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Rapid thymomas induced by Abelson murine leukemia virus

(RNA tumor virus/T cell transformation/target cell)

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ABSTRACT **Abelson murine leukemia virus (A-MuLV) is derived from the thymotropic Moloney leukemia virus. However, injection of mice with A-MuLV by conventional routes results in rapidly arising peripheral and bone marrow lymphomas, not thymomas or T cell tumors. In this study thymomas have been induced by intrathymic injection of A-MuLV into BALB/c and C57BL/Ka mice. In both strains thymomas arose with short latent periods, comparable with the latencies of nonthymic tumors induced by intraperitoneal injection of A-MuLV and significantly shorter than those of thymomas induced by intrathymic injection of Moloney leukemia virus. Cells of the BALB/c thymomas were predominantly Thy-1$^-$; those of C57BL/Ka thymomas were predominantly Thy-1$^+$. Tissue culture lines were established and cloned. Some of these expressed low amounts of Thy-1 and one also expressed Lyt-1. Virus from cloned lines transformed 3T3 cells *in vitro* and induced Abelson disease *in vivo* when injected intraperitoneally into neonates. The A-MuLV p120 protein has been precipitated from metabolically labeled cell lysates of one cloned Thy-1$^+$ line. These results show that A-MuLV can transform cells in the T lymphocyte lineage.**

Abelson virus (A-MuLV) is a defective transforming virus derived from Moloney virus (Mo-MuLV), a slow leukemogenic RNA tumor virus. The emergence of A-MuLV on passage of Mo-MuLV through a prednisolone-treated mouse (1) was marked by two features of viral action that differed from that of the parental virus: (*i*) a reduction in tumor latency from ≈3 mo to 3–5 wk, and (*ii*) an apparent change in tissue tropism, with the parental Thy-1$^+$ thymomas being replaced by Thy-1$^-$ peripheral and bone marrow lymphomas. The demonstration of the acquisition by A-MuLV of a cell-derived oncogene (*abl*) (2, 3) suggested an explanation for the decreased latent period, but the cause and mechanism of the altered tumor phenotype have not been explained. The most obvious interpretation is that the oncogene confers on the virus a different target cell specificity.

This paper shows that thymomas can be induced by the intrathymic (i.t.) injection of A-MuLV into young adult BALB/c (B/c) and C57BL/Ka (BL) mice. The observation extends the known tissue tropism of A-MuLV to include the lineage of cells transformed by the parental Mo-MuLV. The *abl* gene is effective in transforming T as well as B cells and myeloid lineage cells.

MATERIALS AND METHODS

Mice. Breeding pairs of BL and the Thy-1.1 congeneic C57BL/Ka Thy-1.1LB were provided by M. Lieberman (Stanford University). These and B/c mice were bred at the Hall Institute. Male and female mice of all three strains were used.

Cell Lines. Ann-1, a line of NIH-3T3 cells clonally transformed by A-MuLV (4) and superinfected with cloned Mo-MuLV in the laboratory of N. Rosenberg (Tufts University), and NR18.2, an A-MuLV-transformed lymphoid cell line, were provided by R. Coffman (Stanford University). RL12, an x-ray-induced virus-nonproducer BL thymoma cell line (5), was from M. Lieberman. A. McGregor (Commonwealth Serum Laboratories, Melbourne) provided B/c 3T3 cells. XC cells and Mo-MuLV-infected SC-1 cells (6) were from G. Shellam (Department of Microbiology, University of Western Australia, Perth, Australia).

Virus Preparations. Two sources of A-MuLV were used in these studies: (*i*) supernatants of Ann-1 cells that had been infected with cloned Mo-MuLV helper virus, and (*ii*) virus from Ann-1 or from NR18.2 cells after growth of the virus in RL12 cells. For preparation of RL12-passaged virus, the RL12 cell line was infected with supernatants from A-MuLV-producing cells and was cultured for 10 days. Aliquots of infected and uninfected cells were acetone-fixed and their cytoplasms were stained with rat anti-MuLV serum, which crossreacts with Mo-MuLV gp70, followed by fluorescein-conjugated goat anti-rat immunoglobulin antibodies. Cells infected with virus stained positive; control uninfected cells were negative. Freshly harvested cell supernatants from either source of A-MuLV or from Mo-MuLV-infected SC-1 cells were centrifuged at 450 × *g* for 5 min and passed through 0.45-μm filters before inoculation of mice or cell cultures or storage at −85°C.

