PNL2, a New Monoclonal Antibody Directed against a Fixative-Resistant Melanocyte Antigen

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We report the production of a new monoclonal antibody, PNL2, directed against a fixative resistant melanocyte antigen. The analysis of PNL2 immunostaining on a broad range of normal or malignant human tissues and on various melanocytic lesions revealed its high specificity. PNL2 gave a strong cytoplasmic staining of skin and oral mucosae melanocytes, and staining of granulocytes when used at high concentration. PNL2 stained all intraepidermal nevi irrespective of their histologic type, but common intradermal nevi and the dermal component of compound nevi were largely non-reactive as only scattered nevus cells in the papillary dermis were labeled. PNL2 labeled more than 70% of the neoplastic cells in all primary melanomas irrespective of their histologic type. However, PNL2 did not label desmoplastic melanomas. All metastatic melanomas were also stained but the percentage of labeled cells was occasionally lower than the primary tumor. PNL2, as anti–Melan A and HMB-45 antibodies, stained most of the clear cell sarcoma cells, and a few cells in angiomyolipomas and lymphangioleiomyomatosis. None of the other nonmelanocytic lesions tested were labeled. Proteomic approaches showed that the immunoaffinity purified PNL2-binding complexes isolated from melanoma cell lines comprise at least TAP1, Clathrin 17 and prealbumin proteins, but not the gp100 recognized by HMB-45. In conclusion, this new monoclonal antibody, PNL2, is directed against a new fixative resistant melanocyte associated antigen. This antigen is chemically resistant and thus allows immunostaining after melanin bleaching or decalcification. We also demonstrate that it is different from Melan A and from gp100, even if PNL2 and HMB-45 staining patterns are sometimes similar.

KEY WORDS: Clathrin 17, Melanoma, Monoclonal antibody, Immunohistochemistry, Pre albumin, Proteomic, TAP1.

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Melanocyte proliferation often constitutes a diagnostic problem in surgical pathology. The differential diagnosis between benign nevi and malignant melanomas is sometimes difficult and malignant melanomas may mimic other neoplasms such as undifferentiated carcinoma, sarcoma or large cell lymphoma (1). Furthermore, melanocyte-specific antigens expression is of diagnostic value in some soft tissue lesions such as clear cell sarcoma, angiomyolipoma, and lymphangiomyomatosis (2). The increasing number of monoclonal antibodies (Mab) directed against formalin resistant melanoma associated antigens such as gp100 (HMB-45 Mab), Melan A (A103 Mab), CD63 (NKI/C-3 Mab), MAGE 1, MAGE 3, or tyrosinase (T311 Mab) has provided useful diagnostic tools (3–16). However, some melanocytic lesions lack most of these antigens, and non-melanocytic lesions may express melanocyte-related antigens (17–19). The use of a broad range of antibodies is therefore needed for definitive identification.

Furthermore, the increasing interest in sentinel lymph node examination and the development of ultrarapid immunostaining protocols for frozen

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sections emphasize the need of highly sensitive and specific anti-melanocyte antibodies.

At least, antigen-targeted approach in melanoma therapy highlights the interest of new melanoma specific proteins (20–32).

We herein report the production of a new monoclonal antibody, named PNL2, directed against a fixative resistant melanocyte associated antigen. This antibody can be used on routinely processed paraffin sections. A detailed analysis of the reactivity of PNL2 on a broad range of normal or malignant human tissues and on various melanocytic lesions is provided below to reveal its high specificity. The results are compared with those obtained using HMB-45 and anti-Melan A (clone A103) antibodies. These findings point to potential applications of the PNL2 Mab in the immunostaining of melanomas and basic research.

MATERIALS AND METHODS

Production of PNL2 Mab

PNL2 Mab was generated using BALB/C mice immunized by intraperitoneal injections of a recombinant protein corresponding to the C-terminal part of the sub-type 2 of human somatostatin receptor (SST2) coupled with glutathione S-transferase. The non-immunoglobulin-producing myeloma cell line P3 × 63 Ag8-653 was used as a fusion partner. Fusions were performed using standard techniques (33, 34). Supernatants were tested for antibody-binding activity by ELISA and by indirect immunoperoxidase method on frozen sections of human pancreas and tonsil. Isotype characterization was performed using IsoStrip mouse Mab isotyping kit according to manufacturer's protocol.

Immunostaining

Immunohistochemistry was performed on 4-μm routinely processed paraffin sections using a Techmate Horizon slide processor. A prior antigen retrieval based on microwave heating (10 mM citrate buffer, pH 6) was needed for anti–Melan A (clone A103, DAKO, Glostrup, Denmark) and PNL2 antibodies. An enzymatic digestion was performed using Proteinase K according to the manufacturer's protocol (ChemMate, DAKO) before HMB-45 (DAKO) immunostaining. Antibody binding was revealed using a streptavidin-biotin complex reagent (StrepABComplex/HRP Duet, DAKO) on human samples and using the "Animal Research Kit" ARK (DAKO) on mouse samples according to manufacturer's protocol. PNL2 supernatant, anti–Melan A and HMB-45 were used respectively in 1/2, 1/40 and 1/75 dilution. Negative controls were performed for each sample using the supernatant of the cell line P3 × 63 Ag8–653 as the primary antibody.

