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Abelson Virus-Infected Cells Can Exhibit Restricted In Vitro Growth and Low Oncogenic Potential

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We have designed a method for growing bone marrow cells infected with Abelson murine leukemia virus which permits examination of target cell growth early after infection. This culture system increases the efficiency of target cell growth by favoring rapid growth of a mixed population of adherent cells in the primary culture. The nonadherent Abelson virus-infected cell populations expressed pre-B-cell differentiation markers characteristic of Abelson virus-transformed cells (μ-heavy chains of immunoglobulin M and terminal deoxynucleotidyltransferase). Early after infection, these cell populations exhibited restricted in vitro and in vivo growth properties which differed from those of an established Abelson virus-transformed cell line, 2M3. These included a marked dependency upon the adherent cell layer for growth and viability, a lower efficiency of agar colony formation, and a lower capacity for tumor production in syngeneic animals. Growth of the early populations could be maintained in the absence of the adherent cell layer by using conditioned medium from long-term adherent cell cultures established in the absence of viral infection. After passage of the populations for several weeks, the in vitro growth properties gradually shifted toward that of the 2M3 cell line. Twelve-week-old populations grew independently of the adherent cell layer and showed an increased efficiency of agar colony formation. These data indicate that many lymphoid target cells exhibit an intermediate transformed phenotype when infected with Abelson virus. Growth of these cells in culture is mediated via a synergistic interaction between intracellular expression of the viral transforming gene and an exogenous growth-promoting activity which can be provided by cultures of adherent bone marrow cells.

Abelson murine leukemia virus (A-MuLV) is one of several retroviruses that preferentially transform hematopoietic cells at specific stages of differentiation (11, 29). The transforming properties of these viruses have been used to establish cell lines which represent various developmental stages of different hematopoietic cell lineages (11). Whether simple expression of a transforming virus is sufficient to permit growth of cells in agar and tumor production in animals is not clear. In many tumor virus systems, infected target cells have been reported to express a variety of transformed phenotypes ranging from poorly tumorigenic cells to highly tumorigenic cells which vary in their in vitro growth characteristics (8, 16). Hematopoietic cells may also express a range of growth phenotypes when transformed by tumor viruses. We have begun to examine this possibility by studying the in vitro and in vivo growth properties of target cells from mouse bone marrow early after infection with A-MuLV.

A-MuLV is a member of the replication-defective, rapidly transforming group of retroviruses (24) and has the capacity to transform both immature lymphoid cells and 3T3 fibroblasts (1, 18-21, 24). A-MuLV is a recombination between the parental replication-competent Moloney murine leukemia virus (M-MuLV) and specific sequences from the normal mouse genome (designated *abl*) (2, 10). This recombination results in expression of a single, fused protein with an amino-terminal region derived from part of the *gag* gene of M-MuLV and a carboxyl-terminal portion derived from the *abl* sequences (17, 31). The oncogenic effects of A-MuLV appear to be carried out by this single viral protein (23, 30).

The prototype P120 and P160 strains encode proteins with molecular weights of 120,000 and 160,000, respectively. An internal deletion in the *abl* portion has given rise to the transformation-defective P92*td* mutant (30). Other mutants of A-MuLV have been isolated (P85, P90, and P100) which show no reduction in their ability

to transform fibroblasts in vitro (23). However, these mutants show a 10- to 30-fold-reduced frequency of lymphoid cell transformation when the mutant and prototype virus stocks are normalized for fibroblast-transforming titers (22). Clonal cell lines transformed with either the prototype or partially defective strains express the same pre-B-cell differentiation phenotypes (intracellular μ-heavy chains of immunoglobulin M and terminal deoxynucleotidyltransferase) (3, 22, 25, 26). This suggests that the target cell specificity is very similar for all strains but that the efficiency of transformation, as detected by growth in liquid or soft agar cultures, varies markedly. If target cells infected with A-MuLV express a range of growth phenotypes, populations infected with mutant strains may contain a lower frequency of cells expressing the transformed phenotypes that are detectable by our conventional assays.

To examine the growth properties of A-MuLV-infected target cells, we have designed a modified culture system in which adherent layers of mouse bone marrow cells are used. This system was found to be more permissive in supporting A-MuLV target cell growth. Our results indicate that many target cells infected with A-MuLV exhibit restricted in vitro and in vivo growth early after infection. The culture system that we describe provides an efficient method for maintaining these cells in culture and for studying the growth-promoting activity of adherent bone marrow cell cultures.

MATERIALS AND METHODS

Mice. BALB/c mice, 4 to 6 weeks old, were purchased from Cumberland View Farms, Clinton, Tenn.

