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Human Malignant Melanoma: Assay of Tumor Associated Antigens by Use of Rabbit Antisera

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The usefulness of rabbit antisera for the characterization of tumor associated antigens of human melanoma was studied.

Membranes of human melanoma tissue were extracted by 2 M potassium iodide and sonication. Antisera were raised in rabbits against these extracts as well as against melanoma membranes and cultured melanoma cells. Antisera were absorbed with normal human material.

Two of 14 absorbed antisera were specific for tumor associated antigens: they reacted with melanoma extracts (counterimmunoelectrophoresis) and melanoma cells (immune adherence) but not with 20 extracts of normal tissue, erythrocytes, lymphocytes, bacteria and molds (counterimmunoelectrophoresis), not with human fibroblasts (immune adherence), not with human erythrocytes with known blood group antigens (hemagglutination) and normal human skin (immunofluorescence). Rabbit antisera did not contain antibodies against carcinoembryonic antigen and alpha-1 fetoprotein (radioimmunoassay). Reactivity with tumor associated antigens could be directly demonstrated by two-dimensional immunoelectrophoresis.

Two types of melanoma associated antigens could be discriminated: the first type with restricted specificity could be demonstrated by an antiserum that reacted with the extract used to raise it and in addition had a weak reactivity with 1 of 7 other extracts. Broader cross reactivity is characteristic for the second type: the corresponding antiserum reacted with 6 of 8 melanoma extracts.

While studies in experimental tumors suggest a significant influence of specific immunological reactions on the course of the malignant disease, the biological role of tumor antigens in spontaneously arising tumors remains doubtful: Weak immunogenicity and rare total spontaneous regressions have been reported [1]. Cell mediated and humoral antitumor immune reactions have been detected in melanoma patients by various assays [2–18], but their influence on the clinical course has so far not been proven. While the usefulness of isolated tumor antigens for immunotherapy and for the analysis of possible beneficial and harmful immunological reactions in the patient cannot be predicted, tumor associated fetal antigens have already been successfully employed for monitoring the clinical course in patients with hepatomas and gastrointestinal malignancies [19].

Absorbed rabbit antisera have been used for the detection and characterization of tumor associated antigens on cell surfaces and in solution [20–29]. The specificity of these absorbed antisera could be clearly defined in studies with bladder carcinoma antigens by a large number of control tests [28].

We report here on studies with 2 M potassium iodide-ultrasonic extracts of membranes of human malignant melanoma. Tumor associated antigens of these extracts could be characterized with counterimmunoelectrophoresis and 2-dimensional immunoelectrophoresis using heteroantisera from rabbits.

MATERIALS AND METHODS

Tissue

Melanoma tissue was obtained from excision biopsies of metastases and stored at −20°C. Normal liver, spleen, kidney, brain and lung were from autopsies performed 4–24 hr after death.

Antigens of Bacteria and Fungi

Tuberculin (1000 IE/ml tested in 10-fold dilutions down to 0.1 IE/ml) was from Behringwerke (Marburg/Germany). Extracts of bacteria and fungi were from Bencard.

Extraction

Frozen biopsy tissue was thawed, finely minced and homogenized with an Ultraturrax homogenizer (Janke and Kunkel, Staufen i.Br.) in 1 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.25 M sucrose pH 7, 5 [30]. The homogenate was centrifuged at 600 *xg*. Homogenization and centrifugation were repeated once. The combined supernatants of the 600 *xg* centrifugation, free of nuclei as judged by phase contrast microscopy, were spun for 1 hr at 50.000 *xg*. The membrane pellet was suspended in phosphate buffered saline pH 7.2 containing 0.02% sodium azide and stored at −70°C until use. Frozen membranes were thawed, mixed with an equal volume of 4 M potassium iodide [31] and sonicated with a sonicator model W-220F (20 KHz, 130 w, Ultrasonics Inc., Plainview L.I., New York, USA) for 3 × 45 sec. Insoluble material was removed by centrifugation for 1 hr at 180,000 *xg*. The supernatant was dialyzed over night at 4°C against 0.05 M barbiturate-acetate buffer pH 8.6 + 0.02% sodium azide. 4 of 11 tumor extracts and 8 of 19 control extracts were dialyzed against Tris or phosphate buffered saline pH 7.2. Two extracts were dialyzed against nonionic detergents (Be against 0.5% NP-40 (Shell) and Ja against 0.5% Tween 20 in phosphate buffered saline). Precipitates observed after dialysis were removed by centrifugation for 15 min at 5000 *xg*. Protein concentrations were estimated according to Lowry et al [32]. Gel filtration was performed on a 100 × 1.5 cm column of Sephadex G 150 (Pharmacia, Frankfurt) in 0.15 M Tris NaCl pH 7.5 + 0.5% NP-40 + 0.02% sodium azide. The active fraction was rechromatographed on Ultrogel AcA 34 (LKB) in the same buffer.

