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MODULATION OF CELL-CYCLE ASSOCIATED ANTIGEN EXPRESSION BY THE B16 MELANOMA: MULTIPARAMETER ANALYSIS USING MONOCLONAL ANTIBODIES AND FLOW CYTOMETRY

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MODULATION OF CELL-CYCLE ASSOCIATED ANTIGEN EXPRESSION

BY THE B16 MELANOMA: MULTIPARAMETER ANALYSIS USING

MONOCLONAL ANTIBODIES AND FLOW CYTOMETRY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

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* * * * *

The Ohio State University

1985

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B16 Melanoma

The B16 melanoma, that arose spontaneously in a C57BL/6J mouse (Green, 1968), has been extensively studied as model for tumor growth and metastasis (Fidler, 1978; Fidler and Hart, 1982; Poste et al, 1982; Poste and Fidler, 1980; Talmadge and Fidler, 1982). A number of clones of the B16 melanoma have been isolated which differ in their growth and lung colonizing characteristics (Fidler, 1973; Hart, 1979; Poste et al, 1981; Stackpole, 1983). The sublines B16-F1 and B16-F10 were derived by Fidler from the parent B16 line by selection for their ability to form lung colonies following intravenous (i.v.) injection and subsequently established in vitro after one (B16-F1) or ten (B16-F10) cycles of lung colony formation (Fidler, 1973). A number of other sublines such as the B16-BL6 (Hart, 1979) have been selected based on their invasive and metastatic properties (Poste et al, 1981; Stackpole, 1983).

Cells of the B16 melanoma have been shown to be heterogeneous with respect to their ability to form metastases. Metastatic heterogeneity has been assessed principally on the capability of cultured cells to form tumor colonies in the lungs of mice following intravenous injection. These experimental metastasis or lung
colonization assays reflect in some cases, but not always, the capability of a B16 melanoma subline to spontaneously metastasize (Stackpole, 1981; Stackpole, 1983). Stackpole has reported the progressive emergence of clonal variants within primary tumors and metastases of the B16 melanoma. While individual metastases may have been monoclonal in origin, after a period of proliferation they became as heterogeneous as the parent tumor with respect to metastatic potential. Selection pressures within the tumor or from host-tumor interactions may regulate the phenotypic shifts as heterogeneity progresses. The establishment of B16 melanoma sublines which retain 'high' or 'low' lung colonizing or invasiveness capabilities is not inconsistent with the evidence describing heterogeneity within a given tumor. Differential rates of colonizing cell formation have been described for the B16-F1 and B16-F10 variants with the rate of the F10 variant nearly 5 times that of the F1 (Hill et al, 1984).

The immune response to the B16 melanoma involves both humoral and cell-mediated mechanisms (Kato and Marcus, 1971; Bartholomaeus et al, 1974; Fidler, 1974; Baniyash et al, 1982). Syngeneic C57BL/6J mice injected intradermally with viable B16 melanoma cells produce complement-fixing and cytotoxic antibodies to B16 antigens (Kato and Marcus, 1971; Bartholomaeus et al, 1974). These antibodies reacted with both cytoplasmic and cell surface determinants as detected by immunofluorescence. Antibody titers increased over the first two weeks, remained constant during the third week, then declined before death occurred at day 25. Cells of the B16-F1 variant have been shown to express a higher serologically detectable antigenicity than cells
of the B16-F10 line (Baniyash et al., 1982). Immune lymphocytes were
detectable in the spleen and regional lymph nodes of tumor-bearing
mice by the sixth day of tumor growth (Bartholomaeus et al., 1974).
Macrophages in the tumor increased in number during tumor growth.
Immune splenic lymphocytes were capable of inhibiting B16 growth in
vitro while those from the regional lymph nodes stimulated B16 growth.
Mice that were specifically immunized against the B16 melanoma
demonstrated a significant decrease in incidence of experimental
pulmonary metastasis following i.v. injection of tumor cells (Fidler,
1974). Fidler has also shown that immune lymphocytes from the spleen
or lymph nodes decreased the number of experimental metastases formed
when these lymphocytes were mixed (5000:1) with B16 melanoma cells
prior to i.v. injection. Conversely, normal lymphocytes or
lymphocytes from tumor-bearing animals enhanced the number of lung
colonies formed when mixed with B16 melanoma cells. Serum from
tumor-bearing mice converted from inhibitory to stimulatory the action
of immune spleen lymphocytes on B16 growth (Bartholomaeus, 1974).
Loss of serum factors (antibodies or antigen-antibody complexes)
capable of blocking spleen cell cytotoxicity occurred 24 hours after
tumor excision (Bray and Keast, 1975). Cells from regional lymph
nodes which stimulated tumor growth before the excision of a primary
B16 tumor were no longer stimulatory 3 days after tumor excision. It
has been hypothesized that the immune response may have a dual role in
its relationship to the development, progression, and perhaps spread
of cancer (Prehn, 1971; Fidler, 1974).
Monoclonal Antibodies to Tumor-Associated Antigens

The development of monoclonal antibodies against tumor-associated antigens has enormous potential in the analysis, detection, diagnosis, localization and monitoring of primary and metastatic disease. Monoclonal antibodies have been generated against antigens expressed on a wide variety of tumors including malignant melanoma, pancreatic adenocarcinoma, prostate adenocarcinoma, lung squamous cell carcinoma, ovarian mucinous cystadenocarcinoma, and leukemias (Seeger et al., 1981; Metzgar et al., 1982; Starling et al., 1982; Brenner et al., 1982; Bhattacharya et al., 1982; Forster et al., 1982). These antibodies have been used primarily to study the characteristics and expression of tumor-associated antigens in vitro.

Recently, monoclonal antibodies have been utilized as diagnostic and therapeutic tools. Monoclonal antibodies specific for acute lymphoblastic leukemia have been successful in reducing the number of circulating lymphoblasts in a patient with a heavy tumor burden (Ritz et al., 1981). Monoclonal antibodies labeled with radioactive isotopes may be used in radioimmunodetection of tumor cells. Several investigators have successfully detected tumor in patients receiving I-131 labelled anti-carcinoembryonic antigen (CEA) or anti-alpha-fetoprotein (AFP) antibodies (Goldenberg et al., 1980; Kim et al., 1980).

A number of laboratories have developed monoclonal antibodies to human and mouse melanomas. Koprowski and co-workers (1978) were the first to fuse splenic lymphocytes from mice immunized with human melanoma cell lines to a mouse myeloma cell to produce hybridomas that
secreted antibodies against human melanomas. Antibodies that reacted with most melanoma cell lines tested but not with other tumor or normal cell lines were isolated from the cultures. One monoclonal antibody that was extensively studied from this group was designated 69115Nu-4B and was found to react with all melanomas tested as well as two astrocytoma cell lines and at low levels to human embryonal fibroblasts. Yeh et al (1979) produced hybridomas secreting antibodies reactive with human melanoma after immunizing BALB/c mice with M1804 melanoma cells. These monoclonal antibodies showed high levels of binding to 15% of cultured melanomas, intermediate binding to 46% and no binding to 38% in direct binding assays. The antigen detected by these monoclonal antibodies was later found to be expressed not only on melanomas, but also on several normal tissues (Brown et al, 1981).

The identification of the tumor-associated antigen p97 by Woodbury et al (1981) was carried out using monoclonal antibodies from hybridomas produced by immunizing BALB/c mice with SK-MEL 28 cells. Radioimmunoprecipitation studies indicate that 90% of cultured melanomas and 50% of melanoma biopsies contained detectable p97 (Woodbury et al, 1981). While p97 was not detected by this method in a variety of tissues from normal adults, it was found in some human fetal tissues, particularly in fetal colon, leading the investigators to conclude that p97 was an oncofetal or differentiation antigen. Brown et al (1981) later examined normal adult tissues further, using a sensitive radioimmunoassay, and detected p97, often in minute quantities, on all tissues tested. Additional studies of p97 using partial amino acid sequencing showed substantial homology with serum
transferrin and the ability to bind iron (Brown et al, 1982). The p97 gene, like the transferrin and transferrin receptor genes, was found to be located on chromosome 3 (Plowman et al, 1983). To date, p97 is the best-characterized human melanoma-associated antigen with regard to molecular structure and its possible function.

