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Research Article

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Serial Studies of Autologous Antibody Reactivity to Melanoma

Relationship to Clinical Course and Circulating Immune Complexes

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Abstract

The low titer and incidence of autologous antibody to melanoma has hampered its evaluation. Through acid dissociation and ultrafiltration of serum, we have been able to augment the autologous immune response in 9 of 10 patients studied. This result suggests that autologous antibody is present in most patients with melanoma, but is obscured by circulating antigen and the formation of immune complexes. Because native antibody and antibody derived from circulating immune complexes are produced by the host against physiologically relevant antigens, correlations can be made to clinical course. Serological studies of three patients with melanoma were performed with serum samples obtained over many months; these studies demonstrated correlations with tumor progression and clinical course. Serial serologic studies may yet provide one of the better ways to evaluate these relationships. They have the advantage of detecting transient events that may occur with the inception of metastatic disease or autoimmune phenomena, and of avoiding the difficulties encountered in comparing antibody responses between different individuals.

Introduction

Over the past several years various antibodies have been used to detect tumor-associated antigens of melanoma and other tumors. These antibodies have included polyclonal xenogeneic and allogeneic antisera (1-7) and, more recently, monoclonal murine and human antibodies (8-14). Numerous cell surface antigens have been described using these probes, which are beginning to define an "antigenic map" of various tumors. We have focused our efforts on the autologous immune response to melanoma. These host-derived antibodies may detect antigens not previously identified by heteroantisera. Equally important, because autologous antibodies are produced against physiologically relevant antigens, they may increase our understanding of the nature of the host's immune response and its relationship to disease status.

The study of the autologous immune response to melanoma has been hampered by the low incidence and titer of these antibodies. In native sera, we and others have found low-titer autologous antibody to melanoma in one-third of melanoma patients (15-19). Our failure to detect autologous antibody to melanoma more frequently led us to postulate that this might

be due to circulating antigen and the formation of immune complexes. To pursue this theory, we modified a technique reported by Sjogren et al. (20) in order to dissociate antigen from antibody by acid exposure, followed by ultrafiltration. We have recently reported that this technique enhanced autologous antibody reactivity in six of the seven cases we studied (19).

Preliminary work suggests that immune complexes are a relevant and accessible source of autologous antibody and antigen. There is a substantial body of literature describing the presence of elevated levels of immune complexes in malignancy (21-23). Immune complex levels have been correlated with disease status, tumor burden, and prognosis (24, 25). If circulating immune complexes prove to be a reservoir of antibody to autologous tumor, then measurement of circulating immune complex levels may be an important correlate of autologous serological studies.

One of the better ways to analyze the relationship between autologous antitumor antibody titer, immune complexes, and clinical course is through serial studies of individual patients. Serial studies have the advantage of detecting transient events that may occur at the inception of metastatic disease, or the development of associated autoimmune phenomena. In addition, serial studies avoid the difficulties found in comparing antibody responses between different individuals. In this paper, we report our findings relating the autologous immune response to clinical course and the level of circulating immune complexes in three patients over the course of their disease.

Methods

Cell lines

Melanoma cells. Tumor acquisition followed a protocol approved by the institutional review board. Preparation maintenance and identification of tumor lines have been reported previously (19). Melanoma cell line M14 was kindly supplied by Dr. Darwin O. Chee of Scott Laboratories, West Warwick, RI. Cultures established at three or more passages were used in serologic assays.

Nonmelanoma cells

Acquisition and maintenance of nonmelanoma cell lines have been reported previously (19). Additional cell lines used for specificity testing include: breast carcinoma cell line (BCA), supplied by Dr. James Robinson (Yale University School of Medicine, New Haven, CT); renal cell carcinoma cell line (HCRC), supplied by Dr. Neil Bander (Memorial Sloan Kettering Hospital, New York); ovarian (SK-OV) and head and neck (A253) carcinoma cell lines, supplied by Dr. John S. Lazo (Yale University School of Medicine); and T-cell leukemia cell line (CEM), supplied by Dr. William Hait (Yale University School of Medicine).

Serologic methods

Serial serum specimens are routinely obtained on all patients followed by the Yale Melanoma Unit. Aliquots of serum are stored at -70°C until ready for use.

