SOLUBLE MEMBRANE ANTIGENS OF HUMAN MALIGNANT MELANOMA CELLS

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Delayed hypersensitivity reactions to soluble components of the cell membranes of autologous and allogeneic tumors were elicited in a series of patients with malignant melanoma. Two skin reactive antigens were prepared using stepwise low frequency sonication of cell membranes, chromatography of membrane sonicates on Sephadex G-200, and separation by polyacrylamide gel electrophoresis (PAGE). One group of antigens, in Sephadex fraction II, PAGE region a, appears to be melanoma-associated antigen. Sephadex fraction II, and further separated Sephadex fraction II PAGE region a, produced no reactions in 21 of 22 tests in patients with cancers other than melanoma; positive reactions were seen in 17 of 22 patients with early stage melanoma and in 7 of 19 patients with late stage melanoma. The other antigen, from Sephadex fraction III, PAGE region b, was also reactive in 5 of 6 early stage breast cancer patients. Comparable separated proteins from Sephadex fraction III, and Sephadex fraction III PAGE region b, of normal black skin cell membrane sonicates were reactive in 4 of 9 early stage melanoma patients but were negative in 7 late stage melanoma patients. Sephadex fraction III and Sephadex fraction III PAGE region b produced no reactions in patients with renal, cervical, colonic, and head and neck cancer; 9 of 21 patients with early stage melanomas were skin test positive; positive reactions were seen in 13 of 18 patients with late stage melanoma.

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IMMUNOLOGIC REACTIVITY TO HUMAN MALIGNANT melanoma has been a subject of great interest for many years. The possible role of immunologic factors was only inferred from observations on the natural history of the disease.[1,29,30] There has been much discussion about the cause of spontaneous regression. In one recent study, only 2 of 1700 patients with disseminated melanoma had substantial spontaneous regression.[25] However, even in this study, immune reactivity was invoked to explain the extreme variation in the time for appearance of metastases. In another study, patients who remained tumor-free for a long period of time were shown to have particularly strong delayed hypersensitivity reactions to a battery of standard recall antigens.[8]

In the past few years, there has been increasing direct evidence for immunologic reactivity to malignant melanoma. Several investigators have found antibodies to melanoma-associated antigens. In one large study, two classes of antibodies were described.[24] One class reacted against the various antigens on the cell surface membrane, and contained at least some types of autoantibodies which appeared to be specific for each tumor. The other class of autoantibodies reacted with some of the components inside the cell.[24] These latter anti-

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gens were detected on almost all melanoma cells; this study may or may not be similar to previous studies[26,27] showing that melanomas contain a common tumor antigen.

Cellular immune reactions to melanoma antigens have also been described.[5,7,9,10,21] Extracts from melanomas have been shown to produce stimulation of lymphocytes of patients with melanoma and had no such effect on lymphocytes from healthy donors.[3,22,23] Delayed hypersensitivity skin reactions in melanoma patients have been elicited by extracts of autologous tumor cells.[2,4,6,28,31,32] Stewart found only 1 out of 7 patients responded to their melanoma membrane preparations.[31] In a study of 8 melanoma patients in Uganda, 3 patients with localized disease gave positive skin reactions to membrane preparations from autologous tumor cells, while the others, with widespread disease, had negative reactions.[6] In a subsequent study of 16 additional patients, however, no relationship was seen between positive tests and stage of disease;[2] some positive reactions were also elicited by normal skin extracts.

It becomes obvious that malignant melanoma cells contain specific tumor-associated antigens and also normal tissue antigens. It has been possible to separate tumor-associated antigens for studies in animal model systems.[13,14] An application of these methods toward the study of various human cancers has resulted in a systematic attempt to isolate, separate, and directly identify the role of various soluble cell membrane antigens as they relate to the specific human malignancy.[12,15–19] A preliminary study[11] of partially separated soluble melanoma cell antigens indicated the presence of two antigens which elicit delayed hypersensitivity skin reactions. When we understand the role of various separated, carefully delineated antigens, we may be able to understand what measures need to be taken to increase the defence mechanisms and resistance in the host. In the present report, we have applied these methods for further separation and identification of soluble melanoma cell membrane antigens to the study of cellular reactivity to malignant melanoma antigens.