All the tissue samples were fixed in ethanol based Bouin's fluid or in 4% formalin and were wax embedded. They consisted of a wide variety of normal human tissues (Table 1), 96 cases of melanocytic neoplasms (Table 2) including 47 benign lesions and 49 cases of melanoma (the latter included 32 primary cutaneous melanomas of different histologic types and 17 metastatic melanomas), 208 cases of non-melanocytic tumors of different categories (Table 3), and a panel of various tissues from white (Balb/c), black (C57/Bl6) and nude mice (Table 4).

Immunostaining after Enzymatic Treatment of Tissue Sections

The biochemical characterization of the nature of the epitope recognized by PNL2 Mab was performed using neuraminidase digestion before PNL2 immunostaining as previously reported for CNA.42 antibody (35). The CNA.42 Mab, which recognizes a

TABLE 1. Reactivity of PNL2 with Normal Human Tissue on Paraffin-Embedded Sections

Normal Tissue	PNL2 (positive/tested)
Adrenal gland	0/4
Appendix	0/2
Bladder	0/2
Bone marrow	0/4
Breast	0/5
Central nervous system	0/2
Cerebellum	0/1
Colon	0/8
Duodenum	0/1
Esophagus	0/3
Gallbladder	0/2
Heart	0/4
Kidney	0/8
Liver	0/6
Lung	0/5
Lymph node	0/5
Oral mucosa	**4/4**[¤$]
Ovary	0/2
Pancreas	0/3
Peripheral nerves	0/3
Placenta	0/2
Prostate	0/4
Rectum	0/1
Salivary gland	0/4
Seminal vesicle	0/2
Skin	**8/8**[¤]
Spleen	0/9
Stomach	0/4
Testis	0/4
Thymus	0/6
Thyroid	0/8
Tonsil	0/2
Uterine cervix	0/2
Uterus	0/5
Total	**12/135**

[¤] Strong immunostaining restricted to melanocytes.

[$] After formalin fixation, nitric acid-based decalcification was performed in two cases.

The immunostaining of the leukocytes was not taken into account.

TABLE 2. Reactivity of PNL2, HMB-45, and Anti Melan A with Melanocytic Tumors on Paraffin-Embedded Sections

Tumor Type	PNL2 (positive/tested)	HMB-45 (positive/tested)	Anti Melan A (positive/tested)
Benign lesions			
Nevi			
Dermal	9/9*	8/9*	9/9
Junctional	1/1	1/1	1/1
Compound	9/9*	9/9*	9/9
Congenital	1/1*	1/1*	1/1
Spitz	3/3*	3/3*	3/3
Desmoplastic	1/4	1/4	1/4
Pigmented spindle cells (Reed)	3/3	3/3	3/3
Common blue	4/4	4/4	4/4
Lymph node capsular nevi	4/4⊕	1/4⊕	4/4⊕
Dysplastic	1/1	0/1	1/1
Lentigo simplex	**8/8**	**8/8**	**8/8**
Total	**44/47**	**39/47**	**44/47**
Malignant lesions			
Melanomas			
Primary			
Lentigo maligna	7/7	7/7	7/7
Superficial spreading	8/8	8/8	8/8
Acral lentiginous	8/8	8/8	8/8
Nodular	4/4	3/4	3/4
In congenital melanocytic nevus	1/1	1/1	1/1
Mucosal	1/1	1/1	nd
Desmoplastic	0/3	0/3	0/3
Metastatic			
Skin	10/10	9/10	9/10
Lymph node	6/6	6/6	6/6
Pleural effusion	1/1	1/1	1/1
Total	**46/49**	**44/49**	**43/48**
Total	**90/96**	**83/96**	**87/95**

* Intradermal nevi and dermal component of compound nevi were largely non-reactive and only scattered nevic cells in the papillary dermis are labeled with HMB-45 and PNL2.

⊕ PNL2 immunostaining of nodal capsular nevi was weak while the anti Melan A staining was strong and HMB-45 negative or very weak.

nd = not done.

The immunostaining of the leukocytes was not taken into account.

glycosylated epitope expressed by follicular dendritic cells and some mononuclear cells, was used as an internal control for the enzymatic digestion.