Virus stocks. Stocks of the P90, P120, and P160 strains of A-MuLV were prepared by superinfection of nonproducer, transformed NIH-3T3 fibroblasts with the M-MuLV helper virus as previously described (19, 20).

Cell lines. 2M3, a nonproducer lymphoid line infected with the P120 strain of A-MuLV (31), was maintained in tissue culture medium containing 10% newborn calf serum and 5×10^{-5} M 2-mercaptoethanol.

Bone marrow cultures. Femoral bone marrow plugs were extruded into a conical tube, and clumps of cells were dispersed by repeated pipetting. The entire cell suspension, including small clumps, was pelleted and suspended at 2×10^6 cells per ml in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% newborn calf serum. The cell suspension was mixed 1:1 with medium or A-MuLV virus stocks containing 16 μg of Polybrene per ml. After incubation at 37°C for 2 to 4 h, cells were pelleted again and suspended at 10^6 cells per ml in RPMI 1640 supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 100 μg of glutamine per ml, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. After 8 to 10 days of culture, nonadherent cells were replated twice weekly at 5×10^4 to 10×10^4 cells per ml in culture medium containing 5% fetal calf serum. For maximal growth and viability, cells were replated onto the original culture dish which contained a layer of adherent bone marrow cells or onto fresh feeder layers established in the absence of A-MuLV infection.

Conditioned medium. Culture supernatants were collected 4 days after a complete medium change of 1- to 5-month-old cultures of adherent mouse bone marrow cells established in the absence of viral infection. The conditioned medium was sterile filtered and stored at 4°C for up to 1 week or at −20°C for periods greater than 1 week. Before use, the conditioned medium was mixed 1:1 with fresh RPMI 1640 medium containing 5% fetal calf serum.

Metabolic labeling and immunoprecipitation. Nonadherent cells were washed twice in phosphate-buffered saline and suspended at 5×10^6 to 10×10^6 cells per ml in leucine-deficient minimal essential medium (GIBCO Laboratories) supplemented with 50 μCi of tritiated leucine ([^{3}H]leucine, ICN, Irvine, Calif.) per ml. Adherent cells were labeled in the original 6-cm tissue culture dishes (Corning Plastics, Santa Clara, Calif.) by using 2 ml of labeling medium. After incubation at 37°C for 1 h, cell lysates were prepared and clarified as previously described (31). Antisera used for immunoprecipitation of the labeled lysates were as follows: normal rabbit serum, polyvalent rabbit anti-mouse immunoglobulin (a gift from I. Weissman, Stanford University, Stanford, Calif.), rabbit anti-calf terminal deoxynucleotidyltransferase (27), and goat anti-M-MuLV virion proteins (31). Immunoprecipitated proteins were analyzed by fluorography (4) after reduction with 2-mercaptoethanol and separation on a sodium dodecyl sulfate–9% polyacrylamide gel (12).

RESULTS

Permissive culture method for in vitro growth of A-MuLV-infected target cells. The culture system that we designed was a modification of the procedure developed by Rosenberg et al. for in vitro cultivation of bone marrow cells transformed by A-MuLV (19–21). The aim of these modifications was to promote rapid growth and establishment of an adherent cell population in the primary cultures of A-MuLV-infected bone marrow cells. Such adherent cell layers have been shown by several investigators to provide an in vitro environment supportive of the growth of normal myeloid and lymphoid stem cells of both murine and human origin (5–7, 9, 15).

Our procedure differs from that originally published by Rosenberg et al. (19–21) in that all cells flushed from the femur, including small clumps and spicules, were included in the cultures. In addition, the amount of fetal calf serum in the medium was reduced from 20 to 5%. Both modifications greatly enhanced the growth of an adherent feeder layer of cells. This procedure

also differs from the basic Dexter culture system in which bone marrow cells are cultured at 33°C in medium containing 20% horse serum (6, 7). The conditions described by Dexter are conducive to the formation of an adherent feeder cell layer, but the lower temperature has an inhibitory effect on growth of A-MuLV-infected lymphoid cells (N. Rosenberg, O. Witte, and C. Whitlock, unpublished data).

At 37°C, foci of adherent cells appeared in the cultures within 2 days in the presence or absence of infection with the P120 strain of A-MuLV. These foci expanded rapidly, forming a confluent layer of adherent cells by 10 days that showed no gross morphological change with virus infection (Fig. 1A and B). The numbers of nonadherent cells in the mock-infected or M-MuLV-infected cultures (data not shown) declined steadily after 4 days. In contrast, the numbers of nonadherent cells in the A-MuLV-infected cultures initially declined and then increased rapidly to 1×10^6 to 3×10^6 cells per ml by 8 to 10 days. A typical culture of A-MuLV-infected bone marrow cells after passage of the nonadherent cells for 3 weeks on the original culture dish is shown in Fig. 1B. Examination of Wright-stained cytocentrifuge preparations of these nonadherent cells showed that the heterogeneous population of bone marrow cells present at the initiation of culture was replaced in the A-MuLV-infected cultures with a relatively homogeneous population of immature blast cells (Fig. 1C).

