Modulation of B16 Melanoma Antigen Expression by Lymphokines and Dimethyl Sulfoxide

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ABSTRACT

We have developed two monoclonal antibodies, designated 152 El2 D7 and 153 C7 A6, which have reactivity with cell surface antigens expressed on the B16 mouse melanoma. These monoclonal antibodies are produced by hybridomas resulting from the fusion of spleenocytes taken from C57BL/6 mice bearing the B16-F10 tumor. The monoclonal antibodies are of the immunoglobulin M class and have been shown to react with three variants of the B16 and another mouse melanoma but no normal murine tissues. Exposure of B16 melanoma cells to a concanavalin A stimulated spleen cell mixed lymphokine preparation (LK) and to dimethyl sulfoxide (DMSO) enhanced the expression of the cell surface antigens recognized by these monoclonal antibodies. The cultures stimulated with LK or DMSO contained a greater proportion of cells expressing the antigens recognized by monoclonal antibodies 152 El2 D7 and 153 C7 A6 than did unstimulated controls. In addition to increasing the proportion of antigen-positive cells, the antigen expression per cell, as measured by fluorescence intensity, was substantially increased following exposure to LK and DMSO. The effects of treatment with LK or DMSO were apparent after 24 h exposure but did not persist after the agent was removed from the cultures, suggesting that the enhancement of antigen expression was a transient event rather than a permanent differentiation of the melanoma cells.

INTRODUCTION

Monoclonal antibodies reactive with a wide variety of tumor-associated antigens have been generated following xenogeneic or allogeneic immunizations (1-11). Using these monoclonal antibodies, tumor-associated antigens have been shown to be expressed continuously on all cells of some tumor lines but only by certain subpopulations of other tumors.

One of the tumor types that has been extensively studied is malignant melanoma (12-21). The cells express antigens that are recognized by the host immune system resulting in the development of humoral and cell-mediated immunity (22-25). Several monoclonal antibodies to melanoma-associated antigens have been produced by immunizing mice with human or mouse melanoma cells (2, 5, 19, 26, 27, 32).

Monoclonal antibodies generated against TAAs may detect only subpopulations of cells expressing relatively high antigen density (28). To optimally utilize these antibodies, it would be desirable to maximize the expression of antigen on positive cells. The use of agents which both increase the proportion of cells expressing TAAs and the antigen density per cell would facilitate the use of monoclonal antibodies as analytical, diagnostic, and therapeutic tools. Lymphokines have been shown to increase the expression of TAAs and histocompatibility antigens in several systems (29-32). DMSO has been extensively studied as an inducer of differentiation and modulator of cell growth and function (32-37). We have developed two monoclonal antibodies with anti-melanoma activity by producing hybridomas from syngeneic mice bearing the B16-F10 mouse melanoma. The purpose of this investigation was to define the reactivity of these monoclonal antibodies and to explore the use of a lymphokine preparation containing γ-interferon activity or DMSO to enhance the expression of cell surface antigens on B16 melanoma.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice, 4-5 weeks old, were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c mice, used as a source of thymocytes, were purchased at 3 weeks of age from Harlan Sprague-Dawley, Indianapolis, IN.

Cell Lines. The B16 melanoma lines F1, F10, and BL6 were selected by Fidler (12) and Hart (38) for their lung-colonizing and tissue-invasive characteristics. Additional cell lines used to determine the specificity of the monoclonal antibodies were kindly provided by Drs. C. W. Johnson, Department of Pathology, Ohio State University. C57BL/6 lines include: JB/RH, melanoma; JB/MS, melanoma (J. Berkelhammer, AMC Research Center, Lakewood, CO); and the EL-4 lymphoma. Non-H2b tumor target cell lines include: clone M-3 of the CxDBA Cloudman melanoma S91 (ATCC); 410.4, a BALB/c mammary tumor (G. H. Hepner, Michigan Cancer Foundation, Detroit, MI); Neuro 2a, a strain A albino neuroblastoma (ATCC); and SCCPSA 1, a murine teratocarcinoma (ATCC). Cells were grown in tissue culture from frozen stocks in Dulbecco’s minimal essential medium (MA Bioproducts) supplemented with 10% FBS (Sterile Systems, Logan, UT), vitamins, nonessential amino acids, pyruvate, glutamine, and penicillin-streptomycin. Single cell suspensions were obtained by scraping monolayers with a rubber policeman.

