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The value of S-100 immunostaining as a diagnostic tool in human malignant melanomas

A comparative study using S-100 and neuron-specific enolase antibodies

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Summary. The brain proteins S-100 and neuron-specific enolase have been reported by separate groups to be present in human malignant melanomas. There is no systematic study comparing the occurrence of these proteins in the same tumour specimens. We have examined 33 primary malignant melanomas, including 5 which were amelanotic, and 25 metastatic melanomas using immunohistochemical methods with specific, non-cross-reacting antibodies to S-100 and NSE. We found S-100 immunoreactivity to be present in all cases but one, whereas NSE immunoreaction was very weak and patchy, and present in only 6 cases.

S-100 immunoreactivity was not demonstrated in 40 control tumours, either primary or metastatic in skin, including basal- and squamous-cell carcinomas, spindle-cell sarcomas, lymphomas and Merkel cell tumours. All intradermal ($n=4$) and compound ($n=1$) naevi were positive for S-100, 2 blue naevi showing much less reaction. NSE immunoreactivity was detected in Merkel cell tumours ($n=8$), undifferentiated ($n=2$) and small cell ($n=1$) carcinomas, and all melanocytic naevi.

It is suggested therefore that antibody to S-100 is the reagent of choice for demonstration of melanocytic tumours, and may be especially valuable in the diagnosis of amelanotic melanoma or metastatic tumours of doubtful origin where melanoma is suspected.

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S-100 is a small, acidic, Ca^{2+}-binding protein, originally isolated from bovine brain (Moore 1965). It is found in glial elements of the brain (Perez et al. 1970), and Schwann cells and satellite cells of dorsal root and autonomic ganglia in the peripheral nervous system (Stefansson et al. 1982a). It also occurs in other tissues, including Langerhans cells and melanocytes of the skin (Cocchia et al. 1981), interdigitating reticulum cells of human lymph node (Takahashi et al. 1981) and human chondrocytes of both neural crest and mesodermal types (Stefansson et al. 1982c).

Enolase, a glycolytic enzyme, is a dimer of three possible subunits: α, β and γ. The α and β subunits, collectively termed non-neuronal enolase (NNE), are found in liver and epithelial tissues (α), and muscle including heart (β). The γ-γ form, or neuron-specific enolase (NSE), is found exclusively in neurons, peripheral and central neuroendocrine cells (Schmechel et al. 1978; Bishop et al. 1982; Gu et al. 1983) and tumours deriving from them (Tapia et al. 1981).

Complement fixation assay shows that S-100 occurs in melanoma cell cultures (Gaynor et al. 1980) and melanoma tissues (Gaynor et al. 1981), at levels up to 80-fold that found in normal human skin (Cocchia et al. 1981). Immunohistochemical studies have demonstrated S-100 in the majority (Nakajima et al. 1981, 1982; Stefansson et al. 1982b) or all (Gaynor et al. 1981; Clark et al. 1982; Cochran et al. 1982) of melanomas, and in normal melanocytes (Cocchia et al. 1981).

NSE immunoreactivity was detected immunohistochemically (Dhillon et al. 1982) in human malignant melanomas, in benign naevi, except Spitz naevi (Dhillon and Rode 1982), and in some normal melanocytes in skin. However, Royds et al. (1982a, b) found γ-enolase (NSE) immunoreactivity in intradermal naevi and in malignant intradermal melanocytes, but not in normal melanocytes.

In view of these conflicting reports of NSE and S-100 immunoreactivity in melanocytic tumours, we considered it important to look for both proteins in the same specimens using specific and non-cross-reacting antibodies. Such a study has not hitherto been made, and might establish whether an interrelationship exists between NSE and S-100 immunoreactivity in each melanoma specimen, and the relationship of both proteins to histological classification of the tumours compared with naevi and control tumours in skin.

Materials and methods

Tissues

Tissues from 33 patients with malignant melanoma, including 5 amelanotic tumours, were obtained from the files of the Departments of Histopathology at St. Helier Hospital, Carshalton, Surrey, and The Royal Marsden Hospital, Fulham Road, London. The material was all formol-saline fixed and wax-embedded. It included the skin tumour(s) in all cases, and

skin recurrences ($n=5$) and metastases to various sites ($n=25$) if they were available. Seven cases of benign naevi were obtained from the same sources.

Control tissues were normal skin from 20 patients, and 40 biopsies of various primary or metastatic tumours in skin (Table 2). The normal skin specimens were obtained fresh and processed in three ways: formol saline fixation followed by wax embedding, Bouin's fluid fixation followed by wax embedding, and 0.4% p-benzoquinone fixation (Bishop et al. 1978) followed by freezing for cryostat sectioning.

