

TRANSFORMED LYMPHOCYTES FROM ABELSON-DISEASED  
MICE EXPRESS LEVELS OF A B LINEAGE  
TRANSFORMATION-ASSOCIATED ANTIGEN ELEVATED  
FROM THAT FOUND ON NORMAL LYMPHOCYTES

BY GEORGE F. TIDMARSH,\* MORRIS O. DAILEY,<sup>‡</sup>  
CHERYL A. WHITLOCK,\* ERIC PILLEMER,\* AND IRVING L. WEISSMAN\*

*From the \*Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305; and the ‡College of Medicine, Department of Pathology, The University of Iowa, Iowa City, Iowa 52242*

The means by which pre-B lymphoid cells are transformed to malignancy by Abelson murine leukemia virus (A-MuLV)<sup>1</sup> are still unclear. Essential for activity of this rapidly transforming virus is a transduced section of a cellular gene, *c-abl*, labeled *v-abl* in the viral genome, that replaced classical retroviral *pol*, *env*, and a portion of *gag* DNA sequences (1). The replication-defective A-MuLV depends upon coinfection with the competent parent virus, Moloney murine leukemia virus (M-MuLV), which supplies genes encoding reverse transcriptase (*pol*) and viral structural proteins (*gag*, *env*) necessary for assembly of new viral particles (2). The *gag-abl* fusion protein encoded by the A-MuLV genome locates at the inner aspect of the plasma membrane (3) and is a tyrosine phosphoprotein kinase capable of phosphorylating itself (4) and cellular proteins (5). Several known oncogenes (e.g., *v-src*) share this behavior with *v-abl* (6–11). Although *v-abl* expression is necessary for A-MuLV pre-B cell neoplastic transformation, it does not appear to be sufficient for autonomous growth: early, in vitro A-MuLV-infected bone marrow pre-B cells express high levels of *v-abl*, but their growth is nevertheless dependent on bone marrow-derived stromal feeder layer cells (12). Later, without apparent change in *v-abl* expression, malignant clones emerge that grow in a feeder layer-independent fashion. These later malignant clones, but not most *v-abl*<sup>+</sup> cells, which predominate in the premalignant period, express a transformation-associated B lineage tumor antigen detected by the monoclonal antibody 6C3 (mAb 6C3) (13 and Tidmarsh, Whitlock, and Weissman, manuscript in preparation).

Previously (13), we demonstrated that the antigen detected by mAb 6C3 (6C3 Ag) was present on a moderately abundant 160 kD cell-surface glycoprotein (gp160<sup>6C3</sup>) on the surface of all A-MuLV established cell lines and some sponta-

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<sup>1</sup> *Abbreviations used in this paper:* Ag, antigen; A-MuLV, Abelson murine leukemia virus; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; M-MuLV, Moloney murine leukemia virus.

neous B lineage leukemias, but was absent from A-MuLV-transformed fibroblasts (13). In addition, gp160<sup>6C3</sup> is not the product of genes contained in the A-MuLV/M-MuLV complex. Because of the correlation between expression of this nonviral antigen and the transformed phenotype, we proposed that a high level of expression of gp160<sup>6C3</sup> not only identifies the neoplastic state but may play a central role as a normal cellular gene product in the oncogenic process. Thus, it became important to determine (a) if the antigen is expressed on any nontransformed, normal lymphoid cells, and (b) if a positive correlation exists between A-MuLV transformation of lymphoid cells in vivo and high level expression of this antigen.

Towards this goal, we developed more sensitive techniques to analyze lymphoid cells for the presence of the antigen before and after infection with A-MuLV in vivo. Using these techniques, we identify here normal cells that display very low levels of the 6C3 Ag on their surface compared with A-MuLV-transformed cells. We document a tissue-specific increase in cell surface antigen expression in Abelson-diseased mice. Furthermore, in such mice we demonstrate that cells bearing high levels of this antigen are neoplastically transformed and specifically coexpress differentiation markers representative of pre-B cells.

### Materials and Methods

**Cells.** The A-MuLV in vitro lymphoma cell lines L1-2 and 2M3 and the A-MuLV-transformed, non-virus-producing fibroblast line, ANN-1, were originally provided by Naomi Rosenberg (Tufts University, Medford, MA). The rat IgG2a mAb-producing hybridoma, 6C3, was developed as described earlier (13); the control rat IgG2a monoclonal hybridoma R7D4 was obtained courtesy of Ron Levy (Stanford University). mAb R7D4 recognizes an idiotypic determinant on a human B-cell lymphoma. The anti-B220 monoclonal hybridoma 6B2 and the anti-ThB monoclonal hybridoma were kindly provided by Robert Coffman (DNAX Research Institute, Palo Alto, CA). Production of the rat IgG2a mAb 31-11, specific for the constant region of mouse Thy-1, was described elsewhere (14). mAb 10-4.22, a mouse IgM antibody directed towards mouse IgD, was obtained from the American Type Culture Collection, Rockville, MD. All cells were grown in Dulbecco's modified Eagle's medium or RPMI 1640 (JR Scientific, Woodland, CA) with 10% fetal calf serum, and  $3 \times 10^{-5}$  M 2-mercaptoethanol.

