

BALB- AND HARVEY-MURINE SARCOMA VIRUS TRANSFORMATION OF A NOVEL LYMPHOID PROGENITOR CELL

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Acute transforming retroviruses have arisen in nature by recombination of replication-competent type C viruses with cellular genes. By a number of approaches, the cell-derived information of these viruses has been shown to be intimately involved in their transforming functions (for reviews see refs. 1, 2). A limited number of such cell-derived *onc* genes have been incorporated into retroviral genomes. In some cases, different retrovirus isolates have incorporated closely related *onc* genes from the same, or even different species (3–12). One such group of related sarcoma-inducing viruses includes the mouse-derived BALB-murine sarcoma virus (MSV)¹ (13), and rat-derived Harvey-MSV (14). Analysis of the molecularly cloned BALB-MSV genome has revealed that its *onc* gene, designated *bas*, is closely related to the rat-derived *onc* gene, *ras*, of Harvey-MSV (8).

BALB- and Harvey-MSV, like most transforming retroviruses, induce solid tumors in vivo. In tissue culture, study of such viruses has been generally limited to analysis of their transforming effects on fibroblast cell lines. Recent studies have indicated that Harvey-MSV, in addition to its ability to cause sarcomas, can induce proliferation of hematopoietic cells of the erythroid series both in vivo (15) and in tissue culture (16).

Investigation of the diversity of target cells for neoplastic transformation by a particular transforming retrovirus might be expected to provide insights into pathways of transformation, and in particular, the relationship of the differentiated state of the cell to its susceptibility to *onc* gene action. In the present report, we demonstrate the transforming action of BALB- and Harvey-MSV for a unique hematopoietic target cell both in tissue culture and in vivo.

Materials and Methods

Cells and Viruses. Cell lines included NIH/3T3 (17), a clonal BALB-MSV nonproducer transformant of NIH/3T3 cells (8), an Abelson-murine leukemia virus (MuLV) transformed nonproducer clone of NIH/3T3 cells (ANN-1) (18), and a Harvey-MSV transformed nonproducer clone of NRK (S.A. Aaronson, unpublished observations). Mouse type C helper leukemia viruses included clonal strains of Rauscher-MuLV (19), and amphotropic (Amph)-MuLV (20, 21). MuLV pseudotypes of BALB-MSV, Abelson-MuLV, and Harvey-MSV were obtained by

¹*Abbreviations used in this paper:* Amph, amphotropic virus; DMSO, dimethyl sulfoxide; FFU, focus-forming unit; ME, mercaptoethanol; MSV, murine sarcoma virus; MuLV, murine leukemia virus; PBS, phosphate-buffered saline; REV-T, reticuloendotheliosis virus; SRBC, sheep erythrocytes; Staph A, staphylococcus A; TdT, terminal deoxynucleotidyl transferase.

superinfection of appropriate nonproducer cells (22, 23). The titer of the defective transforming viruses, expressed as focus-forming units/ml (FFU/ml), were determined on NIH/3T3 cells as previously described (22).

Murine hematopoietic cell lines included a clonal Rauscher-MuLV transformed NIH/Swiss pre-B cell line, 6EC4, that has been shown to synthesize μ chain (24), a clonal Thy-1 antigen-positive Moloney-MuLV transformed NIH/Swiss T cell line, 19-1-2 (24), and a Friend-MuLV transformed erythroblast line, clone 745, that is dimethyl sulfoxide (DMSO) inducible for hemoglobin synthesis (25).

Hematopoietic Colony Formation Assay. Bone marrow suspensions were prepared from the femurs and tibias of 2-3-wk-old NFS/N mice. Single-cell suspensions were washed and resuspended at a density of 2×10^6 nucleated cells/ml in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) containing 20% heat-inactivated fetal calf serum (Reheis Chemical Co., Phoenix, AZ), and 5×10^{-5} M mercaptoethanol (Sigma Chemical Co., St. Louis, MO).

Single-cell suspensions of NFS/N bone marrow were exposed to virus for 1 h at 37°C in the presence of 4 μ g/ml polybrene (Aldrich Chemical Co., Milwaukee, WI). After centrifugation, the cells were mixed with semisolid agarose medium containing RPMI 1640 medium supplemented with 20% fetal calf serum, 5×10^{-5} M mercaptoethanol (ME), and 0.47% Sea Plaque agarose (FMC Corp., Homer City, PA) and plated at the appropriate concentration into 35-mm petri dishes in a volume of 2 ml. Plates were cooled at 4°C to allow solidification of the agarose, and the cultures were incubated at 37°C in a 5% CO₂ humidified incubator. 1 ml of agarose medium was overlaid at 5-d intervals. Visible colonies were scored at 12-18 d after infection.