Sections (5 μm thick) were mounted on glass slides coated with poly-L-lysine (mol. wt. 350,000) to ensure good adhesion (Huang et al. 1983), and dried overnight at 37° C. One slide from each block was stained with haematoxylin and eosin to study tumour morphology and for correlation with the immunohistochemistry results.

Antibodies

Purified NSE was prepared from rat brain as previously described (Marangos et al. 1978) and antiserum was raised in New Zealand white rabbits (Marangos et al. 1975). Antiserum to S-100 extracted from ox brain was raised in rabbits and characterised according to Zuckerman et al. (1970).

Immunohistochemistry

Dried sections were dewaxed in xylene and taken to water through graded alcohols. Endogenous peroxidase was blocked using a 0.15% solution of hydrogen peroxide in PBS (phosphate-buffered saline – 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3). The staining procedure used was the unlabelled antibody enzyme (PAP) method of Sternberger (1979). Both primary antibodies were used at a dilution of 1:4,000 in PBS containing 0.1% bovine serum albumin, and were applied for 16–18 h at 4° C.

Sections were then incubated with goat anti-rabbit IgG (Miles Laboratories) at 1:200 dilution for 30 min, followed by rabbit PAP complex (UCB Bioproducts) diluted 1:300 for 30 min. Peroxidase activity was revealed by either diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (Graham et al. 1965). The latter gave a brick-red colour which was found to contrast well with melanin, unlike the brown reaction product obtained from DAB. Sections were lightly counterstained with haematoxylin and mounted in glycerine jelly (amino ethylcarbazole) or dehydrated, cleared and mounted in Styrolite (DAB).

Initially, duplicate sections from 10 melanoma cases were immunostained, one with DAB, the other with amino ethylcarbazole as chromogen. This was done for two reasons. Firstly, to ensure that there was no loss of sensitivity when using amino ethylcarbazole rather than DAB. Secondly, since the DAB reaction product is not affected by hydrogen peroxide, the melanin in heavily pigmented tumours could be bleached in order to see whether it was masking any immunostaining. Bleaching was also performed prior to immunostaining, by placing sections in 0.25% aqueous potassium permanganate for 20 min, followed by 1 min in 1% aqueous oxalic acid.

The immunostaining controls used included the incubation of sections with non-immune rabbit serum or PBS instead of primary antibody, and antibody absorbed with homologous antigen (NSE – 2.5 nmol/ml; S-100 – 5.0 nmol/ml of diluted antibody) or cross-absorbed (anti-NSE absorbed with S-100 and anti-S-100 absorbed with NSE, 5.0 and 2.5 nmol/ml respectively).

Results

Haematoxylin and eosin stained sections of melanomas were examined initially without reference to immunostaining results, and tumour grading and staging were evaluated. Results of immunostaining are given in Table 1.

Six of 33 cases of malignant melanoma showed positive immunoreactivity for NSE, and these were amongst the monomorphic tumours. The immu-

Table 1. Intensity of immunostaining for S-100 and NSE in melanomas and melanocytic naevi

Tissue		Number of cases	Immunostaining intensity[a]									
			S-100					NSE				
			0	+	2+	3+	4+	0	+	2+	3+	4+
Melanoma												
Melanotic	1°	28	1	–	4	7	16	22	3	3	–	–
	2°	22	–	1	3	10	8	22	–	–	–	–
Amelanotic	1°	5	–	–	1	2	2	5	–	–	–	–
	2°	3	–	–	1	1	1	3	–	–	–	–
Naevi												
Intradermal		4	–	–	–	2	2	–	–	3	1	–
Compound		1	–	–	–	1	–	–	1	–	–	–
Blue		2	1	–	1	–	–	–	1	1	–	–

[a] 0 signifies no immunostaining; + to 4+ represent increasing number of cells and intensity of immunostaining

nostaining in these cases was usually very patchy and weaker than that for S-100 (Figs. 1 and 2A).

Thirty-two of 33 malignant melanomas, both primary and metastatic, pigmented and amelanotic, were immunoreactive for S-100. Immunostaining of S-100 was strong in most cases (Figs. 2B and 3), more so at the periphery of the tumour adjacent to the dermis (Fig. 3B) and in intradermal tumour cells (Fig. 4). This pattern was consistent in serial sections. The degree of overall staining intensity did not correlate with degree of pleomorphism, level of invasion or absolute thickness of the tumour, or with the amount of melanin present. However, cells containing much melanin were not reactive with antibodies to S-100. Bleaching of melanin, using 10% hydrogen peroxide, after immunostaining, did not reveal further reaction product, showing that there was no masking effect of the pigment. Bleaching the sections with permanganate before immunostaining did not affect the immunoreactivity, and this method again showed S-100 immunoreactivity to be absent from heavily pigmented malignant melanocytes.

