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A Monoclonal Antibody Specific for Cells of the Melanocyte Lineage

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A monoclonal antibody, NKI/beteb, was prepared against membranes from a human melanoma metastasis, and in immunoprecipitates of melanoma cell lysates specific 100- and 7-kd glycoproteins were found. The large glycoproteins were also present in conditioned medium of melanoma cell lines. The antigen is located on the inner side of membranes of (pre)melanosomes and premelanosomelike vesicles. The antibody reacted in the immunoperoxidase test on frozen tissue sections with 27 of 28 nevocellular nevi (15/16 common, 12/12 dysplastic), 39/39 primary melanomas (3 intraepidermal, 24 cutaneous, 12 choroidal), 56/63 melanoma metastases, and 4/4 clear-cell sarcomas (melanoma of soft tissue). With sections of formalin-fixed paraffin-embedded tissues, the reaction was less sensitive. No reactivity was detected with frozen sections of 185 other tumors, except for 1 case of non-Hodgkin's lymphoma in which macrophages were positive. With the exception of melanocytes, all frozen sections of adult tissues that were tested were negative with NKI/beteb. On the basis of its tissue distribution so far, the antigen recognized by NKI/beteb seems to be a specific and sensitive diagnostic marker for cells of the melanocyte lineage. (Am J Pathol 1988, 130:179–192)

THE DIAGNOSIS of poorly differentiated malignant tumors can be improved considerably by the application of monoclonal antibodies.[1] Recently, monoclonal antibodies have been described that recognize preferably tumors from distinct origins, such as colon cancer,[2] lung cancer,[3] pancreatic adenocarcinoma,[4] renal cancer,[5] urinary bladder carcinoma,[6] ovarian tumors,[7] neuroblastoma,[8] and melanoma.[9,10] Unfortunately, virtually none of the antibodies produced has been proven to be absolutely specific for a certain type of tumor; but in practice, this problem can be overcome by using panels of monoclonal antibodies.[11,12] For the differential diagnosis of poorly differentiated malignant tumors, a first panel has been suggested consisting of monoclonal antibodies directed against cytokeratin, an epithelial membrane antigen (EMA), a common leukocyte antigen, protein S-100, and the melanoma-associated antigen recognized by NKI/C-3.[13] These antibodies can all be used on formalin-fixed paraffin-embedded tissue sections. Other authors include in their diagnostic test monoclonal antibodies directed against vimentin and other intermediate filament proteins, followed, when frozen tissue sections are available, by a second screening with a panel of other monoclonal antibodies directed against different melanoma-associated antigens.[14] Monoclonal antibodies directed against a high-molecular-weight melanoma-associated chondroitin sulfate proteoglycan have shown a very restricted tissue distribution,[9] but they are by no means specific for melanoma.[15] A monoclonal antibody, HMB-45, has recently been described that shows high specificity for melanocytic tumors.[16] We have prepared a new monoclonal antibody named NKI/beteb with a same degree of specificity, and this antibody appears to be directed against a different epitope on the antigen recognized by HMB-45. In this paper, we describe the reactivity of antibody NKI/beteb with freshly frozen

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tissue sections of normal tissues, melanocytic lesions, and other tumors, as well as the biochemical characterization and ultrastructural localization of the antigen recognized by the antibody.

Materials and Methods

Cell Culture and Tissues

Cell lines were cultured as described previously.[17] Blood and buffy-coat cells were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Tissues were obtained directly after operation and snap-frozen or fixed immediately. Frozen tissues were stored in liquid nitrogen.

Antibodies

Monoclonal antibody NKI/beteb was prepared from the same fusion and by the same selection procedure as described for NKI/black-13.[18] A BALB/c mouse was immunized by an intraperitoneal and a subcutaneous injection on Day 0 with 0.1 mg protein from a smooth membrane fraction of a fresh melanotic melanoma lymph node metastasis prepared as described earlier[19] and which had been emulsified in complete Freund's adjuvant. Booster injections of 0.1 mg protein were given on Day 7 intraperitoneally (emulsified in incomplete Freund's adjuvant), Day 14 subcutaneously and Days 74, 75, and 76 intravenously. The spleen cells of the mouse were fused 2 days later with the mouse myeloma cell line Sp 2/0.[20] The hybridoma supernatants were screened successively in enzyme immunoassays[19] for absence of reactivity with Nonidet P-40 (NP-40) lysates of ghosts of AB-positive erythrocytes and NP-40 lysates of membranes of buffy-coat cells (pool of 20 donors), as well as for a positive reaction with NP-40 lysates of membranes of melanoma tissue (for preparation of the lysates see Vennegoor et al[19]). The hybridoma cultures were further selected by the immunoperoxidase test based on a positive reaction of their supernatants with melanoma cells in both frozen and formalin-fixed tissue sections, and negative reactions with the blood and control cells in the melanoma sections. After two additional cloning procedures (1 cell/well), stable production of monoclonal antibody was obtained. The antibody was of the IgG2b isotype as determined in an Ouchterlony immunodiffusion test with antisera directed against different mouse immunoglobulin subclasses (IgM, IgG1, IgG2a, IgG2b, and IgG3; Litton Bionetics, Kensington, Md).

The monoclonal antibodies HMB-45,[16] HMSA-2,[21] and 8.2[22], all of the mouse IgG1 isotype, have already been described. One sample of HMB-45 was a gift from Dr. A. M. Gown, Department of Pathology, University of Washington, Seattle, Washington; and a second sample was obtained from Enzo Biochem, Inc., New York, New York. Antibody HMSA-2 was a gift from Dr. K. Jimbow, Department of Dermatology, Sapporo Medical College, Sapporo, Japan. Antibody 8.2 was purchased from Hybritech, Inc., San Diego, California. Rabbit antiserum 66924 (R anti-C3) was prepared with monoclonal antibody NKI/C-3-purified antigen.[19,23] Rabbit antiserum against cathepsin D[24] was a gift from Dr. M. V. Johnsson, Laboratory of Biochemistry, University of Amsterdam, Amsterdam, the Netherlands.

Antigen Detection

For formalin-fixed paraffin-embedded tissue sections and cryostat sections, the indirect immunoperoxidase procedure was used as described previously.[25] Cryostat sections were fixed in acetone for 10 minutes at room temperature before preincubation with bovine serum albumin (BSA). For formalin-fixed tissue, diaminobenzidine (0.05 mg/ml) was used as substrate for peroxidase, whereas on frozen tissues the substrate 3-amino-9-ethylcarbazole was used.

For immunoelectron microscopy, fresh patient material consisting of a melanotic metastasis was fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde. Cryosectioning and immunolabeling were carried out as described previously.[19] Frozen thin sections were incubated with NKI/beteb and 5 nm gold-labeled goat anti-mouse IgG (GAM/Au5). For the simultaneous localization of cathepsin D and the antigen recognized by NKI/beteb, cryosections of MeWo cells were then incubated with rabbit anti-cathepsin D, 10 nm protein A–gold (PA/Au10), NKI/beteb, and GAM/Au5. To control for a possible interference, the incubations were also done in the reverse order, ie, NKI/beteb, GAM/Au5, protein A (0.05 mg/ml), anti-cathepsin D, and PA/AU10.