Dependence on the adherent cell feeder layer for growth of A-MuLV-infected cells. We examined whether growth of the early populations was dependent upon the adherent cell layers which rapidly formed in the primary cultures under our modified culture conditions. Nonadherent cells from P120-infected cultures that had been maintained in their original culture dishes for 2, 4, 8, or 12 weeks were examined. Cells were harvested, washed in fresh medium, and cultured at 10^4 cells per ml in the presence or absence of adherent cells or conditioned medium from adherent cell cultures. After 6 days, cultures were harvested, and the numbers of viable and nonviable cells were determined by trypan blue exclusion (Table 1).

In the absence of an adherent cell layer, the 2- and 4-week-old P120-infected populations grew poorly. Cell numbers increased by only three- to fivefold, and cell viabilities were less than 35% (Table 1). Populations maintained for 8 weeks in culture grew much better. Cell numbers increased 76- and 230-fold in the 8- and 12-week-old cultures, respectively, and viabilities in the 12-week-old cultures were increased to 76%.

In the presence of established adherent cell layers or conditioned medium, a dramatic increase in cell number was seen for the early populations (Table 1). Growth of the 2-week-old populations was enhanced 30-fold in the presence of either conditioned medium or an adherent cell layer. However, cell viabilities were lower in the presence of conditioned medium, indicating that the growth-promoting activity present in the supernatant may have been depleted during the 6-day incubation. Similar results were found for the 4-, 8-, and 12-week-old cultures, but the degree of enhancement de-

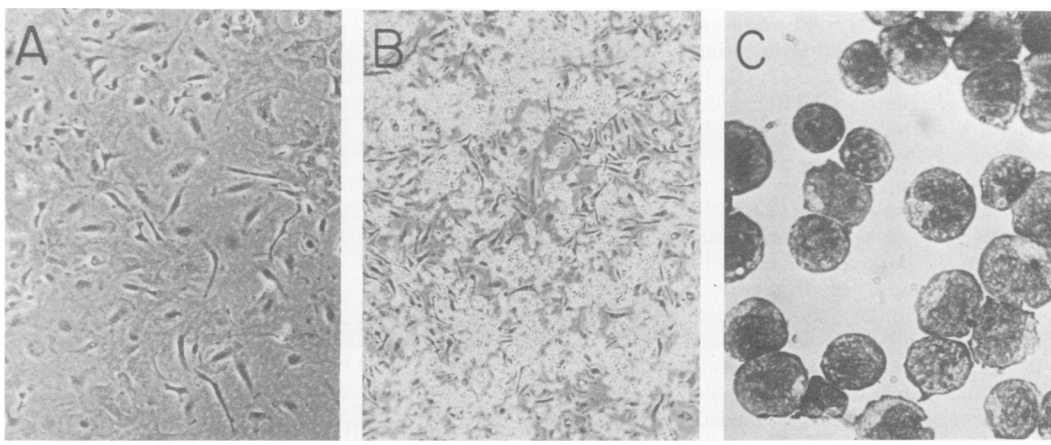

FIG. 1. *Morphologies of bone marrow cultures established in the presence or absence of A-MuLV infection under permissive culture conditions. Mock-infected (A) and A-MuLV-infected (B) cultures of BALB/c bone marrow were photographed after 3 weeks of culture. Wright-stained cytocentrifuge preparations of nonadherent cells from the 3-week-old cultures infected with A-MuLV (C) are also shown. Magnifications: A and B, ×30; C, ×300.*

TABLE 1. *Growth of nonadherent cells from the P120-infected bone marrow cultures in the presence or absence of adherent cell layer*

Age of culture[a] (wk)	Cells per ml × 10^{-5} (% viable)		
	No adherent cells	Adherent cells	Conditioned medium
2	0.3 (33)	9.1 (87)	9.0 (47)
4	0.5 (13)	7.9 (89)	8.5 (58)
8	7.6 (36)	20.0 (90)	28.0 (40)
12	23.0 (76)	50.0 (76)	37.0 (44)

[a] Nonadherent cells from 2-, 4-, 8-, and 12-week-old cultures of P120-infected bone marrow cells, which had been maintained in their original culture dishes, were washed once and suspended at 10^4 cells per ml in RPMI 1640 containing 5% fetal calf serum. Cells were plated onto new culture dishes (no adherent cells) or onto dishes containing adherent cell layers established in the absence of viral infection (adherent cells). In addition, cells were resuspended in a 1:1 mixture of fresh culture medium and conditioned medium from adherent cell cultures and plated onto new dishes. After 6 days, the numbers of viable and nonviable cells were determined by trypan blue exclusion.

creased with the increasing age of the population. As a result, the 12-week-old population showed only a twofold enhancement of growth in the presence of an adherent cell layer, with no increase in viability.