Immunoblotting Analysis

Western blot analysis was performed using the human melanoma derived cell line CAL (our laboratory), the murine melanoma cell line B16F10 and granulocytes obtained from blood samples from healthy volunteer. Negative control consisted of P3 × 63 Ag8–653 cell line. The cell proteins were extracted in lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40), containing a protease inhibitor mixture (protease inhibitor cocktail Complete, Roche Molecular Biochemical, Mannheim, Germany) and boiled in sample buffer (4% SDS, 9% glycerol, 10% β-mercaptoethanol/bromophenol blue in 160 mM Tris HCl pH 6.8). Solubilized proteins were resolved through 10% SDS-polyacrylamide gels, and transferred to PVDF membranes. Blots were incubated with PNL2 supernatant, followed by incubation with horseradish peroxidase conjugated anti-mouse antibody (P0260, Dako). The peroxidase activity was revealed using chemiluminescence reaction (ECL+, Amersham, Little Chalfont, UK).

Immunoprecipitation

An immunoprecipitation technique was applied to melanoma protein extracts using immunomagnetic beads coated with sheep anti mouse IgG (Dynabeads M-450 sheep anti mouse IgG, Dynal, Oslo, Norway). Briefly, PNL2 supernatant was coated to the beads and was incubated with the protein extracts for 2 hours at 4° C. After washing, the beads were reduced in loading buffer and resolved through 10% SDS-PAGE.

Protein Identification by Proteomic Analysis and Mass Spectrometry

The immunoprecipitates were analyzed using one dimensional electrophoresis and visualized by coomassie blue staining. Peptide mass fingerprintings were obtained by using a PE Biosystems MALDI-TOF mass spectrometer (Voyager DE STR, Foster City, CA, USA) from each protein band (36). Unknown proteins were identified using the database fitting program MS-Fit (Protein Prospector,

(http://prospector.ucsf.edu)), searching against all eukaryotic entries in Swiss Prot and NCBI nonredundant protein data bases. We considered the identification positive when a minimum of four measured peptide masses was matched and provided at least approximately 20% sequence coverage (mass accuracy of 10 ppm was obtained with internal calibration).

TABLE 3. Reactivity of PNL2 with Human Non-Melanocytic Tumors on Paraffin-Embedded Sections

Tumor Type	PNL2 (positive/tested)
Haematopoietic neoplasms	
Non-Hodgkin's lymphoma	0/57
Hodgkin's lymphoma of various types	0/15
Carcinomas	
Skin	
Squamous cell	0/3[¤]
Basal cell	0/4[¤]
Lung	
Squamous cell	0/8
Adenocarcinoma	0/6
Gastrointestinal tract	
Esophagus (of various types)	0/7
Stomach	0/5
Small intestine	0/2
Large intestine	0/11
Breast (of various types)	0/7
Endometrial adenocarcinoma	0/1
Pancreas adenocarcinoma	0/1
Liver	0/3
Prostate adenocarcinoma	0/3
Kidney	0/2
Bladder	0/1
Thyroid	
Papillary carcinoma	0/3
Follicular carcinoma	0/2
Medullary carcinoma	0/1
Adrenal cortical	0/2
Neuro endocrine tumors of various types	0/10
Others	
Angiosarcoma	0/1
Clear cell sarcoma	**6/6**
Desmoplastic small cell tumour	0/1
Ewing sarcoma	0/1
Fibrous histiocytoma (cellular)	0/1
Ganglioneuroblastoma	0/1
Ganglioneuroma	0/1
Gastrointestinal stromal tumour	0/1
Glioblastoma	0/5
Granular cell tumor	0/2
Keratoacanthoma	0/1
Leiomyoma	0/2
Leiomyosarcoma	0/1
Lipoma (spindle cell type)	0/1
Liposarcoma	0/1
Lymphangioleimyomatosis	**3/3**
Malignant peripheral nerve sheath tumor	0/2
Nephroblastoma	0/4
Neuroblastoma	0/1
Pheochromocytoma	0/3
Renal angiomyolipoma	**3/3**
Renal oncocytoma	0/1
Rhabdomyosarcoma	0/2
Schwannoma	**1/3[£]**
Synovialosarcoma	0/1
Thymoma	0/3
Yolk sac tumor	0/1
Total	13/208

[¤] Strong immunostaining restricted to non neoplastic melanocytes.
[£] The positive case was a melanotic schwannoma.
The immunostaining of the leukocytes was not taken into account.

RESULTS

PNL2 Hybridoma Selection

PNL2 supernatant strongly stained the melanocytes and the granulocytes of the tonsil sections on frozen sections but unexpectedly did not recognize the recombinant protein in ELISA test. Despite the negativity of the ELISA test, PNL2 was selected for further analysis because the immunostaining appeared to be of interest and persisted on paraffin sections after microwave heating based antigen retrieval.

Isotype characterization showed PNL2 to belong to an IgG1 class but no light chain was detected. Furthermore, PNL2 supernatant resolution through 10% SDS-PAGE showed a 54-kDa band corresponding to the IgG1 heavy chain, but no light chain band was detected (Fig. 1). HHF35, an IgG1κ antibody used as positive control, showed as expected two bands of 54 kDa and 30 kDa respectively, corresponding to heavy and light chains.