Tumor Induction. B/c or BL mice were inoculated, by using a 30-gauge needle, with 50 μl of filtered cell supernatant containing Polybrene at 4 μg/ml (Pierce), either intraperitoneally (i.p.) within 48 hr of birth or i.t. as 5- to 7-wk-old adults.

Tumor Cell Cultures. Tumors were adapted to culture as described (5). Culture medium consisted of RPMI-1640 (GIBCO), 10% fetal calf serum, and 50 μM 2-mercaptoethanol.

Cloning. Cells were cloned in agarose (SeaKem) as described (7), except that no conditioned medium or feeder layers of cells were used. Clones were plucked from plates containing <200 colonies.

Sera. Hybridoma cell lines 30H12 (rat IgG2b, anti-Thy-1.2), 53-7.3 (rat IgG2a, anti-Lyt-1), and 53-6.7 (rat IgG2a, anti-Lyt-2) (8) were provided by P. Bartlett (Hall Institute, Melbourne). Rabbit anti-rat Ig was from I. Clark-Lewis (Hall Institute). Rabbit antibodies and 30H12 antibody were affinity-purified from a rat IgG-coupled Sepharose column and a fetal calf serum-absorbed rabbit anti-rat Ig Sepharose column, respectively, before fluorescein conjugation. Mouse anti-p120 serum was provided by V. Rotter and D. Baltimore (Department of Biology, Massachusetts Institute of Technology). Rat IgG was from J. Goding.

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Abbreviations: A-MuLV, Abelson murine leukemia virus; Mo-MuLV, Moloney murine leukemia virus; *abl*, transforming gene of A-MuLV; B/c, BALB/c AnBradleyWehi mouse strain; BL, C57BL/Kaplan mouse strain; EBSS, Eisen's balanced salt solution; i.p., intraperitoneal(ly); i.t., intrathymic(ally); FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; pfu, plaque-forming unit(s); ffu, focus-forming unit(s).

Fluorescent Antibody Staining and Analysis. All antisera were centrifuged at 100,000 × *g* for 15 min before use. Samples (5×10^5 viable cells) were stained for 60 min on ice in conical plastic tubes with 50 μl of antibody diluted in Eisen's balanced salt solution (EBSS)/5% fetal calf serum/10 mM NaN_3. As necessary, dead cells were removed beforehand by Ficoll/Paque centrifugation or by filtration through cotton wool in low ionic strength buffer (9). All indirect staining included controls that used EBSS/fetal calf serum/NaN_3 alone as the first stage. All direct staining included controls of cells of inappropriate allotype or negative for the antigen. Cells were washed in 9 ml of cold EBSS/fetal calf serum/NaN_3 after each staining stage. Propidium iodide at 10 μg/ml was included in the last stage to counterstain dead cells. Microscopic examination was carried out at a magnification of 1,250. Flow cytometry was performed by using a fluorescence-activated cell sorter (FACS) II (Becton Dickinson) with linear or logarithmic fluorescence readout. Dead cells were excluded by low-angle light scatter and propidium iodide uptake.

Virus Assays. 3T3 focus assays for transforming virus and XC plaque assays for leukemia virus were performed as described (4, 10).

