

T-CELL LYMPHOMA INDUCTION BY RADIATION LEUKEMIA VIRUS IN ATHYMIC NUDE MICE*

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Radiation leukemia virus (RadLV)¹ is a naturally occurring, B-tropic, leukemogenic murine leukemia virus (MuLV) which is obtained from radiation-induced thymic lymphomas of strain C57BL/Ka mice (1, 2). Previous studies have shown that the thymus is essential for the replication of the virus (3) and the neoplastic transformation of infected cells (4, 5). We report here the development of extrathymic lymphoblastic lymphomas in RadLV-inoculated athymic nude mice, the establishment in vitro of a permanent cell line from such lymphoma cells, and the biochemical, serological, and biological characterization of the virus produced by this lymphoma cell line.

We have also characterized the RadLV-induced lymphomas with respect to several immunological and enzymatic markers. These studies demonstrate that the neoplastic cells are of T-lineage despite the absence of the thymus. Further characterization of the stage of differentiation of the T-cell lymphomas reveals that their antigenic profile resembles that of mature peripheral T cells. Alternative hypotheses concerning the origin of the target cells for RadLV lymphomagenesis in athymic mice are discussed.

Materials and Methods

Mice. C57BL/Ka mice bred in this laboratory and congenitally athymic nude (nu/nu) mice on the BALB/c or NIH/Swiss background and their thymus-bearing heterozygote (nu/+) littermates, originally obtained from the National Cancer Institute, Bethesda, Md., were used in these experiments. Mice of either sex were 4 wk old when used.

Viruses. RadLV was prepared as a 20% cell-free extract of RadLV-induced C57BL/Ka thymic lymphomas in phosphate-buffered saline (PBS) as previously described (5). The virus isolate RadLV/VL₃ was obtained from culture fluids of a RadLV-induced C57BL/Ka thymic lymphoma cell line (BL/VL₃) permanently established in culture.² The virus harvested from the supernatant fluids of the nude mouse lymphoma cell line (BALB/Nu₁) described in this report has been designated RadLV/Nu₁.

Cells and Culture Conditions. Suspension cultures of the BALB/Nu₁ lymphoma cell line were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum,

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; DTT, dithiothreitol; IF, immunofluorescence; gp, glycoprotein; MIF, membrane immunofluorescence; MuLV, murine leukemia virus; p, protein; PBS, phosphate-buffered saline; RadLV, radiation leukemia virus; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TdT, terminal deoxynucleotidyl transferase.

² M. Lieberman et al. Manuscript in preparation.

2×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. Cell suspensions from spleen, lymph nodes, and thymus were obtained by mincing each tissue and passing the suspension through a nylon cloth into PBS (pH 7.4) supplemented with 5% fetal calf serum. Viable, nucleated cells were counted and the cell concentration adjusted to levels suitable for the various assays.

Immunofluorescence (IF) Test for Detection of MuLV Antigen. Indirect IF staining of acetone-fixed cells was performed according to Hilgers et al. (6). The antisera used in this assay have been described (7). Values reported for percent MuLV antigen-positive cells were calculated as described by Declève et al. (3).

Membrane IF for Detection of Ig and Thy-1.2 Determinants. Anti-Thy-1.2 serum (AKR anti-C3H) was kindly supplied by Dr. Israel Zan-Bar, Division of Immunology, Department of Medicine, Stanford University. Fluoresceinated rat anti-mouse Ig, IgG, and IgM sera were obtained from Cappel Laboratories, Inc., Cochranville, Pa. All sera were used at dilution 1:10 in PBS. Cells previously washed in PBS with 5% fetal calf serum were incubated with anti-Thy-1.2 serum for 30 min at room temperature. They were then washed, incubated with fluoresceinated serum for 30 min at 4°C, washed again, and placed on the wells of toxoplasmosis slides (Bellco Glass, Inc., Vineland, N. J.). The slides were dried, fixed in acetone for 10 min, mounted with glycerol-PBS 1:1 mixture and examined with a Zeiss fluorescence microscope equipped with a dark field condenser, exciter filter B6 12, barrier filters (50 and 66) and a high pressure mercury bulb (HBO 220 W). The same cell suspension could thus be examined for the presence of both surface Ig and Thy-1.2 antigens: the frequency of cells positive for Thy-1.2 was derived by subtracting the number of cells showing fluorescence after incubation with anti-Ig serum only from those which were positive after sequential exposure to anti-Thy-1.2 and anti-Ig.

Quantitative Absorption Tests and Antisera. The cells used for absorption were obtained from confluent cultures or from actively growing tumors. Cell suspensions were made in PBS, washed twice, and samples of different cell numbers distributed into U-shaped well microtiter plates. The plates were spun at 900 rpm for 10 min and the cells resuspended in 50 μ l of antiserum. The antiserum dilution used for absorption was approximately one twofold dilution below the endpoint of 50% cytotoxicity of each serum. Cells and antiserum were incubated on ice for 1 h with frequent shaking. The plates were then centrifuged at 1,500 rpm for 15 min in the cold and 30 μ l of antiserum was removed and stored at -70°C.

The absorbed antisera were tested in direct cytotoxicity assays by the method of Amos et al. (8), as modified by Frelinger et al. (9). The final results were expressed as:

$$\text{percent specific absorption} = \frac{\% \text{ absorption by test cells} - \% \text{ absorption by negative controls}}{\% \text{ absorption by positive controls}}$$

The antisera used in this assay were heat-inactivated (56°C for 30 min) before use. Anti-Thy-1.2 serum has been described above and was absorbed at dilution 1:2,500, at which it kills 80% of C57BL/Ka thymocytes. Anti-TL serum was raised in our own laboratory against C57BL/TL+ thymocytes in C57BL/Ka mice. For absorption it was diluted 1:64. Anti-Ly-1.2 (C3H/An anti-CE/J thymocytes) and anti-Ly-2.2 [(C3H/An \times C57BL/6-Ly 2.1) F_1 anti-ERLD] sera were obtained from Dr. F.-W. Shen, Memorial Sloan-Kettering Cancer Center, New York. Both were used for absorption at 1:80 dilution. Anti-G_{IX} serum was obtained from Dr. Elizabeth Stockert, Memorial Sloan-Kettering Cancer Center, New York. It was produced in (W/Fu \times BN) F_1 rats against W/Fu leukemia (C58NT)D. It was absorbed with G_{IX} negative thymocytes at 1:10 dilution before use. The final dilution for absorption was 1:640.

Terminal Deoxynucleotidyl Transferase (TdT) Assay. The TdT assay was performed according to Pazmiño et al. (10). The reaction mixture consisted of 2 μ g oligo(dA)₁₂₋₁₈, 0.05 M Tris, pH 8.3, 2 mM dithiothreitol (DTT), 0.6 mM MnCl₂, 210 pmol [³H]dGTP (35,500 cpm/pmol), and 2,100 pmol of cold dGTP, in 100 μ l containing 30 μ l of each phosphocellulose fraction. Under these conditions, the reactivity incorporated for 30 min was linear with enzyme concentration. 1 U of enzyme activity is defined as the amount catalyzing the incorporation of 1 pmol of dGTP per hour into acid insoluble material. Specific activity was calculated from the total enzyme activity recovered in phosphocellulose from 10⁸ viable nucleated cells.

Radiolabeling of the Viral Proteins. Suspension cultures of BALB/Nu₁ cells were seeded at 1×10^6 cells/ml. After 2 days incubation, the culture fluid was changed to L-leucine-free medium with 10% dialyzed fetal calf serum, containing either 0.25 μ Ci/ml of [¹⁴C]L-leucine (284

mCi/mmol) or 10 μ Ci/ml of [3 H]L-leucine (5 Ci/mmol). To label viral RNA, [3 H]uridine (30 Ci/mmol) was used at a concentration of 5 μ Ci/ml. Viruses were harvested from culture after 18–20 h of labeling. All isotopes were obtained from New England Nuclear Corp., Boston, Mass.

