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An investigation of β enolase as a histological marker of rhabdomyosarcoma

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SUMMARY Sections from 21 tumours diagnosed as primary or metastatic rhabdomyosarcoma were stained for α and β enolase. The cases were subdivided into embryonal and alveolar subtypes (38% and 62%, respectively). Positive cytoplasmic staining for α enolase was seen in all but one case, and cytoplasmic staining for β enolase was seen in some cells in 18 of the 21 cases (86% of the total, 88% of the alveolar subgroup, and 85% of the embryonal subgroup). No cells stained positively for β enolase in the control series of neuroblastomas, fibrosarcomas, Wilms' sarcomas, and an osteosarcoma. The results show that β enolase is a sensitive marker of muscular differentiation in rhabdomyosarcoma.

Rhabdomyosarcoma is the commonest soft tissue sarcoma in childhood. Recent improvement in survival of patients with this neoplasm is due to a combination of improved staging of the disease enabling the choice of optimal treatment and more accurate histological interpretation.[1] Diagnostic accuracy is also important for the proper evaluation of different treatment protocols and in assessing prognosis.

The histological diagnosis of rhabdomyosarcoma is based largely on the overall cellular pattern and the presence of rhabdomyoblasts with or without cytoplasmic cross striations.[2] Although a large proportion of tumours may be diagnosed with reasonable confidence using these criteria, many are not well differentiated and, depending on histological pattern, may potentially be confused with a variety of spindle celled, myxoid, and small cell neoplasms.[3]

A variety of special techniques have been used to try to clarify the nature of the more undifferentiated neoplasms, including electron microscopy[4 5] and the identification of tumour markers by enzyme histochemical[6] or immunohistochemical[7] techniques. Electron microscopy has the disadvantage of being subject to sampling error, and the ultrastructural criteria advocated for diagnosis[5] have become so strict that many tumours may go unrecognised if too much reliance is placed on this method. Several cytoplasmic protein constituents of striated muscle such as myosin,[7] actin,[8] desmin[9] and, more recently, myoglobin[10 11] have been investigated as potential markers for identifying immature neoplastic muscle cells. Of these, only myoglobin appears to be specific. The percentage of tumours staining positively for myoglobin is less than the percentage of tumours diagnosed as rhabdomyosarcoma on the basis of overall cellular pattern and cytological characteristics.[11 12] It is for this reason that further markers should be assessed in the hope that at least some of the less well differentiated neoplasms can be shown to contain cellular components that define the cells as muscular in origin. One such marker, not yet studied from this aspect, is β enolase, which occurs mainly in muscle.

Enolase is a dimeric glycolytic enzyme which has three immunologically distinct subunit types, designated α, β, and γ.[13–16] The α subunit, which is found in all adult tissues, is also the fetal form of the enzyme. In adult skeletal muscle the ββ isoenzyme accounts for 3% of the soluble protein, that is 3mg/g wet weight of tissue. Radioimmunoassay of enolase isoenzymes in human tissues[16 17] shows that heart contains only 10% as much β enolase as skeletal muscle and that other tissues have less than 1%. The pattern of immunoperoxidase staining for β enolase is consistent with the results of radioimmunoassay;[16] skeletal muscle fibres show intense staining, whereas smooth muscle stains only weakly.

β enolase measured by immunological inhibition of enzyme activity has already been used as an index of muscle damage in myocardial infarction[18] and muscular dystrophy.[19] It has also been used as a marker of muscle phenotype in studies of the dif-

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ferentiation of cultured mouse teratocarcinoma cells.[20]

The purpose of this study was to determine the usefulness of β enolase as a diagnostic marker of rhabdomyosarcoma using an immunoperoxidase peroxidase-antiperoxidase technique.

Material and methods

PREPARATION OF ENOLASE ISOENZYMES AND ANTISERA

Pure $\alpha\alpha$ enolase was prepared from human brain as previously described[21]; $\beta\beta$ enolase was purified from human muscle according to the procedure used by Rider and Taylor for the rat isoenzyme.[13]

Antisera against these isoenzymes were raised in rabbits by injecting 1 mg of protein in 1 ml Freund's complete adjuvant followed by booster doses of 0·2–0·5 mg in 1 ml Freund's incomplete adjuvant. The rabbits were bled from the marginal ear vein one week after each booster injection. Adsorbed antisera were obtained by successive 30 min incubations with the appropriate purified isoenzyme followed by centrifugation at 20 000 *g* for 15 min.

