Differential Antigenic Expression of the DBA/2 Lymphoma L1210 and Its Sublines: Cross-Reactivity with C3H Mammary Tumors as Defined by Syngeneic Monoclonal Antibodies

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ABSTRACT

The expression of tumor-associated antigens on the DBA/2 lymphoma L1210 and three L1210 sublines, each resistant to a different antileukemic agent [guanazole, methylglyoxal-bis(guanilhydrazine), and 4,4-diacetyldiphenylurea-bis(guanilhydrazine)] was investigated by the use of monoclonal hybridoma antibodies. Hybridomas were produced by the fusion of spleen cells from DBA/2 mice immunized with irradiated L1210 or L1210 subline cells and cells of a non-immunoglobulin-secreting BALB/c myeloma variant. Three clones producing antibodies reacting with L1210 or L1210 subline cells were used to study the antigenic expression of L1210 and L1210 subline cells.

Monoclonal antibodies from anti-L1210 and anti-L1210 subline hybridomas exhibited a greater reactivity with L1210 subline cells than with L1210 cells in complement-dependent cytotoxicity, quantitative absorption, and membrane immunofluorescence experiments, thereby demonstrating a tumor-associated antigen shared by L1210 and the L1210 sublines and an increased expression of this antigen on subline cells. Cross-blocking tests of antibody binding demonstrated that monoclonal antibodies from anti-L1210 and anti-L1210 subline hybridomas recognized the same or very closely situated antigenic determinants on the tumor cell surface. Most syngeneic and allogeneic tumor cells used as controls failed to react with the anti-L1210 and anti-L1210 subline hybridoma antibodies. However, two syngeneic tumors, L5178Y and P388-D1, demonstrated a significant reaction with the monoclonal antibodies. In addition, several spontaneous mammary tumors from C3H/St and DBA/2Ha, both high-frequency mammary tumor strains, reacted in various degrees with anti-L1210 or anti-L1210 subline hybridoma antibodies in absorption tests and in immunofluorescence experiments. On the other hand, liver, kidney, and spleen from normal C3H/St mice, as well as mammary tumors from BALB/c and C3Hf, both low-frequency mammary tumor strains, did not demonstrate significant reactivity in similar experiments. Normal lactating mammary glands from high-frequency mammary tumor mouse strains reacted with the monoclonal antibodies, whereas lactating mammary glands from low-frequency mammary tumor mouse strains were negative by this method. Purified murine mammary tumor virus preparations reacted strongly with the monoclonal antibodies in solid-phase radioimmunoassays, whereas a purified murine leukemia virus preparation failed to do so in similar experiments. These results indicate that the tumor-associated antigen(s), differentially expressed on L1210 and L1210 subline cells, is related to an antigen which is associated with the murine mammary tumor virus.

INTRODUCTION

Previous studies with 3 drug-resistant sublines of the murine DBA/2 lymphoma L1210 demonstrated an increased ability of these subline cells to elicit antibody responses and tumor transplantation immunity in histocompatible hosts (7–9, 17). Our attention was focused on the question of whether the immunogenicity changes in such subline cells are due to qualitative or quantitative changes in the antigenic properties of the tumor cells. The development of the hybridoma technique by Köhler and Milstein (11) for production of monoclonal antibodies provided an ideal tool to study the expression of tumor cell surface antigens. We applied this technique to develop clones of antibody-producing hybridomas raised against L1210 or L1210 subline cells.

This paper describes the results of studies on the differences in the TAA4 expressed by L1210 and L1210 subline cells, which were conducted with monoclonal antibodies raised against parental L1210 and L1210 subline cells. These monoclonal antibodies were shown to recognize a TAA that was expressed in a greater amount on all 3 L1210 sublines than on parental L1210, which also expresses this antigen. In addition, significant reactivities were demonstrated with the monoclonal antibodies and other DBA/2 lymphoid tumors, several spontaneous C3H/St mammary tumors, and normal mammary glands from high-frequency mammary tumor mouse strains. Strong reactivity was also demonstrated with the monoclonal antibodies and purified MuMTV preparations. These findings suggest that the monoclonal antibody-defined TAA expressed in an increased amount on L1210 subline cells may be associated with the MuMTV.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old females of DBA/2Jax, DBA/2Ha-DD [a brown-coated mutant of DBA/2 (18)], C3H/SIHa, C3Hf/HeHa, C57BL/6Ja, AKR/Sn, A/St, BALB/cCr, and lactating females of various mouse strains were obtained from the Roswell Park breeding colony at West Seneca, N. Y. The colony of DBA/2Jax used in this study was derived from the inbred germ-free colony established by Dr. H. W. Walberg at Oak Ridge National Laboratory and has been maintained under conventional conditions at this institute since 1976; this DBA/2Jax colony has