Virus Purification. The supernatant fluid of exponentially growing cell cultures was first clarified at 4°C by centrifugation at 2,000 *g* for 15 min, followed by a second centrifugation at 7,000 *g* for 20 min. Viruses were pelleted at 35,000 *g* for 3 h at 4°C, resuspended after overnight incubation with TNE buffer (20 mM Tris hydrochloride, pH 7.5, 100 mM NaCl, 1 mM EDTA) at 0°C and spun through discontinuous (15–55% wt/vol in TNE) sucrose gradients in a Beckman L5-50 (SW-40 rotor) ultracentrifuge at 40,000 rpm for 3 h. Material sedimenting to the interface was collected by piercing the bottom of the tube, slowly diluted in TNE buffer, layered on a continuous sucrose gradient (15–55% wt/vol in TNE) and spun in a Beckman L5-50 (SW-40 rotor) ultracentrifuge at 40,000 rpm for 3 h. A gradient-collecting device was used to separate 0.5-ml fractions. For detection of radiolabeled macromolecules, 10- μ l aliquots of each fraction were precipitated with 10% trichloroacetic acid (TCA), filtered on Whatman GF/C filter papers, extensively washed with cold 0.5 M HCl, and counted in a Toluene-Omnifluor scintillation fluid. Sucrose densities of the fractions were measured with a Bausch and Lomb Abbe refractometer (Bausch & Lomb, Scientific Optical Products Div., Rochester, N. Y.). Protein content was monitored by adsorbance at 280 nm with a Zeiss PM 6 spectrophotometer (Carl Zeiss, Inc., New York).

Radioimmune Precipitation Assay. The assay for [3 H]L-leucine labeled RadLV/Nu₁ virus was performed with rabbit antisera against the envelope glycoprotein (gp71) of the BALB:virus-2 xenotropic virus, AKR MuLV gp71, and RadLV/VL₃ virus. After a 1-h incubation at 25°C with serial dilutions of the primary antiserum, the secondary antiserum, a goat anti-rabbit IgG (Huntington Research Center, Brooklandville, Md.), was added and the mixture incubated overnight at 4°C. Aliquots were then removed from each sample, precipitated with 10% TCA, filtered on GF/C paper filters, extensively washed with cold 0.5 M HCl, and counted in a toluene-omnifluor based scintillation fluid. The remainder of the precipitation mixture was then spun for 20 min at 3,000 *g* to pelletize the immunoprecipitate, dried at room temperature, dissolved in NCS-tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) at 50°C for 30 min, and counted as above. The values are expressed as percentages of counts recovered from the immunoprecipitate versus the counts recovered from the samples before centrifugation.

Electrophoretic Analysis of Viral Proteins. Radiolabeled viral proteins of isopycally banded viruses were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Witte et al. (11). The differentially radiolabeled proteins of two different viruses were compared by coelectrophoresis on the same gel.

Assay of RNA-Dependent DNA Polymerase (RT). The assay was performed as described by Lieber et al. (12), modified by incubation at 30°C, pH 8.1 with 0.25 A260 U of (rA)_n and 0.025 A260 U of (dT)_{12–18} per ml.

Electron Microscopy. Purified RadLV/Nu₁ viral particles, fixed with osmium tetroxide vapors, were stained with 1% uranyl acetate for 15–30 s, mounted on Parlodion-coated copper grids (Ted Pella Co., Tustin, Calif.), air dried, and examined on a Philips EM 300 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV. Cells were fixed in 2.5% glutaraldehyde in phosphate buffer, postfixed in 2% osmium tetroxide, and processed for dehydration embedding and sectioning as already described (13). Thin sections were examined on a Siemens electron microscope (Siemens, Berlin, W. Germany) at 80 kV, through the courtesy of Dr. K. Bensch, Department of Pathology, Stanford University.

Results

Induction of Lymphomas by RadLV in Athymic Nude Mice. Athymic nude (nu/nu) mice derived on BALB/c and on NIH/Swiss backgrounds were used in these experiments (Table I). RadLV was injected intracranially. Since nude mice are immunodepressed, they show a very high mortality. In the present experiments, 142 NIH/Swiss nude mice were injected, but only eight mice reached the age of 9 mo.

Among the injected BALB/c nude animals, lymphoblastic lymphomas developed

TABLE I
Incidence of Lymphomas in RadLV-Infected Nude Athymic Mice

Virus	Mouse type	Lymphomas/ mice*	Latent pe- riod	
			%	mo
RadLV	BALB/c nude (nu/nu)	3/5	60	3-4
RadLV	NIH/Swiss nude (nu/nu)	1/8	12	8
(Controls)	NIH/Swiss nude (nu/nu)	0/35	—	—

* Denominator is the number of virus-infected nude mice surviving to the age at which the first lymphoma appeared.

in three (60%) of five mice surviving to the age at which the first lymphoma developed. Only one (12%) of eight injected NIH/Swiss nude animals surviving to the corresponding age developed a lymphoblastic lymphoma. As shown in Table I, the latent period for these tumors was much longer in NIH/Swiss nude mice (8 mo) than in BALB/c nude mice (3-4 mo). Because BALB/c mice are of Fv-1^{bb} genotype, these results are consistent with the previously demonstrated B-tropic nature of RadLV (14). At autopsy, the tumor-bearing mice had greatly enlarged lymph nodes and moderately enlarged spleens. Histological examination showed a diffuse lymphoblastic lymphoma involving the lymph nodes and spleens of the tumor-bearing mice (Fig. 1). Indirect immunofluorescence staining demonstrated the presence of MuLV antigens in 100% of the BALB/c nude and NIH/Swiss nude lymphoma cells (Fig. 2).

Establishment of a Virus-Producing Nude Mouse Lymphoma Cell Line in Culture. A permanent cell line was established from a BALB/c nude lymphoma and designated BALB/Nu₁. The cells grow readily in stationary suspension culture. When injected intraperitoneally in mice of different strains, the cells are highly tumorigenic in BALB/c (100% takes progressing to death in 1 mo) and in NIH/Swiss nude (nu/nu) mice, but are poorly tumorigenic in NIH/Swiss nude (nu/+) heterozygotes. They are not tumorigenic in normal NIH/Swiss and C57BL/Ka mice (Table II).

The typical electron micrographic appearance of BALB/Nu₁ cells is shown in Fig. 3. The nucleus of these cells is characterized by diffuse chromatin and by a hypertrophic nucleolus which is generally rectangular in shape. The cytoplasm contains numerous free ribosomes as well as rosettes of polysomes. Mitochondria and lysosomes are present but endoplasmic reticulum is scarce. The Golgi apparatus, when visualized, is well developed. The ultrastructural appearance is therefore very similar to that of a typical stimulated lymphocyte. Numerous viral particles are seen in the extracellular space or budding from the plasma membrane of such cells. Fig. 3 (insert) shows that they are C-type particles, 100 nm in diameter.

Physicochemical and Serological Characterization of RadLV/Nu₁ Virus. Fig. 4 shows the acid precipitable radioactivity of [³H]uridine and of [³H]L-leucine-labeled RadLV/Nu₁ virus, after purification on sucrose gradients by isopycnic banding. RT activity coincided with that of [³H]labeled material in the 1.15-1.17 g/cm³ density region of the gradient. A sample of the peak material, negatively stained and examined with the electron microscope, showed large numbers of typical C-type viral particles.