Antimelanoma sera: These were obtained after immunization of rabbits with cultured melanoma cells (anti SK Mel 25), membranes from melanoma biopsies (anti Ja) or extracts from such membranes (anti-Fr and anti-Ki). Animals were subcutaneously injected twice in 6 weeks with 0.5 mg (protein quantity) of membrane extracts or 1.5×10^8 cultured cells in complete Freund's adjuvant. Animals were bled 2 weeks after the second injection. Antimelanoma sera were absorbed with extracts of normal human tissue, erythrocytes, and with normal human serum. The optimal relative quantities of antisera, extracts of normal tissue and normal human serum were determined by titration.

Some antisera were also absorbed on immunoadsorbent columns prepared from:

a. Homogenized human liver cross-linked with 1% glutardialdehyde and mixed 1:1 with Pevikon C 870 (Serva, Heidelberg);

b. Human serum cross-linked with 1% glutardialdehyde [33].

Counterimmunoelectrophoresis: This was performed in 0.9% agarose (Behringwerke, Marburg, Oreo 14/15-0.05 M barbiturate-acetate buffer

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pH 8.6 with 0.1% sodium azide). For counterimmunoelectrophoresis 2 M KI extracts of membranes from biopsies were used after dialysis. Extracts were diluted with electrophoresis buffer either to a final protein concentration of 0.5 mg/ml or in a ratio of 1:10, if protein determination according to Lowry et al [32] was impossible because of the presence of nonionic detergents. Counterimmunoelectrophoresis was carried out for 15 min at 225 v after antibody was prerun for 25 min. Slides were washed with saline and water for 2 days, dried and stained with Coomassie blue.

Two-dimensional immunoelectrophoresis: This was performed according to Axelsen, Kroll, and Weeke [34] with 5 ul of melanoma extract (after gel filtration on Sephadex G 150) and with liver spleen extract (after dialysis), human serum and human plasma.

Hemagglutination tests: These were performed in microtiter plates with erythrocytes of known blood group specificities (gift of Prof. Dr. Sachs, Institut für Rechtsmedizin der Universität Münster).

Immune adherence tests: These were done with living cultured cells as previously described [17,35].

Indirect immunofluorescence: Tests with normal skin from biopsies were performed with fluorescein-labeled goat antirabbit immunoglobulins (Behringwerke Marburg).

Antibody to carcinoembryonic antigen (CEA): This was determined with a commercial radio-immunoassay kit of Hoffman La Roche (Grenzach). Antisera were tested undiluted and in 2-fold dilution up to 1:16.

Anti-alpha-1 fetoprotein activity: This was determined by use of a commercial radio-immunoassay kit of Serono (Freiburg i.Br., Germany). Antisera were diluted 1/10, 1:100 and 1:1000.

RESULTS

1. Counterimmunoelectrophoresis of Absorbed Rabbit Antisera with Membrane Extracts

14 rabbits were immunized either with 2 M KI-membrane extracts or whole membranes from biopsy tissue or with cultured melanoma cells (Table I). After extensive absorption only five of the 14 sera still reacted in counterimmunoelectrophoresis with melanoma extracts from membranes of biopsies (Table I, Fig 1). Two of the 5 sera were obtained after immunization with extracts from the same tumor (Fr). Antisera absorbed by solid immunoadsorbents (glutardialdehyde treated serum or tissue) gave weaker precipitates than those absorbed with soluble extracts of normal tissue and normal human serum.