Decreased agar growth potential of A-MuLV-infected cells. We examined the agar growth potential of the various ages of P120-infected populations by using the standard agar growth assay developed by Rosenberg and Baltimore (19) (Table 2). In addition, we examined the capacity of the adherent cell layer to enhance agar colony formation by plating cells in agar over an established adherent cell layer or by incorporating conditioned medium into the agar layer. A total of 5,000 cells from each population were plated into 6-cm dishes, and colonies were scored after 10 days by using a dissecting microscope.

Although the P120-infected cell populations of all ages exhibited poor agar colony formation, the efficiency of agar growth did increase with the age of the culture. Of 5,000 cells plated under the standard agar growth conditions, the 2- and 4-week-old populations produced no colonies, and the 8- and 12-week-old populations gave only 2 and 43 colonies, respectively. If cells were plated in agar over adherent cell layers, the number of agar colonies increased dramatically. The younger populations, however, were still deficient in agar colony formation relative to the older populations. The 2-week-old populations produced only 14 colonies, whereas the 12-week-old populations produced 620 colonies. Conditioned medium also enhanced agar colony growth but only for the 8- and 12-week-old populations.

Although the P120-infected 12-week-old bone marrow populations produced significant numbers of agar colonies, the agar growth potential of this population was significantly below that of an established P120-transformed cell line, 2M3. More than 60% of the 2M3 cells cultured in agar produced colonies in both the presence and the absence of adherent cells (data not shown).

These data support the findings of Teich and Dexter who examined growth of A-MuLV-infected bone marrow cells in the standard Dexter culture system (28). A population of blast cells gradually predominated their cultures which also exhibited poor agar growth potential. In addition, growth of these cells was dependent upon the presence of the adherent cell layer for up to 15 weeks. At that time, agar growth efficiency increased, and cells were capable of growing independently of the adherent layer.

Increased efficiency of target cell growth of bone marrow cells infected by the P90 strain of A-MuLV. Although the P90 strain of A-MuLV is capable of transforming NIH-3T3 fibroblasts with a high efficiency, bone marrow cells infected with P90 show a 10- to 30-fold-lower frequency of agar colony transformation with P120 and P160 (22). A lower efficiency of bone marrow transformation with P90 was also observed in liquid cultures, a large fraction of which failed to show detectable growth of target cells even after 17 days of culture (22).

We examined whether our permissive culture conditions allowed more efficient growth of A-MuLV-infected target cells by determining the

TABLE 2. *Decreased agar growth efficiency of P120-infected bone marrow cells*

Age of culture[a] (wk)	Agar colonies per 5,000 cells plated		
	No adherent cells	Adherent cells	Conditioned medium
2	0	14	4
4	0	81	0
8	2	437	40
12	43	620	315

[a] Nonadherent cells from 2-, 4-, 8-, and 12-week-old cultures of P120-infected bone marrow cells were cultured in medium containing 20% fetal calf serum, 0.26% Noble agar, and 5 × 10^{-5} M 2-mercaptoethanol. A total of 5,000 cells were plated onto new culture dishes (no adherent cells) or over established adherent cell layers (adherent cells). In addition, an equivalent number of cells was cultured in medium supplemented with 50% conditioned medium from adherent cell cultures. Agar colonies were counted after 10 days by using a dissecting microscope.

frequency of 1-ml bone marrow cultures containing 10^6 or 10^5 cells that exhibited foci of growth of nonadherent cells after infection with the P90 or P120 strain of A-MuLV (Table 3). At 10 and 17 days after initiation of the cultures, growth of nonadherent cells was monitored by using a phase microscope. After 10 days, mock-infected cultures at either cell density showed growth of adherent cells but did not contain foci of nonadherent cells. In contrast, all cultures initially containing 10^6 cells and infected with either the P90 or P120 strain of A-MuLV showed many foci of dividing nonadherent cells.

At 10^5 cells per ml, all cultures infected with P120 showed large foci of growth of nonadherent cells by 10 days. The P90-infected cultures at the low cell density also exhibited growth (7 of 10 were positive), but the foci of nonadherent cells were somewhat smaller. All cultures infected with P90 were positive for transformation by 17 days. Cultures were not initiated at cell densities below 10^5 per ml, since adherent cell layers did not establish reproducibly at such low cell densities (data not shown).