The superficially spreading melanomas immunostained for S-100 more strongly than the nodular type.

All of 25 metastatic melanomas were immunoreactive for S-100 (Fig. 5B and C). The skin metatases ($n=1$) or recurrences ($n=5$) and the metastases to lung, liver, bone, breast, peritoneum, stomach and thyroid (13 in all) and lymph node ($n=6$) tended to have the same type of staining pattern as the primary tumour. None of the primary melanomas which reacted with anti-NSE had demonstrable metastases.

Intrinsic positive controls in each section were provided by nerves or supportive cells in normal skin adjacent to the tumour. They showed reac-

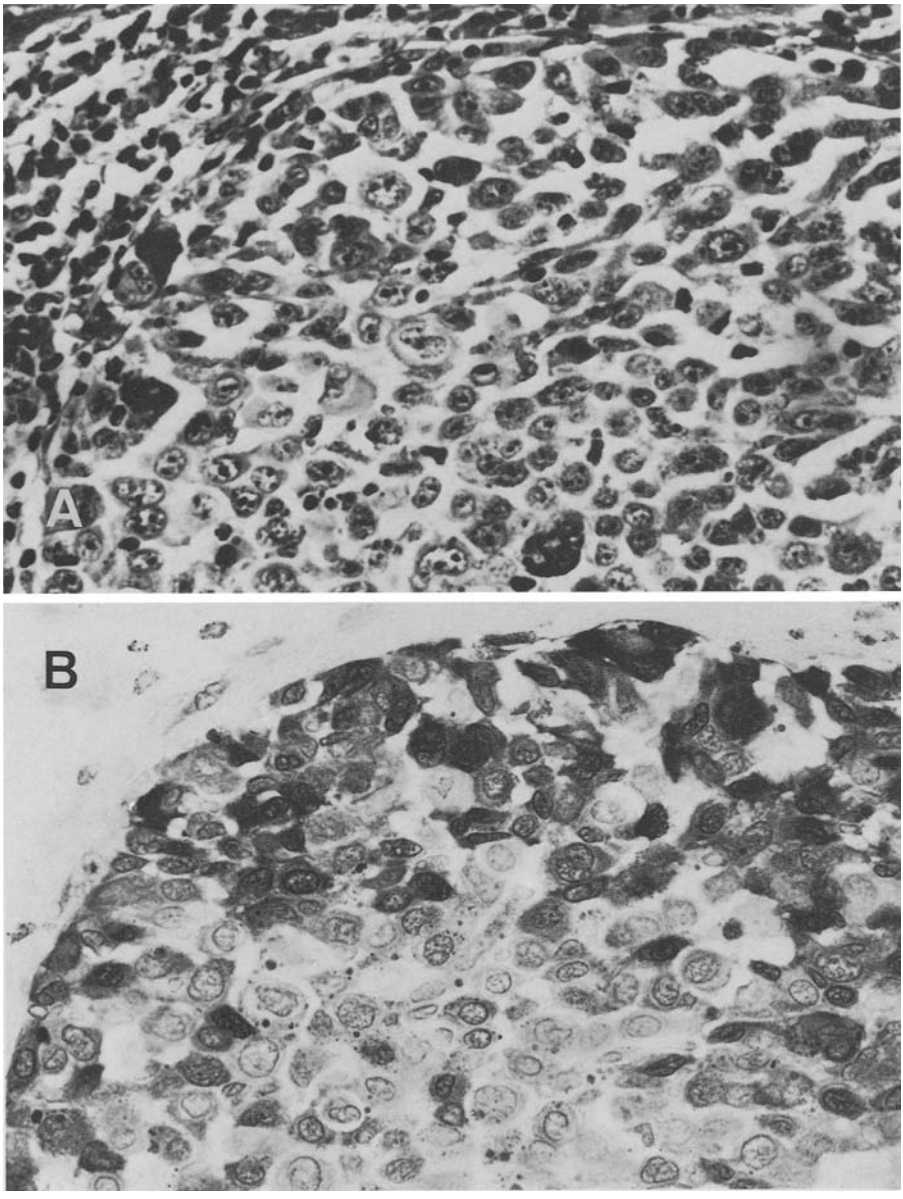

Fig. 1 A, B. Melanoma in skin. Adjacent sections **A** stained by haematoxylin and eosin, **B** immunostained for S-100. The immunostained section is not counterstained and clearly reveals tumour cells in the heavy inflammatory reaction. Compare with NSE immunostaining in Fig. 2. (Both $\times 360$)