Biosynthetic Labeling, Immunoprecipitation, and Electrophoretic Analysis. Cells (10^7) from logarithmic phase cultures were washed in warm Dulbecco's modified Eagle medium lacking methionine and were then preincubated at 37°C in this medium with 2% fetal calf serum for 60 min. Cells were labeled with 100 μCi of [^{35}S]methionine (1 Ci = 3.7×10^{10} becquerels) in 1 ml of medium for 2 hr. After two washes in cold phosphate-buffered saline, cells were lysed at 10^7 cells per ml in buffer containing 0.1 M Tris·HCl (pH 8.3), 0.4 M NaCl, and 0.5% Triton X-100 for 30 min on ice. Cell lysates were brought to 20 mM unlabeled methionine, 2.5 mM phenylmethylsulfonyl fluoride, 10 mM ethylenediaminetetraacetic acid, and were clarified at 100,000 × *g* for 10 min. After preadsorption with fixed *Staphylococcus aureus* Cowan 1 coated with preimmune mouse serum, lysates were allowed to react with antiviral protein sera overnight at 4°C. Antibody–antigen complexes were adsorbed to *S. aureus*, washed three times, eluted, and analyzed on 7.5% polyacrylamide gels with a Tris glycine buffer system (11). Low molecular weight (Pharmacia) and high molecular weight (Bio-Rad) markers were used. Labeled proteins were visualized by fluorography.

RESULTS

A-MuLV Induces Predominantly Thy-1$^-$ Tumors in B/c Mice. Filtered supernatants from Ann-1 cells were injected with Polybrene into B/c mice, either i.p. into neonates or i.t. into 5- to 7-wk-old animals. As summarized in Table 1, i.p. injected mice developed, with the expected latency (1) (23–41 days), tumors of the usual Abelson type—i.e., of bone marrow and peripheral lymph nodes. Mice injected i.t. developed thymomas, with slightly longer latency (28–61 days) and with variable involvement of spleen and lymph nodes. This result was obtained even with a 1:10,000 dilution of the virus. Every mouse that developed a tumor after i.t. injection developed a thymoma, but none of the mice injected i.p. did so. Mice injected i.t. with Mo-MuLV developed thymomas in ≥92 days.

Table 1. Titration of virus i.t. vs. i.p.

Source	Route*	Dilution†	Disease frequency‡	Type§	Latent period, days¶ Mean	Range
Ann-1 (A-MuLV)	i.p.	None	12/12	B/L	30	23–38
		1:10	6/9	B/L	31	23–41
		1:100	1/2	B/L	40	—
		1:10,000	0/2	B/L	—	—
Ann-1	i.t.	None	4/5	T	43	34–61
		1:10	5/6	T	38	28–61
		1:100	5/5	T	48	43–53
		1:10,000	3/5	T	40	33–43
SC1.Mo (Mo-MuLV)	i.t.	None	9/9	T	108	92–137
1.2.1	i.p.	None	12/12	B/L	29	23–53
		1:10	12/16	B/L	28	23–35
		1:100	1/4	B/L	69	—
1.2.1	i.t.	None	10/12	T	38	27–53
		1:10	2/2	T	29	28–30
		1:100	3/5	T	38	37–40

* Fifty microliters of filtered cell supernatant containing Polybrene at 4 μg/ml was administered i.p. within 48 hr of birth or i.t. into 5- to 7-wk-old B/c mice.

† Ann-1 supernatants routinely produce 10^4–10^5 B/c-3T3 focus-forming units (ffu)/ml and 10^5–10^6 XC plaque-forming units (pfu)/ml. SC1.Mo cell supernatants routinely produce 10^4–10^5 XC pfu/ml. None indicates no dilution.

‡ Ann-1- and 1.2.1-infected mice were held for 3 mo.

§ B/L, bone marrow and lymphoid tumors; T, thymoma with or without spleen and lymph node involvement.

¶ Mice with B/L tumors were harvested when paraplegia, swollen cranium, or lymph nodes (or all) were evident. Mice with thymomas were harvested when weight loss, breathing difficulty, or exophthalmia (or all) were evident.

