A QUANTITATIVE ASSAY FOR TRANSFORMATION OF BONE MARROW CELLS BY ABELSON MURINE LEUKEMIA VIRUS*

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Abelson murine leukemia virus (A-MuLV)[1] causes a rapidly progressive lymphosarcoma which does not involve the thymus (1). The tumor appears to originate in the bone marrow, the site of first detectable pathological changes after virus infection (2). The tumors are probably composed of cells related to bone marrow-derived cells (B cells) because some of the tumors have immunoglobulin on their surface (3, 4). The tumor cells all lack θ-antigen (3), an antigen found on thymic tumors (5).

Scher and Siegler (6) have shown that A-MuLV directly transforms NIH/3T3 fibroblastic cells in vitro. They derived a nonproducer A-MuLV transformed cell line thus demonstrating that A-MuLV is defective for replication and requires a helper virus, such as Moloney leukemia virus (M-MuLV), to replicate.

Sklar et al. (7) and Raschke et al. (8) have infected short-term (3–6 days) cultures of mouse splenocytes with A-MuLV and have shown that after inoculation into animals, the cultured cells developed into lymphoid tumors. Animal inoculation was required for transformation to be recognized in these systems.

A-MuLV infection of fetal liver cells in vitro has been shown to induce the continuous growth of populations of cells with lymphoid morphology (9). These lymphoid cells have been maintained in culture for more than 300 generations and are malignant because they cause rapidly invasive lymphoid tumors in animals. A-MuLV is therefore able to carry out in vitro the transformation of lymphoid cells.

We now report the development of a semisolid agarose transformation assay (10) which allows recognition of foci of transformed lymphoid cells. Using this system, the number of lymphoid cell colonies produced was shown to depend linearly on the concentration of A-MuLV. The assay system has been used to study the distribution of A-MuLV-sensitive target cells in lymphoid organs of fetal and neonatal mice and to study the susceptibility of different strains of mice to A-MuLV-induced transformation.

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* Supported by grant no. VC-4G from the American Cancer Society, a contract from the Virus Cancer Program, and a grant no. CA-14051 from the National Cancer Institute.

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[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; BSA, bovine serum albumin; M-MuLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline.

Materials and Methods

Cell Preparation. Fetal liver cell cultures were prepared from the livers of embryos of 12–20-day-pregnant mice. Spleen and thymus cultures were prepared from 3–5-wk-old mice. All tissues were forced through a stainless steel mesh (Falcon Plastics, Division of BioQuest, Oxnard, Calif.), and the cells were suspended in RPMI-1640 medium (Grand Island Biological Company, Grand Island, N. Y.) containing 20% fetal calf serum (Microbiological Associates, Bethesda, Md.) (heat inactivated at 56°C for 30 min) and 2-mercaptoethanol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) at a final concentration of 50 μM. Bone marrow cell cultures were prepared from the marrow of 3–5-wk-old mouse femurs and tibias. The marrow plugs were removed using a syringe and suspended in RPMI-1640 medium. For experiments involving variations in culture medium, all cell suspensions were prepared in phosphate-buffered saline (PBS) medium (200 mg/liter KCl, 200 mg/liter KH_2PO_4, 100 mg/liter $MgCl_2 \cdot 6H_2O$, 8,000 mg/liter NaCl, 1,150 mg/liter $Na_2HPO_4 \cdot 2H_2O$, supplemented with 2% heat-inactivated fetal calf serum). Aliquots of the cell pools were collected by centrifugation at low speed and resuspended in the test medium. Cell viability was monitored by the exclusion of 0.05% trypan blue.

