ABSTRACT

A patient presented with a primary melanoma, Level IV, 2.5 mm thick; 30 excised lymph nodes were all negative for tumor. Four local recurrences followed in the ensuing 17 months. Tumor cells cultured at this time were epithelioid. Autoimmunization was followed by a disease-free interval of 15 months. Postimmunization, the patient's lymphocytes destroyed his melanoma cells in culture and were stimulated in mixed cell culture by his irradiated tumor cells. Cells grown from the relapsing tumor were spindle/dendritic with bizarre morphology and were not attacked by his lymphocytes in culture. Using a C fixation technique, DR antigen profiles of the patient's B-cells and both tumor cell types showed that the immunizing tumor was positive for DR antigens 3, 5, and 8, none of which were present on his B-cells which had DR 2 and 4. Both B-cells and immunizing tumor cells were positive for DQ antigens. The relapsing tumor cells were positive for DR2 and negative for all other D region antigens tested. The evidence suggests that given a melanoma of heterogeneous cell population, autoimmunization against the predominant immunogenic cell inhibits tumor growth but allows the ascendance of a nonimmunogenic tumor cell type.

INTRODUCTION

In 1978 Winchester et al. (1) reported that cultured human malignant melanoma cell lines expressed antigens analogous to immune response (Ia) antigens of mice. This finding was confirmed and the antigens were further characterized serologically and biochemically by Wilson et al. (2). Wolfe and Ferrone (3), using monoclonal antisera to HLA antigens, demonstrated the identity of these melanoma cell antigens with Class II HLA-DR antigens of human B-cells. They found that normal melanocytes, intradermal nevi, and senile freckles lacked DR antigens and suggested that melanoma cells might acquire DR antigens not expressed on their normal counterparts, thus affecting the immunogenicity of the tumor. Albino et al. (4) reported that melanoma metastases from the same patient differed in expression of DR antigens. This finding was confirmed in seven patients studied by Natali et al. (5). Ruiter et al. (6,7) found the expression of DR antigens highest in metastatic melanoma lesions. They suggested that these antigens might mediate the interaction of melanoma cells with T-lymphocytes since DR antigens, in general, play a role in cell-to-cell interactions. Guerry et al. (8) reported that melanoma cells carrying DR antigens stimulated the proliferation of autologous lymphocytes in culture. This property was characteristic of early but not late metastatic lesions and was proportional to the quantitative expression of HLA-DR. Fossati et al. (9) found that autologous lymphocytes were cytotoxic to melanoma cells of primary (2 of 4), but not metastatic (0 of 21) tumors. Among primary tumors autologous lymphocyte stimulation correlated with the presence of DR antigens on melanoma cells, but metastatic lesion cells containing DR antigens did not stimulate autologous lymphocytes.

Our study indicated that autoimmunization with "alien" DR+ epithelioid melanoma cells resulted in strong lymphocyte cytotoxicity against the immunizing cells, specific in vitro tumor cell stimulation of autologous lymphocytes, and interruption of clinical progression which lasted for 15 months. At that time a new metastatic lesion occurred whose cells contained only a DR antigen compatible with the patient's B-cells. These latter melanoma cells were not attacked by autologous lymphocytes in culture and varied from the immunizing tumor cells in morphology and cultural characteristics. Disease progressed rapidly to death. We studied DR antigen profiles of the patient's B-lymphocytes and both types of tumor cells and propose that the major immunological event was the expression on the epithelioid melanoma cell of alien Class II, HLA-D region histocompatibility antigens.