Two patterns of reactivity could be discriminated (Table II): the first type is represented by the extract Fr and the corresponding anti-Fr serum: extract Fr reacted only with the antiserum raised against it. The anti-Fr serum on the other hand reacted with the extract Fr and in addition had a weak reactivity

TABLE I. *Frequency of positive reactions of rabbit antisera before and after absorption with melanoma extracts from biopsies*

Immunization with	Number of positive versus number of tested sera	
	Before absorption	After absorption
Melanoma membrane extracts (5)[a]	7/7	3[b]/7
Cultured melanoma cells (2)[a]	3/4	1/4
Melanoma membrane preparations (3)[a]	2/2	1/2

[a] The number of different preparations used for immunization is given in parenthesis. Each rabbit was immunized with one preparation only.

[b] Two positive absorbed sera were obtained after immunization with the same extract.

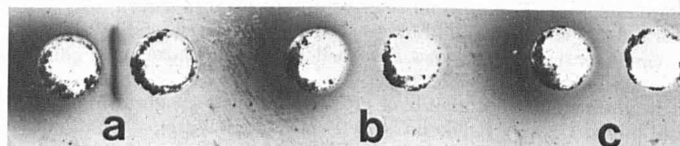

FIG 1. Counterimmunoelectrophoresis of absorbed antimelanoma Fr serum. Antigens: *a*, extract Fr, *b*, extract of human liver and spleen, *c*, human plasma.

TABLE II. *Counterimmunoelectrophoresis of absorbed rabbit antisera with membrane extracts of human melanoma (biopsies)*

Absorbed antiserum[a]	Melanoma extracts[e]							
	Fr	Mo	Ki	Ja	Be	Ra	Li	Ri
Anti-Fr[b]	+	–	–	–	–	–	–	(+)
Anti-Ki[b]	–	–	+	+	+	+	+	+
Anti-Ja[c]	–	–	(+)	+	+	+	(+)	n.d.
Anti-SK Mel 25[d]	–	–	+	+	+	+	(+)	–
Normal rabbit serum	–	–	–	–	–	–	–	–

(+) weakly positive.

[a] Absorbed with human serum and soluble extracts of human liver, spleen and erythrocytes.

Identical results were obtained with anti-Fr and anti-Ki, absorbed with immunoadsorbent (serum and homogenized tissue—insolubilized by glutardialdehyde).

[b] Obtained after immunization with soluble extracts of membrane preparations from melanoma biopsy tissue.

[c] Obtained after immunization with a membrane preparation from melanoma biopsy tissue.

[d] Obtained after immunization with cultured cells of a melanoma long-term line.

[e] Soluble extracts of membrane preparations from melanoma biopsy tissue.

with Ri. In contrary to the restricted specificity of the Fr anti-Fr system broader cross-reactivity was observed with the sera anti-Ki, anti-Ja and anti-SK Mel 25. Extract Mo reacted with none of the tested absorbed antisera. Absorbed antisera were negative with an extract of a squamous carcinoma of the skin (not shown in Table II).

2. Two-Dimensional Immunoelectrophoresis of Melanoma Extract and Rabbit Antiserum

Tumor associated antigen in a partially purified melanoma extract from biopsy-tissue could be directly demonstrated by two-dimensional immunoelectrophoresis with rabbit antiserum: Fig 2*a* is the reaction pattern of the Fr melanoma membrane extract—after gel filtration—with anti-Fr serum. The typical precipitation line on Fig 2*a* is not found in two-dimensional immunoelectrophoresis with liver-spleen extracts (Fig 2*b*) and human serum (Fig 3*a*). This can be demonstrated by electrophoresis of mixtures of tumor extract with normal material (Fig 2*c* and 3*b*).

3. Fetal Antigens in Melanoma Extracts

It could be excluded by radioimmunoassays that rabbit antimelanoma sera contained antibodies against known carcinofetal antigens: neither labeled carcinoembryonic antigen nor labeled alpha-1 fetoprotein from commercially available test kits were bound by rabbit antisera anti-Fr, anti-Ki, anti-Ja, and anti-SK Mel 25. After extensive absorption with normal human serum and normal tissue and erythrocyte extracts anti-Ja still reacted with an extract of fetal internal organs. No antibodies against fetal calf serum could be detected by counterimmunoelectrophoresis in absorbed sera anti-Fr, anti-Ki, anti-Ja and anti-SK Mel 25.

4. Immune Adherence Test with Cultured Human Melanoma Cells

Identical tumor associated antigens could be demonstrated on cultured melanoma cells and in tumor extracts (Table III): serum anti-Ki after extensive absorption reacted with cultured melanoma cells in immune adherence tests but was negative with adult fibroblasts. No reactions were observed with anti-Fr probably due to the fact that anti-Fr is directed against antigens with a more restricted specificity.