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Protein A hemadsorption assay. The protein A hemadsorption assay was modified after a method described by Pfreundschuh et al. (26). Indicator cells were prepared from staphylococcal protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) conjugated to human blood group O Rh(+) erythrocytes with 0.01% CrCl₃, pH 5.0, at equal volumes of packed erythrocytes and protein A. A positive cell was one in which ≥50% of the cell surface is marked by indicator cells. The endpoint of the assay was the last dilution with ≥10% of melanoma cells (+). Absorption analysis was performed as previously described (19), using a minimum of 1.0×10^8 cells/ml of diluted serum.

Acid dissociation and ultrafiltration

The method described by Sjogren et al. (20) was used to dissociate immune complexes. 3 ml of prefiltered (0.45 M; Millipore Corp., Bedford, MA) serum were added to 50 ml of glycine-saline buffer (0.1 M, pH 3.1) in a 60-ml ultrafiltration chamber fitted with an XM-100 membrane (Amicon Corp., Danvers, MA). Ultrafiltration was performed at 4°C under 10 psi N₂ until the original serum specimen volume was reached. This was repeated twice and then washed three times with 50 ml PBS.

Immune complex assays

Raji cell binding assay. The Raji cell assay was modified after the method of Theofilopoulos et al. (27). All tests were done in triplicate. Raji cells were kindly provided by Dr. G. Miller (Yale University School of Medicine). 25 μl of sera were added to each tube. Serial dilutions of aggregated IgG, produced by incubating human IgG (Sigma Chemical Co., St. Louis, MO) for 10 min at 63°C, to which 25 μl of normal human sera was added were used as a positive control. All tubes were incubated at 37°C for 30 min. Raji cells were washed twice in RPMI and resuspended at 2×10^6 cells/50 μl. 50 μl of Raji cells were added to each tube, mixed, and incubated at 37°C for 45 min with agitation. Raji cells were washed with 2 ml RPMI plus 0.5% BSA and spun at 1,500 rpm for 15 min twice. The pellet was gently resuspended in the remaining volume and 100 μl of ¹²⁵I rabbit anti-human IgG (New England Nuclear, Boston, MA) was added at 20,000 cpm per tube. The tubes were incubated 1 h on ice, washed twice in RPMI, and counted. Counts were calculated as follows: % binding = (counts/tube)/(counts/standard) × 100. Binding was compared against a standard curve of aggregated human IgG and 15 normal human sera.

¹²⁵I-C1q binding assay. C1q was isolated from normal human serum using the technique of Zubler and Lambert (28) and radiolabeled with ¹²⁵I by the lactoperoxidase technique. The C1q-binding test was performed following the method of Zubler and Lambert (28). All tests were done in duplicate. 50 μl of test serum was mixed with 100 μl of 0.2 M EDTA, pH 7.5, and incubated 30 min at 37°C. Tubes were then placed on ice and 50 μl of ¹²⁵I-C1q followed by 1 ml of 3% polyethylene glycol were added and allowed to stand for 1 h. All tubes were spun at 1,500 g for 20 min at 4°C. The supernatant was completely discarded and the radioactivity of the precipitate counted. ¹²⁵I-C1q binding was calculated in the same fashion as the Raji cell-binding assay.

Results

Acid dissociation and ultrafiltration of sera. Native sera from 10 patients were subjected to acid dissociation and ultrafiltration. Previously, only three sera demonstrated reactivity against autologous cultured melanoma cells as tested by protein A hemadsorption. After acid dissociation and ultrafiltration, 9 of 10 sera showed enhanced IgG reactivity against melanoma. These results are shown in Table I. As reported previously (19), sera from six normal individuals showed no reactivity to melanoma cells before or after acid dissociation and ultrafiltration.