4 The abbreviations used are: TAA, tumor-associated antigen; MuMTV, murine mammary tumor virus; PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; MulV, murine leukemia virus; FCS, fetal calf serum; FITC-RAMig, fluorescein isothiocyanate-labeled F(ab')2 fragments of rabbit anti-mouse immunoglobulin; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; ARS, p-azobenzesaronate; BSA, bovine serum albumin; NMig, normal mouse immunoglobulin; ML, mammary leukemia.
been found to have a low frequency of mammary tumors as compared to DBA/2Ha-D2, which has a high frequency of mammary tumors.\(^5\) (BALB/c \(\times\) DBA/2F\(_1\), hereafter called CD2F\(_1\)) hybrids of the same age and sex as above were obtained from the Charles River Breeding Laboratories, Wilmington, Mass.

**Mouse Tumors.** The DBA/2 lymphoma, L1210, and the 3 L1210 sublines were the same tumors used in the previous studies (7-9). These L1210 sublines had been established by in vivo selection for resistance, each to a different antileukemic agent, i.e., 4,4-diacyldiphenylurea-bis(guanhydrizone), methylglyoxal-bis(guanhydrizone), and guanazole, and were designated L1210/DDUG, L1210/CH\(_2\)-G, and L1210/GZL, respectively (4, 16). C57BL/6 lymphomas EL-4(G-), EL-4(G+), and RBL5 and the BALB/c lymphoma RL\(_5\)1 were originally obtained from Dr. Ronald B. Herberman, National Cancer Institute, Bethesda, Md. The B10.129 (5M) lymphoma, L5MF22, was originally supplied by the late Dr. Gustavo Cudkowicz, State University of New York at Buffalo, and the DBA/2 lymphoma, L5178Y, was obtained from Dr. Lionel A. Manson, Wistar Institute of Anatomy and Biology, Philadelphia, Pa. BALB/c myeloma MOPC-21 (\(\gamma_\text{M} 1\), \(\alpha\)), MOPC-195 (\(\gamma_\text{M} 2\), \(\alpha\)), and UPC-10 (\(\gamma_\text{M} 2\), \(\beta\)). DBA/2 tumors P388-D1 and P815, strain A lymphoma L No. 2, and C3H lymphoma 6C3HED were maintained in this laboratory. All ascites tumors were maintained routinely by weekly i.p. transfer of approximately 10\(^6\) cells into syngeneic mice, collected on Days 4 through 6 postinjection, and washed 3 times with PBS. Cell viability was determined by trypan blue dye exclusion. Two spontaneous AKR thymomas, SAK 8 and SAK 37, were supplied by Dr. Oliver Rohott of this department. Spontaneous C3H/St, C3H/HeHa, and DBA/2Ha mammary tumors were obtained from the Roswell Park breeding colony at West Seneca, and the BALB/c D2 hyperplastic alveolar nodule-derived mammary adenocarcinoma (15) (BALB/c D2 mammary tumor) was supplied by Dr. Bonnie B. Asch from this institute. These tumors were maintained in syngeneic mice by s.c. transfer of a cell suspension made from the solid tumor. The nature of each mammary tumor was confirmed by histological examination.

**Preparation of Cell Suspensions from Solid Tumors or Normal Tissues.** Solid tumor or normal tissue (spine, thymus, and liver) cell suspensions to be used for absorption experiments were prepared in cold PBS by pressing minced tumor or tissue through a 50 mesh stainless steel screen, followed by passage through a 200 mesh screen. Cell suspensions were centrifuged at 500 \(\times\) g and washed twice in PBS prior to use.

**Mice Tumor Viruses.** C3H MuMTV (341-7) and RII MuMTV (CRF-7), purified from concentrated culture supernatants by continuous-flow equilibrium density gradient with 15 to 60% RNase-free sucrose, were obtained from the Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute. Purified AKR MuLV, obtained from the Viral Oncology Program of the National Cancer Institute, was supplied by Dr. Kenneth F. Manly of this institute.