MATERIALS AND METHODS

Patient History

The patient (C. H.) was a 52-year-old Caucasian male. A primary tumor of the right shoulder was diagnosed as spindle cell malignant melanoma, Level IV, 2.5 mm thick. Thirty excised lymph nodes were all negative for tumor. He subsequently had 4 recurrences in the same general area during the succeeding 17 months. The metastatic lesion removed at that time was an epithelioid tumor which was established in culture and grew in an epithelioid pattern. These cultured cells were used for immunization. Prior to immunization the patient's lymphocytes were not cytotoxic to his cultured tumor cells; following immunization his lymphocytes destroyed his tumor cells. The specificity of lymphocyte response was demonstrated by a positive mixed cell culture technique. After 15 months a new metastasis appeared. The cells grown from this lesion were bizarre in appearance, being mostly spindle and dendritic, highly motile cells resistant to radiation and having a longer (eight times) doubling time in culture than that of the immunizing cells. Cytotoxicity tests showed that these cells were not attacked by the patient's lymphocytes. Some epithelioid cells were found and these were selected; both types of cells recovered from the same metastasis were tested for the presence of DR antigens at the same time with the same panels of antisera. The patient's B-cells were also tested for DR antigens and comparisons were made among the three cell types (epithelioid melanoma, spindle/dendritic melanoma, and B-cells from the same patient).

Cell Culture

Tumor tissue was trimmed free of fat and necrotic tissue, finely minced, and placed in culture in RPMI 1640, 25 µm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid medium containing 24 µm glutamine,
penicillin (100 units/ml), and streptomycin (100 mg/ml) with 10% heat-inactivated fetal calf serum. This medium was used routinely, except in the modified mixed lymphocyte reaction where human AB serum replaced the fetal calf serum. Incubation was uniformly done at 37°C and 7% CO₂ in an air humidified atmosphere. At the sixth cell passage, aliquots were frozen in dimethyl sulfoxide in liquid N₂ for future experiments.

Autoimmunization

Epithelioid melanoma cultures were washed 3 times in RPMI 1640 without serum, irradiated with 5000 R, and harvested by trypsinization. One aliquot (10%) was returned to culture in complete medium for 24 h and examined for adherent cells. If no cells adhered the remaining 90% were frozen in liquid N₂ and used for autoimmunization. Tumor cells were considered suitable for autoimmunization if they were unable to adhere but retained cellular morphology. If the cells were viable or completely disintegrated, the radiation dose was adjusted to meet the above criteria. The dose was 5000 R for the immunizing epithelioid cells and greater than 30,000 R for the spindle/dendritic cells which were not used for autoimmunization. The vaccine inoculum consisted of 6 × 10⁶ irradiated tumor cells mixed with 1/40 ampule of Glaxo strain Bacillus Calmette-Guérin. Vaccination at multiple skin sites was repeated at 2-week intervals for 2 months. At that time in vitro lymphocytoxicity against the immunizing cells was compared with preimmune values.

Determination of In Vitro Lymphocytoxicity Against Autologous Tumor Cells

Lymphocytes were separated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation and passage through a nylon column. Yield was approximately 10⁶ mononuclear cells/ml of whole blood. Viability was >98% by trypan blue exclusion test. Tumor cells from a 48-h culture were harvested with trypsin EDTA (Gibco), counted, and 10⁵ viable cells were distributed in each of six 25-cm² flasks. After 4 h incubation cells had attached and at that time 5 × 10⁶ lymphocytes were added to each of 3 flasks. Incubation was continued for 72 h, at which time all cultures were washed with medium to remove nonadherent cells. The attached tumor cells were then collected by trypsinization and counted. Tests were considered valid if the cell counts within each triplicate set of flasks varied by less than 5% from the mean. A test was considered positive if the control cells had increased in number and the experimental cells were less than 50% of the original inoculum. Cytotoxicity tests were done on lymphocytes before autoimmunization and at intervals of 6 weeks to 2 months following immunization with autologous melanoma cells.

Modified Mixed Lymphocyte Culture Test Using Autologous Melanoma Cells

Lymphocyte sources were (a) patient C. H. and (b) a donor incompatible for all HLA antigens.

