Augmentation of Autologous Antibody to Human Melanoma following Acid Dissociation and Ultrafiltration of Serum

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ABSTRACT

Sera of 22 individuals were examined for immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody to autologous cultured tumor cells. Reactive native IgM was detected by immune adherence in four, and IgG by Protein A hemadsorption in five, sera. Direct and absorption testing of these reactive sera against a range of normal and neoplastic cells revealed one each with specificity for a highly restricted melanoma cell surface antigen and common tumor-associated antigen of melanoma. Given this low prevalence of antibody, we then tested whether IgG antibody might be retrieved from circulating immune complexes in melanoma patients’ sera. Acidification and ultrafiltration of sera from seven patients have enhanced detectable IgG binding to autologous cultured melanoma in six. Characterization of one reactive autologous antibody has detected a common antigen in eight of nine melanoma lines tested. This antibody also detected two neuroblastomas, one of two glioblastomas, one of two sarcomas, and one of two breast carcinomas. The common melanoma antigen detected in these tests may be related to the neuroectodermal, oncofetal differentiation antigens described by others with autologous or allogeneic IgM. Autologous antitumor antibody in circulating immune complexes may provide a source of antibody for serodiagnostic and therapeutic applications relevant to treatment modalities, such as plasmapheresis and plasma perfusion over Protein A in melanoma and other cancers.

INTRODUCTION

Autologous antibody responses to human tumors have been infrequently detected. When found, they have generally been of insufficient titer to resolve questions of specificity. Carey et al. (4, 5) and Shiku et al. (28, 29), using sensitive microsorosological assays, have demonstrated antibodies against cultured autologous tumor cell surface antigens in one-third of the 18 melanoma patients studied. The antigens described have been divided into 3 classes: those restricted to the tumor of a single individual (Class I); those shared among tumors of a similar histotype or ontogeny, but rarely detected in nonneoplastic cells (Class II); or those more widely distributed in nonneoplastic as well as neoplastic cells (Class III). Our initial studies of cultured melanoma cell lines and freshly cryopreserved tumor tissue reported here have confirmed the presence of antibody to antigens representing each of these classes in 22 patients. Autologous antibody responses to cultured melanoma were demonstrated by tests of techniques of Protein A hemadsorption (20) and immune adherence (28, 29). While antibody to autologous tumor was demonstrable in less than one-third of patients, tumor-restricted antibody could be shown in only one-third (2 of 6) of reactive sera, by direct and absorption testing.

We postulated that our inability to detect autologous antibody to cell surface antigens might be due to the presence of circulating antigen and the formation of immune complexes. Increased levels of circulating immune complexes have been detected in a variety of cancers (2, 5, 11, 14, 15, 21, 22, 26, 27, 32, 33). In melanoma, as in other human and experimental cancers, circulating immune complexes have been correlated with prognosis and stage of disease (21, 26).

The mechanism of action for circulating immune complexes in cancer is incompletely understood, but there is ample evidence for a role in the modulation of both cellular and humoral immune responses (2, 33). Sjögren et al. (30) developed a method of analyzing serum inhibition of lymphocyte cytotoxicity by acid dissociation and ultrafiltration of the serum. This method has been applied here to autologous serological study of human melanoma, with detection of an increased incidence of antibody to melanoma in 6 of 7 patients studied.

MATERIALS AND METHODS

Patient Tumor Cell and Normal Cell Cultures. Two primary melanomas and a number of metastatic melanomas from soft tissues, lymph nodes, and visceral sites were studied. Tissue acquisition followed a protocol approved by the Institutional Review Board (HIC 1757). Tumor specimens placed in cell cultures were identified by means of gross pigmentation in monolayer culture or on addition of 1 μg dihydroxyphenylalanine/ml in several specimens which were equivocally pigmented in routine media. The presence of premelanosomes on electron microscopy corroborated the microscopic assessment of melanoma cell cultures in 2 cases.

