Leukemogenicity and Cell Transformation Mechanisms In Vitro by Gross Murine Leukemia Virus: Analysis of Virus Subpopulations

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The leukemogenic activity of Gross murine leukemia virus adapted to rats was tested in W/Fu rats and NIH/Swiss mice. All animals infected with this virus developed thymic and nonthymic T-cell leukemia with a short latency period. It was observed that cell-free extracts from thymic lymphoma tissue of mice and rats, induced by either Gross murine leukemia virus or Gross murine leukemia virus adapted to rats, consisted of both small-plaque-forming and large-plaque-forming viruses, as determined by the XC plaque test. MCF-type virus was found in these virus complexes. Transformed cell foci were induced in SC-1 cell layers by double infection of the cloned MCF-type virus and an ecotropic virus. SC-1 cells containing transformed cell foci were shown to be tumorigenic upon inoculation into nude mice. The formation of transformed cell foci in mink lung cells was also observed after double infection with the cloned MCF-type virus and a xenotropic virus. The possible mechanism of leukemogenesis by endogenous viruses is discussed.

Passage A of Gross murine leukemia virus (G-MuLV complex), which shows high leukemogenic activity in mice and rats (4), has been studied in our laboratory as a prototype of murine leukemia virus (MuLV). The distribution pattern of G-MuLV adapted to rats (G-MuLV-AR complex) correlates well with the leukemogenic process in rats (14). On the other hand, the dissociation between ecotropic virus infectivity and leukemogenicity has been shown in mice (9, 11, 12, 18-20, 26, 31, 32). The endogenous oncoviruses of mice, which exist as the inherited compounds of the normal mouse genome, are generally classified into three groups on the basis of the host range: ecotropic (capable of infecting mouse cells), xenotropic (capable of infecting only xenogeneic cells), and dual tropic (capable of infecting both mouse cells and xenogeneic cells) (1, 2, 7, 16, 24, 30). Only the last group of virus has been considered to be responsible for the pathogenesis of murine leukemia (3, 5, 21, 23).

It has been presumed that G-MuLV consists of heterologous viral populations (19). In this study, we demonstrate the existence of the dual-tropic virus in animals infected with either G-MuLV complex or G-MuLV-AR complex. We also show that the induction of transformed cell foci in SC-1 cell cultures is achieved by collab-

oration of a cloned MCF-type virus with an ecotropic virus and that transformed cell foci in mink lung cell cultures (MLC) are also induced by the coinfection of a cloned MCF-type virus and a xenotropic virus.

MATERIALS AND METHODS

Animals. Randomly bred NIH/Swiss mice were obtained from Y. Takamori (Radiation Center of Osaka Prefecture, Osaka, Japan) in 1973. The colony has been maintained in our laboratory by random breeding. This mouse stock does not develop leukemia spontaneously within the 1st year of life (15). W/Fu rats were sent by the late Jacob Furth (Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York) in 1962. Their offspring have been produced in our laboratory by brother-sister mating. The nude mice, with the genetic background of BALB/c (BALB/c, *nu/nu*, specific pathogen-free), were obtained from the Institute of Medical Science, University of Tokyo, Tokyo, Japan. They were maintained in vinyl plastic isolators in filter-capped cages to prevent microbial infection and given sterilized pellets and water.

Viruses. A stock of G-MuLV complex (passage VR 590) was obtained from the American Type Culture Collection, Rockville, Md. Several litters of NIH/Swiss mice were inoculated with G-MuLV complex within 48 h after birth, and cell-free extracts (CFEs) (passage 5) were used as the source of G-MuLV complex. The stock of G-MuLV-AR complex was obtained

from H. Okano (Cancer Institute of Kyushu University, Fukuoka, Japan) in 1967 and has been maintained in our laboratory through serial cell-free passage in newborn W/Fu rats as previously described (33). MCF 247 virus, xenotropic virus (BALB-IU-1), and ecotropic virus (AKR L1) were the gifts of A. Ishimoto (Institute for Virus Research, Kyoto University, Kyoto, Japan) in 1979.

Cultures. A mouse cell line, SC-1 cells (6), was grown and maintained in Eagle minimal essential medium supplemented with heat-inactivated 5% fetal calf serum. The MLC line (ATCC CCL-64) (10) and mink S⁺L⁻ cells (22), originally derived from CCL-64, were cultured in Dulbecco-Vogt-modified Eagle minimal essential medium containing 10% heat-inactivated fetal calf serum. These cell lines were also obtained by the courtesy of A. Ishimoto in 1979.

Virus assays. Virus assays were performed by the XC plaque assay (27). The procedure for focus induction in SC-1 cells is described below.

Preparation of CFEs. CFEs of various tissues were prepared by the procedure described previously (14).

Induction of leukemia. For the induction of leukemia and the study of MuLV infectivity, 0.1 ml of G-MuLV-AR complex was inoculated subcutaneously into NIH/Swiss mice and intraperitoneally into W/Fu rats within 48 h after birth.

