Immunohistochemical and Histochemical Tools in the Diagnosis of Amelanotic Melanoma

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The histologic diagnosis of (metastatic) oligomelanotic or amelanotic melanoma may be difficult. In most cases this diagnosis can be established with conventional light and electron microscopic examination, supplemented with staining for melanin on ultrathin sections, but in other cases it remains equivocal. Therefore, the melanoma-associated monoclonal antibody NKI/C-3, effective on paraffin sections, was tested with an indirect immunoperoxidase technique. All 19 metastatic melanomas, used as positive controls, were stained. Seventeen of 23 primary melanomas and 8 of 9 initially equivocal eventually unequivocal melanomas (Group I) were stained with a diffuse cytoplasmic and in some cases locally peripheral pattern. Only two large cell undifferentiated carcinomas of 58 histogenetically unrelated but differential diagnostically relevant tumors showed localized staining in few tumor cells. Furthermore, 10 of 20 histogenetically related tumors (neuroendocrine tumors and clear cell sarcomas) were positive. These tumors however, can easily be differentiated from melanomas by other means. Of 15 equivocal melanomas (Group II) 9 cases reacted with NKI/C-3, suggesting that it may be a useful marker for difficult metastatic tumors suspect for amelanotic melanoma. Although sensitivity of NKI/C-3 for metastatic melanomas is high, its specificity is not sufficient. It therefore can be applied most properly in a selected panel of different tumor-associated antibodies that are reactive in formaldehyde fixed, paraffin-embedded tissue.

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IT IS WELL KNOWN that apparently amelanotic melanomas may be difficult to diagnose, due to lack of demonstrable melanin and of premelanosomes.[1-4] These cases may be diagnosed by extensive electron microscopic examination, or by performing the DOPA-reaction on frozen sections. Recently we described a modified Warthin-Starry (WS) procedure for melanin directly applicable on epoxy sections, that appeared to be promising for the diagnosis of oligomelanotic melanoma.[5] Immunohistochemistry with monoclonal antibodies against melanoma-associated antigens could be of great potential value for the diagnosis of amelanotic melanoma. A set of such monoclonal antibodies has been developed by one of the authors (C.V.). Immunohistochemical testing of these monoclonal antibodies revealed a high sensitivity and specificity of monoclonal antibody NKI/C-3 when applied to a series of malignant nevomelanocytic and other tumors in paraffin sections.[6,7] Therefore, we have set up a study in which the diagnostic value of this monoclonal antibody for apparently amelanotic (metastatic) melanoma was tested with an immunoperoxidase procedure. Furthermore, this technique was compared with conventional electron microscopy and with the modified WS procedure on the ultrastructural level.

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Material and Methods

Tumors

Twenty-four predominantly metastatic tumors, 15 of which were collected from the files of the Department of Pathology, University Medical Center, Leiden, and 9 from the files of the Massachusetts General Hospital, Boston, were studied retrospectively. Selection for this study was based on the fact that the cases were submitted to the Units for Diagnostic Electron Microscopy for assessment of the diagnosis malignant melanoma, because histology of these tumors was equivocal, and in part of the cases

TABLE 1. Results of Immunohistochemical, Histochemical and Electronmicroscopic Examination of Cases Eventually Diagnosed as Metastatic Malignant Melanoma; Group I

		Light microscopy				Electron microscopy		Immunoperoxidase procedure with monoclonal antibody NKI/C-3	
Case no.	Age/sex	Melanoma in history	Location of current tumor	Cell type	Melanin staining	Melanosomes	Melanin staining (WS)	Percentage of positive-staining tumor cells	Intensity of staining‡
1	27/F	not known	Brain	EP	−	+*	+	80	+/++
2	53/M	not known	Parotid gland	EP + SP	−	+*	+	100	+/++
3	32/F	yes	Lymph node	SP + EP	+*		SP: + EP: +	SP: 0; EP: 50	+
4	25/F	no	Lymph node	EP	+*	−	+	50	+/++
5	13/F	no	Muscle	EP	−	±†	+	40	+
6	36/F	yes	Ovary	EP	−	+		<5	+
7	40/F	yes	Subcutis	EP	−	+	−	50	+/++
8	80/F	no	Rectum	EP	−	+*	+	40	+/++
9	58/F	no	Palatum	EP	−	+*	−	40	+