For the simultaneous localization of tyrosinase activity and the antigen recognized by NKI/beteb, MeWo cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 15 minutes at 4 C. The cells were washed several times in this buffer and incubated overnight at 4 C with L-DOPA (3,4-dihydroxyphenylalanine, 1 mg/ml) in the same buffer, followed by incubation at 37 C for 1 hour in fresh DOPA solution. DOPA is an intermediate compound in the synthesis of melanin from tyrosine. Tyrosinase oxidizes this substrate, giving a dark reaction product consisting of melanin

or an immediate precursor. The control was incubated without substrate. Frozen thin sections were incubated with NKI/beteb and GAM/Au5.

For the simultaneous localization of the antigens recognized by NKI/beteb and NKI/C-3, cryosections of MeWo cells were incubated sequentially with a rabbit antiserum directed against the antigen recognized by NKI/C-3 (R anti-C3), PA/Au10, NKI/beteb, and GAM/Au5. To control for a possible interference, we also did the incubations in reverse order.

For immunofluorescence cells were cultured on a coverslip in 6-well tissue culture plates (3506 Costar, Cambridge, Mass). The cells were washed in phosphate-buffered salt solution containing 0.8 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-B). When fixed cells were used, the coverslips were incubated in acetone for 10 minutes, air-dried, and washed in PBS-B. The cells were incubated for 1 hour at 4 C with monoclonal antibody (hybridoma supernatant diluted 1:1 in MEM-Hanks' + 0.5% BSA), washed in ice-cold PBS-B, incubated for 1 hour at 4 C with FITC-conjugated goat anti-mouse immunoglobulin (502401 Tago, Burlingame, Calif; dilution 1/80 in MEM-Hanks' + 0.5% BSA), washed in ice-cold PBS-B, and fixed for 10 minutes in methanol. Acetone-fixed cells were counterstained for 5 minutes with 0.006% Evans blue and washed in distilled water. The cells were embedded in 0.075 M NaCl containing 5 mM Tris-HCl, pH 8.6, 1% (wt/vol) 1,4-diazabicyclo(2.2.2)octane, and 50% (vol/vol) glycerol.

For enzyme immunoassays (EIA), polyvinylchloride EIA plates (6675, Costar Europe Ltd., Badhoevedorp, the Netherlands) were used. Unless indicated otherwise, the incubations were 1 hour at room temperature. Cell lysates were prepared by incubating 75 sq cm confluent monolayers of cultured cells that had been washed in PBS at 4 C with 1 ml 1% NP-40, 0.5% sodium deoxycolate (DOC), 0.1% sodium dodecyl sulfate (SDS), 0.01 M triethanolamine-HCl buffer, pH 7.8, 0.15 M sodium chloride, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.02 mg/ml ovomucoid trypsin inhibitor (TI). They were then centrifuged for 15 minutes at 15,000 *g* for removal of nuclear debris. Antigen was coated in the EIA plates by overnight incubation at 4 C with 50 μl of 1/1000 in PBS-diluted lysate of Mel-57 cells per well. The test plates were coated with HPLC-purified NKI/beteb at a concentration of 10 μg/ml as described previously.[18] The plates were washed, incubated with 2% BSA in PBS containing 0.1% sodium azide, and stored at 4 C. To determine whether the different monoclonal antibodies recognize the same epitope as NKI/beteb, we used an additive EIA[26] as well as a test of inhibition of binding of biotinylated NKI/beteb to antigen. For the additive EIA, antigen-coated test plates were incubated with saturating concentrations (10 μg/ml) of various monoclonal antibodies in the presence or absence of NKI/beteb (10 μg/ml). They were then incubated with peroxidase-labeled goat anti-mouse IgG (Tago, Inc., Burlingame, Calif; dilution 1/1000), and 3,3'5,5'-tetramethylbenzidine was added as substrate. The reaction product was measured at 450 nm in a Titertek multiscan spectrophotometer.[18] The inhibition test was similar. Mixtures of different concentrations of inhibiting monoclonal antibody and 6 μg/ml NKI/beteb prepared as described previously[18] were used in the first incubation step. Avidin and biotinylated peroxidase (Vector Laboratories, Burlingame, Calif) were used in the second incubation step. Recognition of a different epitope on the antigen detected by NKI/beteb (IgG2b isotype) was demonstrated in a double-determinant enzyme immunoassay (DDEIA) with EIA plates coated with NKI/beteb. After incubation with lysate of Mel-57 or T24 cells, the plates were incubated with different monoclonal antibodies of the IgG1 isotype (20 μg/ml) and then with peroxidase-labeled goat anti-mouse IgG1 (dilution 1/1000; Nordic, Tilburg, the Netherlands). The subsequent incubation with substrate and measurement of the reaction product were as described above.

Biochemical Characterization of the Antigen

For immunoprecipitation, subconfluent monolayers of melanoma or bladder carcinoma cells were radiolabeled for 20 hours at 37 C with 0.1 mCi/ml L-^{35}S-methionine (specific activity 1000 Ci/mmol; Amersham International, Amersham, England) in Eagle's minimal essential medium containing 10% of the usual concentration of methionine and 10% newborn calf serum dialyzed against PBS. Labeling with L-4,5-^{3}H-leucine (specific activity 155 Ci/mmol; Amersham International, Amersham, England) was performed similarly. For labeling with glucosamine, RPMI-1640 (RPMI-Selectamine kit; Gibco, Paisley, Scotland) was used containing 20% (0.2 g/l) of the usual glucose concentration, 10% newborn calf serum dialyzed against PBS and 0.2 mCi/ml N-acetyl-D 1-^{3}H-glucosamine (specific activity 2.84 Ci/mmol; Amersham International, Amersham, England). Cells were lysed as described above for the EIA, except that SDS was usually omitted. Conditioned tissue culture medium of the labeled cells was adjusted to 1% NP-40, 0.5% DOC, 0.01 M triethanolamine buffer, pH 7.8, 1 mM PMSF, and 0.02% TI. Immunoprecipitation with monoclonal antibody covalently coupled to protein A Sepharose beads was performed

as described.[27] SDS 0.1% was present during the first three washings of the immunoprecipitation beads. After SDS-polyacrylamide gel electrophoresis (PAGE)[28] on a linear gradient of 5–17% acrylamide, the gel was processed with Enlightning (NEN Research Products, Boston, Mass). Kodak XAR-50 film was used for autoradiography at −70C. Prestained protein molecular weight standards (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md) were in the molecular ranges of 14–200 kd and 3–43 kd.

For Western blotting, SDS-PAGE was performed with lysates prepared from unlabeled cells as described above. Blotting to nitrocellulose membrane filters was at 30 V for 15 hours with forced cooling of the Tris/glycine/methanol buffer, resulting in more than 90% transfer of all prestained molecular weight standards.[29] The nitrocellulose sheet was washed and preincubated in 0.01 M sodium phosphate buffer, pH 7.0, 0.15 M NaCl, 0.25% gelatin, and 0.3% Tween-20. The antigen was detected after incubation for 3 hours at room temperature with 2×10^6 cpm/ml of ^{125}I-labeled NKI/beteb (0.96 mol ^{125}I/mol IgG, prepared with chloramine T and ion exchange chromatography[30]) by autoradiography at −70 C (using Kodak XAR-50 films and DuPont Cronex intensifying screens).