Sterile tumor specimens for cell culture were dissociated mechanically or digested in Clostridium histolyticum collagenase (0.1 units/ml; Sigma Chemical Co., St. Louis, MO) and DNase in EDTA (0.5 mM) with trypsin (0.25%); Sigma) for 4 to 12 hr at 37° in a humidified atmosphere of 5% CO2. Preparations were passed through sterile 2-ply gauze, washed by centrifugation, and assessed by trypan blue dye exclusion for viability prior to tissue culture in Ham’s F-10 or Eagle’s minimal essential medium with penicillin (100 units/ml); streptomycin (100 μg/ml); 2 mM L-glutamine; 1% nonessential amino acids (complete medium) supplemented with 30% prescreened FCS (Grand Island Biological Co., Grand Island, NY). Multiple aliquots of 1 x 106 to 5 x 106 viable cells were frozen stepwise for storage in liquid nitrogen with complete medium containing 20% FCS or 5% agamaglobulinemic normal human serum (Connecticut Red Cross, Farmington, CT) with 10% dimethyl sulfoxide added immediately prior to freezing. FCS was reduced to 5 to 10% for established cultures, and 5% prescreened agamaglobulinemic human serum was used in place of FCS for selected direct and absorption tests. Melanoma cell lines Y-Mel 78:010, 80:120, and 81:180 were successfully subcultured in vivo using female BALB/c-nu/nu nude mice (The Jackson Laboratory, Bar Harbor, ME). Cultures were routinely checked for fungal, bacterial, and Mycoplasma contaminants by culture and Hoechst stain (Flow

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Laboratories, Inc., Rockville, MD). Contaminated cultures were discarded. Cultures were not used for serological studies after passage 12, as a consequence of the diminished reactivity observed in late passages.

Peripheral blood lymphocytes were separated upon Ficoll-Hypaque density cushions, and populations of B- and T-cells were separated by rosetting with sheep erythrocytes in a second stage, using neuraminidase-treated erythrocytes according to the method of Henderson et al. (12). B-cells collected from the interface of the secondary Ficoll-Hypaque gradient (1 x 10^6 B-cells in 0.1 ml medium) were infected with Epstein-Barr virus kindly provided by Dr. Robinson (Department of Pediatrics, Yale University School of Medicine) at a titer of 1 x 10^3 to 10^4 to diminish reactivity of 50% tissue culture infective doses per ml. Cells were incubated with the virus stock overnight and seeded for culture in fresh medium the following morning, at 12 to 18 h posttransformation.

Normal fibroblasts were cultured from skin obtained from patients with melanoma or from placental and neonatal foreskin specimens kindly provided by the Department of Obstetrics, Yale University School of Medicine. Fibroblast cultures were maintained as monolayers in complete medium as for melanoma. Normal melanocytes were cultured from fetal foreskins obtained as for fibroblasts by the method of Eisinger and Marko (8) as modified by Dr. R. Halaban, Department of Dermatology, Yale University.

Neuroblastoma cell lines 141N and SKNSH were kindly provided by Dr. June Biedler, Memorial Sloan Kettering Institute, Rye, NY. Two cell culture lines derived from glioblastoma multiforme (GB867, GB5293) were provided by Dr. M. Sauer (Department of Neurology, Yale University). Two osteosarcoma (MG63, G292) and 2 breast carcinoma cell lines COLO355 were kindly supplied by Dr. Chris Benz (Department of Medicine, Yale University). One mouse melanoma cell line (B16) was provided by Dr. Alan Sartorelli (Department of Pharmacology, Yale University).

Preparation of Urinary β2-Microglobulin. β2-Microglobulin was isolated from the urine of a patient with interstitial nephritis following renal transplantation by methods similar to those of Berggard and Beam (3). The resultant material was resuspended and dialyzed against distilled water, concentrated by lyophilization, and stored frozen at -70°, subaliquoted at 10 µg/ml, as determined by radioimmunoassay (Pharmacia Fine Chemicals, Uppsala, Sweden).

Immune complexes of β2-microglobulin: Anti-β2-microglobulin were prepared by 1:1 mixture with a 1,500 dilution of rabbit anti-β2-microglobulin (60 µg/ml; Dako, Westbury, NY) in PBS plus 4% agammaglobulinemic FCS. The mixture was incubated 30 min at 4° and 30 min at 37°, and the presence of complexes was tested by both turbidimetric and Clq binding assays.

Acid Dissociation and Ultrafiltration. The method described by Sjogren et al. (30) was used to dissociate immune complexes. Three ml of prefiltred (0.45 µm; Millipore, Bedford, MA) serum or other test solutions were added to 50 ml of glycine:saline buffer (0.1 M, pH 3.1) in a 60-ml ultrafiltration chamber with XM-100 membrane (Amicon Corp.). Ultrafiltration was performed at 4° under 10 psi N2 until the original serum specimen volume was reached. This was repeated twice and then washed 3 times with 50 ml PBS, the total time involved ranging from 24 to 48 hr, for completion, in total.

Control specimens of serum or artificially prepared immune complexes were held at 4° or acidified without ultrafiltration at 4° and dialyzed to correct pH against PBS using M, 8000 exclusion tubing.