MATERIALS AND METHODS

Preparation of Antigens

The details of the methods used have been described previously.[12–19] Tumor and control skin specimens were obtained from Columbia Hospital for Women and from The Ohio State Medical Center. Because of the limited availability of skin from the melanoma patients and because the white skin of these Caucasian patients might not adequately control for melanoma extracts, some studies were performed with extracts of skin from Negro patients with nonmalignant diseases. The specimens were collected under sterile conditions and washed in Eagle's minimum essential medium or saline, minced finely, washed in saline, pressed through a 60-mesh stainless steel sieve, and washed again. The number of viable cells in the resultant suspension of single cells was determined with the aid of trypan blue dye exclusion. Between $10^{7.5}$ and 10^{9} cells were then frozen at −80C, thawed rapidly in a 37C water bath, and the membranes extracted by stepwise washing with isotonic and hypotonic saline. The membranes were then exposed to low frequency sonication for four sequential periods of 1½ minutes, with centrifugations at 100,000 g for 1 hour after each exposure. The pooled soluble sonicates were then separated on Sephadex G-200 and pooled fractions concentrated by ultrafiltration. Very little protein was recovered from control skin cell membrane sonicates in the elution ranges comparable to Sephadex fractions II and III (see text), and, therefore, five to six times as many viable cells were required to achieve comparable protein concentrations. The centers of the three protein peaks (Ve/Vo ratios 1.18, 2.00, and 3.32 respectively) were not comparable to those for melanoma peaks (Ve/Vo ratios 1.25, 2.7, and 3.6 respectively). The center of the protein peaks of separated soluble melanoma cell membrane preparations was quite reproducible from one material to another. The approximate molecular weight was calculated as 38,500 for Sephadex fraction II and 9950 mw for Sephadex fraction III. The appropriate molecular weight of proteins was calculated by comparing the ratios of elution volume to void volume for the centers of the eluted protein peaks with the centers of protein peaks of standard proteins under the same conditions.[13–15,17] The soluble materials were passed through a Millipore filter to ensure sterility. All materials, membranes and soluble fractions, were tested for sterility by culturing in blood agar and in thioglycollate. The protein concentrations of the materials were determined by the Lowry method. Further separation was by gradient polyacryla-

mide gel electrophoresis, using four stacked gels, as previously described.[16–19]

Patients Studied

Patients were evaluated clinically; the stage of disease is listed in the tables (Stage I = primary tumor; II = regional nodes involved; III = spread to other nodes; IV = metastases). The ability of the patients to manifest a delayed skin reaction was determined just prior to testing with tumor materials by tests with mumps antigen* or SKSD (streptokinase—40 units, streptodornase—10 units).† Only patients who were reactive with one of the antigens were tested with tumor materials. In all skin tests, 0.1 ml was inoculated on the patient's back. The reactions were observed at 20 minutes and at 24, 48, and 72 hours. Reactions were considered positive when the diameter of induration was 5 mm or more at 48 hours. Many of the reactions were biopsied to obtain histologic confirmation of the delayed hypersensitivity reactions. Approval for such tests was obtained from the ethics committees, and all tests were under code, read independently by two observers.

RESULTS

The following materials were used for skin tests: Melanoma and Control—1) cell membrane sonicates, 2) Sephadex G-200 separated soluble cell membrane fractions, and 3) polyacrylamide gel electrophoresed regions of reactive Sephadex fractions.

In tests with Sephadex G-200 separated fractions of the soluble membrane preparations (sonicates), positive reactions were seen to two of the melanoma cell fractions and appeared to test differently in relation to the stage of cancer. None of these patients reacted to tests with melanoma or control cell membrane sonicates prior to separation; we have reported previously the presence of a blocking factor present in the tumor cell material.[18] However, in many of the Sephadex melanoma and control antigens, tested in amounts ranging from 40 to 60 μg, the proteins were not totally separated, although present in relatively different quantities, and further separation was indicated. The results of preliminary tests with the crude Sephadex fractions are shown in Table 1. Sephadex fraction II was positive in 10 of 12 skin tests in patients with early stages of melanoma and in 5 of 12 patients with advanced disease. Sephadex fraction III was positive in 4 of 11 patients with early melanoma and in 9 of 11 patients with later stages of melanoma. Two out of 5 early stage melanoma patients were skin test positive to Sephadex fraction III of black skin cell membrane sonicates. Fifteen patients with cancers other than melanoma were skin test negative to Sephadex fraction II of melanoma; 14 nonmelanoma cancer patients did not react to Sephadex fraction II of black skin membrane preparations. However, 3 of 4 early stage breast cancer patients showed delayed hypersensitivity skin responses to Sephadex fraction III of melanoma and 1 of 3 to Sephadex fraction III of black skin, respectively.

