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Original Paper

Immunostaining of Human Melanomas by a Monoclonal Antibody to B700 Mouse Melanoma Antigen

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Previous studies have shown that B700, an albumin-like murine melanoma antigen, has a human homologue termed H700. Polyclonal antibodies to B700 also bind to all cultured human, swine and hamster melanoma cells, suggesting that B700 is a "pan-melanoma" antigen. The objects of this investigation were: (a) to determine if 2-3-3, a monoclonal antibody to B700, can be used to identify human melanomas in formalin-fixed, paraffin-embedded tissues, and (b) to determine the specificity and potential diagnostic value of 2-3-3. Forty-eight of the 49 human melanomas, including spindle melanoma cells, stained positively, as did five of the eight pigmented naevi including cellular spindle naevi. Twenty-six of the 32 human non-melanomatous lesions were negative for 2-3-3 staining (weakly positive on one breast carcinoma and positive on five neural tumours). These results indicate that 2-3-3, a monoclonal antibody to the mouse melanoma antigen B700, can be used to identify H700 in archival specimens. 2-3-3 may have an advantage over HMB45, which is the most commonly used antibody for melanoma diagnosis, because of its immunoreactivity with spindle melanocytic lesions. Antibodies to B700 may prove to be a useful adjunct in the diagnosis of human melanoma and related lesions.

Key words: melanoma, B700, diagnosis, monoclonal antibody
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INTRODUCTION

Research in tumour pathobiology is rapidly changing. In recent years the identification, isolation and biochemical characterisation of unique, antigenic tumour proteins has become, and continues to be, one of the most extensively explored fields in cancer research. Knowledge regarding tumour antigens has not only broadened studies of basic tumour biology, but has also stimulated clinical research in immunotherapy, immunoprophylaxis and particularly immunodiagnosis.

Human and other animal melanomas are highly aggressive tumours with well-documented antigenic natures [1]. From a research standpoint, many workers in the field have taken a two-stage approach toward the ultimate goal of clinical applications. The first step is to study antigen production by animal tumours, and the second is to study the extent to which the isolated animal antigens have relevant homologues in human tumours of the same histological type [2].

B700, an albumin-like 67-kD glycoprotein, is a murine melanoma antigen. It is the major protein of the melanosomal membrane and can be found on most membranous structures of melanoma cells, including their plasma membrane [3,4]. ELISA studies using rabbit antibodies raised against purified B700 show that the antibodies cross-react with molecules on all xenogeneic cultured melanoma cells tested including those of human, swine and hamster origin [5]. This suggests that B700 and B700-like molecules are candidates for "pan-melanoma" antigens.

H700, the human homologue of B700, is immunologically crossreactive with B700. It has been demonstrated in the melanosomal membrane fraction of human melanoma cells [6], and in the sera and urine of melanoma patients [7]. Given the demonstrated crossreactivity of B700 and H700 proteins, and the demonstration of anti-B700 crossreactivity to cultured xenogeneic melanoma cells, we aimed in this study to determine whether a murine monoclonal antibody (termed 2-3-3)

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to B700 could be used to identify H700 in human melanoma tissue specimens, and if so, whether this strategy would be of value in the immunodiagnosis of human melanoma. As a comparison, two other markers, HMB45 [8] and S100 [9], which are frequently used in melanoma diagnosis, were also investigated.

MATERIALS AND METHODS

Tissue samples

We studied 49 cases of malignant melanoma, eight benign naevi, 32 non-melanomatous tumours and normal skin. These tissue samples were obtained from the files of the Department of Pathology, Georgetown University Hospital and from the Department of Dermatopathology, Armed Forces Institute of Pathology.

Antibody

The mouse monoclonal antibody 2-3-3 was raised against B700 from JB/RH murine melanoma tumours. 2-3-3 specifically recognises intact B16 melanoma cells bound to ELISA plates, but does not crossreact with GL26 glioma cells (which are also derived from the neural crest and the same strain of mice) [10]. 2-3-3 recognizes a distinct epitope on B700 and does not crossreact with serum albumin, even though B700 is an albumin-like molecule [11]. Most specimens were also evaluated immunohistochemically by antibodies HMB45 and S100, which are commonly used in diagnostic pathology for melanoma. The sources and working dilutions of these antibodies are described in Table 1.