Hybridizations were carried out using the X63.Ag8.653 myeloma cell line (Cell Distribution Center, Sloan Institute) as one of the fusion partners. These cells lack hypoxanthine-guanine phosphoribosyl transferase which allows their growth to be inhibited by aminopterin. The myeloma cells were maintained in RPMI 1640 (MA Bioproducts) supplemented with 10% FBS, pyruvate, glutamine, and penicillin-streptomycin.

Production of Monoclonal Antibodies. To produce hybridomas secreting monoclonal antibodies with reactivity against the B16 melanoma, spleen cells were obtained from C57BL/6 mice bearing the B16-F10 tumor. Briefly, 20 male C57BL/6 mice, 5 weeks old, were given intradermal injections of 1 × 10⁶ B16-F10 cells. Spleens were removed from each of four mice after 5, 10, 15, 20, and 25 days of tumor growth. A single cell suspension was prepared and the lymphocytes fused with the X63.Ag8.653 myeloma at a 1:1 ratio using polyethylene glycol 4000 (Fisher) according to the method of Oi and Herzenberg (39). Cells were cultured in 96-well plates using an enriched medium composed of DMEM supplemented with 10% FBS, 10% NCTC-109 (MA Bioproducts), nonessential amino acids, insulin, oxalocacetate, glutamine, pyruvate, and penicillin-streptomycin. Hypoxanthine-aminopterin-thymidine selection against unfused myeloma cells was initiated on day 1 and culture supernatants were screened for antibody production after 3 weeks using a β-galactosidase ELISA (Amersham, Arlington Heights, IL).
Screening of Hybridomas. All hybridoma antibodies were subsequently tested for anti-B16 activity by ELISA. B16-F10 cells were seeded into 96-well Immulon-II plates (Dynatech) at 2 × 10^4 cells/well; the cells were centrifuged at 350 × g, the wells were aspirated, and the cells were air-dried overnight. Prior to addition of hybridoma supernatants, the cells in the prepared ELISA plates were rehydrated by briefly filling the wells with 0.15 M PBS, pH 7.4. All subsequent incubations were at 37°C for 1 h. The tumor cells were then incubated with undiluted hybridoma supernatants, washed with PBS, and incubated with β-galactosidase-conjugated sheep anti-mouse immunoglobulins (1:400). After a washing in PBS, the enzyme substrate o-nitrophenyl β-D-galactopyranoside (3 mM in PBS containing 10 mM MgCl2 and 0.1 M 2-mercaptoethanol) was added to each well. After incubation, the absorbance of each well was determined at 405 nm using a BioTek ELISA reader and antibody concentration was calculated from a standard curve. All hybridoma supernatants were tested against non-B16 cell lines in a similar fashion. The ELISA assay for the presence of antibody in hybridoma supernatants was a modification of the above procedure with the following change. Instead of target cells, goat anti-mouse immunoglobulins (1:500) were coated to the wells with carboxybiotin, washed, and antibodies were then identified using a horseradish peroxidase ELISA isotyping kit from Sterile Systems.

Immunohistochemical Staining. Fresh tissues from normal C57BL/6J mice were quickly frozen on dry ice and 5 μm sections prepared using a cryostat. Endogenous peroxidase activity was inhibited by treatment with 0.3% H2O2 in 0.1 M Tris-HCl buffer, pH 7.6. Tissue sections were rinsed in 0.1 M Tris-HCl buffer, pH 7.6, and incubated overnight at 4°C with the primary antibody. The tissues were rinsed thoroughly with Tris buffer, followed by the addition of the horseradish peroxidase-conjugated goat anti-mouse antibody (1:500, Cappell) for 30 min at room temperature. After the sections were rinsed with Tris buffer, the 3,3’-diaminobenzidine substrate solution Tris-HCl, pH 7.6 (500 μg/ml) 0.1% H2O2 was added for 20 min at room temperature. Excess 3,3’-diaminobenzidine solution was rinsed away, and the sections were counterstained with fast green for 5 min and then progressively dehydrated in ethanol in preparation for permanent mounting. Tissue sections were carefully examined by light microscopy for the presence of specific antibody staining.

