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Antimelanoma Hybridoma Antibodies Against Partially Purified Melanoma Antigen*

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Abstract—*We have immunised BALB/c mice with a melanoma antigen obtained after papain solubilisation of the membranes of a metastatic melanoma tumour and fused the immune spleen cells to the mouse myeloma line P3-NS1/1-Ag4.1. The produced hybridoma antibodies (Mel-PV antibodies) recognised the initial melanoma antigen in haemagglutination, but did not react with any of the HLA phenotypes tested by cytotoxicity on a panel of B lymphocytes with known HLA-A and B phenotypes. We rosetted red blood cells coated with protein A with dispersed cells from fresh melanoma tumours, and a high degree of specificity for human malignant melanocytes was observed. Purified Mel-PV antibodies were also tested by indirect immunofluorescence and found to be oriented towards cytoplasmic components of malignant melanoma cells. These results indicate that the use of melanoma antigens for preparing monoclonal antibodies maintained a satisfactory degree of specificity and may be an adequate starting point for defining common and specific antigenic determinants on human melanoma.*

INTRODUCTION

THE EXPRESSION of malignancy in tumour cells is usually considered to be a multistep process which is accompanied by the appearance of new molecules, generally termed 'tumour antigens'.

Antibodies to tumour antigens of melanoma have been identified from three different sources: natural antibodies from melanoma-bearing patients, xenoantisera raised in laboratory animals against membranous material [1, 2] or monoclonal antibodies secreted by hybridomas.

The characterisation of these tumour antigens has been greatly improved in the last few years by the latter, i.e. the *in vitro* production of monoclonal antibodies using cultured hybridomas [3–6]. The list of hybridomas to melanoma tumour antigens is now quite impressive, and this tumour remains one of the most interesting systems for defining specific and common tumour antigenic markers. The human melanoma antigens so far identified have been recently classified by Houghton *et al.* [7] into three classes according to their molecular weight and their specificity.

We have studied the production of hybridomas against human malanoma by immunising BALB/c mice with a solubilised melanoma antigen described by Viza and Phillips [2]. This antigen is only partially purified by column chromatography. This, however, would seem to be a better starting point than immunisation with whole melanoma cells, which contain several additional antigenic determinants. We report here some properties of two new hybridoma antibodies produced in our laboratory and their specificity on cell recognition.

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MATERIALS AND METHODS

Antigen

The antigen used for immunisation of

BALB/c mice was prepared according to the technique already described [8] based on papain solubilisation of the membranes of a metastatic melanoma tumour (MH23). The extract contained the specificity described in 1975 by two of us [2]. This specificity was found in 20% of melanoma tumour cell extracts and was used to produce xenoantisera in rabbits.

Immunisation

For hybridoma preparation, two BALB/c mice received two injections seven days apart of 0.5 mg antigen in 0.5 ml saline mixed with Freund's complete adjuvant. One of these animals received a third injection three weeks later. After control of efficacy of immunisation by cross-over electrophoresis and immunodiffusion, the mouse which had received three injections was killed six days after the third injection, its spleen removed and a cell suspension prepared immediately.

Cells and hybridoma production

Immune spleen cells were obtained by gentle mechanical dispersion and fused with the mouse myeloma line P3-NS1/1-Ag4.1 (NS-1) [9]. NS-1 was grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Fusion was performed according to the method previously described [10]. Briefly, 2×10^6 myeloma cells and 10^7 mouse spleen cells resuspended in Earle's salts (without Ca^{2+} or Mg^{2+}) were layered on a membrane filter (Nuclepore) by gentle vacuum aspiration. Fusion was induced by contact for 45 sec with a 45% solution of polyethylene glycol (PEG). Hybrid cells were selected in HAT medium (Dulbecco's medium supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine). One hybridoma (Mel-PV-1) was isolated from a single filter maintained for four weeks in HAT medium, and is probably a mixture of clones. A second hybridoma (Mel-PV-2) was selected as a single clone growing in microcavities (Linbro plates) from another filter fusion experiment.