Preparation of tissues

All specimens were fixed in 5% buffered formalin and paraffin embedded. Sections of 5 μm thickness were cut and slides were incubated in a 60°C oven for at least 30 min.

Immunohistochemical staining

The streptavidin-biotin-alkaline phosphatase indirect immunostaining method was used. In some experiments, 10% normal mouse serum was used as a negative control. Dewaxed tissue sections were incubated with the primary antibody for 1 h at room temperature (2-3-3 does not bind at low temperature, i.e. 4°C). A biotinylated antimouse IgG and streptavidin-alkaline phosphatase conjugate (Zymed Lab, California, U.S.A) was used as a detecting system, with fast red (Sigma, Missouri, U.S.A.) as the chromogen. Tissues were then counterstained by Mayer's haematoxylin (Sigma), and mounted in Aqua-Mount (Lerner Lab, Pennsylvannia, U.S.A.).

Evaluation of slides

All slides were reviewed blind by at least three observers. Specimens showing any staining (ranging from clusters of stained cells to the staining of virtually all cells) were scored as positive, while specimens having a complete absence of staining were scored as negative.

RESULTS

2-3-3 immunostaining in malignant melanoma

We studied 53 specimens from 49 melanoma cases (summarised in Table 2). Among the 49 cases, 31 were primary melanomas and 18 were metastatic melanomas. The majority were pigmented; nine were spindle cell and 16 were amelanotic melanomas. 2-3-3 immunostaining indicated positivity in 48 of 49 (98%) human melanomas. Only one melanoma (case 5, a primary melanoma) was not stained by this antibody (Table 2, Figure 1). Of 49 malignant melanomas, 12 tumour samples were derived from lymph nodes, 31 were from skin, two each were from brain and bone, one each from vaginal mucosa and adrenal gland. Multiple metastatic lesions of 2 patients (cases 24 and 27) were removed at the same time and in one patient (case 38) at different times.

Cases 4, 28 and 30 are shown in Figure 2. The staining pattern of 2-3-3 is both membranous and cytoplasmic, and tends to be diffuse throughout the tumour (Figure 3). The intensity of the stain was sometimes heterogeneous in different melanoma specimens, and sometimes variable in different areas of the same specimen. 2-3-3 gave high background on some specimens and occasionally stained sebaceous glands and peripheral nerves.

2-3-3 immunostaining in non-melanomatous specimens

Eight pigmented naevi, positive for HMB45, were studied. Five were positive and three negative for B700 (Table 3). Thirty-two non-melanomatous lesions and normal skin tissues were also tested. These 32 cases consisted of 14 carcinomas, four neurofibromas, three Schwannomas, two germ cell tumours, two malignant lymphomas, one mesothelioma, one dermatofibroma, one fibrothecoma, one meningioma, one ear polyp inflammation, one atypical endometrial hyperplasia and one normal skin. Twenty-six of the 32 non-melanomatous lesions were negative. Among the six non-melanomatous cases which stained positive with 2-3-3, one was a breast carcinoma (weakly positive) and five were neuroectodermal tumours (three Schwannomas and two neurofibromas) (Figure 4). Normal skin samples showed no positive staining of the dermal melanocytes.

Table 1. Antibodies used

Antibody	Specificity	Dilution*	Source
2-3-3 mouse monoclonal antibody	B700 melanoma antigen	1:500	Gersten, 1992 [10]
HMB45 mouse monoclonal antibody	Melanocyte	1:2000	Enzo, New York, U.S.A.
S100 rabbit polyclonal antibody	Melanocyte, chondrocytes, lipocytes, Schwann cell and myoepithelial cells	1:2000	Dako, California, U.S.A.

* Dilutions of antibodies noted are of the antibody as supplied.