Agarose Transformation Assay. Virus stocks of A-MuLV and M-MuLV and conditioned medium have been described (9). 2 million nucleated cells were suspended in 1 ml of medium and treated with 1 ml of virus stock, conditioned medium, or fresh medium. Virus adsorption was carried out in 13 x 100-mm glass test tubes in the presence of 4 μg/ml polybrene (Aldrich Chemical Co., Inc.) at 37°C in a humidified 5% CO_2 atmosphere. Tubes were agitated every 15 min. After a 2.5 h adsorption period, the cells were mixed with agarose medium containing RPMI-1640 in 0.3% agarose (Type II, Sigma Chemical Co., St. Louis, Mo.) supplemented with 20% fetal calf serum, 50 μM mercaptoethanol, and 2 μg/ml polybrene final concentration. 1 million cells were plated in 30-mm plastic Petri dishes (A/S Nunc, Vanguard International, Red Bank, N. J.) in a total volume of 2 ml of agarose medium. Cultures were incubated and fed 1 ml of agarose medium at 5 and 10 days postinfection. Macroscopically visible colonies were scored 12 days postinfection.

Clones of Transformed Cells. Foci of transformed cells were removed from agarose 10–18 days postinfection using a Pasteur pipette, smeared on slides and stained with Wright-Giemsa stain (Harleco, Philadelphia, Pa.) for microscopic examination. Clones were also removed, washed in PBS medium to remove any contaminating agarose, and treated with goat antimouse IgG and IgM (Meloy Laboratories, Springfield, Va.) as described (9). To subculture the clones, foci were removed and plated in 2 ml of liquid RPMI-1640 medium in 30-mm plastic dishes. Cultures were monitored for cell growth. When the cells reached a density of 5×10^5–1×10^6 cells/ml they were transferred at a 1:2 split ratio. After an initial period of about 1 mo during which cell densities were maintained between $1–2 \times 10^5$ and 1×10^6 cells/ml, the cell population doubled every 12–15 h and could be passaged at split ratios of 1:100 every 3–4 days. Such cells have been maintained in continuous culture in excess of 300 generations.

Viral Studies. The presence of both the focus-forming and plaque-forming components of A-MuLV in transformed clones was tested using the NIH/3T3 transformation assay (6) and the XC plaque assay (11). Foci of transformed lymphoid cells were removed from agarose with a Pasteur pipette and added to indicator layers of NIH/3T3 cells. Foci of transformed NIH/3T3 cells were scored 13–15 days postinfection. After the foci were scored, the dishes were irradiated and overlaid with XC cells in standard fashion.

Discontinuous Bovine Serum Albumin Gradients. Fractionation of mouse bone marrow cells was carried out by centrifugation on discontinuous bovine serum albumin (BSA) gradients according to Dicke et al. (12). The gradient was prepared by sequentially layering 1-ml aliquots of BSA solutions in 2% decreasing concentration from 33 to 19% into a 16 x 125-mm plastic test tube (Falcon Plastics). BSA solutions were prepared as described by Kung et al. (13). Bone marrow cells from BSA fractionation were harvested in PBS medium. The cells were collected by centrifugation at low speed and resuspended in 17% BSA before layering on top of the gradient. The gradient was centrifuged at 1,500 *g* for 30 min at 6°C in an International model PR-J centrifuge using the no. 269 rotor (International Equipment, Needham Heights, Mass.). Cell fractions forming at the interfaces of the BSA solutions were removed using a Pasteur pipette and washed with PBS. The cell fractions were resuspended in RPMI-1640 medium, counted, and adjusted to 2×10^6 cells/ml before virus adsorption. Cell fraction 1 was between 17–19% layers, fraction 2 between 19–21% layers, etc.

Mice. NIH/Swiss mice, originally obtained from the Division of Research Services, Veterinary

Resources Branch, National Institutes of Health, Bethesda, Md., were raised in our colony; BALB/c mice were either raised in our colony or purchased from Charles River Breeding Laboratory, Willmington, Mass. A/J, NZB/BINJ, C57L/J, C57BR/cdJ, SWR/J, DBA/2J, and DBA/1J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. C57BL/6 mice were purchased from West Seneca Laboratories, West Seneca, New York. Nude mice were obtained from Dr. J. Beard, Life Sciences, Inc., St. Petersburg, Fla.

Results

Agarose Transformation Assay. To quantitate the transformation process, an assay which allows recognition of single transformation events has been developed using a semisolid agarose culture system. For this assay, bone marrow cells were infected in suspension in RPMI-1640 medium. After a 2.5 h adsorption period, the cells were mixed with agarose medium and plated. Cultures were checked to insure the absence of clumps of cells.

