

In vitro* infection of lymphoid cells by thymotropic radiation leukemia virus grown *in vitro

(RNA C-type virus/murine lymphosarcoma)

MARTIN HAAS[†] AND JO HILGERS[‡]

[†] Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel; and [‡] Department of Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

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ABSTRACT Murine lymphoid cells were infected *in vitro* with purified leukemogenic radiation leukemia virus (RadLV) produced by virus-induced lymphoblast cell lines. Thymocytes were shown to be highly susceptible to infection by the virus, whereas murine or other fibroblasts were refractory to it. Murine bone marrow and spleen cells were shown to be much less sensitive to infection by this thymotropic RadLV. By comparison, a B-tropic RadLV isolate (RadLV*), propagated on a mouse fibroblast cell line, was noninfectious for lymphoid cells but infected fibroblasts. A correlation was shown to exist between *in vitro* infection of thymocytes, as assayed by immunofluorescence, and *in vivo* leukemogenicity of the thymotropic RadLV. This constitutes a rapid *in vitro* test for *in vivo* leukemogenicity of a natural lymphatic leukemia virus.

The endogenous RNA C-type viruses that occur *in vivo* in C57BL/6 (B6) mice after exposure to physical agents (1) are clearly linked etiologically to the lymphatic leukemias induced in these mice. These viruses, collectively designated radiation leukemia virus (RadLV), have been propagated *in vivo* (2). They can be consistently extracted from tumors that have been induced by virus injection, and to a lesser extent from tumors induced by x-irradiation (1, 3). Recent biochemical and genetic evidence has shown that multiple biologically distinguishable C-type viruses are naturally present within the cells of many mouse strains (4, 5). Thus far, almost all of these induced C-type RNA viruses have been shown to lack oncogenicity *in vivo*. Two inducible mouse C-type virus preparations have been demonstrated to cause lymphatic leukemia *in vivo*. Stephenson *et al.* (6) have induced RNA C-type viruses in cell culture fibroblasts which were shown to be leukemogenic *in vivo*. Haas has shown (7) that a purified C-type cell-culture-derived virus originally induced by x-rays in B6 mice is leukemogenic in its original host.

A problem that has hampered progress in murine leukemia virus (MuLV) research involves the decreased leukemogenicity of many murine leukemia viruses after their passage through cultures of mouse embryo fibroblasts (MEF). Long-term passage of MuLV through mouse embryo fibroblasts usually results in the production of "attenuated" virus that has lost most or all of its pathogenic properties; this has been shown to be true for the Friend (8-10) and Rauscher (10-12) erythroleukemia-inducing strains of MuLV, and re-

cently also for the natural lymphatic leukemia-inducing Gross-AKR (10, 13, 14) and the radiation leukemia (RadLV) (15) variants of MuLV. In contrast to the loss of oncogenicity of MEF-grown virus, long-term *in vitro* culture of MuLV has consistently produced potent *in vivo* leukemogenic viruses whenever the virus was cultered in or produced by cells of lymphatic origin (7, 16, 17).

Studies from a number of laboratories have amply demonstrated that both Friend MuLV and Rauscher MuLV (18) are composed of two viral entities with quite different biological properties. The two components together cause the induction of the classical erythroblastic disease pattern described by Friend (19). These virus complexes are composed of a replicating lymphatic leukemia virus (LLV) (20) and a "defective" spleen focus forming virus (SFFV). The two entities have been studied separately by a variety of biological methods, and loss of the "defective" virions is responsible for attenuation of these virus complexes when passaged through mouse fibroblast cultures (see ref. 21 for a comprehensive review). It has not been possible so far to obtain the "defective" SFFV particles free of the helper LLV, so that one could not test whether the "defective" virions have by themselves pathogenic properties, as is seen in combination with the helper LLV.

The basis for attenuation of the LLV grown in fibroblast tissue culture cells is obscure. The observed attenuation may be caused by the selection of viral mutants that grow on fibroblasts but have lost their affinity for lymphoid cells and their leukemogenicity. Alternatively it may be caused by the loss of one of the virus agents which, by analogy, may be present in a heterologous mixture of viruses in the *in vivo* virus preparations.

So far only circumstantial evidence suggests that the natural LLV may be composed of more than one viral entity. That this is a theoretical possibility has been suggested by Lilly and Pincus (ref. 21, p. 272). The loss of Gross-AKR (LLV) pathogenic properties has been reported by passage of this virus through some mouse strains *in vivo* (22). These investigators stressed the independence of the *in vitro* XC-assay and *in vivo* assays of LLV.