Immunoreactivity of PNL2 on Paraffin-Embedded Tissue Sections

PNL2 immunostainings of melanomas were strong with no background staining. When melanin pigment was bleached by $KMnO_4$/sodium bisulfite treatment (the slides were immersed in 0.25% $KMnO_4$ for 2 min, washed and immersed in 2% sodium bisulfite for 2 min), or when a nitric acid-based decalcification was performed, the PNL2 staining was not modified, whereas anti–Melan A and HMB-45 stainings were abolished.

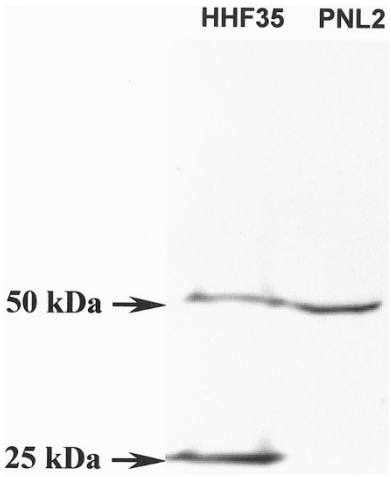

FIGURE 1. SDS-PAGE analysis of PNL2 structure: resolution of PNL2 supernatant and an IgG1 κ Mab (HHF35) through 10% SDS-PAGE. *Lane 1:* HHF35 antibody is used as positive control: two bands are detected at 54 kDa and 30 kDa, corresponding respectively to the IgG1 heavy chain and the κ light chain. *Lane 2:* PNL2 gives only one band at 54 kDa corresponding to the IgG1 heavy chain but no light chain was detected.

Human Normal Tissues

Results of PNL2 immunostainings on human non-neoplastic tissues are summarized in Table 1. PNL2 consistently gave a strong cytoplasmic staining of melanocytes on skin and oral mucosa sections and a moderate staining of granulocytes (Fig. 2). This staining was granular and cytoplasmic and highlighted dendritic prolongements of melanocytes. Except for melanocytes and granulocytes, no other cell was immunostained in the wide variety of normal human tissues tested. When the PNL2 supernatant was diluted, the melanocyte staining remained strong until high dilution were used (*i.e.*, 1/50), whereas leukocyte staining disappeared at low supernatant dilution (*i.e.*, 1/10).

Human Melanocytic Tumors

The staining of PNL2, anti–Melan A, and HMB-45 antibodies on paraffin embedded melanocytic tumors is detailed in Table 2. PNL2 immunostainings were very similar to those obtained with HMB-45. They were always strong with no background staining. About 90 to 100% of the melanocytes were labeled in all benign intraepidermal lesions, irrespective of their histologic type. As observed with HMB-45, common intradermal nevi and dermal component of compound nevi were largely nonreactive and only scattered nevus cells in the papillary dermis were labeled with PNL2 (Fig. 3), whereas anti–Melan A stained all melanocytes of the benign melanocytic lesions. However, spindle-shaped intradermal nevi such as blue nevus and desmoplastic Spitz nevus were consistently and strongly labeled by PNL2.

The PNL2 staining of nodal capsular nevi was weak whereas the anti–Melan A staining was strong, and HMB-45 staining was either negative or very weak.

PNL2 gave a strong immunostaining of 70 to 100% of the melanocytes in all the non desmoplastic primary melanomas, irrespective of their histologic type (Figure 4). However, PNL2 did not label desmoplastic melanomas. All metastatic melanomas were positively stained by PNL2. The percentage of labeled neoplastic cells varied from case to case from 10 to 100% (mean, 45%). However, the staining was always strong, dramatically different from the weak staining of the nodal capsular nevi. The lower percentages of stained cells were most often observed in hypomelanotic lesions. A discrepancy between PNL2, HMB-45, and anti–Melan A staining was observed in two cases: PNL2 gave a strong staining of 10% of the tumors cells whereas, anti–Melan A and HMB-45 were negative.

Human Non-Melanocytic Tumors

The staining of PNL2, anti–Melan A, and HMB-45 antibodies on paraffin embedded non melanocytic tumors is detailed in Table 3. PNL2 and anti–Melan A gave a strong immunostaining of most of the neoplastic cells in clear cell sarcomas, of a few cells in angiomyolipomas (Figure 5) and of spindle cells in lymphangioleiomyomatosis. By contrast, HMB-45 immunostaining was strong in clear cell sarcomas but weak in angiomyolipomas and weak or negative in lymphangioleiomyomatosis. One case of melanotic schwannoma was strongly labeled by the three antibodies. None of the other tumors tested were found to be positive for PNL2.