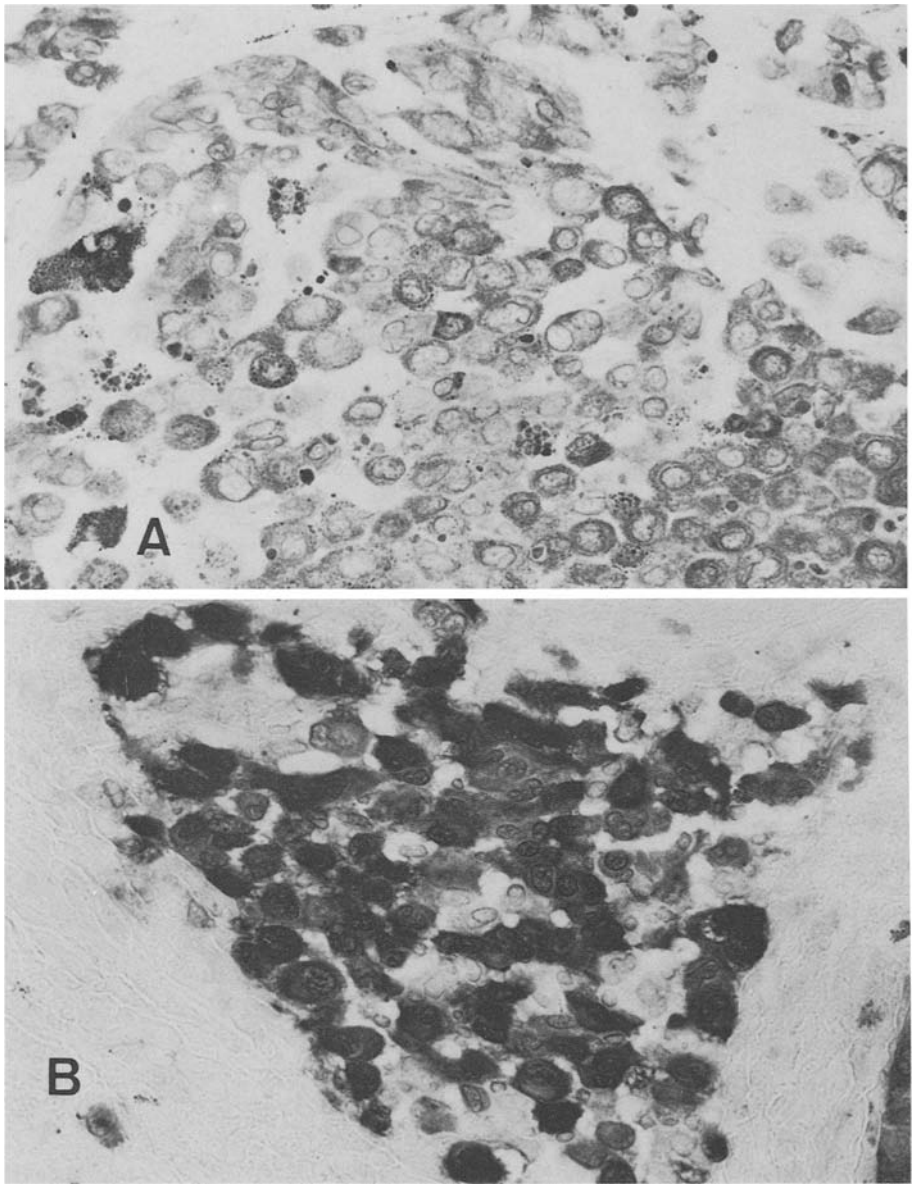

Fig. 2. **A** Adjacent section to Fig. 1, immunostained for NSE. The reaction is much weaker than that with anti-S-100 (Fig. 1). **B** Another area from the same tumour showing strong S-100 immunoreactivity. (Both uncounterstained, **A** ×360, **B** ×225)

tion with the appropriate antibody in all cases. This demonstrated that fixation had not significantly destroyed the antigenicity of the NSE and S-100. Use of non-immune rabbit serum or PBS in place of primary antiserum gave no immunostaining. Absorption controls showed no reaction in tumour or nerves. Cross-absorbed antibodies gave the same reaction as