In this communication we describe a cell-culture-derived LLV of the mouse and some of its biological properties. This virus is leukemogenic *in vivo*, can infect mouse thymocytes *in vitro*, but is totally unable to infect mouse, rat, or rabbit fibroblast cells, whether embryo cells or various cell lines are used. These properties are contrasted with those of the B-tropic MEF-passaged RadLV, which is not leukemogenic, does not infect thymocytes, infects MEF cells, and can serve as a helper virus in various virological tests.

Abbreviations: RadLV, radiation leukemia virus; GLV, Gross leukemia virus; MuLV, murine leukemia virus; LLV, lymphatic leukemia virus; MEF, mouse embryo fibroblast; IFC assay, immunofluorescent cell assay.

[†] Present address: Department of Cell Biology, The Weizmann Institute of Science, Rehovot, Israel.

The name lymphatic leukemia virus (LLV) traditionally designates the lymphatic leukemia-inducing virus obtained *in vivo* and from some mouse cell cultures (20). For the purpose of this discussion and in order to clearly differentiate the various agents, we will use the notation of Declève *et al.* (27), using an asterisk to designate the nonleukemogenic virus selected by passage of RadLV through B6 mouse fibroblasts, RadLV*. The leukemogenic virus that is produced *in vitro* by RadLV-induced thymoma cells we will call thymotropic RadLV.

MATERIALS AND METHODS

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in 60-mm plastic petri dishes. The cells used included clonal lines of continuous contact-inhibited mouse cells, BALB/3T3 (23) and NIH/3T3 (24); a normal rat kidney (NRK) (25) cell line; BL-5, an established line of C57BL/Ka mouse embryo fibroblasts kindly supplied by Dr. Alain Declève; M-6, an established line of B6 mouse embryo fibroblasts, kindly supplied by Dr. E. Heller; SC-1, a wild mouse embryo cell line highly susceptible to infection by N- and B-tropic murine leukemia virus strains, kindly supplied by Dr. A. Hackett. Secondary cultures of embryos of C3HeB, BALB/c, C57BL/6, Lewis rat, and B/N rat strains were prepared by methods described (26).

Cultures of mouse thymocytes, spleen cells, and bone marrow cells were prepared by aseptically removing the respective organs from 2-month-old B6 mice. Thymuses and spleen were passed through a grid to disperse the cells. Single cell suspensions were washed with medium containing 1% fetal calf serum, suspended for 1–2 min at room temperature in a solution of 0.83% ammonium chloride to remove erythrocytes, and washed twice with medium. Viability of the cells was determined by means of trypan blue exclusion. Each 60-mm plate contained 4×10^6 viable mouse cells at the time of infection.

Viruses. Included was the thymotropic virus 136-4, a RadLV produced in tissue culture from a continuous line of B6 RadLV-induced thymoma (7). The virus was concentrated and purified through two sequential bandings in continuous sucrose gradients and had a % IFC titer of 6×10^7 per 0.4 ml when assayed by the % IFC assay of Declève *et al.* (27) on B6 thymocytes. K127, a similar thymotropic virus, was produced by a continuous rat lymphoma cell line K127R, originally induced *in vivo* by injection of P127 RadLV. This virus had a titer of 2×10^8 /0.4 ml by the % IFC assay on B6 thymocytes. RadLV*, *in vitro* passaged RadLV, was harvested from the supernatant fluid of 3-day-old BL5 (RadLV) cell cultures (titer 3×10^7 /0.4 ml) (15). GLV*, *in vitro* passaged Gross leukemia virus, was harvested from the supernatant fluids of 3-day-old C3H (GLV) cell cultures. The last two virus preparations were kindly supplied by Dr. A. Declève. Passage 127 (P127) and passage 136 (P136) are preparations of RadLV prepared as cell-free extracts of RadLV-induced B6 mouse lymphoma tissue, as described (28).

Infection Procedure was the D-P procedure described by Declève *et al.* (27), which involves a 1-hr treatment of the cells with 25 μ g/ml of DEAE-dextran in modified Eagle's medium containing 1% serum, followed by infection with 0.4 ml of virus diluted in the same medium containing 4 μ g/ml of polybrene (Aldrich Chemical Co.). After 2 hr of virus adsorption, modified Eagle's medium containing 10% fetal calf serum and 4 μ g/ml of polybrene was added. After

48 or 72 hr of incubation, the cells were removed (by means of trypsinization in the case of monolayers), and washed three times in Earle's balanced salt solution without Ca^{++} or Mg^{++} ; cell concentration was adjusted to 10^6 cells per ml, and eight spot microscope slides were made for immunofluorescence. Drying, fixing, and keeping of the fixed slides has been described (29). Lymphoid cells in suspension culture were infected by the same procedure, except that DEAE-dextran treatment of these cells was done on the stock suspension in a tissue culture dish. Cells were then diluted and distributed to 60-mm plates at the proper concentration. Medium was removed by careful suction after allowing the plates to rest on the bench for a few minutes so the cells could settle on the bottom of the plates.