* Only eventually were positive results obtained.
† Suggestive melanosomes were seen.
‡ +: moderate/slight; ++: intense; EP: epitheloid; SP: spindle; WS: Warthin-Starry procedure.

a history of primary malignant melanoma was present. The diagnosis malignant melanoma could not be made by light microscopy on the available paraffin sections (H & E, Schmorl, and modified WS staining for melanin[8]), or by electron microscopy on the available conventionally stained epoxy sections. The tumors were divided into two groups:

Group I: The diagnosis metastatic melanoma could be established only after additional extensive sampling of tumor tissue by processing of all available tissue or cutting of additional paraffin and epoxy sections, and by additional examination by three observers (S.G.v.D., D.J.R., and G.R.D.) (9 cases, Table 1).

Group II: The diagnosis (metastatic) melanoma could not be established in spite of these efforts (15 cases, Table 2).

In these cases additional diagnostic methods were applied, namely, an immunohistochemical staining with a monoclonal antibody against melanoma-associated antigens (NKI/C-3, clone MX-49.129.5, Netherlands Cancer Institute, Amsterdam, The Netherlands) and a histochemical staining directly on semithin and ultrathin epoxy

TABLE 2. Immunohistochemical, Histochemical and Electronmicroscopic Examination of Cases Equivocally Diagnosed as Metastatic Malignant Melanoma; Group II

				Electron microscopy			Immunoperoxidase procedure with monoclonal antibody NKI/C-3	
Case no.	Age/sex	Melanoma in history	Location of current tumor	Cell type	Melanosomes	Melanin staining (WS)	Percentage of positive-staining tumors cells	Intensity of staining*
1	79/M	no	Abdominal mass	EP	−		15	+
2	63/M	no	Brain	EP	−	+	70	+
3	62/F	not known	Lymph node	EP		−	0	−
4	56/F	no	Soft tissue	EP	−	−	20	+
5	43/F	yes	Subcutis	EP + SP		−	15	+/−+
6	87/F	no	Soft tissue	EP	−	−	0	
7	35/F	no	Skin	EP	−	−	25	+
8	77/M	yes	Lung	EP + SP	−	−	75	++
			Brain	EP	ND	ND	50	++
9	37/M	no	Skin	SP + EP	−	−	0	−
10	77/F	no	Subcutis	EP	−	−	25	+/++
11	46/F	probably	Lymph node	EP	−	−	20	+
12	52/F	no	Rectum	EP		−	0	−
13	72/F	yes	Subcutis	EP		−	0	−
14	56/M	no	Subcutis	EP	−	−	80	+/++
15	76/F	no	Os ilium	EP	−	−	0	−

* +: moderate/slight; ++: intense; EP: epitheloid; SP: spindle; ND: not done; WS: Warthin-Starry procedure.

TABLE 3. Staining of Monoclonal Antibody NKI/C-3 in Control Tumors

	Positive staining of tumor cells
A: Positive control cases	
Primary cutaneous melanoma	17/23
Metastatic melanotic melanoma	10/10
Metastatic amelanotic melanoma	9/9
Nevocellular nevus (4 dermal, 5 compound)	9/9
B: Negative control cases	
B1	
Carcinoid	5/6
Chemodectoma	2/7
Medullary carcinoma of thyroid	2/2
Poorly differentiated carcinoma with few APUD granules	1/5
Clear cell sarcoma	1/3
B2	
Histiocytic sarcoma	0/10
Histiocytosis X	0/2
Poorly-differentiated squamous cell carcinoma	0/6
Poorly-differentiated adeno carcinoma (5 breast, 4 colon)	0/9
Renal cell carcinoma, clear cell type	0/6
Undifferentiated large cell carcinoma (5 lung, 3 others)	2/8
Undifferentiated small cell carcinoma (6 lung, 2 others)	0/8
Fibrosarcoma	0/4
Glioma	0/7

B1: histogenetically-related tumors; B2: histogenetically unrelated tumors.