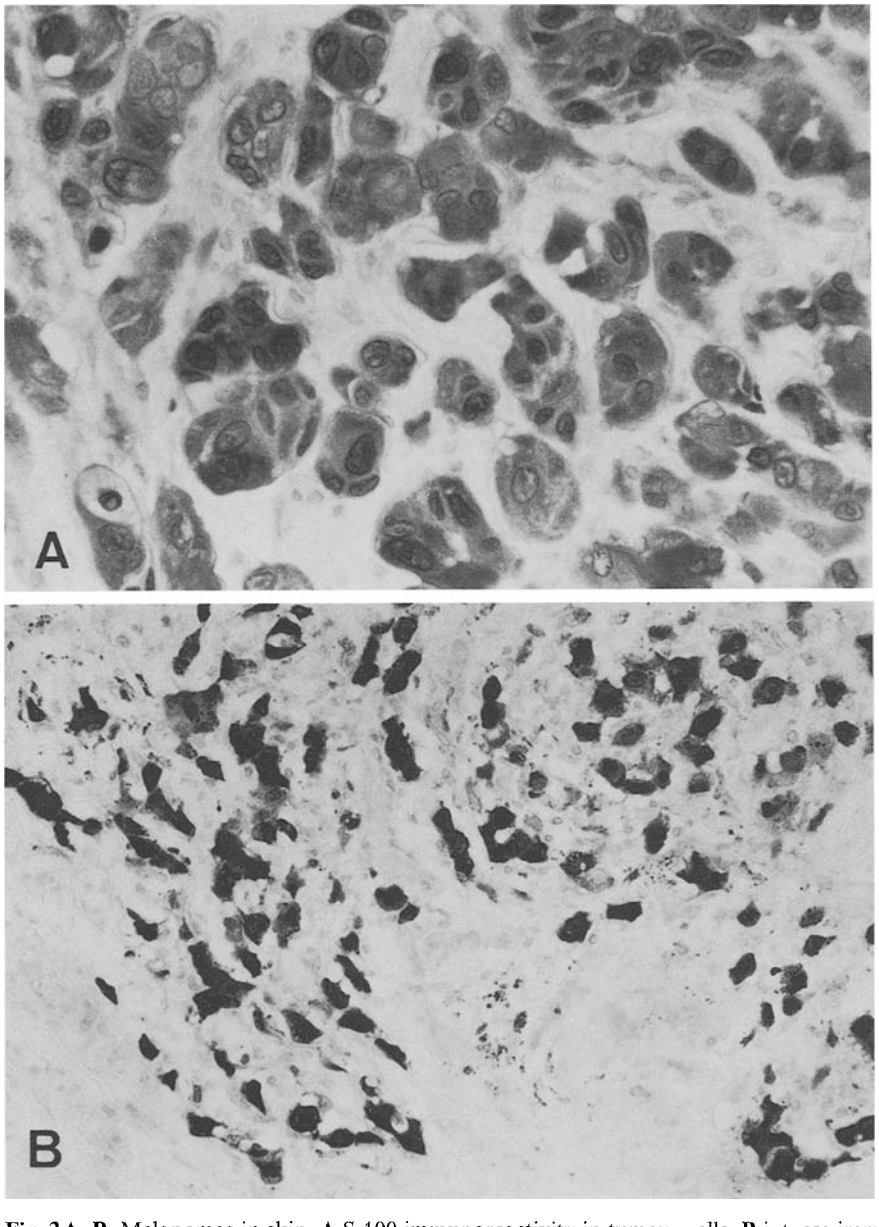

Fig. 3A, B. Melanomas in skin. **A** S-100 immunoreactivity in tumour cells, **B** intense immunostaining of S-100 in tumour cells at the periphery of the tumour. (Both uncounterstained, **A** ×360, **B** ×225)

their unabsorbed counterparts, demonstrating that the antibodies were specific and did not cross-react with inappropriate antigen.