Table 2. Human melanoma specimens used

No.	Tumour location	P/M	Melanin	HMB45	2-3-3	S100
1	Bone acromion	M	–	+	+	+
2	Axillary node	M	–	+	+	+
3	Brain	M	–	+	+	+
4	Groin node	M	+	+	+	+
5	Arm skin	P	+	+	–	+
6	Axillary node	M	–	+	+	+
7	Cystic duct node	M	+	+	+	+
8	Gluteal skin	M	–	+	+	+
9	Vaginal mucosa*	P	–	+	+	+
10	Axillary lymph node	M	+	+	+	+
11	Femoral skin	M	–	+	+	+
12	Lymph node	M	+	+	+	+
13	Bone	M	+	+	+	+
14	Brain	M	+	+	+	+
15	Axillary lymph node	M	–	–	+	+
16	Adrenal gland	P	+	+	+	+
17	Supraclavicular node	M	–	Not done	+	+
18	Skin*	P	–	–	+	+
19	Skin*	P	–	–	+	+
20	Parotid lymph node*	M	+	+	+	+
21	Skin	P	+	+	+	+
22	Skin	P	+	+	+	+
23	Skin	P	+	–	+	+
24a	Skin	P	+	+	+	+
24b	Skin	P	+	+	+	+
25	Skin	P	+	+	+	+
26	Skin	P	+	+	+	+
27a	Skin	P	+	+	+	+
27b	Skin*	P	+	+	+	+
27c	Skin	P	+	+	+	+
28	Skin	P	+	+	+	+
29	Skin	P	–	+	+	+
30	Parotid lymph node	M	+	+	+	+
31	Skin*	P	+	+	+	+
32	Skin	P	+	+	+	+
33	Skin	P	+	+	+	+
34	Skin	P	+	+	+	+
35	Skin	P	+	+	+	+
36	Skin	P	+	+	+	+
37	Skin	P	+	+	+	+
38a	Tracheal lymph node	M	–	+	+	+
38b	Mediastinal lymph node	M	–	+	+	+
39	Skin*	P	+	+	+	+
40	Skin	P	+	+	+	+
41	Skin	P	–	+	+	+
42	Parotid lymph node	M	+	+	+	+
43	Skin	P	+	+	+	+
44	Skin	P	+	+	+	+
45	Skin	P	+	+	+	+
46	Skin	P	+	+	+	+
47	Skin	P	+	+	+	+
48	Skin*	P	–	–	+	+
49	Skin*	P	–	–	+	+

P, primary; M, metastatic. * Spindle cell tumour.

HMB45 and S100 staining

These two antibodies are commonly used in diagnostic pathology for the demonstration of melanocytic differentiation. In this study, all malignant melanomas showed a positive reaction for S100 protein. Forty-two of 48 (case 17 was not tested for HMB45) melanomas showed focal to diffuse positive staining for HMB45. Four of the six specimens which did not stain with HMB45 were spindle cell melanomas.

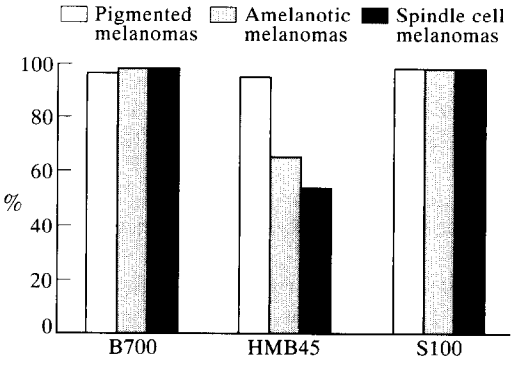


Figure 1. Immunostaining results of malignant melanomas.

DISCUSSION

In the present study, we demonstrated that the monoclonal antibody 2-3-3 reacts with H700 antigen in human primary and metastatic melanomas in formalin-fixed paraffin-embedded tissues. The specificity of 2-3-3 was generally restricted to melanoma samples and to some pigmented naevi. Reaction with melanocytes in the normal skin tissues and with non-melanomatous tumours was generally negative except for some neuroectodermal tumours (Figure 4). The results of this study stress several important immunobiological properties of H700 that are recognised by 2-3-3. The demonstration of 2-3-3 binding by 98% of human melanoma specimens confirms previous studies which implicate B700 as a crossspecies "pan-melanoma" antigen. This finding parallels other studies of melanoma-associated antigen crossspecies reactivity [12–14]. The value of demonstrated crossspecies reactivity among tumour antigens is that they validate the use of animal models, which in turn are useful for developing immunotherapeutic approaches for melanoma. Correlation between expression of melanoma-associated antigens and biological behaviour of melanoma cells in various animal species will contribute to our understanding of the functional role of these antigens. It should also be noted that the intensity of the stain with 2-3-3 was not uniform in the examined melanoma tissues. The non-uniformity of staining within and among specimens can be attributable either to heterogeneity in the levels of H700 expression, to authentic antigenic diversity, or to both. Antigenic heterogeneity is, indeed, a likely occurrence in this system given that appreciable amino-terminal variation in the B700 amino acid sequence has been observed [15]. The restricted specificity of monoclonal antibodies to a single epitope can be a confounding factor in instances of antigenic diversity.