Analysis of system Y-Mel 83:070. Before acid dissociation

Table I. Titers of Melanoma Patients' Sera Tested by Protein A Hemadsorption before and after Ultrafiltration against Autologous Cultured Tumor

Melanoma	Sera	Native serum	Acidified serum	Acidified ultrafiltered serum
81-370	998	0	0	0
81-060	1,041	0	0	1:8
81-090	1,071	0	0	1:64
81-180	1,375	0	0	1:128
81-170	1,276	0	0	1:256
81-120	870	1:4	1:4	1:16
78-010	781	1:64	1:64	1:128
83-070	2,362	1:32	1:32	1:128
82-550	2,280	0	0	1:128
81-710	1,969	0	0	1:128

and ultrafiltration, serum S2362 showed reactivity to only 1:32 against autologous cultured melanoma Y-Mel 83:070. After acid dissociation and ultrafiltration, enhanced reactivity was noted to 1:512, the highest titer yet observed (Table I). As seen in Fig. 1, addition of autologous serum eluate from ultrafiltration obtained within the first 30 min ablated enhanced reactivity. Less of a reduction in enhanced reactivity was seen with the addition of fraction 2, obtained at 60 min, and no reduction was seen with fractions 3 and 4. Reactivity of acid-dissociated and ultrafiltered serum was also reduced by the addition of spent autologous tumor culture media and native serum. One possible interpretation of this experiment is that circulating antigen is removed by acid dissociation and ultrafiltration of serum and that it is also present in spent media and native serum.

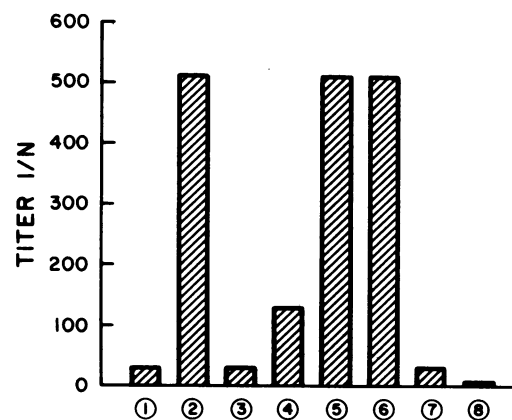


Figure 1. Absorption of autologous S2362 reactivity by antigen eluted from immune complexes. This illustrates the reactivity of serum S2362 against autologous cultured melanoma Y-Mel 83:070. Native sera S2362 (lane 1) gave a titer of 1:32. Acid dissociated and ultrafiltered sera S2362 (lane 2) resulted in a titer of 1:512. Addition of an equal volume of autologous eluate obtained within the first 30 min of ultrafiltration ablated enhanced reactivity (lane 3). Less of a reduction in enhanced reactivity was seen with fraction 2, obtained at 30-60 min (lane 4). No reduction in reactivity was seen in fractions 3 (60-90 min) and 4 (90-120 min) (lanes 5 and 6). Enhanced reactivity was also ablated by the addition of equal volumes of autologous spent tumor culture media (lane 7) and native sera S2362 (lane 8).

Specificity analysis of sera. To determine the specificity of acid-dissociated and ultrafiltered sera, we performed direct and absorption analyses on selected sera from the three autologous systems. Reactivity of serum S1375, from autologous system Y-Mel 81:180, against various normal and malignant cell lines has been previously reported (19). S1375 was found to detect an antigen common to melanoma, sarcoma, glioblastoma, neuroblastoma, and breast carcinoma cell lines. Specificity analysis of sera S2280 and S1969 from respective autologous systems Y-Mel 82:550 and Y-Mel 81:710 are summarized in Table II.

Serial autologous studies

Serial autologous studies were performed on three autologous systems. Sera were obtained at 1–4-mo intervals and tested before and after acid dissociation and ultrafiltration against

Table II. Serological Definition of Systems Y-MEL 82:550 and Y-MEL 81:710 by Direct* and Absorption‡ Analysis

Cell line	Serum			
	S2280		S1969	
	Direct	Absorption	Direct	Absorption
Human melanoma				
Y-Mel 82:550§	+	+	+	+
Y-Mel 81:710	+	+	+	+
Y-Mel 81:180	+	+	+	+
Y-Mel 84:255	+	+	+	+
M14	+	+	+	+
Human nonmelanoma				
Breast carcinoma, BCA	+	+	+	+
Colon carcinoma, HCT-8	0	0	0	0
Renal cell carcinoma, HCRC	0	+	0	+
Sarcoma, MG-63	0	0	0	0
Head and neck carcinoma, A253	0	+	+	+
Ovarian carcinoma, SK-OV	0	0	0	0
T-Cell leukemia, CEM	0	0	0	0
Fetal fibroblasts	0	0	0	0
Autologous lymphocytes		0		0
Pooled platelets		0		0
Xenogeneic				
Sheep RBC		0		0
Fetal calf serum		0		0
Bacillis Calmette-Guerin		0		0