Results

Reactivity of NKI/beteb With Tissue Sections

A comparison of the reactions of monoclonal antibody NKI/beteb with freshly frozen and formalin-fixed paraffin-embedded tissue sections of melanoma is shown in Table 1. In general, the reaction of NKI/beteb with sections of formalin-fixed melanomas was inferior to that with frozen sections. As many as 60% of the tumors became completely negative with NKI/beteb after fixation in formalin. Compared with monoclonal antibody NKI/C-3 (which retains its reactivity with melanoma after formalin fixation[13,19,25]), only slightly fewer positive cells were found when NKI/beteb was applied on freshly frozen melanoma sections. If only a limited number of cells reacted with NKI/beteb, a spotted staining of dispersed positive cells was observed (Figure 1E). All freshly frozen primary melanomas were positive with NKI/beteb (Table 2). The antibody reacted with 100% of the melanotic and with about 60% of the amelanotic melanoma metastases (Table 2). In melanoma *in situ*, all tumor cells were positive. In primary cutaneous melanomas, both the junctional and subepidermal components were stained after incubation with NKI/beteb and frequently the deeper parts of the tumor as well (Figure 1C).

Table 1—Reactivity of Monoclonal Antibody NKI/beteb on Sections of Melanoma Metastases Compared With the Reactivity of Monoclonal Antibody NKI/C-3 in the Immunoperoxidase Test

	Staining results*		
	NKI/beteb		NKI/C-3
Tumor	Freshly frozen tissue	Formalin-fixed tissue	Formalin-fixed tissue
1	<10	0	>90
2	>90	0	50
3	50	0	>90
4	50	10	50
5	90	90	>90
6	50	10	>90
7	100	10	100
8	<1	<1	100
9	90	0	70
10	10	0	5
11	90	0	90
12	50	50	70
13	0	0	80
14	100	0	50
15	50	0	100

*Percentage of melanoma cells stained.

Monoclonal antibody NKI/beteb also reacted with nevocellular nevi. Common compound nevi were stained almost exclusively in the junctional region; in dysplastic nevi the junctional and also the subepidermal parts were stained (Figure 1A and B). Moreover, clear cell sarcomas were also recognized by NKI/beteb (Figure 2). This type of tumor has recently been defined as melanoma of soft tissue.[31] All other tumors tested were negative with NKI/beteb, as were the sections of freshly frozen normal tissues, except for adult and fetal skin melanocytes and intralesional macrophages in one case of non-Hodgkin lymphoma (Tables 2 and 3; Figure 3). No difference was found in reactivity of NKI/beteb with the melanocytes of black, compared with white, skin. On very rare occasions, a few melanophages and dendritic cells in primary melanomas stained with NKI/beteb.

Comparison of the Reactivity of NKI/beteb and HMB-45 on Tissue Sections

In a few frozen sections of melanoma metastases and normal skin, the reaction of NKI/beteb was compared with the reactivitv of HMB-45. In melanoma metastases, the distribution of HMB-45-positive cells was similar to that of NKI/beteb-positive cells. In contrast to the reactions of NKI/beteb in normal skin, however, a weak staining with HMB-45 was observed at the basement membrane between the epidermis and dermis, whereas melanocytes were generally negative. With HMB-45 some staining was found on de-

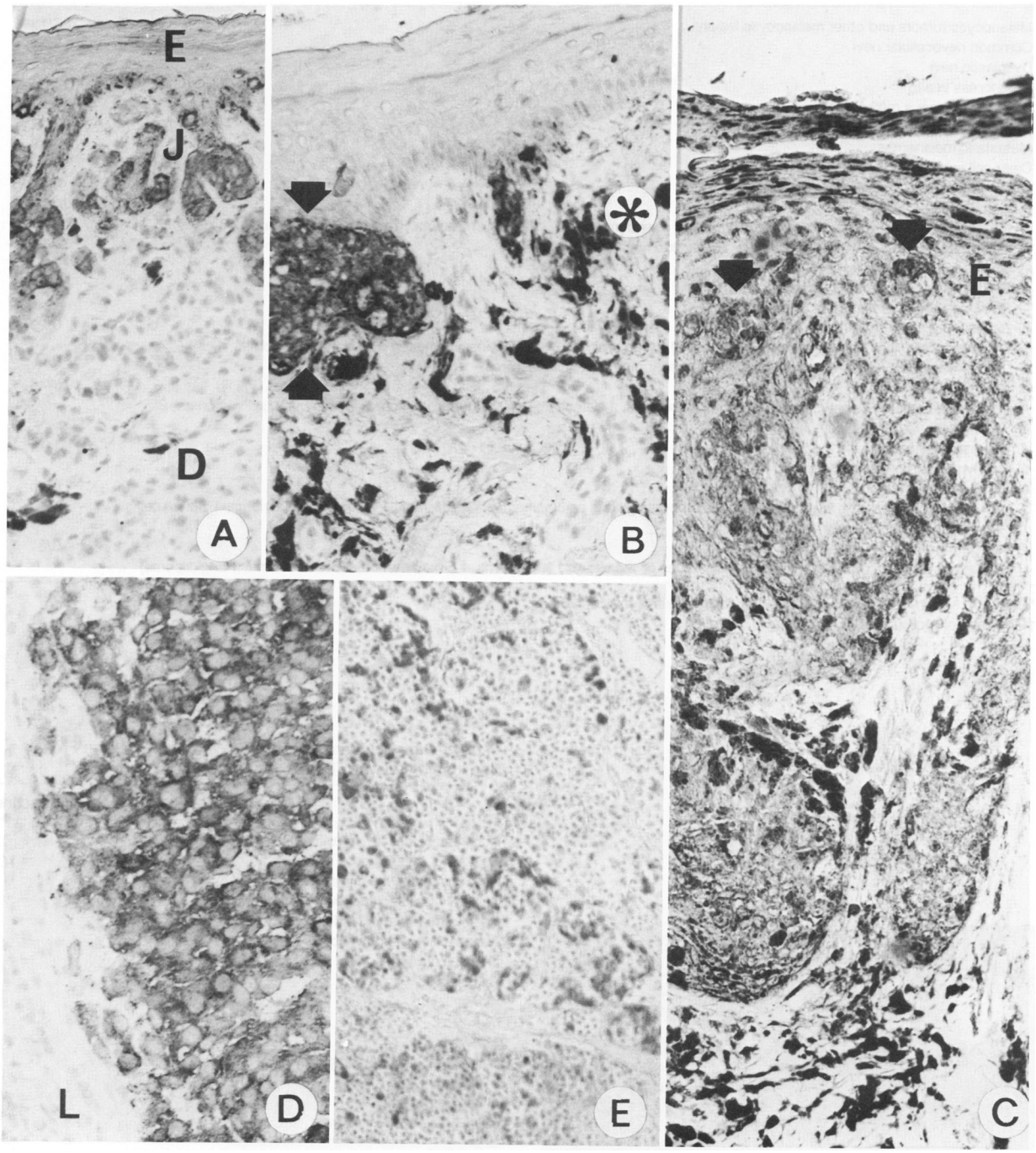

Figure 1—Reaction with NKI/beteb in the immunoperoxidase test of frozen tissue sections (**A–D**) and a formalin-fixed and paraffin-embedded tissue section (**E**). The tissues were counterstained with hematoxylin. **A**—Compound nevocellular nevus. Note that especially the junctional component (*J*) is stained. *E*, epidermis; *D*, dermal component. (×240) **B**—Dysplastic nevus showing staining of a junctional nest (*arrows*). The melanophages (*) in the adjacent dermis do not stain. (×400) **C**—Primary cutaneous melanoma. Note staining of the ascending intraepidermal (*arrows*) and subepidermal tumor cells. *E*, epidermis. (×600) **D** and **E**—Melanoma metastases in lymph nodes from different patients. **D**—Marked cytoplasmic staining of tumor cells. Adjacent lymphocytes (*L*) are negative. (×600) **E**—Several tumor cells are intensely stained; others are negative or weakly stained. (×64)