Preparation of Fluorescein-conjugated Monoclonal Antibodies. Monoclonal antibodies were fluorescently stained according to the method of Mishell and Shiigi (40) for use in direct fluorescent antibody staining and competitive binding experiments. Briefly, antibodies were precipitated from hybridoma supernatants using 40% saturated ammonium sulfate, dialyzed exhaustively against PBS, and further purified by passage over a G-100 gel filtration column (Pharmacia). The prepared antibodies were then dialyzed against 0.5 M carbonate-bicarbonate buffer, pH 9.2, and fluoresceinated by dialysis in this buffer containing fluorescein isothiocyanate (100 μg/ml; Sigma) for 14 h. The fluoresceination reaction was stopped by dialyzing the antibodies against 0.15 M PBS, pH 7.0, for 2 h.

Flow Cytometry Analysis. Single cell suspensions were prepared from freshly isolated B16 tumors by pressing tumors through a stainless steel screen, followed by repeated passage through a 21-gauge syringe needle. All cultured cells to be examined by flow cytometry for IFA staining were taken from 24-h log phase cultures. Cells were harvested from monolayers using a rubber policeman, dispersed into single cells by vortexing and washing in Ca²⁺- and Mg²⁺-free SSBS. Cell viability was typically greater than 95% when harvested this way. The cells were then incubated with either hybridoma supernatants or monoclonal antibodies prepared from ammonium sulfate precipitates of culture supernatant fluids for 1 h on ice. Cells were washed 3 times with SSBS and incubated with a 1:20 dilution of FITC-conjugated goat anti-mouse IgM (Tago) for 1 h on ice. Cells were washed 3 times with SSBS and examined directly or were fixed prior to analysis in 70% ethanol for 15 min on ice.

Cell fluorescence was quantified using a Cytofluorograf 505 flow cytometer (Ortho Diagnostics, Westwood, MA) equipped with an argon laser tuned to 488 nm. The green FITC (520 nm) fluorescence emissions from each cell were measured and the data were stored on a disc using the Ortho 2150 computer. All staining reagents were used under saturating conditions; therefore green cell fluorescence is linearly related to the quantity of surface antigen. Fluorescence intensity expressed as the percentage of mean cell fluorescence above background was calculated according to the formula

\[
\% \text{ of } F1 > \text{bkgd} = \frac{(F1 \text{ test}) - (F1 \text{ bkgd})}{(F1 \text{ bkgd})} \times 100
\]

where F1 test is the mean fluorescence intensity of the population stained with the test antibody and F1 bkgd is the mean fluorescence intensity of the cells stained with unrelated antibody.

Cycloheximide Treatment. B16 cells were seeded onto 100-mm tissue culture dishes (Corning) at 2 × 10^6/dish in complete DMEM with 10% FBS. After 12 h in culture, cycloheximide (Sigma) was added to a final concentration of 3 μg/ml and the cells were cultured for an additional 12 h. Cells were then harvested and examined by flow cytometry using IFA.

Lymphokine Preparation. The LK used in this study was prepared from mitogen-stimulated spleen cell cultures. Briefly, C3H/HeN splenocytes were cultured for 3 days at 5 × 10^6/ml in RPMI 1640, without FBS, supplemented as above containing concanavalin A (2 μg/ml; Sigma) and 5 × 10^-3 M 2-mercaptoethanol (Sigma). The supernatant was collected, filtered through a 0.22-μm filter (Nalgene), and stored at 4°C. The lymphokine was standardized by determining the dilutions required to prime macrophages for antitumor activity. The antitumor activity was assessed following triggering with lipopolysaccharide using the P815 mastocytoma as a target cell in a chromium release assay. The active lymphokine preparations were always active in modulating the B16 antigens. This lymphokine preparation was diluted to the proper concentration in complete DMEM-10% FBS immediately before addition to B16 melanoma cultures.

RESULTS

The 20 fusions between mouse myeloma cells and splenocytes obtained at 5 time intervals from C57BL/6 mice with the B16-F10 tumor growing intradermally resulted in 1765 master wells with antibody producing hybridomas. Of these, 168 (9%) secreted antibodies which reacted with B16-F10 cells in an ELISA assay. A summary of binding studies using these two monoclonal antibodies is given in Table 1.

