Use of Monoclonal Antibodies in Detection of Melanoma-Associated Antigens in Intact Human Tumors

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The use of antimelanoma monoclonal antibodies on tissue sections using a two-step indirect immunoperoxidase technique is reported. Antibodies 691-13-17 and 691-I5-Nu4B reacted with dysplastic nevus cells and all melanomas tested, but not with normal skin melanocytes, intradermal nevi, or lentigines. Antibody 691-13-17, directed against DR antigen, reacted also with Langerhan's cells, macrophages, and a subpopulation of lymphocytes. Antibody 691-I5-Nu4B reacted *only* with melanomas. The technique allows analysis of the expression of antigens by tumor cells *in situ*. (Am J Pathol 1982, 107:357-361)

THE USE of anti-human antibodies as immunodiagnostic reagents for delineation of the specificity of tumor antigen(s) present on freshly removed fragments of tumor tissue has been seriously hampered up to now by the lack of antibody directed against specific determinants expressed only by tumor cells. Monoclonal antibodies against human melanoma antigens[1] are now available that bind specifically to melanoma cells maintained in long-term culture,[1] freshly obtained from patients,[2] or maintained in short-term cultures.[3]

A two-step indirect immunoperoxidase method that allows monoclonal antibodies to be used with frozen tissue sections and permits antibody specificity studies to be done on a variety of neoplastic and normal tissues is reported here. The ability to use monoclonal antibodies on tissue sections permits the localization of melanoma-associated antigens in anatomically intact tissue and may permit their use as precise immunodiagnostic reagents in the immediate future.

Materials and Methods

Antibodies

Monoclonal antibodies 691-I5-Nu4B (Nu4B) and 691-13-17 (13-17), which bind to melanoma cells, have been described previously.[1] Nu4B antibody has been shown to immunoprecipitate a glycoprotein antigen with four polypeptide chains, with molecular weights of 116,000, 95,000, 29,000, and 26,000 daltons.[4,5] Antibody 13-17 has been shown to react with an epitope on the light (B) chain that is common to all human DR alloantigens and to precipitate all antigens detectable with a xeno-anti-DR antiserum in immunoprecipitation studies.[5]

Tissue

Specimens of intradermal nevi, normal skin, and malignant melanomas were obtained from the Pigmented Lesion Clinic, the Division of Surgical Pathology, and the Division of Autopsy Pathology of the Hospital of the University of Pennsylvania. These were immediately embedded in OCT compound (Tissue-Tek II, Miles Laboratories, Naperville, Ill), quick-frozen in liquid nitrogen, and stored at −70 C.

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Immunoperoxidase Technique

Frozen sections were cut on a Lipshaw cryostat and picked up on 22-mm coverslips. These sections were immediately placed in Columbia jars (Arthur H. Thomas, Philadelphia, Pa) containing 0.01 percent glutaraldehyde (Sigma, St. Louis, Mo) in phosphate-buffered saline (PBS) (0.15 M with calcium and magnesium, Dulbecco modified, pH 7.4), and fixed for 10 minutes. Following fixation, sections were treated with 0.1 M glycine (Fisher Scientific, King of Prussia, Pa) in PBS for 10 minutes. The coverslips were given three 5-minute rinses in PBS. After the third rinse, they were placed in 10% normal goat serum in PBS for 30 minutes. They were then placed in supernatant medium from antibody-secreting hybridomas 13-17 or Nu4B diluted 1:3 with PBS (primary antibody step). One percent normal goat serum was added.

The supernatant medium from cultures of mouse myeloma P3 × Ag8 (P3) was used as negative control. Further, each run contained known samples that did and did not react with Nu4B and 13-17 antibodies.

The tissue was incubated for 24 hours at 4 C, and was washed for 5 minutes three times as before. The first wash was performed with PBS containing 1.0 M NaCl. The second and third washes were with PBS alone. For the secondary antibody step. IgG fraction goat anti-mouse IgG (heavy- and light-chain-specific) conjugated to horseradish peroxidase (Cappell Laboratories, Cochranville, Pa) was diluted 1:100 in PBS. The coverslips were incubated for 1 hour at 4 C and then were washed in cold PBS four times for 5 minutes each using 1.0 M NaCl in the first wash step as before.