Mouse Normal Tissues

PNL2 strongly stained numerous melanocytes in the hair bulb of the C57/Bl6 and nude mice whereas the staining was moderate in Balb/c mice. Furthermore, it gave a moderate immunostaining of the

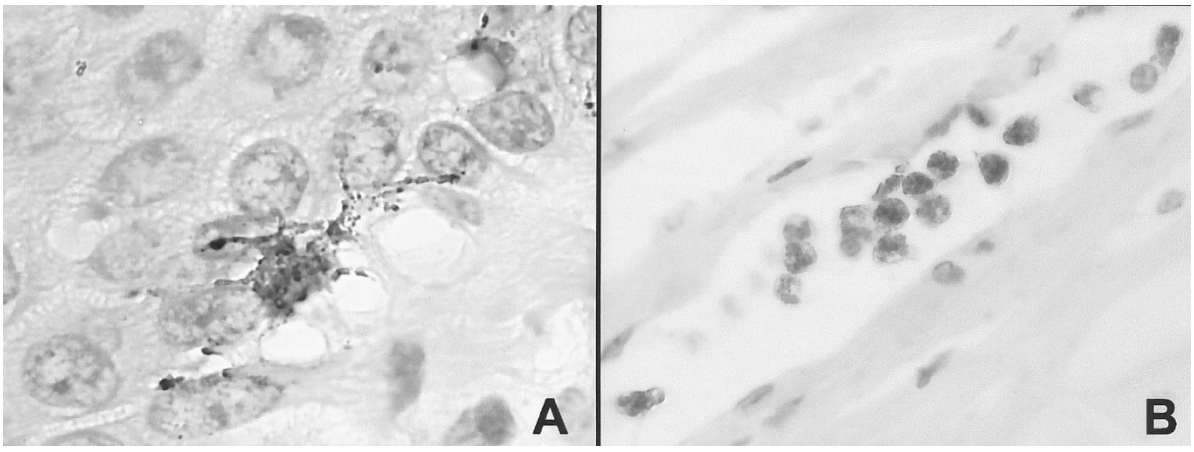

FIGURE 2. PNL2 immunostaining of normal tissues is limited to melanocytes (**A**) and leukocytes (**B**): the staining of the skin melanocytes (**A**) is cytoplasmic and dendritic in a granular pattern. It remained strong up to a supernatant dilution of 1/50. The staining of leukocytes (**B**) is also cytoplasmic in a granular pattern but it rapidly disappears at low supernatant dilution (*i.e.,* 1/10).

