# Intermediate Filaments in Malignant Melanomas

## IDENTIFICATION AND USE AS MARKER IN SURGICAL PATHOLOGY

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ABSTRACT Intermediate-sized filaments have been studied in human malignant melanomas and in normal melanocytes by immunofluorescence microscopy with antibodies directed against keratin, vimentin, desmin, neurofilament protein, and glial filament protein. Both human melanotic and amelanotic tumor cells and tumor metastases as well as normal melanocytes in human skin and in the rat eye contain exclusively intermediate filaments of the vimentin type. No reaction was seen with antibodies to keratin, desmin, neurofilaments, or glial filaments. These latter four antisera, however, gave strong reactions in epidermis and other epithelial tissues, muscle, or neural tissues, respectively. The results favor a mesenchymal character of melanocytes, although a neuroectodermal origin in an early developmental stage is possible. The finding that melanomas contain exclusively vimentin intermediate filaments may prove useful in differential diagnosis of melanomas from other tumor types.

## INTRODUCTION

The origin of melanocytes, the pigment-producing cells in the basal layer of the epidermis and in the uveal tract of the retina, is still a subject of discussion and controversial interpretations in the literature (1–4). Their morphology may vary from an epithelioid or dendritic shape to a fibroblastlike appearance. The most commonly accepted theory at present is that melanocytes originate from the neuroectoderm. This theory is substantiated by the facts that melanomas contain S-100, a protein claimed to be specific for nervous tissue (3), and that the ancestral cells that form the melanocytes may have a dendritic shape and can be stained specifically for Dopa (5). Therefore, one would expect other markers for neural tissue, including neurofilaments or glial filaments, the intermediate filament types present in neurons or astrocytes, respectively, to be present in these cells (6–8). In electron microscopic studies (9), intermediate-sized filaments (10-nm thick) have been reported for melanin-forming neuroectodermal tumor cells and have been referred to as tonofilaments (10) or neurofilaments (11). In general, the type of intermediate-sized filaments present in specific cell types seems to depend primarily on their differentiation and embryological origin (4). With antibodies to the main protein constituents of these intermediate filaments (i.e., keratins, vimentin, desmin, the neurofilament proteins, and the glial filament acidic protein) one can distinguish between cells of epithelial, mesenchymal, myogenic, neural, and astrocytic origin, respectively, in the immunofluorescence microscope (9–12). Neoplastic outgrowths of these tissue types can also be characterized using these antisera because solid malignant tumors seem to retain their original intermediate filament type and do not develop additional intermediate filament systems when malignant degeneration occurs (13–22). Because of our interest in characterizing different tumors and normal cell types for their classes of intermediate filaments in order to use them as histochemical markers in surgical pathology, as well as our interest concerning the development and expression of these filaments during embryogenesis, we have studied normal and malignant human tissues. In this study, we report the occurrence of vimentin intermediate filaments in human malignant melanomas.

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## METHODS

*Tissues.* Normal and malignant human tissues were obtained immediately after surgery and frozen in liquid nitrogen. In some cases the tissue was used directly for preparation of frozen sections. The melanomas used in this study include melanotic and amelanotic tumors. These tumors were: (*a*) a malignant melanoma of the superficial spreading type, located in the shell of the ear, (*b*) a malignant melanoma of the vulva, (*c*) an amelanotic metastasis of a malignant melanoma in an inguinal lymph node, (*d*) a nodular malignant melanotic melanoma from the skin overlying the ankle, and (*e*) a metastasis of an amelanotic melanoma in the lung.

Routine light microscopic diagnosis of these tumors was made on hematoxylin-eosin-stained, formalin-fixed, and paraffin-embedded material. Specific silver staining of melanocytes and (melanotic) tumor cells by the method of Masson using Fontana's solution was performed as described in detail elsewhere (23).