Table 2—Immunoperoxidase Test With NKI/beteb on Frozen Tumor Sections

Lesion tested	No. positive/ no. tested
1. Melanocytic tumors and other melanocytic lesions	Total: 126/134
Common nevocellular nevi	15/16
Dysplastic nevi	12/12
Melanomas in situ	3/3
Primary cutaneous melanomas	24/24
Choroideal melanomas	12/12
Metastatic melanomas	56/63
Clear-cell sarcomas	4/4
2. Other tumors	Total: 1/185*
a. Carcinomas	0/86
Adenocarcinoma (32 breast,† 7 ovary, 1 tube, 1 endometrium, 1 uterus, 1 rectum, 10 colon, 2 stomach, 2 kidney, 2 prostate, 2 lung, 6 thyroid, 1 adenocystic carcinoma)	
Squamous-cell carcinomas (4 lung, 7 others)	
Large-cell undifferentiated carcinomas (3)	
Small-cell undifferentiated carcinomas (4)	
b. Neuroendocrine tumors	0/20
(3 carcinoids, 3 medullary thyroid carcinomas, 1 parathyroid carcinoma, 3 insulinomas, 4 phaeochromocytomas, 1 chemodectoma, 3 neuroblastomas, 1 Merkel cell tumor, 1 ganglioneuroma)	
c. Sarcomas	0/45
(7 liposarcomas, 6 leiomyosarcomas, 2 fibrosarcomas, 1 osteosarcoma, 5 chondrosarcomas, 7 Schwann cell sarcomas, 8 malignant fibrous histiocytomas, 9 others)	
d. Malignant lymphomas	1/11*
(6 lymphocytic, 5 histiocytic)	
e. Other malignant tumors	0/15
(4 nephroblastomas, 1 testicular teratoma, 1 hepatocellular carcinoma, 5 glioblastomas, 1 embryonal carcinoma, 1 Sertoli cell tumor, 2 mesotheliomas)	
f. Benign tumors	0/8
(2 adenofibromas of the breast, 2 cystadenomas of the ovary, 1 leiomyoma, 1 Schwannoma, 1 neurofibroma, 1 angiolipoma)	

*One case of non-Hodgkin's lymphoma contained positive macrophages.
†One case of Morbus Paget of the nipple.

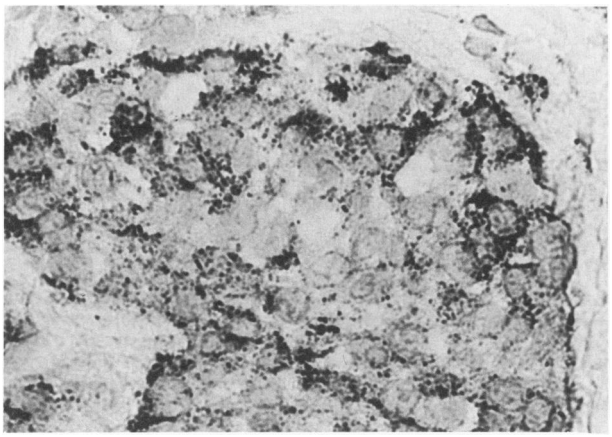

Figure 2—Reaction with NKI/beteb in the immunoperoxidase test on a frozen section of a clear-cell sarcoma (melanoma of soft tissues). Note the distinctly granular intracytoplasmic staining. The tissue was counterstained with hematoxylin. (×980)

Table 3—Immunoperoxidase Test With NKI/beteb on Frozen Sections of Normal Tissues

Tissue	Reaction
Adult and fetal skin melanocytes	+
Epidermis, hair follicle	–
Sebaceous glands, sweat glands	–
Salivary glands (submandibulary, parotis)	–
Resting mammary gland	–
Lung, alveolar and bronchial cells	–
Liver, all cell types	–
Gall bladder	–
Kidney	–
Pancreas	–
Adrenals	–
Gastrointestinal tract (rectum, colon, ileum, duodenum, stomach)	–
Ovary, tube, vagina	–
Testis, epididymis, prostate	–
Brain, gray matter of cortex, cerebellum	–
Hypophysis	–
Peripheral nerves	–
Parasympathetic ganglia	–
Muscle (smooth, striated and heart muscle)	–
Thymus	–
Spleen	–
Peripheral blood cells	–
Lymph nodes (including activated nodes)	–
Connective tissue	–
Blood and lymph vessels	–

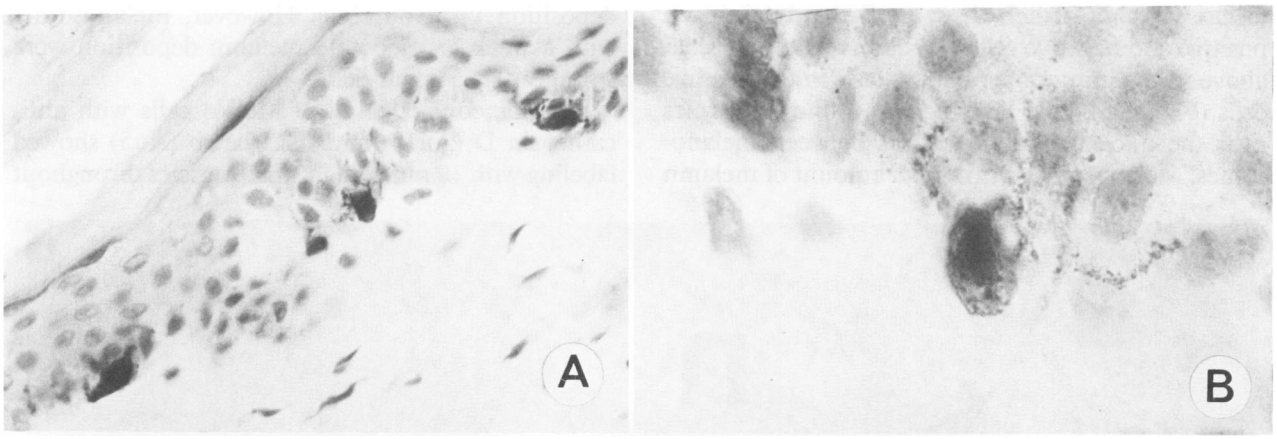

Figure 3—Reaction with NKI/beteb on a frozen section of normal skin of the breast. Only the melanocytes are stained with the antibody. The tissue was counterstained with hematoxylin. (**A**, ×400; **B**, ×1000)

generating tissue which was not observed with NKI/beteb.

Reactivity of NKI/beteb With Cultured Cells

In the immunofluorescence test, cultured cells showed primarily a cytoplasmic reaction with NKI/beteb (Table 4). About 50% of the melanoma cell lines reacted with this antibody, but none of the control cell lines was positive. The cytoplasmic fluorescence was located in small vesicles around the nucleus (Figure 4A). Sometimes a membrane fluorescence was also seen, usually very weak. Quite strong membrane fluorescence was observed occasionally (Figure 4B).