Purification of Mel-PV gamma-globulin

The mouse gamma-globulin contained in the supernatants from Mel-PV hybridoma cultures were extracted by affinity chromatography using an adsorbent of goat anti-mouse gamma-globulin fixed to CNBr-activated Sepharose 4B (Pharmacia). The eluants used were 3 M NH_4SCN or 4 M Mg_2SO_4, which gave equivalent results.

Tests of hybridoma antibodies

Haemagglutination. Sheep or human red blood cells were coated with antigen: melanoma antigen, embryonic antigen (a papain-solubilised extract of a 6-week-old human embryo) [2] or soluble HLA antigen [8], using a solution of $CrCl_3$ according to the technique of Gold and Fudenberg [11] modified by Imai and Ferrone [12].

Cytotoxicity. For cytotoxicity tests, frozen human fibroblast cells of known HLA A and B phenotypes were thawed and resuspended in phosphate-buffered saline (PBS) with 20% human AB serum. Cells were washed once and dispensed in microcavities (1 μl per cavity from a suspension of 2×10^6 cells per ml) presoaked with mineral oil (Merck). One microlitre of Mel-PV antibodies were added to the cells and incubated for 30 min at 37°C. Rabbit complement (5 μl per cavity) was then added and incubated for 1 hr at 37°C. Cells were then stained with 5% eosin (1 μl per cavity). Cytotoxicity was read 1 hr later under a phase-contrast microscope and was considered positive in the wells with more than 15% dead cells.

Rosette test with protein A. Cell suspensions from six primary malignant melanomas (PMM), pigmented and amelanotic, ranging from level III to level V, and five amelanotic or pigmented lymph node metastases (LNM) were tested. Three dermal benign naevi (BN), one case of squamous cell carcinoma and a suspension of pooled lymphocytes (see 'Miscellaneous' in Table 2) were used as controls. The method used to make the rosettes was the micromethod described by Imai and Ferrone [12]. Briefly, sheep red blood cells (SRBCs) or human O^+ red cells (HO^+RC) were coated with protein A using the $CrCl_3$ method. The tumour antigen (Tag) consisted of tumour cell suspensions obtained by mechanical dispersion of fresh tumours, then washed in physiological saline and fixed in 10% formalin at room temperature overnight, according to techniques already described [13]. These cells were kept at +4°C before use for up to five months. Cells (5×10^5) were incubated with purified Mel-PV-2 at the concentration of 1 mg protein (evaluated by the Lowry's method) per ml PBS for 1 hr at room temperature, washed in isotonic saline and then mixed with the protein A-coated SRBCs.

For scoring, the following rules were adopted: due to the fact that the yield of target cells from tumour tissue is low, especially when malignant primary and benign tumours are used, we had to set the limit of counted cells to 100 or less instead of 200, as in the study by

Imai and Ferrone, which was done with cultured cells. Another reason for setting the counts at this relatively low value was that it allowed the test to be duplicated or triplicated instead of 'consuming' all available target cells in one single experiment. The number of RBCs per rosette was also considered. In scoring controls, only the rosettes with 3 RBCs or more were counted. PMM and LNM were scored first on the basis of 3-RBC rosettes and secondly on that of 5-RBC rosettes. Readings were made by at least two independent observers.

Before reading, tumour cell suspensions were mixed with 0.1% methylene blue. Only rosettes with stained cells having a diameter double that of red cells were taken into account. In some cases a dioxyphenylalanine (Dopa) reaction was performed after the rosetting process to count tyrosinase-positive cells (Fig. 1).

Immunofluorescence. Direct immunofluorescence was performed using FITC-labelled hybridoma antibody on frozen sections and cell suspensions from a primary melanoma, two melanoma skin metastases and one metastatic melanoma from a lymph node. The controls consisted of choroidal melanocytes from normal eyes obtained on autopsy and from surgically removed, injured eyes. In addition, the hybridoma antibody was tested against normal human skin and a panel of normal tissues used for autoantibody screening.

RESULTS

Haemagglutination

Coated human red blood cells (group O^+) were tested for haemagglutination with increasing dilutions of Mel-PV antibodies. The results presented in Table 1 indicated that the anti-melanoma antibodies tested recognised the MH23 antigen, which was used for the immunisation of the mice from which the spleen cells for hybridisation were obtained, and that the Mel-PV-2 antibody did not react with the embryonic or HLA antigens tested.