* Direct testing was performed by protein A hemadsorption. A positive cell was one in which 50% of the cell surface was marked by indicator cells. A positive test (+) was one, at a serum dilution of 1:4, where 10% of the cells were (+).

‡ Absorption analysis was performed by mixing cells, at a ratio of 1:1 (vol/vol) (1×10^8 cells/ml minimum), with the serum to be tested, at a serum dilution two doublings below the endpoint of the titration for the serum. A positive (+) absorption test was one that resulted in at least a twofold drop in antibody titer when compared to unadsorbed serum.

§ Corresponding autologous serum: S2280.

^{||} Corresponding autologous serum: S1969.

autologous melanoma cells by protein A hemadsorption. Results were then correlated with clinical status.

System Y-Mel 81:180. In this patient with progressive metastatic disease, no rise in reactivity was seen in native serum. After acid dissociation and ultrafiltration persistently elevated titers were seen. The one significant drop in titer may be related to a specific episode. These results are illustrated in Fig. 2.

System Y-Mel 82:550. In this patient with resected stage II melanoma, a rise in autologous antibody detectable in native serum paralleled the clinical appearance of vitiligo. The titer of the acid-dissociated and ultrafiltered serum fraction remained elevated throughout as illustrated in Fig. 3.

System Y-Mel 81:710. Serial studies of this patient with metastatic melanoma were performed. While native sera showed no rise in autologous antibody titer, acid-dissociated and ultrafiltered sera demonstrated a rise in titer that paralleled disease progression. Of note are two significant drops in titer of the derived serum; both followed the administration of human leukocyte interferon for periods of 7 wk at dosages of $5-20 \times 10^6$ U/d i.m. (29). These results are illustrated in Fig. 4.

Immune complex detection

The Raji cell assay was used to detect immune complexes in all three serial studies. In all three cases no significant rise in circulating immune complex levels was seen when compared to 15 normal controls. The ¹²⁵I-C1q binding assay failed to detect elevated levels of circulating immune complexes in system Y-Mel 82:550. Finally, serial immunoglobulin levels obtained from Y-Mel 81:710 failed to show any significant rise (29).

Discussion

The definition and characterization of melanoma cell surface antigens has been the goal of studies using monoclonal,

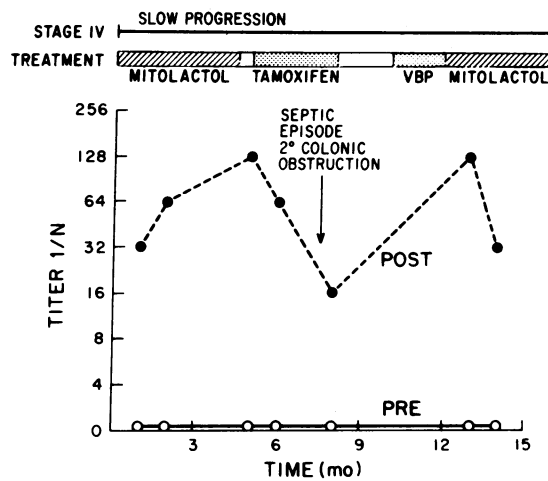


Figure 2. Serial autologous antibody titers Y-Mel 81:180. Serial serum samples were obtained from this patient with progressive metastatic melanoma. No significant rise was seen in native (pre) autologous antibody titers. Dissociated serum (post) titers demonstrated no significant change except for a drop at month 8, which might have been secondary to a septic episode. Note also that hormonal or chemotherapeutic agents had no effect on dissociated antibody titers. VBP, velban, bleomycin, cis-platinum.