Microscopic colonies of 25–500 cells appeared in both control and infected cultures 3–5 days after plating. The number of colonies varied from 300–500/plate. These are presumably the same type of colonies reported by Metcalf et al. (14) and do not appear related to the presence of A-MuLV.

In plates of cells infected with A-MuLV, colonies appeared that grew progressively and became macroscopically visible (1–2 mm in diameter) by 10–12 days postinfection (Fig. 1). The number of visible colonies did not increase after this time, although most of the colonies continued to increase in size if the feeding schedule was extended. No macroscopic colonies were seen in control cultures or cultures infected with M-MuLV alone. The microscopic colonies in the control and M-MuLV-infected cultures began to die 7–10 days after plating.

Colonies of Transformed Cells. Colonies from A-MuLV-infected cultures were transferred with a Pasteur pipette to microscope slides and stained with Wright-Giemsa stain. Light microscope examination showed that the foci were composed of cells of lymphoid morphology (Fig. 2). The cells had a large nucleus with a poorly defined nucleolus. The scant cytoplasm contained no granules but was frequently vacuolated. Mitotic figures were observed. Although the size of the foci varied, the morphology of the cells was uniform from colony to colony.

Both large and small foci were removed from agarose and tested for the presence of surface immunoglobulin. The cells were washed with PBS medium to remove the agarose and treated with goat antimouse IgG and IgM. None of the cells in the 20 colonies tested had immunoglobulin on their surface detectable by this assay.

Many of the foci could be transferred from agarose with a Pasteur pipette and grown in RPMI-1640 medium. Colonies that become permanent cell lines reach densities of 5×10^5–1×10^6 cells/ml by 10–14 days after transfer from agarose. Cells from colonies that do not grow continuously in liquid medium do not increase in number significantly after transfer. Colony size in agarose was related to the ability of the cells to replicate continuously in liquid medium. Greater than 90% of the larger colonies could be maintained by serial passage, while only 20–40% of the smaller foci became established cell lines.

Optimum Conditions for Transformation. The dependence of A-MuLV-induced transformation on the concentration of several components in the assay mixture was studied. For these experiments pools of bone marrow cells were

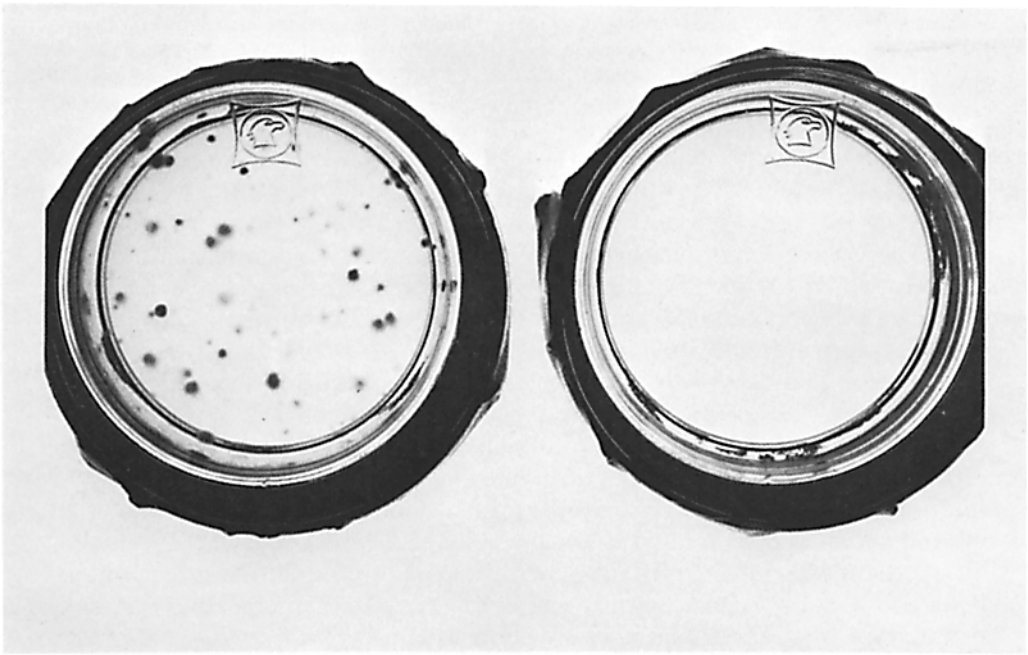

FIG. 1. Agarose transformation assay plates. (A, plate on left) Bone marrow culture 15 days after infection with A-MuLV. (B, plate on right) Control bone marrow culture 15 days after plating.