Dippold et al (1980) developed monoclonal antibodies following immunization with human melanoma cells which recognized a 150kd glycoprotein and two other antigens with characteristics of gangliosides. The gp150 was distributed on a wide variety of normal and malignant cell types while the glycolipid antigens were restricted mostly to melanomas and astrocytomas. Other investigators have described the development of additional monoclonal antibodies with reactivity to a variety of antigens associated with human melanoma cells (Imai et al, 1981; Morgan, 1982; Galloway et al, 1981; Bumol and Reisfeld, 1982).

Monoclonal Antibodies to B16 Melanoma-Associated Antigens

While a large number of monoclonal antibodies have been developed against human melanoma-associated antigens, relatively few have been specifically generated with reactivity to the B16 mouse melanoma. A cross-reacting, melanoma-specific antigen termed B700, which is produced by the B16 melanoma has been identified by several investigators (Klingler et al, 1976; Gersten and Marchalonis, 1979; Gersten et al, 1981). B700 is expressed on the cell surface and is eventually shed into the culture medium. The B700 antigen has a molecular weight of approximately 70 kd and has been purified and
partially sequenced. The data indicate that there is significant sequence homology between B700 and serum albumin (Marchalonis et al., 1984; Hearing et al., 1985). This situation is analogous to the relationship between the human melanoma antigen p97 and transferrin (Brown et al., 1982). Tomita et al. (1985) have developed 8 monoclonal antibodies in the rat system which have reactivity to the B700 antigen. The hybridomas were prepared from splenocytes taken from rats which were repeatedly injected with the B700 antigen in complete Freund's adjuvant. The resulting monoclonal antibodies were screened against purified B700 and various albumins using the ELISA technique. These monoclonal antibodies cross-react with murine albumin but not albumins from other sources. Analysis of the dissociation kinetics have demonstrated that the monoclonal antibodies have less affinity for murine albumin than for the B700 antigen suggesting structural differences in the epitopes of these two antigens.

Wakabayashi et al. (1984) have developed monoclonal antibodies with reactivity to two different determinants expressed on the B16 melanoma. The hybridomas were prepared using splenocytes from C57BL/6 mice which had been immunized at weekly intervals for 10 weeks with mitomycin C treated B16 melanoma cells. The M562 antibody identifies a mouse melanoma (B16) specific determinant whereas the M2590 antibody recognizes the interspecies cross-reactive determinant shared among mouse, hamster and human melanoma cells. These monoclonal antibodies were reported to be specific for melanoma cells since the antibody activity was not absorbed with normal tissues. The amounts of the cross-species determinant expressed on the cell surface were found to
be different from each melanoma cell line. The B16 melanoma and
certain human melanoma cell lines bound much more of the M2590
antibody than did the hamster melanoma or other human melanoma lines.
Immunocchemical analyses have shown that the melanoma specific
determinants recognized by M562 are proteinaceous in nature whereas
the cross-species determinant was composed of a sugar moiety involving
a terminal sialic acid.

Lymphokines

Lymphokines are the glycoprotein hormones that maintain the
homeostasis of the immune system and have the potential to influence
the development of cancer. Some common lymphokines are
lymphocyte-activating factor (LAF), lymphotoxin, macrophage-activation
factor (MAF), T-cell growth factor (TCGF), and leukocyte or
alpha-interferon and immune or gamma-interferon (IFN). Lymphokines
are secreted primarily by lymphocytes although some are released by
nonlymphoid cells such as the production of alpha-IFN by fibroblasts.
While all of the lymphokines have the potential to influence the
growth and development of tumors through their indirect action on
effector cells of the immune system, a few may act directly at the
tumor cell level.

Lymphokine preparations from the conditioned media of
mitogen-stimulated lymphocyte cultures have been shown to affect the
growth, differentiation, and antigen expression of tumor cells
(Fidler, 1980; Ralph et al, 1983; Kim et al, 1983). Supernatants of
concanavalin A-activated spleen cells selectively modulated the
expression of Ia and H-2D antigens on lymphoid cell lines (Kim et al 1983). It was hypothesized that at least one of the factors in the lymphokine preparation was interferon-like in nature. The human monoblast leukemia line U937 was growth-inhibited and induced to develop maturation markers by lymphokine preparations (Ralph et al, 1983). The expression of Fc receptors, complement receptors, Mac-1 and Mac-3 antigens as well as phagocytic and antibody-dependent cellular cytotoxicity (ADCC) functions by the U937 cells followed treatment with lymphkine preparations. Fractions of the lymphokine preparation containing gamma-IFN were capable of inducing Fc receptors, Mac-1, and cytostasis, whereas induction of ADCC required unfractionated lymphokine.

The interferons are a family of glycoproteins which are synthesized by cells in response to viral infection, immune stimulation and a variety of chemical inducers (Toy, 1983). The direct effects of interferons on tumor cells include alterations of the cell membrane, changes in the cytoskeleton, loss of tumorigenicity, and lengthening of the cell cycle. Alterations in the phospholipids of the cell membrane following IFN treatment have been shown to result in a more rigid membrane in sarcoma S-180 cells (Chandrabose et al, 1981). The recombinant human leukocyte interferon, IFN-alpha A/D, has been shown to inhibit differentiation of the B16 mouse melanoma as measured by inhibition of melanogenesis (Fisher et al, 1984). Recombinant human alpha-IFN has been shown to enhance the expression of tumor-associated antigens on human breast and colon tumor cells and the expression of HLA class I antigens (Greiner et al,
1984; Rosa et al., 1983). Natural alpha-IFN has been shown to affect human hematopoietic cell lines and leukemic cells by effecting and accumulation of cells in the G0/G1 phase of the cell cycle (Roos et al., 1984). Some cell lines also exhibited a decreased rate of progress through S-phase. Other investigators have shown alpha- and beta-IFN's to be antiproliferative for a variety of human cell lines including lymphomas, myelomas, lymphoid leukemias, breast carcinomas, osteo- and soft-tissue sarcomas, ovarian carcinomas, melanomas, and urinary tract cancers (Borden et al., 1982).

Gamma-interferon has also been shown to directly affect the growth, differentiation and antigen expression of tumor cells. Recombinant gamma-IFN induced the expression of antigens characteristic of monocytes as well as changes in morphology consistent with monocytoid differentiation in cells of the HL-60 promyelocytic leukemia cell line (Ball et al., 1984). These changes included induction of enzymes, increased cell size, a decrease in azurophilic granules, and de novo protein synthesis as measured by antigen expression. Colony formation by the mouse bladder tumor MBT-2 was inhibited by gamma-IFN in a liquid clonogenic assay (Ratliff et al., 1984). Inhibition of proliferation was both dose and time dependent. The expression of HLA-DR antigens by human peripheral blood monocytes has been enhanced by treatment of the cells with gamma-IFN (Sztein et al., 1984; Virelizier et al., 1984). Monocytes cultured with gamma-IFN had enhanced accessory cell function and were capable of initiating a significantly greater mixed lymphocyte response than control cells (Sztein et al., 1984). The human myelomonocytic cell lines HL-60, U937,
and THP-1 were shown to have enhanced HLA-DR antigen expression following culture with as little as 2 units of recombinant human gamma-IFN (Virelizier et al, 1984). The enhancement of antigen expression was an effect only of gamma-IFN and was not observed with alpha- or beta-IFN's. The effect of gamma-IFN could be abrogated with anti-gamma-IFN antibodies.

Dimethyl Sulfoxide (DMSO)

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. The Friend erythroleukemia cells are induced to differentiate along the erythrocytic pathway by DMSO which is marked by morphological and biosynthetic changes (Friend and Freedman, 1978). Differentiation and the expression of the erythroid phenotype in cells treated with DMSO was determined to be independent of cell division (Tsiftsoglou and Sartorelli, 1979). The phospholipid composition and membrane dynamics of Friend leukemia cells were also altered following DMSO treatment (Tapiero et al, 1983). HL-60 cells cultured in the presence of DMSO differentiate into more mature cells of the myeloid lineage, displaying functional characteristics associated with normal peripheral blood granulocytes (Collins et al, 1979).

DMSO has been shown to increase doubling time, change cellular enzyme content, and alter carcinoembryonic antigen expression in human adenocarcinoma cells (Tsao et al, 1982). Differentiated functions such as reduced cell growth and enhanced melanin synthesis have been
reported in human melanoma cells following exposure to DMSO (Huberman et al., 1979). DMSO induced the differentiation of the rat mammary stem cell line, Rama 25, into doming (alveolar-like) cells releasing casein into the medium (Rudland et al., 1982). These differentiated cells were reduced in their neoplastic potential when injected into nu/nu mice. Low-metastatic Lewis lung carcinoma cells were found to have enhanced lung-colonizing ability following in vitro treatment with DMSO (Takenaga, 1984). Treatment of the cells with DMSO resulted in an increase in homotypic aggregation and adhesiveness which may have enhanced their retention in the lungs.