FACS analysis (Fig. 1) and microscopic examination of individual thymomas resulting from A-MuLV i.t. injection of B/c mice showed high proportions of Thy-1$^-$ cells. Fig. 1 shows FACS analyses of two independent B/c thymomas, stained with fluorescein isothiocyanate-conjugated monoclonal anti-Thy 1.2 (FITC-anti-Thy-1.2). Positive controls of normal thy-

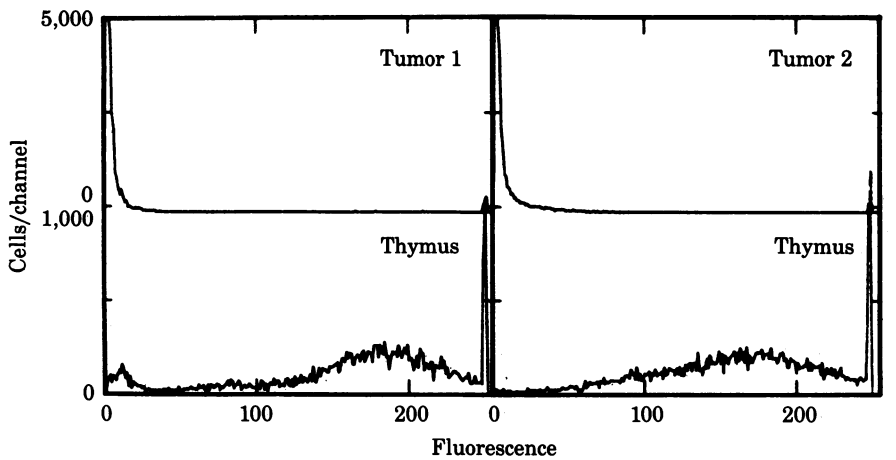

FIG. 1. FACS analysis of FITC-anti-Thy-1.2-stained B/c A-MuLV thymomas. Each tumor is accompanied by a positive control, FITC-anti-Thy-1.2-stained normal thymus, analyzed at the same fluorescence gain. Note linear fluorescence readout.

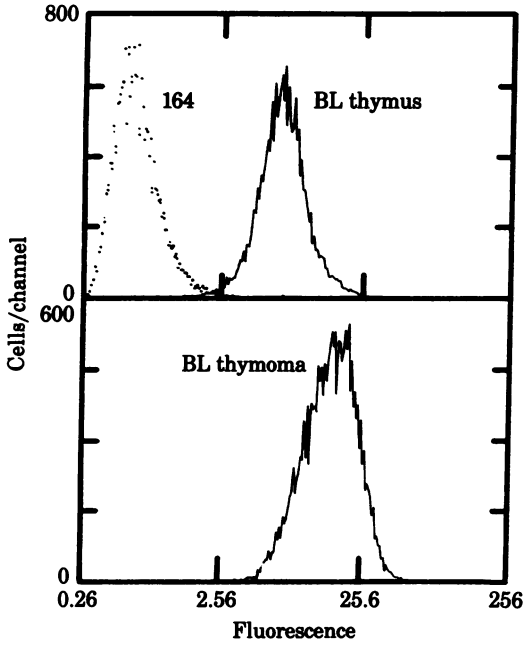

FIG. 2. FACS analysis of FITC-anti-Thy-1.2-stained BL A-MuLV thymoma. (*Upper*) A Thy-1$^-$ cell line, 164 (dotted) and Thy-1.2 thymus (solid). (*Lower*) BL A-MuLV thymoma. Correction of staining intensities for cell size as described in legend of Table 2 adjusts the apparent relative staining of 2.28 for tumor/thymus to 1.48.

mocytes showed Thy-1 profiles with >95% of cells brighter than 30 fluorescent units. By comparison, both of the A-MuLV B/c tumors appeared Thy-1$^-$, with >95% of cells below 30 fluorescent units. Seventeen B/c A-MuLV thymomas were examined microscopically after FITC-anti-Thy-1.2-staining. The mean value was 24% Thy-1$^+$ cells, with the highest proportion 43% positive.

A-MuLV Induces Thy-1$^+$ Thymomas in BL Mice. When BL mice were injected i.t. with filtered Ann-1 supernatants, thymomas arose rapidly (40–42 days), most of which were Thy-1$^+$. Seven tumors were stained with FITC-anti-Thy-1.2. By microscopic analysis, six of the tumors consisted of >85% Thy-1$^+$ cells. FACS analysis of one of the tumors appears in Fig. 2. The majority of its cells had even higher levels of Thy-1 than was the modal value for normal thymocytes.