Establishment of BALB-MSV- and Harvey-MSV-Transformed Hematopoietic Cell Lines. Individual colonies were removed from the agarose plates and suspended in 1 ml of RPMI growth medium in 24-well microtiter plates (Costar, Data Packaging, Cambridge, MA). When the cell number in an individual well reached $\sim 10^6$ cells, the cells were transferred at a 1:2 split ratio. After an initial adaptation period of 1-6 wks, the suspension lines could be maintained by transferring at 2-3-d intervals (split ratio of 1:20-1:100). In some cases, bone marrow cell-conditioned medium or secondary adherent bone marrow cell feeder layers were used during an initial adaptation period needed to establish hematopoietic cell lines. Any clones that were exposed to normal bone marrow feeder layers were recloned in soft agar before further characterization.

For preparation of feeder layers or conditioned medium, NFS/N bone marrow cell suspensions (10^6 nucleated cells/ml) were plated in 60-mm tissue culture plates in RPMI 1640 medium supplemented with 20% horse serum (Flow Laboratories, Rockville, MD) and 5×10^{-5} ME. Nonadherent cells and medium were removed after 5 d, and medium containing 15% fetal calf serum was used thereafter. Conditioned medium was collected at 5-d intervals starting 10 d after initial cell plating. Secondary adherent bone marrow cultures were generated from 15-d-old primary cultures. Cells were trypsinized and plated at a final concentration of 2×10^5 cells/ml in 60-mm tissue culture dishes.

Analysis of Tumor Induction. Clonal hematopoietic cell lines were washed twice in phosphate-buffered saline (PBS) and resuspended at a final concentration of 2×10^7 cells/ml. For tumorigenicity testing, NFS/N weanlings were inoculated subcutaneously with 5×10^6 cells in a volume of 0.25 ml. Virus alone was inoculated subcutaneously in the same volume.

Histochemical Staining. The presence of α -naphthyl acetate esterase was determined by cytochemical staining as described by Yam et al (26) using the Sigma research kit. Hemoglobin-containing cells were detected by benzidine staining according to the method of Peterson and McConkey (27). In some cases, cells were treated with 1.5% DMSO (Fisher Scientific Co., Pittsburgh, PA) or 0.3 U erythropoietin (step 3) (Connaught Laboratories, Ontario, Canada) for 4 d before staining.

Nonspecific Phagocytosis. The ability of cells to phagocytize latex beads was determined essentially as described by Raschke et al. (28). Cells (2×10^6 /ml growth medium) were incubated for 1 h with 10^9 latex beads/ml (0.81 μ m Diam) (Sigma Chemical Co.). Cells were washed three times with PBS, and a wet mount slide was observed under the light microscope at 400 \times magnification for evidence of cellular uptake of latex beads. Cells with at least 10 internalized latex beads were scored as positive.

Lysozyme Analysis. Lysozyme was assayed by the lysoplate assay as described by Osserman and Lawlor (29). Egg white lysozyme was used as a standard. Levels of lysozyme were expressed in $\mu\text{g}/10^6$ cells.

Detection of Thy-1 Antigen. The presence of Thy-1 antigen was determined by an antibody-dependent cytotoxicity assay. The complement-mediated lysis assay was performed essentially as described by Reddy et al. (24). Briefly, 2×10^6 cells were incubated with a 1:25 dilution of monoclonal Thy-1 antigen (New England Nuclear, Boston, MA) for 1 h at room temperature. Cells were washed in PBS and incubated for 1 h at 37°C with undiluted guinea pig serum (Grand Island Biological Co.) as the source of complement. Trypan blue (Flow Laboratories) diluted in PBS was added to the cells, and the percentage of lysed cells was determined by microscopic examination.

Detection of Fc Receptors. The presence of Fc receptors was determined by rosette formation essentially as described by Kerbel et al. (30). Briefly, 2×10^6 sheep erythrocytes (SRBC) per ml were incubated in RPMI medium with an equal volume of a subagglutinating titer (1:1000) of heat-inactivated sheep hemolysin for 30 min at 37°C with occasional shaking. A suspension of washed, sensitized SRBC (4×10^7 cells in 0.2 ml) was mixed with an equal volume containing 4×10^5 test cells. Sodium azide was added at a final concentration of 0.1%. The cell mixture was centrifuged at 100 g for 5 min, and the pellet was gently resuspended. Percent rosette formation was determined by counting with a hemocytometer. Cells with at least three attached SRBC were scored as positive.

Radioimmunoassay for Immunoglobulin μ Chain. The amount of mouse immunoglobulin μ chain present in cells was determined by competition radioimmunoassay as previously described (24).

Terminal Deoxynucleotidyl Transferase (TdT) Assay. The TdT enzymatic assay was performed according to the method of Barton et al. (31) with minor modifications. Briefly, 10^6 cells were washed three times in PBS, and the pellet was resuspended in 1 ml of potassium phosphate buffer at a final concentration of 0.25 M (pH 7.5). Cell extracts were prepared by sonification and clarified at 100,000 g for 1 h. Terminal transferase activity in cell extracts was assayed in a reaction mixture containing 0.5 mM [^3H]deoxyguanosine triphosphate (1,000 cpm/pmol), 10 mM oligo (dA)₁₂₋₁₈ (P-L Biochemicals, Inc., Milwaukee, WI), 8 mM MgCl_2 , 0.1% bovine serum albumin, 0.2 M potassium cacodylate (pH 7.3), and 1 mM 2-mercaptoethanol. The 0.25 ml reaction mixture, containing 1–10 μl of cell extract, was incubated at 37°C for 1 h. Trichloroacetic acid precipitates were collected on millipore filters, washed, and dried, and the amount of radioactivity was determined by liquid scintillation. TdT activity was expressed in units equivalent to 1 nmol of dGTP incorporated per h.