Table 1 Summary of antibody reactivity

<table>
<thead>
<tr>
<th>Cell and tissue</th>
<th>152 E12 D7</th>
<th>153 C7 A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16 BL6</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>F10</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>F1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>JB/RH</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>JB/MS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EL-4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neuro 2a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCC-PSA 1</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Clone M3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>401.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Striated muscle</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thymus</td>
<td>+/-</td>
<td>+/-</td>
</tr>
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1. Evaluation of reactivity above background with all cell lines reflects intensity of staining by ELISA whereas activity against normal tissues was measured by immunoperoxidase staining of frozen sections. Both D7 and A6 are IgM antibodies which recognize all three variants of the B16 melanoma tested as well as JB/RH, another C57BL/6 melanoma. Only a slight amount of activity could be detected with the SCC-PSA 1 teratocarcinoma or normal thymic tissue.

Several lines of evidence suggest that the antigens detected by D7 and A6 are protein in nature but efforts to specifically immunoprecipitate metabolically labeled antigen have not been successful. Cells fixed in methanol prior to IFA staining with D7 and A6 exhibited no loss in either fluorescence intensity or the percentage of positive cells, suggesting the antigens are not soluble lipids. Additionally, when cells were treated with the protein synthesis inhibitor cycloheximide for 12 h, a 65% reduction in fluorescence intensity was observed (data not shown).

The D7 and A6 monoclonal antibodies appear to detect two distinct epitopes, either on the same or on two different molecules. Incubating B16 cells first with the D7 antibody followed by FITC-labeled A6 did not reduce the fluorescence intensity of the cells compared to medium-treated controls (Table 2). Preincubation of B16 cells with A6 before the addition of FITC-A6 reduced the fluorescence intensity by 80% and the percentage of positive cells by 50%. The reciprocal experiment yielded similar results (data not shown).

Antibodies D7 and A6 were tested for reactivity against freshly isolated B16 tumor cells (Table 3). Cells taken from relatively small tumors (10 days) reacted well with A6 but not with D7. Nearly twice as many cells of the BL-6 variant expressed the antigen detected by A6 compared to the F10 cells. However, when cells from 25-day-old tumors were analyzed, each variant of the B16 had virtually the same percentage of cells expressing both the D7 and the A6 antigens. The level of expression of D7 was quite low after 10 days of tumor growth. After 25 days, the tumor cells expressed the D7 antigen at the same or greater densities than A6.

Table 2 Monoclonal antibodies A6 and D7 recognize distinct epitopes

<table>
<thead>
<tr>
<th>Activity with fluoresceinated A6</th>
<th>BL-6</th>
<th>F10</th>
<th>F1</th>
</tr>
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<tbody>
<tr>
<td>Cells preincubated with</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>518</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td>D7</td>
<td>505</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td>A6</td>
<td>91</td>
<td>25</td>
<td>71</td>
</tr>
</tbody>
</table>

Table 3 Reactivity of monoclonal antibody with freshly isolated tumor cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Days of tumor growth</th>
<th>% of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BL-6</td>
<td>F10</td>
</tr>
<tr>
<td>152 E12 D7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>141</td>
</tr>
<tr>
<td>25</td>
<td>43</td>
<td>215</td>
</tr>
<tr>
<td>153 C7 A6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>46</td>
<td>377</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>144</td>
</tr>
</tbody>
</table>

Exposure of the B16 melanoma cells to DMSO resulted in an increase in antigen expression that was both dose and time dependent (Fig. 1). The fluorescence intensity of cells treated with 2.5% DMSO and stained with A6 increased after the first 24 h by more than 150% of control levels (Fig. 1B). The increase in fluorescence intensity of those cells stained with D7 was more modest; an increase of only 30% over control levels was observed after 2 days of treatment (Fig. 1A). There was a rapid increase in the percentage of B16 cells reacting with D7 or A6 after 24 h exposure to DMSO; 85% of the cells treated with 2.5% DMSO reacted with D7 and 95% reacted with A6 after 24 h of treatment (Fig. 1, C and D). Continued exposure to DMSO increased these percentages to 95 and 98%, respectively, by the third day of treatment.