Injection i.t. of BL mice with RL12-passaged virus showed a similar effect. Thymomas developed rapidly (44–75 days) in 9 of 32 mice injected. Four of these tumors were stained and analyzed by FACS for Thy-1.2. All were >90% positive (unpublished data). Thus, Abelson virus from either source administered i.t. to this strain of mice produces predominantly Thy-1$^+$ thymomas (9 of 10 examined) in contrast to the predominantly Thy-1$^-$ B/c thymomas.

Tissue Culture Lines. One B/c and four BL tumors were established in continuous tissue culture. The lines produce tumors in syngeneic mice at the site of subcutaneous injection. The origins and some of the surface markers of the lines are summarized in Table 2. Only one of the five lines bears significant levels of Lyt-1 or Lyt-2: the BL line 1.4.4.5 is Lyt-1$^+$ (Table 2 and Fig. 3). Although the BL tumors and short-term cultures stained positive for Thy-1 (Fig. 2), long-term (>8 wk) tissue culture lines and clones derived from them showed levels of Thy-1 lower than that of normal cortical thymocytes. As shown in Fig. 4, only two lines—both from BL thymomas—bore significant levels of Thy-1. After correction for cell surface area, these values correspond to 0.18 and 0.20 of the mean staining of normal Thy-1.2 thymocytes (Table 2). The other three lines showed Thy-1.2 levels comparable with the mean of a Thy-1.1 thymocyte control (0.03) and so were considered negative.

The binding of anti-Thy-1.2 to the lines 1.4.2.69 and 1.4.4.5 is unlikely to be mediated by Fc receptors. All sera were centrifuged at 100,000 × *g* to remove aggregated Ig before use. The results shown were unaffected by the presence of rat IgG up to 1 mg/ml during the staining incubation and have been confirmed by indirect staining with 30H12 and fluorescent rabbit anti-rat Ig serum. Controls that used rat IgG at 1 mg/ml as the first stage were negative. When 93% of Thy 1.2 (B/c) thymocytes were stained (<2% in negative control), the results for the cloned cell lines were: 1.4.4.5, 85% (negative control, <1%); 1.4.2.69, 87% (4.4%); 1.2.1.3, <1% (<1%); and for Thy 1.1 thymocytes, <0.5% (<0.5%).

The cell lines appear lymphoid by size and morphology. Comparison of FACS II-determined 0° scatter profiles (Table 2) showed that the cells of the thymic lines had cross-sectional areas similar to those of peripheral A-MuLV-transformed lines and x-irradiation-induced thymoma cells. Giemsa-stained preparations appeared lymphoid, having a high nucleus/cytoplasm ratio and no detectable granules in the cytoplasm. No Ig μ chain was detectable by immune precipitation of [^{35}S]methionine-labeled cell lysates (unpublished data).

Virus Production in Cloned Lines. To identify the virus responsible for transformation of the lines, filtered supernatants of cloned lines were assayed for transformation *in vivo* and *in vitro*. Table 1 compares the i.p. and i.t. administration of supernatant from one line to B/c mice. Pathology was in each case

Table 2. Thy-1, Lyt phenotypes of A-MuLV thymoma cell lines

Cell line*	Strain	Transforming virus	Thy-1.2†	Lyt-1	Lyt-2	Relative size‡
1.4.4.5	BL	Ann-1 (RL12)§	0.20 ± 0.06 (+)	+	−	92
1.4.2.69	BL	Ann-1 (RL12)	0.18 ± 0.09 (+)	−	−	131
1.2.1.3	BL	NR18.2 (RL12)	0.02 ± 0.02 (−)	−	−	108
289	BL	Ann-1	0.03 (−)	−	−	104
164	B/c	Ann-1	0.01 (−)	−	−	102
RL12	BL	¶	0.625 (+)			80

* All lines, except 289 and 164, are cloned.

† Values (±SD) are relative intensities of FITC-anti-Thy-1.2 stain compared with normal thymus, corrected for cell size as measured by FACS II:

$$\frac{\text{mean experimental } \alpha\text{-Thy-1.2}}{\text{mean thymocytes } \alpha\text{-Thy-1.2}} \times \frac{\text{mean thymocyte 0° scatter}}{\text{mean experimental 0° scatter}}.$$

‡ As measured by FACS 0° light scatter units.