sections with the modified WS procedure for melanin.[5] As positive controls sections of 23 primary cutaneous malignant melanomas, 10 metastatic melanotic melanomas and nine metastatic amelanotic melanomas were used (group A, Table 3). Furthermore nine nevocellular nevi (four dermal and five compound) were examined. As negative controls, sections of 83 malignant tumors with a histologic appearance mimicking malignant melanoma were used (group B, Table 3). These cases were divided into a group of 23 tumors that can be considered as histogenetically related to melanoma, *i.e.*, 20 neuroendocrine tumors (APUD-omas),[9,10] and three clear cell sarcomas, including one of the melanotic type[11] (group B1, Table 3) and 60 cases that do not belong to this category (group B2, Table 3). The diagnosis of undifferentiated carcinoma was always confirmed with conventional electron microscopy. The WS procedure on ultrathin epoxy sections was performed in these cases to exclude the diagnosis of melanoma as much as possible.

Monoclonal Antibody

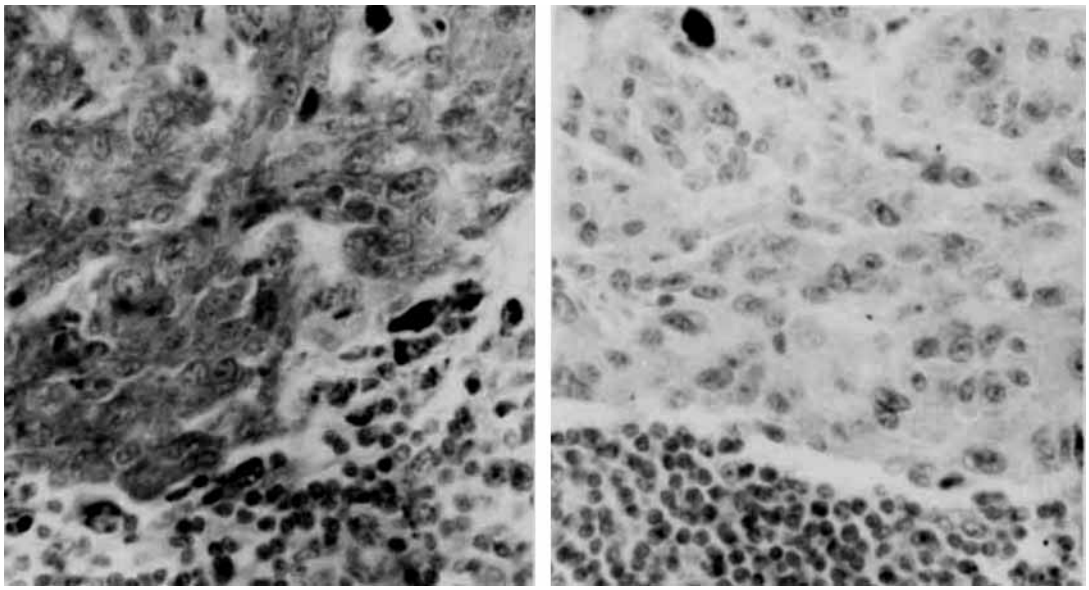
The monoclonal melanoma-associated antibody NKI/C-3 was raised against a melanoma cell membrane fraction, and obtained as tissue culture supernatant fluid.[6,7] Staining with this antibody with an immunoperoxidase procedure on formaldehyde-fixed paraffin-embedded tissue had shown positive results on sections of melanoma. Positive staining of mast cells and sweat glands among others, also was found.

Immunoperoxidase Procedure

An indirect immunoperoxidase procedure at room temperature was used. In short, 4-μ-thick paraffin sections were mounted on albumen coated slides, and stored overnight at 37°C. After preincubation with 1% bovine serum albumin in phosphate buffered saline (PBS) for 30 minutes, the sections were incubated with a 1:20 dilution of the tissue culture supernatant containing the monoclonal antibody for 60 minutes, and subsequently with a 1:80 dilution of a peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako Immunoglobulines A/S, Copenhagen, Denmark). The sections were washed with PBS between incubations, except for the last washing before staining, which was done in an acetate buffer solution (pH 5.0). Staining was achieved with a solution of 40 mg 3-amino-9-ethylcarbazol (Aldrich Chemical Co. Inc., Milwaukee, WI), 10 ml dimethylformamide, and 100 μl hydrogen peroxide (30%) in 200 ml of the acetate buffer solution. The staining reaction was terminated after 3 to 5 minutes with distilled water. The sections were counterstained with hematoxylin for 10 seconds and mounted with aquamount (Hopkin and Williams, Chadwell Heath, United Kingdom). At least two sections per case were stained. Mouse control ascites fluid (Bethesda Research Laboratories Inc., Gaithersburg, MD) was used for each case as a negative control for the monoclonal antibody. Of each section treated with NKI/C-3, the percentage of tumor cells that stained was estimated by three observers. Staining was scored as intense (++) or moderate/slight (+). Staining intensity was correlated with the cell type of the tumor, epitheloid or spindle. The staining distribution within the tumor cells was also noted.