Treatment of B16 melanoma cells with LK also increased both the amount of antigen expressed per cell and the percentage of cells expressing antigen. The results from experiments with B16-BL6 are illustrated; B16-F10 and B16-F1 showed similar patterns of response. An increase in the amount of antigen expressed per cell relative to untreated controls was observed (Fig. 2A). Cells treated with a 1:10 dilution of LK also demonstrated an increase in the percentage of D7- and A6-positive cells during the first 2 days (Fig. 2B). Expression of the antigen detected by A6 increased over 200% after 3 days exposure to LK compared to an increase of only 70% for the antigen detected by D7. The percentage of positive cells increased approximately 30% for both antibodies following 3 days exposure to LK (Fig. 2B).

The ability of the tumor cells to maintain antigen expression following treatment with LK or DMSO for 48 h was examined. Removal of LK or DMSO resulted in the loss of the antigens recognized by A6 or D7 (Fig. 4). Cells examined by IFA with D7 rapidly lost up to 60% of their fluorescence intensity after removal of either LK or DMSO for 24 h (Fig. 4, A and B). In contrast, removal of LK or DMSO resulted in a decrease in fluorescence intensity of less than 20% on cells stained with A6 after the first 24 h (Fig. 4, A and B). However, the decrease in fluorescence intensity to the same levels as the D7-stained cells occurred during the second 24-h period. The percentage of cells expressing D7 or A6 declined after the removal of LK or DMSO (Fig. 4, C and D).

The coexpression of the antigens recognized by D7 and A6 was assessed by measuring fluorescence intensity of B16-F10 and B16-F1 cells stained with a mixture of D7 and A6 (Fig. 5). The fluorescence intensity of LK- and DMSO-treated B16 cells was increased when the cells were stained with a mixture of D7 and A6 compared to the individual controls.

Microscopic examination of B16 melanoma cultures containing concentrations of LK or DMSO sufficient to modulate antigen expression suggested that these cells did not divide as frequently as untreated cells. Measurement of [3H]thymidine incorporation revealed that treatment of B16 cultures with LK or DMSO inhibited cell proliferation over time (Table 4). Incorporation of [3H]thymidine into LK-treated cells was re-
MODULATION OF B16 MELANOMA ANTIGENS

Fig. 1. Effect of DMSO treatment on B16-BL6 melanoma antigen expression. Replicate plates of B16-BL6 cells were grown in vitro in the presence of various concentrations of DMSO (1.0 to 2.5%, v/v) for up to 3 days. Cells from each series were harvested daily, stained with D7 or A6 and FITC-anti-IgM, and evaluated for immunofluorescence by flow cytometry. Change in fluorescence intensity (FI) (A and B) is expressed as percentage of change relative to control untreated cells stained with D7 (A) or A6 (B). C and D, percentage of cells stained with D7 (C) or A6 (D) with fluorescence intensity greater than that of cells stained with control antibody and FITC-anti-IgM.

Reduced by 65% after 24 h exposure and 69% after 48 h. Cells treated with DMSO were affected similarly.

The effect of LK and DMSO exposure on protein synthesis in B16 melanoma cells was also determined by measuring the incorporation of [35S]methionine into cellular protein (Table 4). Treatment of cells with LK reduced the incorporation of [35S]methionine by 20% after 24 h exposure and by more than 55% after 48 h. Incorporation of [35S]methionine into cells exposed to DMSO was reduced by more than 40% after 24 h and more than 60% after 48 h.

DISCUSSION

Monoclonal antibodies have been generated against a variety of antigens expressed on human and mouse tumor cells. These antibodies have been used primarily to study the characteristics and expression of tumor-associated antigens in vitro (6–9, 27). A number of investigators have produced monoclonal antibodies to human or mouse melanomas by utilizing the spleen cells from mice that had been immunized with cultured cells. The approach described in this report used splenocytes taken from syngeneic mice bearing the B16-F10 tumor at various stages of growth. Baniyash et al. (23) have hypothesized that the presentation of antigenic determinants on the membrane of tumor cells may be different in tumor-bearing animals compared to normal animals artificially immunized with these determinants. The production of hybridomas using splenocytes from tumor-bearing animals may yield monoclonal antibodies that reflect the animal’s humoral immune response to the tumor. The
MODULATION OF B16 MELANOMA ANTIGENS

Fig. 4. Antigen expression requires the presence of LK or DMSO. Replicate plates of B16-F10 and BL6 cells, previously grown in vivo, were treated with 1.0 µg of [3H] thymidine or [35S] methionine per culture. Cells were harvested after labeling for 12 h using a MASH II unit and incorporated radioactivity was determined. The values listed indicate the percentage reduction of [3H] thymidine or [35S] methionine incorporation relative to untreated controls.