§ Ann-1 virus grown in RL12 cells. See *Materials and Methods*.

¶ RL12, an x-ray-induced thymoma cell line, is included for comparison.

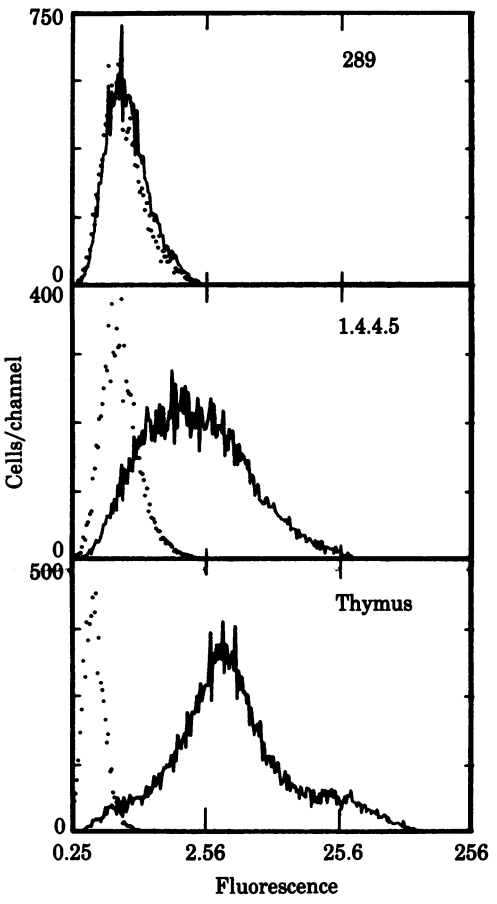

FIG. 3. FACS analysis of anti-Lyt-1 stain of two cell lines. Stain is anti-Lyt-1 followed by FITC-rabbit anti-rat Ig. (*Top*) Line 289; (*Middle*) line 1.4.4.5; (*Bottom*), normal thymus. Dotted profiles, 2nd stage controls.

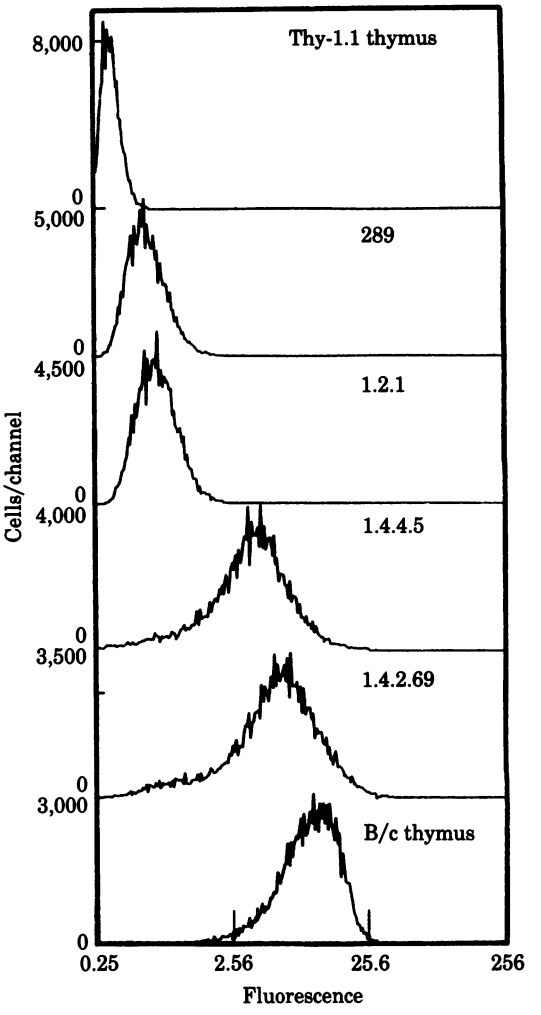

FIG. 4. FACS analysis of FITC-anti-Thy-1.2-stained thymoma cell lines. Lines 1.2.1, 1.4.4.5, and 1.4.2.69 are cloned; 289 is not cloned. Thy-1.1 and B/c are thymus controls.