Biosynthetic Labeling and Immunoprecipitation. The procedures for metabolic labeling with ^{35}S -methionine and immunoprecipitation were as previously described (11). Briefly, 10^7 transformed hematopoietic or normal thymus cells were labeled with 200 μCi ^{35}S -methionine (New England Nuclear, Boston, MA) for a 3-h period. The cells were disrupted in 1 ml of staphylococcus A (Staph A) lysing buffer, clarified, and 0.2-ml samples were incubated with 10 μl goat anti-calf TdT serum (P-L Biochemicals, Inc.) or normal goat serum overnight at 4°C . Antigen-antibody complexes were precipitated with protein A sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) by agitation for 15 min at 4°C , washed three times in Staph A buffer, resuspended in sample buffer, and boiled for 2 min. Reduced samples were analyzed on sodium dodecyl sulfate-polyacrylamide gels. Gels were then fixed, dried, and exposed to X-Omat R film (Eastman Kodak Co., Rochester, NY).

Results

BALB-MSV Induces Growth of Hematopoietic Colonies in Infected Bone Marrow Cultures. Several transforming retroviruses, including avian erythroblastosis (32), myelocytomatosis (33), myeloblastosis (34), and reticuloendotheliosis viruses (35), as well as mammalian Abelson (36) and myeloproliferative viruses (37) preferentially cause hematopoietic tumors. In some cases, tissue culture assays for transformation of hematopoietic target cells by such viruses have been developed. To investigate the effects of BALB-MSV on hematopoietic cells, we used a colony-forming assay devel-

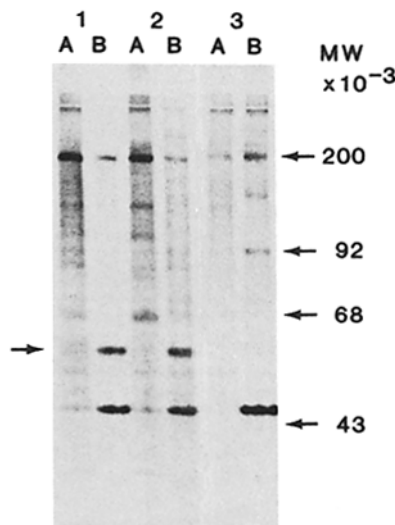


FIG. 1. Morphology of BALB-MSV-induced colonies. (A) Colony at 14 d post-infection (bar equals 0.1 mm). (B) Wright-Giemsa-stained preparations of cells from BALB-MSV-induced colonies (bar equals 5 μ m).

oped by Rosenberg and Baltimore (38) for detection of hematopoietic cells transformed by Abelson murine leukemia virus (MuLV). Bone marrow cell suspensions obtained from 2–3-wk-old NFS/N mice were infected with BALB-MSV and suspended in soft agar as described in Materials and Methods. By 7–10 d, large numbers of colonies were observed in BALB-MSV-infected but not in control cultures.

The colonies appeared golden and iridescent under the microscope, were round or oblong in shape, and possessed a compact morphology (Fig. 1 A). Cells within the colonies were round in shape and nonadherent. By light microscopy, such cells exhibited an undifferentiated blast cell morphology (Fig. 1 B). The majority of cells had a large eccentric nucleus with a scanty cytoplasmic rim lacking any granules. Occasionally, cytoplasmic vacuoles were observed. There were no apparent morphologic differences in cells from 20 different colonies analyzed, although some variations in cell size were noted.

The length of virus exposure required for optimal colony formation was found to be 1 h. Incubation for longer periods appeared to decrease cell viability and did not increase the number of colonies detected. Variation in the cell number plated into soft agar from 5×10^5 to 3×10^6 cells correlated directly with the number of colonies formed (data not shown). As shown in Fig. 2, there was a linear relationship between the number of colonies formed and the amount of virus inoculated. The one-hit titration pattern suggested that colony formation was dependent on infection with a single virus particle. Colony formation was considerably less efficient than was focus formation in fibroblasts by the same BALB-MSV stock (Fig. 2). This may be due to a limited number of susceptible target cells for infection and/or transformation within the bone marrow cell population.