Serological Methods. Immune adherence assays for the detection of IgM were performed by the method of Nelson as applied by Shiku et al. (5, 29) with minor modification. Tests were read as follows: +, if ≥50% of cell surface is marked by indicator cells, or ≥3 erythrocytes are attached per target cell; −, if indicator erythrocytes are bound to <50% of the cell surface or <3 erythrocytes are attached per target cell. The last dilution at which ≥10% of target cells were positive was taken as the end point. Control sera and saline blanks gave background readings of <1% (+) cells. Positive control rabbit antiserum to β2-microglobulin (Dako) was included in each test, yielding a titer of 1:250 to 1:500.

Assays were performed and confirmed in duplicate. Sera which were positive at low titer (≤1:8) were analyzed only by direct tests.

The PAHA was carried out with serum incubations as for IA. Indicator cells for PAHA were prepared from staphylococcal Protein A (Pharmacia Fine Chemicals) conjugated to selected human Blood Group O, Rh-positive RBC in 0.01% CrCl3 at pH 5.0, at equal volumes of packed RBC and Protein A (1 mg/ml) (20). The end point of the assay, read as for IA, is the last well with ≥20% of melanoma cells (+).

Absorption tests were performed as described by Carey et al. (4, 5), at 2 doubling dilutions before the end point of sera with titers >1:8. A minimum of 1.0 x 10^6 cells was used per ml of diluted serum. Cells utilized for absorption were detached mechanically or by brief exposure to 0.5 mM EDTA, without use of trypsin to avoid potential destruction of protease-sensitive antigens. The ultrafiltered specimen and freshly thawed serum or anti-β2-microglobulin were tested in parallel.

RESULTS

Reactivity of Native Serum. Autologous serological reactions were detected by IA testing at 4° and 37° against 4 tumors. Three of these were optimal at 37° while one was optimal at 4°. Two of the 3 reactions observed at 37° were found with sera positive also by PAHA. Two other autologous reactions, to Y-Mel 79:090 and 81:180, were never stronger than 1:8 and did not allow reliable absorption analysis or further detailed examination of specificity. Sporadic reactions first detected in autologous whole serum tested against Y-Mel 81:180 by IA appear to follow a pattern which was more readily analyzed with the acid-dissociated and ultrafiltered Specimen S1375 as below.

Y-Mel 80:120 exhibited a cell surface antigen detected by autologous sera (especially S973) by direct and absorption tests.4 Direct studies of S973 gave positive results against a single other melanoma (Y-Mel 78:010), but no other malignant or nonmalignant cells tested (Table 1). Absorption of S973 with Y-Mel 78:010 failed to diminish the reaction against Y-Mel 80:120. The melanoma Y-Mel 78:010 was also tested for absorption of S973 with a line of B-cells established from the donor of Y-Mel 78:010. The S973 antibody reaction against Y-Mel 78:010 was removed by this absorption. Thus, HLA antigens appear to be the basis of reactivity of S973 with Y-Mel 78:010. Serial serum specimens tested for autologous antibody to Y-Mel 80:120 during the course of this patient’s life revealed a preterminal drop in titer from 1:16 to 1:4.

Acid Dissociation and Ultrafiltration of β2-Microglobulin: Anti-β2-Microglobulin. Y-Mel 81:180 was found to be the most reactive of 4 melanoma culture lines derived in this laboratory against anti-β2-microglobulin antisera with titers of 1:128,000. This titer was reduced to 1:16,000 with the addition of β2-microglobulin. Acid dissociation and ultrafiltration of this preparation restored the previous titer. Exposure to glycine:saline buffer and dialysis failed to alter the titer. Control mixtures held at 4° or freshly thawed for the hemadsorption assay failed to show a rise in titer. The results are summarized in Table 2.

Acid Dissociation and Ultrafiltration of Sera. Sera from 7 patients were subjected to acid dissociation and ultrafiltration. Only 2 of these sera showed any reactivity against autologous cultured melanoma cells when native specimens were tested. Following ultrafiltration, 6 of 7 sera showed enhanced reactivity

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4 Y-Mel 80:120 was a spindle-shaped, sparsely pigmented melanoma taken from a lymphadenectomy specimen of a 49-year-old multiparous woman; 2 months following resection of a deep midpocordial cutaneous primary. Cryopreserved viable lymph node tumor cells and a cell line derived from the lymph node tumor were tested in studies reported here.
against autologous melanoma. These results are shown in Chart 1. Serum from 6 normal donors failed to show any reactivity against melanoma cell line Y-Mel 81:180 before or after acid dissociation and ultrafiltration. We have confirmed the enhanced titer of ultrafiltered autologous sera by immunofiltration with enzyme-linked immunosorbent assay in separate tests against Y-Mel 81:180 (data not shown).