similar to that produced by A-MuLV—i.e., all tumors resulting from i.t. injections were thymomas and i.p. injection produced bone marrow and peripheral lymphomas. Supernatants from all three cloned lines were tested for B/c 3T3 transformation, as a measure of transforming virus (expressed as ffu/ml), and for XC plaques, as a measure of helper leukemia virus (expressed as pfu/ml). The results were: 1.2.1.3, 111 ffu/ml and 911 pfu/ml; 1.4.4.5, 91 ffu/ml and 696 pfu/ml; 1.4.2.69, <1 ffu/ml and <1 pfu/ml; control medium, <1 ffu/ml and <1 pfu/ml.

To confirm the presence of A-MuLV sequences, cells of the 1.4.2.69 clone and of Ann-1 were metabolically labeled with [^{35}S]methionine. Lysates precipitated with anti-p120 or anti-gp70 were analyzed on polyacrylamide gels (Fig. 5). A band corresponding to $M_r \approx 120{,}000$ was visible in both Ann-1 and 1.4.2.69 extracts that were precipitated with anti-p120 serum (Fig. 5*A* and *B*). The anti-p120 serum is known to contain anti-gp70 activity, which accounts for the second major band in the Ann-1 lysate (lane A). The equivalent band was greatly decreased in 1.4.2.69 (lanes B and C). This and its negative results in 3T3 and XC assays were interpreted to mean that this clone was transformed by A-MuLV, with subsequent loss of some or all of its helper virus expression.

DISCUSSION

The data presented here demonstrate that i.t. injection of A-MuLV into B/c or BL mice induced thymic leukemias that were rapidly arising. The latent period of these tumors was similar to that of bone marrow and peripheral lymphomas induced by other routes of A-MuLV inoculation and was approximately 50 days less than the latency of thymomas induced by i.t. injection of Mo-MuLV.

The tumors are of lymphoid morphology and of at least two cell types. The cells of A-MuLV-induced B/c thymomas were predominantly negative for Thy-1 expression, and the one tissue culture line derived from a B/c thymoma was Thy-1$^-$, Lyt-1$^-$, Lyt-2$^-$. Most of the BL tumors were predominantly Thy-1 positive, as were two of four cell lines derived from these tumors. Although the Thy-1 levels on the positive lines were low, the detection of Thy-1 and Lyt-1 on cells of lymphoid morphology indicates that at least some of the transformed lines were of the T cell lineage.

It was confirmed that the tumors described here were transformed by A-MuLV. The Ann-1 virus stock and virus shed from the cloned thymoma cell lines were shown to transform 3T3 cells *in vitro* and to cause typical A-MuLV lymphoma pathology *in vivo*. Immune precipitation of metabolically labeled cell lysates demonstrated the *abl* gene product, p120, in one Thy-1-bearing line.

The phenomenon reported here is surprising for two reasons. (*i*) Abelson virus administered via other routes does not induce thymomas or peripheral T cell tumors (1). This may be explained by the very low frequency of A-MuLV target cells per thymus (12) and the speed with which tumors of more numerous