5. Specificity Controls

a. Counterimmunoelectrophoresis: Specificity of absorbed antisera anti-Fr, anti-Ja, anti-Ki and anti-SK Mel 25 was checked with human plasma, 11 extracts of normal human

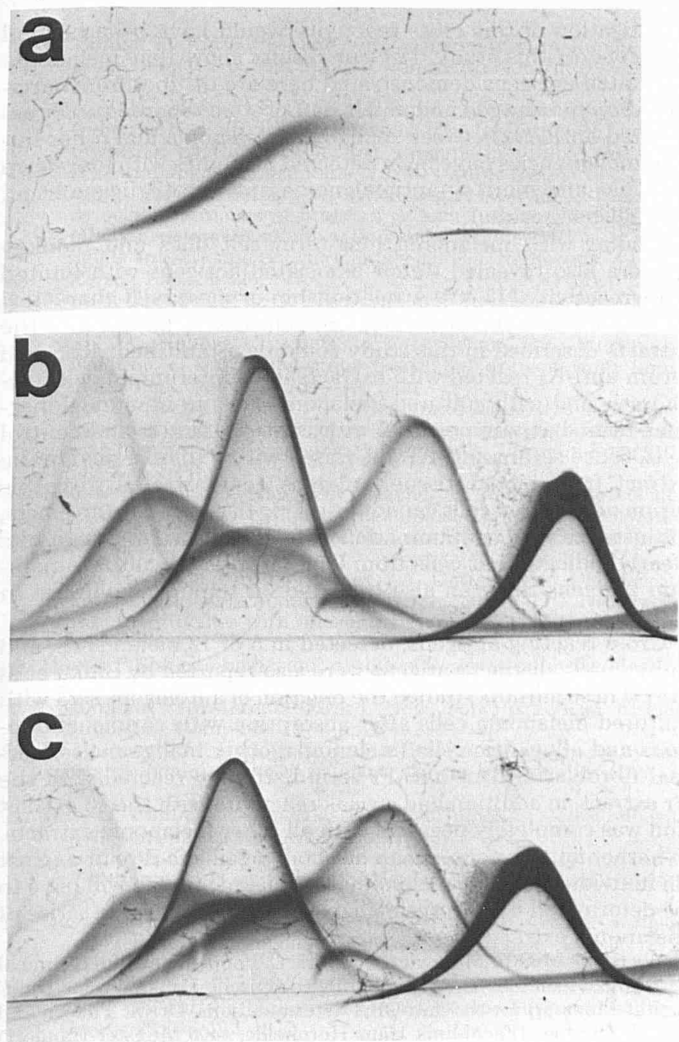

FIG 2. Two-dimensional immunoelectrophoresis with anti-Fr serum. Antigens: *a*, melanoma extract Fr after gel filtration on Sephadex G 150. *b*, Extract of human liver and spleen. *c*, Mixture of gel filtrated melanoma extract and liver spleen extract. The precipitation line of the melanoma extract (*a*) cannot be obtained with the control extract (*b*) as seen by two-dimensional immunoelectrophoresis with the mixture (*c*).

tissues and cells (Table IV) and also with 9 antigens of bacteria and fungi including tuberculin.

b. Hemagglutination: Extensively absorbed sera anti-Fr, anti-Ki, anti-Ja and anti-SK Mel 25 did not agglutinate several erythrocyte preparations that contained at least once the following blood group specificities: A_1, A_2, B, O, M, N, P, S, s, C, D, E, c, d, e.

c. Immunofluorescence: Absorbed rabbit anti-Fr and anti-Ki did not contain antibodies against normal human skin as judged by immunofluorescence. In contrary, antibodies against antigens of normal human epidermis were observed in rabbit sera anti-Ja and anti-SK Mel 25: anti-Ja reacted with cytoplasmatic substances of the basal layer, with suprabasal cells, and with peripheral cells of hair follicles. Serum anti-SK Mel 25 after extensive absorption still reacted with antigens of the intercellular spaces of the lower epidermis and the peripheral part of hair follicles.