Melanoma Sephadex fraction II and III and normal black skin cell membrane sonicates were selected for further separation by polyacrylamide gradient gel electrophoresis (PAGE). The proteins in each material migrated differently under comparable conditions. Fraction II contained traces of protein in PAGE region b, and fraction III contained traces in PAGE region a (Figs. 1 and 2A). The stability of the antigens kept at −70C prior

* Eli Lilly and Co., Indianapolis, IN.
† Varidase, Lederle Laboratories, Pearl River, NY.

TABLE 1. Skin Tests of Melanoma-Soluble Antigens Partially Separated by Sephadex G-200

Patients	Number of positive reactions*/Number of patients tested Sephadex fraction II		Sephadex fraction III	
	Melanoma	Black skin	Melanoma	Black skin
Melanoma Stages I and II	10/12	0/5	4/11	2/5
Melanoma Stages III and IV	5/12	0/5	9/11	0/5
Breast cancer Stage I	0/4	0/3	3/4	1/3
Breast cancer Stages III and IV	0/3	0/3	0/3	0/3
Other cancers	0/8	0/8	1/8	0/8

* >5 mm induration at 48 hours.

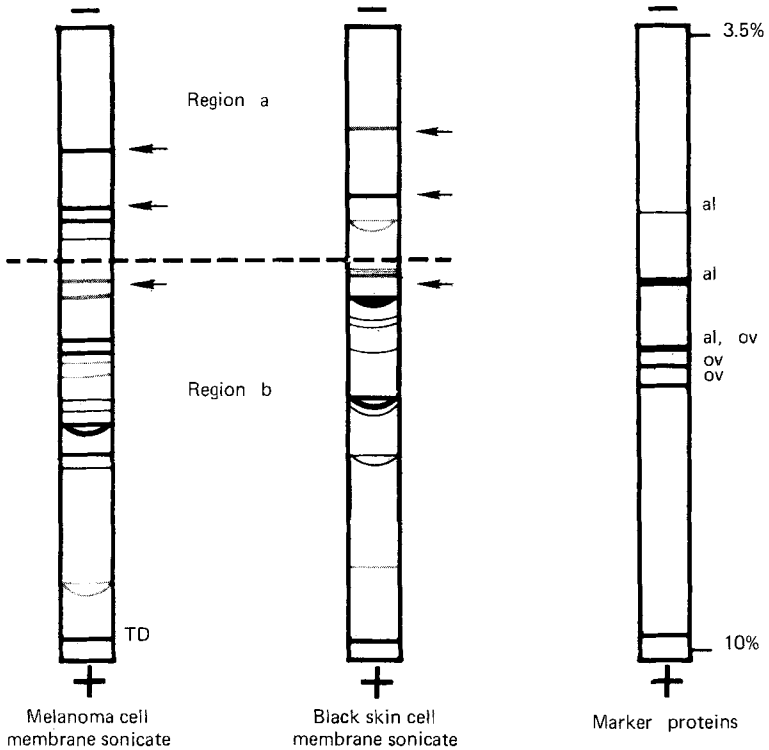

FIG. 1. Melanoma cell membrane sonicate and normal black skin cell membrane soluble sonicate fractionated by gradient polyacrylamide gel electrophoresis (3.5, 4.75, 7, and 10% stacked gels). A bromphenol blue tracking dye (TD) was used in each preparation. One hundred twenty-seven micrograms of melanoma sonicate and 110 μg black skin sonicate protein were run on separate gels under the same conditions. Non-enzymic protein molecular weight markers ovalbumin (2 × cryst.) (OV) and albumin (bovine) cryst. (AL) from Mann Research Laboratories, NY, kit number 20900-8109 were also run on separate gels but are shown together. Protein bands which stained with Coomassie brilliant blue are shown. Arrows point to bands which also stained with periodic-acid Schiff stain, indicating glycoproteins. Region a proteins predominate in PAGE separations of Sephadex fraction II and region b proteins predominate in PAGE separations of Sephadex fraction III.