The substrates used for localization of binding included 3,3'-diamino-benzidine tetrahydrochloride (DAB) (Sigma, St. Louis, Mo),[6] or 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, Mo).[7] For DAB staining, sections were incubated at room temperature in 0.05 g/dl solution of DAB in 0.05 M TRIS buffer (pH 7.6), containing 0.3 percent H_2O_2 for 8 minutes. After rinsing once in PBS, sections were dehydrated in graded alcohols and xylene and mounted in Preservaslide (MCB Reagents, Cincinnati, Ohio). Positive staining is a brown, granular precipitate. For AEC staining, a 0.4 g/dl solution of this reagent in dimethylformamide (Sigma, St. Louis, Mo.) was diluted 1:20 with 0.05 M acetate buffer (pH 5.0), and 0.03% H_2O_2. The slips were incubated for 4 minutes, rinsed in PBS, and mounted in water-soluble mounting media.[8] A positive reaction gives an orange to red, granular precipitate.

Evaluation

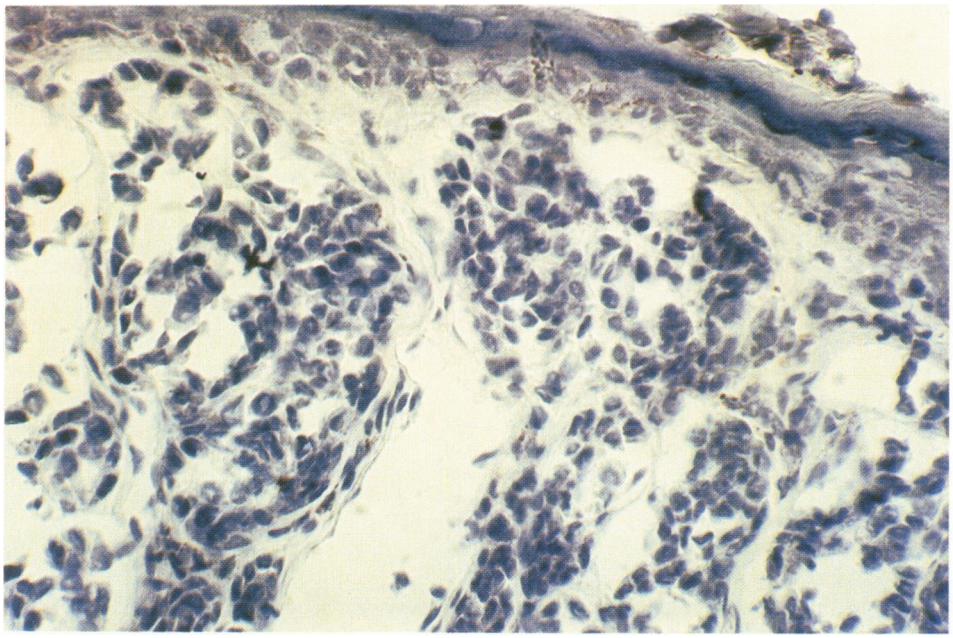
The slides were evaluated by light microscopy with the following criteria: 1+, barely discernable from background staining; 2+, clearly but faintly different from background; 3+, clearly different from background; 4+, markedly different from background. In addition, the percentage of cells reacting in a population was roughly estimated.

As a test of method specificity, 4 cell lines previously tested by mixed hemadsorption assay (MHA) and radioimmunoassay (RIA) were tested in a blind fashion. These four lines were harvested by trypsinization, made into a pellet by centrifugation, and frozen in the same manner as the tissue samples. When tested blindly by the immunoperoxidase method, the two known DR-positive and DR-negative cell lines gave the same results as found in MHA and RIA.

All slides were initially evaluated by one observer (JJT). Subsequently they were coded and were evaluated by a second observer (DEE). Slides were then reevaluated by both observers. On the first series of observations (64 total observations) 11 instances of disagreement were noted. In 7 of these, the error was of one grade difference (3+ instead of 4+), the grading of Observer 2 being consistently lower. In the other four instances, the difference was of two grades (2+ instead of 4+ in 3 cases, and 4+ instead of 2+ in 1 case). Both observers agreed on all scores of 0 that were given. Individual cases were discussed mutually, and then each observer regraded the slides independently. On reevaluation, 7 instances of differences were noted, these all being of one grade (3+ instead of 4+). In 6 instances Observer 2 was lower by one grade. In the last instance, Observer 1 gave the lower grade. Comparison of evaluations 1 and 2 by the same observer showed a difference in 4 instances out of 64 for Observer 1, and 7 out of 64 for Observer 2. In all cases, the difference was of one grade (lower for Observer 1, higher for Observer 2), a consistent variation. Results listed in Tables 1 and 2 are those of Observer 1 on reevaluation.