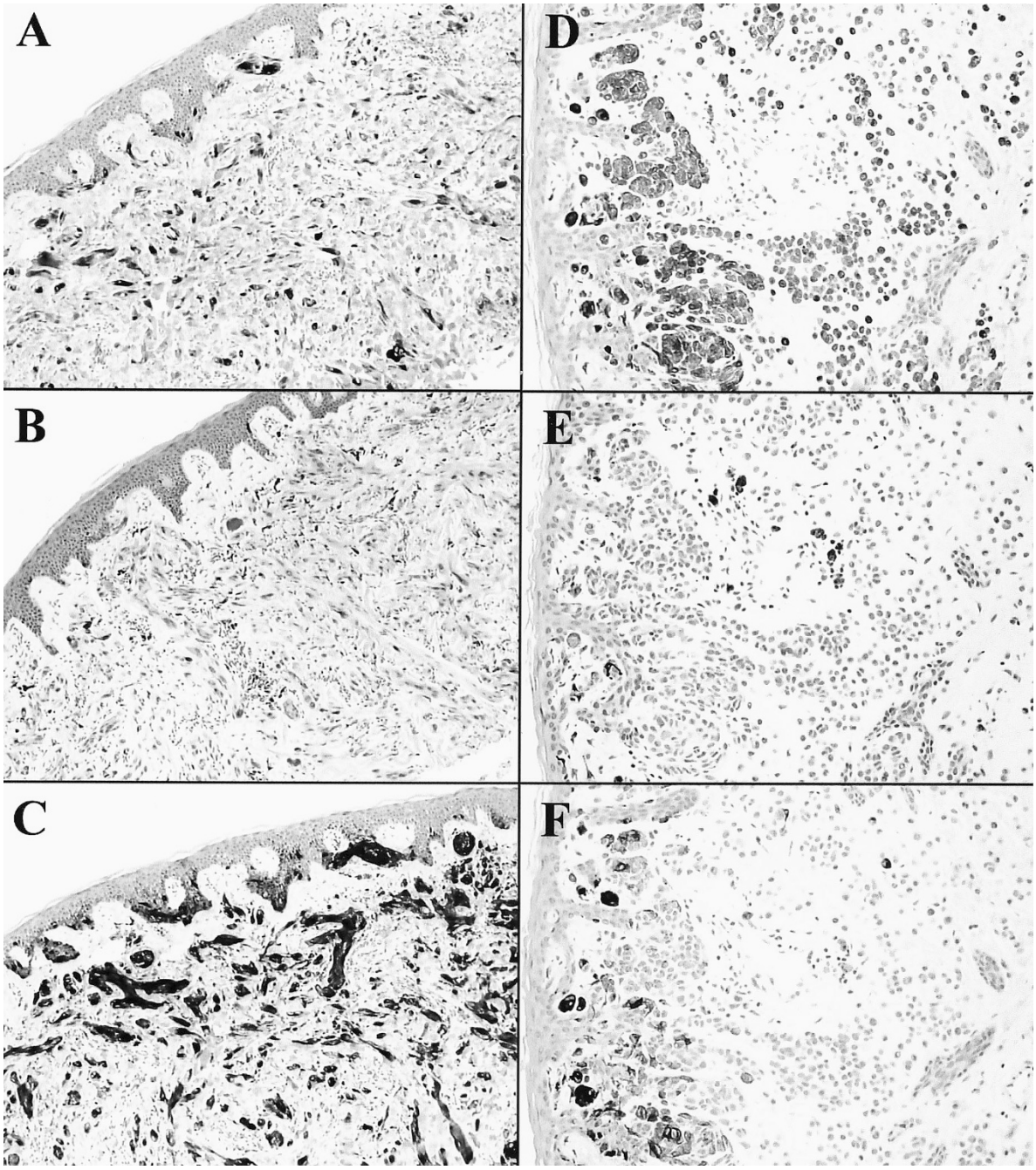

FIGURE 3. PNL2 immunostaining of benign melanocytic lesions, comparison with anti–Melan A and HMB-45 immunostaining. *Left side:* adjacent sections from a Spitz nevus. Whereas Melan A (**A**) and HMB-45 (**B**) stainings are heterogeneous, PNL2 (**C**) strongly stains all the cells of the nevus. *Right side*: adjacent sections from a compound nevus. Melan A is expressed by all the nevi cells (**D**), whereas the intradermal component of this nevus is largely non painted by HMB-45 (**E**), and PNL2 (**F**), with only scattered nevus cells labeled in the papillary dermis.

granular cell layer of the cornified esophagus epithelium. Such a staining was observed in all mice studied.

Immunostaining after Enzymatic Treatment of Tissue Sections

The neuraminidase digestion of sections from lymph node metastatic melanoma before immunostaining had no influence on the PNL2 reactivity but abolished, as expected, CNA.42 staining, suggesting that PNL2 did not recognize a glycosylated epitope.

Immunoblotting Analysis

PNL2 produced three bands with an apparent molecular weight of 160 kDa, 100 kDa, and less than 10 kDa in CAL melanoma cell line, whereas it produced three bands with an apparent molecular weight of 80 kDa, 70 kDa, and less than 10 kDa in B16F10 cell line.