The expression of Class I MHC antigens on mouse lung carcinoma cells was recently reported to be substantially increased following exposure to DMSO (Bahler and Lord, 1985). This is believed to be the first report demonstrating that DMSO can induce synthesis of MHC-encoded antigens. DMSO-treated murine hepatoma cells (BW77-1) exhibited a dose dependent reduction in population density and an increase in overall protein synthesis accompanied by increased albumin synthesis (Higgins and O'Donnell, 1982). DMSO treatment initiated changes in morphology and F-actin cytoarchitecture of BW77-1 cells prior to the onset of albumin accumulation (Higgins, 1984). An early reorganization of the cytoskeletal microfilament system, in response to DMSO, may be involved in subsequent programmatic and quantitative changes in gene expression.
CHAPTER II

CELL-CYCLE ASSOCIATED ANTIGEN EXPRESSION IN THE B16 MELANOMA
IDENTIFIED BY MONOCLONAL ANTIBODIES

Introduction

Monoclonal antibodies reactive with a wide variety of tumor-associated antigens have been generated following xenogeneic or allogeneic immunizations (Morgan and McIntyre, 1983; Dippold et al., 1980; Herlyn et al., 1979; Young et al., 1981; Koprowski et al., 1978; Seeger et al., 1981; Metzgar et al., 1982; Starling et al., 1982; Brenner et al., 1982; Kupchik et al., 1981; Kim et al., 1980). 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. The expression of several normal and tumor-associated antigens has been shown to be associated with the different stages of the cell cycle (Gerdes et al., 1984; Lanier and Warner, 1981; Jakesz et al., 1984; Brooks et al., 1985; Czerniak et al., 1984).

One of the tumor types that has been extensively studied is malignant melanoma (Fidler, 1973; Banks et al., 1977; Poste et al., 1982; Stackpole, 1981; Moshakis et al., 1981; Albino et al., 1981; Natali et al., 1983; Hellstrom et al., 1983; Ruiter et al., 1984; Atkinson et al., 1984). The cells express antigens that are recognized by the host immune
system resulting in the development of humoral and cell-mediated immunity (Bartholomaeus et al., 1974; Baniyash et al., 1982; Colnaghi et al., 1982; Poskitt et al., 1974). Several monoclonal antibodies to melanoma-associated antigens have been produced by immunizing mice with human or mouse melanoma cells (Koprowski et al., 1978; Dippold et al., 1980; Dippold et al., 1984; Hellstrom et al., 1983; Wakabayashi et al., 1984). We have developed two monoclonal antibodies with anti-melanoma activity by producing hybridomas from syngeneic mice bearing the B16-F10 mouse melanoma. The expression of the antigens detected by these antibodies was heterogeneous within the three variants of the B16 examined. The relationship between levels of antigen expression and DNA content were studied using multiparameter flow cytometry.
**Materials and Methods**

**Animals** C57BL/6J male 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 (1973) and Hart (1979) for their lung colonizing and tissue invading characteristics. Additional cell lines used in screening assays were kindly provided by Dr. C.W. Johnson, Dept. of Pathology, OSU. C57BL/6 lines include: JB/RH, melanoma; JB/MS, melanoma (J. Berkelhammer, AMC Research Ctr., 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. Heppner, Michigan Cancer Foundation, Detroit, MI); Neuro 2a, a strain A-albino neuroblastoma (ATCC); and SCC-PSA1, 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, non-essential 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, Salk Inst.) as one of the fusion partners. These cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) which allows their growth to be inhibited by aminopterin. The myeloma cells were maintained in RPMI 1640 medium (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 utilized from C57BL/6 mice bearing tumor of the F10 variant. Briefly, 20 C57BL/6 male mice, 5 weeks old, were injected ID with $1 \times 10^5$ B16F10 cells. On days 5, 10, 15, 20, and 25, spleens were removed from four mice, a single cell suspension 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 (Oi and Herzenberg, 1980). Cells were plated in 96-well plates using an enriched medium composed of DMEM supplemented with 10% FBS, 10% NCTC-109 (MA Bioproducts), non-essential amino acids, insulin, oxaloacetate, glutamine, pyruvate, and penicillin-streptomycin. HAT selection (hypoxanthine, aminopterin, thymidine) against unfused myeloma cells was initiated on day 1 and culture supernatants screened for antibody production after 3 weeks using a beta-galactosidase ELISA (Amersham, Arlington Hts, IL).
Screening of Hybridomas All hybridoma antibodies were subsequently tested for anti-B16 activity by ELISA. B16-F10 cells were seeded into 96-well plates (Dynatech) at 2x10^4 per well, the cells centrifuged at 350 x g, the wells aspirated, and the cells air-dried overnight. Prior to addition of hybridoma supernates, the cells in the prepared ELISA plates were rehydrated by briefly filling the wells with 0.15M phosphate-buffered saline (PBS), pH 7.4. All subsequent incubations were at 37°C for 1 hour. The target cells were then incubated with undiluted hybridoma supernatants, washed with PBS, and incubated with a beta-galactosidase conjugated, sheep antimouse immunoglobulins (1:400). After washing in PBS, the enzyme substrate o-nitrophenyl beta-d-galactopyranoside (ONPG) (3mM in PBS containing 10mM MgCl₂ and 0.1M 2-mercaptoethanol) was added to each well. After incubation, the OD of each well was determined at 405nm using a Bio-Tek EIA reader and antibody concentration 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 antimouse immunoglobulins (1:500) were coated to the wells with carbonate-bicarbonate buffer, pH 9.6. Antibody isotypes were identified using a horseradish peroxidase ELISA isotyping kit from Sterile Systems.

Immunohistochemical Staining Fresh tissues from normal C57BL/6J mice were quick frozen on dry-ice and 5μm sections prepared using a
cryostat. Endogenous peroxidase activity was inhibited by treatment with 0.3% H₂O₂ in 0.1M Tris-HCl pH 7.6 buffer. Tissue sections were rinsed in 0.1M Tris-HCl pH 7.6 buffer 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 antimouse antibody (1:500, Cappell) for 30 minutes at room temperature. After rinsing the sections with Tris buffer, the 3,3'-diaminobenzidine (DAB) substrate solution (500µg/ml Tris-HCl pH 7.6, 0.1% H₂O₂) was added for 20 minutes at room temperature. Excess DAB solution was rinsed away, the sections were counterstained with fast green for 5 minutes 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 fluoresceinated according to the method of Mishell and Shiigi 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 0.5M carbonate-bicarbonate buffer, pH 9.2 and fluoresceinated by dialysis in this buffer containing 100µg/ml fluorescein isothiocyanate (Sigma) for 14 hours. The fluoresceination reaction was stopped by dialyzing the antibodies
against 0.15M PBS pH 7.0 for two hours.

**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 21g syringe needle. All cultured cells to be examined by flow cytometry for indirect fluorescent antibody staining (IFA) or cell-cycle analysis were taken from 24 hour, log-phase cultures. Cells were harvested from monolayers using a rubber policeman, washed with Seligman's balanced salt solution (SBSS), and incubated with either hybridoma supernatants or monoclonal antibodies prepared from ammonium sulfate precipitates of supernatants for 1 hour on ice. Cells were washed 3 times with SBSS and incubated with a 1:20 dilution of FITC-conjugated goat antimouse IgM (Tago) for 1 hour on ice. Cells were washed 3x with SBSS and examined directly or were fixed prior to analysis in 70% ethanol for 15 minutes on ice.

For some experiments, B16 cells were fixed in methanol prior to staining with monoclonal antibodies. Briefly, cells were harvested from log-phase cultures, washed with SBSS, and resuspended in methanol for 10 minutes on ice. The cells were then gently washed in SBSS and stained with monoclonal antibodies and fluorescent second antibody as described above.

For DNA staining, cells were fixed in ethanol, resuspended in 0.2 ml RNase A, 40ug/ml (Worthington Biochemicals) for 20 minutes, followed by the addition of 0.3 ml propidium iodide (PI, 40ug/ml in PBS; Sigma) solution. Cells were allowed to equilibrate in the PI for
at least 15 minutes prior to analysis.