HISTOLOGY

Material from 21 previously diagnosed cases of rhabdomyosarcoma were retrieved from the pathology files of the Children's Hospital, Sheffield. The tissue had been fixed in 4% formol-saline for between 24 and 48 h and processed in paraffin wax. Standard 6μm sections were cut and stained with haematoxylin and eosin, Masson's trichrome stain, phosphotungstic acid haematoxylin, the periodic acid Schiff technique, and for α and β enolase by the peroxidase-antiperoxidase technique using 3,3′-diaminobenzidine as the final indicator.[21]

The diagnostic criteria used were the site of the tumour, the overall histological pattern, and evidence of skeletal muscle differentiation such as cells thought to be rhabdomyoblasts, with or without cytoplasmic cross striations. A variable distribution of rhabdomyoblasts set against a background of small, undifferentiated cells was the usual pattern,

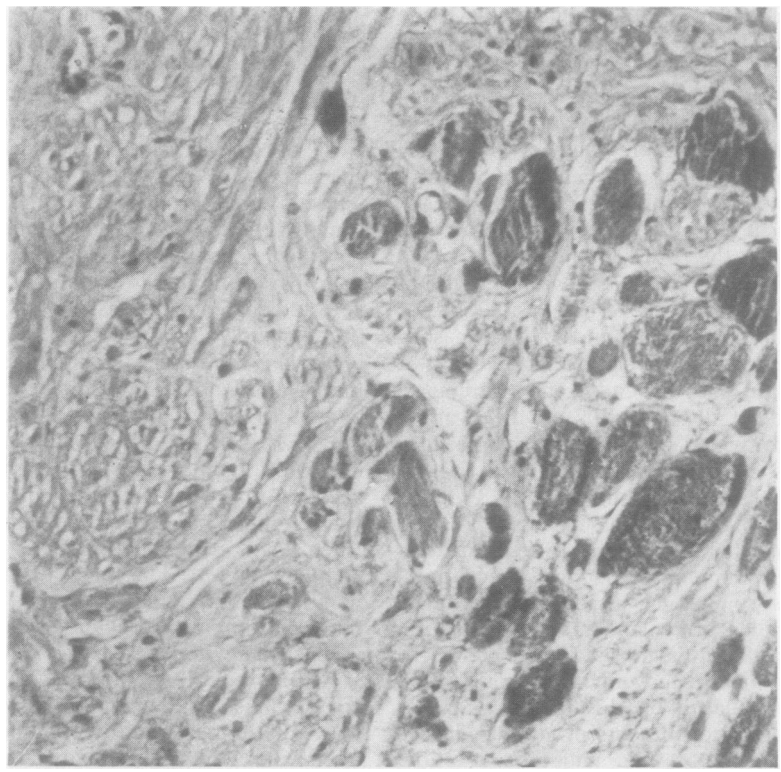

Fig. 1 *Section of the edge of a normal prostate gland stained for β enolase. Note the strong staining of skeletal muscle fibres of the pelvic floor in contrast to weak staining of the smooth muscle of the prostate stroma. Original magnification* × 750.