BALB-MSV, like other replication defective transforming retroviruses, can only be propagated in the presence of a type C helper virus. As shown in Table 1, helper virus alone produced no detectable colonies, further indicating that the colony-forming

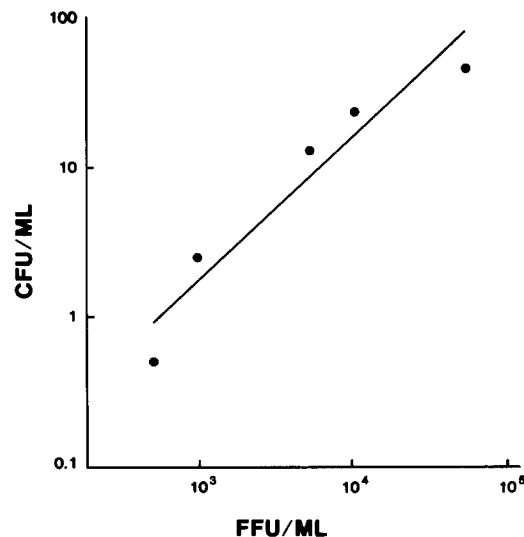


FIG. 2. Dose-response curve for BALB-MSV induction of compact bone marrow cell colonies. Bone marrow cells were infected with serial dilutions of BALB-MSV and suspended at 2×10^6 cells per plate in semi-solid agar medium as described in Materials and Methods. Colonies were scored 18 d after infection. Each point represents the mean result obtained from two plates.

TABLE I
In Vitro Hematopoietic Colony Formation Induced by BALB- and Harvey-MSV

Virus source	Colonies/ 10^6 fibroblast FFU*
Amph-MuLV	$<10^0$
Rauscher-MuLV	$<10^0$
BALB-MSV (Amph-MuLV)	2.5×10^3
Harvey-MSV (Amph-MuLV)	1.1×10^3
Harvey-MSV (Rauscher-MuLV)	4.5×10^2
Abelson-MuLV (Amph-MuLV)	1.6×10^3

* Bone marrow cell suspensions from NFS/N weanlings were infected with varying virus dilutions and plated at a concentration of 2×10^6 nucleated cells into soft agar as described in Materials and Methods. Hematopoietic colony formation was scored at 18 d.

activity must be due to BALB-MSV. We also investigated the ability of Harvey-MSV and Abelson-MuLV to induce hematopoietic colonies. Harvey-MSV is a rat-derived sarcoma virus (39, 40) whose *onc* gene is closely related to that of BALB-MSV (8). Like BALB-MSV, Harvey-MSV was found to induce the formation of hematopoietic colonies (Table I). Under the same assay conditions, Abelson-MuLV induced colonies with indistinguishable morphology and at a comparable efficiency.

In Vivo Infection with BALB- and Harvey-MSV Leads to Induction of Similar Hematopoietic Colonies. In view of our findings that BALB- and Harvey-MSV induced hematopoietic cell colonies in tissue culture, we investigated whether such colonies could be detected in virus inoculated animals. Moribund mice, killed 2–3 weeks after infection as newborns, demonstrated grossly enlarged spleens containing many pale, poorly defined foci. We also observed gross enlargement of the thymic region, which has not

TABLE II
*Colony Formation by Bone Marrow Cells from BALB- and
 Harvey-MSV-infected Mice*

Virus inoculated	Colonies/ 10^6 nucleated bone marrow cells plated*
BALB-MSV (Amph-MuLV)	1.6×10^2
Harvey-MuSV (Amph-MuLV)	3.9×10^1
Abelson-MuLV (Amph-MuLV)	9.6×10^2
Amph-MuLV	$<10^0$

* Newborn NFS/N mice were inoculated intraperitoneally with 5×10^4 FFU of each transforming retrovirus. Bone marrow was removed from moribund mice at 2-3 wk, and single-cell suspensions were plated in soft agar as described in Materials and Methods. Colony formation was scored at 12 d. The results represent mean values obtained from three individual animals in each group. Amph-MuLV-infected mice, killed at 3 wk, demonstrated no evidence of pathology.

been previously reported. This pathology was not observed in helper virus-infected or -uninfected animals. When bone marrow cells from BALB- or Harvey-MSV infected animals were plated into soft agar, we observed the formation of compact colonies as early as 3-4 d after plating. As shown in Table II, the frequency of colony-forming cells ranged from 3.9×10^1 to 1.6×10^2 colonies per 10^6 nucleated bone marrow cells plated. The frequency of colony-forming cells in the bone marrow cells of Abelson-MuLV infected mice sacrificed at the same time was 5-15-fold greater.

Establishment and Characterization of Transformed Lines from BALB- and Harvey-MSV-induced Colonies. The ability of a transforming virus to induce hematopoietic colony formation in tissue culture might reflect its direct transforming action or result from some indirect effect of virus infection such as the production of soluble factors that induce normal cellular proliferation. To differentiate between these two alternatives, it is necessary to determine whether such colonies are capable of continued, independent proliferation. Moreover, if cell lines can be established, it is possible to characterize their phenotype with respect to malignant potential and stage of differentiation. Thus, efforts were undertaken to develop clonal cell lines from colonies induced by BALB- and Harvey-MSV.

