and Kurokawa (1980). Human spinal nerve roots (approximately 10 g) were collected from autopsy cases. The nerves were transversely minced with a blade and treated in a hypotonic solution consisting of 10 mM Tris-HCl containing 1 mM EDTA (PH 7.2) and then centrifuged. The supernatant was layered on 1 M sucrose and then centrifuged at 30,000 rpm for 15 h at 4° C. The final pellet was used for further purification as crude neurofilaments. These were electrophoresed for 4 h at 4° C with a voltage of 120 V on 7.5% SDS-polyacrylamide gel. Next, each band of the triplet (68Kd, 160Kd, 200Kd) was cut out and proteins were eluted from the gel. The purified NFP was emulsified with Freund's complete adjuvant and injected into New Zealand white rabbits. Antisera were applied to a column of Sepharose 4B coupled with human serum proteins. Specificity of antisera was checked by Ouchterlony's double diffusion.

Antisera against NSE were prepared in New Zealand white rabbits by injection of water

Table 1. Peroxidase-anti-peroxidase (PAP) method for demonstrating NFP and NSE proteins in paraffin sections

1. Deparaffinize in xylol
2. Block endogenous peroxidase with 0.3% H_2O_2 in methanol for 30 min. at room temp.; rinse in PBS
3. Treat with normal swine serum (1:10) for 60 min. at room temp.; rinse in PBS
4. Incubate with rabbit anti-68K serum (1:150) or anti-160K serum (1:200) or anti-200K serum (1:150) or anti-NSE serum (1:100) overnight at 4° C; rinse in PBS
5. Incubate with anti-rabbit IgG (1:20, DAKO) for 60 min. at room temp.; rinse in PBS
6. Incubate with PAP reagent (1:20, DAKO) for 60 min. at room temp.; rinse in PBS
7. Add DAB-H_2O_2 solution for 5 min. at room temp.; rinse in PBS
8. Osmicate, haematoxylin counterstain, dehydrate and mount

in an oil emulsion of purified bovine NSE (Wako pure chemical industries, LTD) and Freund's complete adjuvant. The purity of the antisera was examined by Ouchterlony's double diffusion. The antisera were absorbed with human liver powder.

The immunohistochemical procedure was followed according to Sternberger's peroxidase-antiperoxidase (PAP) method. Formalin-fixed, paraffin-embedded tissues were sectioned and stained (Table 1). Anti-68Kd antiserum, anti-160Kd antiserum, anti-200Kd antiserum and anti-NSE antiserum were used at dilutions of 1:150, 1:200, 1:150 and 1:100, respectively. Normal rabbit serum and antisera absorbed with each specific antigen were used as controls for immunostaining specificity.

In stained sections of some cases, the nuclear size of tumour cells were measured.

Results

All cases of GN were positive for both NFP and NSE. NFP was observed in the axons and cell bodies of some ganglion cells. In the axons all three NFP were present, whereas in cell bodies of ganglion cells, very little 200Kd protein was detected (Fig. 1a–c). Almost all the axons and cell bodies of