*Antisera.* Keratin was isolated from human foot epidermal calluses essentially as described by Franke et al. (24). The keratin antiserum was prepared as described previously (13). Vimentin was isolated from calf lenses by preparative gel electrophoresis and antibodies prepared as described (13). Desmin was isolated from chicken gizzard by using modifications of the methods described by Geisler and Weber (25) and Franke et al. (26). Essentially, chicken gizzard muscle tissue was minced in phosphate-buffered saline (PBS) for 10 min at 4°C (all subsequent steps were performed at 4°C) and homogenized with an Ystral X10/20 homogenizer (Ystral GmBH, Ballrechten-Dottingen). The homogenate was stirred for another 10 min, centrifuged at 6,000 rpm in a GSA-rotor (E. I. Du Pont de Nemours & Co., Inc./Sorvall Instruments Div., Newtown, CT) for 10 min, the pellet rehomogenized in EDTA-buffer (1 mM EDTA, 0.01% β-mercaptoethanol, 0.5% Triton X-100, and 10 mM Tris/HCl, pH 7.4) and extracted for 30 min. After recentrifugation, the pellet was washed once with EDTA-buffer, homogenized in KCl-buffer (1 M KCl, 0.01% β-mercaptoethanol, 0.5% Triton X-100, and 10 mM Tris/HCl, pH 7.4) and extracted for 3 h. This suspension was centrifuged again and the pellet extracted for 3 h with KI-buffer (0.6 M KI, 0.01% β-mercaptoethanol, 0.5% Triton X-100, and 10 mM Tris/HCl, pH 7.4). The pellet obtained after this extraction was washed once with PBS and used for desmin purification by using preparative sodium dodecyl sulfate (SDS) gel electrophoresis essentially as described for vimentin (13).

New Zealand white rabbits were immunized subcutaneously with ~0.2 mg of vimentin, 0.4 mg keratin, or 0.25 mg of the desmin (in SDS-sample buffer) with equal volumes of Freund's complete adjuvant. Rabbits were boosted repeatedly (up to three times with 3-wk intervals) with equal amounts of protein in incomplete Freund's adjuvant and the sera collected 3 wk after the last booster. Before immunization, the rabbits were thoroughly tested for autoimmune antibodies. Only rabbits with preimmune sera that gave a negative reaction on frozen sections from human skin were used for immunization. The preimmune sera of these rabbits were occasionally used in parallel incubations on tumor sections as controls. Furthermore, vimentin antiserum absorbed with a purified vimentin preparation was used as a control. The antisera prepared as described above were tested for their specificity on frozen sections of human tissues. In addition, tissues from rat and hamster, as well as cultured cells, were used for quality screening as described earlier (13).

Antineurofilament antibodies were directed against the 68,000-mol wt neurofilament protein isolated from calf brain and were a kind gift from Dr. Denise Paulin (see ref. 27). Another neurofilament antiserum raised in rabbits was also directed against bovine brain 68,000-mol wt polypeptide isolated from preparative SDS gel electrophoresis and absorbed with glial filament protein from bovine brain.[1] Antibodies to glial filaments were prepared in rabbits and directed against the 51,000-mol wt glial filament acidic protein isolated from bovine brain with preparative gel electrophoresis.[1] The two latter sera were kindly provided by Dr. Chris Pool. When these two latter sera were used, frozen sections were fixed with 2% paraformaldehyde for 2–3 min at room temperature instead of the methanol/acetone fixation; this was followed by washes in water for 15–20 min and in PBS for 10 min.

The keratin and vimentin antibodies were further tested by using the immunoblotting technique. The keratin antiserum could be shown to recognize several keratin bands ranging in mol wt between 40,000 and 66,000 in cytoskeletal preparations from rat epithelial tissues. This antiserum did not detect a protein band comigrating with vimentin. The antivimentin antiserum gave a reaction with protein bands ranging in mol wt between 40,000 and 57,000 in a cytoskeletal preparation from a human theka cell fibroma. These protein bands were recognized as vimentin and its typical breakdown products by using two-dimensional gel electrophoresis. Also, in a cytoskeletal preparation from a human stomach carcinoma, only these bands were observed. Other, even more pronounced bands were not recognized by the antivimentin antiserum.