to separation was determined (Fig. 2B). Two patients of the same sex were selected for skin tests: one a Caucasian with primary melanoma, and another a Negro patient with Stage I cervical cancer. Sixty micrograms each of melanoma Sephadex fractions II and III and 110 μg of normal black skin cell membrane sonicate were separated on each of six gels, with one gel of each set again stained for comparison with the electrophoretic patterns obtained with the first separations. The remaining five gels of each of these preparations were sliced, each of the two regions pooled (Fig. 1) and eluted with sterile saline at 4C for 50 hours, concentrated by ultrafiltration, rediluted 100-fold, and reconcentrated. The eluates were divided into two aliquots, coded, and immediately tested for skin reactivity in the two patients. As shown in Table 2, antigens of fraction II PAGE region a and of fraction III PAGE region b produced delayed skin reactions in the melanoma patient, but comparable material from black skin cells was negative.

Further tests were conducted using antigens separated by gel filtration and gel electrophoresis from allogeneic pools of selected primary tumors. The amounts of purified antigens were sharply limited since it required a large amount of primary tissue for the series of separations required for the isolation of the skin test antigens.

As shown in Table 3, six of nine early Stage I and II melanoma patients reacted to Sephadex fraction II gel region a; five of these early stage patients reacted to Sephadex fraction III gel region b. Two of seven later Stage III and IV nonanergic patients reacted to Sephadex fraction II gel region a; four of these seven patients reacted to Sephadex fraction III gel region b. Several patients did not react to either antigen.

Part of the low reactivity in these patients was due to a restriction in the protein content used: about 30 μg protein content in each test antigen. However, there was again (Table 1) an indication of decreased skin reactivity against the antigen from Sephadex fraction II in the later stages of disease (66% positive in early stages and 28% positive in later stages).

Control black skin PAGE region a produced no delayed skin reactions in five melanoma patients; however PAGE region b produced one positive and one partial skin response in early-stage melanoma patients but was negative in another early stage and in two late stage melanoma patients.