Table 4—Immunofluorescence With NKI/beteb

Cell Line	Immunofluorescence	
	Membrane	Cytoplasmic
Melanoma		
IGR-39	−	−
Mel-1	−	−
Mel-2a	+/±*	+++†
M-5	−	−
Mel-57	±‡	++
MeWo	−§	+++
SK-Mel-25	−	±‖
Bro	−	−
M14	−§	++
A375	−	−
Control		
BAT (normal adult skin fibroblasts)	−	−
D_3 (normal embryonal lung)	−	−
HCV-29 (normal bladder)	−	−
CCL-13 (normal liver)	ND	−
T_{24} (bladder carcinoma)	−	−

The symbols indicate the intensity of fluorescence (±, weak; +, positive; ++, moderately positive; +++, markedly positive reaction).
*Sixty-six percent of the cells were +, 33% −.
†Sixty-six percent of the cells were +++, 33% ± or −.
‡Five percent of the cells were ++, 95% ±.
§Five percent of the cells were ±, 95% −.
‖Less than 5% of the cells were ++, 95% ±.

Ultrastructural Localization of the Antigen Detected by NKI/beteb

In an immunoelectron microscopic study on ultrathin frozen sections of a melanotic melanoma me-

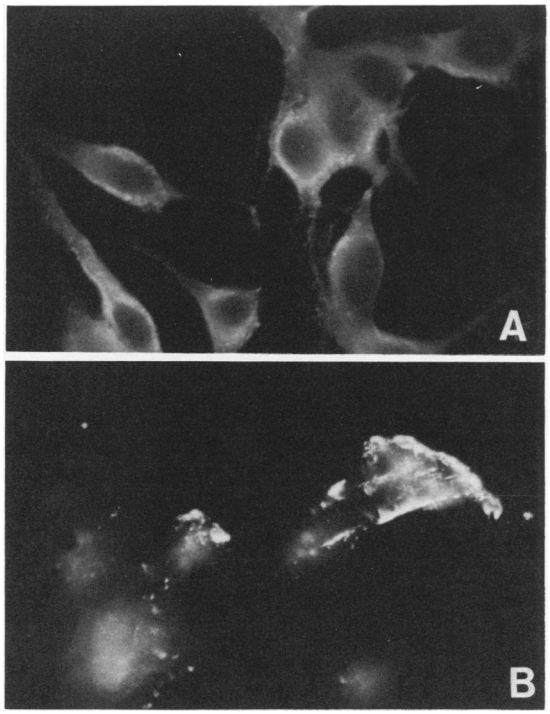

Figure 4—Immunofluorescence of melanoma cells (Mel-57) with NKI/beteb. **A**—Acetone-fixed cells. **B**—Living cells. (×590)

tastasis with NKI/beteb, the same diversed staining of positive and negative cells was observed as described above in the immunoperoxidase test. In the positive cells (Figure 5), gold labeling was found in vesicles near the nucleus and in vesicles between melanosomes. Melanosomes with a high amount of melanin deposition were negative. However, melanosomes with a weak or moderate melanin deposition were strongly or partly labeled.

Simultaneous labeling of MeWo cells with anti-cathepsin D (Au10) and NKI/beteb (Au5) showed labeling with 10 nm gold in small vesicles throughout

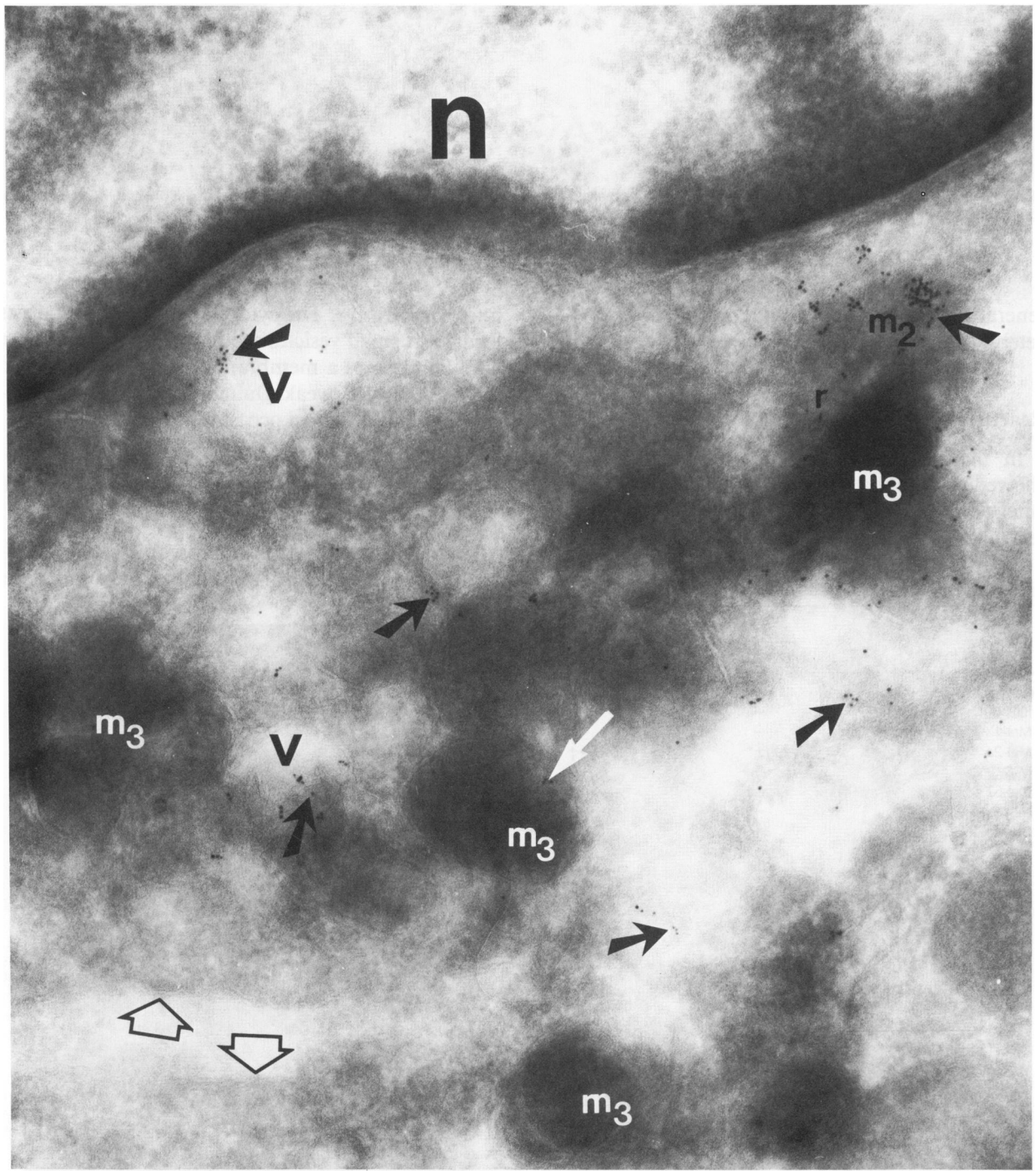

Figure 5—Frozen thin section from a melanotic melanoma metastasis after incubation with NKI/beteb and GAM/Au5. The labeling (*arrows*) is found in vesicles (*v*) near the nucleus (*n*) and between melanosomes (*m*). Melanosomes with a high amount of melanin deposition (*m3*) are negative or partly labeled (*white arrow*). Melanosomes with a weak or moderate melanin deposition (*m2*) are strongly or partly labeled. No labeling was seen on the plasma membrane (*open arrows*). The tissue section was counterstained with uranyl acetate. (×80,000)

the cytoplasm (Figure 6A), melanosomes with a large amount of melanin (Figure 6B), and lysosomes. Labeling with 5 nm gold was found in large vesicles and in premalanosomes with weak melanin deposition. Premelanosomes with the characteristic arrangement of the matrix were often strongly labeled (Figure 6A). Co-labeling was found only in some melanosomes (Figure 6B) and lysosomes; and in these cases, labeling with anti-cathepsin D was higher than with NKI/beteb. The same staining pattern was obtained with both labeling sequences described in Materials and Methods.