Indirect Immunofluorescence was used throughout. The methods have been extensively described (29,†), as has the percent immunofluorescent cells test (% IFC) which was used as described (27). The antisera used were of rats carrying a murine GLV-induced syngeneic tumor W/Fu(C58NT)D with an endpoint immunofluorescent titer of 512, and a murine RadLV-induced regressing tumor of Lewis rats, Lewis (K127R), having an endpoint immunofluorescent titer of 1024. The specificity of these antisera for virus-related antigens has been described (29,†). % IFC tests were determined with these antisera at a dilution of 1:8, and using goat antibody against rat IgG (Nordic Pharmaceuticals, Holland) at a dilution of 1:20 in saline.

In Vivo Virus Injection was done intrathymically in 2-month-old female B6 mice and has been described (30). The latent period refers to the time after virus injection when a palpable tumor was found.

XC Plaque Assays were used as described (31, 32), and a reverse XC plaque procedure was used (33). The XC cell line was kindly supplied through the courtesy of Dr. A. Hackett, and showed no spontaneous syncytium formation.

RESULTS

Infection of fibroblast cell cultures with the thymotropic RadLV and control virus RadLV*

Infection of fibroblast cells was followed by the appearance of virus-related antigens in the cytoplasm of 11 different cell lines and in secondary mouse and rat embryo cultures. The production of MuLV gs-antigen was assayed by indirect immunofluorescence, three days after infection by GLV*, RadLV*, and two preparations of sucrose gradient-purified thymotropic RadLV. The infections and % IFC assays on the cell lines BALB/c-3T3, NIH-3T3, and M-6 were done with the thymotropic virus preparations and RadLV* concurrently. Other fibroblast cultures were infected a number of times with four different purified preparations of thymotropic 136-4 virus (the *in vivo* leukemogenicity of which was determined for each preparation) and with four different purified preparations of K127R virus. The results are summarized in Table 1. Titration of the MuLV* preparations, GLV* and RadLV*, clearly showed GLV* to be an N-tropic virus (yielding a one-hit titration curve on NIH cells) and RadLV* a B-tropic virus (yielding a one-hit titration curve on BALB/c cells), as has been shown by Pincus *et al.* (41) and by Declève *et al.* (42). In no experiment was there any evidence of infection of mouse or rat fibroblasts by the thymotropic viruses.

The cell line SC-1 is highly sensitive to both N- and B-, as well as NB-tropic MuLV. Nevertheless it has not been possi-

† M. Haas (1975), submitted for publication.

Table 1. Infection of mouse and rat embryo cells and cell lines with thymotropic RadLV and MuLV grown in mouse embryo cells

Virus dilution	Percentage of IF-positive cells 3 days after virus inoculation																	
	GLV*			RadLV*			136-4-T RadLV and K127R-T RadLV											
	BALB/c-3T3	NIH-3T3	M-6	BALB/c-3T3	NIH-3T3	M-6	BALB/c-3T3	NIH-3T3	M-6	SC-1	NRK	SIRC	BL-5	C3HeB ME cells	BALB/c ME cells	C57BL ME cells	Lewis rat E cells	B/N rat E cells
10 ⁰	35	80	—†	82	42	90	—	—	—	0	—	—	0	0	0	0	0	0
10 ⁻¹	30	82	24	85	40	90	0	0	0	0	0	0	0	0	0	0	0	0
10 ⁻²	0.4	60	0.3	78	0.8	82	0	0	0	0	0	0	0	0	0	0	0	0
10 ⁻³	0.1	6	—	8	—	10	—	—	—	—	—	—	0	0	0	0	0	0
10 ⁻⁴	—	0.8	—	1.0	—	1.2	—	—	—	—	—	—	0	0	0	0	—	—
10 ⁻⁵	—	0.1	—	—	—	—	—	—	—	—	—	—	0	0	0	0	—	—
No virus‡	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

† Dash means assay not done.

‡ Cells were infected with virus in dilution medium containing polybrene.

ble to show replication of the thymotropic viruses in this sensitive indicator cell line. Since the thymotropic RadLV might be a xenotropic (X-tropic or S-tropic) virus (34, 35), we tried to infect NRK and SIRC cells, known to support replication of xenotropic viruses of murine origin. In no case was there evidence of infection of NRK or SIRC cells with thymotropic RadLV.