**Virus.** Virus was harvested from confluent, clonal M-MuLV-producing cells originally provided by O. N. Witte (University of California, Los Angeles). This virus was then used to rescue the A-MuLV genome from a confluent culture of ANN-1. After 16 h, culture supernatant was harvested, filtered through a 0.45  $\mu$ m filter, and stored at  $-70^{\circ}\text{C}$  until use. This stock was titrated in susceptible BALB/c mice. All mice developed typical signs of Abelson disease 3–5 wk after retroorbital intravenous injection of 0.1 ml of virus, whereas only 30% became symptomatic after injection with 0.025 ml. An injection of 0.1 ml was used throughout this study.

**Mice.** All mice used for induction of Abelson tumors were BALB/c and no older than 4 wk at the time of injection. All normal mice were 3–8-wk-old, BALB/c mice bred in our colony. There is no observed difference in A-MuLV susceptibility between sexes (15), so both males and females were used for injection. There was an observed difference in the quantity of antigen in the thymus of female vs. male normal young mice. Where a comparison has been made, animals are sex matched.

**Immunofluorescence Reagents.** mAb was purified from Nu/Nu mouse ascitic fluid using DEAE Affi-gel Blue chromatography (Bio-Rad Laboratories, Richmond, CA) (16). Both rabbit and goat anti-rat second-stage reagents were used without any difference in result. Rabbit anti-rat serum was produced in our laboratory. Goat anti-rat was obtained from Pel-Freez Biologicals (Rogers, AR). Immunoglobulin was purified by protein A chroma-

tography. Purified immunoglobulin was coupled to fluorescein isothiocyanate by the established method of Goding (17), after further purification to high specificity for rat immunoglobulin by several passages over a Sepharose 4B (Pharmacia, Inc., Piscataway, NJ)-coupled mouse immunoglobulin column. As a further precaution, the fluoresceinated second-stage reagent, when used, contained 10% (final concentration) normal mouse serum. All antibodies and the normal mouse serum were centrifuged for 15 min in a Beckman Airfuge (Beckman Instruments, Inc., Irvine, CA) at 20 psi before use. For detection of biotinylated reagents, Texas Red-coupled avidin (Cappel Laboratories, Cochranville, PA) was used as a second-stage reagent.

**Cell Suspensions.** Lymphoid organs, both primary (thymus and femoral bone marrow) and secondary (lymph nodes and spleen), were removed from sacrificed animals. After scalpel sectioning of both ends of all bones, marrow plugs were gently removed via expulsion with a syringe of cold suspending medium (Hank's balanced salt solution, 5% fetal calf serum, 0.1%  $\text{NaN}_3$ ). Other organs were teased apart with forceps and gently filtered through a 200-gauge stainless steel screen. Bone marrow suspensions were routinely centrifuged at room temperature (20 min, 2,000 rpm) underlaid with Lympholyte M (Cedarlane Laboratories Ltd., Ontario, Canada). Cell counts were determined in the presence of trypan blue to exclude dead cells or with Turk's solution to exclude nonnucleated cells (bone marrow).

**Immunofluorescence Staining.**  $10^6$  cells of each cell suspension were placed in a Falcon No. 2052 tube (Becton, Dickinson & Co., Cockeysville, MD) tube for each antibody. After a centrifugation wash through newborn calf serum and aspiration of supernatant, each pellet was resuspended in 0.025 ml appropriately diluted test antibody solution for 10–15 min on ice, before a second wash. If a second stage reagent was required, samples were resuspended in 0.025 ml appropriately diluted reagent and incubated 10 min on ice before staining dead cells with propidium iodide as described before (18). After a third wash, each sample was brought up in 0.1 ml suspension medium for FACS analysis.

**FACS Analysis.** Fluorescence-activated cell sorter (FACS) analysis of immunofluorescence was accomplished using a FACS II (Becton Dickinson Immunocytometry Systems, Mountain View, CA) for one color and a modified dual laser FACS for two color analysis (17). This machine was made available through the FACS shared users group at Stanford University.

**Analysis of Immunofluorescence Data.** After collection of mAb R7D4 background fluorescence data on one sample of each lymphoid tissue, a fluorescence gate was chosen to include ~99% of the cells in that sample below the chosen gate. This gate is termed the tissue background gate. Once established, the tissue background gate was used in the analysis of all stainings of that tissue to avoid introducing bias to the analysis. The percentage of mAb 6C3-positive cells for each tissue staining is defined as: (percentage cells exceeding tissue background gate in mAb 6C3 sample) – (percentage cells exceeding tissue background gate in mAb R7D4 sample). Mean fluorescence was obtained only for those cells in the test sample that fell above the chosen gate. It should be noted that the calculated percentage is not an absolute value but only a number to be used for comparison relative to percentages obtained by the same procedure, for the same tissue, from a different mouse.