*Indirect immunofluorescence microscopy.* Small pieces of normal and tumor tissue were frozen in liquid nitrogen immediately after surgery. Air-dried cryostat sections (4–7 μm thick) were fixed for 5 min with methanol at −20 C and thereafter dipped in acetone and the indirect immunofluorescence technique was applied as described previously (13).

For experiments on ocular melanocytes, eyes from 3-mo-old rats were treated exactly as described above for tumor tissue.

## RESULTS

The melanomas described here include (*a*) a superficial spreading malignant melanoma in the shell of the ear, (*b*) a malignant melanoma of the vulva, (*c*) an amelanotic melanoma metastasis in an inguinal lymph node, (*d*) a nodular malignant melanoma of the skin covering the ankle, and (*e*) an amelanotic melanoma metastasis in lung. Many of the cells in these tumors showed pigment granules in the silver staining procedure. Normal melanocytes were also stained strongly with this method, showing the typical distribution of the pigment granules.

Although the tumor cells showed a rather strong shape variability, in all tumors most of the cells showed a polygonal epithelioid appearance, sometimes with focal collections of spindle-shaped cells. The results obtained by using the antisera to intermediate filament proteins on normal and tumor tissues are summarized

[1] Heyting, C., C. W. Pool, and W. van Raamsdonk. Manuscript in preparation.

in Table I. The rabbit antibodies directed against human keratin did not react with the tumor cells (Fig. 1 a and c). A strong reaction, however, was seen in the epidermis (Fig. 1 a and b; compare with Fig. 1 d and e), in hair follicle cells (Fig. 1b), in sweat glands, and in sebaceous glands (Fig. 1b). In Fig. 1a it can be seen that the superficially spreading tumor cells (case a) destroy the integrity of the epidermis, especially of the basal layer. No reaction is seen with the keratin antibodies in dermal connective tissue. In contrast, the antibodies to vimentin did show a prominent staining in this dermal layer (Fig. 2a). Also some dendritic cells present in the spinosal and basal layer were positive for vimentin. These cells represent melanocytes and dermal Langerhans cells as shown with other techniques (28). In general, a strong reaction with the vimentin antiserum was seen in all cases of the melanocytic tumors (Fig. 2b). The intensity of the fluorescence staining in the cells of melanotic malignant melanomas (cases b and d) was, however, dependent on the amount of melanosomes present in a cell. In the cells showing high concentrations of pigment granules, vimentin fluorescence is less predominant (compare Figs. 3 a and b), whereas in malignant tumor cells that are (almost) devoid of pigment, strong vimentin fluorescence is seen (Fig. 3c). As compared with lymphocytes, amelanotic metastatic tumor cells in a lymph node (case c) show a considerably stronger reaction to antibodies directed against vimentin. When the vimentin antiserum was preabsorbed with a purified vimentin preparation from calf lens, no staining was observed (not shown).

No significant staining of the tumor cells was seen with antibodies to chicken gizzard desmin (Fig. 4 a and b). These antibodies reacted strongly with smooth muscle cells present in the skin, in particular the arrector pili muscle.

Antibodies directed against neurofilament proteins

TABLE I

*Reactions of Antibodies Directed Against the Five Types of Intermediate Filament Proteins on Normal Tissues and Malignant Melanomas*