prepared in PBS medium, aliquoted, and centrifuged at low speed. The cells were then suspended in the various test media. This procedure did not affect cell viability. Transformation frequency was dependent on the concentration of fetal calf serum in the incubation medium, with concentrations of less than 20% resulting in fewer foci (Table I). Foci in cultures maintained at 10% serum were also much smaller than those in cultures maintained in 15 and 20% serum.

Mercaptoethanol was required for transformation in this system. 50 μM was the optimum concentration with 10-fold increases or decreases resulting in 100-fold reductions in transformation frequency.

Use of polybrene to enhance virus infectivity (15) increased the transformation frequency by approximately 5-fold. The presence of polybrene in the adsorption and incubation medium at the concentrations used did not affect cell viability.

Agarose was a more efficient supporting medium for transformation than agar (Bacto-agar, Difco Laboratories, Detroit, Mich.). Transformation frequency was reduced approximately 50% in medium containing 0.3% agar.

Dilution Curve for A-MuLV Transformation. The agarose suspension assay was used to determine the relationships of A-MuLV fibroblast focus-forming units to lymphoid focus-forming units. Bone marrow cells were infected with serial dilutions of A-MuLV and plated in agarose suspension cultures. Macroscopically visible colonies of transformed cells were counted 12 days postinfection. Enumeration of the foci at various virus dilutions showed that the number

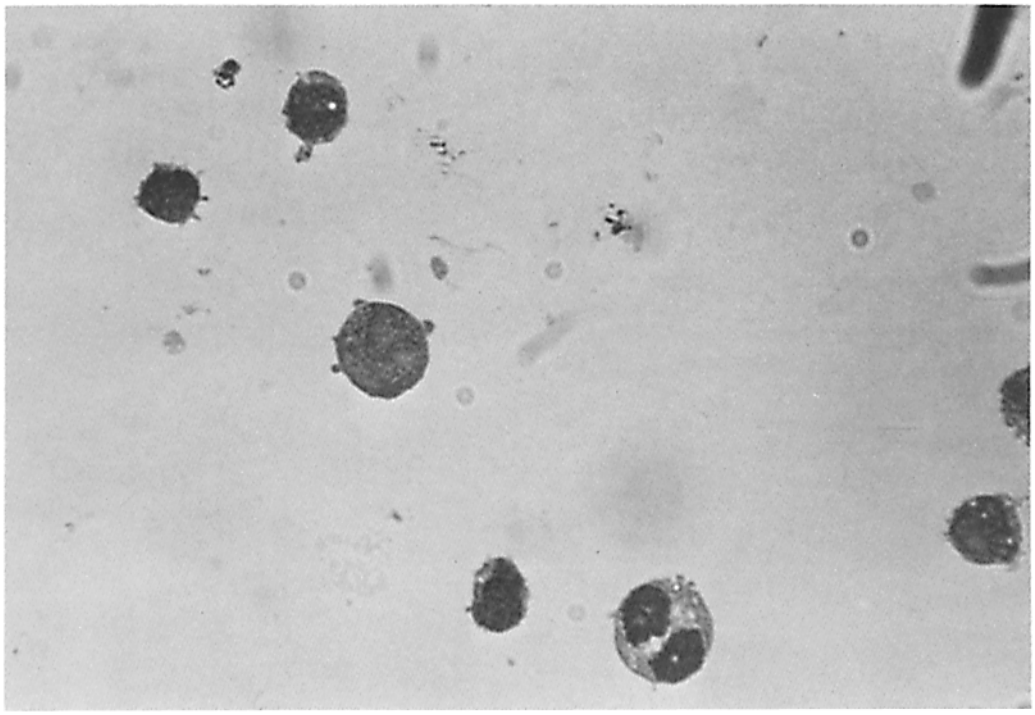

FIG. 2. Cells from an A-MuLV transformed focus showing typical lymphoid morphology. Note the mitotic figure (Wright-Giemsa × 400).