Histochemical Procedure for Melanin on Epoxy Sections

A modified WS procedure, an argyrophilic reaction at low pH was performed on ultrathin epoxy sections as described by van Duinen and associates.[5] In short, ultrathin epoxy sections (50–80 nm) were cut using a LKB III or IV ultramicrotome. The sections were mounted on nickel grids and impregnated in silver nitrate for 30 minutes at 43°C. Then they were put on a drop of developing solution for 45 seconds at 54°C and quickly washed in hot tap water (±60°C), followed by distilled water. Finally, the sections were counterstained with uranyl acetate and lead citrate, and examined and photographed in a Philips 201 EM. All routinely used fixation methods, with ex-



FIGS. 1A AND 1B. Lymph node metastasis of malignant melanoma (positive control case). Parallel sections were stained with monoclonal antibody NKI/C-3 (A, left) and negative control mouse ascites (B, right). In A, a diffuse cytoplasmic staining pattern of tumor cells is observed. Melanophages are not stained. Notice in B that few tumor cells are only slightly pigmented (hematoxylin counterstaining, ×400).

ception of primary osmium tetroxide (OsO_4) fixation, are acceptable for this WS procedure. Postfixation with OsO_4 did not affect the staining results and even deparaffinized and subsequently epoxy-embedded tissue gave satisfactory results with the WS procedure.

Results

Paraffin sections, treated with the monoclonal antibody NKI/C-3 showed red cytoplasmic staining (Fig. 1). This immunoperoxidase staining sometimes was located at the periphery of the tumor cell, giving the impression of membrane staining (Fig. 2). In addition, mast cells and necrotic tumor cells often stained with NKI/C-3. This was not due to the presence of endogenous peroxidase as staining occurred even after blocking of this endogenous peroxidase with H_2O_2 in methanol, and no staining was observed in the negative control section. Positive staining of melanosomes with the WS procedure on ultrathin epoxy sections was visualized as distinct silver grains (5–10 nm), which may form larger aggregates (15–50 nm), when much melanin is present. Not only pigmented melanosomes, but also mast cell granules and the inner membrane of lipid droplets showed silver deposits.

All 19 cases unequivocally diagnosed as metastatic melanoma (group A, Table 3), that were used as positive controls and 8 of 9 cases in group I (initially equivocal), eventually diagnosed as metastatic melanoma (Table 1), stained positively with the monoclonal antibody NKI/C-3; in Case 6 only a few tumor cells reacted. Seventeen of 23 primary cutaneous malignant melanomas, and all

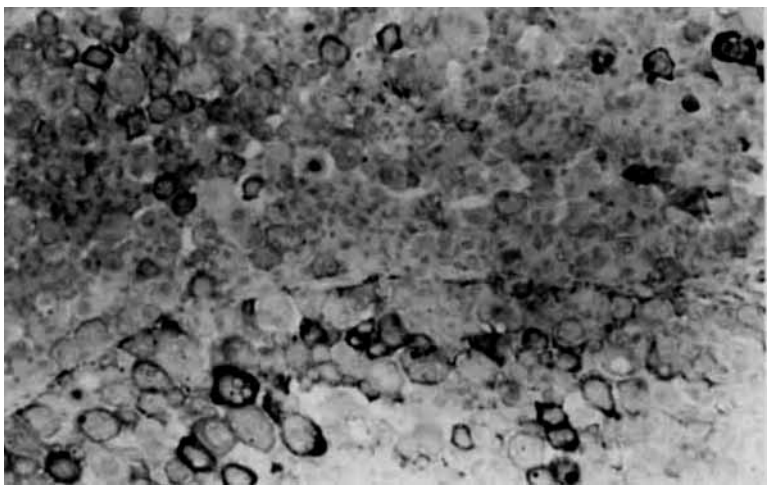


FIG. 2. Large epitheloid cells in a metastatic melanoma (positive control case) showing peripheral cytoplasmic staining with monoclonal antibody NKI/C-3 (hematoxylin counterstaining, ×160).