Fig. 5 shows the protein patterns observed when [³H]L-leucine-labeled RadLV/Nu₁ was coelectrophoresed with [¹⁴C]L-leucine-labeled RadLV/VL₃ in a 10% polyacrylamide gel. The major core proteins (p30) of both viruses migrate in identical positions, but the major envelope protein (gp71) of RadLV/Nu₁ is not well demonstrated. For

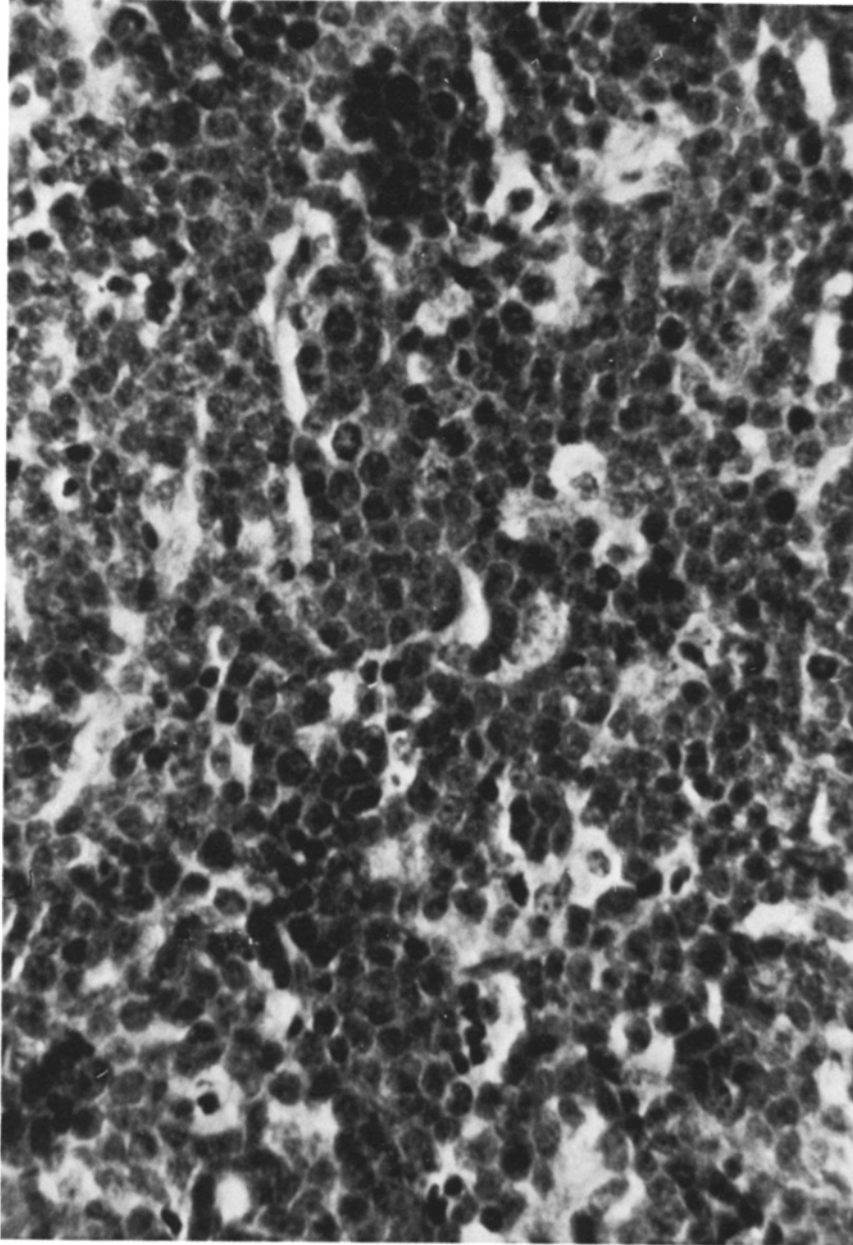


Fig. 1. BALB/c nude mouse lymphoma 4 mo after RadLV inoculation. Peripheral lymph node extensively infiltrated by a monotonous population of medium-sized and large lymphocytic tumor cells. H and E, X 1175.

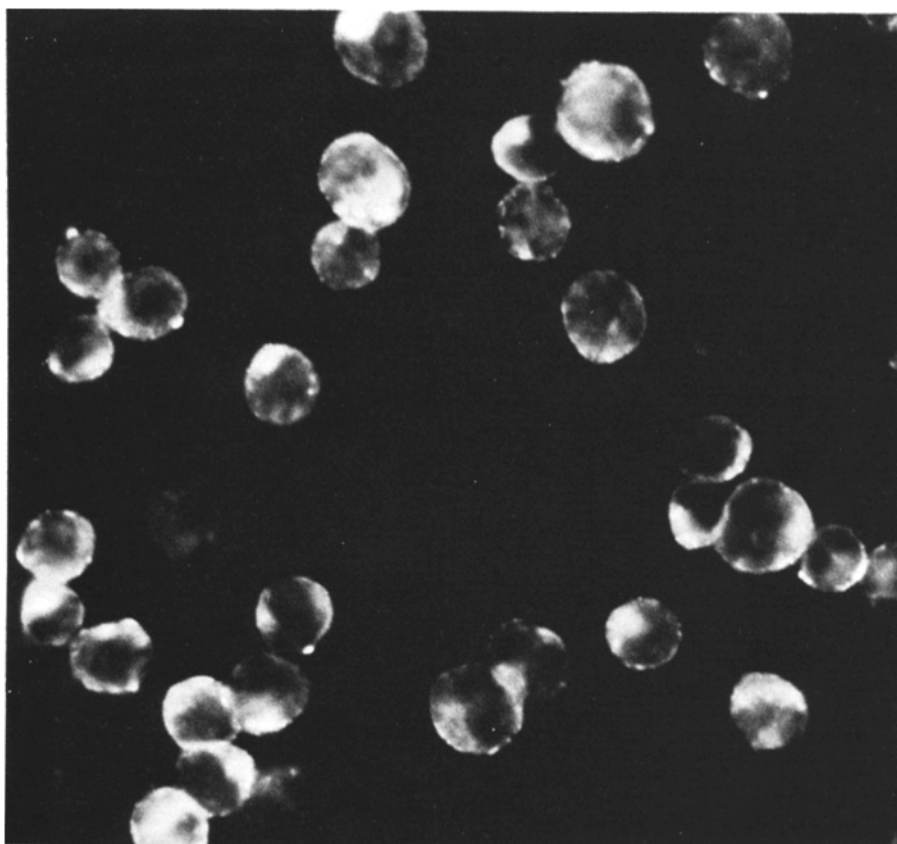


FIG. 2. Immunofluorescent staining of NIH/Swiss nude tumor cells with α -MuLV serum for the detection of cytoplasmic viral antigens. The lymphoma cells were stained by indirect immunofluorescence as described in Materials and Methods and examined by fluorescence microscopy.

TABLE II
Transplantability of BALB/Nu₁ Lymphoma Cells

Strain	Mice	Lymphomas	Latent period
		No. (%)	
C57BL/Ka	15	0	—
NIH/Swiss nude (nu/nu)	15	15 (100)	2-3 wk
NIH/Swiss nude (nu/+)	28	6 (20)	2-3 wk
BALB/c	10	10 (100)	2-3 wk
NIH/Swiss	10	0	

both viruses, the positions of p15, p12, and p10 are essentially identical. Recently, it has been reported that oncornaviruses can be typed into defined subgroups by tryptic digest peptide mapping of the major core protein (p30) (15). By this method, the p30 tryptic peptide profiles of RadLV/VL₃ and RadLV/Nu₁ were both found to belong to the 12B subtype (J. Gautsch, personal communication).