Cell fluorescence was quantified using a Cytofluorograf 50H cell sorter (Ortho Diagnostics, Westwood MA) equipped with an argon laser tuned to 488nm. The green FITC (520nm) and red PI (600nm) fluorescence emissions from each cell were measured simultaneously by separate photomultipliers and the data stored on disk using the Ortho 2150 computer. Because all staining reagents were used under saturating conditions, green cell fluorescence is linearly related to the quantity of surface antigen. Fluorescence intensity expressed as the percent mean cell fluorescence above background was calculated according to the formula:

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

where FI test is the mean fluorescence intensity of the population stained with the test antibody and FI bkgd is the mean fluorescence intensity of the cells stained with unrelated antibody. The proportion of cells in the G1, S, and G2 + M compartments was determined using cell-cycle analysis computer programs provided with the 2150.

**Cycloheximide Treatment**  B16 cells were seeded onto 100mm tissue culture dishes (Corning) at $2 \times 10^6$ per dish in complete DMEM with 10% FBS. After 12 hours in culture, cycloheximide (Sigma) was added to a final concentration of 3ug/ml and the cells cultured for an additional 12 hours. Cells were then harvested and examined by flow cytometry using IFA and cell-cycle analysis as described above.
Results

The twenty fusions between mouse myeloma cells and splenocytes obtained at five time intervals from C57BL/6 mice with the B16-F10 tumor growing ID resulted in 1765 master wells with antibody producing hybridomas (Table 1). Of these, 168 (9%) secreted antibodies which reacted with B16-F10 cells in an ELISA assay. Following expansion, 52 retained reactivity and 44 were subcloned by limiting dilution to yield 187 monoclonal hybridomas secreting antibodies with reactivity to the original tumor. Several of these hybridoma lines remained stable antibody producers and were tested for reactivity to normal tissues and other tumor cell lines.

The two selected hybridomas, designated 152 E12 D7 (D7) and 153 C7 A6 (A6), were the result of separate fusions involving splenocytes taken from mice 15 days after ID injection with viable B16-F10 cells. The results of binding studies using these two monoclonal antibodies are listed in Table 2. 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. The intensity of staining with A6 was greater than that of D7 while the BL-6 and F10 variants of the
Table 1. Hybridoma production summary. Fusions were performed using the X63.Ag8.653 myeloma and splenocytes from C57BL/6 bearing the syngeneic B16-F10 melanoma for the number of days indicated. Antibody production and reactivity with B16-F10 cells was determined by ELISA. Numbers in parentheses indicate the percentage relative to the value in the previous column.

<table>
<thead>
<tr>
<th>Day</th>
<th>Total Master wells</th>
<th>Total Ab producers</th>
<th>Total F10 positive</th>
<th>F10 pos at 1ml</th>
<th>Subcloned</th>
<th>F10 pos subclones</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>384</td>
<td>374 (97)</td>
<td>31 (8)</td>
<td>5</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>384</td>
<td>376 (98)</td>
<td>39 (10)</td>
<td>15</td>
<td>7</td>
<td>38</td>
</tr>
<tr>
<td>15</td>
<td>384</td>
<td>293 (76)</td>
<td>28 (10)</td>
<td>7</td>
<td>7</td>
<td>41</td>
</tr>
<tr>
<td>20</td>
<td>384</td>
<td>383 (99)</td>
<td>47 (12)</td>
<td>16</td>
<td>16</td>
<td>74</td>
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<tr>
<td>25</td>
<td>384</td>
<td>339 (88)</td>
<td>23 (7)</td>
<td>9</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>Totals</td>
<td>1920</td>
<td>1765 (92)</td>
<td>168 (9)</td>
<td>52</td>
<td>44</td>
<td>187</td>
</tr>
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</table>
TABLE 2
SUMMARY OF ANTIBODY REACTIVITY

<table>
<thead>
<tr>
<th>Cell / Tissue</th>
<th>152 E12 D7</th>
<th>153 C7 A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16 BL-6</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-PSA1</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Clone M3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>410.4</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>Str. Muscle</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Thymus</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Table 2. Reactivity of monoclonal antibodies with B16 melanomas, other tumor lines, and normal mouse tissues. Symbols (+ or -) indicate the degree of reactivity above background controls based on ELISA results for tumor cells and immunohistochemical staining for normal tissue sections.
B16 melanoma reacted to a greater degree than did the F1. Only a slight amount of activity could be detected with the SCC-PSA1 teratocarcinoma, 410.4 mammary tumor 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 them for identification have been unsuccessful. 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 (data not shown). Cells treated with the protein synthesis inhibitor cycloheximide at 3μg/ml for twelve hours demonstrated a 65% reduction in fluorescence intensity and a 20-50% reduction in the percentage of positive cells (Table 3).

The D7 and A6 monoclonal antibodies appear to detect two different epitopes, either on the same or on two different molecules. Incubating B16 cells first with the D7 antibody followed by FITC-labelled A6 did not reduce the fluorescence intensity of the cells compared to medium treated controls (Table 4). Pre-incubation of B16 cells with A6 before the addition of FITC-A6 reduced the fluorescence intensity by 80% and percentage of positive cells by 50%. Additionally, when F10 and F1 cells were incubated with a mixture of D7 and A6, there was an increase in fluorescence intensity of more than 20% compared to each of the monoclonal antibodies alone (Figure 1). There was no additive effect observed with the B16-BL6 variant. The percentage of positive cells was the same for cells analyzed with the monoclonal antibodies used singly or in combination.
**TABLE 3**

CYCLOHEXIMIDE BLOCKS ANTIGEN EXPRESSION IN G0/G1 AND G2+M CELLS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ab</th>
<th>G0/G1</th>
<th>S</th>
<th>G2+M</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>untreated</td>
<td>D7</td>
<td>227</td>
<td>229</td>
<td>242</td>
<td>231</td>
</tr>
<tr>
<td>cycloheximide</td>
<td>D7</td>
<td>69</td>
<td>183</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>untreated</td>
<td>A6</td>
<td>117</td>
<td>92</td>
<td>149</td>
<td>118</td>
</tr>
<tr>
<td>cycloheximide</td>
<td>A6</td>
<td>39</td>
<td>71</td>
<td>56</td>
<td>41</td>
</tr>
<tr>
<td>%+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>untreated</td>
<td>D7</td>
<td>22</td>
<td>25</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>cycloheximide</td>
<td>D7</td>
<td>17</td>
<td>24</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>untreated</td>
<td>A6</td>
<td>22</td>
<td>23</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>cycloheximide</td>
<td>A6</td>
<td>9</td>
<td>20</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 3. The effect of cycloheximide on antigen expression. Replicate plates of B16-BL-6 cells were cultured for 12 hours in complete medium prior to the addition of cycloheximide (3.0ug/ml) to the cultures for an additional 12 hours. The fluorescence intensity (FI) is expressed as the percent mean cell fluorescence above background as described in Materials and Methods. The percentage of positive cells (%+) is corrected for background levels of cells stained with control antibody and FITC-anti-IgM.


**TABLE 4**

**ACTIVITY WITH FLUORESCINATED A6**

<table>
<thead>
<tr>
<th>cells preincubated with:</th>
<th>BL-6 FI</th>
<th>%+</th>
<th>F10 FI</th>
<th>%+</th>
<th>F1 FI</th>
<th>%+</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>518</td>
<td>45</td>
<td>446</td>
<td>41</td>
<td>502</td>
<td>64</td>
</tr>
<tr>
<td>D7</td>
<td>505</td>
<td>49</td>
<td>423</td>
<td>38</td>
<td>473</td>
<td>57</td>
</tr>
<tr>
<td>A6</td>
<td>91</td>
<td>25</td>
<td>71</td>
<td>17</td>
<td>65</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 4. Monoclonal antibodies A6 and D7 do not compete for binding. Replicate plates of B16 melanoma cells were cultured for 24 hours in complete medium. Cells were harvested and incubated with either D7 or A6 followed by staining with fluoresceinated A6 and flow cytometry analysis. Fluorescence intensity is expressed as mean cell fluorescence and the percentage of positive cells (%+) is corrected for background levels of cells stained with unrelated FITC-conjugated antibody.
Figure 1. Enhanced fluorescence intensity of cells stained with a mixture of D7 and A6. Replicate plates of B16 melanoma cells were cultured for 24 hours in complete medium. Cells were harvested and stained with D7 alone, A6 alone, or a mixture of the two monoclonal antibodies followed by FITC-anti-IgM. All antibodies were used at saturating concentrations. Cells were analyzed by flow cytometry with values expressed as mean cell fluorescence.
FIGURE 1