TABLE I
Agarose Transformation Assay Conditions

		Transformation
		%
Serum concentration	20%	100
	15%	68
	10%	45
Mercaptoethanol concentration	500 μM	1
	100 μM	54
	50 μM	100
	10 μM	16
	5 μM	<0.2
	1 μM	0
	0	0
Polybrene concentration	8 μg/ml	100
	4 μg/ml	100
	0	20

Bone marrow cells from NIH/Swiss mice were infected with A-MuLV under quantitative conditions. The maximum number of foci obtained under the various regimen was taken as 100%.

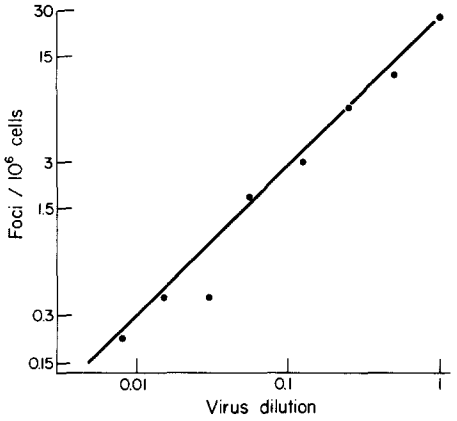

FIG. 3. Dilution curve of A-MuLV. Bone marrow cells were infected with serial dilutions of A-MuLV and plated under quantitative conditions. Foci were scored 12 days postinfection.

of foci was linearly related to the virus concentration (Fig. 3). Approximately 10^3 fibroblast focus-forming units were required to produce a focus of lymphoid cells. Numbers of foci on replicate plates or in duplicate experiments normally varied less than 20%.

Susceptibility of Different Mouse Strains to A-MuLV. Bone marrow cells from different strains of mice vary in susceptibility to A-MuLV-induced transformation and can be classified as giving a high, medium, or low response to the virus (Table II). NIH/Swiss and BALB/c bone marrow cells were most susceptible to A-MuLV. Bone marrow cells from DBA/2J, DBA/1J, and nude mice on an NIH/Swiss background were very resistant to A-MuLV in vitro. In most cases, the foci arising in DBA/2J, DBA/1J, or nude mouse cultures were abnormally small, and attempts to serially passage foci from DBA/2J and DBA/1J cultures have been unsuccessful.

Presence of A-MuLV in Transformed Clones. The association of the focus-forming and plaque-forming components of A-MuLV with foci of transformed cells was monitored by plating the colonies directly onto indicator monolayers of NIH/3T3 cells. Foci of transformed NIH/3T3 cells were scored 13–15 days after plating at which time the plates were treated with XC cells in standard fashion. Both large and small foci were tested. Under the assay conditions a small percentage of both the large and small lymphoid foci grew continuously. Most lymphoid cells died within 48 h of plating on the NIH/3T3 cells. In cultures where the lymphoid cells were growing, both NIH/3T3 foci and plaques were found in 66% of dishes with large foci and 100% of dishes with small foci (Table III). In cultures where the lymphoid foci did not grow both NIH/3T3 foci and plaques were found in 60% of dishes with large foci and 50% of dishes with small foci. 10% of cultures lacking growing lymphoid cells were negative for foci but had XC cell plaques. No dishes containing replicating lymphoid cells lacked foci but showed plaques. Dishes containing foci but no plaques were not found and would not be expected because A-MuLV is helper dependent.