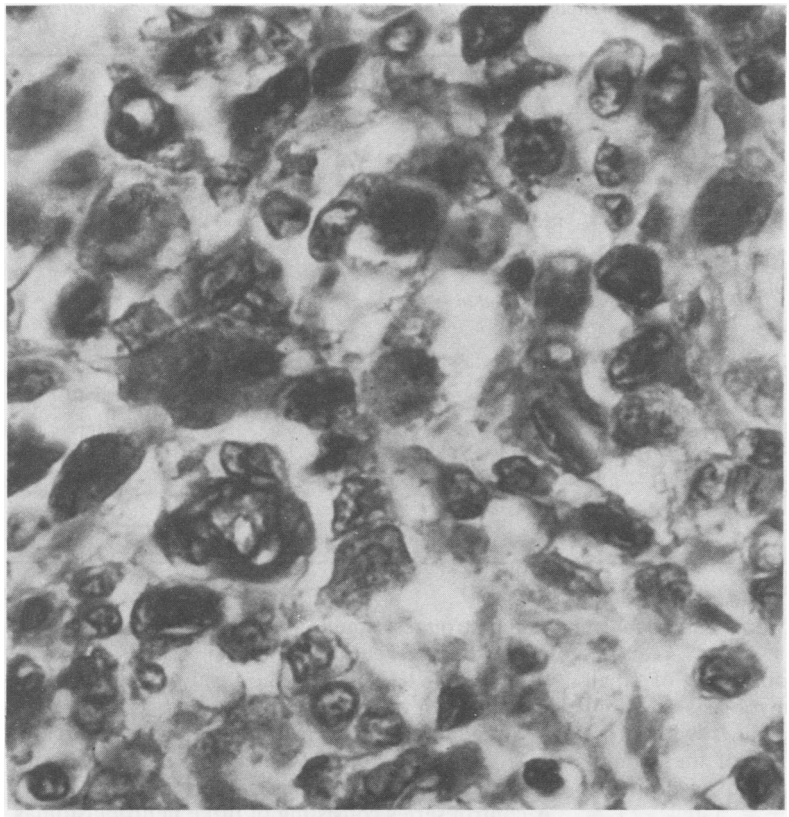

Fig. 2 *Section of a rhabdomyosarcoma stained for β enolase showing marked variation in the intensity of staining of rhabdomyoblasts. Original magnification × 1200.*

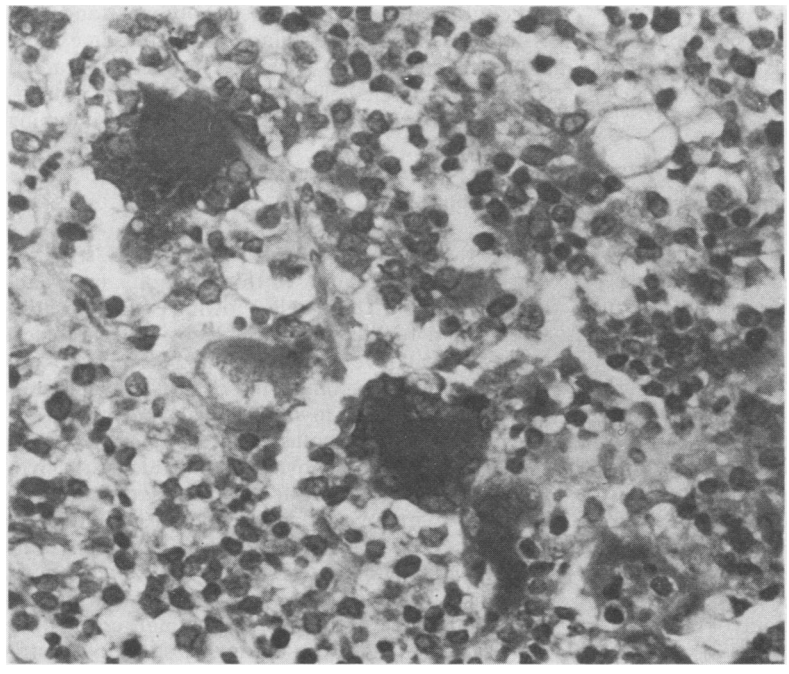

Fig. 3 *Section of rhabdomyosarcoma stained for β enolase showing very strong staining in multinucleate cells with large peripheral nuclei. Original magnification × 750.*

Table *Degree of cytodifferentiation related to intensity of staining for α and β enolase in alveolar and embryonal patterns of rhabdomyosarcoma*