| Tissues | Antibodies to | | | | |
|---|---|---|---|---|---|
| | Keratin | Vimentin | Desmin | Neurofilaments | Glial filaments |
| Skin: | | | | | |
| Epidermis | +++ | – | – | – | – |
| Melanocytes | – | ++ | – | – | – |
| Langerhans cells | – | ++ | – | – | – |
| Dermis (fibroblasts) | – | ++ | – | – | – |
| Smooth muscle cells | – | – | +++ | – | – |
| Hair, hair roots, and hair follicles | +++ | – | – | – | – |
| Sebaceous glands | ++ | – | – | – | – |
| Sweat glands | +++ | – | – | – | – |
| Nerve cells/endings | – | – | – | +++ | – |
| Melanoma cells | – | ++/+++ | – | – | – |
| Vagina: | | | | | |
| Epithelium | ++ | – | – | – | – |
| Proper lamina: | | | | | |
| Fibroblasts | – | ++ | – | – | – |
| Endothelium | – | ++ | – | – | – |
| Vascular smooth muscle cells | – | ++ | ++ | – | – |
| Muscle layer | – | – | +++ | – | – |
| Melanoma cells | – | ++/+++ | – | – | – |
| Lymph node: | | | | | |
| Lymphocytes | – | +/++ | – | – | – |
| Reticular (trabecular) connective tissue | – | ++ | – | – | – |
| Melanoma cells | – | +++ | – | – | – |
| Lung: | | | | | |
| Glandular epithelium | ++ | – | – | – | – |
| Melanoma cells | – | ++ | – | – | – |
| Rat eye: | | | | | |
| Pigment epithelium | – | ++ | – | – | – |
| Choroidal melanocytes | – | ++ | – | – | – |