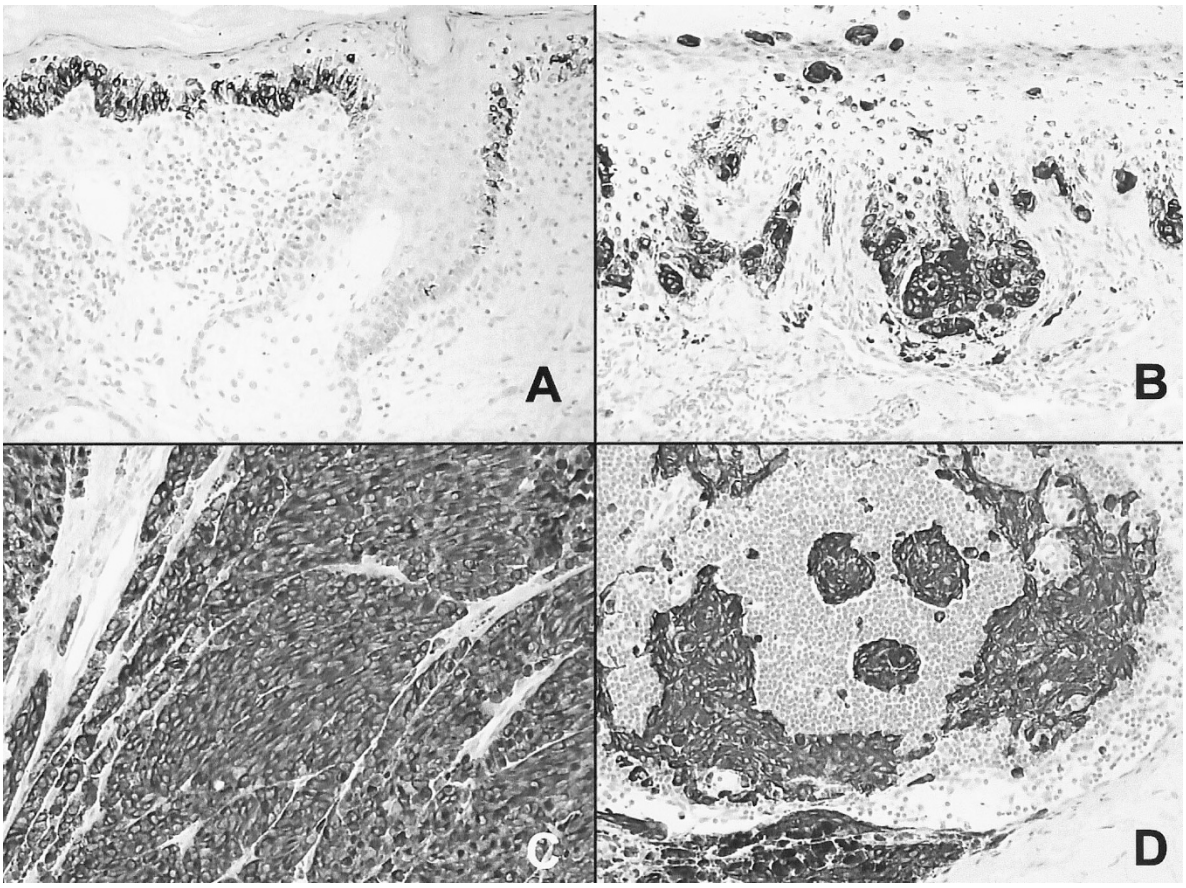

FIGURE 4. PNL2 immunostaining of malignant melanocytic lesions: strong immunostaining of the melanocytes in a lentigo maligna melanoma (**A**), an acral lentiginous melanoma (**B**), a nodular melanoma (**C**), and an achromic melanoma metastasis in a lymph node (**D**).

A protein with an apparent molecular weight of less than 10 kDa was also detected with the PNL2 Mab in leukocyte protein extracts. No band was observed in P3 × 63 Ag8–653 myeloma cell line used as negative control.

Protein Identification by Proteomic Analysis and Mass Spectrometry

In human melanoma, identification of the immunoaffinity purified PNL2-binding complexes showed that the 160 kDa protein was the clathrin 17 and that the 100 kDa protein was the vesicular docking protein TAP1. In the B16F10 cell line, the 70 kDa protein was identified as an albumin precursor. The identification of the low molecular weight molecule was not possible in both human and murin melanomas.

DISCUSSION

PNL2 is a new and interesting anti-melanocyte antibody suitable for use on paraffin sections. On human samples, PNL2 stains only melanocytic lesions, clear cell sarcoma, melanotic schwannoma, angiomyolipoma, and lymphangioleiomyomatosis. Overall, PNL2 has the same specificity that anti-Melan A and HMB-45 antibodies. However, we noted that PNL2 staining is stronger and more consistent than that of HMB-45 antibody. Indeed, occasional melanomas (two in the present study) negative for HMB-45 were found to be strongly positive for PNL2. Staining of non-melanocytic lesions such as lymphangioleiomyomatosis or angiomyolipomas is also stronger than with HMB-45 antibody which often shows a weak and focal staining. In addition, PNL2 staining is not altered by KMnO4/sodium bisulfite melanin bleaching or by a nitric acid-based decalcification. These points are of interest in lesions rich in highly pigmented macrophages or for the immunodetection of bone metastasis. The staining of leukocytes does not cause a diagnostic problem and constitutes a good intrinsic positive control of the immunostaining quality.

The exact nature of the antigen recognized by PNL2 remains to be defined. Even if the staining obtained with PNL2 is very similar to that obtained with HMB-45, the protein recognized is not the gp100. Mass spectrometry analysis identified the clathrin 17 and TAP1 in the PNL2-binding complexes purified by immunoaffinity. However, these

