Histochemical demonstration of acetylcholinesterase in neuroblastoma

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SUMMARY  The presence of acetylcholinesterase in the tumour cells of neuroblastoma has been shown by enzyme histochemistry. For comparison, some other tumours likely to be found in children and commonly presenting histologically as small cell tumours have also been studied. Acetylcholinesterase activity was seen in rhabdomyosarcoma, but, compared with neuroblastoma, the activity was focal and sparse. One Ewing’s tumour and a lymphoblastic lymphoma were negative for the enzyme reaction.

Some of the ultrastructural features of neuroblastoma are correlated with the presence of this enzyme. Acetylcholinesterase enzyme histochemistry may provide a useful adjunct in the distinction of neuroblastoma from other small cell tumours.

Choice of treatment and prognosis in childhood malignancy are determined largely by tumour cell type. Childhood tumours often present histologically as small cell tumours, which may be difficult to resolve by conventional microscopy alone. Rosette formation, neurofibrillary material, and early ganglionic differentiation are light microscopic features which favour a diagnosis of neuroblastoma. These criteria are not always present, however, and, consequently, neuroblastoma may be difficult to distinguish from Ewing’s sarcoma, rhabdomyosarcoma, and lymphoma. Electron microscopy, immunocytochemistry for neurone specific enolase, and induced catecholamine fluorescence, as well as monoclonal antibodies, have all been recommended to aid histological diagnosis, but many of these procedures are lengthy, are not without technical problems, and are often not specific for neuroblastoma.

The concept of “one nerve cell, one neurotransmitter” has been challenged recently. Morphological and pharmacological evidence exists in animals that distinction between neurone groups with regard to their content of neurotransmitter is not clear cut. Until quite recently, catecholamine secretion had generally been assumed to be the only biochemical expression of neuroblastoma. But several studies on neuroblastoma have now shown biochemically the presence of an acetylcholine synthesising enzyme, choline acetyltransferase, and one study has shown the coexistence of acetylcholinesterase and catecholamines cytochemically in cultured murine neuroblastoma. Neuroblastoma now has recognised status among the neuroendocrine tumours, and one of the many characteristics of that tumour group is the ability to express acetylcholinesterase. As far as we know, acetylcholinesterase activity has not been harnessed for clinical diagnosis of neuroblastoma, and the aim of the present study was to show by enzyme histochemistry its presence in two cases of human neuroblastoma. For comparison, the enzyme reaction was also evaluated in some other small cell tumours.

Patients and methods

One of the neuroblastomas was removed from a six month old baby girl who presented with an abdominal mass (case 1). Urinary concentration of vanilmandelic acid and homovanillic acid were increased and her blood pressure was raised. The second tumour was resected from the retroperitoneal region of a four month old baby boy, whose urine also contained a high concentration of vanilmandelic acid (case 2).

For electron microscopy, fresh tissue from both tumours was excised, diced into 1 mm cubes, and fixed in phosphate buffered 2% paraformaldehyde-2.5% glutaraldehyde pH 7.3 at 0-4°C (buffer osmolarity 288 mOsm/l). In both cases the tissue was post-fixed in osmium tetroxide, processed by standard methods, and embedded in Araldite.

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Sections (1 μm thick), stained with toluidine blue, were assessed to ensure representative sampling, and ultrathin sections, stained with uranyl acetate and lead citrate, were studied in an AEI Corinth 275 electron microscope.

Fresh tissue was also snap frozen in liquid nitrogen and stored (in liquid nitrogen) until required. Tissue was then sectioned in a cryostat at 10 μm thickness and stained for acetylcholinesterase activity using a modification of the method of Karnovsky and Roots. The remainder of the tumour was processed as for routine microscopy, embedded in paraffin wax, cut at 6 μm, and stained with haematoxylin and eosin.

The same histochemical procedure for acetylcholinesterase was also performed on rhabdomyosarcoma (four cases), lymphoblastic lymphoma (one case), and Ewing’s tumour (one case). Of the four rhabdomyosarcomas, three conformed to the alveolar subtype; the other was of the embryonal pattern. The latter tumour was fairly well differentiated with many large rhabdomyoblasts and frequent cytoplasmic cross striations. The alveolar rhabdomyosarcomas were generally composed of small, undifferentiated cells with an occasional early rhabdomyoblast. Rare cytoplasmic cross striations were found in only one of these tumours.

**Fig. 1 Neuroblastoma from case 1, showing an essentially undifferentiated small cell pattern but with several characteristic neurofibrillary rosettes. Haematoxylin and eosin. Original magnification × 145.**

**Fig. 2 Neuroblastoma from case 2, showing early ganglionic differentiation, patches of neurofibrillary material and focal calcification. Haematoxylin and eosin. Original magnification × 230.**

**Results**

Neuroblastoma was confirmed by conventional microscopy and electron microscopy in both tumours. In the tumour from case 1 this was generally poorly differentiated, but neurofibrillary rosettes, characteristic of neuroblastoma, were occasionally seen (Fig. 1). The tumour from case 2 showed moderate differentiation with focal calcification, early ganglionic differentiation, and patches of neurofibrillary matrix (Fig. 2). Both tumours showed variable acetylcholinesterase activity, which occurred as a brown granular precipitate mainly surrounding individual nuclei, which were pale by contrast (Fig. 3). Larger tumour cells seemed to show a more intense reaction (Fig. 4), but most of the smaller cells were also positive, albeit to a lesser degree. Anuclear patches of fine positive granularity, apparently corresponding to areas of neurofibrillary matrix seen in sections stained with haematoxylin and eosin, were also present. The centres of rosettes in the tumour from case 1 showed a similar reaction.