Monoclonal Antibodies. Production of monoclonal antibodies followed the procedure of Köhler and Milstein (11). Female DBA/2/Jax mice, which received 6 weekly i.p. injections of 10\(^7\) L1210 or L1210/GZL irradiated cells (4000 rads), using a \(^{137}\text{Cs}\) source (GammaCell 40; Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada), were sacrificed 4 or 5 days after the last injection. Immune spleen cells (10\(^6\) cells) and a non-immunoglobulin-secreting BALB/c myeloma variant, P3-NS1/1-Ag4-1 (26) (10\(^7\) cells), were suspended in 0.2 ml of 30% (v/v) polyethylene glycol (M, 1000), incubated at room temperature for 30 sec, and diluted stepwise in Dulbecco’s modified Eagle’s medium, followed by centrifugation at 500 \(\times\) g for 10 min at room temperature and resuspension of the cell pellet in Dulbecco’s modified Eagle’s medium containing 1.0 \(\times\) 10\(^{-4}\) \(\times\) hypoxanthine, 4.5 \(\times\) 10\(^{-8}\) m aminopterin, 1.5 \(\times\) 10\(^{-8}\) m thymidine, 10\(^{-5}\) FCS, 2 mm L-glutamine, streptomycin (50 \(\mu\)g/ml), and penicillin (50 units/ml). One million spleen cells and 100 myeloma cells suspended in 1 ml of this medium were dispensed into each well of a 24-well tissue culture plate (Linbro; Catalog No. 76-003-05) and were incubated at 37\(^\circ\) in a 5% CO\(_2\) humidified incubator.

Culture supernatants from wells showing hybridoma growth were tested for antibody production by complement-dependent cytotoxicity in agar gel as described previously (7), using L1210, L1210/GZL, and P815 cells as targets. Two of 5 fusions performed as described above resulted in stable hybridomas secreting antibodies which reacted with L1210 or L1210/GZL cells. In one fusion, which involved spleen cells immunized against L1210/GZL, 10 of 48 wells showed hybridoma growth, and of these hybridomas produced antibodies which reacted with L1210/GZL cells and not with P815 cells; 3 of these antibody-positive hybridomas later stopped producing antibodies. In a second fusion, which involved spleen cells immunized against L1210, 16 of 84 wells showed hybridoma growth, resulting in only one stable antibody-positive hybridoma.

Hybridomas secreting antibodies reacting with L1210 and L1210/GZL cells were cloned at least twice by a limiting dilution method in the presence of peritoneal exudate cells from normal CD2F\(_1\) mice, as a feeder layer (2.5 \(\times\) 10\(^7\) peritoneal exudate cells/well). Two cloned hybridomas established against L1210/GZL cells, 2B2/2D1/4G3 and 1C3/2D2/2E12/2G3, and one cloned hybridoma established against L1210 cells, 3A6/2B2/1D2, were maintained in cultures or in ascites form in CD2F\(_1\) mice, and will be referred to as 2B2, 1C3, and 3A6, respectively. Monoclonal antibody heavy- and light-chain isotypes were determined with rabbit antisera directed against mouse \(\kappa\) or \(\lambda\) chains and \(\gamma_1\), \(\gamma_2\), \(\gamma_2\), \(\gamma_\alpha\), \(\alpha\), \(\omega\) chains (Lilton Biometrics, Kensington, Md., or Miles Laboratories, Inc., Elkhart, Ind.) in Ouchterlony double diffusion analysis.

**Complement-dependent \(^{51}\text{Cr} \text{Release Cytotoxicity Assay.** Monoclonal antibody activity was measured by a complement-dependent \(^{51}\text{Cr} \text{release assay described previously (9). Briefly, \(^{51}\text{Cr} \text{labeled target cells (2.5} \times\) 10\(^5\) cells in 50 \(\mu\)l of Roswell Park Memorial Institute Tissue Culture Medium 1640:5% FCS) were incubated in triplicate with 50 \(\mu\)l of the monoclonal antibody preparation and 50 \(\mu\)l of the appropriate dilution of rabbit serum preabsorbed with mouse lymphoma cells (1) [complement (C) source] in a 96-well microtiter tray (Linbro; Catalog No. 76-031-03) for 2 hr at 37\(^\circ\) in a 5% CO\(_2\) humidified incubator, followed by centrifugation at 500 \(\times\) g for 5 min. Supernatants were collected with the Titertek supernatant collection system (Flow Laboratories, McLean, Va.). The amount of radioactivity present in each supernatant was determined by an Auto-Gamma counter (Packard Instrument Co., Downers Grove, Ill.). The percentage of cytotoxicity was calculated as follows.

\[
\text{% of cytotoxicity} = \frac{\text{experimental release} - \text{release with C only}}{\text{maximum release with 0.5% non-ionic detergent} - \text{release with C only}} \times 100
\]