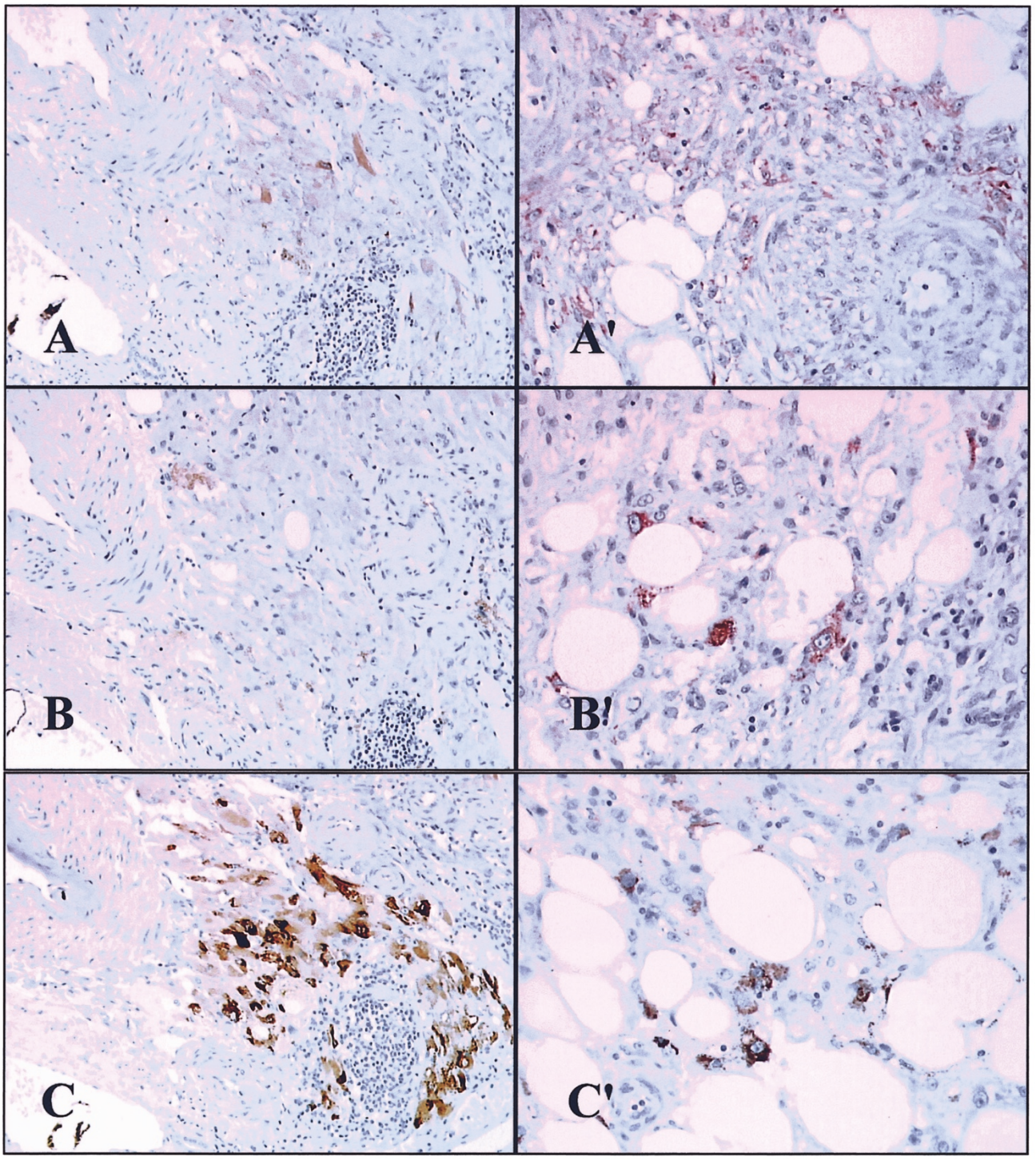

FIGURE 5. PNL2 immunostaining of angiomyolipoma, comparison with anti–Melan A and HMB-45 immunostainings. Adjacent sections from an angiomyolipoma. HMB-45 (**A** and **A'**), anti–Melan A (**B** and **B'**), and PNL2 (**C** and **C'**) stain the same tumor cells, but PNL2 staining is stronger and more intense.

proteins are probably not the antigens recognized by PNL2. Indeed the clathrin 17 is present in most cells and TAP1 has been reported in numerous cell types, not labeled by PNL2, such as keratinocytes, lymphocytes, breast epithelial cells and trophoblastic cells (37–41). One can hypothesize that the 100-kDa protein immunoprecipitated by PNL2 is not TAP1 but a melanocytic-related TAP variant because a TAP1 variant has been recently described in rabbit retina, a melanin rich organ (Database references EMBL BC016069; swissprot Q91WE7). It is also possible that the antigen recognized by PNL2 has the same molecular weight that TAP1 or the Clathrin 17 and is masked by these abundant pro-

teins. Indeed, the proteomic analysis was performed on 1 D gel electrophoresis, and it is well known that more than one protein can be present in a single band with this resolution technique. Finally, we cannot exclude that the very low molecular weight molecule detected by immunoblotting is not a degradation product but the antigen recognized by PNL2. Further studies are needed to identify this low molecular weight product.

The lack of reactivity of PNL2 antibody with the SST2 recombinant protein used as immunogen suggests that splenocytes secreting such an antibody were not immunized B cells, but B cells secreting an auto-antibody directed against a murine associated-melanocyte antigen. The immunostaining obtained in mouse skin supports this hypothesis because PNL2 recognizes Balb/c mouse melanocytes. The negativity of ELISA analysis but the detection of PNL2 antibody in the supernatants emphasizes the value of immunohistochemistry as a primary screening method for hybridoma supernatants (42) because it allows the detection of unexpected but interesting antibodies.

In conclusion, we report here the production of a new monoclonal antibody, PNL2, directed against a new fixative-resistant melanocyte-associated antigen. This antigen which is expressed by human and mouse melanocytes remains to be characterized but we demonstrate that it is different from those recognized by anti–Melan A and HMB-45.

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