The lack of infection of fibroblasts with thymotropic RadLV obtained by the % IFC assay was verified using the XC-syngyia assay, using an AKR virus extract from leukemic AKR spleens as positive control. No evidence for thymotropic RadLV multiplication was obtained after infecting the following cell lines: SC-1, M-6, and secondary embryo cells prepared from the following animal strains: C3HeB, BALB/c, B6, Lewis rat, and B/N rat.

Infection of mouse thymocytes with thymotropic RadLV and control virus RadLV*

Single cell suspensions of mouse thymus cells from 2-month-old B6 mice were infected with a series of 10-fold dilutions of thymotropic RadLV and RadLV* preparations. Table 2 shows that as many as 80% of the thymocytes became MuLV gs-antigen positive 48 hr after infection. RadLV*-infected thymus cells remained MuLV gs-antigen negative, as did mock-infected cells. The spontaneous induction of MuLV

Table 2. Infection of mouse thymocytes with thymotropic RadLV and MuLV grown in mouse embryo fibroblasts

Virus dilution	Percent of IF-positive cells 48 hr after virus inoculation			
	RadLV*	136-4-T RadLV	K127R-T RadLV	No virus
10 ⁰	0	—†	—	
10 ⁻¹	0	70	80	
10 ⁻²	0	5	75	<0.1‡
10 ⁻³	—	0.4	82	
10 ⁻⁴	—	0	19	

† A dash means assay not done.

‡ Noninfected thymocytes were incubated with polybrene-containing medium.

gs-antigen in the thymus cells as reported by Lonai *et al.* (36) was not observed with cells of B6 mice from either the Netherlands Cancer Institute or the Weizmann Institute. The different culture conditions used in this study as compared to those of Lonai *et al.* may account for the difference. The fluorescence found in fixed thymotropic-RadLV-infected thymocytes appeared as many distinct fluorescent dots all over the cell, unlike the fluorescence of MuLV*-infected cells in which the fluorescence appeared to be continuous rather than dotted.

Time course of infection of cells from mouse lymphatic organs with thymotropic RadLV

Mouse thymus, bone marrow, and spleen cells were infected with two preparations of thymotropic RadLV, and the kinetics of appearance of MuLV gs-antigen positive fluorescent cells was determined. Table 3 shows that the percent of immunofluorescent thymus cells rose within hours after infection and reached a plateau of 60% in K127R virus-infected thymus cells and 30% in 136-4 virus-infected cells 48 hr after infection. At this time viability of the cells was approximately 55%. Infection of bone marrow and spleen cells from the same animals, done concurrently, was lower and reached a maximum of 10% IF-positive cells. Viability of bone marrow cells and spleen cells was similar to that of thymus cells up to 48 hr, after which their viability dropped more rapidly. It is not yet known which subpopulation of cells in each organ tested is susceptible to the virus, although it is clear that a high proportion of thymus cells is susceptible to infection with thymotropic RadLV. A smaller fraction of bone marrow and spleen cells is susceptible to the thymotropic RadLV. After infection of cells from these organs with undiluted virus, a maximum of 10% of these cells became MuLV gs-antigen positive.

Correlation between percent immunofluorescent thymocytes and *in vivo* leukemia induction

A preparation of 136-4 thymotropic virus purified by sucrose gradient centrifugation was tested by intrathymic injection of virus at various dilutions into 6-week-old female B6 mice, and by *in vitro* thymocyte infection as assayed by percent immunofluorescent cells. Table 4 shows that a correlation

Table 3. Time course of infection of cells from C57BL lymphatic organs with T RadLV viruses 136-4 and K127R†

Hr after virus infection	% Viable cells	Percentage of IF-positive cells at various times after virus inoculation								
		Thymocytes			Bone marrow cells			Spleen cells		
		136-4-T RadLV	K127-T RadLV	No virus	136-4-T RadLV	K127-T RadLV	No virus	136-4-T RadLV	K127-T RadLV	No virus
2	85	2	10	0	0	2	0	0	0	0
5	85	18	21	2	2	0	0	5	5	0
22	80	18	38	0	5	8	0	14	8	0
32	70	—‡	—	0	8	10	0	10	0	0
49	55	32	60	1	—	12	0	—	10	3
70	50	28	64	0	5	9	0	11	14	0

† Both virus preparations were used at a dilution of 1:30 of the sucrose gradient purified stock described under *Materials and Methods*.