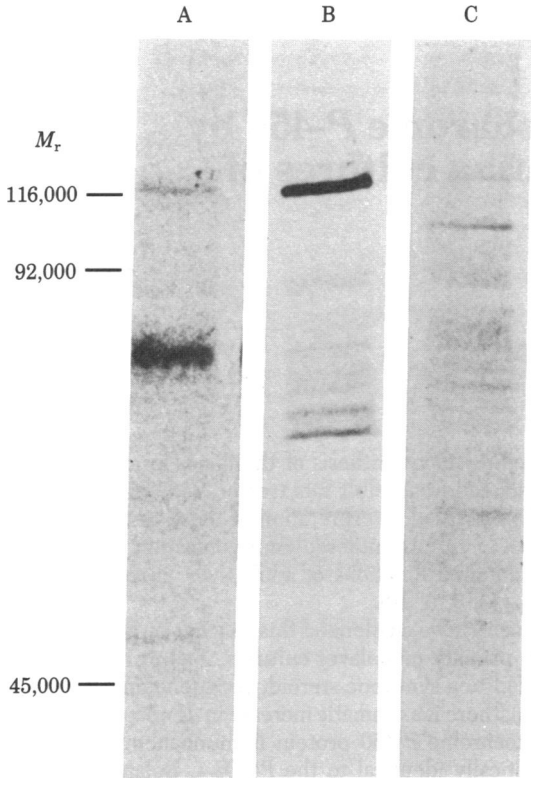

FIG. 5. Clone 1.4.2.69 is p120 positive. Cells were labeled with [^{35}S]methionine, lysates were precipitated, and gels were run as described. Lane A, Ann-1, anti-p120; lane B, 1.4.2.69, anti-p120; lane C, 1.4.2.69, anti-gp70. Marker proteins are indicated at left. The upper and lower sections of the gel, in which no bands appeared, have been removed.

and accessible peripheral target cells kill the animal. (*ii*) In normal thymus populations up to 99% of cells bear high levels of Thy-1 on their surfaces (13), yet the B/c thymomas described here are predominantly Thy-1$^-$ and the BL tissue culture lines exhibit only low levels of this antigen. Candidates for normal equivalents of the cell lines described here do exist in the thymus. Among the thymocytes that are Thy-1$^-$ (≈1.5%), a subpopulation of ≈1% lacks surface Ig (14). The Thy-1$^-$ tumors and cell lines could be derived from this population, and thus could be pre-B, pre-T, or nonlymphoid cells. Cells bearing low levels of Thy-1 are found in adult thymus medulla, in peripheral lymphoid organs and blood ("mature" T cells), and in fetal thymus. In 14-day fetal thymus, most cells are Thy-1$^+$, Lyt-1$^-$, 2$^-$, with lower levels of Thy-1 than adult thymocytes (R. Scollay, personal communication). Mathieson *et al.* (15) have described Thy-1$^+$, Lyt-1$^+$,2$^-$ cells appearing in day-15 to day-16 fetal thymus before Thy-1$^+$, Lyt-1$^+$,2$^+$ cells arise. The Thy-1$^+$ cell lines derived from BL thymomas in the present study have Thy-1 and Lyt phenotypes matching these two classes of immature thymocytes. Further characterization of the cell lines is in progress.

The outgrowth of Thy-1$^-$ and low Thy-1$^+$ cell lines from Thy-1$^+$ BL tumors in culture presumably reflects selection on a heterogeneous starting population. Whether this heterogeneity is generated by polyclonal transformation of targets at various differentiation states or by differentiation after a single transformation event is not clear. The contrast in Thy-1 phenotypes of B/c and BL thymomas suggests a strain difference in this differentiation process. Risser *et al.* (16) have described a multigene-determined difference between B/c and C57BL strains, resulting in susceptibility in B/c and resistance in C57BL to nonthymic Abelson lymphoma after intravenous injection of A-MuLV into adults. The thymomas reported here demonstrate that resistance is broken in the BL thymus, though a strain difference remains in the phenotype of transformants. Moreover, the results of the present study are consistent with the description by Boyer *et al.* (17) of a B/c versus C57BL/10 strain difference in Mo-MuLV transformants—B/c tumors frequently being Thy-1$^-$, whereas C57BL/10 tumors were Thy-1$^+$. Though this last observation needs to be confirmed with cloned Mo-MuLV, the concordance among the three studies is striking. One interpretation is that A-MuLV and Mo-MuLV have at least overlapping target cell populations.

The known tissue specificity for transformation by A-MuLV now includes myeloid (18), B lymphocyte (19, 20), and T lymphocyte lineages. One critical question addressed by others (21, 22) has been whether A-MuLV transformants arise from a single oligopotential hematopoietic precursor target cell or from A-MuLV targets in the committed myeloid and lymphoid lineages. Shinefeld *et al.* (21) present evidence that myeloid and B cell lineage targets are distinct. A-MuLV may now prove a useful probe in defining the branchpoint of B and T lymphoid precursor pathways.

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