Quantitative Absorption of Monoclonal Antibodies with Various Tumors or Normal Tissues. The antibody dilution corresponding to the titer of the antibody preparation was chosen for use in all absorption experiments. Culture supernatants from 3A6, 2B2, or 1C3 hybridomas (1:9, 1:9, and 1:2 dilutions, respectively) were used as a source of monoclonal antibodies. Graded numbers of washed ascites tumor cells, thymus or spleen cells, were centrifuged at 8000 \(\times\) g for 2 min in an Eppendorf microcentrifuge and incubated with the appropriate dilution of monoclonal antibody for 2 hr at 4\(^\circ\) with continuous mixing. Cell pellets were prepared from mammary tumor or normal tissue cell suspensions by centrifugation at 8000 \(\times\) g for 2 min, and were resuspended and incubated with monoclonal antibodies or anti-H2.2 alloantisemur (1:50 dilution) as a control [(B10.AKMK129) anti-B10.A serum recognizing a private specificity of H-2\text{D}^d, prepared by Dr. G. D. Snel], The Jackson Laboratory, and obtained from the Transplantation Immunology Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md.] at a ratio of 2 volumes diluted antibody:1 volume packed cells for 1 hr at 4\(^\circ\) with continuous rotation. This mixture was centrifuged at 8000 \(\times\) g for 2 min, an aliquot of the absorbed antibody was removed for assay, and a second absorption was repeated as described above. The residual cytotoxicity of the absorbed antibody samples was determined by a
complement-dependent $^5$Cr release assay using L1210/GZL cells as targets. The percentage of cytotoxic activity remaining was calculated as follows.

\[
\text{% of cytotoxic activity remaining} = \frac{\text{% of cytotoxic activity after absorption}}{\text{% of cytotoxic activity before absorption}} \times 100
\]

**Membrane Immunofluorescence.** Immunofluorescence staining was accomplished by the indirect method, using PBS containing 5% FCS and 3 x 10^-7 M NaN₃ as an antibody diluent as described previously (6). Unless otherwise described, all incubations were conducted at 37° for 30 min, followed by 2 successive washings with PBS and centrifugations at 500 x g. Briefly, 5 million tumor cells were incubated with 100 μl of 3A6, 2B2, or MOPC-195 ascites fluid (1:500 dilution), washed, stained with 100 μl of the appropriate dilution of FITC-RAMlg, and analyzed, using a fluorescence activated cell sorter (FACS II; Becton, Dickinson FACS Systems, Sunnyvale, Calif.) (6, 13).

In the blocking experiments, 1 x 10⁶ tumor cells were incubated with 50 μl ascites fluid of 3A6, 2B2, or 1C3 (1:250 dilution) for 30-57, an anti-H-2Ld hybridoma (22) supplied by Dr. K. Ozato, NIH; 30-H12, an anti-Thy 1.2 rat-mouse hybridoma (12), obtained from the Immunology Program of the National Cancer Institute and grown in nu/nu BALB/c mouse; or MOPC-195 myeloma, as controls, washed, and then incubated with 50 μl of 3A6 (1 μg), 2B2 (2 μg), 1C3 (2 μg), or 30-H12 (0.2 μg) antibody conjugated to ARS as described previously (25). After washing, the tumor cells were incubated with the appropriate dilution of FITC-rabbit anti-ARS sera prepared as described previously (2). Washed and stained cell preparations were examined with a Leitz Ortholux II fluorescent microscope.

**Immunofluorescence for Tumor or Normal Tissue Sections.** Fresh tumor or normal tissues were frozen and cut into 5- to 10-μm sections with a Cryo-Cut II microtome (American Optical, Buffalo, N. Y.), mounted on slides, fixed in −20° acetone for 30 to 90 sec, and used immediately for immunofluorescence staining or stored at −70° until used. After fixing, sections were rinsed in PBS containing 5% FCS, followed by staining with 50 μl of 3A6-ARS, 2B2-ARS, or NMIG-ARS (1:100, 1:50, and 1:50 dilutions, respectively; 1.5 mg protein/ml), and finally, 50 μl of the appropriate dilution of FITC-rabbit anti-ARS antisera. All incubations were conducted in a humidified chamber at room temperature for 30 min, followed by rinsing of the slides in PBS. Stained tissue sections were observed as described above.