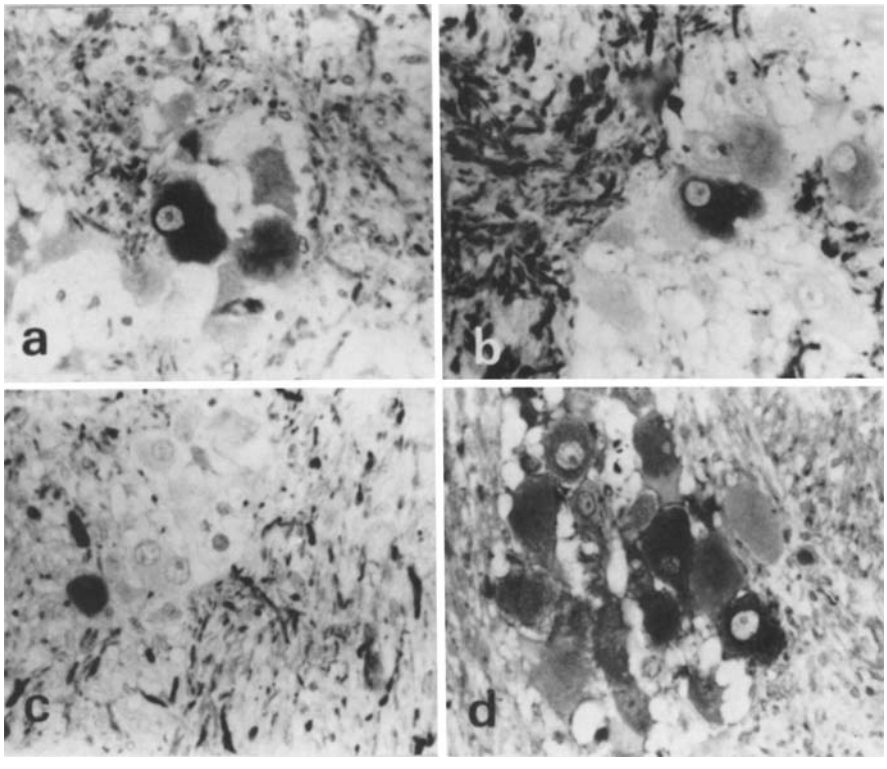

Fig. 1a–d. Ganglioneuroma. Immunoperoxidase staining with **a** neurofilament 68Kd antiserum (1:150), **b** neurofilament 160Kd antiserum (1:200), **c** neurofilament 200Kd antiserum (1:150) and **d** NSE antiserum (1:100). The axons are clearly stained with all three NFP. Note some ganglion cells are negative for NFP-reactivity , especially for 200Kd polypeptide. The perikarya of ganglion cells are diffusely stained with NSE antiserum, but the staining intensity varied from cells to cells. Counterstain with haematoxylin. Magnifications **a–d** ×278

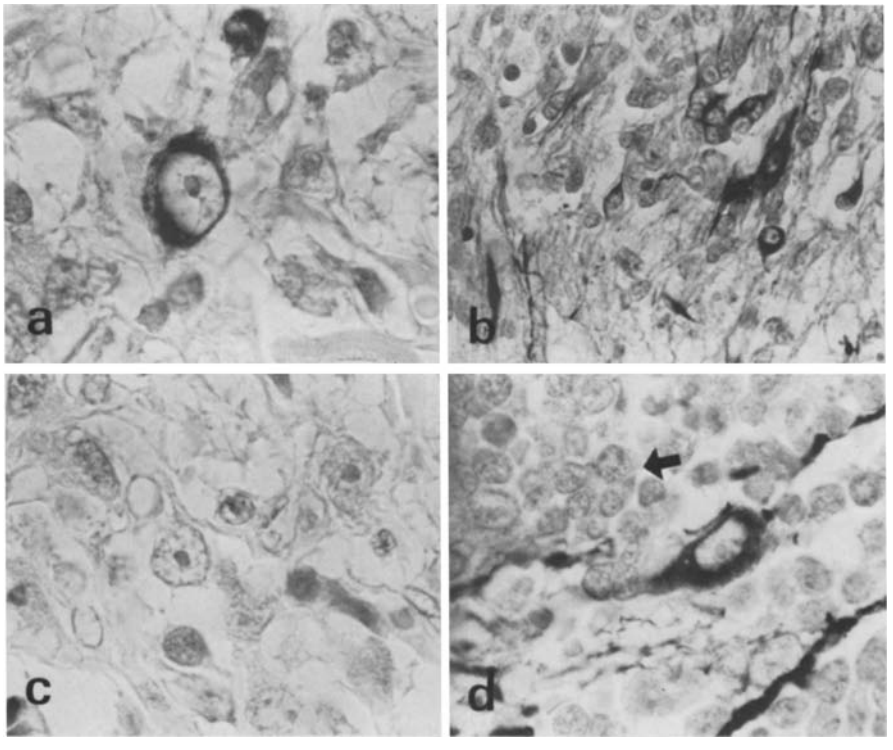

Fig. 2a–d. Ganglioneuroblastoma. Immunoperoxidase staining with **a, b** neurofilament 68Kd antiserum (1:150), **c, d** neurofilament 200Kd antiserum (1:150). 68Kd is present in the perikarya and cytoplasmic processes of immature ganglion cells. 200Kd-positive cells are occasionally detected **d**, but in the same case **a, c**, immature ganglion cells are positive for 68Kd and negative for 200Kd. Neuroblasts are not stained with 200Kd antiserum (arrow). Counterstain with haematoxylin. Magnifications **a, c, d** × 798, **b** × 302

ganglion cells reacted with anti-NSE antiserum (Fig. 1d). No staining for NFP or NSE was noted in Schwann cells or satellite cells. Some spheroid-like bodies in tumour tissues were positive for NFP and NSE.