DISCUSSION

Monitoring the clinical course of tumor patients by use of immunological assays requires reproducible test methods and easily available reagents. We were interested to know whether rabbit antisera can be used for the detection of tumor associated

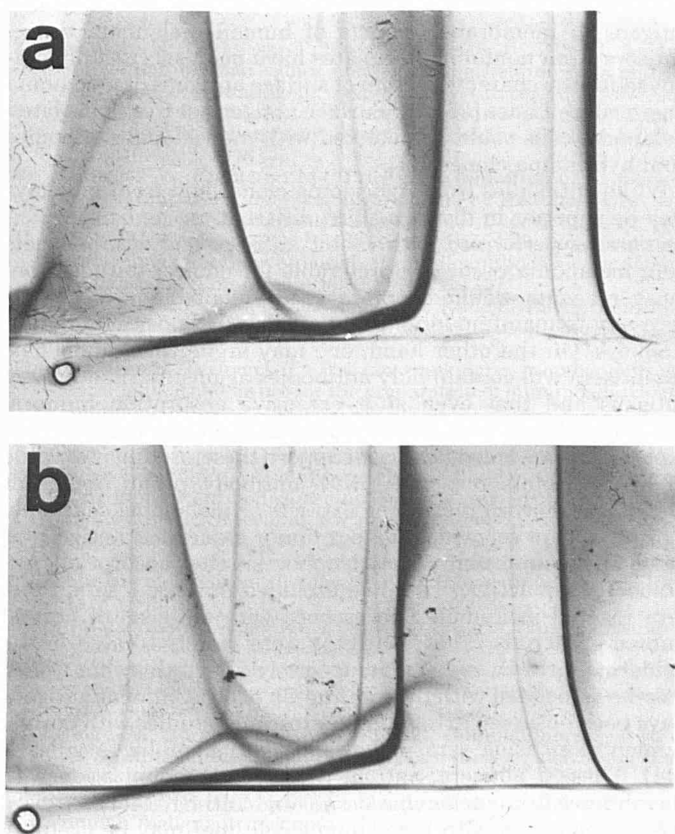

FIG 3. Two-dimensional immunoelectrophoresis with anti-Fr serum. Antigens: (*a*) human serum, *b*, mixture of gel filtrated melanoma extract Fr and human serum. The precipitation line of the melanoma extract Fr (Fig 2*a*) cannot be obtained with human serum (*a*) as demonstrated with the mixture of human serum and the Fr extract (*b*).

TABLE III. *Reactivity of absorbed rabbit anti-tumor-antisera with long-term melanoma cell lines in immune adherence tests*

Cell line	Anti-Ki[a]	Anti-Fr[a]
SK Mel 25	+	–
SK Mel 25 subcl. 7	+	–
SK Mel 25 subcl. 9	+	–
Mel 57	(+)	–
Teratoma	(+)	–
Adult fibroblasts	–	–
Embryonic lung fibroblasts	–	–

[a] Obtained after immunization with soluble extracts of membrane preparations from melanoma biopsy tissue.

TABLE IV. *Counterimmunoelectrophoresis of absorbed rabbit antisera with normal human material and extracts of bacteria and fungi*

Absorbed antimelanoma sera anti-Fr, anti-Ki, anti-Ja and anti-SK Mel 25 were negative with:

1. normal human serum
2. 11 extracts of normal human tissues and cells:

a. Liver and spleen	3	d. Kidney	1
b. Liver	3	e. Erythrocytes (26 donors)	1
c. Brain	2	f. Lymphocytes (15 donors)	1

3. 9 extracts of bacteria and fungi (Bencard):
   a. *E. coli* d. *Streptococci*
   b. *Staph. albus* e. *Altern. tenuis*
   c. *Staph. aureus* f. *Cand. albicans*
   g. Penicillia
   h. Botrytis cinerea
   i. Aspergilli
   j. Tuberculin

antigens in membrane extracts of human melanoma tissue. Antisera from nonhuman primates have been successfully employed for the characterization of surface antigens of melanoma long-term cell lines [36–42]. Surface antigens of freshly isolated melanoma cells could be detected with monoclonal antibodies from hybridoma clones [43].