Cellular activity for acetylcholinesterase was present in three of the four rhabdomyosarcomas. This was conspicuous in the tumour with the embryonal pattern and prominent rhabdomyoblasts
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Fig. 3 Acetylcholinesterase in tumour from case 1, showing a generalised positive reaction with patchy accentuation. Acetylcholinesterase. Original magnification × 145.

Fig. 4 Acetylcholinesterase in tumour from case 2, showing positive clumps of moderate sized cells corresponding to areas of early ganglionic differentiation. Acetylcholinesterase. Original magnification × 250.

(Fig. 5). Two of the three tumours of the alveolar subtype showed positive staining, which was focal and sparse (Fig. 6). The remaining alveolar tumour was negative. The Ewing's tumour and lymphoma also showed no discernible reactivity for acetylcholinesterase.

Ultrastructurally, both neuroblastomas contained dense cored vesicles as well as small clear vesicles within their neurite processes. Cored vesicles generally measured 100–120 nm in diameter, being about twice the size of the clear vesicles (Fig. 7), which were 50–80 nm in diameter. Small clear vesicles were easily distinguishable from cross sectioned neurotubules, which measured 20 nm in diameter.

Discussion

Until 1972 it was generally assumed that neuroblastoma originated in sympathetic neurones and that catecholamine was its sole biochemical expression. Choline acetyltransferase, an acetylcholine synthesising enzyme, has since been shown biochemically in cultured murine neuroblastoma, cultured human neuroblastoma, and also, more directly, in...
Fig. 6  Alveolar pattern of rhabdomyosarcoma with sparse focal acetylcholinesterase activity. Small rhabdomyoblasts were discernible only occasionally in routine stained sections. Acetylcholinesterase. Original magnification × 230.

Human tumour. Acetylcholinesterase also has been shown biochemically in cultured murine neuroblastoma, and one report has referred to its cytochemical demonstration in murine cultured neuroblastoma. These investigations all support the biochemical diversity of the cell type of neuroblastoma. Acetylcholinesterase activity in our two randomly selected cases suggests that this is a common integral feature of this neoplasm. The combination of acetylcholinesterase activity, as shown here, and the clinical evidence in the two patients of catecholamine secretion, support further the existence of biochemical diversity of the neoplasm.

On the basis of the relative activity of two neurotransmitter synthesising enzymes, including choline acetyltransferase, three cell types in neuroblastoma have been defined. Choline acetyltransferase activity was variable and even absent in one tumour. In cultured murine tumour, acetylcholinesterase activity may be enhanced by restricting cell division or by adding acetylcholine to the culture medium. The former is apparently associated with concurrent morphological differentiation but the latter is independent of such differentiation. These experiments show that in vitro the enzyme is under the control of several factors. It is therefore difficult to predict the true incidence of histochemically demonstrable

Fig. 7  Electron microscopic view of neurite processes in tumour from case 1, showing a mixture of dense cored vesicles (arrow) and clear vesicles (arrowhead). Uranyl acetate and lead citrate × 62500.
acetylcholinesterase activity in a larger sample of
tumours.

Electron microscopy of both tumours showed
three main types of intracytoplasmic vesicles: small
cored vesicles, large cored vesicles, and small clear
vesicles. Of these, only small cored vesicles have
been shown to contain catecholamine,16 but
Yokoyama et al17 could not exclude a similar func-
tion for the large cored vesicles in their ultrastruc-
ture study. While the additional presence of small
clear vesicles has been described in neuroblastoma,
no definite functional role seems to have been
assigned to them. Small clear vesicles in axon
profiles of mammalian tissue have been related to
cholinergic neuronal transmission.5 It has been sug-
gested that their presence in neuroblastoma rep-
resents the morphological aspect of a related func-
tion.18

The histochemical procedure for showing the
presence of acetylcholinesterase is used widely in
diagnosing Hirschsprung’s disease,11 and most
laboratories dealing with childhood tissues are
therefore already familiar with its application. The
method is straightforward and can be executed in a
few hours. Of the two tumours described here, one
was moderately differentiated and, under ordinary
circumstances, would present little difficulty with
histological diagnosis. The other was generally
poorly differentiated, however, and the demonstra-
tion of acetylcholinesterase activity in that tumour
probably reflects a substantial amount of the enzyme
in early neuroblasts. By contrast, the positive acetyl-
cholinesterase activity in the rhabdomyosarcomas
appeared to be associated with rhabdomyoblastic
differentiation, whereas the small, undifferentiated
cells were negative. This resulted in focal cellular
positivity in two of the alveolar rhabdomyosar-
comas, in which the cellular pattern was essentially
undifferentiated.

Red blood cells contain acetylcholinesterase,11
and haemorrhage into tumours may be a potential
source of concern for accurate interpretation; how-
ever, cytoplasmic localisation of the enzyme, which
is easily discernible, should resolve any difficulty.
Application of the method to a larger number of
tumours will gauge its real value as a histochemical
marker. The technique also has a potential to distin-
guish between neuroblastoma and other small cell
tumours as well as providing a convenient method
by which to study further the nature and behaviour
of this clinically unpredictable neoplasm.

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