FLUORESCENT INTENSITY

B16BL-6  B16F10  B16F1

D7  D7 + A6  A6
To determine if there was a relationship between antigen expression and cell position in the cell cycle, 2-parameter analysis (D7 or A6 versus DNA content) was performed. Figure 2 depicts a representative cytogram of B16F10 cells stained with the D7 monoclonal antibody and PI. While there is a greater number of cells in the G1 phase, both the fluorescent intensity and percentage of positive cells are greater in the G2+M population. Analysis of cells in each phase demonstrates that as the cells progress through the cell cycle the antigen density on the cell surface increases for both D7 and A6 (Table 5). The increase in fluorescence intensity seen in later phases following staining with D7 or A6 and the FITC reagent was not due exclusively to an increase in cell size since cells of all diameters were observed in each cell-cycle phase. The BL-6 and F10 variants had similar percentages of positive cells but the antigen density on the BL-6 cells was much higher than the other two. The fluorescence intensity of cells of the F1 variant and particularly the percentage of positive cells were much lower than that measured with the F10 and BL-6 lines. A similar pattern of cell-cycle related antigen expression was observed in cells isolated from growing tumors of all three variants (not shown).

Cycloheximide treatment affected only the cells in the G0/G1 and G2+M phases and not S-phase cells (Table 3). Fluorescence intensity was reduced by more than 60% in G0/G1 and G2+M cells following incubation in the presence of cycloheximide. The percentage of non-S phase cells binding A6 was reduced by more than 60% while the percentage binding D7 was approximately 70% of control levels.
Figure 2. Cytogram representing FITC (x-axis) fluorescent intensity, corresponding to antigen concentration, and PI (y-axis) fluorescent intensity, corresponding to DNA content, of individual B16-F10 cells. Cells were harvested, stained with D7 and FITC-anti-IgM, fixed in 70% ethanol, and stained with propidium iodide before multiparameter flow cytometry analysis. Distribution of FITC fluorescence on cells incubated with the D7 monoclonal antibody in the basic cell cycle compartments. The increase in antigen expression detected by D7 is evident in the G2+M phase.
Table 5. Cell-cycle associated antigen expression. Replicate plates of B16 melanoma cells were cultured for 24 hours in complete medium. Cells were harvested, stained with D7 or A6 and FITC-anti-IgM, fixed in 70% ethanol, and stained with propidium iodide before multiparameter flow cytometry analysis. The FITC fluorescence intensity (FI) of cells in each cell-cycle compartment was analyzed and is expressed as percent mean cell fluorescence above background. The percentage of positive cells (%+) is corrected for background levels.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Antibody</th>
<th>GO/G1 FI</th>
<th>GO/G1 %+</th>
<th>S FI</th>
<th>S %+</th>
<th>G2+M FI</th>
<th>G2+M %+</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL-6</td>
<td>152 E12 D7</td>
<td>375</td>
<td>29</td>
<td>368</td>
<td>30</td>
<td>407</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>153 C7 A6</td>
<td>368</td>
<td>42</td>
<td>366</td>
<td>52</td>
<td>447</td>
<td>60</td>
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<tr>
<td>F10</td>
<td>152 E12 D7</td>
<td>207</td>
<td>39</td>
<td>250</td>
<td>40</td>
<td>337</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>153 C7 A6</td>
<td>163</td>
<td>52</td>
<td>230</td>
<td>59</td>
<td>250</td>
<td>65</td>
</tr>
<tr>
<td>F1</td>
<td>152 E12 D7</td>
<td>138</td>
<td>5</td>
<td>206</td>
<td>12</td>
<td>172</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>153 C7 A6</td>
<td>171</td>
<td>7</td>
<td>231</td>
<td>12</td>
<td>190</td>
<td>13</td>
</tr>
</tbody>
</table>
Antibodies D7 and A6 were tested for reactivity against freshly isolated B16 tumor cells (Table 6). 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 A6 antigens. Whereas at day 10 the level of antigen expression for D7 was quite low, by day 25 the tumor cells were expressing the D7 antigen at the same or greater densities than A6.
TABLE 6

REACTIVITY WITH FRESHLY ISOLATED TUMOR CELLS

<table>
<thead>
<tr>
<th>Antibody</th>
<th>BL-6</th>
<th>%+</th>
<th>F10</th>
<th>FI</th>
<th>%+</th>
<th>F1</th>
<th>%+</th>
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</thead>
<tbody>
<tr>
<td>10 day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>152 E12 D7</td>
<td>141</td>
<td>6.3</td>
<td>63</td>
<td>8</td>
<td></td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>123 C7 A6</td>
<td>377</td>
<td>45.5</td>
<td>135</td>
<td>23</td>
<td></td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>25 day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>152 E12 D7</td>
<td>215</td>
<td>42.5</td>
<td>127</td>
<td>44</td>
<td>184</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>123 C7 A6</td>
<td>144</td>
<td>47.5</td>
<td>165</td>
<td>44</td>
<td>101</td>
<td>34.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Expression of antigen on freshly isolated B16 melanoma cells detected by D7 and A6 monoclonal antibodies. Single cell suspensions of tumor cells taken from 10-day-old or 25-day-old growing ID tumors were prepared and the cells stained with D7 or A6 and FITC-anti-IgM. Cells were analyzed by flow cytometry for FITC fluorescence intensity. The FITC fluorescence intensity (FI) of each cell was analyzed and is expressed as percent mean cell fluorescence above background. The percentage of positive cells (%+) is corrected for background levels.
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 (Seeger et al., 1981; Metzgar et al., 1982; Starling et al., 1982; Brenner et al., 1982; Wakabayashi et al., 1985). 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. (1982) 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 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 rises.
after 12 days, and peaks at about day 22 (data not shown). Therefore, the use of day 15 splenocytes for the production of hybridomas corresponds to a rapidly developing humoral immune response.

The specificity of monoclonal antibodies D7 and A6 was tested by ELISA and IFA with cultured cells and by immunohistochemical staining of frozen sections. The reactivity of both monoclonal antibodies was greater with the B16 melanoma than with other cultured cell lines and normal tissues. The high lung colony forming and spontaneously metastasising variants F10 and BL-6 reacted with greater fluorescence intensity and larger percentage of positive cells than did the lower colony forming F1 variant. B16 cells isolated from actively growing tumors were also stained by D7 and A6 using IFA, demonstrating 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 the 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 been unsuccessful at immunoprecipitating the antigens recognized by D7 and A6, experimental evidence suggests that these antibodies detect antigens composed, in part at least, of protein. Traditional alcohol fixation procedures dissolve the methanol-soluble membrane lipids, leaving the 'fixed' cellular proteins. When methanol fixed B16 melanoma cells were examined with D7 and A6 by IFA and flow cytometry, there was no loss of antigen expression either as fluorescence intensity or percentage of positive cells. Secondly, the expression of the antigens detected by D7 and A6 were effectively reduced following incubation of B16 cells in the presence of cycloheximide, a potent protein synthesis inhibitor.