Individual colonies were transferred to liquid medium in microtiter wells and grown up to mass culture. The success rate for establishment of continuous cell lines could be improved with the initial use of conditioned medium or feeder layers. After an adaptation period of 1-6 wk, the transformed lines could be propagated independently. Any cell line established in the presence of a feeder layer was recloned in soft agar before its further characterization.

The established lines grew to saturation densities of $\sim 2 \times 10^6$ cells/ml and possessed generation times of 10-12 h. Individual cell lines formed colonies in soft agar at efficiencies ranging from 10^{-3} to 10^{-1} (data not shown). Some clonal lines have been in continuous culture for >8 mo. Each cell line tested was found to release transforming virus. Under the assay conditions used, many of the colonies selected were likely to have been infected with both sarcoma and helper virus. Nonetheless, we would have expected to obtain some nonproductively transformed lines from colonies that arose at low multiplicities of infection. Thus, there may be some selective advantage for

productively infected cells to develop into established lines.

To analyze the malignant potential of the cell lines, we inoculated NFS/N weanling mice subcutaneously with 5×10^6 cells from representative BALB- and Harvey-MSV transformed lines. Each cell line tested was oncogenic, forming very large tumors within 1–2 wk. Histopathologic analysis revealed the tumors to be comprised of undifferentiated hematopoietic blast cells. In contrast, subcutaneous inoculation of weanlings with 5×10^5 FFU of virus alone yielded either no detectable tumors or very small sarcomas, rather than hematopoietic tumors. To confirm the donor origin of the hematopoietic tumors, cells from BALB- and Harvey-MSV-transformed hematopoietic clones derived from male mice were injected subcutaneously into 5-wk-old female mice. The tumors that formed displayed a male karyotype. Moreover, a metacentric chromosomal marker identified in one of the donor clones was shown to be present in the tumor cells derived from the recipient mouse. All of these results established that the hematopoietic lines derived from BALB- and Harvey-MSV colonies were leukemogenic.

Phenotype of BALB- and Harvey-MSV Hematopoietic Transformants. To define the phenotype of BALB- and Harvey-MSV-transformed hematopoietic cells, we analyzed clonal lines for markers that help to distinguish cells of various hematopoietic lineages. There was no evidence of hemoglobin synthesis as detected by benzidine staining (25), nor could the cells be induced to synthesize hemoglobin after exposure to DMSO or erythropoietin (Table III). The cells also lacked detectable nonspecific esterase or the ability to phagocytize latex beads, markers of cells within the myeloid series (Table III).

To further define their stage of hematopoietic cell differentiation, we analyzed a number of individual clonal transformants for markers found associated with lymphoid cells. ME has been shown to enhance normal lymphoid cell survival and growth in tissue culture (41, 42), as well as the growth capacity of certain lymphoblastic and myeloblastic tumor lines (43). ME has also been shown to be critical for hematopoietic cell colony formation by Abelson-MuLV and growth of Abelson-MuLV transformed lymphoid cells in culture (44, 38). The growth of BALB- and Harvey-MSV transformants was generally found to be dependent on the presence of ME (Table III).

As shown in Table III, none of the lines induced by BALB-MSV and Harvey-MSV demonstrated detectable Thy-1 antigen as determined by complement-mediated lysis and fluorescence-activated cell sorting analysis (T. Storch and T. Chused, personal communication). The presence of Fc receptors on the cell surface is a characteristic of many hematopoietic cell types, including B cells of lymphoid origin (45). When assayed for Fc receptors by the rosette assay (30), a low percentage of rosette formation was observed in some of the lines. The presence of low levels of Fc receptors on these lines was confirmed by fluorescence-activated cell sorting analysis (T. Storch and T. Chused, personal communication). However, other lines lacked detectable Fc receptors when analyzed by either technique.

Pre-B lymphoid cells, which synthesize heavy μ chain in the absence of detectable light chain (46), appear to be the preferential target for Abelson-MuLV transformation (47). When BALB- and Harvey-MSV transformants were analyzed for expression of mouse μ chain, none contained detectable levels as determined by competition radioimmunoassay. In contrast, 5 out of 12 Abelson-MuLV in vitro transformed hematopoietic lines established in our laboratory produced readily detectable amounts

TABLE III
Characterization of BALB- and Harvey-MSV Hematopoietic Cell Lines

Marker	Hematopoietic cell lines transformed by	
	BALB-MSV	Harvey-MSV
	<i>number positive/number tested</i>	
Erythroid*		
Hemoglobin	0/6	0/6
+ DMSO	0/6	0/6
+ erythropoietin	0/6	0/6
Myeloid‡		
Lysozyme	0/6	0/6
Nonspecific esterase	0/6	0/6
Nonspecific phagocytosis	0/6	0/6
Lymphoids§		
Mercaptoethanol dependence for growth	6/6	5/6
Thy.1 antigen	0/6	0/6
Fc Receptors	4/6	3/6
Cytoplasmic μ	0/6	0/6
TdT	5/6	6/6

* Hemoglobin synthesis was determined by benzidine staining as described in Materials and Methods. Cells were treated with 1.5% DMSO or 0.3 U/ml erythropoietin at the time of cell seeding. Staining was performed after 4 d of treatment. A Friend-MuLV erythroblast line, clone 745, was used as a positive control in this assay system.