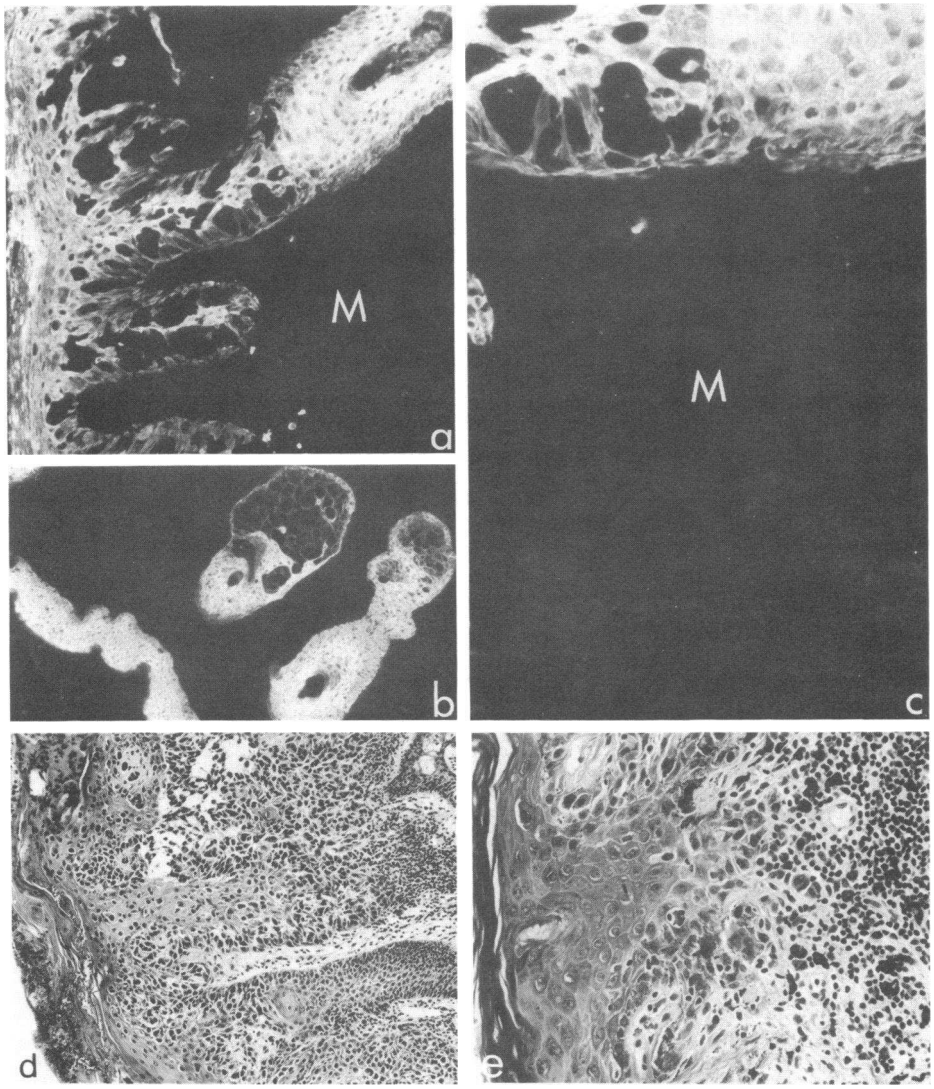


FIGURE 1 Immunofluorographs showing the reaction of antibodies directed against keratin with a melanoma in the human skin. Note strong positive reactions in the epidermis (a, b), hairs and follicles (a, b), and sebaceous glands (b, c). No reaction with these antibodies is seen in melanoma cells (M; a, c). d, hematoxylin-eosin-stained paraffin section from the same tumor. e, Tumor section stained after Masson using Fontana's solution. a, ×80; b, ×50; c, ×190; d, ×50; and e, ×120.

(Fig. 4c, d, and e) or glial filament antibodies did not stain tumor cells, whereas nerve fibers and fine nerve endings in the skin showed a bright fluorescence with the neurofilament antisera (Fig. 4 c and d). The latter antibodies (antidesmin, antineurofilament, and anti-glial filament) did not stain epithelial or mesenchymal tissues in the skin or in lymph nodes.

As far as normal melanocytes in the rat eye are concerned, we were able to confirm the data cited above for the skin melanocytes and could only detect vimentin in these ocular cells (compare also ref. 29).

## DISCUSSION

Melanocytes in the skin and retina are by some authors believed to be derived from the neural crest during an early stage in embryogenesis (1, 30). Other theories, however, suggest that they are either modified basal

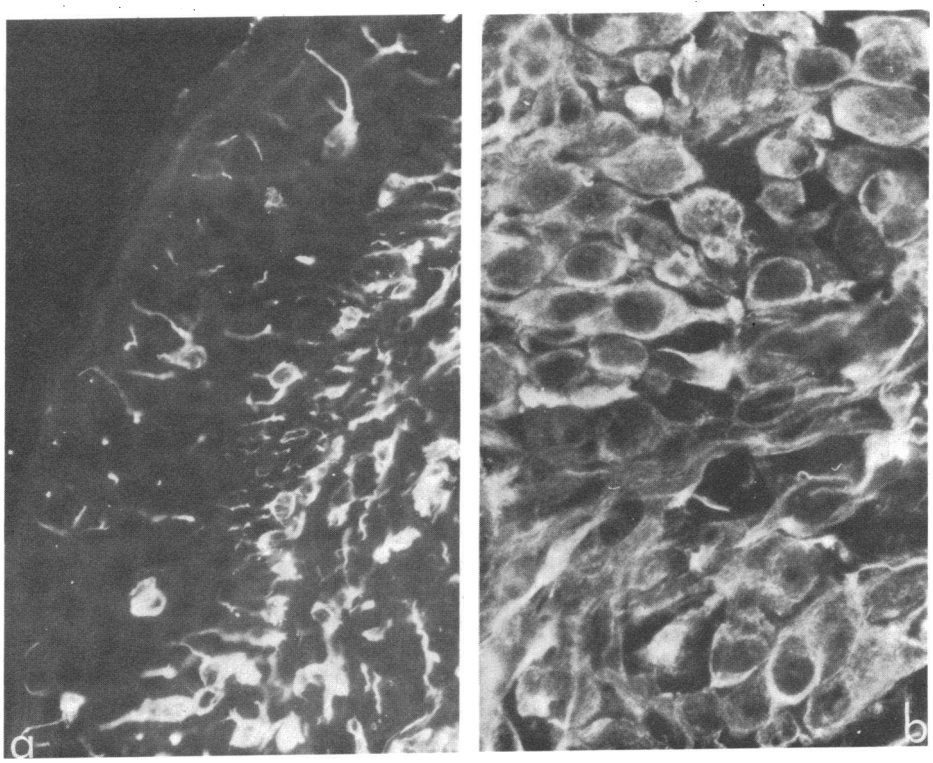

FIGURE 2 Immunofluorographs showing reactions of antibodies to vimentin with Langerhans cells, melanocytes, and fibroblasts in normal human skin (a). Strong reaction is also seen in cells of a malignant melanoma (b). No reaction is seen in the epidermis (a). a, ×300; b, ×480.