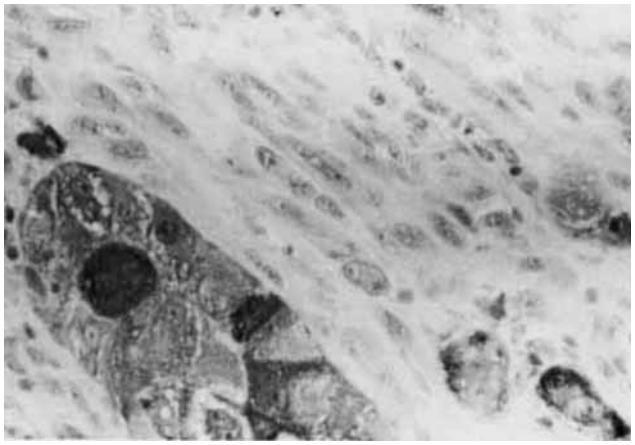


FIG. 3. Malignant melanoma (positive control case) showing marked staining with monoclonal antibody NKI/C-3 in epitheloid cells whereas the spindle cells in the center of the figure only show slight staining (hematoxylin counterstaining, original magnification ×400).

nine nevocellular nevi showed positive staining. In all nevi, staining was intense in the superficial part, and weak or absent in the deep dermal areas. The percentage of tumor cells that stained and the intensity of this staining varied between the various cases studied, but was generally higher in the metastatic melanomas than in the primary melanomas and the nevocellular nevi.

In all melanomas cytoplasmic staining was diffusely granular, moreover, 15 cases showed markedly peripheral staining as seen in Figure 2. Staining intensity was relatively higher in large cell epitheloid melanoma cells, that were mainly seen in metastases, than in other types of melanoma cells (Fig. 3). In all cases, at least 40% of these large tumor cells were stained. Positive staining of melanoma cells generally was not associated with the presence of melanin, although in Case 3 (Table 1) the staining was largely confined to a small focus of melanin-containing epitheloid cells within a spindle cell melanoma.

The WS procedure on ultrathin sections was positive in six of nine cases of Group I. In areas with positive staining, premelanosomes could convincingly be identified ultrastructurally, whereas they were not found in areas without silver staining.

Nine of 15 cases in Group II (equivocally diagnosed as metastatic melanoma) showed positive staining with the monoclonal antibody NKI/C-3 (Table 2). In three cases 50% or more of the tumor cells stained, in the remaining six cases a minority was positive. The intensity and distribution of staining were similar to that of the Group I cases.

The WS procedure on ultrathin sections was positive in only one case. Silver deposits were focally found on atypical melanosomes in a few tumor cells as can be seen in Figure 4.