**Solid-Phase Radioimmunoassay.** A solid-phase radioimmunoassay described previously was modified for use in this study (3). Briefly, 0.1 ml of a purified C3H MuMTV, RII MuMTV, or AKR MuLV suspension (30 μg/ml in PBS) was added to each well of a 96-well polystyrene microtiter plate (Costar; Catalog No. 2596). After incubation at 4° overnight, the plates were washed 5 times with PBS and incubated with 5% BSA-PBS for 1 hr at 37°. After washing, 0.1 ml of a globulin fraction of 3A6, 2B2, or 1C3 antibodies (5 mg protein/ml) serially diluted in 1% BSA-PBS, or as controls, serially diluted MOPC-195, UPC-10, rabbit anti-C3H MuMTV serum (R168, obtained from Dr. Larry O. Arthur, Frederick Cancer Research Facility, National Cancer Institute, Frederick, Md.), or monoclonal anti-MuLV gp 70 (14) (NEI-013A; New England Nuclear, Boston, Mass.) was added to duplicate wells. After incubation at 4° overnight, the wells were washed with PBS, and the wells were incubated for 30 min at 37° with 0.1 ml of 1% BSA-PBS containing 50,000 cpm 125I-protein A. After washing with PBS, the wells were cut apart by a hot wire cutter and monitored for the presence of bound 125I-protein A with an Auto-Gamma scintillation spectrometer (Packard Instrument Co.).

**RESULTS**

Production of 3A6, 2B2, and 1C3 Monoclonal Antibodies. Culture supernatants from wells showing hybridoma growth were screened for antibody production by complement-depend-

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Table 1

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<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Immunizing tumors</th>
<th>Immunoglobulin isotypes</th>
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<tr>
<td>3A6</td>
<td>L1210</td>
<td>( \gamma_m ), ( \kappa )</td>
</tr>
<tr>
<td>2B2</td>
<td>L1210/GZL</td>
<td>( \gamma_m ), ( \kappa )</td>
</tr>
<tr>
<td>1C3</td>
<td>L1210/GZL</td>
<td>( \gamma_m ), ( \kappa )</td>
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CANCER RESEARCH VOL. 43
Increased TAA Expression on L1210 Sublines

Membrane Immunofluorescence Analysis of L1210 and L1210 Sublines with 3A6 or 2B2 Monoclonal Antibodies. L1210 and L1210 subline cells were stained with 3A6 or 2B2 antibody and FITC-RAMLg and analyzed with the FACS. As shown in Chart 3, L1210/DDUG and L1210/GZL tumor cells demonstrated stronger staining with the monoclonal antibody and the FITC conjugate than did L1210 cells. Staining with control myeloma protein (MOPC-195) and FITC-RAMLg did not show significant fluorescence as compared to the staining with FITC-RAMLg alone (data not shown). Approximately 65 and 70% of the L1210/GZL cells were highly fluorescent, and approximately 20 and 15% of the L1210 cells showed moderate to high fluorescence after reaction with 3A6 and 2B2 antibodies, respectively, whereas less than 5% of the tumor cells were fluorescent in the controls.

Blocking of Binding of 3A6-, 2B2-, or 1C3-Hapten Conjugate by 3A6, 2B2, and 1C3 Monoclonal Antibodies. To determine whether the 3 monoclonal antibodies recognize the same or different antigenic determinants, 3A6, 2B2, or 1C3 antibody, or control ascites bearing MOPC-195, 30-5-7 (anti-H-2Ld hybridoma), or 30-H12 (anti-Thy 1.2 hybridoma) was incubated with L1210/GZL, followed by staining with 3A6-ARS, 1C3-ARS, or 2B2-ARS, and finally FITC-rabbit anti-ARS. As shown in Table 2, preincubation of L1210/GZL cells with 3A6, 2B2, or 1C3 antibody inhibited the binding of 3A6-ARS, 1C3-ARS, or 2B2-ARS conjugate, as indicated by a decrease in fluorescent staining. In contrast, 30-5-7, 30-H12, and MOPC-195 control antibodies did not significantly interfere with the binding of 3A6-ARS, 1C3-ARS, or 2B2-ARS conjugate to L1210/GZL cells, whereas 30-H12 did inhibit the binding of 30-H12-ARS to Thy-1-positive EL-4 cells. These data suggest that 3A6, 2B2, and 1C3 recognize the same antigenic determinant or that the determinants are situated very close to each other.