Two patients with breast cancer were se-

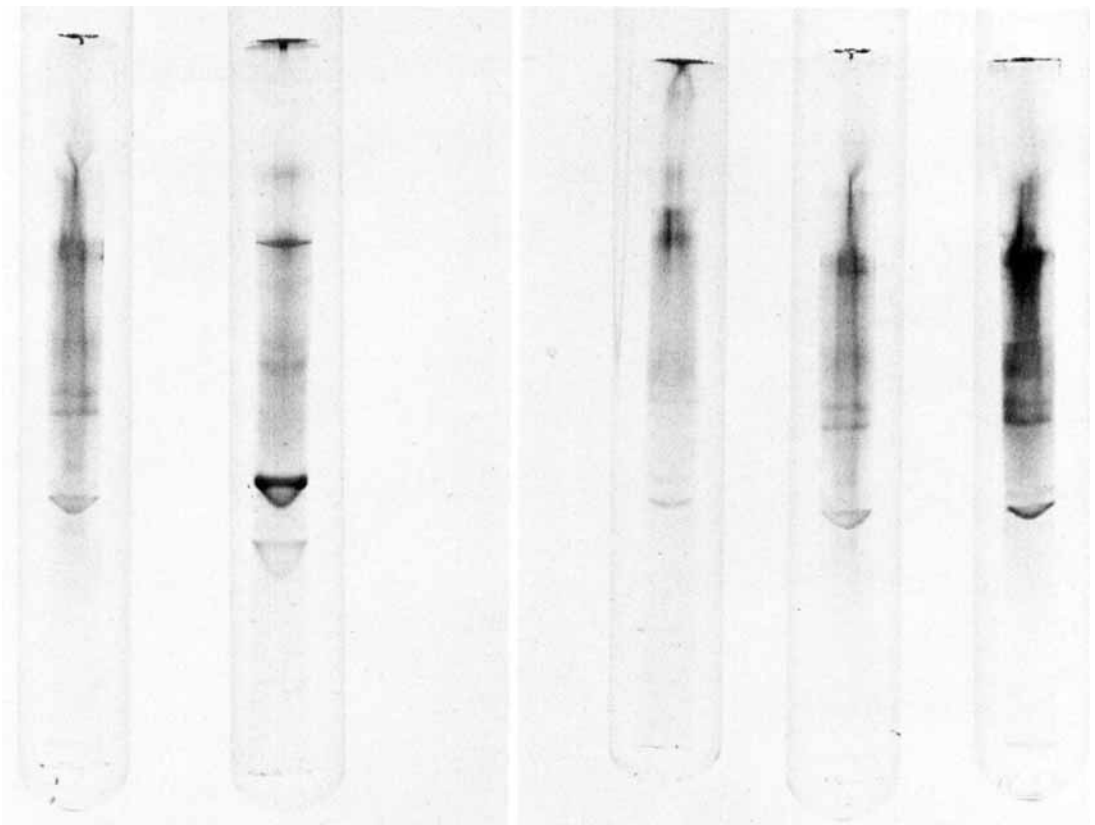

FIG. 2A (*left*). Melanoma cell sonicate (left) and black skin cell sonicate (right) PAGE separation (see Fig. 1). B (*right*). Gel separations. On left is shown PAGE separation of 127 μg protein melanoma sonicate preserved for 3 months in acetic acid and in the dark. In center is a fresh PAGE separation of 127 μg of the same preparation. On the right is a separation of 254 μg protein concentration to illustrate difficulty in separation if too much protein is used.

TABLE 2. Skin Tests of a Patient with Primary Melanoma Localized in the Right Lower Extremity and of a Patient with Stage I Squamous Cell Carcinoma of the Cervix with Melanoma Cell Membrane PAGE Soluble Antigens, Counterpart PAGE Regions, and Comparable PAGE Regions of Black Skin Cell Membrane Sonicate (Fig 1.)

PAGE preparation tested	Skin test results (induration: mm diameter)* Cervical cancer patient 24 hours	48 hours	Melanoma patient 24 hours	48 hours
Melanoma Sephadex fraction II				
gel region a	10	0	10	8
gel region b	7	0	9	3
Melanoma Sephadex fraction III				
gel region a	8	0	10	4
gel region b	7	4†	13	11
Black skin cell membrane				
sonicate gel region a	7	0	9	3
gel region b	6	0	5	0
Control antigens				
Mumps	10	11	8	10
SKSD	10	12	10	16

* A reaction of 5 mm or greater at 48 hours is considered positive.
† Shallow induration.

TABLE 3. Further Skin Tests of Melanoma-Soluble Antigens Separated by Sephadex G-200 and Further Separated by Polyacrylamide Gel Electrophoresis

Melanoma patient		Stage	Sex	Age	Recall antigens tested	Response to melanoma antigens (48-hour induration >5mm = +; >4mm = ±) Sephadex fx II PAGE region a	Sephadex fx III PAGE region b
Cot		I	F	54	PPD− Mumps+ SKSD+	+	0*
Tim		I	F	67	Mumps+	0*	0*
Gos		II	F	72	PPD+	+	+
Roj		II	M	52	Mumps+	+	+
Rob		II	F	46	NT	+	+
Car		II	M	55	PPD− Mumps−	+	±
Lit		II	F	64	PPD+ Mumps+ Histo+	0*	0*
Cra†		II	F	35	PPD+	0	0
Vil		II	M	41	PPD− Mumps+ Candida+ Varidase+	+	+
Smi-L		III	F	45	PPD+ Mumps+ Histo+	0	0
Del		IV	F	43	PPD− Candida+	0	0
Gra		IV	M	42	PPD+	0	0
Jah		IV	M	53	Mumps+	0	+
Poc		IV	M	51	PPD+	0	+
Cut		IV	F	62	PPD+ Mumps− Histo−	+	±
Des		IV	F	63	PPD+ Mumps+ Candida− Varidase−	+	+
						Response to black skin antigens PAGE region a	PAGE region b
Pat		I	F	51	Mumps+ SKSD+	0	+
Oto		II	M	60	Mumps− SKSD+	0	±
Cra		II	F	35	PPD+	0	0
Hil		IV	F	69	PPD+ Mumps+	0	0
Bel		IV	M	58	Mumps− SKSD+	0	0
	Control Patients						
Sha†	Cancer kidney	III	M	53	PPD+ Histo−	0	0
Woo†	Colon carcinoma Dukes' B		F	64	PPD+ Histo−	0	0
Spi	Breast cancer	II	F	47	PPD+ Mumps+ Histo+	0	+
Smi-R	Breast cancer	IV	F	30	PPD+ Mumps+ Histo+	0	0

TABLE 3. (*Continued*)