For statistical comparison of values obtained in this way, we used the Mann-Whitney rank test. Here, values from two groups of stainings, e.g., bone marrow from normal and M-MuLV-injected mice, are ranked together in order of their relative value. A serial rank number is then assigned to each value (1 through combined total  $n$  of both groups). The actual value is lost and has no further meaning in the analysis. The summed rank of each group is compared mathematically, and, based on the number of samples in each group, a statement of confidence can be made as to whether the mean values of the two groups are different.

**Radioimmunoassay.** 500  $\mu\text{Ci}$   $^{125}\text{I}$  (New England Nuclear, Boston, MA) was coupled to 10  $\mu\text{g}$  of purified mAb 6C3 or R7D4 using chloramine T. Briefly, 0.005–0.02 ml of antibody in phosphate-buffered saline was added to a mixture of 0.01 ml 1 M phosphate buffer, pH 7.4 and 0.01 ml  $^{125}\text{I}$  diluted previously to 1 mCi/0.02 ml with 0.5 M NaOH.

At  $t = 0$  at room temperature, 0.002 ml of a 5 mg/ml solution of chloramine T (Eastman Kodak Co., Rochester, NY) was added and the reaction allowed to proceed for 1 min. At that time, 0.002 ml of a 5 mg/ml solution of sodium metabisulfite was added to stop the reaction. 0.07 ml of phosphate-buffered saline supplemented with 5% newborn calf serum was added and the mixture applied to a 0.5 ml column of Sephadex G-25 (Pharmacia, Inc., Piscataway, NJ) prewashed with phosphate-buffered saline/5% newborn calf serum. Fractions with detectable radioactivity were collected to a total volume of 0.3 ml. A measured volume of each labeled antibody was analyzed for radiation using a Beckman Biogamma counter (Beckman Instruments, Inc.). This analysis was performed so that an equal quantity of counts per minute from labeled mAb 6C3 or R7D4 could be used in the subsequent binding studies. Triplicate samples of  $10^6$  cells from bone marrow, spleen, thymus, or a log phase culture of L1-2 were incubated in separate wells of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) for 20 min on ice, suspended in 0.025 ml of control or test antibody.  $^{125}\text{I}$ -coupled mAb 6C3 had been previously titrated to a binding plateau on  $10^6$  L1-2 cells. After four washes with ice-cold phosphate-buffered saline/5% newborn calf serum, the microtiter plate was dried under a heat lamp and the individual wells cut out before determining bound counts per minute. The mean count from triplicate samples and the standard error of the mean are presented.

**Agar Colony Assay.** Bone marrow cells were obtained from mice that had previously been injected intravenously with A-MuLV and showed typical Abelson disease symptoms. After FACS analysis of indirect mAb 6C3 immunofluorescence, appropriate gates were set on the FACS machine to sort the brightest 25% staining cells from the dimmest 25%. These cells were then compared for their ability to grow in agar as originally described by Rosenberg and Baltimore (19).

## Results

*6C3 Ag Is Present at Low Levels on Cells Suspended from Normal Lymphoid Tissue, Most Predominantly Bone Marrow and Thymus.* It seemed unlikely that an antigen such as that recognized by mAb 6C3, expressed at high levels on the surface of pre-B and B cell lymphomas, would be absent from all normal tissues. To search for expression of the 6C3 Ag by normal cells we began by using a more sensitive technique—FACS analysis of indirect immunofluorescence on cell suspensions—than used previously (13). Expecting extremely low-level expression of the antigen, we controlled for mAb isotype as well as for purification technique and protein concentration for the staining. Fig. 1A displays representative FACS plots comparing mAb 6C3-specific and mAb R7D4 background stainings on a normal bone marrow cell population.

Each of the eight stainings on untreated BALB/c bone marrow yielded a similar result. Table I gives numerical data obtained from comparable immunofluorescence stainings of three groups of mice. Based on these numbers, the appropriate background control, and the high reproducibility, we conclude that there exists a subpopulation of normal BALB/c bone marrow cells that display a low level of 6C3 Ag. We designate these cells 6C3Ag<sup>lo</sup> by comparison to the mean indirect mAb 6C3 immunofluorescence of the Abelson lymphoma cell line (L1-2), which is designated 6C3Ag<sup>hi</sup>.

Additionally, the normal female BALB/c thymus contains a population of 6C3Ag<sup>lo</sup> cells. Fig. 2A illustrates FACS plots comparing mAb R7D4 background with mAb 6C3-specific staining. Again, each of the four experiments yielded a nearly identical result. Analysis done on normal lymph node and spleen cells shows that these tissues do not contain levels of the antigen distinguishable from the background level.