The slower appearance of nonadherent foci in the P90-infected cultures may indicate that this virus transforms fewer target cells or that the cells transformed by this virus have a slower growth rate. The modified culture conditions that we describe provide a more efficient method for detecting growth of the mutant-infected cells. These culture conditions were not sufficient, however, to permit growth of cells infected with the P92*td* mutant (data not shown), further confirming the transformation-defective nature of this mutant strain of A-MuLV (30).

Mass populations of cells from cultures infected with the P90 mutant and the P160 wild-type strains of A-MuLV were also examined for their dependence on the adherent cell layer for growth and viability (data not shown). Both populations were similar to the P120-infected populations in that they showed a marked dependence on the adherent cell layer for growth early after infection. Populations maintained in culture for several weeks were capable of growing independently of the feeder layer.

Lymphoid phenotype of A-MuLV-infected cells in adherent layer-dependent cultures. Two major differentiation markers have been shown to be characteristic of A-MuLV-transformed lymphoid cells, synthesis of μ-heavy chains of mouse immunoglobulin M in the absence of immunoglobulin light chain synthesis and synthesis of terminal deoxynucleotidyltransferase (3, 25, 26). We examined the expression of these two markers by the mass populations of P90- and P120-infected cells maintained in adherent layer-dependent cultures for 3 weeks (Fig. 2).

Cultures of P90-infected (Fig. 2A, adherent layer; Fig. 2C, nonadherent layer) and P120-infected (Fig. 2B, adherent layer; Fig. 2D, nonadherent layer) bone marrow cells were labeled for 1 h with [^{3}H]leucine. Cells were lysed and clari-

TABLE 3. *Increased efficiency of growth in P90-infected bone marrow cultures*

Virus strain	No. of cells per culture	Frequency of positive cultures[a]	
		Day 10	Day 17
Mock	10^6	0/4	0/4
	10^5	0/10	0/10
P90	10^6	4/4	4/4
	10^5	7/10	10/10
P120	10^6	4/4	4/4
	10^5	10/10	10/10

[a] Bone marrow cells from BALB/c mice were mock infected or infected with the P90 or P120 strain of A-MuLV. After viral adsorption, cells were cultured in 1-ml volumes at 10^6 or 10^5 cells per ml. Cultures were fed twice weekly and monitored for foci of nonadherent cells. The data represent the frequency of positive cultures after 10 and 17 days.

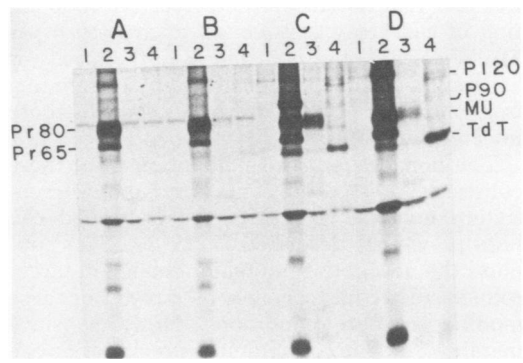

FIG. 2. *Differentiation markers expressed by the nonadherent and adherent cell populations of P90- and P120-infected bone marrow cultures. Three-week-old P90-infected (A and C) and P120-infected (B and D) cultures were divided into adherent (A and B) and nonadherent (C and D) fractions and metabolically labeled with [^{3}H]leucine as described in the text. Clarified lysates were immunoprecipitated with normal rabbit serum (lane 1) or antisera specific for M-MuLV virion proteins (lane 2), mouse immunoglobulin light and heavy chains (lane 3), or terminal deoxynucleotidyltransferase (TdT) (lane 4). Immunoprecipitated proteins were analyzed by fluorography after reduction with 2-mercaptoethanol and separation on a sodium dodecyl sulfate–9% polyacrylamide gel.*

fied, and samples were immunoprecipitated with normal rabbit serum (lane 1), goat anti-M-MuLV virion proteins (lane 2), rabbit anti-mouse immunoglobulin (lane 3), and rabbit anti-terminal deoxynucleotidyltransferase (lane 4). Proteins released from immunoprecipitates were separated on sodium dodecyl sulfate-9% polyacrylamide gels and examined by fluorography (Fig. 2).

The nonadherent cells from the virus-infected cultures synthesized large amounts of the 90,000-dalton (Fig. 2C) and 120,000-dalton (Fig. 2D) A-MuLV-encoded proteins and the $Pr80^{env}$ and $Pr65^{gag}$ proteins encoded by the M-MuLV helper virus (lane 2). Both nonadherent populations also synthesized μ-heavy chains of mouse immunoglobulin M (lane 3) and significant amounts of terminal deoxynucleotidyltransferase (lane 4). Trace amounts of immunoglobulin light chain synthesis could be detected on longer exposure of the fluorogram (data not shown). We believe that this derives from small numbers of contaminating mature B cells which are capable of being maintained under these culture conditions for long periods of time (Whitlock and Witte, manuscript in preparation).