Case no	*Small cells*	*Rhabdomyoblasts*	*Cross striations*	*α enolase*	*β enolase*
		Alveolar rhabodmyosarcoma (n = 8)			
1	3+	2+	0	2+	3+
2	3+	2+	0	4+	3+
3	3+	0	0	trace	0
4	3+	1+	1+	3+	3+
5	3+	1+	0	4+	2+
6	2+	1+	0	2+	2+
7	3+	0	0	2+	3+
8	3+	0	0	4+	2+
		Embryonal rhabdomyosarcoma (n = 13)			
1	3+	0	0	trace	0
2	3+	0	0	0	0
3	3+	1+	0	4+	3+
4	3+	1+	0	4+	1+
5	3+	2+	1+	4+	1+
6	3+	2+	1+	4+	1+
7	2+	3+	3+	2+	1+
8	3+	1+	0	1+	1+
9	2+	2+	1+	3+	2+
10	3+	2+	1+	2+	1+
11	3+	0	0	2+	trace
12	3+	1+	1+	3+	1+
13	3+	1+	0	5+	1+

and cytoplasmic cross striations were seen in fewer than half of the tumours.

In each case the relative proportions of small undifferentiated cells, rhabdomyoblasts without cross striations, and rhabdomyoblasts with cross striations were evaluated and each of these modalities was arbitrarily assigned a symbol according to prevalence: 0 = not present; 1+ = a few; 2+ = moderate in number; 3+ = many.

Sections of normal skeletal and smooth muscle were stained for β enolase to illustrate the relative staining intensities of the two muscle types (Fig 1).

Results

In 21 tumours diagnosed as primary or metastatic rhabdomyosarcoma, sections were stained for α and β enolase. The cases were subdivided into embryonal and alveolar subtypes (38% and 62%, respectively). Cross striations were present in 20% of the alveolar and 50% of the embryonal rhabdomyosarcomas (Table).

Positive cytoplasmic staining for α enolase was seen in all but one case, a small celled embryonal type containing no rhabdomyoblasts or cells with cross striations. Cytoplasmic staining for β enolase was seen in all tumours containing rhabdomyoblasts, and staining was strongest in the cells with cross striations. β enolase staining was also seen in several tumours in which no cross striations could be detected by light microscopical techniques. Intensity of staining for β enolase in rhabdomyoblasts varied from weak (1+) to intense (3+) (Fig. 2), and some of the strongest staining was seen in multinucleate cells with large peripheral nuclei (Fig. 3).

The Table summarises the results for intensity of staining seen in the two main tumour groups.

No staining was seen in control sections treated with normal rabbit serum or with antiserum adsorbed with α or β enolase as appropriate (Fig. 4). A control series consisting of three neuroblastomas, three fibrosarcomas, one osteosarcoma, and five Wilms' sarcomas were stained using the same techniques. None of the Wilms' sarcomas contained convincing muscle cells by light microscopical techniques. In no case was there any evidence of staining for β enolase.

Discussion

These results show that β enolase is a sensitive marker of muscular differentiation in rhabdomyosarcoma. Those cells showing evidence of differentiation readily recognisable by morphological criteria in conventional staining material also showed a strong staining for β enolase. Cells less readily classified as myoblastic showed more variable staining for β enolase.

In 1982 Brooks[11] showed the usefulness of myoglobin as a tumour marker for rhabdomyosarcoma using an immunoperoxidase technique. Eighty nine per cent of tumours diagnosed as rhabdomyosarcoma by conventional light microscopical techniques contained immunoreactive myoglobin regardless of histological type. Not only was the marker sensitive but it also proved to be extremely specific for stri-