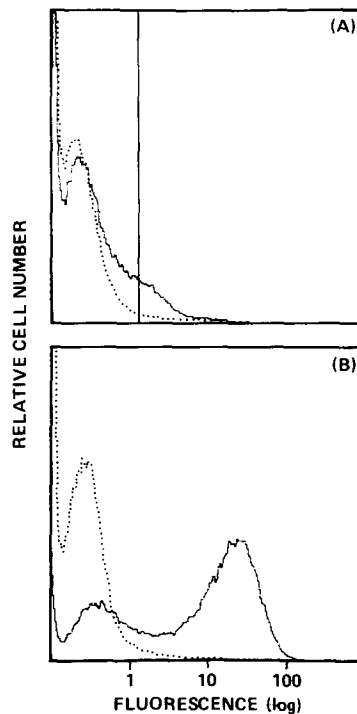


FIGURE 1. mAb 6C3 immunofluorescence of bone marrow cells from normal and A-MuLV-infected animals. Representative FACS-generated profiles comparing mAb 6C3-specific (—) and mAb R7D4 (---) background immunofluorescence staining on bone marrow cells from a normal (A) or an Abelson-diseased (B) BALB/c mouse. Cell surface-bound mAb was detected using a fluorescein isothiocyanate-conjugated anti-rat immunoglobulin. The vertical line in A represents the tissue background gate, described in Materials and Methods, used for numerical analysis of data in Table I.

To quantitate the amount of 6C3 Ag found in normal lymphoid tissue, we coupled  $^{125}\text{I}$  directly to purified mAb 6C3 and mAb R7D4, as outlined in Materials and Methods, and used the labeled antibodies in a direct cell-binding radioimmunoassay. Fig. 3 depicts a graph comparing mAb R7D4-specific and mAb 6C3-specific radioactivity bound to either normal lymphoid or L1-2 cells. Normal spleen bound a barely detectable level of mAb 6C3 while the thymus bound more than bone marrow; both bone marrow and thymus bound significant levels of Mab 6C3.

*A-MuLV Infection Leads to the Appearance of 6C3Ag<sup>hi</sup> Bone Marrow Cells in Susceptible Mouse Strains.* Infection of 3–4-wk-old BALB/c mice with A-MuLV caused an acute lymphoma within 3–5 wk that was rapidly lethal. Hind limb paralysis is a confirming diagnostic sign (15). In all lymphoid organs analyzed from diseased mice, an altered distribution of the 6C3 Ag was observed. 6C3Ag<sup>hi</sup> cells, never found in normal animals, appeared in the bone marrow. In the thymus of diseased animals, 6C3Ag<sup>lo</sup> cells were decreased in number compared with normal counterparts while 6C3Ag<sup>hi</sup> cells were never observed. Figs. 1B and 2B each compare FACS plots of mAb 6C3 and R7D4 immunofluorescent stainings on bone marrow and thymus cells, respectively, of an animal previously

TABLE I  
Summary of mAb 6C3 Immunofluorescence

Tissue	Percentage mAb 6C3-positive cells in mice infected with:			Mean fluorescence (above background)		
	A-MuLV	None	M-MuLV	A-MuLV	None	M-MuLV
Bone marrow	50.7 (22.6-72)	5.9 (3.5-9.4)	12.9 (9-17.3)	16.1 (9.5-22.2)	2.7 (2.2-3.1)	3.5 (3.2-4)
Thymus	15.9 (7.8-27)	31.8 (28-36.8)	40.6 (26.6-53)	1.5 (1-2)	1.2 (1-1.3)	1.2 (1-1.4)
Spleen	16.6 (9.3-28.9)	1.8 NA	0 NA	13.6 (12.1-14)	NA NA	NA NA
Lymph nodes	16.7 (0-43.9)	0.07 NA	0 NA	9.1 (8.5-10)	NA NA	NA NA
L1-2	100 100	NA NA	NA NA	39 (29-45)	NA NA	NA NA

Lymphoid tissue distribution of the transformation-associated antigen recognized by mAb 6C3 in normal, M-MuLV-infected, and A-MuLV-infected mice. Data represent average values (ranges in parenthesis). Indirect immunofluorescent stainings were performed as described in Materials and Methods on cells suspended from the indicated tissues. Positively staining cells were determined as those exceeding background mAb R7D4 staining. After gating for mAb 6C3-staining cells, we derived a mean fluorescence for the population. Only an approximate comparison of the mean fluorescence data is practical because this data was collected on two different FACS machines. NA, not applicable.