Results

DAB was abandoned as a substrate in favor of AEC during the evolution of our technique. DAB reactivity was found to resemble melanin pigmentation, and thus was a source of confusion and possible error in heavily pigmented lesions. AEC was found to be preferable because of its contrasting color to melanin. In addition, AEC was found to be a



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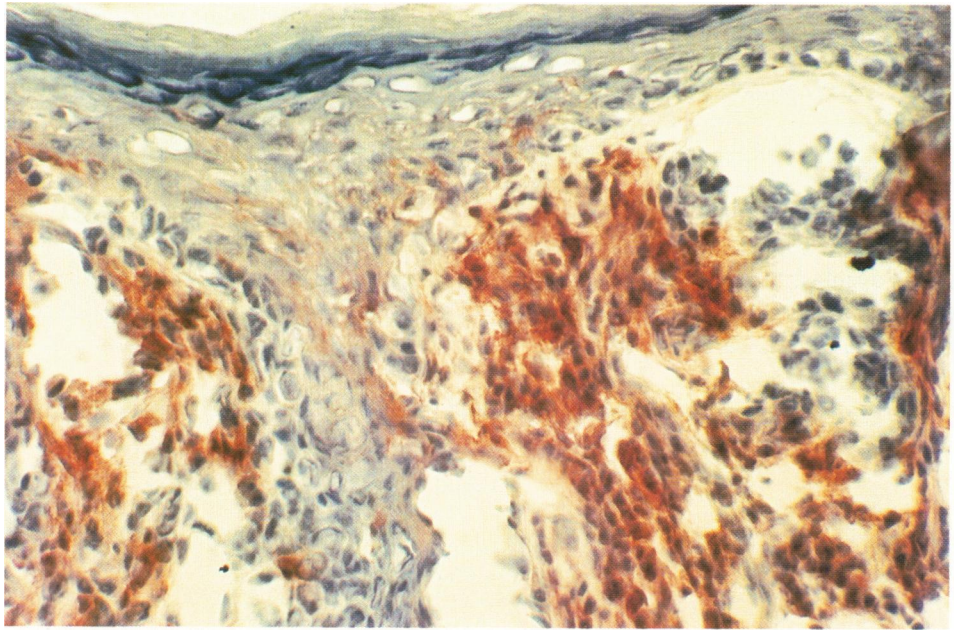

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Figure 1 — Primary superficial spreading melanoma, radial and vertical growth phase, incubated with P_3 control, goat anti-mouse peroxidase conjugate, aminoethylcarbazole, and counterstained with Harris' hematoxylin. (×300) **Figure 2** — Primary superficial spreading melanoma, radial and vertical growth phase, incubated with 691-I5-Nu4B, goat anti-mouse peroxidase conjugate, aminoethylcarbazole, and counterstained with Harris' hematoxylin. (×300) Positive staining is a reddish orange granular precipitate.

relatively poor substrate for endogenous peroxidases and catalases found in neutrophils, macrophages, mast cells, and the granular cell layer of normal skin. When used as indicated, the background with AEC was almost entirely absent, a factor giving greater sensitivity.

Both 13-17 and Nu4B antibodies reacted with all melanomas tested. Not all malignant cells within a lesion were positive; the proportion positive varied from roughly 20–90% (Table 1). Neither antibody bound to normal melanocytes, nevus cells from intradermal nevi, or benign lentigines (Table 2).

Table 1—Summary of 13-17 and Nu4B Antibody Reactivity with Primary and Metastatic Melanomas

Patient	Age	Sex	Diagnosis	13-17		Nu4B	
				Degree	%	Degree	%
Primary melanomas							
LM 45	83	M	Lentigo maligna	4+	20%	3+	40%
LM 46	83	M	Lentigo maligna	3+	20%	3+	40%
TC 103	56	M	Lentigo maligna	4+	50%	NT	NT
WB 36	69	M	SSM	4+	20%	4+	50%
AK 39	42	M	SSM	4+	40%	3+	30%
AR 90	53	M	SSM	4+*	40%	4+	50%
EB 29	67	M	SSM	4+	30%	NT	NT
CM 32	65	F	Acral lentiginous	4+	40%	4+	40%
Metastatic melanomas							
AN 40	–	F	Lymph node met	4+	70%	3+	80%
MC 66	57	F	Lymph node met	4+	90%	4+	90%
BE 25	54	F	Lymph node met	3+	90%	3+	80%
EB 29	67	M	Lymph node met	3+	90%	2+	60%
CM 67	–	F	Lymph node met	4+	80%	NT	NT
MT 38	–	M	Lymph node met	4+	80%	4+	70%
TM 84	27	M	Lymph node met	4+	50%	2+	60%
EM 109	59	F	Lymph node met	4+	60%	NT	NT
LR 122	36	F	Liver metastasis	4+	80%	4+	80%

* Seen on radial and vertical growth phase cells and on dysplastic nevus cells in a patient with dysplastic nevus syndrome.[10]
SSM = superficial spreading melanoma.
NT = not tested.