Analysis of B16 melanoma cells by multiparameter flow cytometry demonstrated a heterogeneity of antigen expression within a given population of cells. The effect of cell volume (surface area) on antigen expression was examined and failed to demonstrate any relationship between cell volume and fluorescence intensity of D7 or A6 stained cells. Data from simultaneous measurement of DNA content (PI staining) and IFA staining suggest that cell-cycle related events may be responsible for heterogeneity in antigen expression. B16 cells in the S phase, and to a greater degree, G2+M phases of the cell cycle demonstrated both an increase in fluorescence intensity and a greater percentage of cells expressing the antigens detected by D7 and A6. Additionally, when B16 cells were cultured in the presence of cycloheximide, the reactivity of cells in the G0/G1 and G2+M stages was dramatically reduced whereas the S phase cells were relatively
unaffected. Since the cells were exposed to cycloheximide for 12 hours prior to flow cytometry analysis, these data suggest that antigen synthesis occurs primarily in the G2+M phases and during the time prior to analysis, the cells with cycloheximide-reduced antigen expression progressed into the G0/G1 compartment. Biosynthesis of several other antigens, receptors, and secreted products have been associated with particular phases of the cell cycle (Czerniak et al, 1984; Brooks et al, 1985; Tsukada et al, 1975). While the cell-cycle related antigen expression in the B16 melanoma is interesting, it remains to be determined if it is significant in the growth and metastasis of this tumor line.
CHAPTER III

Introduction

Monoclonal antibodies generated against tumor-associated antigens (TAAs) may detect only a subpopulation of cells expressing an antigen at relatively high density (Greiner et al, 1984). 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 (Attallah et al, 1979; Imai et al, 1981; Liao et al, 1982; Ng et al, 1983). DMSO has been extensively studied as an inducer of differentiation and modulator of cell growth and function (Tsiftsoglou and Sartorelli, 1979; Tsao et al, 1982; Bahler and Lord, 1985; Huberman et al, 1979; Higgins and O'Donnell, 1982). In this study we describe the use of a mixed lymphokine preparation and DMSO to enhance the biosynthetic expression of cell-surface antigens on B16 melanoma.
Materials and Methods

Cell lines The B16 melanoma lines F1, F10 and BL6 were selected by Fidler (1973) and Hart (1979) for their lung colonizing and tissue invading characteristics. Cells were grown in tissue culture from frozen stocks in Dulbecco's Minimal Essential Medium (DMEM, MA Bioproducts) supplemented with 10% fetal bovine serum (FBS, Sterile Systems, Logan UT), vitamins, non-essential amino acids, pyruvate, glutamine, and penicillin-streptomycin (MA Bioproducts). Single cell suspensions were obtained by scraping monolayers with a rubber policeman.

Antibodies Monoclonal antibodies 152 E12 D7 (D7) and 153 C7 A6 (A6), both of the IgM class, were developed from syngeneic mice bearing the B16-F10 tumor as previously described. Monoclonal antibodies were partially purified by precipitation with saturated ammonium sulfate followed by extensive dialysis against 0.15M phosphate buffered saline (PBS). FITC-conjugated goat anti-mouse IgM was obtained from Tago and was diluted 1:20 in PBS prior to use.

Lymphokine preparation The mixed lymphokine preparation (LK) used in this study was prepared from mitogen stimulated spleen cell cultures. Briefly, C3H/HeN splenocytes were cultured for 3 days at 5x10^6/ml in
RPMI 1640, without FBS, supplemented as above containing 2μg/ml concanavalin A (Sigma) and 5x10^-5 M 2-mercaptoethanol (Sigma). The supernatant was collected, filtered through a 0.22μm filter (Nalgene) and stored at 4°C. This lymphokine preparation was diluted to the proper concentration in complete DMEM, 10% FBS immediately before addition to B16 melanoma cultures.

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 21g syringe needle. All cell cultures to be examined by flow cytometry for direct or indirect fluorescent antibody staining (IFA) or cell-cycle analysis were cultured in complete medium 24 hours before the addition of medium containing lymphokine or DMSO. Cells were harvested from monolayers using a rubber policeman, washed with Seligman's balanced salt solution (SBSS), and incubated with monoclonal antibodies (prepared from ammonium sulfate precipitates of supernatants) for 1 hour on ice. Cells were washed 3 times with ice cold SBSS and incubated with a 1:20 dilution of FITC-conjugated goat antimouse IgM for 1 hour on ice. Cells were washed 3 times with SBSS and examined directly or were fixed prior to analysis in 70% ethanol for 15 minutes on ice.

For some experiments, B16 cells were fixed in methanol prior to staining with monoclonal antibodies. Briefly, cells were harvested from log-phase cultures, washed with SBSS, and resuspended in methanol for 10 minutes on ice. The cells were then gently washed in SBSS and
stained with monoclonal antibodies and fluorescent second antibody as described above.

For DNA staining, cells were fixed in ethanol, resuspended in 0.2 ml RNase A (40ug/ml in PBS, Worthington Biochemicals) for 20 minutes, followed by the addition of 0.3 ml propidium iodide (PI, 40ug/ml in PBS) solution. Cells were allowed to equilibrate in the PI for at least 15 minutes prior to analysis.

Cell fluorescence was quantified using a Cytofluorograf 50H cell sorter (Ortho Diagnostics, Westwood MA) equipped with an argon laser tuned to 488nm. The green FITC (520nm) and red PI (600nm) fluorescence emissions from each cell were measured simultaneously by separate photomultipliers and the data stored on disk using the Ortho 2150 computer. Because all staining reagents were used under saturating conditions, green cell fluorescence is linearly related to the quantity of surface antigen. Fluorescence intensity expressed as the percent mean cell fluorescence above background was calculated according to the formula:

$$\% \text{FI} > \text{bkgd} = \frac{(\text{FI test}) - (\text{FI bkgd})}{(\text{FI bkgd})} \times 100$$

where FI test is the mean cell fluorescence of cells stained with D7 or A6 and FI bkgd is the background mean cell fluorescence of cells stained with an unrelated antibody. The proportion of cells in the G1, S, and G2 + M compartments was determined using cell-cycle analysis computer programs provided with the 2150.
Cycloheximide Treatment  B16 cells were cultured in 100mm tissue culture dishes (Corning) at 2x10^6 per dish in complete DMEM with 10% FBS. After 12 hours in culture, cycloheximide (Sigma, St. Louis, MO) was added to a final concentration of 3.0ug/ml and the cells cultured for an additional 12 hours. Cells were then harvested and examined by flow cytometry using IFA and cell-cycle analysis as described above.

Incorporation of H-3TdR and S-35 methionine  Incorporation of tritiated thymidine (H-3TdR) and S-35 methionine (Amersham, Arlington Hts, IL) into B16 cells was used to estimate DNA and protein synthesis, respectively. B16-F10, F1 and BL-6 cells were seeded into 96-well plates (Costar) at 3x10^3/well and allowed to adhere prior to the addition of LK or DMSO. Cells were cultured for 24 or 48 hours in LK or DMSO dilutions followed by the addition of 1.0uCi H-3TdR or S-35 methionine (Amersham, Arlington Hts. IL). Radiolabelled cells were harvested 12 hours later using a MASH II unit and incorporated radioactivity determined by liquid scintillation counting.
Results

We have previously described two monoclonal antibodies, designated D7 and A6, which recognize antigens expressed on the surface of the B16 mouse melanoma. The antigens recognized by D7 and A6 are detected on a subpopulation of cells in log-growth comprising 30% to 60% of the total population and appears to increase, on a given positive cell, as the cell progresses through the cell cycle.

B16 melanoma cells exposed to DMSO demonstrated an increase in antigen expression that was both dose and time-dependent (Figure 3). The fluorescence intensity of cells treated with 2.5% DMSO and stained with A6 increased after the first 24 hours by more than 150% of control levels (Figure 3b). 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 (Figure 3a). There was a rapid increase in the percentage of B16 cells reacting with D7 or A6 after 24 hours exposure to DMSO. 85% of the cells treated with 2.5% DMSO reacted with D7 and 95% reacted with A6 after 24 hours of treatment (Figure 3c,d). Continued exposure to DMSO increased these percentages to 95% and 98% respectively by the third day of treatment.
Figure 3. 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 (panels a and b) is expressed as percent change relative to control untreated cells stained with D7 (panel a) or A6 (panel b). Panels c and d depict the percentage of cells stained with D7 (panel c) or A6 (panel d) with fluorescence intensity greater than that of cells stained with control antibody and FITC-anti-IgM.
FIGURE 3
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 (Figure 4a). 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 two days (Figure 4b). 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 (Figure 4b).

The rapid increase in antigen expression by DMSO-treated cells after 24 hours prompted us to examine the expression of antigen during the first 24 hours of exposure to DMSO. Cells were examined for reactivity to D7 and A6 after 1, 3, 6, 9, 12, and 24 hours of DMSO treatment. The fluorescence intensity of cells stained with either D7 or A6 increased after 1 hour exposure then returned to control levels before increasing again between 12 and 24 hours (Figure 5a). The percentage of positive cells also increased after 1 hour, returning to control levels and then increasing between 12 and 24 hours (Figure 5b).