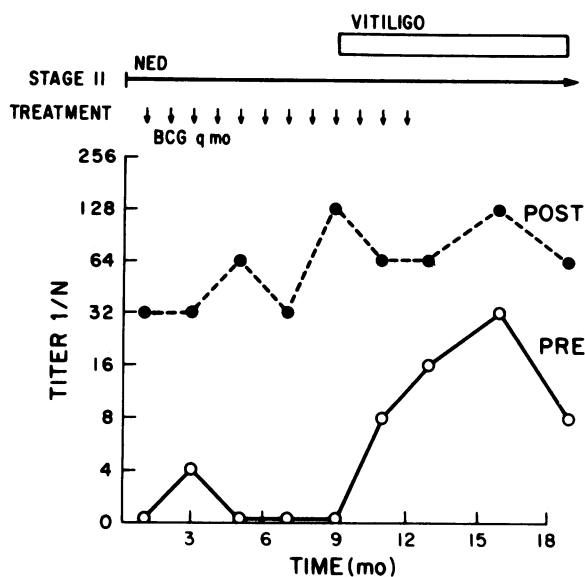


Figure 3. Serial autologous antibody titers Y-Mel 82:550. This is from a patient with resected stage II melanoma. Sera obtained at 2-3-mo intervals were subjected to acid dissociation and ultrafiltration and tested against autologous melanoma by protein A hemadsorption. A rise in autologous antibody detectable in native serum (pre) paralleled the clinical appearance of vitiligo. Acid dissociated ultrafiltered serum (post) revealed no significant change in retrievable antibody over this period. The total pool of antibody contained free in serum and bound in immune complexes may thus be relatively constant, and the free antibody (pre) reflects a decrease in antigen. NED, no evidence of disease.

heterologous, and, as in this report, autologous antibody. Heterologous tumor antibodies to human cancer have generally been found, when sufficiently studied, to react against normal

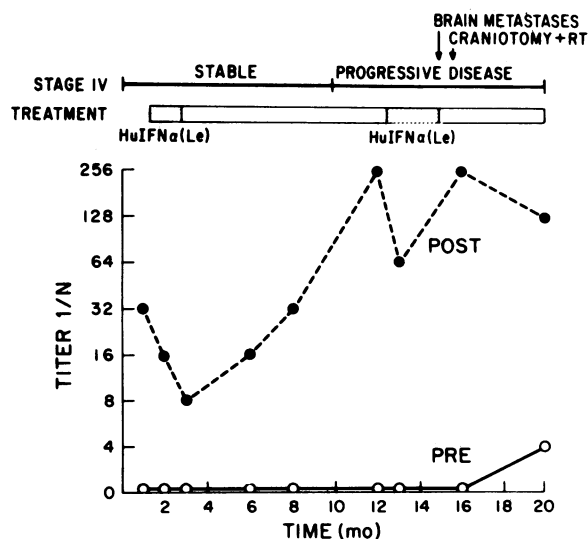


Figure 4. Serial autologous antibody titers Y-Mel 81:710. This is from a patient with stage IV metastatic melanoma. Sera was obtained and treated as in Fig. 2 and tested for autologous antibody. No significant rise in antibody was detected in native serum (pre). Dissociated serum (post) demonstrated a rise in antibody titer that paralleled disease course. Of note were two significant drops in dissociated antibody titer that occurred after the administration of human leukocyte interferon (HuIFN α (Le)).

cell constituents (30). We have elected to pursue the autologous immune response because it offers a greater likelihood of detecting tumor-restricted antigens (15-19). Equally important, autologous antibody titers may be correlated with the clinical course. Serial studies of autologous antibody may allow the detection of transient events that are not apparent when only individual samples are examined.

This report expands our previous experience (19), documenting results in 10 autologous systems, all but one of which has demonstrated enhanced reactivity after acid dissociation and ultrafiltration of serum (Table I). The results of these studies suggest that antibody directed against autologous tumor cell surface antigens occurs in most patients with melanoma, but is obscured by circulating antigen that forms immune complexes. Enhanced autologous antibody reactivity after acid dissociation and ultrafiltration was ablated not only by the acid eluate, but by spent media of melanoma tissue culture and also by the addition of native autologous serum. We conclude that antigen detected by autologous antibody is (a) shed by tumor cells in tissue culture, as reported by others (31), and (b) present in the serum of melanoma patients. Detection and quantification of tumor antigen present in sera may provide a new means of tumor detection and assessment of tumor burden.