All GNBL showed positive reaction for NFP and NSE. GNBL contained a variable number of differentiating neuroblasts which showed different degree of differentiation. Fine cell processes and cell bodies of immature ganglion cells were stained with anti-68Kd and 160Kd antisera (Fig. 2a, b), but few were stained with anti-200Kd antiserum (Fig. 2c). In a small number of immature ganglion cells, there were 200Kd polypeptide as well as 68Kd and 160Kd polypeptides (Fig. 2d). Some NFP-positive cells were seen in areas of neuroblastic tumour cells, but fewer than in areas of differentiating tumour cells. Anti-NSE antiserum stained nearly all the tumour cells in GNBL. Ganglion cells had the most intense staining for NSE. However, some ganglion cells and differentiating neuroblasts were not detected with the anti-NSE antiserum (Fig. 3).

Of 14 NBL, 8 showed positive staining for NFP and 12 positive for NSE.

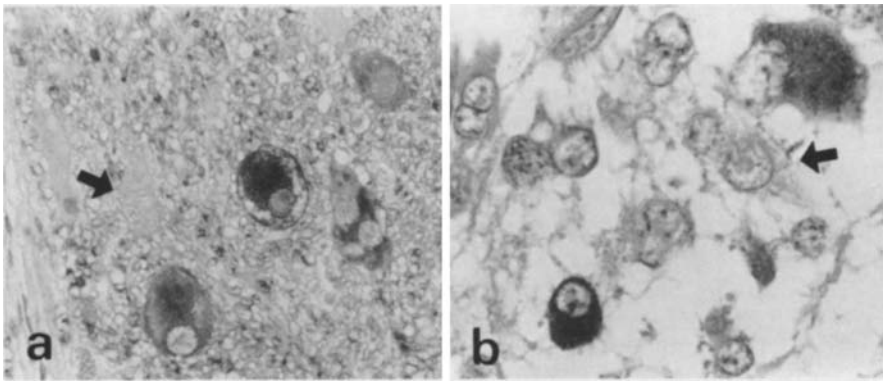

Fig. 3a, b. Ganglioneuroblastoma stained with NSE antiserum (1:100). Some ganglion cells and differentiating neuroblasts showed strong reaction for NSE. Note some differentiated tumour cells either negative or slightly positive for NSE-reactivity (arrow). Immunoperoxidase-haematoxylin. Magnifications **a** ×300, **b** ×733