2-3-3 reacts with an epitope of the glycoprotein which is heat stable and resistant to formalin fixation and alcohol treatment. Thus, 2-3-3 is amenable to immunohistochemical studies using routinely processed and archival surgical pathology specimens. It is not yet known whether the epitope recognised by 2-3-3 is the carbohydrate portion of B700 (this might be suspected because the epitope survives fixation and dehydration with alcohol and xylene).

In this study, 2-3-3 showed positive staining in all spindle and melanotic melanoma samples. In contrast, four of the nine spindle cell melanomas and five of the 16 amelanotic melanomas did not react with HMB45. Therefore, 2-3-3 may

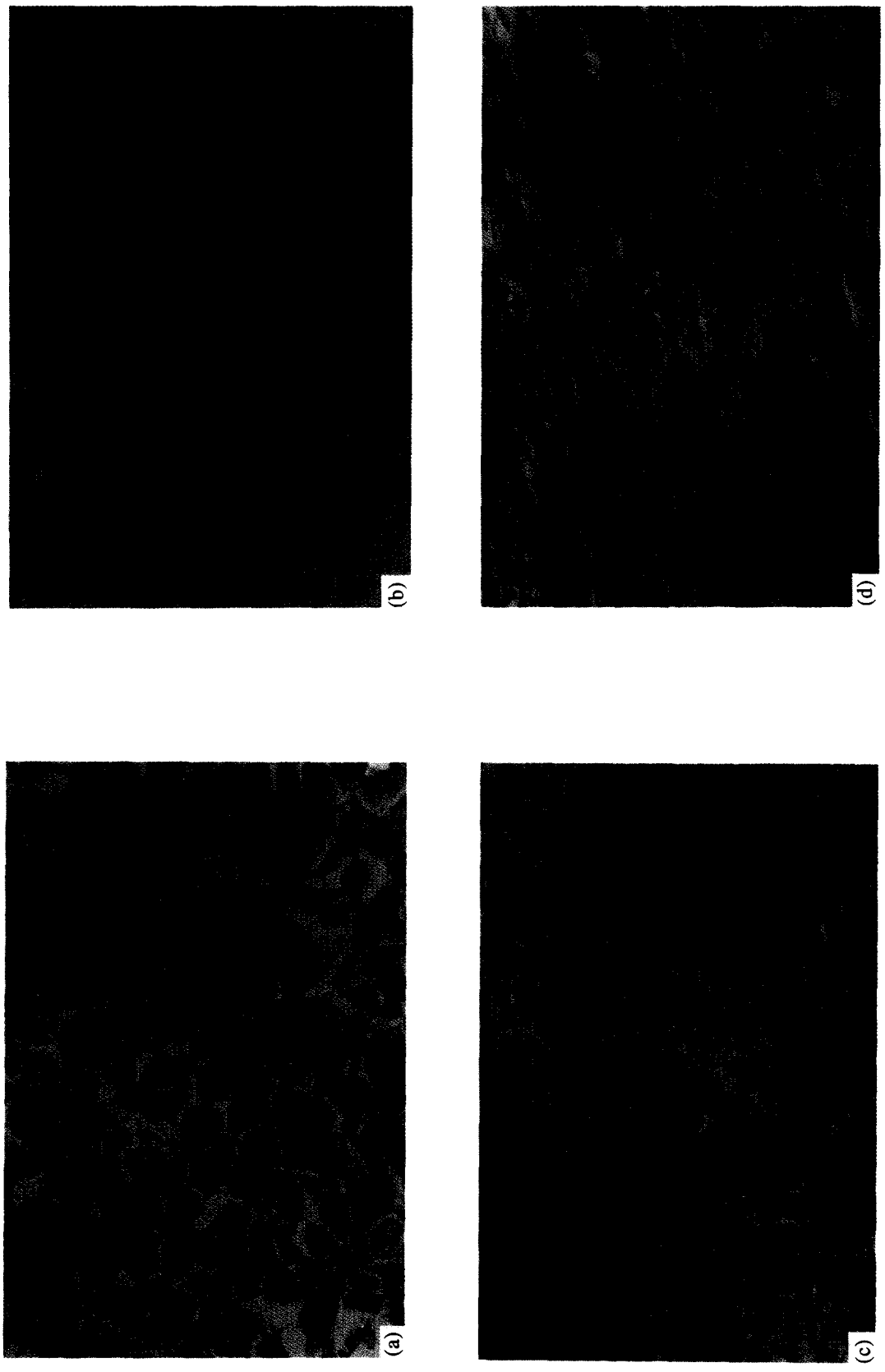
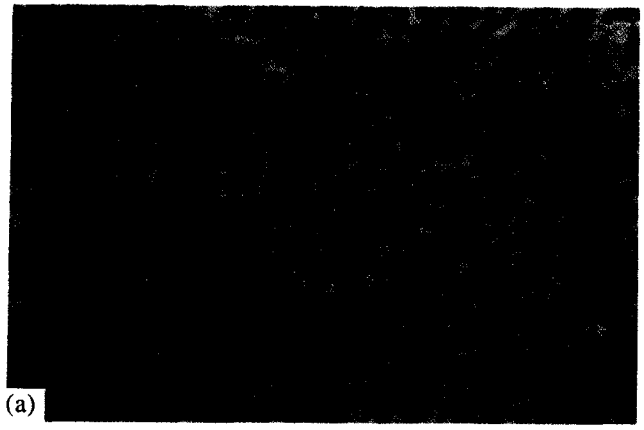