Cells in the adherent layer (Fig. 2A and B) synthesized large amounts of M-MuLV proteins. However, no synthesis of the A-MuLV-encoded P90 or P120 protein was detected. This population of cells may restrict the expression of A-MuLV either by controlling integration or expression of the viral genome or by eliminating from the mass population those cells which are infected with A-MuLV.

The nonadherent cell population from these cultures expressed the same lymphoid differentiation markers as A-MuLV-transformed cell lines previously described (3, 25, 26). This supports the notion that no major change in target cell specificity has occurred as a result of these modified culture conditions. However, since metabolic labeling and immunoprecipitation are averages of the entire mass population, we cannot be sure from such data that every cell in the culture expresses both μ-heavy chains and terminal deoxynucleotidyltransferase. In preliminary observations, clonally derived cell lines that retain adherent layer dependence for growth showed continued expression of intracellular μ-heavy chains and terminal deoxynucleotidyltransferase (data not shown). Whether these cell lines represent a rare subpopulation or a representative sampling of the early mass population remains to be determined.

Decreased oncogenicity of the A-MuLV-infected lymphoid cells. We examined the oncogenic potential of the A-MuLV-infected cells in the early mass populations by comparing the capacity of these cells and the established A-MuLV transformed line 2M3 to form tumors in animals. Syngeneic BALB/c mice were injected intraperitoneally with 5×10^3 to 1×10^7 2M3 cells that had been superinfected with M-MuLV or cells from 2- to 4-week-old mass populations of bone marrow cells infected with the P90 or P160 strain of A-MuLV. The appearance of tumors was monitored for each set of mice, and the time of death of each animal is shown in Fig. 3.

Tumors appeared rapidly in mice injected with 5×10^6 2M3/M cells, and death of the animals occurred within 10 to 12 days. Lower numbers of cells gave only slightly longer survival times, with animals receiving 5×10^3 cells surviving for 17 to 20 days. The time course of tumor production and animal death with the 2M3/M cell line was similar to that found for other clonal cell lines transformed with the P120 and P160 strains of A-MuLV (N. Rosenberg and O. Witte, unpublished data).

A total of 10^7 P160-infected cells resulted in death for all animals injected, but the animals survived for approximately 30 days rather than for 10 to 12 days. The time course of tumor appearance with 10^7 P160-infected cells resembled more closely that seen upon injection of 5×10^3 2M3/M cells. Once the animals began to show signs of tumor growth (approximately 20 to 25 days after injection), tumor progression

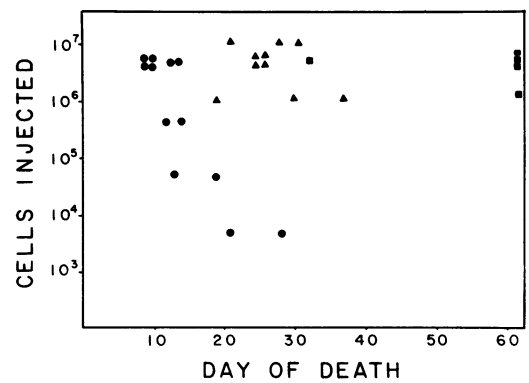

FIG. 3. *Reduced oncogenic potential of cells from the early mass cultures of P90- and P160-infected bone marrow cells. A-MuLV-infected cells from the P90-infected (■) and P160-infected (▲) cultures 3 weeks of age were washed in RPMI 1640 without serum and suspended in serum-free medium for injection. BALB/c mice (6 to 8 weeks old) were injected intraperitoneally with 0.5 ml of the cell suspensions and monitored for the appearance of tumors and day of death. Control mice were injected with lymphoid cell line transformed with the P120-infected strain of A-MuLV, 2M3/M (●).*

and death proceeded in a manner which was similar to that seen with 2M3/M. The slower appearance of tumors may have been due to a low frequency of truly oncogenic cells in the mass population of P160-infected cells.

This lower oncogenic potential of the A-MuLV-infected populations was most prominent for the population of bone marrow cells infected with the P90 mutant strain of A-MuLV. Mice injected with 10^7 P90-infected cells developed tumors after approximately 55 days and died after more than 60 days. These results parallel the results of previous work in which clones of P90-infected lymphoid cells were shown to vary extensively in their ability to produce tumors in animals (N. Rosenberg and D. Clark, personal communication).