**Analysis of Serum S1375.** One serum which had exhibited no reactivity prior to ultrafiltration showed a titer of 1:128 against autologous melanoma cell line Y-Mel 81:180 after ultrafiltration (Chart 1). Direct and absorption tests of specificity were performed on autologous and allogeneic cultured tumor cells. In addition, absorption analysis was performed with *Bacillus Calmette-Guérin*, FCS, and freshly thawed autologous melanoma cells. These results are summarized in Table 3 and are consistent with a Class II antigen.

Absorption of ultrafiltered Serum S1375 with autologous cultured melanoma cells (Y-Mel 81:180) posed a problem we have not seen before. Autologous absorption testing with this melanoma at cell numbers up to $1 \times 10^6$ cells/ml failed to reduce the titer against autologous test cells as compared with an unabsorbed control (Chart 2). To test the possibility that Protein A-reactive material was shed from viable, cultured tumor cells during absorption or that membrane antigenic changes (*i.e.*, modulation) might have spuriously negated the test, cultured melanoma cells were fixed with 0.1% glutaraldehyde before absorption. Fixed cells completely absorbed reactivity from the autologous ultrafiltered serum. Absorption tests with viable and fixed cells of 2 cultured fibroblast lines, one neuroblastoma, 2 breast carcinoma lines, and cultured fetal melanocytes yielded identical results (Table 3). Similar studies of cells from 4 additional melanoma cell lines gave identical absorption results in 2 and enhanced absorption after glutaraldehyde fixation in 2 cases (Y-Mel 81:170 and 81:370), while one which failed to absorb (Y-Mel 81:180) could not be retested. To assure that the antigen detected by S1375 was not an artifact of culture, we tested freshly-thawed autologous melanoma cells by direct and absorption techniques with viable and glutaraldehyde-fixed cells. These tests were all positive (Chart 2).
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Conclusion that melanoma rarely displays cell surface antigens

DISCUSSION

The detection of tumor antigens restricted to human melanoma is a prerequisite to all immunological strategies for the diagnosis and therapy of this disease. Carey et al. and Shiku et al. (28, 29) have reported the infrequent detection of serological responses to autologous human tumors tested and analyzed by sensitive microserological tests. This study of serial specimens of native sera from 22 individuals initially found 27% reactive against autologous tumor, of which one detects a highly restricted melanoma cell surface antigen (Y-Mel 80:120), confirming the work of Carey et al. and Shiku et al. (4, 28). Beyond the possible conclusion that melanoma rarely displays cell surface antigens recognized in the human host, we asked whether the formation of immune complexes might obscure antibody to tumor cell surface antigens. Hybridoma technology for the production of murine monoclonal antibody to human melanoma reagents has not yet proven able to detect highly restricted Class I melanoma cell surface antigens or any widely accepted serodiagnostic or therapeutic reagents. Human B-cell-derived hybridomas or B-cell lines established with Epstein-Barr virus may provide reagents of higher resolution, although these remain speculative. The study of autologous serology will provide a useful guidepost to the selection of lymphocyte donors for creation of B-cell or human hybridoma monoclonal antibody and will provide the only direct evidence for a role of the natural history of immunity in human cancer short of effective immunological therapy.

Our examination of immune complexes as a potential reservoir of antibody to melanoma has utilized the method of acid dissociation and ultrafiltration reported by Sjögren et al. (30). This technique was first shown effective in a model system using cell surface β2-microglobulin and a heterologous rabbit antibody. β2-Microglobulin was subsequently used as a positive control in all autologous serological assays. Acid dissociation and ultrafiltration of serum from 6 of 7 patients have allowed detection of autologous antibody responses against melanoma cell surface antigens by the PAHA assay, with reactivity enhanced as much as 8-fold. In 5 of these 7 sera, no autologous reactivity could be detected by either of the 2 sensitive microserological assays used here for detection of native IgG or IgM, and in 2, there were low-titer IgG antibodies, the titers of which rose after serum treatment as we have described.