The envelope antigens of RadLV/Nu₁ were identified by the radioimmunoprecipitation assay, using [³H]L-leucine-labeled virus and rabbit antisera raised against

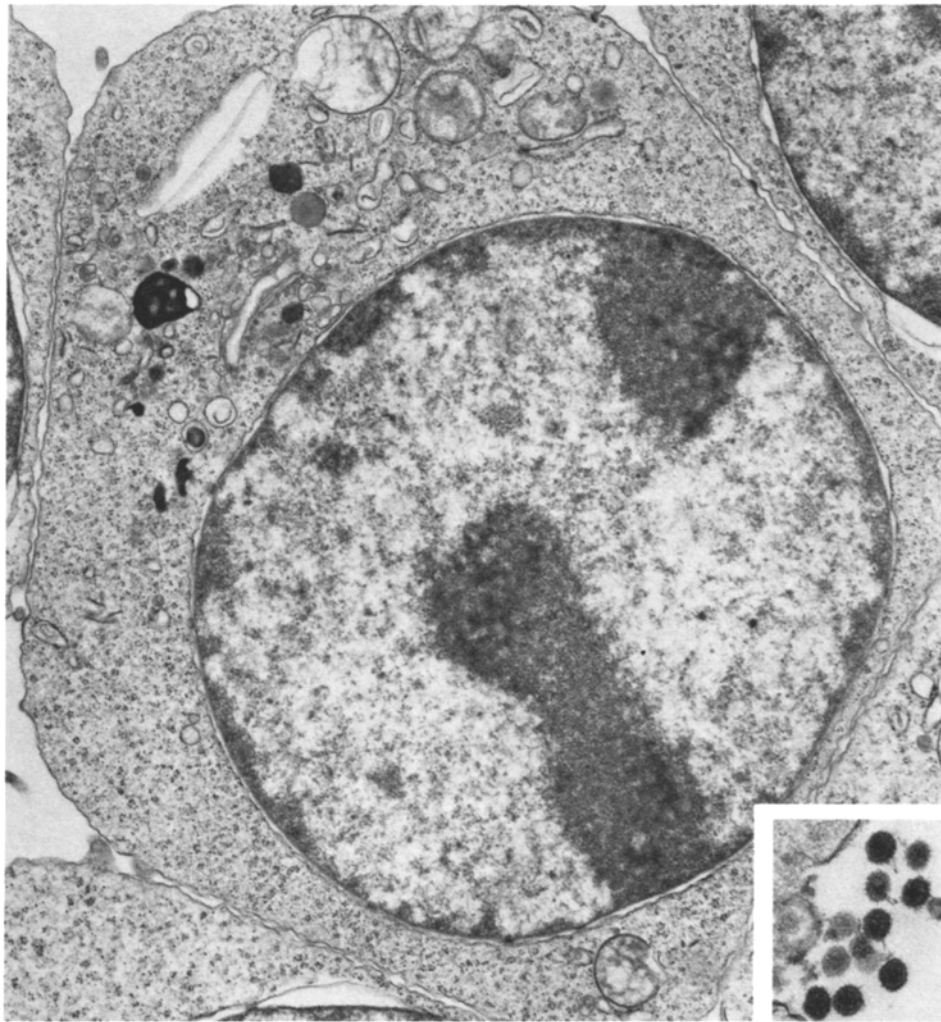


FIG. 3. Electron micrograph of BALB/Nu₁ cells. The ultrastructural appearance is very similar to that of a typical stimulated lymphocyte. Viral particles, easily detected in the extracellular space, are shown in the insert. They are C-type particles, 100 nm in diameter. (magnification: $\times 10,000$; insert, $\times 41,500$)

intact RadLV/VL₃ virions, and against the gp71 of a xenotropic virus, BALB:virus-2, and of ecotropic AKR-MuLV (16). The resulting titration curves (Fig. 6) indicate the presence, on the RadLV/Nu₁ envelope, of antigens common to, or cross-reacting with, the envelope antigens of RadLV/VL₃, and to a lesser extent, of xenotropic and ecotropic MuLVs.

Further evidence of cross-reactivity between the gp71 determinants of RadLV/Nu₁ and RadLV/VL₃ was obtained by use of the membrane immunofluorescence (MIF) technique. BALB/Nu₁ cells were incubated with a 1:10 dilution of a rabbit antiserum raised against purified gp71 of RadLV/VL₃, followed by treatment with a fluoresceinated goat anti-rabbit serum. As shown in Fig. 7, the cells producing RadLV/Nu₁ were stained by the RadLV/VL₃ gp71 antiserum, while control preparations exposed

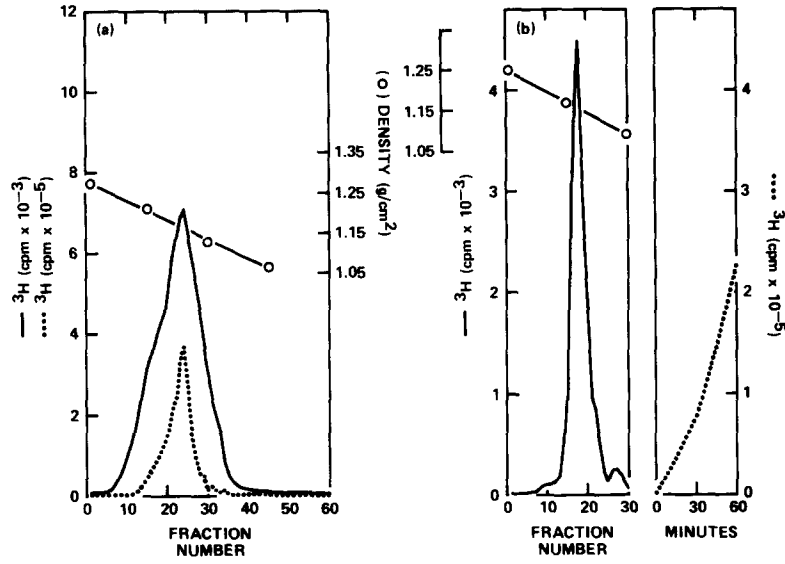


FIG. 4. Physicochemical characterization of RadLV/ Nu_1 by isopycnic banding on sucrose gradients. a) [^3H]uridine labeled RadLV/ Nu_1 acid precipitable radioactivity [—] and reverse transcriptase activity [.....]. b) [^3H]L-leucine labeled RadLV/ Nu_1 acid precipitable radioactivity [—] and reverse transcriptase activity of the peak fractions as a function of the incubation time [.....].

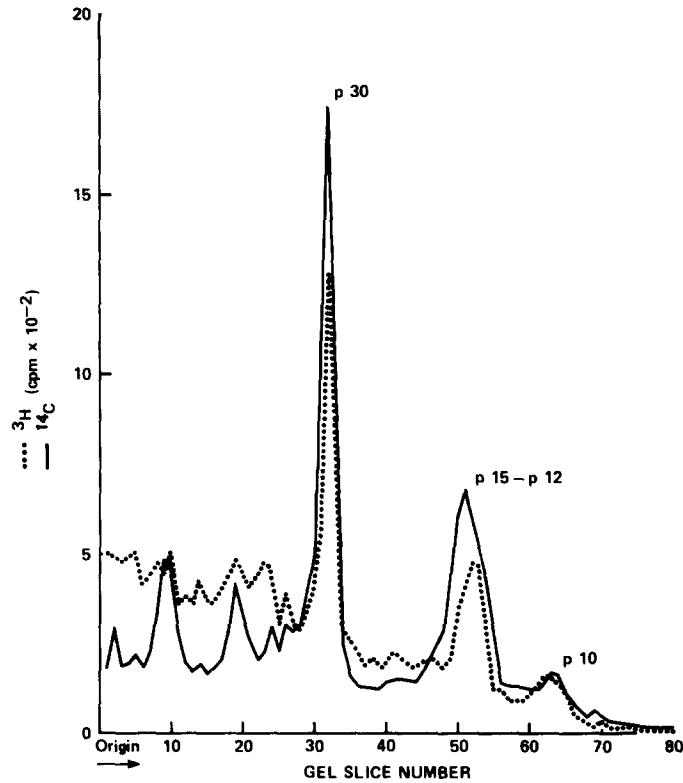


FIG. 5. 10% SDS-PAGE coelectrophoresis of purified radiolabeled viral proteins. [^3H]L-leucine labeled RadLV/ Nu_1 [.....] [^{14}C]L-leucine labeled RadLV/ VL_3 [—].