	Control patient	Stage	Sex	Age	Recall antigens tested	Response to melanoma antigens (48-hour induration >5mm = +; >4mm = ±) Sephadex fx II PAGE region a	Sephadex fx III PAGE region b
Lat	Bronchogenic cancer	III	M	63	PPD+ Mumps+ Candida+ Varidase−	0	0
You	Squamous cancer, pharynx	II	M	65	PPD+ Mumps+ Candida− Varidase−	0	0
Tre	Colon carcinoma		M	67	PPD+ Mumps−	0	0
Jon	Breast cancer	I	F	43	Mumps− SKSD+	0	+
						Response to black skin antigens PAGE region a	PAGE region b
Det	Squamous cancer, larynx	II	M	57	PPD+ Mumps+	0	0
Woo	Colon carcinoma Dukes' B		F	64	PPD+ *Histo*	0	0
Jon	Breast cancer	I	F	43	Mumps− SKSD+	0	0
Ore	Breast cancer	II	F	54	Mumps+ SKSD+	0	0
Sha	Cancer kidney	III	M	53	PPD+ Histo−		

* >8mm erythema.
† Also tested with black skin PAGE regions a and b: negative results.

lected for study, one with primary disease and the other with disseminated disease, since tests of other breast cancer patients with Sephadex fractions of melanoma soluble cell membrane preparations (Table 1) had indicated a cross-reaction between melanoma and breast cancer antigens. Therefore, the further separated gel regions were skin tested (Table 3) in order to see whether the PAGE separated antigens would produce these reactions. Fraction III b produced a positive reaction of >5 mm induration at 48 hours in Stage II breast cancer. This was repeated in a patient with Stage I breast cancer. There were no positive reactions to PAGE region a of fraction II in eight control nonmelanoma cancer patients. The PAGE separated black skin regions produced no delayed skin responses in five nonmelanoma cancer patients (Table 3).

DISCUSSION

It is of interest that soluble proteins which have been separated away from other components on the cell membrane appear to elicit cell-mediated immune responses. It is also of interest that these two melanoma soluble membrane antigens appear to differ in their ability to elicit positive responses at various stages of cancer. Although the relationship is not invariable, it may be useful to test additional patients to determine whether this apparent correlation with disease state is a general phenomenon and to determine the relationship of reactivity to these antigens to resistance against disease progression.

The nature of the detected antigens is a question of considerable interest and importance. Some antigens appeared to have produced specific delayed skin reactions in the melanoma patients. It is unlikely that the reactions seen were nonspecific, since some patients, although not anergic, did not react to these materials. In addition, patients with cancer of the cervix, kidney, colon, breast, and head and neck did not react to one of these antigens. Antigens common to many tumors of the same type might be tissue- or

organ-specific antigens, fetal antigens, or virus-induced antigens. We have no evidence to support the relationship to fetal antigens or to a virus. Such a possibility is suggested by the cross-reactivity seen in breast cancer patients with one of these antigens. It seems quite possible that tissue-specific antigens in addition to tumor-specific antigens may be detected. It was previously observed that membrane extracts of normal autologous skin could produce positive reactions.[2] Two of five melanoma patients tested with Sephadex fraction III from allogeneic black skin and two of six with PAGE region b gave positive reactions. These reactions were seen using concentrated proteins with different elution characteristics fractionated from the same region as the melanoma antigens. Reactivity to these control preparations might be due to the presence of small quantities of the reactive antigens in some preparations of normal skin. Alternatively, the antigens in melanomas and in normal skin might be different. A further separation by gradient polyacrylamide gel electrophoresis indicated that there are some differences in the banded soluble protein patterns of melanoma and black skin cells. It is of interest that this further purified material did not produce skin reactions in head and neck, mammary, cervical, colonic, or renal cancer patients.

The problem of distinguishing between reactivity to tumor-associated melanoma antigens and to normal tissue antigens is also seen in lymphocyte microcytotoxicity studies.[10] Both melanoma patients and many normal Negroes have in vitro reactivity against tissue culture cells derived from melanoma.

The rarity of spontaneous regression in patients with established melanoma and the disappointing results of attempted therapy with autoimmunization or irradiated whole tumor cells,[20,33] might possibly indicate that there are blocking factors or interfering factors present in whole membranes and whole cell extracts which prevent the recognition of any new specific or nonspecific proteins present on the malignant cell membrane. The appearance of reactivity to the separated antigens indicates that there may be blocking or inhibitory factors in the unfractionated materials which prevent skin reactivity. We have described the presence of a blocking factor(s) in unseparated melanoma sonicates as well as in other cancer cell membrane sonicates.[18]

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