‡ Lysozyme levels were assayed by the lysoplate technique as described in experimental procedures. Amounts of lysozyme were determined per 10^6 cells added, using purified egg white lysozyme as a standard. The sensitivity of detection was 0.5 μ g. Histochemical staining for nonspecific esterase was performed as described (26). Phagocytosis of latex beads was determined as described in Materials and Methods. Cultures were incubated for 1 h at an input of 500 beads/cell. Cells that internalized ≥ 10 beads were scored as positive.

§ Exponentially growing clonal lines were transferred at a 1:20 split ratio into separate plates. ME was omitted from the growth medium in one plate. Cultures were passaged every 3rd d and maintained for several weeks. Viability was monitored every 3rd d by trypan blue staining. Cultures were discarded when viability decreased to $<5\%$. The presence of Thy-1 antigen was determined by complement-mediated lysis as described in Materials and Methods. Complement or anti-Thy-1 antibodies alone did not exhibit any cytotoxicity ($<2\%$ cell death). A Moloney-MuLV lymphoma cell line exhibited $>99\%$ lysis, whereas a Rauscher-MuLV pre-B lymphoma cell line demonstrated $<2\%$ cell death. The presence of Fc receptors was determined by the rosette assay as described in Materials and Methods. A cell line was considered positive if 5–10% rosette formation was reproducibly detected. SRBC alone or a Moloney-MuLV T lymphoma cell line did not yield any rosettes whereas a Rauscher-MuLV pre-B lymphoma cell line demonstrated 65% rosette formation. Cytoplasmic μ chain synthesis was analyzed by radioimmunoassay as described (24). The sensitivity of μ chain detection was ~ 0.3 ng/mg of soluble cell protein. Cell lines were considered positive for TdT if they contained >0.1 U/ 10^8 /h as determined by enzymatic assay.

of μ chain (data not shown). The lack of μ chain expression among the BALB- and Harvey-MSV-transformed hematopoietic cell lines suggested that these cells might be at an earlier stage of differentiation than pre-B lymphoid cells transformed by Abelson-MuLV.

TdT activity is a characteristic of immature lymphoid cells of both T and B cell lineage (48–50). As shown in Table IV, BALB- and Harvey-MSV transformants demonstrated TdT activity at levels ranging from 0.72 to 4.83 U/ 10^8 cells/h among

TABLE IV
*TdT Activity in BALB- and Harvey-MSV-transformed
 Bone Marrow Cell Lines*

Cells	TdT activity* <i>U/10⁸ cells/h</i>
Normal thymus‡	2.19
BALB-MSV	
Clone 1	1.31
Clone 2	4.83
Clone 3	2.38
Harvey-MSV	
Clone 1	2.44
Clone 2	0.72
Clone 3	2.56
Abelson-MuLV	
Clone 1	0.04
Clone 2	0.02
Friend-MuLV	
Clone 745	<0.01

* TdT values represent the average of duplicate samples. 1 U is equivalent to the incorporation of one nmol of dGTP into acid insoluble material in 1 h at 37°C.

‡ Normal cells were obtained from 2-3-wk-old NFS/N weanling mice.

several independent transformants analyzed. These levels were as high or higher than the TdT activity detected in normal thymus cells from 2-3-wk-old NFS/N mice (Table IV) (51). In contrast, Abelson-MuLV-transformed cell lines of both μ^+ and μ^- phenotypes contained very low level of TdT, ranging from 0.02 to 0.01 U/10⁸ cell/h, and TdT activity was undetectable in a Friend erythroblast cell line, clone 745 (Table IV). To examine the specificity of the enzymatic assay, cell extracts were incubated with antiserum to TdT before performing the enzymatic assay. This decreased incorporation of dGTP into acid-insoluble polymer by two- to threefold. A similar decrease was observed when purified TdT was tested under the same conditions (data not shown).

Murine TdT is known to have a molecular weight of 60,000 (52). As an independent means of identifying and quantitating the TdT activity in BALB- and Harvey-MSV transformants, we performed radioimmunoprecipitation analysis. When ³⁵S-methionine-labeled cell extracts were exposed to anti-TdT serum, a 60,000-mol wt protein was precipitated from a BALB-MSV transformant (Fig. 3). Similar results were observed with extracts from normal thymus. In contrast, TdT was not detectable in an Abelson-MuLV transformed pre-B cell line tested under the same conditions. These findings help to establish the identity of our BALB- and Harvey-MSV-hematopoietic transformants as early cells within the lymphoid differentiation pathway as well as distinguishing them from any previously reported targets of transforming retroviruses.