Of the control tissues (Table 2), all melanocytic naevi but 1 were immunoreactive for both proteins; intradermal and compound naevi were more strongly reactive to S-100 antibodies than the blue naevi, and all types

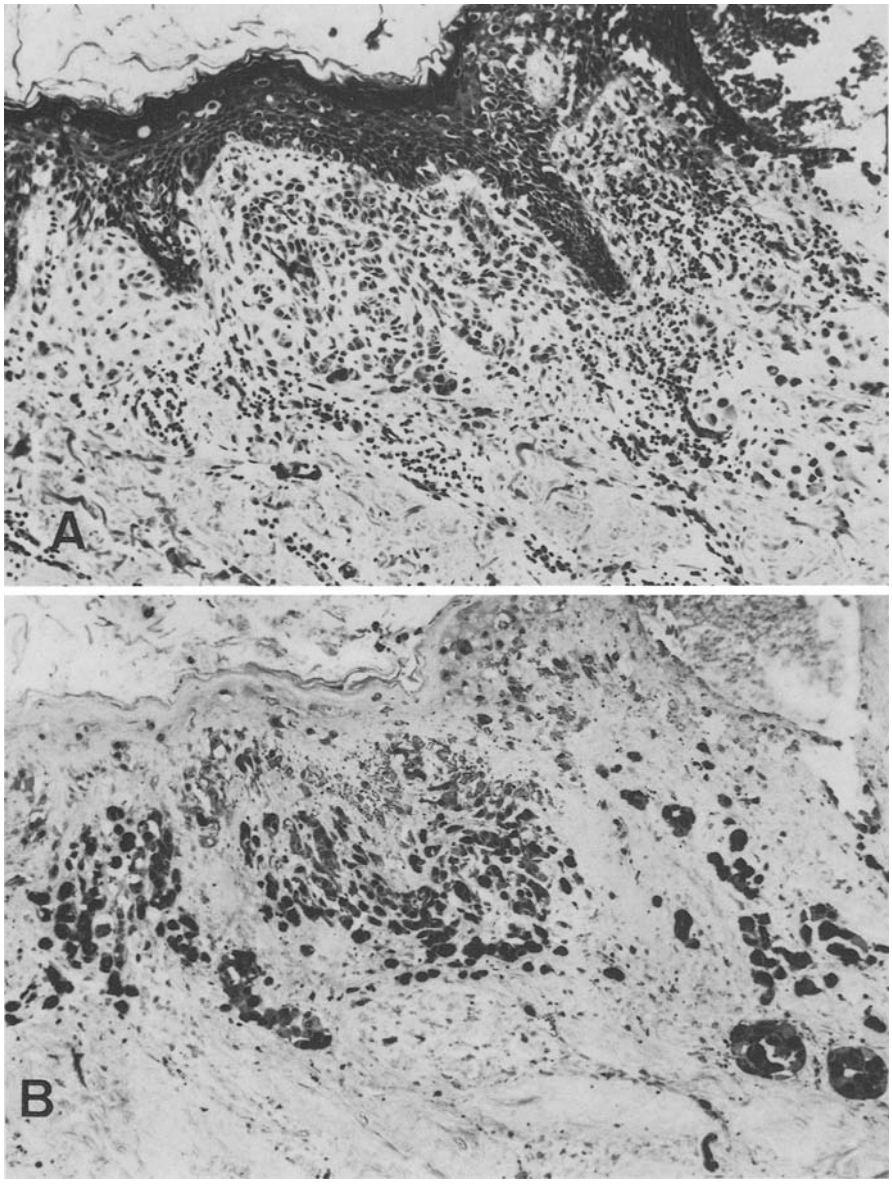

Fig. 4A, B. Intradermal malignant melanocytes at the lateral edge of a melanoma in skin. **A** H&E, **B** uncounterstained section immunostained for S-100. The intradermal tumour cells are intensely stained. (Both ×95)

were less strongly reactive to anti-NSE than to anti S-100. Eight of 11 Merkel cell tumours were immunoreactive for NSE, but all were S-100 negative. The lymphomas and normal lymph nodes were negative for both proteins (except interdigitating reticulum cells which were S-100 immunoreactive), as were squamous cell carcinomas, basal cell carcinomas and spindle cell sarcomas.