Cytotoxicity

The reaction of the anti-melanoma antibodies with HLA was also studied by cytotoxicity on a panel of B lymphocytes with known HLA-A and B phenotypes in the presence of complement (see Materials and Methods). Mel-PV-2 showed no reaction with any of the phenotypes tested, while Mel-PV-1 reacted weakly with four cell HLA phenotypes: 2.9/7.12.

Rosette test with protein A

As shown in Table 2, a high degree of specificity for human malignant melanocytes was observed with purified Mel-PV-2 antibody when protein A-coated red blood cells were rosetted with dispersed cells from fresh tumours. This table shows the percentages of 5-RBC rosettes in PMM, LNM and controls. Five out of six primary tumours (Tags 2, 4, 5, 18 and 27) were found positive. Three out of five LNM were positive (Tags 3, 29, 34). Tag 37 was found positive on one occasion and negative in another. Counting rosettes with three or more RBCs, three benign naevi remained below 10%, as were also non-melanocytic cells such as lymphocytes and squamous carcinoma cells. Among tumour cells, 8 out of 11 cases ranged from 5 to 50% and were scored as positive. The experiments were repeated two or three times for lymph nodes and benign tumours, while the low numbers of tumour cells extracted from primary tumours did not allow repetition. The invasive vertical growth phase was used for the primary tumours except in one case.

Table 3 shows the influence of counting only 5-RBC rosettes instead of 3-RBC rosettes upon the final score.

Lymphocytes present in the tumour infiltrate were observed to be coated with a few SRBCs but were eliminated on the criterion of size.

To eliminate the possible role of tumour cells coated with naturally occurring immunoglobulin, reactions without application of Mel-PV-2 were performed and were constantly found to be negative.

Table 1. *Haemagglutination of human O^+ RBC with anti-melanoma antibodies*

Antibody	Haemagglutination titre of HO^+ RBC coated with: MH23 antigen	Embryonic antigen	Soluble HLA
Mel-PV-1	1/32	1/4	—
Mel-PV-2	1/16	—	—
Purified Mel-PV-2	1/64	—	—

*Table 2. Rosetting of malignant melanoma cells coated with melanoma antibodies (Mel-PV-2)**

Origin of cell suspensions	Experiment 1 % ≥ 5 RBC	Experiment 2 % ≥ 5 RBC	Experiment 3 % ≥ 5 RBC
1. *Primary malignant melanoma (PMM)*			
Tag 2 SSM 4 (amelanotic)	5(+)	/	/
Tag 4 SSM 5 (horizontal growth phase)	30(+)	45(+)	/
Tag 5 (LMM 5 (pigmented)	/	10(+)	/
Tag 16 MN 5 (pigmented)	0(−)	/	/
Tag 18† SSM 5 (pigmented)	/	/	43(+)
Tag 27 SSM 5 (amelanotic)	/	18(+)	40(+)
2. *Lymph node melanoma metastasis (LNM)*			
Tag 3 (amelanotic)	50(+)	35(+)	/
Tag 28 (amelanotic)	/	/	0(−)
Tag 29 (pigmented)	/	41(+)	29(+)
Tag 34 (pigmented)	/	10(+)	20(+)
Tag 37 (amelanotic)	/	8(−)	42(+)
	% ≥ 3 RBC	% ≥ 3 RBC	% ≥ 3 RBC
3. *Benign naevi*			
Tag 1	3	/	/
Tag 26	2	0	10
Tag 31	/	/	11
4. *Miscellaneous*			
Tag 32 squamous cell carcinoma	/	7	/
TBL1 Human lymphocyte pool	1	/	/
5. *Without incubation with Mel-PV-2*			
Tag 3	/	7	/
Tag 34	/	/	6
Tag 37	/	/	3

/Not tested; RBC: red blood cell.

*No mean values were calculated for positive tumours since the degree of recognition of Mel-PV-2 antibody varies for each tumour. Rosetting (≥ 3 RBC) mean values in eleven negative controls were: experiment 1 = 2.0 ± 1.0; experiment 2 = 5.25 ± 0.02; experiment 3 = 7.5 ± 0.73.