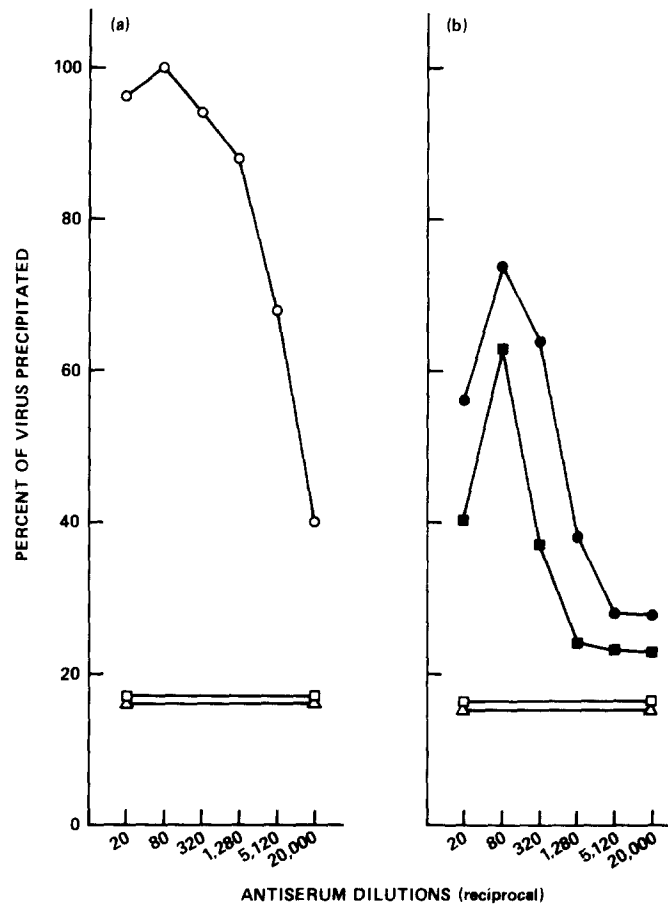


FIG. 6. Immunoprecipitation of [3 H]L-leucine labeled RadLV/ Nu_1 by different rabbit antisera (\square) normal rabbit serum, (\diamond) goat α -rabbit serum. a) Rabbit α -RadLV-VL₃ serum (\circ); b) rabbit α -gp71 BALB:virus-2 (\bullet) rabbit α -gp71 AKR (\blacksquare).

to normal rabbit serum and fluoresceinated secondary serum remained negative.

Biological Characteristics of RadLV/ Nu_1 Virus. RadLV/ Nu_1 has been assayed for leukemogenicity in vivo and for tropism both in vivo and in vitro. The virus was obtained from fresh supernatant fluids of BALB/ Nu_1 cells, which were clarified by centrifugation at 8,000 g for 15 min and filtration through 0.22 μ m Millipore filters. The in vivo experiments were performed in C57BL/Ka and NIH/Swiss nude (nu/nu) and heterozygotic ($nu/+$) mice. The results are summarized in Table III. RadLV/ Nu_1 had thymotropic (T+) and leukemogenic (L+) activity only in C57BL/Ka mice (Fv-1^{bb} genotype). Although the intrathymic, intravenous, and intracranial routes of inoculation all yielded lymphomas, the incidence was much greater and the latent period for lymphoma induction was shorter when the virus was inoculated intrathymically. Neither NIH/Swiss nude homozygotes (nu/nu) nor heterozygotes ($nu/+$) were susceptible to RadLV/ Nu_1 .

When assayed for infectivity by the in vitro immunofluorescence assay (7) on fibroblastic cell lines of Fv-1ⁿⁿ and Fv-1^{bb} genotype, RadLV/ Nu_1 replicated poorly on Fv-1^{bb} fibroblasts and failed to replicate on Fv-1ⁿⁿ fibroblasts (Table IV). It also

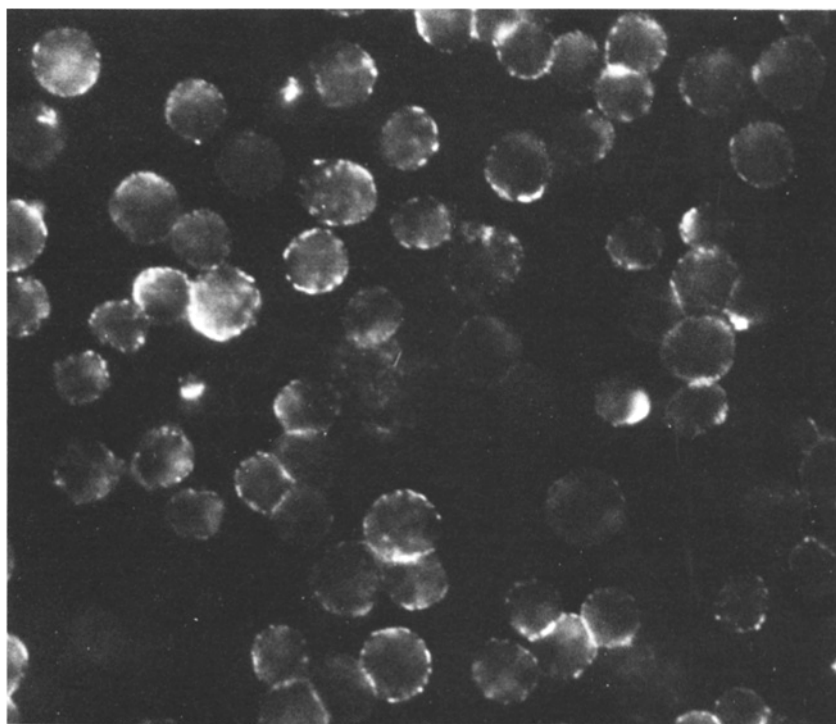


FIG. 7. Immunofluorescent staining of BALB/Nu₁ cells with antiserum to RadLV/VL₃ gp71. The cells were stained by indirect immunofluorescence as described in Materials and Methods and examined by fluorescence microscopy.

TABLE III
Leukemogenicity and Tropism of RadLV/Nu₁

Mouse strain inoculated	Mice	Injection route	Lymphoma incidence (%)	Tumor localization	Latent period (mo)
C57BL/Ka	10	Intravenous	20	Thymic	6-9
C57BL/Ka	8	Intrathymic	80	Thymic and disseminated	3-6
C57BL/Ka	11	Intracranial	9	Thymic	9
NIH/Swiss nude (nu/nu)	39	Intracranial	0	—	—
NIH/Swiss nude (nu/nu)	17	Intravenous	0	—	—
NIH/Swiss nude (nu/+)	10	Intrathymic	0	—	—
NIH/Swiss nude (nu/+)	7	Intracranial	0	—	—

replicated, albeit poorly, on fibroblastic cells derived from other species, such as mink lung cells, and thus has some xenotropic activity. When assayed for infectivity on thymic lymphoma cell lines of Fv-1ⁿⁿ and Fv-1^{bb} genotype, RadLV/Nu₁ replicated to high titers in Fv-1^{bb} cells, and failed to replicate in Fv-1ⁿⁿ lymphoma cells. Thus, it is clearly a B-tropic, ecotropic, and thymotropic virus, again resembling RadLV and RadLV/VL₃ (16-18).