Table 4 Inhibition of DNA and protein synthesis by lymphokine or DMSO

|       | LK  | DMSO  
<table>
<thead>
<tr>
<th></th>
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</tr>
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<tbody>
<tr>
<td>[% of inhibition]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[3H]Thymidine</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>69.2</td>
</tr>
<tr>
<td>[35S]Methionine</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>44.4</td>
<td>66.2</td>
</tr>
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</table>

monoclonal antibodies D7 and A6 examined in this study are produced by hybridomas resulting from the fusion of splenocytes taken from mice 15 days after intradermal injection of B16-F10 tumor cells. The level of circulating antibody reactive with the B16 melanoma rapidly rose after 12 days of tumor growth and peaked at about day 22 (data not shown). Therefore, the use of day 15 splenocytes for the production of hybridomas corresponded with a rapidly developing humoral immune response.

The specificity of monoclonal antibodies D7 and A6 was tested by ELISA and indirect fluorescence with cultured cells and by immunohistochemical staining of frozen sections. The monoclonal antibodies reacted with the B16 melanoma, with another syngeneic melanoma, and only very weakly or not at all with other cultured cell lines and normal tissues. The high lung colony-forming and spontaneously metastasizing variants of the B16 melanoma, F10 and BL6, reacted with greater fluorescence intensity and a higher percentage of positive cells than did the lower colony-forming, F1, variant. An enhancement of fluorescence intensity was observed when F10 and F1 cells were examined with D7 and A6 together. This was not observed with the BL6 cells and may suggest that the greater overall antigen density measured on the BL6 cells may cause steric hindrance resulting in saturation of binding with fewer antibody molecules.

B16 cells isolated from actively growing tumors were also reacted with D7 and A6. This demonstrated that the antigens detected by these monoclonal antibodies are also expressed in vivo. The level of antigen binding by A6 was much greater on cells from relatively small (10-day) tumors compared to the binding of D7. However, this difference was slightly reversed on cells taken from very large (25-day) tumors. The differential reactivity of freshly isolated tumor cells with D7 suggests two possible explanations. One hypothesis is that the rapidly growing melanoma cells in a small tumor do not express the antigen detected by D7 until later stages when the growth rate may decrease due to limitations in blood supply and necrosis. A second possible explanation may be the presence of host-derived antibody on the surface of the freshly isolated tumor cells masking the antigen detected by D7 in the small tumor. As the tumor burden becomes very large, the host immune response may be reduced leaving the antigen available for binding with D7.

While we have not been successful at immunoprecipitating the antigens recognized by D7 and A6, experimental evidence suggests that these antibodies detect different epitopes and may be protein. D7 did not block the binding of A6 suggesting that the antibodies recognize different epitopes. The binding of D7 and A6 was also examined following traditional alcohol fixation, which dissolves the methanol-soluble membrane lipids, with no loss of antigen expression. The expression of the antigens following trypsin treatment decreased with the length of time of trypsin exposure. Finally, the expression of the antigens following trypsin treatment decreased with the length of time of trypsin exposure. Finally, the expression of the antigens detected by D7 and A6 was reduced following incubation of B16 cells in the presence of cycloheximide, a potent protein synthesis inhibitor (data not shown).

Many of the monoclonal antibodies reacting with antigens expressed on human and mouse tumor cells have been used to study the characteristics and expression of TAAas in vitro (6–9, 27). In order to utilize these monoclonal antibodies most effectively, it is desirable to maximize the expression of the recognized TAAas. Agents which would increase the proportion of cells expressing TAAas in a heterogeneous tumor cell population as well as increase the antigen density per cell would facilitate the use of monoclonal antibodies as diagnostic and therapeutic tools.

In this study, we have demonstrated that exposure of B16 melanoma cells to a mixed lymphokine preparation and to DMSO enhanced the expression of cell surface antigens recognized by monoclonal antibodies. The cultures stimulated with LK or DMSO contained a greater proportion of cells expressing...
the antigens recognized by monoclonal antibodies D7 and A6 than did unstimulated controls. Cultures exposed to DMSO for 2 to 3 days became nearly 100% positive. In addition to increasing the proportion of antigen-positive cells, the antigen density per cell, as measured by fluorescence intensity, was substantially increased following exposure to LK and DMSO. However, lower concentrations of DMSO, while inducing the expression of antigens recognized by A6 and D7 by cells not previously expressing the antigen, did not increase the amount of antigen expressed by those cells. Thus the percentage of cells reacting with the antibody increased while the fluorescence intensity remained unchanged.