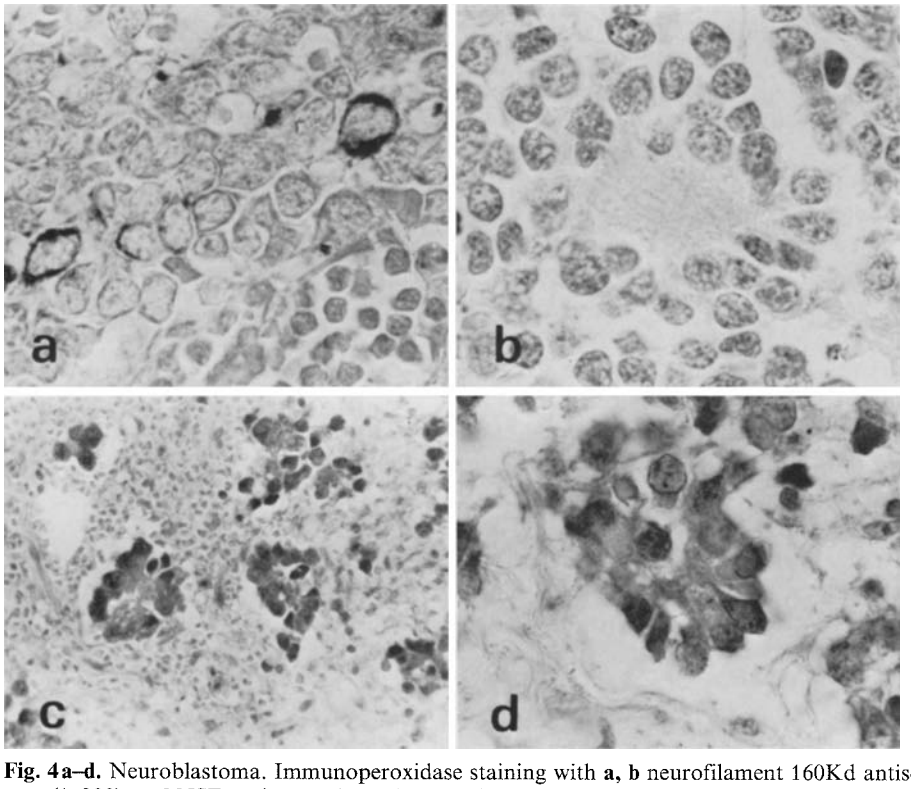

Fig. 4a–d. Neuroblastoma. Immunoperoxidase staining with **a, b** neurofilament 160Kd antiserum (1:200), **c, d** NSE antiserum (1:100). 160Kd is seen in the perikarya of some neuroblastic tumour cells, but not in the center of pseudorosette **b.** NSE is present in the cytoplasm of almost all neuroblastic tumour cells, their processes and pseudorosettes **c, d.** Counterstain with haematoxylin. Magnifications **a–d** ×722

In NFP-positive cases, 5–50% of the neuroblastic tumour cells were stained. 68Kd and 160Kd polypeptides were seen in neuroblasts which had enlarged pale nuclei of more than 6 μm in diameter. Both polypeptides were located mainly in cell processes of tumour cells, but occasionally in scanty cytoplasm (Fig. 4a). No staining of tumour cells was seen with anti-200Kd antiserum. In 10 cases, pseudorosettes were found in tumour tissues; few of them showed a positive reaction for NFP (Fig. 4b). Staining for NSE was demonstrated in tumour cell cytoplasm and in the fibrillary stroma. Positively stained cells showed an even cytoplasmic distribution of reaction products. In NSE-positive cases, all of the immature, small round cells showed a weak, positive reaction for NSE. The center of the pseudorosettes was stained with anti-NSE antiserum (Fig. 4c, d).

In NFP and NSE staining, tumours at post-mortem showed only slightly positive, or negative staining.

Discussion

In the present study, NFP was detected in a high percentage of peripheral neuronal tumours immunohistochemically, using formalin-fixed and paraffin-embedded materials.

NFP, an intermediate filament protein, is specific for most, but not all, neurons. The tissue and cellular distribution of NFP has been studied with polyclonal or monoclonal antibodies in various tumours. Antibodies to NFP have been shown to stain NBL, GNBL, GN, phaeochromocytoma, medulloblastoma and oat cell carcinoma (Osborn et al. 1982; Trojanowski and Lee 1983; Lehto et al. 1983; Rorke 1983; Carlei et al. 1984). However, results with NBL are still equivocal. NBL is less sensitive against NFP when tested in paraffin-embedded materials (Osborn et al. 1982; Trojanowski and Lee 1983; Gown and Vogel 1984). Osborn and Weber (1983), however, showed that all NBL were positive for NFP, using frozen sections. It is not yet clear whether these differences can be attributed to the different methods used or the histological type of tumours, which were not described in detail. Recently, Carlei et al. (1984) reported that NFP was detected in 21 of 30 neuroblastic tumours (ganglioneuroblastoma, neuroblastoma) with the PAP method using formalin-fixed and paraffin-embedded specimens.

The present study showed that all cases of GN and GNBL were positive for NFP. In these tumours, NFP was confined to neuronal elements, such as axons and ganglion cells. Moreover, it was found that the area of mature tissues contained more NFP-positive cells when compared with the area of less mature tissues.

Antibodies against NFP reacted with a higher percentage of NBL than previous reports. Many of the NBL we examined consisted of neuroblasts with some sign of maturation. Signs of maturation in NBL have been listed as the appearance of nucleoli, enlargement of the nuclei, development of cytoplasm, formation of cytoplasmic processes, and pseudorosette formation etc. (Beckwith and Martin 1968; Hughes et al. 1974; Mäkinen 1972).

Of these signs, enlargement of the nuclei can be evaluated objectively. Our results showed that relatively matured neuroblasts with enlarged nuclei of more than 6 μm in diameter contained NFP more often and that NFP reaction did not occur in immature, small round cells. Moreover, NFP was detected in cytoplasmic processes. These findings are consistent with the data of Carlei et al. (1984). Therefore, the presence of NFP seemed to be related to the degree of tumour cell differentiation.