Tyrosinase activity in MeWo cells as judged by the DOPA reaction was present in vesicles in the Golgi region, in premelanosomes (Figure 6C), and in large lysosomes. The gold labeling revealing the antigen

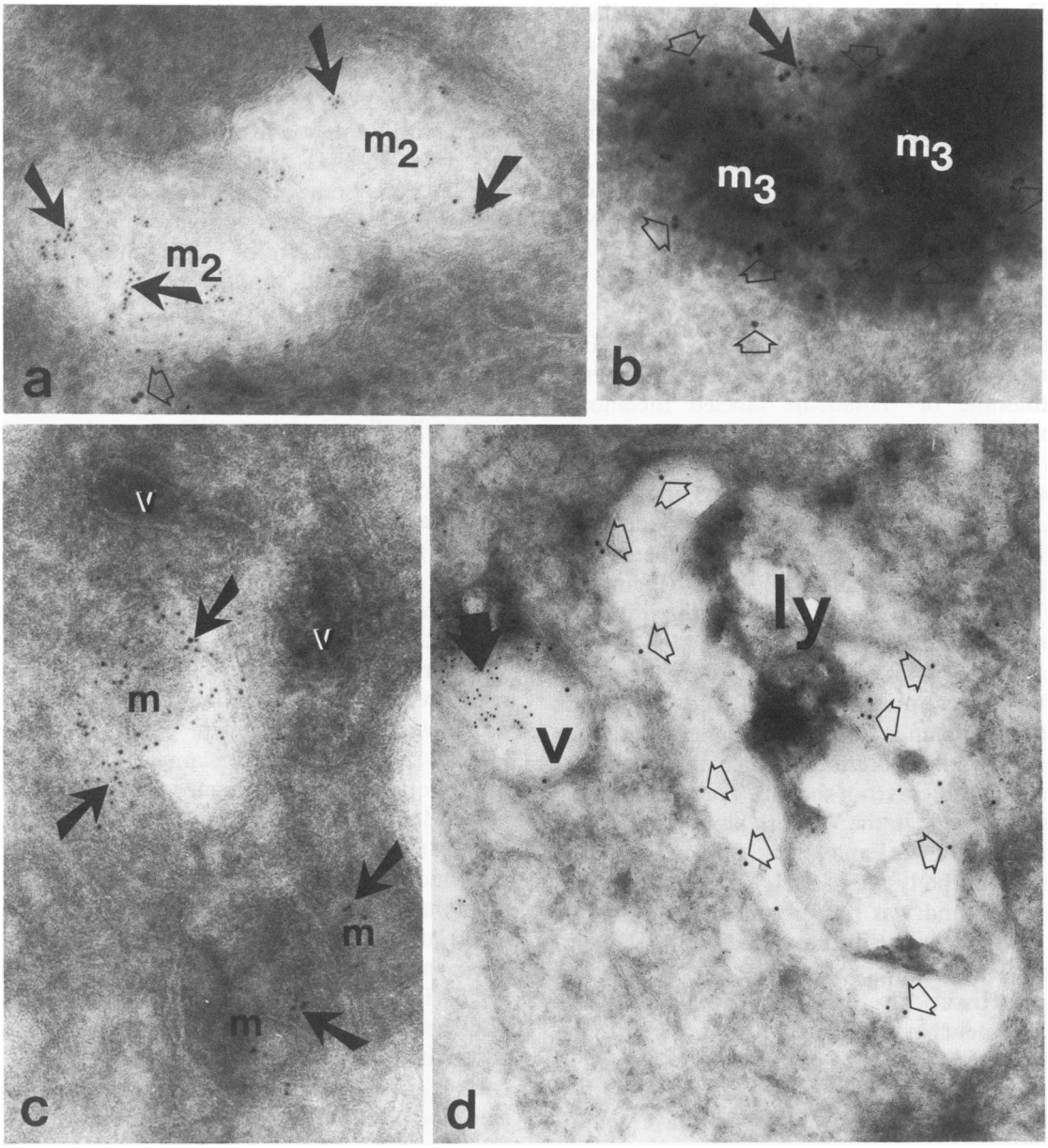

Figure 6A and **B**—Frozen thin sections from melanoma cell line MeWo were incubated with anti-cathepsin D and PA/Au10 (*open arrows*) and with NKI/beteb and GAM/Au5 (*closed arrows*). Small vesicles are labeled with anti-cathepsin D. Melanosomes with a weak melanin deposition (*m2*) are mainly labeled with NKI/beteb, whereas melanosomes with a high amount of melanin deposition (*m3*) are mainly labeled with anti-cathepsin D. **C**—MeWo cells were incubated with DOPA. Frozen thin sections were incubated with NKI/beteb and GAM/Au5. Tyrosinase activity is shown in vesicles (*v*) and in premelanosomes (*m*). Gold labeling (*arrows*) is found only in premelanosomes. **D**—Frozen thin section from MeWo cells incubated with rabbit anti-C3 and PA/Au10 (*open arrows*) and with NKI/beteb and GAM/Au5 (*closed arrow*). The smaller vesicle (*v*) is labeled with NKI/beteb. A lysosome (*ly*) that has phagocytosed a melanosome is labeled with anti-C3. All sections were counterstained with uranyl acetate. (**A** and **B**, ×95,000; **C**, ×100,000; **D**, ×64,000) Note that the size of 5 nm gold is not uniform; the distribution ranges from 3.2 to 6.4 nm.

reacting with NKI/beteb was in some premelanosomes associated with tyrosinase activity (Figure 6C). No codistribution was found in other kinds of vesicles. In the control without substrate, melanin granules were found only in melanosomes.

MeWo cells that had been double-stained with NKI/beteb (Au5) and R anti-C3 (Au10) showed strong labeling in vesicles that were located mainly around the nucleus. The stained vesicles contained either just antigen detected by NKI/beteb or both antigens. Double labeling was also found in premelanosomes, vesicles near the periphery of the cells, and lysosomes. Premelanosomes were strongly labeled with NKI/beteb and weakly with R anti-C3, whereas the peripheral vesicles and the lysosomes contained predominantly the antigen recognized by R anti-C3 (Figure 6D). No difference in the staining pattern was found with both labeling sequences described in Materials and Methods.