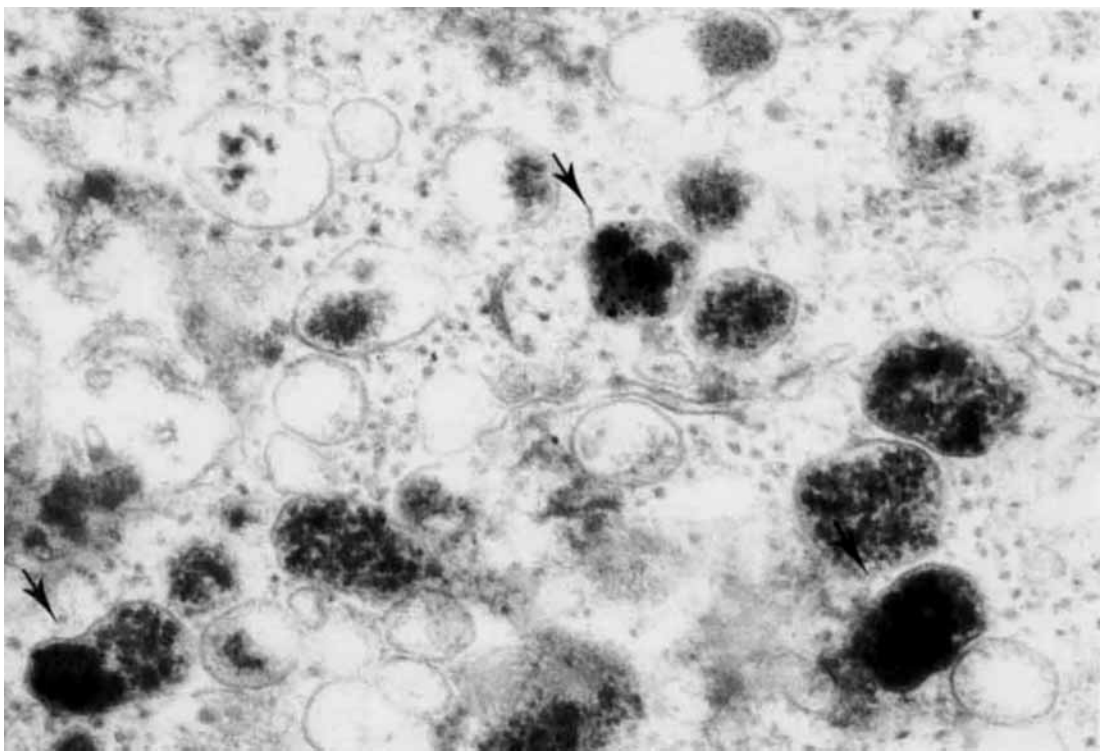


FIG. 4. Electron micrograph of a melanoma cell (Case 2, Group II). Ultrastructurally, three equivocal melanosomes can be identified with certainty by the deposition of silver granules as indicated by arrows (WS procedure, uranyl acetate, and lead citrate, ×63,000).

Of the histogenetically related tumors, five of six carcinoid tumors, two of seven chemodectomas, both medullary carcinomas of the thyroid, one of five poorly differentiated carcinomas with few neurosecretory granules, and the melanotic clear cell sarcoma showed positive staining with the monoclonal antibody NKI/C-3 (group B1, Table 3). Staining was weak in all cases, except for one carcinoid tumor and one medullary carcinoma of the thyroid. Three of the five positive carcinoid tumors showed tubular structures, in which the staining was strikingly confined to the bases of the tumor cells (Fig. 5). Both clear cell sarcomas of the synovial type remained unstained.

Of the 60 histogenetically unrelated tumors (group B2, Table 3) two undifferentiated large cell carcinomas, *i.e.*, a lung carcinoma and a metastasis of uncertain origin, showed slight staining in a minority (<5%) of tumor cells. The staining in these tumors was arranged in a very discrete, mostly paranuclear pattern, which was also observed in four carcinoid tumors and the melanotic clear cell sarcoma (Fig. 6). Peripheral staining as in nevomelanocytic tumor cells was not encountered in other tumors.

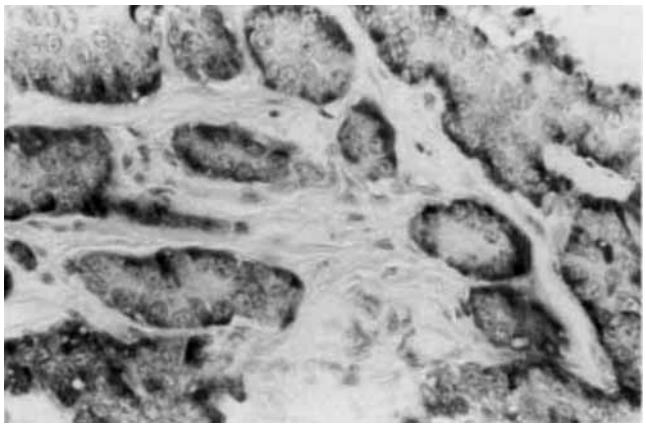


FIG. 5. Carcinoid tumor showing confinement of staining with the monoclonal antibody NKI/C-3 to the basal part of the tumor cells (Hematoxylin counterstaining, original magnification ×450).