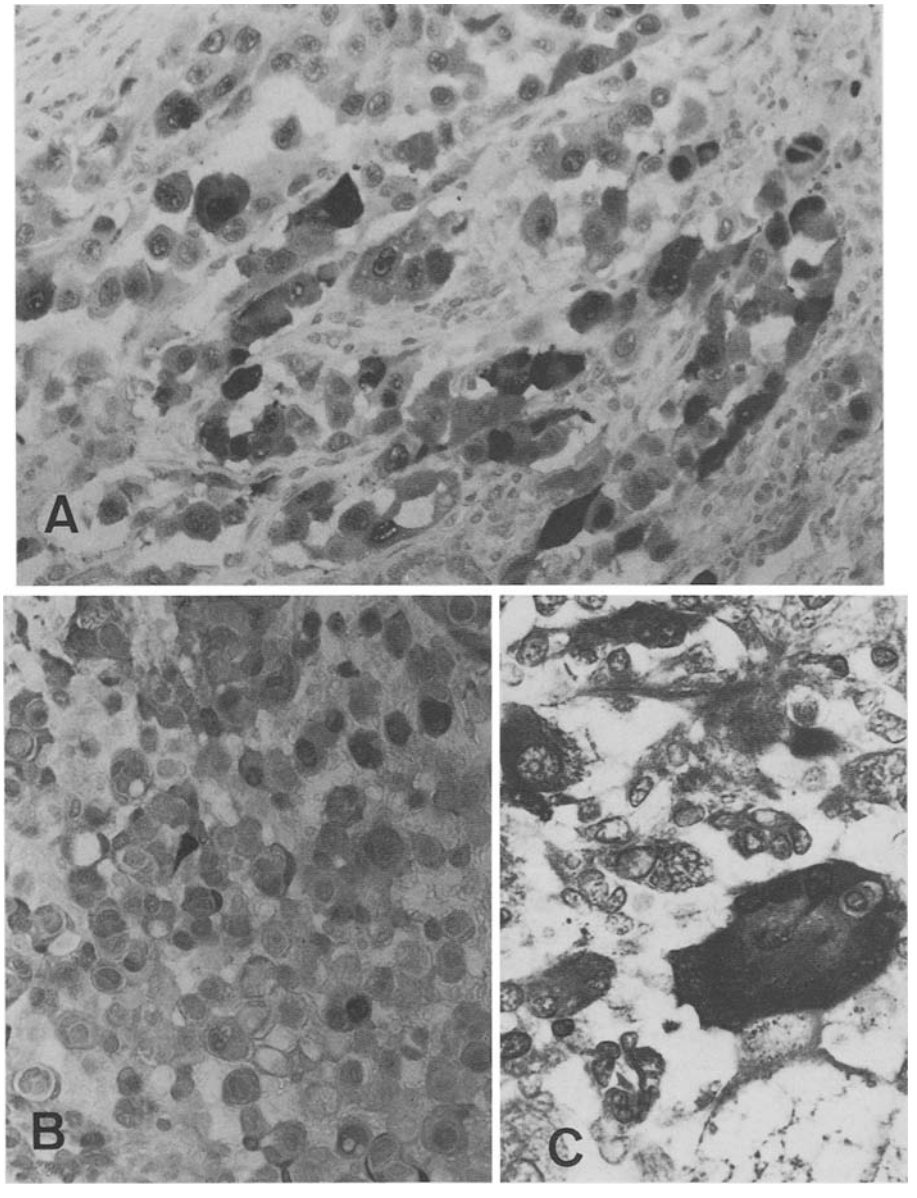

Fig. 5. **A** Pleomorphic melanoma in skin, haematoxylin counterstained showing strong immunoreactivity for S-100; **B** and **C** metastatic melanoma in lymph node immunostained for S-100. (**A**, **B** ×225, **C** ×360)

In 1 normal skin specimen and 6 melanomas, normal melanocytes in the epidermis or hair follicles were immunostained by S-100 antibodies, but never by anti-NSE. This was true of normal skin whether fixed in formol saline, Bouin's fluid or benzoquinone solution.

Table 2. Immunostaining of control tumours in skin for S-100 and NSE

Tissue	Number		Number with positive staining	
			S-100	NSE
Undifferentiated Ca	2		–	2 (trace)
Small cell Ca (bronchus)	1		–	1 (trace)
Merkel cell tumour	11		–	8
Squamous cell carcinoma	3		–	–
Spindle cell sarcoma	2		–	–
Breast carcinoma	1		–	–
Adenocarcinoma, site unknown	1		–	–
Solar keratosis with CIS	1		–	–
Lymphoma	9		–	–
Basal cell papilloma	1		–	–
Dermal fibroma	1		–	–
Condyloma acuminata	2		–	–
Basal cell carcinoma	5		–	–
Normal skin (various sites)	20	Melanocytes	1	–
		Langerhans cells	20	–