Characterization of the Antigen

On autoradiographs obtained after SDS-PAGE of immunoprecipitates of NKI/beteb and cell lysates of ^{35}S-methionine or ^{3}H-leucine labeled melanoma cells, specific peptide bands of about 7 kd were visible, both in the nonreduced state and after reduction with 2-mercaptoethanol (Figure 7A, Lanes 3 and 10, Figure 7B, Lanes 4, 6, 8, and 10). These peptides were absent in the immunoprecipitate of NKI/beteb with cell lysate of bladder carcinoma T24 cells (Figure 7B, Lane 2). In addition, in about 50% of the immunoprecipitations of MeWo, Mel-57, and Mel-2a cells, specific protein bands of about 100 kd (reduced and nonreduced) were present. The 100 and 7-kd bands were also present after labeling of the melanoma cells with ^{3}H-N-acetylglucosamine, which indicated that they are glycoproteins (not shown). These bands were NKI/beteb-specific in the Western blot of melanoma cell lysate (Figure 7C, Lane 1). In the latter Figure the 31-kd band in the lysate of Mel-57 cells is aspecific, because this band was not present in the lysate of MeWo cells, nor in the immunoprecipitates of melanoma cells. In immunoprecipitates of NKI/beteb and conditioned tissue culture medium of melanoma cells a specific protein band of about 110 kd was seen in the nonreduced state, and of about 90 kd after reduction with 2-mercaptoethanol (Figure 7A, Lanes 7 and 12). The 7-kd peptide band, however, was not present in the conditioned medium of melanoma cells.

Comparison of NKI/beteb With Other Monoclonal Antibodies

Several monoclonal antibodies against melanoma-associated antigens have been described that show characteristics resembling antibody NKI/beteb. We have compared these antibodies in immunoprecipitation experiments and in the EIA.

Antibody HMB-45 has been described as precipitating a 10-kd peptide from melanoma cell lysates.[32] In our hands, HMB-45 precipitated a 7-kd peptide with Mel-57 cells, as does NKI/beteb. However, the 100-kd protein precipitated with NKI/beteb was not observed in the immunoprecipitate of HMB-45, not even after prolonged autoradiography (reduced and nonreduced) (Figure 7A, Lane 2). From conditioned medium of melanoma cells, HMB-45 precipitated the same molecular weight protein bands as did NKI/beteb (Figure 7A, Lane 6). These observations, combined with the tissue distribution of the antigen detected by HMB-45 as described earlier,[16] suggested that both antibodies recognize the same antigen. In the additive EIA, an additivity index of 91% was found for the mixture of HMB-45 and NKI/beteb. HMB-45 inhibited binding of biotinylated NKI/beteb to antigen poorly (−2 and 27% inhibition at 9 and 36 μg/ml HMB-45, respectively, compared with 49% and 67% inhibition at the same concentrations of unlabeled NKI/beteb). These results indicate that the two antibodies recognize different epitopes. In the DDEIA with NKI/beteb as catcher antibody and HMB-45 as tracer antibody, an absorbance of 0.476 was measured with lysate of Mel-57 cells, compared with no reaction with lysate of T24 cells or other IgG1 catcher antibodies. Therefore, HMB-45 and NKI/beteb must recognize different epitopes on the same antigen.

Other antibodies with which NKI/beteb may be compared are those antibodies precipitating the melanoma-associated transferrin p97[33] and antibody HMSA-2 raised against melanosomal proteins.[21] In immunoprecipitates of lysates of metabolically labeled Mel-57 cells, antibody 8.2 precipitated a single protein (78 kd nonreduced, see Figure 7A, Lane 8; 88 kd reduced) which is different from the 100 kd protein precipitated by NKI/beteb. In conditioned medium of melanoma cells, the melanoma-associated transferrin showed the same molecular weight as in the cell lysate (Figure 7A, Lane 8). In the additive EIA with NKI/beteb, the antibodies 8.2 and HMSA-2 had an additivity index of 76% and 74%, respectively. Antibody 8.2 and HMSA-2 did not inhibit binding of biotinylated NKI/beteb to antigen, and the results in the DDEIA with NKI/beteb as catcher antibody and 8.2 or HMSA-2 as tracer antibody were negative.

Discussion

Monoclonal antibody NKI/beteb reacts only with melanocytes and melanocytic lesions. Reactions with