Figure 2. Immunostaining of malignant melanoma by 2-3-3. (a) Metastatic malignant melanoma epitheloid cells (600×) (b) Malignant melanoma in skin. 2-3-3 staining was positive in the melanoma cells but negative in the neighbouring keratinocytes (600×) (c) Metastatic malignant melanoma infiltrating lymph node. 2-3-3 staining was positive in melanoma cells but negative in lymphoid cells (600×) (d) Metastatic malignant melanoma spindle cells (400×).



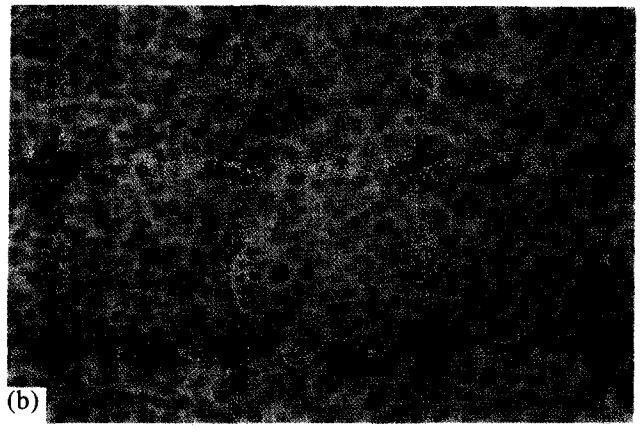


Figure 3. Immunostaining of human malignant melanoma. (a) Human metastatic malignant melanoma in a lymph node stained by 2-3-3. The positivity was diffuse throughout the tumour and the staining patterns were both membranous and cytoplasmic (400×) (b) Human metastatic malignant melanoma in a lymph node stained by HMB45. Focal positivity with cytoplasmic staining (400×).

Table 3. Human naevus specimens examined

No.	Description	2-3-3 staining
1	Blue naevus	+
2	Blue naevus	–
3	Naevus, compound	+
4	Naevus, cellular blue	–
5	Naevus, deep penetrating	+
6	Naevus, congenital	+
7	Naevus, compound	–
8	Naevus, compound	+

have an advantage over HMB45 since it has been reported that HMB45 does not reliably stain spindle melanomas [16]. Thus, 2-3-3 is a useful adjunct in the diagnosis of human melanomas and related lesions. However, none of the immunostains available for melanoma diagnosis are entirely satisfactory. Antibodies to S100 react with all the melanomas, but with other tissues and tumours as well [17]. HMB45 appears to be a pigment-associated antigen, and antibodies to HMB45 are insensitive to the detection of spindle and amelanotic melanomas.