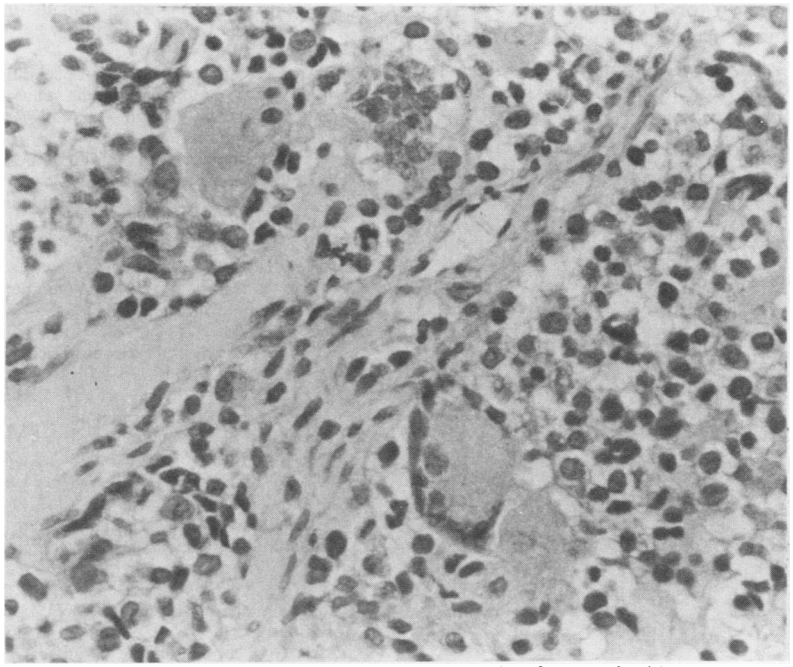

Fig. 4 *Control section of rhabdomyosarcoma stained with normal rabbit serum showing negative reaction in all tumour cell types including multinucleate cells with peripheral nuclei. Original magnification × 750.*

ated muscle histogenesis. No other soft tissue tumour or carcinoma examined was myoglobin positive. Similarly, Corson and Pinkus[22] reported similar results in a series of 30 soft tissue sarcomas; in their series 76% of tumours diagnosed as rhabdomyosarcoma stained positively for myoglobin.

Kahn *et al*[12] attempted to increase the diagnostic accuracy in childhood rhabdomyosarcoma by using antibodies against myoglobin, the MM isoenzyme of creatine kinase, desmin, "calcium magnesium-dependent adenosine triphosphatase of sarcoplasmic reticulum," and calcequestrin. They used an immunoperoxidase staining technique and the results were compared with electron microscopical studies aimed at showing features of skeletal muscle differentiation. Their series consisted of 65 cases of childhood rhabdomyosarcoma, 53 of which were embryonal and 12 alveolar. Cross striations were seen on light microscopy in 23% of the embryonal types and in 33% of the alveolar tumours. Myoglobin was detected in 30% of the embryonal and in 67% of alveolar rhabdomyosarcomas; creatine kinase was demonstrated in 60% of embryonal rhabdomyosarcomas; and the remaining three antibodies were less useful. Of 13 cases studied by electronmicroscopy (two alveolar and 11 embryonal), four showed cross striations and were positive for myoglobin. Thus 64% of the embryonal and 78% of the alveolar rhabdomyosarcomas showed either positive immunostaining or ultrastructural features of rhabdomyosarcoma. They concluded that a combination of immunohistochemical staining, using antimyoglobin and anticreatine kinase (MM isoenzyme) antibodies, and electron microscopy are useful in the diagnosis of childhood rhabdomyosarcomas.

In the present study positive staining for β enolase was seen in some cells in 18 of the 21 cases studied (86% of the total, 88% of the alveolar rhabdomyosarcomas, and 85% of the embryonal rhabdomyosarcomas). Staining was strongest in cells with cross striations but was also seen in several tumours in which no striations could be detected by light microscopical techniques. Intensity of staining for β enolase in rhabdomyoblasts varied from weak to intense, and some of the strongest staining was seen in multinucleate cells with large peripheral nuclei. This isoenzyme therefore appears to be a sensitive marker of myoblastic differentiation. Staining is variable within individual tumours, and in general the intensity of staining of individual cells correlates well with the degree of cytodifferentiation, small cells with a high nuclear to cytoplasmic ratio show-

ing no staining reaction for β enolase.

The three tumours showing no evidence of staining for β enolase in any cell type were composed entirely of small undifferentiated cells. It may be that tumours composed of undifferentiated cells with no markers of skeletal muscle differentiation should be regarded as a separate category. Since differentiation can vary from one part of a tumour to another, it is important to assess different areas. Further work needs to be done to assess the sensitivity of β enolase as a marker of rhabdomyosarcoma in comparison with myoglobin and the MM isoenzyme of creatine kinase. It may be that differentiation of the glycolytic pathway occurs at an earlier stage of cellular development than myoglobin or creatine kinase.

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