‡ The dash means not tested.

exists between the % IF-thymocytes *in vitro* assay and *in vivo* leukemogenicity. Infection of B6 thymocytes *in vitro* with thymotropic RadLV shows a one-hit dose-response relationship (Table 4), reflecting a high level of susceptibility of these cells for thymotropic RadLV infection (37). A high level of susceptibility of B6 thymocytes for naturally occurring, *in vivo* RadLV has been previously shown by injection of that virus intrathymically *in vivo* (43, 44). These authors also demonstrated a correlation between the IF assay in thymocytes infected *in vivo* and the *in vivo* leukemogenicity of the virus. Leukemias induced by lymphatic leukemia viruses are of T-cell type (38, 39); T-lymphocytes seem to be the cells susceptible to RadLV oncogenicity. The thymotropic RadLV derived *in vitro* induces leukemias *in vivo* in cells apparently the same as those susceptible to this virus *in vitro*. Thus it is plausible that the % IFC test of 136-4 virus-infected thymocytes indeed represents a valid *in vitro* test for *in vivo* leukemogenicity. Therefore, these data may be a demonstration of a rapid, direct *in vitro* test for leukemogenicity (40).

DISCUSSION

Attenuation of oncogenic mouse C-type viruses by passage through fibroblast cultures

Loss or "attenuation" of murine leukemia virus *in vivo* activity by passage of the virus through fibroblast tissue culture cells has been observed repeatedly (8-15, 21). Indeed, passage of Gross or RadLV lymphatic leukemia viruses through sensitive fibroblast cell cultures and establishment of continuous virus producing fibroblast cell lines causes the total attenuation of these viruses (14, 15). On the other hand, upon passage of GLV through susceptible cells of lymphoid origin (16, 17) or production of RadLV in similar cells (7), the virus retains *in vivo* leukemogenicity, while virus infectivity on fibroblast cells is completely lost, as is shown here. The data presented in this paper therefore show that *in vitro* thymocyte passage of RadLV selects a thymotropic, leukemogenic virus that has little predisposition for growth on murine or other fibroblasts. Two possible interpretations may account for this selection phenomenon. One is that *in vitro* passage of natural RadLV, which has been shown to be leukemogenic (41) and thymotropic (‡, 43, 44), is initially capable of replication on fibroblasts (15), though perhaps with some difficulty (42). Selective passage on fibroblast cultures

would tend to select variants that grow better on fibroblasts (RadLV*) but that have lost their leukemogenicity and thymotropism, whereas converse passage on thymocytes tends to select variants that may have lost their capacity to grow on fibroblasts (i.e., the thymotropic RadLV under discussion), but retain leukemogenicity and thymotropism. A second hypothesis is also entirely plausible. According to this alternative hypothesis, natural *in vivo* RadLV would be composed of a heterologous mixture of two different viruses, a thymotropic agent and a virus capable of infecting fibroblasts only. Propagation of the natural virus on either cell type would favor selection of only one of the two virus components. The loss of either agent would accentuate the particular properties of the remaining, propagated virus.

The data presented here do not distinguish between these two possible interpretations. Natural tumor-derived RadLV is thymotropic *in vivo* (43, 44), and the *in vitro* derived thymotropic RadLV 136-4 is thymotropic *in vivo*‡ and *in vitro* (this paper). It seems therefore plausible that the thymotropic RadLV represents the transforming vector whereas the B-tropic RadLV*, which is sometimes present in radiation-induced thymomas, is either a passenger virus or performs a hitherto unknown function in leukemia induction. By analogy, some support for this interpretation comes from the lymphotropism of the Abelson murine leukemia virus, with

Table 4. Correlation between percent immunofluorescent C57BL thymocytes infected *in vitro* for 48 hr with 136-4-T RadLV at various dilutions, and *in vivo* leukemogenicity in C57BL mice injected intrathymically with 0.02 ml of virus at various dilutions

Virus dilution	% IFC	% Leukemic mice†	L.P.‡
10 ⁰	—§	90 (9/10)	88 ± 16
10 ⁻¹	70	60 (6/10)	94 ± 22
10 ⁻²	5	25 (3/12)	113 ± 29
10 ⁻³	0.4	10 (1/10)	132
10 ⁻⁴	0	0 (0/10)	—

† The numbers in parentheses give the number of leukemic mice out of the total injected mice in the group. The period of observation was 150 days.

‡ Latent period of tumor appearance ± standard deviation, in days.

§ Dash means not determined.

which direct *in vitro* transformation of lymphoid cells has been demonstrated (45).

The correlation between *in vitro* infection of thymocytes with 136-4 thymotropic RadLV and *in vivo* induction of leukemia by this virus, as shown in Table 4, suggests that the *in vitro* infection of B6 thymocytes assayed by immunofluorescence described in this paper indeed represents a function similar to an *in vivo* step leading to RadLV-induced leukemia.

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