Discussion

In this study we tested the possibility of whether the diagnostic workup of metastatic melanoma on routinely embedded material could be improved. Therefore, nine cases of metastatic melanoma that had been diagnosed only after extensive sampling (Group I) and 15 cases equivocally diagnosed as such (Group II) were studied. This was done using a monoclonal antibody directed against a melanoma-associated antigen in an indirect immunoperoxidase technique on paraffin sections and using a modified WS staining procedure for melanin directly on ultrathin epoxy sections. The latter method was found to be very useful in localizing oligomelanotic areas in a largely amelanotic melanoma, and in staining melanosomes with atypical morphology or poor preservation.[5] It was found that all metastatic melanomas (group A, table 3), used as positive controls, stained with NKI/C-3. Moreover, eight of nine cases of Group I were clearly positive for this monoclonal antibody whereas in the remaining case only a few tumor cells stained; six of nine cases stained with the WS procedure on ultrathin sections. This indicates that application of NKI/C-3 may facilitate the diagnosis of these difficult cases. Fifty-eight of the 60 histogenetically unrelated and differential diagnostically important tumors were negative with NKI/C-3; both large cell undifferentiated carcinomas that were positive, characteristically only focally showed a localized staining pattern with a slight intensity. In contrast, the staining pattern of melanoma cells was mostly diffuse and sometimes peripheral. Furthermore, staining of 50% of the other tumors histogenetically related to malignant melanoma indicate that NKI/C-3 is not specific for nevomelanocytic cells. Clear cell sarcomas of the synovial type will not frequently give rise to diagnostic confusion with malignant melanomas.[11] However, the melanotic type cannot easily be differentiated from malignant melanoma, but its association with tendons and aponeuroses will elucidate its nature in most cases. The diagnosis of neuroendocrine

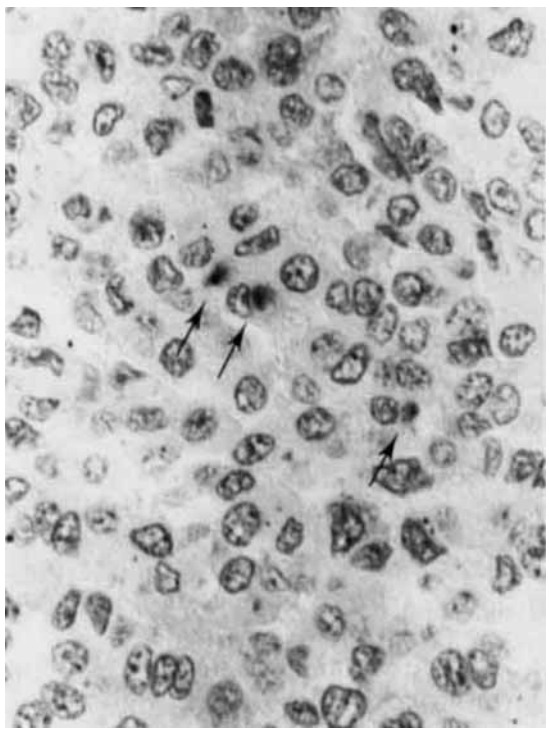


FIG. 6. Oligomelanotic clear cell sarcoma showing three tumor cells staining with the monoclonal antibody NKI/C-3 with a discrete focal cytoplasmic pattern (arrows). Scattered, few melanine granules are found (Hematoxylin counterstaining, ×550).

(APUD) tumor can usually be made by its typical histologic appearance, its argyrophilia with Grimelius' silver method, and in part of the cases by the immunohistochemical presence of peptide hormones. Additional electron microscopic examination will demonstrate neurosecretory granules that do not stain with the modified WS procedure,[5] making the diagnosis of melanoma highly unlikely.

One of five poorly differentiated large cell carcinomas with few neurosecretory granules showed focal staining with NKI/C-3 in less than 5% of the tumor cells, whereas none of six poorly differentiated small cell carcinomas (oat cell carcinomas) of the lung did. As a result, poorly differentiated tumors that stain with NKI/C-3 have to be examined by electron microscopy in order to exclude neuroendocrine tumor other than malignant melanoma. The high incidence of NKI/C-3-positive neuroendocrine tumors indicate that this monoclonal antibody could well be directed against an antigen for APUD (tumor) cells, and further supports the view that melanoma can be considered a tumor of the APUD-series.[9,10]

Based on the previous considerations, we propose the following conditions for the application and interpretation of the NKI/C-3 staining in poorly differentiated metastatic tumors:

1. Sampling should be preferentially of epitheloid cell areas, because these cells tend to stain most intensely.
2. Neuroendocrine (APUD) tumors should be excluded, because they also may react.
3. At least 15% of the tumor cells should be stained, because in two large cell undifferentiated carcinomas few (<5%) of the tumor cells reacted.
4. The cytoplasmic staining pattern should be diffuse or peripheral and not only focal, as observed in several nonmelanocytic tumors.