Presence of A-MuLV-Susceptible Cells in Lymphoid Tissues. Lymphoid

TABLE II
Susceptibility of Mouse Bone Marrow to Transformation

Susceptibility	Mouse strain
High, >30 foci/10^6 cells	NIH/Swiss
	BALB/c
	A/J
	C57L/J
Medium, 5–30 foci/10^6 cells	C57BL/6
	C57BR/cdJ
	NZB/BINJ
	SWR/J
Low, <5 foci/10^6 cells	DBA/2J
	DBA/1J
	nu/nu (NIH/Swiss)

Bone marrow cells from 3–5-wk-old mice were infected with A-MuLV under quantitative conditions. Susceptibility was evaluated based on at least two experiments with each strain.

TABLE III
Recovery of A-MuLV from Transformed Clones

Clone size	Replicating clones		Nonreplicating clones	
	Focus-forming* virus	Plaque-forming virus	Focus-forming virus	Plaque-forming virus
Large	4/6	4/6	20/30	26/30
Small	2/2	2/2	9/18	10/18

Virus was detected by plating the lymphoid clones on NIH/3T3 cells using the standard A-MuLV focus assay and XC plaque assay.
* Number of clones positive for virus/total clones.

organs from fetal and adult mice were infected with A-MuLV under standard conditions. Foci were scored 12 days postinfection. Adult splenocytes, but not thymocytes, were susceptible to A-MuLV. Fetal liver cells could also be transformed using the agarose assay. Splenocytes were more resistant to A-MuLV than either fetal liver or bone marrow cells (Table IV).

Morphologic studies of the cells in the foci from spleen and fetal liver cultures could not distinguish these cells from those appearing in A-MuLV-infected bone marrow cultures. Colonies arising in spleen and fetal liver cultures could be transferred to RPMI-1640 medium and maintained by serial passage.

BSA Gradient Fractionation. Bone marrow cells were fractionated on discontinuous BSA gradients to study the distribution of A-MuLV-sensitive cells in the population. Cells were harvested in PBS medium, centrifuged at low speed, and resuspended in 17% BSA before layering on the gradient. Cell viability remained in excess of 90% throughout the procedure as monitored by exclusion of trypan blue. Recovery of cells from the gradient was greater than 80% in most experiments. The distribution of nucleated cells and A-MuLV-sensitive cells is shown in Fig. 4. Most of the A-MuLV-sensitive cells occurred in fractions 4 and

TABLE IV
Susceptibility of Lymphoid Tissues to A-MuLV

Tissue	Foci/10^6 cells
Bone marrow	40–60
Fetal liver	40–60
Spleen	10–20
Thymus	0

A representative experiment in which single cell suspensions from all tissues were infected with A-MuLV and plated under quantitative conditions. Foci were scored 12 days after infection.

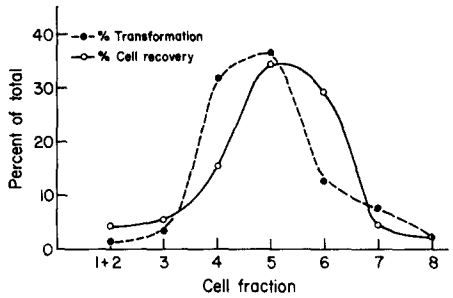

FIG. 4. A-MuLV transformation of bone marrow cells fractionated on BSA gradients. Values are averages of six experiments.

5. The majority of nucleated cells were found in fractions 5 and 6 overlapping the distribution of A-MuLV-sensitive cells.

Discussion

We have described here a quantitative assay for transformation of lymphoid cells by the A-MuLV. The assay exploits the earlier observation that A-MuLV can induce indefinite growth in mass cultures of fetal liver cells (9). Using the focal transformation assay, we have shown that target cells for A-MuLV exist in mouse bone marrow and spleen as well as in fetal liver, but are absent from the thymus.

The assay gives a linear response to dilution of an A-MuLV stock. This behavior shows that the potential target cells in bone marrow are not saturated by our most concentrated A-MuLV stock. A minimum of 100–150 target cells/10^6 nucleated bone marrow cells are present in some mice. The linearity of the assay also shows that either one particle is sufficient to give a transformed focus or that the stocks contain such a huge excess of helper virus that the assay is always saturated for the second virus. We consider it unlikely that the assay is saturated by the helper because the M-MuLV in our stocks is only two to five-fold excess, and therefore, we conclude that infection with defective A-MuLV by itself is able to give rise to a focus. The existence of transformed cells not producing focus-forming virus (Table III) is consistent with this interpretation.