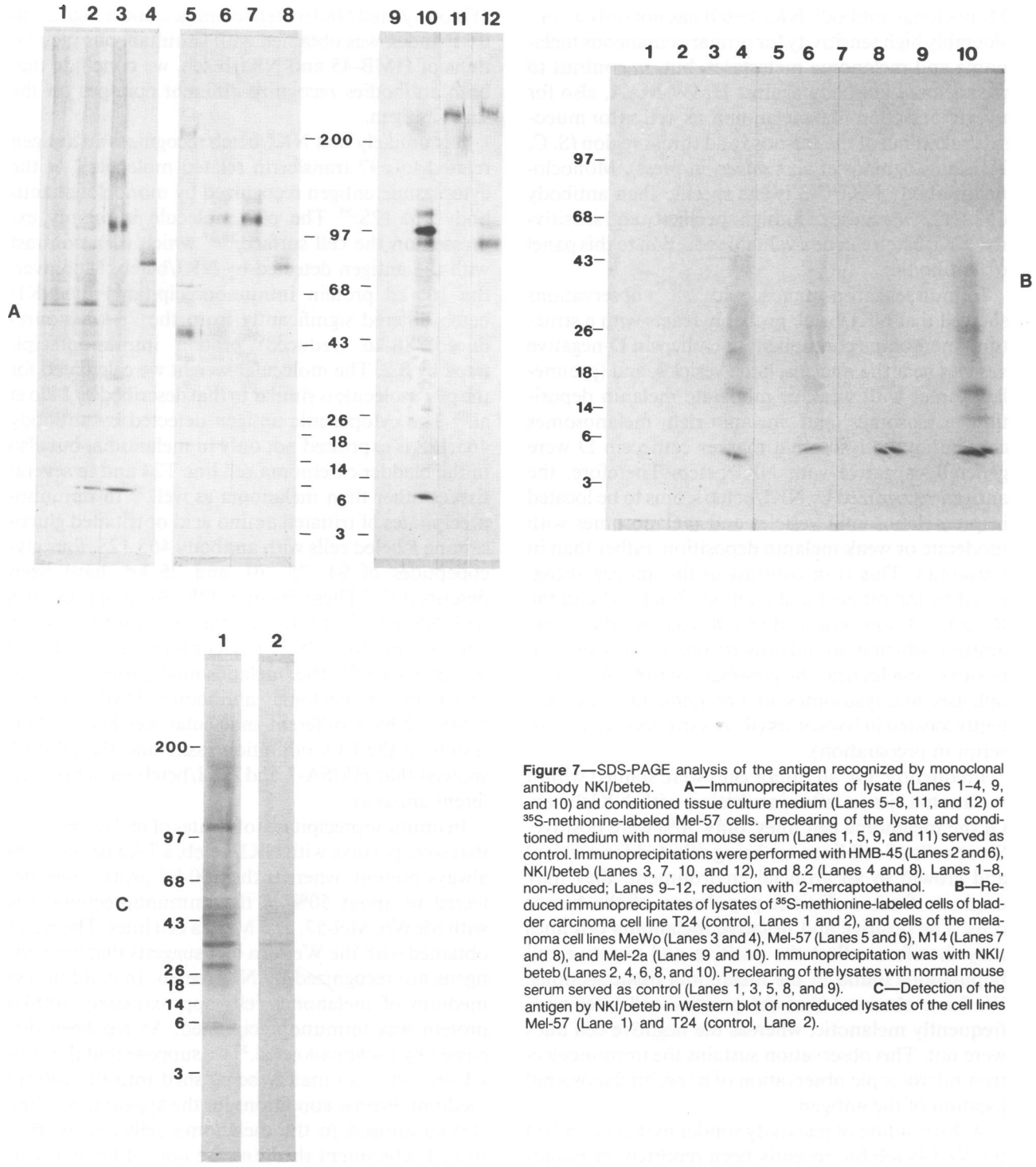

Figure 7—SDS-PAGE analysis of the antigen recognized by monoclonal antibody NKI/beteb. **A**—Immunoprecipitates of lysate (Lanes 1–4, 9, and 10) and conditioned tissue culture medium (Lanes 5–8, 11, and 12) of ^{35}S-methionine-labeled Mel-57 cells. Preclearing of the lysate and conditioned medium with normal mouse serum (Lanes 1, 5, 9, and 11) served as control. Immunoprecipitations were performed with HMB-45 (Lanes 2 and 6), NKI/beteb (Lanes 3, 7, 10, and 12), and 8.2 (Lanes 4 and 8). Lanes 1–8, non-reduced; Lanes 9–12, reduction with 2-mercaptoethanol. **B**—Reduced immunoprecipitates of lysates of ^{35}S-methionine-labeled cells of bladder carcinoma cell line T24 (control, Lanes 1 and 2), and cells of the melanoma cell lines MeWo (Lanes 3 and 4), Mel-57 (Lanes 5 and 6), M14 (Lanes 7 and 8), and Mel-2a (Lanes 9 and 10). Immunoprecipitation was with NKI/beteb (Lanes 2, 4, 6, 8, and 10). Preclearing of the lysates with normal mouse serum served as control (Lanes 1, 3, 5, 8, and 9). **C**—Detection of the antigen by NKI/beteb in Western blot of nonreduced lysates of the cell lines Mel-57 (Lane 1) and T24 (control, Lane 2).

other tissues, normal cells, and cancerous cells have never been observed. Moreover, the antibody reacts with all primary melanomas, probably all melanotic metastases, and about 60% of amelanotic metastases. Therefore, we may define the antibody as specific for cells of the melanocytic lineage.

Recently, monoclonal antibody 653.40S, which recognizes a high-molecular-weight melanoma-associated antigen (HMW-MAA[9]), and antibody NKI/C-3 have been advocated to be used in a panel of monoclonal antibodies for the diagnosis of melanoma in poorly differentiated malignant tumors.[14]

Monoclonal antibody NKI/beteb has not only a considerably high sensitivity for primary cutaneous melanoma and melanoma metastases, but, in contrast to monoclonal antibody against HMW-MAA, also for uveal[34] and choroidal melanoma, as well as for mucosal melanoma of the ear-nose and throat region (S. C. Henzen-Logmans et al, *Cancer*, in press). Monoclonal antibody NKI/C-3 is less specific than antibody 653.40S.[19] Because of its high specificity and sensitivity, NKI/beteb can be a valuable addition to this panel of antibodies.

Immunoelectron-microscopic observations showed that NKI/beteb probably reacts with a structural membrane component in cathepsin D-negative vesicles near the nucleus, large vesicles, and (pre)melanosomes with weak or moderate melanin deposition. Lysosomes and melanin-rich melanosomes containing the lysosomal marker cathepsin D were generally negative with NKI/beteb. Therefore, the antigen recognized by NKI/beteb seems to be located in premelanosomal vesicles and melanosomes with moderate or weak melanin deposition, rather than in lysosomes. This is in contrast to the antigen recognized by the monoclonal antibody NKI/C-3 and the R anti-C3 antiserum directed against the same antigen, which is abundantly present in cathepsin D-positive vesicles near the periphery of the cell, melanophages, and lysosomes, and consequently is prevalently located in lysosomes (R. A. Gruters et al, manuscript in preparation).

Frequently, not all tumor cells were stained within melanoma tissues after incubation with NKI/beteb. Of the melanoma cell lines, only 50% were positive. Since cells of cell lines are being selected for survival and growth in an artificial environment, they may originate from a distinct population of the tumor cells. The positive and negative melanoma cell lines may therefore reflect the positive and negative cell populations found in the melanoma tissue sections. We have observed that the positive cell lines were frequently melanotic, whereas the negative cell lines were not. This observation sustains the immunoelectron microscopic observation of a (pre)melanosomal location of the antigen.

A distribution of reactivity similar to that described for NKI/beteb has recently been reported for monoclonal antibody HMB-45.[16] However, in contrast to NKI/beteb, antibody HMB-45 was negative with adult melanocytes. Except for the 100-kd protein in the Mel-57 cell lysate, HMB-45 immunoprecipitated proteins similar to those precipitated by NKI/beteb. Results from the DDEIA showed that NKI/beteb and HMB-45 indeed recognize the same antigen. However, because HMB-45 poorly inhibited the binding of biotinylated NKI/beteb to antigen and a high additivity index was obtained with simultaneous incubations of HMB-45 and NKI/beteb, we conclude that both antibodies recognize different epitopes on the same antigen.

It is unlikely that NKI/beteb recognizes an antigen related to p97 transferrin related molecule[33] or the cytoplasmic antigen recognized by monoclonal antibody 465.12S.[35] The p97 molecule is mainly expressed on the cell surface,[36,37] which is in contrast with the antigen detected by NKI/beteb. Moreover, the 100-kd protein immunoprecipitated by NKI/beteb differed significantly from the 78-kd (nonreduced)/88-kd (reduced) protein immunoprecipitated by 8.2. The molecular weight we calculated for the p97 molecule is similar to that described by Liao et al.[38] The cytoplasmic antigen detected by antibody 465.12S is expressed not only in melanoma, but also in the bladder carcinoma cell line T24 and in several tissues other than melanoma as well.[39] In immunoprecipitates of tritiated amino acid or tritiated glucosamine labeled cells with antibody 465.12S, four glycopeptides of 94, 75, 70, and 25 kd, have been described.[35,39] These features differ from our findings with NKI/beteb. Moreover, the cytoplasmic antigen detected by 465.12S is not confined to vesicles or melanosomes.[40] The melanosomal proteins recognized by monoclonal antibodies HMSA-1 and HMSA-2 have different molecular weights.[10,21] Our results in the EIA inhibition assay and the DDEIA showed that HMSA-2 and NKI/beteb recognize different antigens.

In immunoprecipitates of lysates of melanoma cells that were positive with NKI/beteb, a 7-kd peptide was always present, whereas the 100-kd protein was detected in about 50% of the immunoprecipitations with MeWo, Mel-57, and Mel-2a cell lines. The result obtained with the Western blot suggests that both antigens are recognized by NKI/beteb. In conditioned medium of melanoma cells approximately 100-kd protein was immunoprecipitated. As has been discussed by Esclamado et al,[32] we suppose that the 100-kd protein is ultimately being shed into the culture medium. Precise conditions for the appearance of the 100-kd antigen in the melanoma cells and its presumed subsequent shedding are not yet known. Unintentional variations of culturing conditions before and during cell labeling could have been responsible for the fluctuations in the amount of 100-kd antigen observed in the cell lysates. Because of shedding of the antigen from melanoma cells and because of the predominantly intracytoplasmic location of the antigen, antibody NKI/beteb seems not to be suited for imaging or immunotargeting purposes. However, the fact

that the antigen is shed from the melanoma cells suggests the possibility of developing a specific immunoassay for circulating antigen in melanoma patients.

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