Detailed serological studies have focused upon an autologous IgG antibody to Y-Mel 81:180, which was obtained at a titer of 1:128 after acid dissociation and ultrafiltration of one native serum, which was weakly positive by IA test for native IgM antibody and negative by PAHA tests for IgG. The antigen detected by the autologous IgG antibody (S1375) to Y-Mel 81:180 is broadly represented in 8 of 9 melanomas we have tested and fits the description of a Class II antigen advanced by Pfundenschuh et al. (20). Only one melanoma line we have tested (Y-Mel 80:120) failed to show a reaction by either direct or absorption testing. Coincidentally, this melanoma line is the one which we had detected by an autologous antibody (S973) which appears uniquely restricted in its reactivity, corresponding to a Class I system (20). The 2 antigenic systems we have identified (Y-Mel 80:120 and Y-Mel 81:180) thus appear to be distinct.

Our finding of improved absorption reactivity following fixation of tumor is compatible with results in viral systems (23). The specificity of absorption reactions observed against fixed cells has been demonstrated by negative tests upon similarly fixed.

Table 3

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<tr>
<th>Cell line</th>
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<td></td>
<td>Viable cultured or fresh cells</td>
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<td>Human melanoma cultures</td>
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<tr>
<td>Y-Mel 81:180β</td>
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<tr>
<td>Y-Mel 81:180*</td>
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<tr>
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<td>Y-Mel 78:010</td>
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<tr>
<td>Y-Mel 81:390</td>
<td>+</td>
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<tr>
<td>Human nonmelanoma cultures</td>
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<tr>
<td>Neuroblastoma</td>
<td>SKNSH 0</td>
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<tr>
<td>141N</td>
<td>0</td>
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<tr>
<td>Glioblastoma</td>
<td>GB887 +</td>
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<td></td>
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<tr>
<td>Sarcoma</td>
<td>MG63 0</td>
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<td>G232 +</td>
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<tr>
<td>Bacillus Calmette-Guerin</td>
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* Autologous cultured melanoma cell line.
β Autologous freshly thawed melanoma cell line.
* Autologous autologous lymphocytes.

Chart 2. Absorption analysis of S1375 reaction against autologous melanoma (Y-Mel 81:180). Cultured viable melanoma (Δ) failed to absorb antibody as compared with unabsorbed control (○). Cells fixed with 0.1% glutaraldehyde prior to absorption and freshly thawed viable cells absorbed antibody.
fibroblasts, melanocytes, and nonreactive breast carcinoma. Absorption results with cultured viable cells were similar at 37°C and at 4°C, arguing against antigenic modulation (16, 19). Capping and swift extrusion of melanoma antigens has been reported with heterologous antibody but would not be expected at 4°C (33). The improved absorption reactivity of some fixed cultured cells observed here argues for more frequent use of fixed cells in the future.

The antigenic heterogeneity of melanoma may contribute to difficulties we and others have experienced in attempting to detect autologous antitumor antibody. Cultured tumor cells have shown antigenic variation related to the site and time of biopsy, age of the cell line, confluence, and the acquisition of other culture artifacts (1, 7, 13, 34). However, the antibody we have age of the cell line, confluence, and the acquisition of other culture artifacts (1, 7, 13, 34). However, the antibody we have

Several investigators have characterized tumor-associated antigens with a molecular weight of less than 100,000. The earliest biochemical studies of the autologous Class I melanoma cell surface antigen (AU) suggested a glycoprotein with a molecular weight of 20,000 to 50,000 (5). The limited quantity and low titer of autologous antibody to AU precluded more precise identification. The method we have applied to autologous melanoma is well-suited to preparation of larger quantities of antigen with a molecular weight of 100,000 for immunochemical study. The serum antibody reactivity detected by acid dissociation and ultrafiltration has thus far detected a common rather than a highly restricted cell surface marker. Autologous antibody to tumor has not been identified previously in any studies of melanoma or other tumor patients’ circulating immune complexes (30). Biochemical studies of the antigen eluted from serum immune complexes will provide the best evidence for specificity, and serial studies within patients will allow us to determine the role of antigen and other immunoregulatory factors present in immune complexes in the natural history of the tumor (6, 9, 10, 16–19, 24, 25, 31).

The well-known elevation of circulating immune complexes in melanoma patients studied by C1q binding, Raji cell binding, or polyelectrolyte glycol turbidimetric assays suggests that antibody to cancers may be more prevalent when studied by the methods we have applied here to melanoma. Acid dissociation and ultrafiltration of serum provide a simpler method for enhancing previously undetectable or weak antibody against autologous melanoma, available in monolayer quantities, than do previous reagents. This technique also provides a reasonable avenue for study of the mechanism of plasmapheresis and Protein A column perfusion therapy.

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