RadLV/Nu₁ has also been tested for its capacity to induce syncytium formation in rat XC cells after 3 days of cocultivation with BALB/Nu₁ cells, using the reverse XC assay described by Niwa et al. (19). Small, clear plaques composed of multinucleated

TABLE IV
In Vitro Replication of RadLV/Nu₁

Cell line	Fv-1 genotype	Tropism of infecting virus	Titer
Fibroblasts			
NIH/3T3	Fv-1 ⁿⁿ	N-tropic, ecotropic, fibrotropic	0
BL-5	Fv-1 ^{bb}	B-tropic, ecotropic, fibrotropic	10 ²
Mink lung cells	—	Xenotropic	10 ³
Thymocytes*			
BL/RL ₁₂	Fv-1 ^{bb}	B-tropic, ecotropic, thymotropic	10 ⁸
C57L	Fv-1 ⁿⁿ	N-tropic, ecotropic, thymotropic	0

* Lymphoma cell lines established from radiation-induced lymphoid tumors of C57BL/Ka and C57L mice, respectively.²

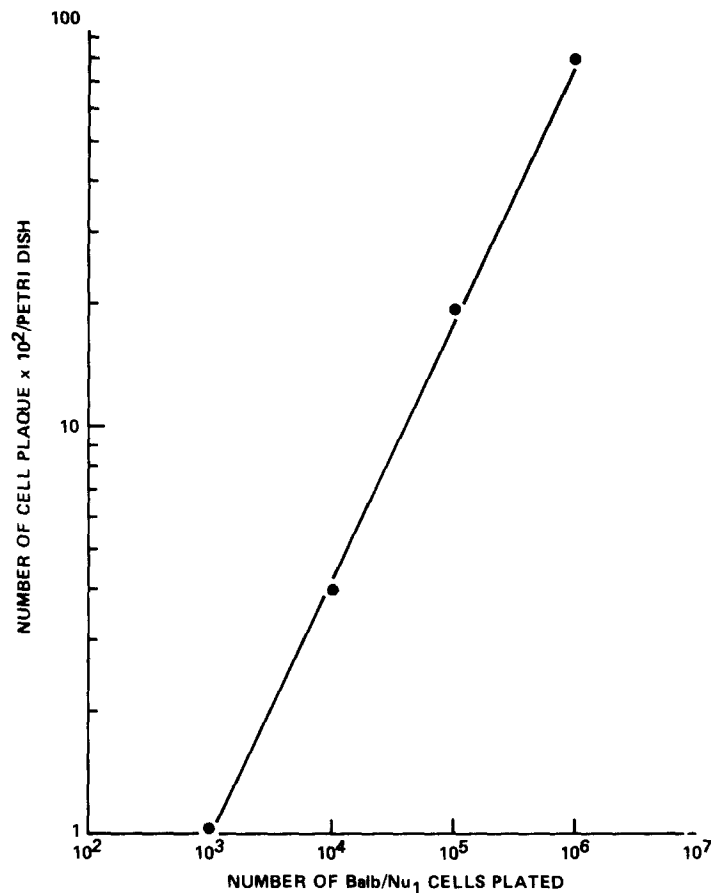


FIG. 8. Correlation between the number of infected BALB/Nu₁ cells plated and XC cell plaque number after 3 days of cocultivation.

giant-cell syncytia were observed. There was a linear correlation between the number of virus-producing BALB/Nu₁ cells plated and the number of plaques formed (Fig. 8).

Characterization of Lymphoma Cells in Nude Mice by Enzymatic and Immunological Markers. Previous studies of RadLV-induced leukemogenesis in C57BL/Ka mice

TABLE V
TdT Activity of Nude Mouse Lymphomas and of Normal Lymphoid Tissues

Cell line	Original tissue	Original strain	Age	Enzyme units*/10 ⁶ cells		
				Peak I	Peak II	Total
—	Spleen	NIH/Swiss nude	1	6	3	9
—	Spleen and nodes (Lymphoma)	NIH/Swiss nude	8	14	78	92
BALB/Nu ₁	Spleen and nodes (Lymphoma)	BALB/c nude	4	53	29	82
—	Thymus (Lymphoma)	C57BL/Ka	4	315	546	861
BL/VL ₃	Thymus (Lymphoma)	C57BL/Ka	4	221	565	786
—	Thymus	C57BL/Ka	4	298	332	630

* 1 U of enzyme activity is defined as the amount catalyzing the incorporation of 1 pmol of dGTP per hour into acid insoluble material.

have shown that the thymus is essential for the replication of the virus (3) and the transformation of infected cells (4, 5). The occurrence of extrathymic lymphoblastic lymphomas in RadLV-inoculated athymic nude mice suggested the possibility that RadLV may be able to transform a different type of target cell under these conditions. Therefore, experiments were designed to characterize the T- or B-cell nature of the nude mouse lymphoma cells and of the derived cell line.

Detection of TdT Activity. This enzyme is selectively localized in the thymus of various animal species (20–22) and therefore appears to be a specific marker for thymocytes. Recently, it has also been detected at very low levels in mouse bone marrow (23, 24). When marrow cell preparations are fractionated on discontinuous bovine serum albumin (BSA) gradients, levels of TdT activity similar to those seen in thymocytes are detected in one fraction, A (24). In contrast, lymphoid organs of athymic nude mice, even after cellular fractionation on BSA gradients, showed TdT values which were only 10% of those observed in normothymic nude heterozygotes (25).

Normal lymphoid cells of nude mice, as well as the lymphomas induced by RadLV inoculation, were tested for TdT activity by the phosphocellulose chromatography technique (10). The typical TdT profile obtained with C57BL thymocytes revealed two peaks of enzyme activity. Peak I eluted at approximately 0.22–0.24 M KCl and peak II eluted at approximately 0.28–0.30 M KCl. The two peaks were consistently observed and maintained their relative difference in elution positions.

The results obtained with nude mice are summarized in Table V. Normal spleen cells from NIH/Swiss nude mice showed extremely low amounts of peak I and peak II TdT activities, compared with those of C57BL/Ka thymocytes. The total enzymatic activity of lymphoma cells obtained from spleen and lymph nodes of nude mice was about 10-fold higher than that of control nude spleen cells; when peak activities were considered separately, the lymphoma cell peak II activity was 26-fold higher. Similar levels were found in the BALB/Nu₁ lymphoma cell line. The fact that both nude mouse lymphomas exhibited an approximately 10-fold increase in TdT activity

TABLE VI
Pattern of Cell Surface Antigens as Detected by MIF

Mouse strain	Cell type	Thy-1.2 ⁺	Ig ⁺	IgG ⁺	IgM ⁺ cells
BALB/c nude	BALB/Nu ₁	88*	0	0	0
NIH/Swiss nude	Lymphoma cells	95	5	4	2
	Lymph node cells	4	78	75	58
	Spleen cells	2	48	45	32
C57BL/Ka	BL/VL ₃	98	0	0	0
	Thymocytes	95	2	2	0
	Spleen cells	30	52	48	30

* Percent positive cells.

relative to normal young NIH/Swiss nude spleen cells suggests that the tumor cell population, which would be expected to be much more homogeneous, may be specifically enriched for a cell type that bears low but significant TdT activity. However, the TdT activity of nude mouse lymphomas was much lower than that of RadLV-induced C57BL/Ka thymic lymphomas (Table V). It has been demonstrated that the involved spleen and lymph nodes of lymphoma-bearing AKR or C57BL mice express TdT activities (for peak I) about equal to those in the thymus. Thus, it seems unlikely that the low values in the nude mouse lymphomas are due to the fact that spleen and lymph nodes, rather than thymus, were assayed. If the nude mouse lymphomas had resulted from the transformation and clonal proliferation of a rare pre-existing thymocyte, the lymphoma cells might have been expected to be as highly TdT positive (for peak I) as thymic lymphomas arising in C57BL/Ka mice. Conversely, the fact that the nude mouse lymphomas demonstrate unequivocally positive, though low, TdT activity indicates that the tumor cells are not of B-cell lineage. In fact, it has been reported that normal lymph nodes and spleens of thymus-bearing mice are consistently TdT negative (10).