While antibodies from hybridoma clones and from monkeys may be superior in distinguishing different antigenic specificities, assays performed with rabbit antisera and with extracts from melanoma tissue are preferable for clinical purposes because reagents would be more easily available without the necessity to maintain long-term tissue cultures or to immunize monkeys. On the other hand, one may argue that rabbit heteroantisera will contain only antibodies against normal human antigens and that even after extensive absorption humoral activity against normal human substances may be left. Our own experience can indeed in part support these arguments: while 13 of 14 rabbits responded with antibody production after immunization with melanoma extracts or melanoma cells, only 5 of these sera seemed to detect tumor associated antigens as judged by counterimmunoelectrophoresis after absorption. Antiblood group activity could be excluded in these 5 sera, but 2 sera reacted in immunofluorescence with antigens of normal human epidermis. These antigens were mainly located in the epidermal growth zone or immediately above this zone. They may be associated with the maturation of cells. Similar antigens have been observed in immunofluorescence studies with normal human skin using sera of patients with pemphigus vulgaris [44]. These 2 antisera, anti-Ja (raised by immunization with membranes from melanoma tissue) and anti-SK Mel 25 (raised by immunization with a melanoma cell line) may be useful in an operational sense because after absorption their reactivity in counterimmunoelectrophoresis was restricted to tumor associated material. Moreover, absorbed anti-Ja and anti-SK Mel 25 did not agglutinate human erythrocytes with various blood group specificities.

Only 2 of the absorbed antisera (Anti-Fr and anti-Ki) exhibited a reaction pattern in all assays that suggested specificity for tumor associated antigens. These 2 sera were raised with soluble membrane extracts of melanoma biopsy tissue. Reactivity with tumor-associated material not present in control extracts and human serum was directly demonstrated by two-dimensional immunoelectrophoresis.

Our antisera did not react in counterimmunoelectrophoresis with extracts of bacteria and fungi including tuberculin. This result cannot completely exclude shared antigens between mycobacteria and human melanoma as reported by Minden, Sharpton, and McClatchy [45]. Such a cross-reactivity was not found in absorption experiments with monkey antisera and cultured cells by Liao et al [42] and Brüggen, Sorg and Macher [40].

The results presented here clearly demonstrate the usefulness of rabbit antisera for the detection of melanoma associated antigens. With regard to this conclusion, these data support previously published results [21,24], although different methods were used in this study.

With respect to their antigenic specificities, 2 types of melanoma tissue could be discriminated: one is characterized by antigens with limited cross reactivity and is represented by extract Fr. Probably extract Mo, which did not react at all with absorbed antisera, contained an antigen of this type with a different specificity. The other type of melanoma tissue—as represented by extract Ki—contains antigens of broader cross reactivity. Both types of antigens are distinct from carcinoembryonic antigen and alpha$_1$-fetoprotein.

Whether the type of antigen, as defined in these studies, is associated with biological features of the tumor cells, for instance malignancy, will have to be elaborated in future studies.

Absorbed antisera did not react with one extract from a squamous cell carcinoma. This does not exclude cross reactivity of melanoma antigens with antigens of other malignancies. The investigation of this cross reactivity would have been beyond the scope of this study, but our results show that melanoma associated antigens demonstrated here are distinct from carcinoembryonic antigen and alpha$_1$-fetoprotein. Shared tumor associated antigens between malignant melanoma and other human malignancies have been detected in studies with long-term cell lines and monkey antimelanoma antisera (Brüggen et al, unpublished results).

Studies with melanoma long-term cell lines and monkey antisera also revealed tumor associated antigens with limited cross reactivity [17,40]. A relationship of these well-characterized tumor associated surface components to antigens of the extracts described in this study could be established: absorbed serum anti-Ki reacted with extracts in counterimmunoelectrophoresis and with cultured melanoma cells in immune adherence tests, but was negative with control extracts and control cells. Since serum anti-Ki was raised with a soluble membrane extract from biopsy tissue, antigens demonstrated with this serum on cultured cells cannot be due to tissue culture artefacts, for instance viral contamination or fetal calf serum. Our results clearly indicate that cells from long-term tissue cultures maintain antigens that can also be found on tumor membranes *in vivo*.

Cross reacting antigens, detected in 5 of 12 melanomas, and individually distinct antigens were also reported by Shiku et al [18]. These authors studied the reaction of autologous sera with cultured melanoma cells after absorption with various autologous and allogeneic cells (melanoma, other malignancies, normal fibroblasts). Our anti-Fr serum strongly reacted with the Fr extract, in addition had a weak reactivity with the Ri extract and was completely negative with all other melanoma extracts. Whether anti-Fr contains an antibody against a rare or against an individual specific melanoma associated antigen will have to be determined by absorption experiments with a large series of melanoma extracts.

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