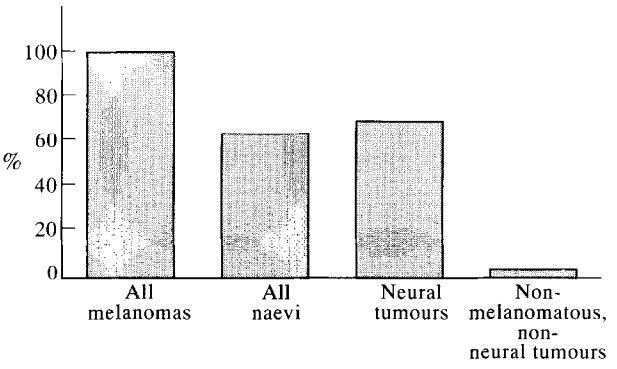


Figure 4. Comparison of staining results.

The limitations of 2-3-3 staining are that a high background is sometimes obtained and that sebaceous glands and neuroectodermal tumours are occasionally stained. As possible explanations for the high background and crossreactivity of 2-3-3, the following points should be considered: (1) melanocytes and Schwann cells are derived from neural crest and thus melanomas, neurofibromas and Schwannomas are neuroectodermal in origin. Neuroectodermal tumours stained positively by 2-3-3 should be further studied in a larger series to determine whether B700 is generally expressed by those tumours. (2) B700 protein is an albumin-like protein and, although ELISA experiments show that 2-3-3 does not cross react with purified murine serum albumin, the potential cross reactivity in other tumours containing albumin-like antigens or in tissues containing human albumin is uncertain. Additionally, we cannot account for potential antigen shedding by the tumours and this may also contribute to high background staining. (3) 2-3-3 may recognise a common epitope which is partially homologous among several different cells [18]. The same carbohydrate on a different protein backbone has been proposed for crossspecies melanoma antigens [19]. Moreover, albumin-like antigens have been reported for neural tumours [20,21] and thus positive staining of neural tumours might be expected.

While the occasional appearance of high background makes the use of 2-3-3 less than ideal, many antibodies currently in use in immunohistochemistry suffer from the same problem. For example, in the context of dermatopathology, S100 can also stain dendritic cells and fat. Antibodies to smooth muscle actin, in our experience, can also give a high background and leave a haze. Nevertheless, the use of these immunostains in combination with observations of pattern and intensity proves valuable.

The subject of melanoma-associated antigens has been extensively investigated, and efforts to search for a more specific melanoma-associated antigen show no signs of ending. A number of laboratories have produced monoclonal antibodies to melanoma-associated antigens, but unfortunately, many of them also react with non-melanocytic tumours, and formalin fixation and paraffin embedding can destroy these cell membrane antigens [22–29]. In many cases, absolute specificity is not required for diagnostic purposes and other factors, such as antigen density, may be more important. In addition, the same melanoma can express several antigens, which implies that a combination of monoclonal antibodies to different antigens would be useful in the identification of most melanomas. Also, a combination of antibodies may be needed

for diagnostic and therapeutic procedures, in view of the finding that the antigens can undergo clonal variation within the same tumour [30]. In this context, the results from this study are promising because the antigen can be demonstrated in 98% of melanoma cases. It would, therefore, be of value to search for another epitope on the B700 and/or H700 antigen that could be detectable. For finding the ideal epitope on B700 and/or H700, the future availability of complete sequencing data would help us identify a suitable, unique oligopeptide.

In summary, we have demonstrated the density and the distribution pattern of H700 antigen in human melanoma cells in routine formalin-fixed and paraffin-embedded tissues by using the monoclonal antibody 2-2-3. This result also confirms the crossspecies reactivity of B700 with its human homologue protein. Therefore, the B700/H700 antigens are candidates for "pan-melanoma" antigens. Further insight into the biological characteristics of malignant melanoma can be elucidated with these antibodies. The recognition of malignant melanocytes and benign proliferative melanocytic lesions, but not normal melanocytes, by these antibodies implies that the B700/H700 antigen may be associated with the process of cellular activation. Expression of the epitope recognised by 2-3-3 may be an early event in melanocytic tumour progression. Studies directed toward additional molecular and biochemical characterisation of B700/H700 antigens will provide a better understanding of their significance and biological role as they relate to the immune response and natural history of this aggressive tumour.

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