The three serial studies described suggest clinical correlations between autologous antibody titer and disease course. No generalization can be drawn, but system Y-Mel 82:550 provides the most dramatic clinical correlation. A rise in native antibody titer against autologous melanoma was noted to occur with the onset of vitiligo. These findings lend support to recent work of others who report that vitiligo is associated with serum antibodies directed at melanoma, as well as cultured melanocytes (32-34). It is unclear what the incidence of antibodies to melanocytes or melanoma is among melanoma patients. Prior investigations have only rarely found antibody to melanoma or melanocytes among patients with vitiligo. The development of vitiligo in patients with melanoma has been considered to be an indication of improved prognosis (35). This might be due to the development of a host immune response directed against both melanocytes and melanoma cells. Notably, native serum antibody titer rose against autologous melanoma, Y-Mel 82:550, while no rise in the titer of serum antibody dissociated by exposure to acid and ultrafiltered, was found. One explanation may be that in this setting, the total pool of antibody that is contained free in serum and bound in immune complexes may be relatively constant, and the appearance of free antibody may reflect a decrease in antigen.

Studies of melanoma Y-Mel 81:710 demonstrate a relationship of autologous antibody to progressive disease and interferon therapy in a patient with metastatic melanoma (Fig. 4). A large burden of tumor antigen may have obscured changes in native antibody titer. Two significant drops in the titer of antibody retrieved after acid dissociation and ultrafiltration occurred following therapy of this patient with human leukocyte interferon (29). Similar reductions of retrieved-antibody titer during interferon treatment on two occasions makes it doubtful that these were random fluctuations or unrelated events. Interferon is capable of inhibiting antibody production and other protein synthesis by B cells. Humoral and cellular immunity have been shown to be susceptible to interferon modulation in a time-dependent fashion (36). No drop in retrieved antibody titer was seen with conventional

chemotherapy or hormonal therapy in the Y-Mel 81:180 system. Only a septic episode reduced antibody reactivity.

The antigens detected by the three autologous antibodies reported here are broadly represented in melanoma and some other tumors (Table II) (19). Comparison between the three systems reveals a similar range of reactivity. Cross-reactivity between all three antibodies is noted as well. Pfreundschuh et al. (26) noted cross-reactivity of antibody against the astrocytoma antigen (AJ) when surveyed against allogeneic astrocytomas, neuroblastomas, sarcomas, and a mixed mesodermal tumor. Cross-reactivity between the AJ astrocytoma antigen and the AH melanoma antigen described by Shiku et al. (17) was implied, although a direct comparison has not been presented. It is possible that the three autologous systems reported here are detecting a similar antigen and that this antigen is also related to those reported by others (17, 26). This may be determined by immunochemical analysis of these antigens that is currently underway.

Circulating immune complex levels in these patients exhibited no simple relationship to antibody titers. This may be a reflection of the imperfection of the assays available at present for immune complexes. Most assays detect larger, complement-binding immune complexes preferentially. No assay currently available reliably detects small noncomplement binding immune complexes as might be found in patients with malignancy and large quantities of circulating antigen. Alternative approaches of potential value currently under evaluation include precipitation of immune complexes by polyethylene glycol and subsequent acid dissociation and ultrafiltration or adsorption by lectins, followed by elution using appropriate sugars or physical means.

The role autologous antibody and circulating immune complexes play in the host response to cancer remains to be determined. Circulating immune complexes and tumor antigen have been implicated in immunosuppression (37-39). It is unknown whether the antigen and antibody detected in this study are a reflection of the host's response to malignancy or are causally involved in the modification of that response. These results demonstrate that autologous antibody reactivity is detectable, but obscured in the serum of most patients with melanoma. Further, new results reported here correlate clinical course with the serum studies of native and derived autologous antibody. Serial studies provide a unique probe through which we will be able to define the physiologic relevance of autologous antibody in malignant disease.

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