Preparation of Lymphocytes. Lymphocytes were recovered from 20 ml of heparinized blood as described above and suspended in RPMI 1640 medium with 10% human AB serum. After washing 3 × in complete medium, the cell pellet was suspended in 5 ml of culture medium and cell concentration was adjusted to 10⁶ cells/ml. Viability was determined by trypan blue exclusion.

Preparation of Melanoma Cells. Melanoma cells prepared as for autoimmunization (see above) were washed 3 times in complete medium containing 10% human AB serum instead of 10% fetal calf serum. Cell concentration was adjusted to 10⁶ cells/ml.

Procedure. CHL, IAL, and autologous melanoma cells were then treated similarly. In each case one-half of the cells was irradiated with 2000 R and designated “stimulator” cells. The nonirradiated half was designated “responder” cells. Responder and stimulator cells (100 µl each aliquot) were dispensed in triplicate in tissue culture trays in a matrix which tested all combinations of cells. After 96 h of incubation, 1 µCi of [³H]thymidine was added to each well and incubation was continued for 18 to 20 h. Cells were then harvested on glass fiber filters, dried 1 h at 56°C, transferred into 3 ml of scintillation fluid, and counted for 5 min in a liquid scintillation counter. With each test, 3 H standards were run, as were radiation controls consisting of appropriate combinations of irradiated cells.

HLA-D Region Identification by C° Fixation

Trays containing antisera to HLA-D region antigens were obtained commercially from Pel Freez Biologicals, Rogers, AZ (tray series DR72-4) and UCLA (Lots 15 and 16; Terasaki, Los Angeles, CA). The antisera were operationally mono-, duo-, and trispecific for HLA-DR antigens 1 to 10, DRW 52 and DRW 53 (previously MT2 and MT3), and the DQ antigens 1 to 3. Panels of 6 to 10 antisera for each specificity were included. Each well contained 1 µl of the specific antiserum and 5 to 10 µl of heavy mineral oil. As a preservative 0.1% sodium azide was added. Positive and negative controls were included in the tray package. The reaction was measured by viewing the test microscopically at ×150 with phase contrast illumination and a vital stain. Tests were read by a qualified laboratory technician.

B-Cells. B-cells were obtained by a standard nylon column technique (10). Residual RBC were lysed with ammonium chloride and the B-cells were suspended in Hanks’ balanced salt solution at a concentration of 2 × 10⁶ cells/ml. Using a 50-µl syringe, 1 µl of lymphocyte suspension (approximately 2000 lymphocytes) was added directly into the antiserum in each well. After a 60-min incubation at 37°C, 5 µl of rabbit complement was added to the antiserum-lymphocyte mixture and incubation continued for 2 h. Two µl of filtered 5% aqueous eosin dye were added to each well and after 3 to 5 min, 5 µl of filtered 37% neutralized formalin were added. Cells were allowed to settle for 15 min and then read by estimating the percentage of dead cells.

Modification of DR Antigen Test for Melanoma Cells. Melanoma cells were harvested by brief (30-s) exposure to trypsin-EDTA (Gibco), tapping to dislodge cells, and immediate addition of 7 ml of complete medium to neutralize trypsin. A cell pellet was obtained by light centrifugation, washed 3 times in medium, and the count was adjusted to 3 × 10⁶ cells/ml. Viability was determined and 1-µl aliquots of cells were added to each well of a Terasaki microtitre plate. One ml of medium was added to the edges of the plate to keep the atmosphere moist. Sterile conditions were maintained during these manipulations. Plates were covered and incubated 18 to 20 h, after which they were examined under inverted phase scope for cell growth and adhesion. Cells were then washed 3 times by adding 10 µl of RPMI 1640, incubating 10 min, and flicking to remove medium containing unattached dead cells. Fresh RPMI 1640 was added to each well to prevent drying. Using a 3-µl Eppendorf pipet, antiserum and oil were removed from each well of the commercial tray and transferred to the corresponding well of the cultured cell plates. It usually required two commercial trays to obtain enough antiserum for one cell line. During transfer care was taken to ensure that oil did not interfere with antiserum on the cell line. Positive and negative controls were included on each tray. A 37°C incubation period was required. Antisera were operationally mono-, duo-, and trispecific for HLA-DW antigens 1 to 10, DW 52 and DW 53 (previously MT2 and MT3), and the DQ antigens 1 to 3. Panels of 6 to 10 antisera for each specificity were included. Each well contained 1 µl of the specific antiserum and 5 to 10 µl of heavy mineral oil. As a preservative 0.1% sodium azide was added. Positive and negative controls were included in the tray package. The reaction was measured by viewing the test microscopically at ×150 with phase contrast illumination and a vital stain. Tests were read by a qualified laboratory technician.