Detection of Ig and Thy-1.2 Determinants by Membrane Immunofluorescence. The results are summarized in Table VI. Although almost all BALB/Nu₁ cells were positive for Thy-1.2 (Thy-1.2⁺), the intensity of the label was weak and the distribution on the cell surface spotty and granular, rather than homogeneous (Fig. 9). Conversely, BALB/Nu₁ cells did not express Ig determinants either on their surface or in the cytoplasm (as detected by prefixation of the cells in acetone). The NIH/Swiss nude mouse lymphoma cells gave similar results; 95% were fluorescent after treatment with anti-Thy-1.2 serum. The very small percentage of Ig⁺ cells is attributed to a minor contaminating B-cell population in the involved tissues. Much higher percentages of Ig⁺ cells were noted in normal nude mouse lymph nodes and spleens, whereas these tissues contained very low levels (2-4%) of Thy-1.2⁺ cells, in agreement with previously published observations (26-28). These results may be compared with those obtained with normal C57BL mice and with BL/VL₃ lymphoma cells (Table VI). All BL/VL₃ cells were fluorescent when treated with anti-Thy-1.2 serum and failed to fluoresce when treated with anti-Ig sera.

Antigen Testing by Absorption. The presence of T-cell markers (Thy-1, TL, Ly-1, and Ly-2) on BALB/Nu₁ cells in vitro and after in vivo passage, as well as on the NIH/Swiss nude mouse lymphoma in vivo, was tested by their ability to absorb antibody from specific antisera (as described in Materials and Methods). C57BL/Ka

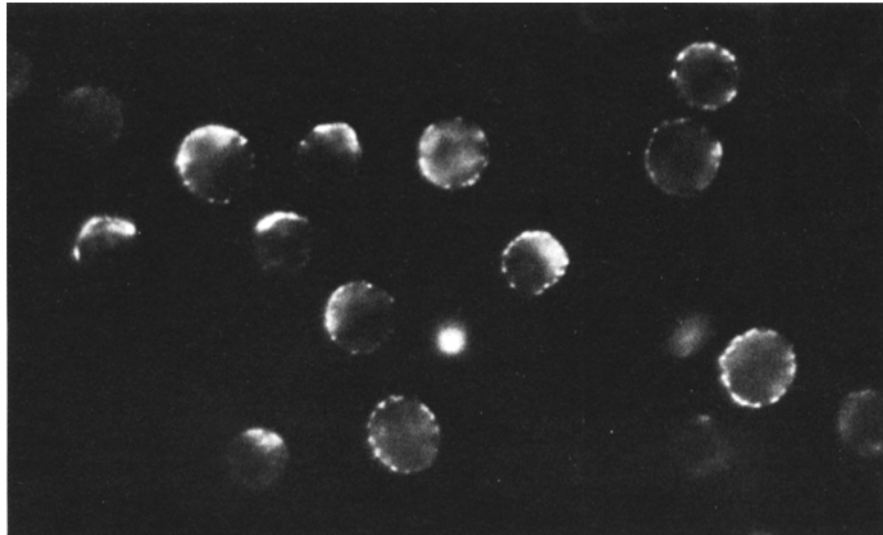


FIG. 9. Immunofluorescent staining of BALB/Nu₁ cells with α -Thy-1.2 serum.

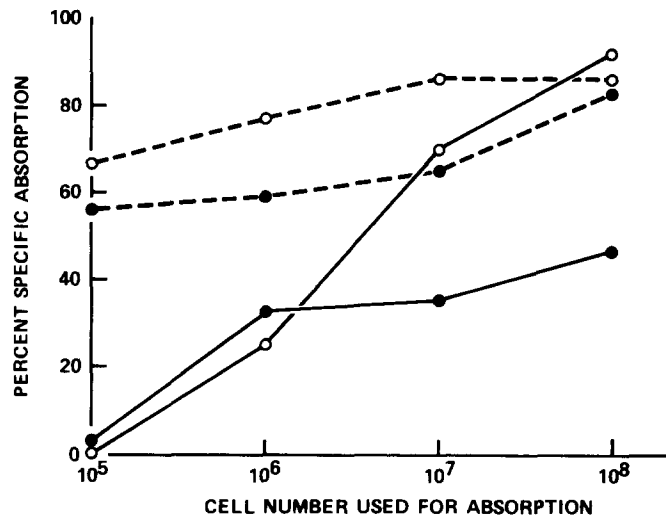


FIG. 10. Specific absorption of anti-Thy-1.2 antiserum by the BALB/Nu₁ cell line in vitro (●—●), and in vivo (●---●), and by NIH/Swiss nude lymphoma, primary tumor (○—○), and first transplant (○---○).

thymocytes used at the same range of cell concentrations (10^5 – 10^8) as the tested tumor cells absorbed over 90% of anti-Thy-1.2 antiserum activity even at the lowest cell concentration, and thus served as a maximal absorption control. The nude mouse spleen had no detectable absorption activity and served as the negative control. Fig. 10 shows the specific absorption data for the nude mouse lymphoma cells. Both the BALB/Nu₁ cell line and the in vivo derived tumor showed moderately high levels of Thy-1.2 antigen as evidenced by their capacity to absorb anti-Thy-1.2 antibody activity. After passage in nu/nu recipients, the level of Thy-1.2 antigen on BALB/Nu₁ lymphoma cell transplants increased significantly. Approximately 10^8 BALB/Nu₁

TABLE VII
Presence of T-Cell Markers in Nude Mouse Lymphomas

Cells used for absorption*	Antisera absorbed				
	α Thy-1.2	α Ly-1.2	α Ly-2.2	α TL	α G _{IX}
BALB/Nu ₁ in vitro	++++‡				
	++	-	+++	-	+
BALB/Nu ₁ transplant in NIH/(nu/nu)	++++	-	++	-	+
BALB/Nu ₁ transplant in NIH/(nu/+)	++++	-	++	-	+
NIH/Swiss nude lymphoma	++++	NT§	NT	NT	NT
NIH/Swiss nude tumor 1st transplant	++++	-	+++	-	+

* 1×10^8 cells used for absorption.

‡ 0-25% specific absorption = +; 25-50% = ++; 50-75% = +++; 75-100% = ++++.

§ NT, not tested.

cells in vitro were required to absorb 50% of anti-Thy-1.2 activity, as compared with less than 10^5 cells after in vivo passage. The NIH/Swiss nude mouse lymphoma showed levels of Thy-1.2 surface antigen higher than those expressed by the cell line in vitro. Approximately 5×10^6 cells sufficed for 50% absorption of anti-Thy-1.2 activity. As in the case of the cell line, however, after transplantation of the original tumor into secondary recipients, higher levels of Thy-1.2 were observed, less than 10^5 cells being required for 50% absorption. This may be due to selection of Thy-1.2 positive cells from the initially heterogeneous cell population in the primary tumor.