epidermal cells (31) or even cells of mesenchymal origin (32). Some morphological and biochemical aspects of melanocytes and of cells in malignant melanomas favor the assumption of a neural or neuroectodermal origin. In addition to the dendritic appearance of some melanocytes and their ancestors, the presence of the S-100 protein may be an indication of such an origin (3). However, recent reports in the literature have shown that cells of mesenchymal origin can also contain this constituent. Stefansson et al. (33) have demonstrated the S-100 protein in mesodermal chondrocytes, whereas Cocchia et al. (28) have found this protein in cells with morphological features of Langerhans cells, which are believed to be of mesenchymal origin (34). Therefore, we wondered what type of intermediate-sized filaments these latter cells as well as melanocytes and their malignant derivatives possess, especially as the nature of intermediate filament proteins seems to correlate with the embryonic origin of the cell type (35). In recent reports, Franke et al. (12) and Sieinski et al. (36) have shown the absence of keratin filaments in cultured melanocytes and in paraffin-embedded sections of malignant melanoma, respectively. Franke et al. (32) have demonstrated vimentin intermediate filaments in normal melanocytes from rat and cow. We could confirm these data on frozen sections from normal and neoplastic human melanocytes in vivo. Furthermore, it is obvious that neurofilaments and glial filaments are also absent from these cells, whereas, as already mentioned, strong reactivity was found with antibodies to vimentin. This reaction was slightly variable in intensity, most probably due to the fact that some melanocytes or melanoma cells contain high concentrations of pigment granules. In the amelanotic tumor metastasis in the lymph node, a very strong reaction with vimentin antibodies was seen. A similar observation with electron microscopy was made by Warner et al. (37) who saw dense arrays of 10-nm filaments (intermediate-sized filaments) in metastatic deposits from neurotropic melanomas of the lip and vulva. Cutler et al. (9) could also show that in melanotic neuroectodermal tumors of infancy, 10-nm filament networks were more pronounced in cells with few or no melanin granules. In cells that contained melanin granules, the filaments seemed to be associated with the granules (see also 30). Because the presence and abundance of the vimentin cytoskeleton may influence mitotic or motile activity (38) it is tempting