Karyotype Analysis

Tumor cells were cultured at 37°C in RPMI 1640 supplemented with 10% fetal calf serum, 2% penicillin-streptomycin solution (Gibco), and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The cells were harvested by routine procedure for chromosome composition analysis. Two to 3 h before harvest, Colcemid at a concentration of 25
ng/ml was added to accumulate metaphases. Single cells were obtained from monolayer cultures hypotonically treated with 0.075 M KCl for 15 min and fixed in methanol:acetic acid (3:1; v/v). Slides were air dried and treated to obtain trypsin G-banding by the method of Wang et al. (11). Twenty-five metaphase spreads were analyzed for chromosome composition.

Monoclonal Antibody Studies

Monoclonal antibody tests to identify cells of mononuclear cell infiltrate observed in relapse tumor tissue sections were stained with anti-Leu 4 antibody, which is a pan T-cell antibody; anti-Leu 3A, which stains helper/inducer subset T-cells; anti-Leu 2A, which stains cytotoxic/suppressor T-cell subsets; and anti-Leu 6, which stains thymocytes and Langerhans' cells. Sections were counterstained with goat anti-mouse fluorescin-tagged antibody.

RESULTS

Fig. 1A illustrates the pattern in culture of the cell line grown from the fourth recurrence 17 months following excision of the primary and regional lymph node dissection. These cells were used for immunization. Fig. 1B demonstrates the pattern of the cell line cultured from the next recurrence which occurred after 15 months, during which there was no clinical disease. Fig. 1C shows the patient’s mononuclear cells attached to an immunizing cell when they were cocultured 6 weeks after immunization in the lymphocytopoietic test. Fig. 1, D and E shows spindle cells of the cell line derived from relapsing tumor.

Table 1 illustrates that prior to autoimmunization, the patient's epithelioid melanoma cells tripled in number from $1 \times 10^5$ to $3.3 \times 10^5$ when cultured for 72 h in the presence of $5 \times 10^6$ autologous lymphocytes. This number did not differ significantly from the control ($3.0 \times 10^5$). The lymphocyte:tumor cell ratio was 50:1. Six weeks after immunization, the control CHE cells increased 3.2 times in 72 h, but the CHE cells in the presence of CHL (CHL:CHE ratio, 50:1) decreased in number to $0.4 \times 10^5$. At 72 h, the number of melanoma cells in the experimental flasks containing lymphocytes had decreased to 40% of the original inoculum and was only 12.5% of the number of control cells grown without autologous lymphocytes. During the next 15 months, the patient was clinically free of disease.
and his lymphocytes destroyed the immunizing tumor cells in culture when tested at 6-week to 2-month intervals. After 15 months a recurrent tumor appeared which was excised and established in culture. These cells grew more slowly since the controls only increased from $1 \times 10^3$ to $1.3 \times 10^3$ in 72 h. The presence of autologous lymphocytes had no significant effect on their growth rate, since the experimental number at 72 h was $1.4 \times 10^3$. It was assumed that natural killer cells or other lymphocytes responding to calf serum would have attacked both tumor cell types.