The presence of A-MuLV-susceptible cells in fetal liver, bone marrow, and spleen, but not in thymus, suggests that A-MuLV transforms cells that are related to B cells. Abelson disease does not involve the thymus (1), and A-MuLV has been shown to cause B-cell tumors under certain conditions (3, 16).

The A-MuLV-sensitive cells are likely to be primitive, relatively undifferentiated cells, similar to the B-cell precursors detected in fetal liver, bone marrow, and spleen by others (17–19). The reduced number of A-MuLV-sensitive cells in spleen, which has been shown to contain fewer undifferentiated cells than bone marrow or fetal liver (20), also suggests that A-MuLV transforms a relatively undifferentiated cell. The inability to detect immunoglobulin on most of the A-MuLV-transformed cells or on A-MuLV-induced tumors (3, 9) without highly specific radiolabeling techniques (4) indicates that most of the transformed cells are not differentiated to any degree. The cells sensitive to A-MuLV in spleen, bone marrow, and fetal liver are likely to be similar to one another. The ability to transform only certain populations of cells in vitro may reflect the in vivo situation and indicates that tumor development is controlled in part by the virus' ability to interact only with a select group of cells.

The possibility that more than one type of cell is stimulated to focus formation after A-MuLV infection can not be completely eliminated. Not all foci can be maintained by serial passage. A proportion of these failures can be attributed to low cell number and poor cell viability. Functional differences between colonies may exist even though morphologic studies indicate a relatively uniform cell type.

The virus-producing status of the foci vary. A significant number of the foci produce no detectable focus-forming or plaque-forming virus. Studies are in progress to determine if these cells contain rescuable A-MuLV. The presence of a small number of clones in which only the plaque-forming component of A-MuLV was detected probably reflects the low number of viable cells in the culture and the usual balance of focus-forming and plaque-forming virus produced by A-MuLV-infected cells. None of the foci that replicate continuously produce only plaque-forming virus.

Studies with bone marrow cells from different strains of mice revealed a range of sensitivity of the cells to A-MuLV. Differences between highly susceptible cells, such as those from NIH/Swiss or BALB/c mice, and resistant cells, i.e. those from DBA/2J or DBA/1J, are most striking. Abelson and Rabstein (1) reported that DBA/2 mice were quite susceptible to Abelson disease. In contrast, they found that C57BL/6 mice, whose cells are moderately sensitive to A-MuLV in vitro, are most resistant to A-MuLV. These data suggest that susceptibility of mice to Abelson disease is only partially controlled at the level of lymphoid cell-A-MuLV interaction, but how an animal with resistant cells can be sensitive to the virus in vivo must be investigated further.

Summary

A quantitative Abelson murine leukemia virus (A-MuLV) lymphoid cell transformation assay has been developed using a semisolid agarose culture system. Under these conditions lymphoid cell transformation was shown to vary linearly with the dose of A-MuLV used. The susceptibility of bone marrow cells from different strains of mice to A-MuLV-induced transformation can be estimated using the agarose assay. Strains with bone marrow cells of high, medium, and low susceptibility to A-MuLV can be identified. The assay has been used to study the susceptibility of cells from lymphoid organs of fetal and adult

mice to A-MuLV. Cell suspensions from fetal liver, adult bone marrow, and adult spleen are susceptible to A-MuLV, while thymocytes are resistant to A-MuLV-induced transformation. Bovine serum albumin gradient fractionation of bone marrow cells before infection with A-MuLV demonstrates that the majority of A-MuLV-sensitive cells are recovered in a broad band partially overlapping the majority of the nucleated cells. The agarose assay system allows study of A-MuLV–lymphoid cell interaction at the level of single cell–single virus particle interaction.

We are grateful to Dr. P. C. Kung for his advice on the use of bovine serum albumin gradients.

Received for publication 9 February 1976.

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