†This material was kept frozen at −30°C in 20% glycerol, decongelated and fixed in 10% formalin before use.

Immunofluorescence

Purified Mel-PV antibodies were also tested by indirect immunofluorescence on four melanomas, two choroidal melanocyte preparations from the human eye and two normal skin sections. As shown in Table 4, Mel-PV-2 antibodies were oriented towards cytoplasmic components of malignant human cutaneous melanoma, and only a few cells of the melanoma deposits in human lymph nodes showed some positive membrane fluorescence. Table 5 presents a list of negative controls.

DISCUSSION

Several hybridoma antibodies against human melanoma have been described in the last few years [3, 6], [14–18].

These hybridomas were all obtained by fusion of spleen cells of BALB/c mice immunised with melanoma-derived cells. In most instances melanoma cells in long-term cultures were used [3–5, 14, 17], or in some cases primary short-term cultures [6, 15].

In the present study we immunised BALB/c mice with an antigen obtained by solubilisation of membrane components from a fresh metastatic tumour. In addition, instead of using either whole cells or total membrane fractions, papain was used to solubilise the membrane determinants, which were subsequently semi-purified by gel filtration on column chromatography. It is interesting to see that the gamma-globulins from the hybridoma Mel-PV-2 produced *in vitro* in our system did not react with any of the HLA extracts or for HLA specificities tested. This absence of reactivity may in part be due to this purification. In some instances [3, 19] antibodies have been produced to Ia-like antigens commonly present on some human malignant melanoma cell lines.

Evidence that Mel-PV-2 recognised the initial melanoma is based on positive im-

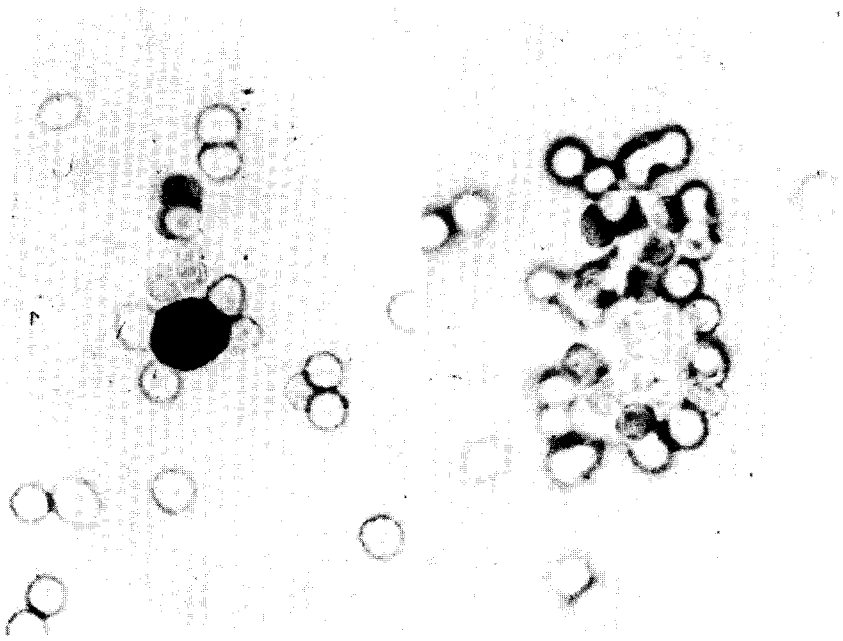

Fig. 1. Two examples of the rosette reaction. Left, methylene blue stains the melanoma cells. Right, Dopa without counter-stain of the rosetting melanoma cells. × 800.