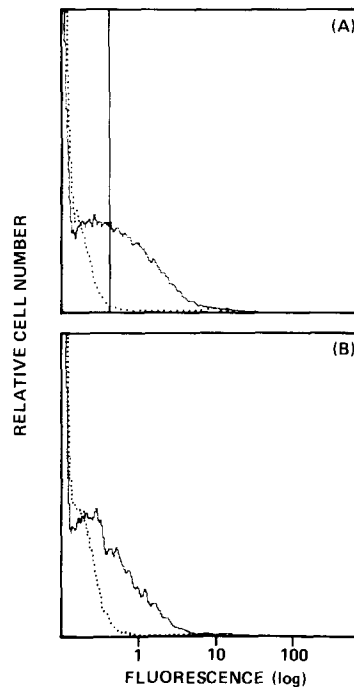


FIGURE 2. mAb 6C3 immunofluorescence of thymocytes from normal and A-MuLV-infected animals. Representative FACS-generated profiles comparing mAb 6C3-specific (—) and mAb R7D4 background (---) immunofluorescence staining on thymocytes from a normal (A) or an Abelson-diseased (B) BALB/c mouse. Cell surface-bound mAb was detected using fluorescein isothiocyanate-conjugated anti-rat immunoglobulin. The vertical line in A represents the tissue background gate used for analysis of data.

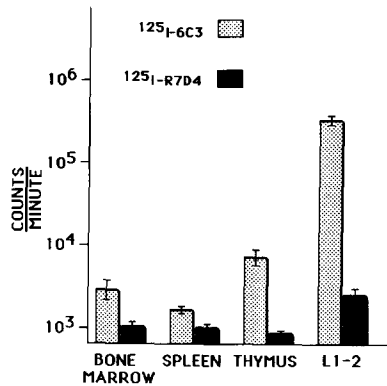


FIGURE 3. Normal lymphoid tissue mAb 6C3 radioimmunoassay. A whole cell radioimmunoassay was performed to quantitate the transformation-associated antigen recognized by mAb 6C3 on the surface of normal lymphoid cells and the A-MuLV cell line, L1-2. As described in Materials and Methods, both mAb 7D4 and 6C3 were purified on DEAE Affi-gel blue and subsequently labeled with <sup>125</sup>I. At 0°C,  $2 \times 10^6$  cpm of either labeled antibody was incubated with triplicate samples of  $10^6$  cells. After four washes with ice-cold phosphate-buffered saline, cell pellets were analyzed for bound gamma radiation. Note the log scale for this plot.

infected with A-MuLV. The numerical analysis of all experiments is displayed in Table I.

Most importantly, the bone marrow of all eight overtly lymphomatous mice contained a subpopulation of cells whose density of mAb 6C3 staining approximates that of the Abelson lymphoma, L1-2 (6C3Ag<sup>hi</sup> cells). A rough correlation was observed between the severity of the disease symptoms and the percentage of 6C3Ag<sup>hi</sup> cells in the bone marrow. In addition, five of six enlarged lymph nodes and four of four enlarged spleens from the mice contained a subpopulation of 6C3Ag<sup>hi</sup> cells. Further analysis of the thymus staining data showed that the percentage of 6C3Ag<sup>lo</sup> cells was actually decreased from that found in the average normal thymus (Table I); 6C3Ag<sup>hi</sup> cells were never observed. This tissue distribution of the 6C3 Ag in A-MuLV-infected animals generally correlates with previous pathological analyses of the disease (15, 20).

*Appearance of 6C3Ag<sup>hi</sup> Cells in Infected Mice Is Dependent on A-MuLV and Independent of the Helper, M-MuLV.* As noted earlier, Abelson virus is defective for viral production and thus requires a competent helper virus for production of new virus particles (2). It was, therefore, crucial to test whether the observed tissue-specific amplification of surface 6C3 Ag expression was A-MuLV dependent or was due to the helper virus, M-MuLV, used to prepare the A-MuLV stock. Culture supernatants from either clonal M-MuLV-producing cells or from ANN-1 cells superinfected with M-MuLV were injected intravenously into susceptible mice. Lymphoid tissues were removed and analyzed by indirect immunofluorescence 3–4 wk after injection, the approximate time of onset for symptoms of Abelson disease.

The data from this experiment are incorporated into Table I. None of the M-MuLV-infected mice developed a subpopulation of 6C3Ag<sup>hi</sup> cells in any lymphoid