The NIH/Swiss nude mouse lymphoma and the BALB/Nu₁ cell line were devoid of TL antigen (Table VII). Normal C57BL/Ka TL⁺ thymocytes served as positive absorption control and as target cells, and the TL⁻ congenic strain was used as a negative control. Unexpected results were obtained with anti-Ly-1.2 and anti-Ly-2.2 antisera. As shown in Table VII, neither the tumors nor the cell line showed the presence of detectable Ly-1.2 surface antigen. However, they all had significant levels of Ly-2.2 antigen. Approximately 5×10^7 cells absorbed 50% of anti-Ly-2.2 antiserum activity. Thus, these tumor cells appear to have the antigenic profile of a mature T lymphocyte subpopulation: Thy-1⁺, Ly-1⁻, Ly-2⁺, TL⁻. Nonetheless, their levels of T-cell antigens are considerably lower than those on the peripheral T cells of normal mice.

Discussion

The successful induction of extrathymic lymphoblastic lymphomas in RadLV-inoculated athymic nude mice was an essential prerequisite to the study of RadLV replication patterns in congenitally athymic animals. However, the relatively low incidence and the long latent period of these tumors would not have permitted meaningful investigations if it had not been possible to establish a permanent cell line from one of these tumors. The BALB/Nu₁ cell line was successfully established and has been extensively characterized. The cells are highly tumorigenic and 100% positive when assayed by indirect immunofluorescence staining for the detection of MuLV antigens. Morphologically they have the typical ultrastructural appearance of stimulated lymphocytes (29). Large numbers of type C viral particles are released into the extracellular spaces. From the supernatant fluid of these in vitro cultures, a virus designated RadLV/Nu₁ has been isolated and characterized. RadLV/Nu₁ is an RNA virus which labels with [³H]uridine, bands in isopycnic gradients at a density of

1.15–1.17 g/cm³, and exhibits reverse transcriptase activity in the banded region. Protein analysis by the SDS-PAGE technique showed the presence of the major polypeptides typically observed in other MuLV and a profile identical to that of RadLV/VL₃, a virus produced by a RadLV-induced C57BL/Ka thymic lymphoma cell line permanently established in culture (16, 18).

Moreover, when [³H]L-leucine labeled RadLV-Nu₁ was assayed by immunoprecipitation with a rabbit anti-RadLV/VL₃ serum, it was completely precipitated. The same technique demonstrated limited activity with antisera to the purified gp71 of xenotropic virus and AKR-MuLV. We also observed the ability of RadLV/Nu₁ to cross-react with the gp71 of RadLV/VL₃, using the membrane immunofluorescence technique on the virus producing cells. Recently, it has been demonstrated by competition radioimmunoassay with purified virion polypeptides that the gp71 of RadLV/VL₃ is distinctively different from that of several other MuLVs (18). Thus, the results obtained with RadLV/Nu₁ indicate the apparent serological identity of the two RadLV-derived viruses.

The biological characteristics of RadLV/Nu₁, determined both in vivo and in vitro, demonstrate that RadLV/Nu₁ has thymotropic (T⁺) and leukemogenic (L⁺) activity and is B-tropic. RadLV/VL₃ virus shows identical properties (18). When injected into C57BL/Ka mice, RadLV/Nu₁ induces thymic lymphoblastic lymphomas which have the same characteristics as those obtained after RadLV/VL₃ injection.

The antigenic profile of the NIH/Swiss nude mouse lymphoma cells and of the BALB/Nu₁ cell line, as detected by specific absorption, was found to be Thy-1⁺, Ly-1.2⁻, Ly-2.2⁺, and TL⁻. Thy-1.2 antigen was also detected by membrane immunofluorescence staining, but at much lower intensity levels than those exhibited by normal thymocytes. Ig determinants could not be detected by this technique on the NIH/Swiss nude mouse lymphoma or the BALB/Nu₁ cells. Taken together, these results suggest that these cells have developed along the T-cell differentiation pathway. Moreover, their antigenic profile places them further along the maturation scale than the prothymocyte.

It has been reported (30) that a rabbit antiserum obtained by immunization with nude mouse spleen cells has strong lytic activity against cytotoxic T-cells (Ly-2,3) of thymus-bearing mice but that it does not recognize T helper cells (Ly-1). This suggests the possibility that Ly-2,3 cells normally exist in the spleens of nude mice. If so, such cells might undergo transformation and clonal expansion into a tumor after infection with a leukemogenic virus. In preliminary studies (data not shown) we have detected low levels of absorption of anti-Ly-2.2 activity by nude mouse spleen lymphocytes. However, these results would not in any case exclude the alternative possibility of virus-induced differentiation of prothymocytes, leading to the expression of Thy-1.2 and Ly-2.2. Precedent exists for such a response, because it has been reported (31) that viral hepatitis infection can greatly increase the amount of Thy-1.2 antigen in nude mouse spleens. Similar increases can be induced by treatment with thymopoietin, ubiquitin, cyclic AMP, or levamisole (25, 31, 32).

It has been demonstrated that the presence of a thymus is necessary for the expression of high levels of TdT activity. In nude mice this enzyme has been found mainly in bone marrow cells fractionated on BSA gradients. Even then, the activity was only about 10% of that detected in similar preparations from thymus-bearing animals (25). These lower values may not be solely the result of a smaller number of TdT producing cells, but may also reflect a decreased synthesis of this enzyme per

cell. This is likely to be the case in the nude mouse lymphomas which, though homogeneous with respect to cell type, showed TdT values only some fivefold higher than those found in nude mouse bone marrow cells after fractionation.

Finally, it is of interest that the same virus (RadLV), inoculated into different hosts (C57BL/Ka or nude mice), is able to replicate in and transform target cells which, though of T-cell lineage, are nonetheless different with respect to their stage of differentiation. Despite their target cell differences, cell lines derived from these two types of lymphomas originating in different tissues continue to produce progeny viruses *in vitro* which have serological, biochemical, and biological characteristics apparently identical to those of the parental inducing virus.

Summary

We report the development of extrathymic lymphoblastic lymphomas in RadLV-inoculated congenitally athymic nude mice. Thus, a leukemogenic virus which appears to require the presence of a thymus for its replication in normothymic mice can infect and transform target cells in the absence of this organ in the athymic host. The cells of one of these lymphomas have been established *in vitro* as a permanent cell line, BALB/Nu₁. This cell line as well as a lymphoma induced in NIH/Swiss nude mice exhibit several T-cell markers, including terminal deoxynucleotidyl transferase activity, Thy-1.2, and Ly-2.2, but not Ly-1.2 nor TL. Ig determinants were not detected. The characteristics of the tumor cells support the view that cells with T-cell markers may normally exist in nude mice and undergo neoplastic transformation and clonal expansion after infection with a leukemogenic virus. The alternative possibility that virus-induced differentiation of prothymocytes may lead to the expression of Thy-1.2 and Ly-2.2 antigens is also considered. BALB/Nu₁ cells release large numbers of type C viral particles. The virus, designated radiation leukemia virus (RadLV)/Nu₁, has RTase activity and the protein profile characteristic of murine leukemia virus (MuLV). In radioimmunoassays, it cross-reacts completely with RadLV/VL₃, a virus obtained from RadLV-induced C57BL/Ka thymic lymphoma cells in culture, and slightly with a xenotropic virus (BALB: virus-2) and with AKR MuLV. On inoculation into C57BL/Ka mice it has thymotropic and leukemogenic activity. *In vitro* it is B-tropic, poorly fibrotropic, and has limited xenotropic activity. Thus, RadLV/Nu₁ appears to be biologically and serologically similar or identical to its parent virus, RadLV.

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