Discussion

BALB- and Harvey-MSV comprise a family of retroviruses whose mouse- and rat-derived *onc* genes are closely related and whose 21,000-mol wt gene products have been causally implicated in their respective transforming functions (11, 53, 54). In the

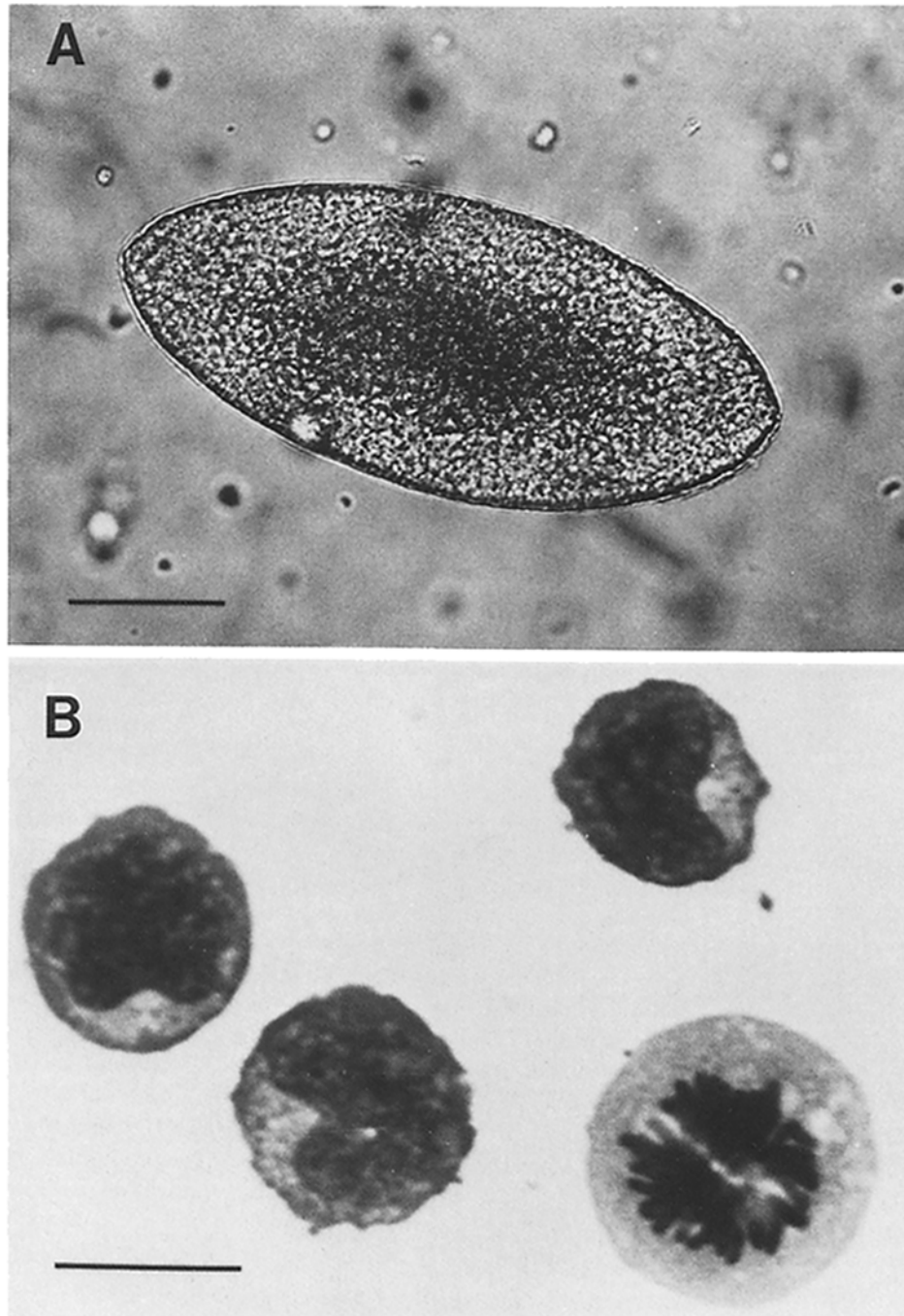


FIG. 3. Analysis of terminal deoxynucleotidyl transferase synthesis in normal and leukemic cells by immunoprecipitation. Cell extracts from ^{35}S -methionine labeled (1) normal thymus; (2) BALB-MSV transformed hematopoietic blast cells; and (3) Abelson-MuLV transformed pre-B cells were treated with normal rabbit serum (lane a) or anti-terminal transferase (lane b) as described in the Materials and Methods. Arrow at left identifies the position of TdT. Molecular weight markers are indicated at the right.

present report, we demonstrate that these viruses transform a novel hematopoietic target cell. Evidence that transformation was a direct result of BALB- and Harvey-MSV infection was derived from the one-hit titration pattern for bone marrow colony formation, the absence of any detectable effect of helper virus alone, and the ability to demonstrate the presence of sarcoma virus in cell lines established from such colonies. BALB- and Harvey-MSV induced colonies could be established in culture as continuous clonal lines with high proliferative capacity. Their ability to form rapidly growing tumors of donor origin and with the same hematopoietic blast cell morphology confirmed their malignant potential.