Two observations suggest that the late-appearing tumors in the animals injected with the P90-infected mass populations may result from virus spread to target cells in the host tissues. The first is that although the tumors which appeared resemble the disseminated lymphomas which appeared in the mice receiving 2M3/M cells or cells from the P160-infected mass population, needle-track tumors were rarely seen in the P90-infected mice. Subcutaneous tumors at the site of injection were common in the former two cases. The second is that the time course of tumor appearance in the mice injected with the P90-infected cells was similar to that found upon injection of mice with P90 virus preparations (22). We are presently examining the frequency of tumors that arise which are of host or donor origin by using F_1 mice as recipients of the P90- and P160-infected BALB/c lymphocytes.

DISCUSSION

Our data suggest that interaction of A-MuLV with target lymphocytes may result in a continuum of transformed states ranging from untransformed cells to fully transformed cells capable of tumor progression. Cells expressing the more restricted growth phenotypes predominated in the culture early after infection but gradually became the minor population upon continued passage of the cultures. Two mechanisms can be used to explain the decreased dependency upon the adherent cell layer and increased agar growth efficiency of the long-term passaged populations. Cells capable of unrestricted growth may be present as a minor subpopulation at the initiation of the culture and gradually become the predominant population through a slight growth advantage. Alternatively, the A-MuLV-infected cells may undergo secondary changes which further alter their in vitro growth properties.

In either case, measurements of the efficiency of bone marrow transformation by A-MuLV depend strongly on the stringency of the growth parameters used to determine transformation. Our ability to improve the efficiency of in vitro growth of bone marrow cells infected with the P90 strain of A-MuLV by using our modified culture conditions supports this concept. The distribution of transformed states expressed by the P90-infected cell population may be shifted toward the more restricted growth phenotypes. As a result, under stringent growth conditions, such as growth in agar, the P90 mutant is observed to be much less efficient than the P120 and P160 strains at transforming lymphoid cells (22).

The major question which arises is how cells that express various transformed phenotypes differ at the molecular level. Mutation of the A-MuLV genome is unlikely, since virus released from populations expressing these growth characteristics retains the same biological properties as the input virus. These include the sizes and relative kinase activities of the virus-encoded proteins, the efficiencies of fibroblast and lymphoid cell transformation, and the ability to reproduce the same range of growth phenotypes upon infection of fresh bone marrow populations (data not shown). The range of growth phenotypes appear, instead, to be due to virus-cell interactions which differ among individual target cells. The site at which A-MuLV integrates into the host cell genome may be important since integration can affect the expression of cellular genes (13, 14). Alternatively, the molecular environments within the individual target cells may differ before infection. The growth properties expressed after infection may be dictated by how the A-MuLV-transforming protein functions in these different environments.

To examine these hypotheses, clones expressing the various growth phenotypes must be isolated. We recently succeeded in obtaining cell populations by limiting dilution that have expressed a marked dependency upon the adherent cell layer for over 3 months. We are attempting to reclone and analyze these populations by using conditioned medium from adherent cell cultures. In addition to being useful for examining the molecular interactions involved in A-MuLV-induced growth and oncogenicity, these clones will be valuable for characterizing the growth-promoting activity of the adherent feeder layer of bone marrow cells.

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ADDENDUM

We recently obtained data comparing the tumorigenic potentials of cells from the 4-week-old and 12-week-old P120-infected cells described in Tables 1 and 2. Animals injected with 5×10^6 cells from the 12-week-old culture died between 8 and 12 days, whereas animals injected with cells from the younger culture survived for 29 to 30 days.