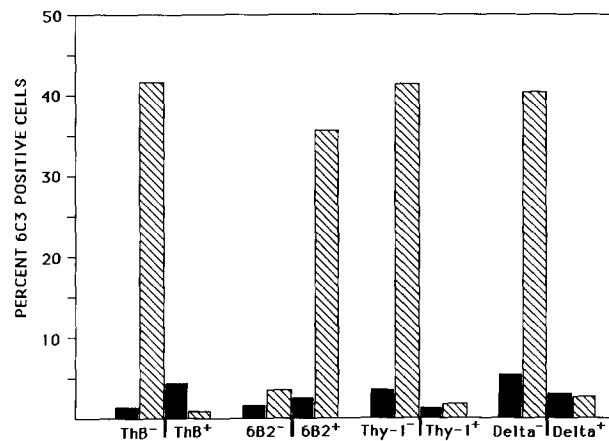


FIGURE 4. Two-color bone marrow cell immunofluorescence. Two-parameter immunofluorescence stainings were performed on bone marrow cells from either a normal BALB/c mouse (solid bars) or a BALB/c mouse previously infected with A-MuLV (hatched bars). Fluorescein isothiocyanate-coupled mAb 6C3 was used to identify cells expressing the transformation-associated antigen; biotinylated ThB, 6B2, anti-Thy-1, or anti-delta mAb were used to define these cells further. Texas Red-labeled avidin was used to detect cells with bound biotinylated antibody after unbound antibodies were removed by centrifugation through fetal calf serum. The normal bone marrow sample was determined to have 19.6% ThB<sup>+</sup> cells, 32.9% 6B2<sup>+</sup> cells, 9.1% Thy-1<sup>+</sup> cells, and 16.3% delta-positive cells. These percentages agree with published data (21).

organ tested. In addition, 5–6 mo after injection we analyzed the thymus and bone marrow of lymphomatous mice by radioimmunoassay (data not shown). The data confirm a previous finding (13) that the 6C3 Ag is not present on M-MuLV-transformed lymphoid (presumably T) cells. We conclude that the amplification of the surface 6C3 Ag observed in the Abelson-diseased state is dependent on A-MuLV and independent of infection with the slowly transforming parent retrovirus, M-MuLV.

However, these data show that M-MuLV alone can induce a significant increase in the percentage of 6C3Ag<sup>lo</sup> cells in bone marrow. Using the Mann & Whitney rank test, we can conclude with >99% confidence that the percentage of 6C3Ag<sup>lo</sup> cells in bone marrow was increased by injection of M-MuLV alone. M-MuLV increased the number of 6C3-positive bone marrow cells without altering the surface antigen level of each cell, based on mean fluorescence. Although the average percentage of 6C3Ag<sup>lo</sup> cells in the thymus appears to be increased after infection with M-MuLV, the increase and the sample size are not great enough to conclude this with >95% confidence.

*6C3Ag<sup>hi</sup> In Vivo-derived A-MuLV Bone Marrow Cells Bear Surface Markers Characteristic of Early Pre-B Cells.* Fig. 4 displays data obtained by two parameter immunofluorescence analysis of in vivo A-MuLV-infected and normal bone marrow cells. Fluorescein isothiocyanate-coupled mAb 6C3 was used to determine cells bearing the transformation-associated 6C3 Ag, while biotinylated anti-Thy-1, -ThB, -IgD heavy chain (anti-delta), and 6B2 (anti-B220) mAb were also used to phenotype this subpopulation. In normal mouse bone marrow, 6B2 marks all known pre-B and B cells; ThB marks only late pre-B and B cells, and



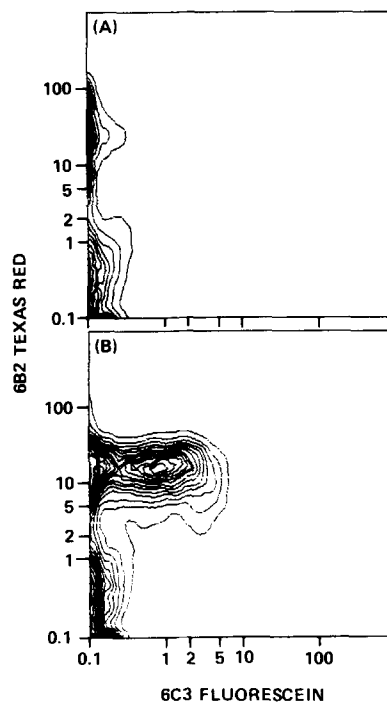


FIGURE 5. mAb 6B2 vs. 6C3 two-parameter immunofluorescence of bone marrow cells from normal and A-MuLV-infected mice. Two-color immunofluorescence stainings were performed on bone marrow cells from either a normal BALB/c mouse (A) or a BALB/c mouse previously infected with A-MuLV (B). As in Fig. 4, fluorescein isothiocyanate-coupled mAb 6C3 was used to identify cells expressing the transformation-associated antigen; biotinylated mAb 6B2 was used to define the B220<sup>+</sup> cells. Texas Red-labeled avidin was used to detect cells with bound biotinylated antibody after unbound antibodies were removed by centrifugation.

delta, only mature B cells and Thy-1, mainly T cells (21). Infection with A-MuLV resulted in the appearance of 6C3Ag<sup>hi</sup>-bearing cells that were homogeneous with respect to the other lymphoid markers. All 6C3Ag<sup>hi</sup> cells also bore the 6B2 antigen but lacked ThB, Thy-1, and delta surface antigen expression, a phenotype characteristic of early pre-B cells (Fig. 4). Fig. 5, A and B compares representative mAb 6B2 vs. 6C3 two-color FACS plots of normal and Abelson-diseased bone marrow, respectively. Although the 6C3Ag<sup>lo</sup> cell in normal bone marrow are apparently equally distributed in the B220<sup>+</sup> and B220<sup>-</sup> fractions, in Abelson-diseased mice the B220<sup>+</sup> cells alone contain the 6C3Ag<sup>hi</sup> cell population. This phenotype has been found on four of four tumors for B220 and two of two tumors for the other markers and it agrees with the pre-B cell phenotype observed for most A-MuLV-derived tumors.