Table 2 shows that the patient's lymphocytes incorporated significant amounts of [3H]thymidine when cultured for 96 h in the presence of inactivated epitheliod autologous melanoma cells. The amount of [3H]thymidine was 3.4 times the amount incorporated when CHL were cocultured with the same number of inactivated CHL (6975 versus 2088 cpm). The [3H]thymidine values obtained at 24-h intervals showed that peak incorporation occurred between 48 and 72 h of culture. The serum component of the medium was 10% human AB serum, thus excluding the possibility that the CHL cells were making a major response to calf serum. CHL responded in expected fashion to lymphocytes from a completely HLA incompatible donor. Stimulator cells were irradiated (2000 R) CHL, autologous melanoma cells, and lymphocytes from an HLA incompatible donor. Responder cells were non-irradiated cells from the same sources. Stimulation was measured by [3H]thymidine uptake after 96 h of culture. Triplicate counts varied by less than 5%. A positive test was 2.5 times the count of a mix of irradiated and nonirradiated identical cells.

Table 1 Selective autolymphocytotoxicity versus immunizing melanoma cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Preimmunization</th>
<th>Postimmunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHE</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>CHE + CHL</td>
<td>3.3</td>
<td>0.4</td>
</tr>
<tr>
<td>CHS</td>
<td>NA*</td>
<td>1.3</td>
</tr>
<tr>
<td>CHS + CHL</td>
<td>NA</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* NA, not available.

Table 2 Stimulation of DR 2, 4 positive lymphocytes following autoimmunization with DR 3, 5, 8 positive melanoma cells

<table>
<thead>
<tr>
<th>Responder cells</th>
<th>Stimulating cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHL</td>
<td>CHL*</td>
</tr>
<tr>
<td></td>
<td>CHE</td>
</tr>
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<td></td>
<td>IAL</td>
</tr>
</tbody>
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* x, X-irradiated.

Some of these common chromosomal markers indicates the clonal original of the two different cell types. In contrast, there are 15 marker chromosomes specific for CHS and there are 4 markers specific for CHE, respectively. The existence of these subline specific markers suggests the dynamic chromosomal evolution occurring during the progression and differentiation of the tumor cell population. Monoclonal antibody studies with antisera to various T-cell subsets showed that the moderate cellular infiltrate of the relapsing tumor stained positively with anti-Leu 4, a pan T-cell antibody. Most cells present were of the helper/inducer subset with a rare cytotoxic/suppressor cell. There was no mononuclear cell infiltrate in the earlier metastatic lesion whose cells were used for autoimmunization (Fig. 3).

DISCUSSION

Day et al. (13) made an exhaustive study of prognostic factors in melanoma. They found that among primary lesions $\geq 3.65$ mm in depth lymphocytic infiltrate at the base of the tumor was a favorable prognostic factor with a P of 0.0001. This finding and general observations on the clinical course of the disease have encouraged numerous attempts to define and exploit immunological response mechanisms in melanoma, particularly since this tumor is so refractory to other adjunctive therapy (chemotherapy and radiation). Discussing immunotherapy of malignant melanoma, Terry (14) stated that the putative cancer antigen must be present and recognizable at clinical onset of the disease, must stimulate an effector response
Fig. 2. Karyotype of cell types CHS (A) and CHE (B) showing common marker chromosomes (CHR.) (underlined) as well as subline specific marker chromosomes.
in the host, and must serve as the site for antibody or cell-mediated injury to the cell. He commented that “such an antigen has not been found on any cancer cell in a human being” (15). Although several melanoma-associated proteins have been described (16–18), none to date has fulfilled the above criteria.