Antibody 13-17 (Table 1) bound to 8 of 8 primary and 9 of 9 metastatic melanomas. In the primary lesions, the antibody bound to 4 of 4 superficial spreading, 1 of 1 acral lentiginous, and 3 of 3 lentigo maligna melanomas. Both radial and vertical growth phase cells[9] were positive in these lesions. In one superficial spreading melanoma associated with a dysplastic nevus,[10] dysplastic melanocytes were also positive. None of 11 intradermal nevi, 2 lentigines, or 12 normal skin specimens showed positive binding to melanocytes or nevus cells (Table 2). Binding was noted on dendritic cells located high up in the epidermis and felt to represent Langerhan's cells. Macrophages (melanophages) and a proportion of lymphocytes also gave positive staining results.

Antibody Nu4B (Table 1) bound to 6 of 6 primary and 5 of 5 metastatic melanomas. In the primary lesions, 3 of 3 superficial spreading, 1 of 1 acral lentiginous, and 2 of 2 lentigo maligna melanomas were positive. In all of these lesions, reactivity was found

Table 2—Summary of 13-17 and Nu4B Antibody Reactivity with Intradermal Nevi, Normal Skin, and Benign Lentigines

Patient	Age	Sex	Diagnosis	13-17		Nu4B	
				Degree	%	Degree	%
Intradermal nevi							
SK 64	50	M	6 Intradermal nevi	0*	0	0	0
MB 93	45	F	3 Intradermal nevi	0*	0	0	0
LR 125	36	F	2 Intradermal nevi	0*	0	0	0
Normal Skin							
AG 17	23	F	Normal skin	0*	0	0	0
BH 28	28	F	Normal skin	0*	0	0	0
WB 36	69	M	Normal skin	0*	0	0	0
LM 45	83	M	Normal skin	0*	0	0	0
CM 32	65	F	Normal skin	0*	0	0	0
AK 39	42	M	Normal skin	0*	0	0	0
AR 90	53	M	Normal skin	0*	0	0	0
TC 103	56	M	Normal skin	0*	0	0	0
LR 122	36	F	Normal skin	0*	0	0	0
MB 93	45	F	Normal skin	0*	0	0	0
ST 64	50	M	Normal skin	0*	0	0	0
EB 29	67	M	Normal skin	0*	0	0	0
Lentigines							
MB 93	45	F	Lentigo	0*	0	0	0
LR 125	36	F	Lentigo	0*	0	0	0

* Found on dendritic cells high in the epidermis (Langerhan's cells), macrophages, and a proportion of lymphocytes.

in both the radial and vertical growth phase. Melanocytes and other cell types in 11 intradermal nevi, 2 lentigines, and 12 specimens of normal skin were negative (Table 2). This antibody reacted only with melanoma cells.

Discussion

Our results indicate that monoclonal antibodies that bind to melanoma cells in RIA or MHA can be successfully used to mediate staining in indirect immunoperoxidase assay of frozen tissue specimens. We have provided evidence that antibodies 691-I5-Nu4B and 691-13-17 react with melanoma cells but do not cross-react with melanocytes from normal skin or nevi in frozen sections.

Although in RIA and MHA we previously demonstrated binding of these antibodies to cells from melanomas but not from Spitz tumors or giant hairy nevi,[2] the technique of obtaining normal skin melanocytes is not available for the performance of these assays. Thus, by using immunoperoxidase staining of frozen sections, we were able for the first time to eliminate the possibility that normal skin melanocytes may express antigens defined by hybridoma-secreted monoclonal antibodies with specificity for malignant melanocytes.

One of these antigens, Nu4B, is also expressed by astrocytoma cells[3] and may represent an oncofetal protein present on neural tube cells from which astrocytes and melanocytes originate. If this assumption is correct, then on the basis of the results obtained in this study, the Nu4B antigen is suppressed in normal melanocytes and reexpressed in malignant cells.

As far as DR antigen is concerned, the results show that normal melanocytes do not express the antigen; but malignant melanocytes, Langerhan's cells, macrophages, and lymphocytes do express it. The acquisition of the antigen by the malignant cells may be of significance in endowing them with the capacity for unlimited growth by a mechanism that is at present under study.[11]

Two limitations of our method should be noted. First, the sensitivity of our method has not yet been formally defined. Second, the use of frozen sections limits the technique, because resolution becomes inferior during the prolonged exposure to buffer and other media involved in the staining procedure. We have not achieved useful staining reactions in paraffin-embedded or routinely formalin-fixed tissue, no doubt because of the lability of the membrane-associated antigen. We hope to resolve this problem with further technical advances.

The use of monoclonal antibodies to detect melanoma-related antigens should prove to be a useful tool for investigation of antigen expression in various stages of melanocytic neoplasia.

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