Reactivity of 3A6, 2B2, and 1C3 Antibodies with Various Syngeneic and Allogeneic Lymphoid Tumors. Various syngeneic and allogeneic lymphoid tumors were tested for reactivity with the monoclonal antibodies in complement-dependent cytotoxicity or quantitative absorption experiments. Among those tumors tested were 3 DBA/2 tumors, L5178Y, P388-D1, and P815; 4 C57BL/6 tumors, L5MF22, RBL5, EL-4(G+), and EL-4(G−); BALB/c tumor RUÃŽ1; strain A tumor L No. 2; C3H tumor 6C3HED; and 2 spontaneous AKR thymomas, SAK 8 and SAK 37. Two of the syngeneic tumors, L5178Y and P388-D1, reacted significantly with these antibodies in complement-dependent cytotoxicity tests, i.e., the maximum cytotoxicity of L5178Y with 3A6, 2B2, and 1C3 was 33, 27, and 55%, respectively; the maximum cytotoxicity of P388-D1 with 3A6, 2B2, and 1C3 was 23, 20, and 20%, respectively. As shown in Chart 2, A and B,
L5178Y moderately absorbed the cytotoxic activity of 3A6 and 2B2 antibodies. Other tumors, including those expressing the MuLV-associated antigens [RBL5, RL61, EL-4(G+)], SAK 8, and SAK 37], did not show significant reactivity with these antibodies.

Reactivity of 3A6 and 2B2 Antibodies with Spontaneous C3H Mammary Tumors. Seven spontaneous mammary tumors from a mouse strain demonstrating high mammary tumor incidence, C3H/St, were used for 2 consecutive absorptions of 3A6 or 2B2 antibodies (or anti-H2.4 antibody as a control). Table 3 shows the percentage of cytotoxicity activity remaining after each consecutive absorption of these antibodies with the designated tumor cells. The C3H mammary tumors were shown to be capable of absorbing 3A6 or 2B2 antibody activity in various degrees; Mammary Tumors 2, 3, and 4 strongly absorbed the activity of 3A6 antibody, even after only one absorption. In contrast, Mammary Tumors 6 and 7 did not show much absorbing capacity. The cytotoxic activity of 2B2 was also removed by these tumors to a degree similar to that of 3A6. The absorptions of 3A6 and 2B2 appeared to be specific because absorption of anti-H2.4 antiserum (a private specificity of H-2*) failed to significantly reduce its cytotoxic activity against L1210/GZL. Although Tumor 6 was negative in absorption, an ascites form of this tumor reacted with 3A6 in a complement-dependent cytotoxicity test (data not shown). Liver and spleens from normal C3H mice did not significantly remove the activity of 3A6 and 2B2 antibodies.

The above results were confirmed by Hapten sandwich immunofluorescence experiments conducted with frozen sections from the C3H mammary tumors and the monoclonal antibodies. As shown in Table 4, all 7 C3H tumors reacted with the 3A6-ARS and 2B2-ARS antibody conjugates in varying degrees. Two of the 7 C3H mammary tumors, Tumors 2 and 4, demonstrated very strong cytoplasmic as well as cell surface staining with 3A6-ARS and 2B2-ARS, whereas C3H Mammary Tumors 3 and 5 demonstrated moderate reactivity, and Tumors 1, 6, and 7 demonstrated a weak but significant reactivity with these antibodies. The reactivity of the monoclonal antibodies with the C3H...
mammary tumors appeared specific, because all 7 tumors failed to be stained with NMig:ARS and FITC-conjugated rabbit anti-ARS. Similarly, in immunofluorescence experiments conducted with spontaneous mammary tumors from another high-frequency mammary tumor strain, DBA/2Ha, 5 of 6 tumors demonstrated strong to moderate staining with 3A6-ARS and 2B2-ARS; one mammary tumor failed to react with these antibodies. In contrast, mammary tumors from low-frequency mammary tumor strains, BALB/c and C3Hf, were not stained with 3A6-ARS or 2B2-ARS. Frozen tissue sections obtained from C3H liver, kidney, and spleen were also negative by this method.

These results indicate that the monoclonal antibody-defined TAA expressed by L1210 and L1210 subline cells cross-reacts with an antigen(s) expressed by spontaneous C3H/St and DBA/2Ha mammary tumors.

**Reactivity of 3A6 and 2B2 Antibodies with Lactating Mammary Glands.** Normal lactating mammary glands from various mouse strains were sectioned and stained with the monoclonal antibodies, using the Hapten sandwich immunofluorescence technique. As shown in Table 4, normal lactating mammary glands from high-frequency mammary tumor strains, C3H/St and DBA/2Ha, demonstrated strong immunofluorescent staining with 3A6-ARS and 2B2-ARS, whereas normal lactating mammary glands from low-frequency mammary tumor strains, C3Hf, BALB/c, and DBA/2Jax, were not similarly stained.