The pseudorosette is a characteristic pattern in NBL and is often emphasized in histological diagnosis. The central area of pseudorosette is explained to be mostly composed of neurofibrils under light microscopy and as aggregates of cytoplasmic processes ultrastructurally. In electron microscopic studies of NBL, cytoplasmic processes were shown to contain neurosecretory granules, small vesicles, microtubules and neurofilaments (Misugi et al. 1968; Tazawa et al. 1971; Nakayama et al. 1975; Romansky et al. 1978). However, it has been reported that microtubules and neurofilaments were not consistently present in processes (Taxy 1980). In this study, most pseudorosettes were negative for NFP. Our results indicate that they are composed of immature cytoplasmic processes without neurofilaments. Furthermore, there was no correlation between NFP-staining and pseudorosette formation in our cases. It seems inconsistent that the pseudorosette is identified as evidence of neuronal differentiation of tumour tissues (Beckwith and Martin 1968). As mentioned above, the localization of NFP seemed to correspond considerably with the degree of tumour cell differentiation from immature neuroblasts to mature ganglion cells. Therefore, it is possible that pseudorosette formation implies that tumour tissues resemble immature nervous tissues, not neuronal differentiation.

Comparing the localization of each polypeptide of NFP in tumours, 68Kd and 160Kd polypeptides were present nearly in parallel; whereas the 200Kd polypeptide was detected only in highly differentiated tumour cells. Shaw and Weber (1982) reported that 200Kd polypeptide appeared later in the development of rat nervous system than did 68Kd and 160Kd polypeptides. Our results seem to be consistent with their view.

Some researchers have reported that there is a high percentage of NSE in neuronal tumours (Tapia et al. 1981; Dhillon et al. 1982; Nakajima et al. 1983; Carlei et al. 1984; Vinores et al. 1984). Nakajima et al. (1983) reported that ganglion cells in GN and GNBL usually showed more intense immunoreaction for NSE than immature neuroblasts. Our results were compatible with their findings. Although the amount of antigens can not be estimated by the staining intensity in different sections, ganglion cells are considered to contain a higher level of NSE than neuroblasts in sections of GNBL.

In NBL, immature, small round cells showed a weak NSE immunoreactivity. The staining intensity varied among specimens. However, no correlation was observed between NBL cell morphology and NSE reactivity. Two NBL, both autopsy cases, were negative for NSE. This is assumed to be a result of an inappropriate fixation process during the post-mortem period, not tumour cell morphology.

Recently, the concentration of serum or tissue NSE in patients with

NBL was determined. Odelstadt et al. (1981) suggested that the NSE level in NBL reflects the degree of histological differentiation of the tumour. In contrast, Ishiguro et al. (1983) showed that the level of γ subunit or the γ: $(\alpha+\gamma)$ ratio in tissues was not related to the type of NBL. This disagreement might be explained by differences in necrosis and presence of non-neoplastic cells. In our cases, necrosis, haemorrhage and stroma were observed in various amounts. In addition, the present study showed that many ganglion cells were not stained with NSE antiserum in GN and GNBL and almost all the neuroblasts reacted positively with NSE within tumours. The localization of NSE in tumour cells demonstrated immunohistochemically may reveal the level of tissue NSE.

Finally, let us compare NFP staining with NSE staining and discuss the value for histological tumour diagnosis. In general, GN and GNBL are easily diagnosed by routine Haematoxylin-Eosin stain. Both NFP and NSE can be useful markers for supporting the diagnosis because all GN and GNBL are positive for both proteins. In the NBL we examined, positive staining for NFP occured less often than for NSE. However, NSE staining results were difficult to judge due to poor contrast. Moreover, NSE has been detected immunohistochemically in a number of non-neuronal tumours including glioma, meningioma, neurinoma, carcinoma and fibroadenoma of the breast and renal cell carcinoma (Vinores et al. 1984). However, it has been reported that no reaction was seen with antibodies against NFP in the tumours of non-neuronal derivation, except oat cell carcinoma. At present, NFP seems to be a more relevant marker than NSE for evidence of neuronal differentiation in tumours. The immunohistochemical application of NFP and NSE antibodies may provide a useful tool for the diagnosis of peripheral neuronal tumours. Also NFP may be useful in the evaluation of cyto-differentiation of NBL. However, so far it seems that the presence of NFP and NSE, particulary NSE, does not imply a neuronal origin for tumours in the differential diagnosis of undifferentiated sarcomas. It is necessary to investigate the localization of NFP in various types of tumours in order to determine its specificity.

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