With these restrictions, this monoclonal antibody will not detect all melanomas, but certainly will facilitate the diagnosis in most cases. The fact that 6 of 23 primary melanomas did not show staining could indicate that the antigen recognized by NKI/C-3 is not present in all melanomas in detectable concentrations. The number of cases of primary melanoma studied is too low to judge a possible prognostic implication of the presence or absence of this antigen. However, false-negative results due to fixation and embedding procedures cannot be excluded with certainty. In formalin-fixed paraffin sections the antigen that is recognized by NKI/C-3 is expressed markedly by nevocellular nevi, most malignant melanomas and about 50% of other well differentiated neuroendocrine tumors. It is conceivable that this antigen may be present in other tumors in low amounts. Moreover, formalin fixation and paraffin embedding might introduce an artificial threshold for the detection of the antigen by immunohistochemistry. Biochemical investigations performed thus far have indicated that the antigen is probably not a protein or a glycolipid. The antigen is expressed markedly in junctional and upper dermal areas of nevocellular nevi, where the nevus cells are mostly epitheloid and DOPA-positive. However, in deeper dermal areas with spindle cells (neuroid differentiation) the antigen is present in low amounts or absent, and the DOPA reaction is negative. The difference in the presence of the antigen between epitheloid and spindle cells in malignant melanomas might be an expression of the same phenomenon. The differences in staining intensity of individual tumor cells within one tumor is probably related to the phenomenon of tumor heterogeneity.[12-14]

Of the 15 cases equivocally diagnosed as (metastatic) melanoma after exclusion of certain other tumors (Group II), 9 cases showed staining with NKI/C-3; only one case was positive with the WS procedure on ultrathin sections. Therefore, NKI/C-3 seems to be valuable in the diagnosis of difficult cases of amelanotic melanoma. However, the diagnosis of the Group II amelanotic melanomas has to be confirmed, presumably with a set of melanoma-associated monoclonal antibodies that is reactive in paraffin embedded tissue or by clinical follow-up. In oligomelanotic tumors the WS procedure on ultrathin sections can confirm the diagnosis of melanoma as illustrated by the results of Group I (Table 1). Melanin staining on light microscopic[1] and on electron microscopic levels may facilitate the identification of premelanosomes. Most other techniques, used to establish the diagnosis of melanoma, require a special treatment of the tissue. Fresh tumor tissue is needed for both the tissue culture technique[15,16] and the well-known DOPA reaction. Furthermore, most melanoma-associated monoclonal antibodies, that are known, were only tested on cell lines and few on frozen sections,[17,18] but reactivity on paraffin sections has not yet been described. Only neurone-specific enolase (NSE)[19] and S-100 protein[20] have recently been demonstrated in paraffin-embedded melanomas, but their specificity appeared less high than NKI/C-3.

The combination of several diagnostic modalities probably will yield a very sensitive and specific approach for identifying metastatic amelanotic melanomas. We currently advocate the following policy for the diagnostic workup of an apparently undifferentiated tumor that is suspect for amelanotic melanoma:

> Staining for melanin in paraffin sections of all available blocks (Schmorl, WS procedure).
>
> If no melanin can be demonstrated, electron microscopy should be performed, preferably on properly fixed epoxy embedded material; the monoclonal antibody NKI/C-3 can be applied, and the reactivity evaluated under the above mentioned conditions.

If the epoxy embedded tissue is not representative or if no premelanosomes are found, a small amount of tissue from the paraffin blocks should be processed for electron microscopic examination.

When by electron microscopic examination structures are found that are only suggestive for melanosomes, the WS procedure on ultrathin sections should be performed.

In conclusion, the melanoma-associated monoclonal antibody NKI/C-3 could be useful for the diagnostic workup of difficult cases of (metastatic) melanoma, preferentially in a panel of different tumor-associated antibodies that are reactive in paraffin sections.

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