LITERATURE CITED

1. **Abelson, H. T., and L. S. Rabstein.** 1970. Lymphosarcoma: virus-induced thymic-independent disease in mice. Cancer Res. **30**:2213-2222.
2. **Baltimore, D., A. Shields, G. Otto, S. Goff, P. Besner, O. Witte, and N. Rosenberg.** 1979. Structure and expression of the Abelson murine leukemia virus genome and its relation to a normal cell gene. Cold Spring Harbor Symp. Quant. Biol. **44**:849-854.
3. **Boss, M., M. Greaves, and N. Teich.** 1979. Abelson virus-transformed haematopoietic cell lines with pre-B cell characteristics. Nature (London) **278**:551-553.
4. **Chamberlain, J. P.** 1974. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98**:132-135.
5. **Cline, M. J., and D. W. Golde.** 1979. Cellular interactions in haematopoiesis. Nature (London) **277**:177-181.
6. **Dexter, T. M., and L. G. Lajtha.** 1974. Proliferation of haemopoietic stem cells *in vitro*. Br. J. Haematol. **28**: 525-530.
7. **Dexter, T. M., and L. G. Lajtha.** 1976. Proliferation of hemopoietic stem cells and development of potentially leukemic cells *in vitro*, p. 1-5. *In* J. Clemmensen and D. S. Yohn (ed.), Comparative leukemia research. Karger, Basel.
8. **Gallimore, P. H., and R. C. Paraskeva.** 1979. A study to determine the reasons for differences in the tumorigenicity of rat cell lines transformed by Adenovirus 2 and Adenovirus 12. Cold Spring Harbor Symp. Quant. Biol. **44**:703-713.
9. **Gartner, S., and H. S. Kaplan.** 1980. Long-term culture of human bone marrow cells. Proc. Natl. Acad. Sci. U.S.A. **77**:4756-4759.
10. **Goff, S. P., E. Gilboa, O. N. Witte, and D. Baltimore.** 1980. Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell **22**:777-785.
11. **Graf, T., and H. Beug.** 1978. Avian leukemia viruses—interaction with their target cells *in vivo* and *in vitro*. Biochim. Biophys. Acta **516**:269-299.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
13. **Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang, and S. M. Astrin.** 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell **23**:323-334.
14. **Payne, G. S., S. A. Courtneidge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus.** 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell **23**:311-322.
15. **Phillips, R. A.** 1978. Stem-cell heterogeneity: pluripotent and committed stem cells of the myeloid and lymphoid systems, p. 9-120. *In* B. Clarkson, P. A. Marks, and J. E. Till (ed.), Differentiation of normal and neoplastic hematopoietic cells, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Pollack, R., A. Lo, B. Steinberg, K. Smith, H. Shure, G. Blanck, and M. Verderame.** 1979. SV40 and cellular gene expression in the maintenance of the tumorigenic syndrome. Cold Spring Harbor Symp. Quant. Biol. **44**:681-688.
17. **Reynolds, F. H., T. L. Sacks, D. Deobagkar, and J. R. Stephenson.** 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polyprotein containing structural and nonstructural components. Proc. Natl. Acad. Sci. U.S.A. **75**:3974-3978.
18. **Risser, R., M. Potter, and W. P. Rowe.** 1978. Abelson virus-induced lymphomagenesis in mice. J. Exp. Med. **148**:714-726.
19. **Rosenberg, N., and D. Baltimore.** 1976. A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. J. Exp. Med. **143**:1453-1463.
20. **Rosenberg, N., and D. Baltimore.** 1978. The effect of helper virus on Abelson virus-induced transformation of lymphoid cells. J. Exp. Med. **147**:1126-1141.
21. **Rosenberg, N., D. Baltimore, and C. D. Scher.** 1975. *In vitro* transformation of lymphoid cells by Abelson murine leukemia virus. Proc. Natl. Acad. Sci. U.S.A. **72**:1932-1936.
22. **Rosenberg, N. E., D. R. Clark, and O. N. Witte.** 1980. Abelson murine leukemia virus mutants deficient in kinase activity and lymphoid cell transformation. J. Virol. **36**:766-774.
23. **Rosenberg, N., and O. Witte.** 1980. Abelson murine leukemia virus mutants with alterations in the virus-specific P120 molecule. J. Virol. **33**:340-348.
24. **Scher, C. D., and R. Siegler.** 1975. Direct transformation of 3T3 cells by Abelson murine leukemia virus. Nature (London) **253**:729-731.
25. **Siden, E. J., D. Baltimore, N. Rosenberg, and D. Clark.** 1979. Immunoglobulin synthesis by lymphoid cells transformed *in vitro* by Abelson murine leukemia virus. Cell **16**:389-396.
26. **Silverstone, A. E., N. Rosenberg, V. L. Sato, M. P. Scheid, E. Boyse, and D. Baltimore.** 1978. Correlating terminal deoxynucleotidyl transferase and cell surface markers in the pathway of lymphocyte ontogeny, p. 433-453. *In* B. Clarkson, P. A. Marks, and J. E. Till (ed.), Differentiation of normal and neoplastic hematopoietic cells, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Silverstone, A., L. Sun, O. N. Witte, and D. Baltimore.** 1980. Biosynthesis of murine terminal deoxynucleotidyltransferase. J. Biol. Chem. **255**:791-796.
28. **Teich, N. M., and T. M. Dexter.** 1978. Effects of murine leukemia virus infection on differentiation of hematopoietic cells *in vitro*, p. 657-670. *In* B. Clarkson, P. A. Marks, and J. E. Till (ed.), Differentiation of normal and neoplastic hematopoietic cells, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Van Zaane, D., and H. P. J. Bloemers.** 1978. The genome of the mammalian sarcoma viruses. Biochim. Biophys. Acta **516**:249-268.
30. **Witte, O. N., S. Goff, N. Rosenberg, and D. Baltimore.** 1980. A transformation-defective mutant of Abelson murine leukemia virus lacks protein kinase activity. Proc. Natl. Acad. Sci. U.S.A. **77**:4993-4997.
31. **Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore.** 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblasts and lymphoid cells. Proc. Natl. Acad. Sci. U.S.A. **75**:2488-2492.