*6C3Ag Marks the Transformed Cell Population in the Bone Marrow of Animals Infected with A-MuLV.* Because of the absolute correlation between overt Abelson disease and the appearance of 6C3Ag<sup>hi</sup> cells in bone marrow, it seemed reasonable to test whether 6C3Ag<sup>hi</sup> cells are transformed, as assayed by in vitro agar clonability (19). Cells were FACS sorted for the 6C3 Ag from bone marrow cells pooled from three animals with overt Abelson disease. Fig. 6 shows the

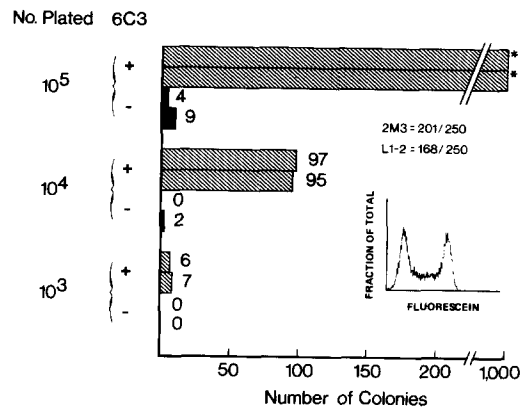


FIGURE 6. Agar colony assay of bone marrow cells sorted by FACS for the 6C3 Ag from A-MuLV-infected mice. Autonomous growth capacity of bone marrow cells from A-MuLV-infected mice was assessed in relation to their expression of the transformation-associated antigen recognized by mAb 6C3. As described in the text, bone marrow cells from mice previously infected with A-MuLV were removed, indirectly tagged with mAb 6C3, and FACS sorted for the 25% highest and 25% lowest antigen-expressing cells. The inset gives the ungated plot derived before sorting. Both populations were plated in agar at 37°C for 10 d before enumeration of colonies. For comparison, A-MuLV established cell lines were included. (\*) Colonies too numerous to count (900–1,000).

results of this experiment. A comparison of the number of colonies reveals that 6C3Ag<sup>hi</sup> cells are 100-fold more likely to form an agar colony than cells with low or no surface antigen. We conclude that mice with symptoms of Abelson disease have a subpopulation of transformed cells in their bone marrow and that these cells express higher levels of surface 6C3 Ag than found on normal bone marrow cells.

### Discussion

The evidence presented here leads us to conclude that the 6C3 Ag is present at low levels on the surface of a subset of normal lymphoid cells in the thymus and bone marrow. This has been shown by FACS analysis of immunofluorescence (Figs. 1A and 2A) and radioimmunoassay (Fig. 3). It will be important to test whether these 6C3Ag<sup>lo</sup> cells express this antigen by endogenous synthesis rather than by passive adsorption. Elsewhere we show that a subset of dendritic stromal cells in the thymus express very high levels of the 6C3 Ag (Adkins, Tidmarsh, and Weissman, manuscript in preparation). In addition, clonal bone marrow stromal cell lines that support the growth and differentiation of pre-B cells to B cells express the 6C3 antigen (unpublished observations). All attempts to immunoprecipitate labeled protein from thymocytes metabolically labeled with high specific activity, radioactive amino acids with mAb 6C3 have failed whereas mAb 6C3 immunoprecipitated gp160<sup>6C3</sup> from a number of L1-2 cells displaying, in aggregate, less surface 6C3 Ag (Adkins, Tidmarsh, and Weissman, manuscript in preparation). We propose that the transfer of the 6C3 Ag from closely associated 6C3Ag<sup>hi</sup> thymic stromal cells to thymocytes during preparation of cell suspensions might account for the detection of 6C3Ag<sup>lo</sup> thymocytes. This type of adsorption is known to occur for Ia antigens within the thymus (22).

Prior infection of susceptible hosts with A-MuLV leads to an increase in the amount of this antigen in bone marrow, lymph nodes, and spleen, but not thymus. This increase in total amount of 6C3 Ag can be ascribed to both an increase in the number of cells bearing a detectable quantity of the antigen as well as a greater amount of 6C3 Ag per cell (Table I). We conclude that mAb 6C3 detects a normal cell antigen that becomes aberrantly induced and/or amplified during neoplastic transformation by A-MuLV.