**Reactivity of 3A6, 2B2, and 1C3 Antibodies with C3H MuMTV.** As shown in Chart 4A, 3A6, 2B2, and 1C3 antibodies demonstrated strong reactivity with the purified C3H MuMTV preparation. The amount of 3A6, 1C3, and 2B2 antibodies necessary to demonstrate 50% maximum binding to the C3H MuMTV was 0.1, 0.01, and 0.005 μg, respectively. Similar experiments conducted with a purified RII MuMTV preparation and 3A6, 2B2, and 1C3 antibodies also demonstrated a similar pattern of strong reactivity (data not shown). Rabbit anti-C3H MuMTV serum, which was used as a positive control, demonstrated strong reactivity with the C3H MuMTV preparation, even at a dilution of 1:10,000 (data not shown). On the other hand, as much as 100 μg of control antibodies UPC-10 or MOPC-195 demonstrated little binding to the virus. This observed binding of the antibodies to the MuMTV appeared to be specific, since as much as 100 μg of 3A6, 2B2, or 1C3 antibody (or control antibodies MOPC-195 and UPC-10) did not show significant binding to a purified preparation of AKR MuLV (Chart 4B). In the same experiments, approximately 0.005 μg of monoclonal anti-MuLV gp 70 produced 50% maximum binding to the AKR MuLV preparation. These results suggest that the antigen(s) recognized

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**Table 4**

<table>
<thead>
<tr>
<th>Tumors or normal tissues</th>
<th>Immunofluorescence</th>
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<tbody>
<tr>
<td></td>
<td>3A6-ARS</td>
</tr>
<tr>
<td>C3H/St mammary tumors</td>
<td>++</td>
</tr>
<tr>
<td>DBA/2Ha mammary tumors</td>
<td>++</td>
</tr>
<tr>
<td>BALB/c D2 mammary tumor</td>
<td>-</td>
</tr>
<tr>
<td>C3Hf/HeHa mammary tumor</td>
<td>++</td>
</tr>
<tr>
<td>Normal lactating mammary glands from high-frequency mammary tumor strains (C3H/St and DBA/2Ha)</td>
<td>-</td>
</tr>
<tr>
<td>Normal lactating mammary glands from low-frequency mammary tumor strains (BALB/c, C3Hf/HeHa, DBA/2Jax)</td>
<td>-</td>
</tr>
<tr>
<td>C3H/St normal tissues (liver, kidney, spleen)</td>
<td>-</td>
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- Symbols indicate increasing intensity of immunofluorescence staining.
- Six tumors were tested; 5 were positive.
- A single tumor was examined.
- Three to six mice per strain were tested.
L. Rapp and H. Fuji

by 3A6, 2B2, and 1C3 antibodies is related to an antigen which is associated with the MuMTV.

**DISCUSSION**

This paper describes the specificity of the monoclonal antibodies produced against L1210, i.e., 3A6, or against L1210/GZL, i.e., 2B2 and 1C3, as determined by complement-dependent cytotoxicity, quantitative absorption, and membrane immunofluorescence. These monoclonal antibodies demonstrated a TAA that is expressed in greater amounts on the 3 drug-resistant L1210 sublines than on the parental L1210. Membrane immunofluorescence and FACS analysis of L1210 cells with 3A6 antibody (immunogen, L1210) revealed that 65% of these tumor cells were highly fluorescent, while approximately 20% of parental L1210 cells showed moderate to high fluorescence. The differential expression of the TAA on L1210 and its drug-resistant sublines, as demonstrated by these monoclonal antibodies, confirmed our previous results with conventional antiserum (9). Furthermore, this study provides additional evidence for the existence of a small number of cells within the parental L1210 population which express this TAA.