The hematopoietic blast cell transformants of BALB- and Harvey-MSV described in this report possessed none of the markers that identify mature cells within the known hematopoietic lineages. The low level of Fc receptors detected on some clones does not place them in any specific lineage or stage of maturation (45). Analysis of BALB- and Harvey-MSV transformants did not reveal any that contained detectable immunoglobulin or thymus antigen. In contrast, lymphoid cells transformed by Abelson-MuLV in tissue culture appear to be at an early stage of B cell differentiation. The majority of such transformants synthesize immunoglobulin heavy μ chain in the absence of light chain (47). In the avian system, reticuloendotheliosis virus (REV-T) transforms more immature lymphoid cells that generally lack immunoglobulin expression (55). Both Abelson-MuLV and REV-T lymphoid transformants have been reported to possess low or undetectable levels of TdT (56, 55). In contrast, BALB- and Harvey-MSV transformed hematopoietic blast cell lines contained very high levels of this enzyme, comparable to that of normal thymus. The different phenotype of BALB- and Harvey-MSV transformants compared with that of Abelson-MuLV-transformed lymphoid cells induced under identical assay conditions demonstrates the exquisite specificity that retroviral transforming genes can have for cells at different stages of differentiation, even within the same lineage.

The growth-promoting actions of BALB- and Harvey-MSV for hematopoietic cells in culture is not limited to their transforming effects on early lymphoid cells reported here. These viruses also induce erythroid burst formation (16) and macrophage colony formation in infected bone marrow cell cultures (57). In neither case have permanent, malignant cell lines as yet been established. Thus, whether the growth-promoting activities of BALB- and Harvey-MSV on erythroid and macrophage cells is directly caused by virus transformation or the result of some indirect effect of the virus remains to be determined.

Infection of newborn mice with Harvey-MSV has been shown to result in erythroleukemia (15). We have confirmed the striking splenomegaly associated with Harvey-MSV infection and observed similar gross pathology in BALB-MSV inoculated animals. We have also observed tumor involvement of the thymic region in infected animals that has not been previously reported. When we analyzed bone marrow cells from such animals, we were able to detect hematopoietic colony-forming cells with the same blast cell phenotype observed after *in vitro* infection. In fact, the numbers of such colonies was only ~ 10 -fold less than that observed with Abelson-MuLV. These findings indicate that the hematopoietic tumors induced by BALB- or Harvey-MSV may have a more complex etiology than has previously been supposed.

The occurrence of TdT in prelymphoid cells has been well documented (48, 49). TdT is considered to be a specific marker for early lymphocytes because it is low or

absent in mature lymphocytes. The existence of a TdT⁺ Thy-1⁻ bone marrow cell population has been indicated by findings that elimination of the Thy-1⁺ population by complement-mediated cytolysis does not significantly reduce TdT activity in the bone marrow (58). That some of Thy-1⁻ TdT⁺ bone marrow cells are committed to T cell differentiation has been indicated by the reduction in TdT activity after exposure to thymopoietin followed by anti-Thy-1 complement-mediated lysis (58). Our BALB- and Harvey-MSV transformants possessed a stable TdT⁺ Thy-1⁻ phenotype. Thus, these cells appear to represent the malignant counterpart of a normal lymphoid progenitor cell. It will be of interest to determine whether these cells have the capacity to differentiate along either T or B cell developmental pathways after transformation by temperature-sensitive mutants or exposure to agents that promote differentiation in other systems (25, 58-60). If so, these transformants should provide a useful approach for analysis of lymphoid cell ontogeny and the possible function of TdT in normal lymphocyte development.

Summary

BALB- and Harvey-murine sarcoma viruses (MSV) comprise a family of retroviruses whose mouse- and rat-derived *onc* genes are closely related. These viruses induce sarcomas and erythroleukemias in susceptible animals. An in vitro colony assay that detects transformation of lymphoid cells by Abelson-murine leukemia virus was used to demonstrate that BALB- and Harvey-MSV transform a novel hematopoietic cell both in culture and in vivo. Bone marrow colony formation was sarcoma virus dependent, followed single-hit kinetics, and required the presence of mercaptoethanol in the agar medium. BALB- and Harvey-MSV-induced colonies could be established in culture as continuous cell lines that demonstrated unrestricted self-renewal capacity and leukemogenicity in vivo. The cells had a blast cell morphology and lacked detectable markers of mature cells within the myeloid or erythroid series. They also lacked detectable immunoglobulin μ chain or Thy-1 antigen, markers normally associated with committed cells of the B and T lymphoid lineages, respectively. However, the transformants contained very high levels of terminal deoxynucleotidyl transferase (TdT), an enzyme believed to be specific to early stages within the lymphoid differentiation pathway. This phenotype distinguishes these BALB- and Harvey-MSV transformants from any previously reported hematopoietic targets of transforming retroviruses, including the pre-B lymphoid cell transformed by Abelson-MuLV under identical assay conditions. These newly identified lymphoid progenitor cell transformants may provide an important means of studying early stages of lymphoid ontogeny and the possible role of TdT in lymphoid development.

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