*Table 3. Quantitative variation in rosette microassay**

Cell type	No. of melanoma cells				
	Negative	≥3 RBC/TC	≥5 RBC/TC	% ≥ 3 RFC	% ≥ 5 RFC
1. *PMM*					
Tag 4	32	72	45	78.5	30.2
Tag 5	90	29	12	22.1	9.2
Tag 18	25	39	28	60.9	43.8
Tag 27	43	78	48	64.5	39.7
2. *LNM*					
Tag 3	32	70	36	78.8	36.1
Tag 28	100	2	0	1.96	0
Tag 29	28	55	34	47.0	29.1
Tag 34	73	32	11	27.1	9.5
Tag 37	33	65	42	66.3	42.9
3. *Benign naevi*					
Tag 26	82	11	8	10.9	7.9
Tag 31	67	9	3	11.4	3.9
4. *Miscellaneous*					
Tag 32	70	5	2	6.6	2.6
5. *Without incubation with Mel-PV-2*					
Tag 3	96	7	0	6.8	0
Tag 34	130	8	1	5.76	0.72
Tag 37	150	5	2	3.2	1.27

*Results obtained in three different experiments in which Mel-PV-2 antibodies were used. RBC: red blood cell; TC: tumour cell. Tumour cells coated with 3 or more red blood cells (3 RBC/TC) and at least 5 red blood cells (5 RBC/TC) were counted separately. Their percentage of the total number of tumour cells is calculated.

Table 4. Immunofluorescence with hybridoma anti-melanoma antibodies on human tissue sections

Test material	Reaction of Mel-PV2	
	Membrane-directed	Cytoplasmic-directed
Primary human melanoma (two)	−	− + and +
Cutaneous human melanoma (two metastases)	−	+
Melanoma deposits in human lymph node	+ some cells	+
Melanocytes from a normal human eye	−	−
Melanocytes from an injured human eye	−	−
Sections from normal human skin (two)	NT	−

NT: not tested.

munodiffusion and haemagglutination reactions. The specificity of the gamma-globulin from the hybridoma Mel-PV-2 is further documented by its positive reaction with human malignant melanocytes, either extracted from different tumours or by direct reaction on frozen tumour sections. If Bystryn and Smalley had documented [20] the shedding of melanoma membrane antigens complexed with glycocalyx and the phagocytosis of this glycocalyx by melanoma cells *in vitro*, it is not surprising that melanoma cells *in vivo* do present cytoplasmic fluorescence with Mel-PV-2 antibodies although these antibodies were obtained against antigen(s) prepared from membrane extracts.

Concerning the reaction with mechanically isolated cells, it is interesting to see that the formalin used to preserve the cells did not block their antigenic properties. This means that a reference tumour cell panel could be envisaged, as the reactivity of these cells can be preserved for many months. This has already been reported for other types of human cells by Pizza *et al.* [21]. Whether or not frozen sections can also be preserved by formalin treatment is presently under investigation.

Three other laboratories kindly cooperated

Table 5. Negative autoantibody screening of Mel-PV-2

Tissue or factor	Fluorescence
Antinuclear factor:	
double-stranded DNA	negative
single-stranded DNA	negative
RNA	negative
Anticanicular factor	negative
Colon mucosa	negative
Glomerular basement membrane	negative
Heart muscle	negative
Kidney mitochondria	negative
Nerve myelin	negative
Stomach parietal cells	negative
Smooth muscle	negative
Striated muscle	negative
Choline esterase receptors	negative
Thyroid (microsomal)	negative
Thyroid (colloid)	negative
Parotid duct lining	negative
Skin basement membrane	negative
Skin intracellular substance	negative

with us in testing our antibodies on human melanoma cell lines using radioimmunoassay techniques. Dr. S. Carrel (Lausanne, Switzerland) found a positive reaction with three different melanoma cell lines (IGR3, ME-43, and ENB 1) and Dr. M. Reynier (INSERM U 119, Marseille, France) found a positive reaction of Mel-PV-1 with the melanoma cell line IGR.39. However, Dr. Hellström, (Heidelberg, West Germany) found practically no reaction between Mel-PV antibodies and the 1477 melanoma tissue culture cell line under the conditions in which their P97 antibodies reacted strongly [18]. Preliminary results from our laboratory using immunoprecipitation suggest that Mel-PV antibodies indeed correspond to antigenic determinants other than P97 or the glycoprotein–proteoglycan complex defined by Bumol and Reisfeld [22].

By the rosetting test, Mel-PV-2 monoclonal antibody detected antigenic determinants in 9 out of 11 melanoma tumours tested so far and was not present on cells isolated from benign naevi, squamous cell carcinoma or human lymphocytes.

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