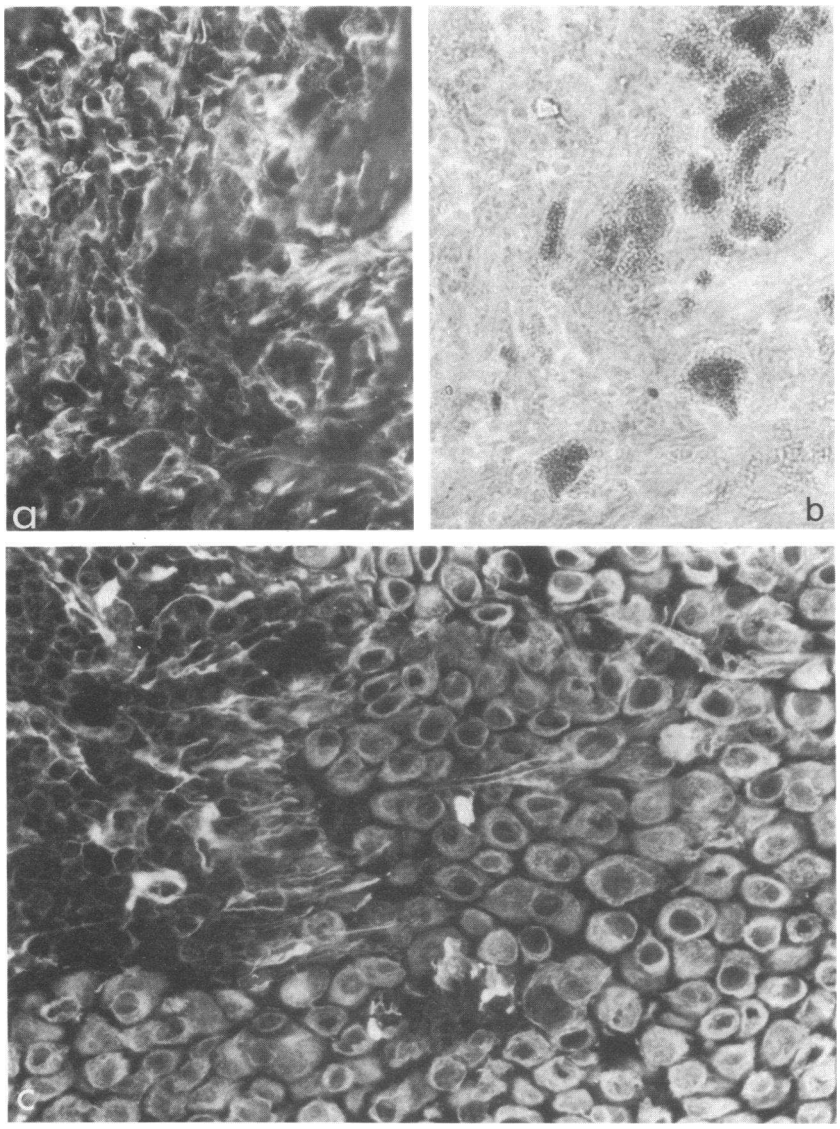


FIGURE 3 Reaction of vimentin antibodies with a melanotic melanoma (a, indirect immunofluorescence; b, phase contrast of the same area showing pigment granules) and a lymph node metastasis of an amelanotic melanoma (c). Note the strong reaction in the metastatic, amelanotic cells as compared with both those containing pigment granules, and with lymphocytes. a, ×325; b, ×325; c, ×350.

to speculate about a possible role of this matrix in metastasis and spreading. Studies in the future shall be directed at the correlation of these observations. No desmin-containing intermediate filaments could be detected in melanomas.

The present results favor the theory that melanocytes are of mesenchymal origin, because vimentin intermediate filaments have been shown to be specific for mesenchymal cells in adult animals. However, recent reports in the literature (39–41) have shown that at a certain stage of embryonic development, neuroepithelial cells, precursor cells to both neurons and glia, contain vimentin, which is replaced by or supplemented with neurofilaments or glial filaments in subsequent stadia of embryonic development. Therefore, our data certainly do not exclude a neuroectodermal origin of melanocytes. The stage in embryogenesis at which cells obtain their definitive function