Discussion

Using routinely fixed histological material, we have detected S-100 immunohistochemically in all but one of 33 cases of melanoma examined, including amelanotic tumours, and in all of 5 intradermal and compound naevi. These results agree with those of other workers (Gaynor et al. 1981; Clark et al. 1982; Cochran et al. 1982; Nakajima et al. 1981, 1982; Stefansson et al. 1982b). It is also predictable since we found some normal melanocytes in normal skin and skin adjacent to melanomas to immunostain for S-100, as was also shown by Cocchia et al. (1981); tumours deriving from other S-100-containing cells also contain the protein (e.g. Schwannomas – Stefansson et al. 1982b). Why normal melanocytes are not all immunoreactive for S-100 is uncertain; possibly the protein is only present in amounts detectable by immunohistochemistry at certain stages of cell life or of activity. That melanin-containing cells in melanomas do not react with anti-S-100 supports this view, as does the strong S-100 immunoreactivity of most superficial spreading melanomas, malignant melanocytes towards the edges of nodular melanomas, and intradermal malignant melanocytes. The strength of this reaction may mean that such cells are of a more active type than those in the middle of a nodular tumour. Alternatively it may reflect the availability of nutrients to the tumour cells, those cells showing greater S-100 immunoreactivity are nearer to the nutrient supply than those in the middle of nodular tumours. However, although there is an increase in the amount of immunostaining of S-100 in the progression from normal melanocytes to naevocytes and malignant melanocytes, our results do not show that there is any correlation of S-100 immunoreactivity with the degree of pleomorphism or melanisation of the malignant melanomas.

We have found NSE immunoreactivity in only 6 of 33 malignant melanomas examined. These were amongst the more monomorphic specimens. None of the NSE-immunoreactive tumours had metastases, suggesting that they may be more benign.

Dhillon et al. (1982) found NSE to be detectable immunohistochemically in 15 out of 16 cases of melanoma, and observe that NSE immunostaining increases in strength from benign to malignant lesions (Dhillon and Rode 1982). This was also suggested by Royds et al. (1982b). However, the latter authors found NSE immunoreactivity in 18 of 20 melanomas, whereas only 6 of our 33 cases reacted with antibodies to NSE.

This variation between our findings and those of Royds and Dhillon and their co-workers could be due to differences in fixation of tissues (although we also observed NSE-immunoreactive nerves in all our sections), or a difference between antibodies or the dilutions used for immunohistochemistry. We raised the concentration of our anti-NSE serum in an attempt to increase the degree of immunostaining, but even at a dilution of 1:800 no improvement was obtained.

In common with Royds et al. (1982b), we were not able to detect NSE immunoreactivity in normal human melanocytes. NSE immunoreactivity was also absent from cat and rat melanocytes (Gu et al. 1981). Identical results were obtained with formol-saline-, Bouin-, or benzoquinone-fixed material. All naevi reacted with antibodies to NSE. Our results suggest that NSE immunoreactivity is present in transformed melanocytes and then decreases with progression to malignancy.

The most obvious pointer to the diagnosis of melanoma in a skin tumour is melanin, but this does not exclude other tumours which have taken up the pigment and thus mimic a melanoma. With non-pigmented melanomas the differential diagnosis of tumours including spindle-cell sarcomas, squamous and basal cell carcinomas, and skin metastases of undifferentiated carcinomas becomes important, as does the site of the primary tumour in metastases. Thus a marker for distinguishing melanoma in these situations would be valuable.

We feel that since we have not found S-100 immunoreactivity in non-melanocytic (control) tumours this protein is a potentially useful marker. As well as its use for differential diagnosis it is valuable in highlighting small groups of tumour cells either spreading superficially or invading the dermis.

Immunohistochemical detection of S-100 has other advantages. Diagnostic tests for melanoma such as formalin-induced fluorescence and enzyme cytochemistry (Rost et al. 1973), the tyrosinase method of Fitzpatrick and Clark (1964) using tissue slices, and biochemical assay of S-100 mostly require fresh or specially fixed tissue. This study has shown that routine fixation and embedding does not destroy the antigenicity of S-100 in melanomas. This is most important for the routine histopathology department, permitting as it does the application of the test in cases where fresh material is no longer available when suspicion of melanoma arises, or in retrospective studies. Thus the need to use non-routine techniques 'just in case' is ob-

viated. However, work in our laboratory (unpublished results) shows that the antigenicity of both NSE and S-100 is diminished if tissue is left in fixative for long periods (but up to 1 week in formol saline is satisfactory) before blocking in wax. The time of storage of wax blocks seems to be unimportant for NSE and S-100 immunoreactivity.

If S-100 should prove to be released into the blood stream from the tumour, the assay of the protein in the circulation may be of value in monitoring the effectiveness of surgical ablation or other therapy, or the subsequent growth of metastases, in patients having melanoma.

In conclusion, S-100 immunostaining appears to be a convenient and specific marker for melanocytic tumours, thus distinguishing these lesions from a variety of other tumours.

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