The ability of the tumor cells to maintain antigen expression following treatment with LK or DMSO for 48 hours was examined. Removal of LK or DMSO resulted in the loss of the antigens recognized
Figure 4. Effect of LK treatment on B16-BL6 melanoma antigen expression. Replicate plates of B16-BL6 cells were grown in vitro in the presence of LK (1:10) 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 (panel a) is expressed as percent change relative to control untreated cells stained with D7 or A6. Panel b depicts the percentage of cells stained with D7 or A6 having fluorescence intensity greater than that of cells stained with control antibody and FITC-anti-IgM.
FIGURE 4
Figure 5. 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 for up to 24 hours. Cells from each series were harvested at the indicated times, stained with D7 or A6 and FITC-anti-IgM and evaluated for immunofluorescence by flow cytometry. Change in fluorescence intensity (panel a) is expressed as percent change relative to control untreated cells stained with D7. Panel b depicts the percentage of cells stained with D7 or A6 with fluorescence intensity greater than that of cells stained with control antibody and FITC-anti-IgM.
FIGURE 5
by A6 or D7 (Figure 6). Cells examined by IFA with D7 rapidly lost up to 60% of their fluorescence intensity after removal of either LK or DMSO for 24 hours (Figures 6a,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 hours (Figures 6a,b).

However, the decrease in fluorescence intensity to the same levels as the D7 stained cells occurred during the second 24 hour period. The percentage of cells expressing D7 or A6 declined after the removal of LK or DMSO (Figures 6c,d).

Expression of the antigens recognized by D7 and A6 was examined simultaneously with cell-cycle analysis following exposure to LK and DMSO. The results, depicted in Figure 7, show cells analyzed with A6 following 24 hour exposure to DMSO. A similar observation was made concerning the expression of D7. The fluorescence intensity of cells in G2+M was greater than those in G0/G1 (Figure 7a). The enhancement of antigen expression measured on cells exposed to increasing concentrations of DMSO occurred in all cell-cycle phases (Figure 7b).

The effect of the protein synthesis inhibitor cycloheximide on antigen expression following treatment with LK and DMSO was evaluated. Cycloheximide reduced the fluorescence intensity as well as the percentage of cells reacting with D7 and A6 (Table 7). The inhibitory effect of cycloheximide reduced the fluorescence intensity of LK treated cells in the G0/G1 and G2+M compartments by more than 65%. In contrast, the expression of antigen by S-phase cells was reduced by only 10-18%. Similarly, cycloheximide reduced the fluorescence intensity of DMSO-treated cells in S-phase by only
Figure 6. Antigen expression requires the presence of LK or DMSO. Replicate plates of B16-BL6 cells, previously grown in vitro in the presence of LK (1:10) or DMSO (2.0%) for 48 hours, were grown in complete medium for up to 2 days. Cells from each series were harvested daily, stained with D7 or A6 and FITC-anti-IgM and evaluated for immunofluorescence by flow cytometry. Percent FI remaining (panels a and b) is expressed relative to 48-hour treated cells stained with D7 or A6. Panels c and d depict the percentage of cells stained with D7 or A6 having fluorescence intensity greater than that of cells stained with control antibody and FITC-anti-IgM.
FIGURE 6

A

PERCENT FL REMAINING

100
90
80
70
60
50
40
30

DAYS AFTER TREATMENT

A6

D7

B

PERCENT FL REMAINING

100
90
80
70
60
50
40
30

DAYS AFTER TREATMENT

FIGURE 6
FIGURE 6 (continued)
Figure 7. Cell-cycle associated antigen expression is enhanced following treatment with 2.0% DMSO. Replicate plates of B16-BL6 cells were cultured for 24 hours in the presence of various concentrations of DMSO. Cells were harvested, stained with A6 and FITC-anti-IgM, fixed in 70% ethanol, and stained with propidium iodide before multiparameter flow cytometry analysis. The green (FITC) fluorescence intensity of cells in each cell-cycle compartment was analyzed (panel a) and is expressed as percent change relative to control untreated cells stained with A6. Panel b depicts the percentage of cells stained with A6 having fluorescence intensity greater than that of cells stained with control antibody and FITC-anti-IgM.
FIGURE 7
Table 7. The effect of cycloheximide on LK- and DMSO-enhanced antigen expression. Replicate plates of B16-F10 cells were cultured for 12 hours in medium alone or in medium containing LK (1:10) or DMSO (2.0%) prior to the addition of cycloheximide (chx, 3.0ug/ml) to the cultures for an additional 12 hours. The fluorescence intensity (FI) is expressed as the percent mean cell fluorescence above background as described in Materials and Methods. The percentage of positive cells (%+) is corrected for background levels of cells stained with control antibody and FITC-anti-IgM. Numbers in parentheses indicate the percent reduction following treatment with cycloheximide.
### TABLE 7

**EFFECT OF CYCLOHEXIMIDE ON ANTIGEN EXPRESSION**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GO/G1</th>
<th>S</th>
<th>G2+M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FI</td>
<td>%+</td>
<td>FI</td>
</tr>
<tr>
<td>medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+chx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td>D7</td>
<td>211</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(59)</td>
<td>(71)</td>
</tr>
<tr>
<td>lymphokine</td>
<td></td>
<td>452</td>
<td>89</td>
</tr>
<tr>
<td>+chx</td>
<td></td>
<td>169</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(65)</td>
<td>(64)</td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td>432</td>
<td>90</td>
</tr>
<tr>
<td>+chx</td>
<td></td>
<td>275</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(36)</td>
<td>(42)</td>
</tr>
<tr>
<td>medium</td>
<td>A6</td>
<td>188</td>
<td>75</td>
</tr>
<tr>
<td>+chx</td>
<td></td>
<td>94</td>
<td>27</td>
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<tr>
<td></td>
<td></td>
<td>(50)</td>
<td>(64)</td>
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<td>DMSO</td>
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<td>285</td>
<td>92</td>
</tr>
<tr>
<td>+chx</td>
<td></td>
<td>148</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(48)</td>
<td>(63)</td>
</tr>
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</table>
10-15%. The percentages of cells in G0/G1 and G2+M reacting with D7 and A6 were also reduced following treatment with cycloheximide.

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 (Figure 8). 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 H-3TdR incorporation revealed that treatment of B16 cultures with LK or DMSO inhibited cell proliferation over time (Table 8). Incorporation of H-3TdR into LK-treated cells was reduced by 65% after 24 hours exposure and 69% after 48 hours. Cells treated with DMSO were similarly reduced in their ability to incorporate H-3TdR.

The effect of LK and DMSO exposure on protein synthesis in B16 melanoma cells was determined by measuring the incorporation of S-35 methionine into cellular protein (Table 8). Treatment of cells with LK reduced the incorporation of S-35 methionine by 25% after 24 hours exposure and by more than 55% after 48 hours. Incorporation of S-35 methionine into cells exposed to DMSO was reduced by more than 40% after 24 hours and more than 60% after 48 hours.
Figure 8. Enhanced fluorescence intensity of cells stained with a mixture of D7 and A6. Replicate plates of B16-F10 and B16-F1 cells were cultured for 24 hours in the presence of LK (1:10) or DMSO (2.0%), stained with D7 alone, A6 alone, or a mixture of the two monoclonal antibodies. All antibodies were used at saturating concentrations. Cells were analyzed by flow cytometry with values expressed as arbitrary units representing mean channel fluorescence.
Table 8. Inhibition of H-3Tdr and S-35 methionine incorporation following incubation with LK or DMSO. B16-F10 cells were incubated in 96-well plates in the presence of LK (1:10) or DMSO (2.0%) for 24 or 48 hours prior to the addition of 1.0uCi H-3Tdr or S-35 methionine per well. Cells were harvested after labelling for 12 hours using a MASH II unit and incorporated radioactivity determined. The values listed indicate the percent reduction in H-3Tdr or S-35 methionine incorporation relative to untreated controls.
Discussion

Monoclonal antibodies have been generated against a variety of antigens expressed on human and mouse tumor cells. Many of these antibodies have been used to study the characteristics and expression of tumor-associated antigens (TAAs) in vitro (Seeger et al, 1981; Metzgar et al, 1982; Starling et al, 1982; Brenner et al, 1982, Wakabayashi et al, 1985). In order to utilize these monoclonal antibodies most effectively, it is desirable to maximize the expression of the recognized TAAs. Agents which would increase the proportion of cells expressing TAAs 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 several 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. The increase in antigen density occurred in cells analyzed from each phase of the cell cycle, moreover, those cells in the later phases continued to express antigen at higher densities than cells in the early phases, as previously reported. The effects of treatment with LK or DMSO were apparent after 24 hours 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. The effect of cycloheximide was very pronounced on cells in the G0/G1 and G2+M phases but small on S-phase cells. Treatment of cells with LK or DMSO also reduced the incorporation of H-3TdR and S-35 methionine suggesting that exposure to these agents decreased both cell proliferation 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 (Attallah et al., 1979; Imai et al., 1981; Liao et al., 1982; Ng et al., 1983). Interferons have been shown to increase the expression of histocompatibility as well as tumor-associated antigens in several systems (Greiner et al., 1984; Basham et al., 1982; Dolei et al., 1983; Fellous et al., 1979). The enhanced antigen expression and cytostatic 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 (Evans, 1983, Namba and Waksman, 1975; Sawada et al., 1975).
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 (Tsiatsos et al., 1979). DMSO has been shown to increase doubling time, change cellular enzyme content, and alter carcinoembryonic antigen expression in human adenocarcinoma cells (Tsao et al., 1982). The expression of Class I MHC antigens on mouse lung carcinoma cells was recently reported to be substantially increased following exposure to DMSO (Bahler and Lord, 1985). Differentiated functions such as reduced cell growth and enhanced melanin synthesis have been reported in human melanoma cells following exposure to DMSO (Huberman et al., 1979). 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 baseline levels following its removal. This suggests that this was a transient effect rather than irreversible differentiation.