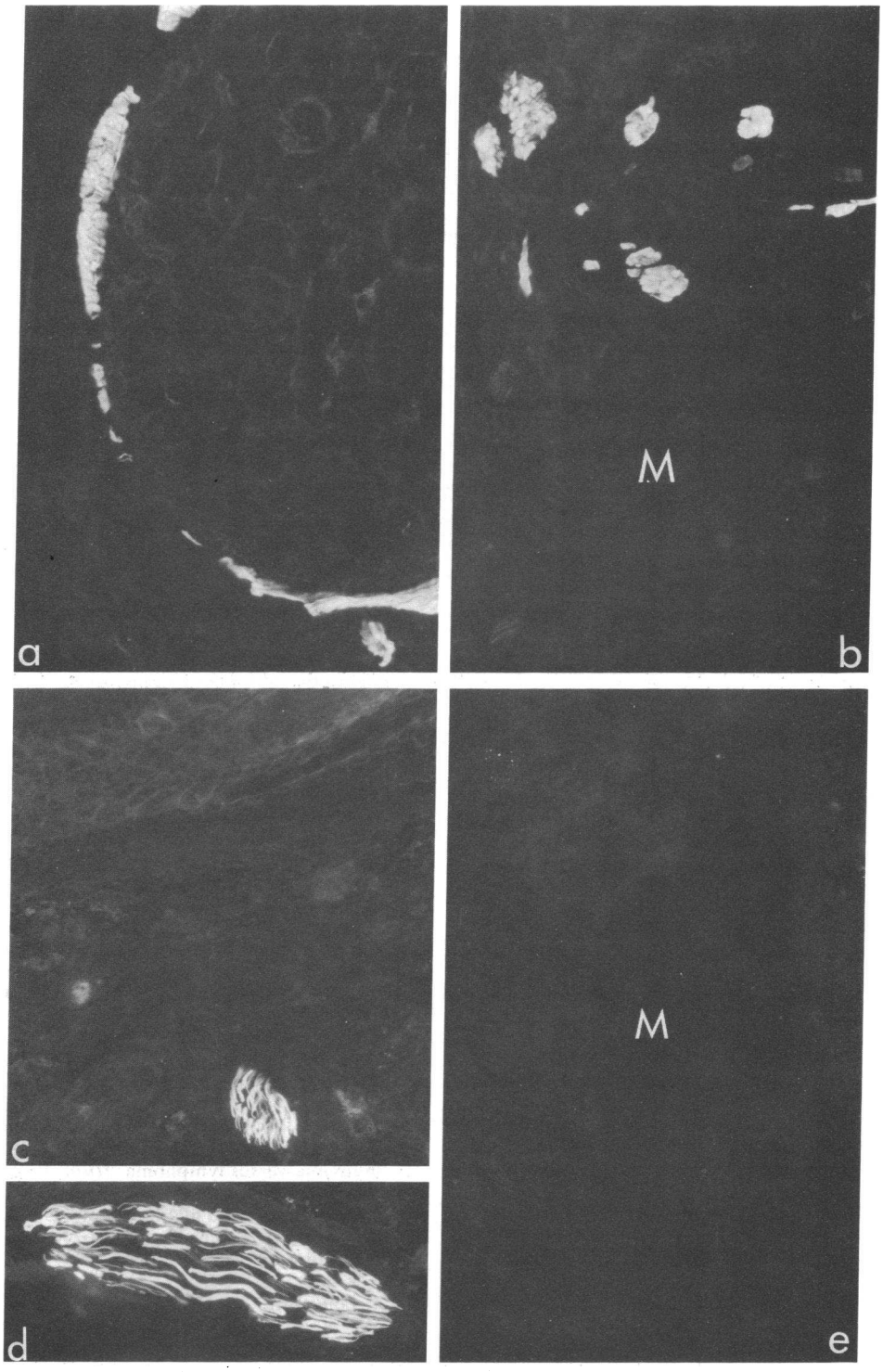


FIGURE 4 Immunofluorographs (×170) of human skin and melanoma (M) incubated with antibodies to desmin (a, b) and neurofilament protein antibodies (c–e). No reaction with these sera is seen in the tumor cells (M; b, e), whereas strong staining is seen in smooth muscle cells with desmin antibodies (a, b) and in nerve cells with antibodies to neurofilament protein (c–d).

and location may determine their type of intermediate-sized filaments.

In agreement with Cutler et al. (9) our observation that intermediate filaments in melanomas are exclusively of the vimentin type further allows us to state that real desmosomes cannot be present as junctions between these cells, because these would involve keratin-containing tonofilaments (42).

The finding of vimentin intermediate filaments in melanomas may prove to be useful in routine practice of surgical pathology whenever it is difficult to determine the origin of tumors first seen as metastases. Malignant melanoma may be confused with malignant lymphoma, sarcoma, and carcinoma (43). Especially in the case of differential diagnosis of anaplastic carcinomas and amelanotic melanomas, antibodies to keratin and vimentin may be helpful tools. The method may be useful also in cases where melanogenic organelles occur in epithelial cells and their tumors. Chumas and Lorelle (44), for example, have reported the presence of pigment granules in an adenocarcinoma of the anorectum. In such cases and in cases of toxin (arsenic, colchicine) induced pigmentation (45, 46), accuracy in diagnosis of neoplasms may be increased and misinterpretation of such lesions as "melanomas" at unusual sites may be avoided by this technique.

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