Furthermore, A-MuLV-injected mice develop Abelson disease and a transformed subpopulation of cells within their bone marrow bearing high levels of 6C3 Ag as well as lymphoid differentiation markers typical of pre-B cells. The surface differentiation phenotype of 6C3Ag<sup>hi</sup> cells from an Abelson-diseased mouse depicted in Fig. 4 corresponds to an early pre-B cell stage of differentiation. In addition, the 6C3Ag<sup>hi</sup> cells present in the bone marrow of diseased mice represent the transformed population of cells, as measured by agar clonability (Fig. 6). Although just one characteristic of transformation, agar colony formation is a good measure of autonomous growth capacity. We do not know if the increased expression of the antigen is a cause or a consequence of A-MuLV transformation. We can conclude, though, that the tumor-specific expression of the antigen recognized by mAb 6C3 marks, specifically, A-MuLV-transformed pre-B cells in diseased mice.

The transformation-specific expression of the 6C3 Ag will allow further investigation of the *in vivo* neoplastic process. Although it appears that a majority of A-MuLV-induced lymphomas are B lineage (23), an analysis of all *in vivo* tumors has not been performed. In fact, mAb 6C3 may provide the means to study pre-B and B cell neoplasms from a variety of sources. NFS mice injected with the Cas-Br-M (NS-1) retroviral complex rapidly develop B lineage lymphomas. A tissue-specific increase in the transformation-associated antigen recognized by mAb 6C3 has been documented in all mice analyzed so far (K. Holmes and H. Morse, personal communication). mAb 6C3 should also prove useful in the study of mouse strains genetically susceptible or resistant to A-MuLV-induced neoplasia. Because of the tissue and expression level specificity of this antigen, A-MuLV tumors/mAb 6C3 could also serve as a model system for mAb diagnosis and treatment.

We do not know if the 6C3Ag<sup>hi</sup> cells that appear in lymphomatous mice derive directly from 6C3Ag<sup>lo</sup> or 6C3 Ag-negative precursor cells during A-MuLV lymphomagenesis. It is possible that A-MuLV infection induces 6C3 Ag expression *de novo* in lymphoid cells as an inappropriate expression of what may be a stromal cell surface molecule that normally participates in lymphoid progenitor cell maturation or proliferation. On the other hand, the 6C3Ag<sup>lo</sup> subpopulation of normal bone marrow cells could contain the A-MuLV target cell, and A-MuLV amplifies antigen expression. Because time course experiments show that detectable numbers of 6C3Ag<sup>hi</sup> cells do not appear before 10 d after A-MuLV infection (data not shown), it is conceivable that a small number of early-infected targets express high levels of 6C3 Ag and expand exponentially, or that a larger number of targets become infected and there is a significant lag-time from infection to 6C3Ag<sup>hi</sup> expression. The 6C3 Ag phenotype of the A-MuLV target cell is under current investigation.

Although amplification of surface 6C3 Ag is independent of M-MuLV alone, there is a significant increase in the percentage 6C3Ag<sup>lo</sup> bone marrow cells after infection with the helper virus (Table I). If the 6C3Ag<sup>hi</sup>-transformed cell population derives directly from precursor 6C3Ag<sup>lo</sup> cells, the helper virus may enhance tumor production by increasing precursor frequency as well as providing the necessary viral structural proteins for the A-MuLV genome. Alternatively, it is conceivable that bone marrow stromal cells respond to infection with M-MuLV by increasing in number and/or expression of 6C3 Ag, thereby amplifying pre-B cell generation and the production of A-MuLV target cells. However, we can only conclude with confidence from these experiments that the increase in 6C3 Ag observed in A-MuLV-infected mice is dependent upon the *gag-abl* gene product, supplied by the A-MuLV genome and not by the M-MuLV genome.

Overall, these results substantiate the hypothesis that 6C3Ag<sup>hi</sup> expression is an inevitable consequence of A-MuLV transformation of pre-B cells in vivo. Coupled with the fact that we have been unable to select mAb 6C3-negative tumor cells from A-MuLV-infected in vitro bone marrow cultures or established A-MuLV cell lines, these experiments suggest that the gene product recognized by mAb 6C3 is essential for many B lineage neoplasms. It is unclear if *v-abl* or augmented *c-abl* expression is a necessary prerequisite for such 6C3 Ag expression. Therefore, a direct examination of the oncogenic potential of 6C3 Ag-encoding genes is warranted, as are experiments designed to establish the relationship between *v-abl* or *c-abl* expression and the 6C3Ag<sup>hi</sup> phenotype.

### Summary

Animals injected with Abelson murine leukemia virus (A-MuLV) rapidly develop fatal bone marrow-derived lymphosarcomas. In all such diseased animals tested, a subpopulation of bone marrow cells expressed a monoclonal antibody-defined, B lineage transformation-associated antigen (6C3 Ag) at levels increased from that detected on normal lymphocytes. Cells bearing a high level of this antigen were found to be transformed as measured by clonal growth in agar, and they expressed surface antigen markers characteristic of early pre-B cells. High-level antigen-expressing cells were found in the bone marrow, lymph nodes, and spleen, but never in the thymus of diseased animals. This distribution agrees with the published pathology of Abelson disease.

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