Complement-dependent cytotoxicity and absorption experiments conducted with various syngeneic and allogeneic tumors indicated that the antigen recognized by the monoclonal antibodies was also expressed by 2 other DBA/2 lymphoid tumors and that this TAA did not seem to be related to known MuLV-associated antigens. However, 7 spontaneous C3H mammary tumors reacted with these antibodies in varying degrees in absorption and immunofluorescence experiments. Since normal C3H tissues, such as liver, kidney, and spleen, did not react with 3A6 or 2B2 antibodies, the observed cross-reaction did not seem to be directed to cell surface alloantigens present on these C3H mammary tumors. Furthermore, 5 spontaneous mammary tumors of DBA/2Ha, as well as normal lactating mammary glands from C3H/ST and DBA/2Ha, both high-frequency mammary tumor strains, exhibited a significant reaction with the monoclonal antibodies, whereas mammary tumors and normal lactating mammary glands from low-frequency mammary tumor strains, i.e., BALB/c and C3Hf, did not react with these antibodies. Interestingly, lactating mammary glands from DBA/2Jax mice failed to react with the monoclonal antibodies (Table 4), and this correlates with a low mammary tumor frequency within the DBA/2Jax colony maintained in the animal breeding facility at this institute.5 These results suggest that the cross-reacting antigens expressed on C3H mammary tumors may be associated with MuMTV. Subsequent solid-phase radioimmunoassay conducted with the monoclonal antibodies and purified C3H and RII MuMTV have indeed demonstrated a strong reactivity between the virus preparations and the monoclonal antibodies. This reactivity appears to be specific, since the monoclonal antibodies fail to react significantly with an unrelated AKR MuLV, and suggests that the antigen recognized by the monoclonal antibodies may be a structural component of the virus. Furthermore, the observation of strong cytoplasmic staining of the C3H/ST and DBA/2Ha mammary tumors and normal lactating mammary glands by the monoclonal antibodies may indicate that this antigen is synthesized in the cytoplasm of the cell. Complement-dependent cytotoxicity experiments conducted with the monoclonal antibodies and an ascites form of C3H Mammary Tumor 6, as well as 2 culture cell lines established in vitro from C3H Mammary Tumors 1 and 4 (data not shown), demonstrate that this antigen is also expressed on the cell surface membrane of the mammary tumor cells, suggesting that the expression of this antigen on the tumor cell membrane may be associated with the budding of the mature MuMTV from the cell membrane.

The reactivity of the monoclonal antibodies with C3H mammary tumors and normal mammary tissue from high-frequency mammary tumor strains observed in this study corresponds to those originally reported by Stück et al. (24), which described the ML antigen and its expression on several DBA/2 lymphomas, C3H mammary tumors, and normal mammary tissue from high-frequency mammary tumor strains. This antigen has been reported to be an antigenic component associated with the MuMTV (19–21, 27). In a recent study, using conventional anti-ML antiserum and the same drug-resistant L1210 sublines used in our experiments, Strzadala et al. (23) have also provided evidence that these L1210 sublines have a higher expression of ML antigen as compared to L1210 cells. However, the exact relationship of the TAA expressed on L1210 sublines and the ML antigen is still not clear, and experiments are currently under way to answer this question.

The cytotoxic reactivities of 3A6, 2B2, and 1C3 antibodies with L1210 and L1210 sublines suggest that these 3 monoclonal antibodies recognize the same antigen. This is further supported by cross-blocking immunofluorescence experiments, which indicate that the antigenic determinants recognized by these antibodies are identical or are situated very close to each other. The facts that the 3 monoclonal antibodies were derived from 2 separate fusions and that they appear to be directed to the same antigenic determinant(s) suggest that the determinant(s) is highly immunogenic in terms of stimulating the antibody response. Previous studies have also demonstrated an increased expression of a cytotoxic T-cell-defined TAA on L1210/GZL cells, as compared to parental L1210 cells (6). Since L1210 sublines are efficient in eliciting transplantation immunity in syngeneic animals (9) and are susceptible to genetically controlled host resistance in histocompatible F1 hybrids (10), it is important to delineate the nature of these antigens and their functions in host resistance against the tumor.

It is clear from this study that the TAA defined by the monoclonal antibodies is not unique to the drug-resistant L1210 sublines; it is shared by parental L1210 as well as by certain other tumors and appears to be associated with the MuMTV. However, the mechanism(s) underlying the increased expression of a TAA on the drug-resistant L1210 sublines is still not clear. The present data appear to be consistent with the hypothesis that the immunogenic drug-resistant tumor sublines arise through selection (7, 17). We have initiated studies on the differential expression of the antibody-defined as well as the T-cell-defined TAA among clones isolated in vitro from parental L1210 cells. Information that will be obtained from such studies will help to understand the immunological functions of differential antigenic expression as well as the relationship that may exist between increased tumor immunogenicity and drug resistance.

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2598

CANCER RESEARCH VOL. 43
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Differential Antigenic Expression of the DBA/2 Lymphoma L1210 and Its Sublines: Cross-Reactivity with C3H Mammary Tumors as Defined by Syngeneic Monoclonal Antibodies

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