The effects of treatment with LK or DMSO were apparent within 48 h of exposure but did not persist after the agent was removed from the cultures, suggesting that the enhancement of antigen expression was a transient event rather than a transformation of the melanoma cells. The effect of LK or DMSO on antigen expression was blocked by the protein synthesis inhibitor cycloheximide. Treatment of cells with LK or DMSO also reduced the DNA and protein synthesis.

Previous studies on the effects of mixed and partially purified lymphokines have demonstrated the ability of several lymphokines to enhance the expression of tumor-associated antigens (29–32). Interferons have been shown to increase the expression of histocompatibility as well as tumor-associated antigens in several systems (28, 41–43). The enhanced antigen expression and cytotoxic activity observed with the LK preparation used in this study may be due to the action of one or more biologically active components including lymphotoxins and the interferons (44–46).

The results obtained in this study of B16 melanoma cells treated with DMSO are consistent with observations made in other tumor cell systems. The polar organic compound DMSO has been extensively studied as a differentiation inducer in several cell lines including the Friend murine erythroleukemia, and the HL-60 human promyelocytic leukemia. Differentiation and expression of the erythroid phenotype in Friend erythroleukemia cells treated with DMSO was determined to be independent of cell division (33). DMSO had been shown to increase doubling time, change cellular enzyme content, and alter carcinoembryonic antigen expression in human adenocarcinoma cells (34). The expression of class I major histocompatibility complex antigens on mouse lung carcinoma cells was recently reported to be substantially increased following exposure to DMSO (35). Differentiated functions such as reduced cell growth and enhanced melanin synthesis have been reported in human melanoma cells following exposure to DMSO (36). We have observed that the enhanced antigen expression detected by monoclonal antibodies D7 and A6 was dependent on the presence of DMSO in the culture medium such that antigen expression returned to base-line levels following its removal. This suggests that this was a transient effect rather than irreversible differentiation.

The monoclonal antibodies D7 and A6 appear to recognize different epitopes expressed on two different membrane molecules. In the absence of conclusive immunoprecipitation data, two lines of evidence lead to this conclusion. (a) Analysis of LK- or DMSO-treated cells with D7 or A6 alone yields fluorescence intensities that are lower than when the cells are analyzed with a mixture of the two monoclonal antibodies. Since all analytical antibodies are used at saturating concentrations, this suggests that the monoclonal antibodies D7 and A6 recognize different epitopes. (b) The kinetics of antigen expression during LK or DMSO exposure and subsequent loss of antigen following removal of the inducing agent appears to follow different patterns when the cells are analyzed with D7 compared to A6. Lymphokine-exposed cells analyzed with D7 demonstrate an increase in antigen expression but not to the same extent as when the cells are analyzed with A6. Similarly, cells treated with DMSO appear to require 48 h to demonstrate a substantial increase in the antigen expression detected by D7 whereas these same cells show a rapid increase in antigen within 24 h when analyzed with A6. The loss of the antigen recognized by D7 occurs rapidly within 24 h of removal of LK or DMSO but analysis of those cells with A6 reveals more than 80% of the antigen remaining after 24 h without LK or DMSO and the rapid reduction in antigen density not occurring until an additional 24 h have passed.

The potential of monoclonal antibodies as diagnostic and therapeutic tools is only beginning to be realized. Studies of monoclonal antibodies that recognize tumor-associated antigens has revealed that some of these antigens are expressed at very low densities on the surface of cells making detection difficult. Effective utilization of monoclonal antibodies that detect such TAAs may require concurrent use of agents that increase the level of antigen expression as well as the proportion of tumor cells expressing the antigen. Treatments which maximize the expression of TAAs facilitate detection of such antigens and may lead to increased analytical and therapeutic applications of monoclonal antibodies.

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MODULATION OF B16 MELANOMA ANTIGENS


Modulation of B16 Melanoma Antigen Expression by Lymphokines and Dimethyl Sulfoxide

Kevin L. Trimpe and Bruce S. Zwilling


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