The expression of the antigens recognized by D7 and A6 was related to the position of a given cell in the cell cycle. The increase in antigen expression measured as cells progressed into the later phases leading to cell division suggested either constant antigen synthesis and accumulation in the cell membrane, or an
increase in antigen synthesis during the later phases. The latter explanation may be correct in light of the data obtained from cycloheximide treated cells. In these cells, antigen expression was reduced in all phases except S, suggesting that perhaps these cells were expressing maximum antigen density at the G2+M stage of the cycle when the cycloheximide was added 12 hours prior to analysis. After the addition of cycloheximide, the synthesis of new antigen was greatly reduced and those G2+M cells with high antigen density progressed into the S-phase in the ensuing 12 hours. Further research into the cell-cycle associated expression of these antigens is needed to completely elucidate this relationship.

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. First, IFA and flow cytometric 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. Second, the kinetics of antigen expression during LK or DMSO exposure and subsequent loss of antigen following removal of the inducing agent appear 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 density but not to the same extent as when the cells are analyzed with A6. Similarly, cells treated with
DMSO appear to require 48 hours to demonstrate a substantial increase in the antigen detected by D7 whereas these same cells show a rapid increase in antigen density within 24 hours when analyzed with A6. The loss of the antigen recognized by D7 occurs rapidly within 24 hours of removal of LK or DMSO but analysis of those cells with A6 reveals more than 80% of the antigen remaining after 24 hours without LK or DMSO and the rapid reduction in antigen density not occurring until an additional 24 hours 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 TAA's may require concurrent use of agents that increase the antigen density as well as the proportion of tumor cells expressing the antigen. Treatments which maximize the expression of TAA's facilitate detection of such antigens and may lead to increased analytic and therapeutic applications of monoclonal antibodies.
Summary

We have developed two monoclonal antibodies, designated 152 E12 D7 (D7) and 153 C7 A6 (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 splenocytes taken from C57BL/6 mice bearing the B16-F10 tumor. These monoclonal antibodies are of the IgM class and have been shown to react with three variants of the B16 and another mouse melanoma but not normal murine tissues. Multiparameter flow cytometric analysis revealed the expression of antigens recognized by D7 and A6 to be cell-cycle related in that cells in the later stages of the cycle had greater antigen density and a greater percentage were antigen positive. The expression of the antigens detected by these monoclonal antibodies was heterogeneous between the three variants of the B16 examined.

Exposure of B16 melanoma cells to a Con-A stimulated spleen cell mixed lymphokine preparation (LK) and to 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 D7 and A6 than did unstimulated controls. Cultures exposed to DMSO for several days became nearly 100% positive
when assayed by IFA with D7 and A6. 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. The increase in antigen density occurred in cells analyzed from each phase of the cell cycle, moreover, those cells in the later phases continued to express antigen at higher densities than cells in the early phases.

The effects of treatment with LK or DMSO were apparent after 24 hours exposure but did not persist after the agent was removed from the culturing assays were kindly provided by Dr. C.W. Johnson, Dept. of Pathology, OSU. C57BL/6 lines include: JB/RH, melanoma; JB/MS, melanoma (J. Berkelhammer, AMC Research Ctr., Lakewood CO) and the EL-4 lymphoma. Non-H2b tumor target cell lines include: Cloest that monoclonal antibodies D7 and A6 recognize different epitopes found on two separate cell surface molecules. The effect of LK or DMSO on antigen expression was blocked by the protein synthesis inhibitor cycloheximide. The effect of cycloheximide was very pronounced on cells in the G0/G1 and G2+M phases but limited on S-phase cells. Treatment of cells with LK or DMSO also reduced the incorporation of H-3Tdr and S-35 methionine indicating that exposure to these agents decreased both cell proliferation and overall protein synthesis.

The use of monoclonal antibodies as diagnostic and therapeutic tools in cancer research shows great promise for the future. More monoclonal antibodies are being developed against tumor-associated antigens giving researchers an increasing library of antibodies for tumor cell analysis. Through the use of these monoclonal antibodies,
investigators may be able to gain insight into the metabolic processes of tumor growth and metastasis. The observation that tumor cell populations are heterogeneous with respect to antigen expression predicts that subpopulations may exist which will not bind monoclonal antibodies to these antigens in sufficient quantities to be effective in diagnosis and therapy. Enhancement of tumor-associated antigen expression by various biological response modifiers may provide a means to increase the effectiveness of monoclonal antibodies in these applications.

Additional research is needed to further evaluate the effects of biological response modifiers on tumor-associated antigen expression. The results of this investigation of the B16 murine melanoma suggest that other tumor cells, including human lines, should be examined for similar types of responses using monoclonal antibodies which recognize M-3 of the CxDBA Cloudman melanoma S91 (ATCC); 410.4, a BALB/C mammary tumor (G.H. Heppner, Michigan Cancer Foundation, Detroit, MI); Neuro 2a, a strain A-albino neuroblastoma (ATCC); and SCC-PSA1, a murine teratocarcinoma (ATCC). Cells were grown in tof biological response modifier treatment and injection of radiolabeled monoclonal antibodies may provide a method for in vivo detection and/or destruction of formerly low-antigen-density metastatic tumor cells. Further research in these areas may realize the potential of monoclonal antibodies as diagnostic and therapeutic tools.
Table 9
SUMMARY OF ANTIBODY REACTIVITY

<table>
<thead>
<tr>
<th>Cell / Tissue</th>
<th>Background</th>
<th>152 E12 D7</th>
<th>153 C7 A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16 BL-6</td>
<td>&lt;10</td>
<td>157±10</td>
<td>256±23</td>
</tr>
<tr>
<td>B16 F10</td>
<td>&lt;10</td>
<td>148±21</td>
<td>244±16</td>
</tr>
<tr>
<td>B16 F1</td>
<td>&lt;10</td>
<td>105±17</td>
<td>162±30</td>
</tr>
<tr>
<td>JB/RH</td>
<td>&lt;5</td>
<td>52±7</td>
<td>66±18</td>
</tr>
<tr>
<td>JB/MS</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>EL-4</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Neuro 2a</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>SCC-PSA1</td>
<td>&lt;5</td>
<td>5±2</td>
<td>7±4</td>
</tr>
<tr>
<td>Clone M3</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>11±8</td>
</tr>
<tr>
<td>410.4</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Table 9. Reactivity of monoclonal antibodies with B16 melanomas and other tumor lines. Values indicate the equivalent amount (ng/ml ± std. dev.) of monoclonal antibody bound to target cells based on an ELISA standard curve of mouse IgG.
LIST OF REFERENCES


Higgins, P.J. Protein accumulation in cultures of hepatic tumor cells exposed to DMSO. Oncology 41: 338-342, 1984.


Roos, G., Leanderson, T., Lundgren, E. Interferon-induced cell-cycle changes in human hematopoietiks of age from Harlan Sprague-Dawley, Indianapolis, IN.

Cell lines The B16 melanoma lines F1, F10 and BL6 were selected by Fidler (1973) and Hart (1979) for their lung colonizing and tissue invading characteristics. Additional cell lines used in screeen induceable by interferon. Eur. J. Immunol. 13: 495-499, 1983.