Accepting the premise that the putative antigen would be an autoantigen, we have for many years attempted autoimmunization using cutaneous injection of cultured autologous melanoma cells mixed with Bacillus Calmette-Guérin. On numerous occasions the patient’s lymphocytes converted from negative to positive in our cytotoxicity test described above. In this test the tumor cell number was large (10^5 cells) and we set a rigid criterion for a positive test, i.e., greater than 50% tumor cell kill in 72 h when the lymphocyte:tumor cell ratio was 50:1. This rigid criterion was used because we were seeking evidence of tumor cell killing on a scale that might be expected to be clinically relevant. In some instances this was associated with periods of remission. A perplexing point was that at relapse the patient’s lymphocytes were usually still cytotoxic to the immunizing cell. In two recent cases we grew cells from the relapsed tumor and found that the patient’s lymphocytes did not attack these cells; although they continued to be cytotoxic to the original immunizing tumor cell (Table 1). Since these tests were run simultaneously in the same medium, we felt that we could probably exclude reactions to media immunogens, i.e., calf serum and also natural killer cells which would have attacked both tumor cell types. We therefore began a search to determine (a) specificity of the reaction to the immunizing tumor cells and (b) antigenic differences between the two cell types which might explain the lymphocyte nonresponsiveness to the relapse tumor cells.

The morphological and cultural characteristics of the two cell types were very different (Fig. 1). In both cases the immunizing cells grew in an epithelioid pattern. The doubling time was approximately 24 h and they were resistant to mitomycin C (30% survival when exposed to 0.75 mg/ml). Radiation sensitivity corresponded to that of a normal fibroblast control. Cells grown from the relapsing tumor were bizarre, with the majority being spindle or dendritic, highly motile, and only a few showing cell-to-cell contact in an epithelioid pattern. Doubling time was 8 days and they were extremely resistant to radiation, some growth continuing after exposure to 30,000 R. Histologically the two tumors were similar except that the relapsing tumor had within it a moderate lymphocytic infiltrate. When this infiltrate was stained with antibodies to T-cells in general (anti-Leu 4), helper/inducer T-cells (anti-Leu 3A), cytotoxic/suppressor T-cells (anti-Leu 2A), thymocytes and Langerhans’ cells (anti-Leu 6), results showed that the infiltrate was composed exclusively of Leu-3A positive cells suggesting that the patches of lymphocytic infiltrate represented an immune response to some member of the tumor cell population. Histological sections of tumor taken before autoimmunization showed no lymphocytic infiltrate (Fig. 2). As a further test of specificity, we chose the standard mixed lymphocyte culture test for transplant donors, into which we introduced the epithelioid immunizing cells as one test cell along with autologous lymphocytes using as positive control lymphocytes from a donor who shared no HLA antigens with the patient. The results (Table 2) showed clearly that the patient’s lymphocytes were stimulated by his melanoma cells used for immunization. By testing at 24-h intervals, we could show that the peak response to the melanoma cells occurred between 48 and 72 h, which is characteristic of a secondary or immune response, whereas his response to the allogeneic donor cells peaked at 96 h, characteristic of a primary response. The patient’s melanoma cells did not stimulate the allogeneic donor cells. This finding agrees with that of Pollack et al. (12) and awaits clarification. Radiation controls were all negative. Fetal calf serum was presumed not to be a factor, since the cells were thoroughly washed and the growth medium contained 10% human AB serum as serum supplement. The results of this test suggested that the epithelioid cells used for immunization contained an HLA-D antigen alien to that of the host.

Recent findings that melanoma cells frequently contain immune response (DR) antigens led us to examine the DR profiles of the CHB and the two types of tumor cells, CHE and CHS. These two types of tumor cells were separated from the same metastatic lesion by taking advantage of their different cultural characteristics, chiefly, rates of attachment and growth. Once separated each type was stable in subculture. Difficulties in determining individual DR specificities by complement fixation were related to the fact that melanoma cells adhere to culture flasks. Removal mechanically by spatula or the use of EDTA both resulted in unacceptably high levels of dead cells in negative controls. Removal by trypsinization destroyed surface proteins, some of which were DR antigens. It was also essential to ensure a complement source nontoxic to the melanoma cells. In particular the epithelioid melanoma cells were very sensitive to the toxicity of various lots of rabbit complement. These problems were solved by the method de-
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Immunological Heterogeneity in Human Melanoma: Immunogenic Alloantigen Expression in Autologous Host


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