RESULTS

Induction of leukemia in W/Fu rats and NIH/Swiss mice with G-MuLV-AR complex. The leukemogenic activity of G-MuLV-AR complex in rats and mice is shown in Table 1. The virus displayed a marked leukemogenicity in both species. Leukemias developed in 100% of the neonatally infected rats, with a mean latent period of 65 days, and all cases were macroscopically thymic. Every virus-inoculated mouse also developed leukemia, with a mean latent period of 66 days, and 7 of 16 cases were macroscopically nonthymic, whereas the remaining cases were thymic. The cytotoxicity test, using brain-associated θ antiserum in mice and C3H anti- θ -AKR serum in rats, showed that all leukemias were of the T-cell type.

Difference in XC plaque size. We noticed that the XC plaques produced by CFEs from animals inoculated with G-MuLV-AR complex

were smaller than those produced by CFEs from animals inoculated with G-MuLV complex. Therefore, the sizes of XC plaques formed by CFEs of thymic lymphomas which were induced in NIH/Swiss mice and W/Fu rats by either G-MuLV complex or G-MuLV-AR complex were compared (Fig. 1).

Most of the plaques produced by CFEs from tissues of G-MuLV complex-infected mice were large, measuring from 2 to more than 10 mm in diameter. In contrast, the majority of plaques produced by CFEs from tissues of G-MuLV-AR complex-infected mice or rats were more homogeneous and much smaller than those observed in specimens of G-MuLV complex-infected mice, measuring less than 1 mm in diameter. These small plaques each appeared to be a single syncytium.

Isolation and characterization of an MCF-type virus and an ecotropic virus from G-MuLV complex and G-MuLV-AR complex. The CFE from the thymus of a leu-

FIG. 1. Frequency distribution of XC plaque size. Each group consisted of five leukemic thymic CFEs.

TABLE 1. Induction of leukemia with G-MuLV-AR in rats and mice^a

Species (Strain)	Incidence of leukemia (%)	Type of leukemia		Latency (days) ^b
		Thymic	Non-thymic	
Rat (W/Fu)	9/9 (100)	9	0	65 ± 6
Mouse (NIH/Swiss)	16/16 (100)	9	7	66 ± 10

^a Animals were inoculated intraperitoneally or subcutaneously with G-MuLV-AR within 48 h after birth.

^b Mean ± standard deviation.

kemic mouse, previously infected with G-MuLV complex, was used to infect MLC. Cytopathic focal changes on MLC (Fig. 2) appeared after several transfer generations of the infected MLC cultures. The lesion was morphologically similar to that induced by AKR-MCF 247 (8). The virus recovered from the seventh culture was injected into NIH/Swiss mouse embryo cells and MLC alternatively, twice each, to eliminate the ecotropic, xenotropic, and phenotypically mixing viruses (13). Finally, after these steps, the virus was carried through two cycles of limiting dilution on MLC. The MCF-type virus from G-MuLV-AR complex was obtained by a similar procedure. The viruses obtained by this procedure (G-MCF 1302 and G-MCF-AR 3341) were free from the large-XC plaque-forming virus. They showed the dual host range and focus-forming ability in mink S⁺L⁻ cells. MLC cultures preinfected with xenotropic virus (BALB-IU-1) were completely refractory to the induction of cytopathic effect-like focus by either G-MCF-1302 or G-MCF-AR-3341. SC-1 cells preinfected with an ecotropic virus (AKR L1) were also resistant to focus induction as determined by the UV mink cell procedure.

The large-XC plaque-forming ecotropic virus was isolated from G-MuLV complex or G-MuLV-AR complex by carrying through two cycles of limiting dilution on SC-1 cells (ecotropic G-MuLV 1302 or ecotropic G-MuLV-AR 3341, respectively).

Focus production on SC-1 cell layers by CFEs of thymic lymphoma tissue. We found that some of the CFEs from thymic lymphoma tissue of mice and rats, induced by either G-MuLV complex or G-MuLV-AR complex, produced altered cell foci directly on the SC-1 cell layer (Table 2). The focal lesions morphologically resembled transformed cell foci. Petri dishes (60 mm) were seeded with 3×10^5 SC-1 cells in Eagle minimal essential medium containing 5% heat-inactivated fetal calf serum. On the next day, SC-1 cells were pretreated with $20 \mu\text{g}$ of Polybrene (Aldrich Chemical Co.) ml^{-1} ; then 0.2 ml of 20% (wt/vol) CFE of the thymic lymphoma tissue was added. The medium was changed at 2- to 3-day intervals. Altered cell foci appeared 4 to 6 days after CFE inoculation. Focus productivity seemed to depend on the infectious virus titer, as quantitated by the XC test. A few to approximately 100 foci appeared in a dish. Infected SC-1 cells and culture fluids overlaying altered cells also produced similar foci on the next passage. The virus infectivity of culture fluids overlaying altered cells was examined by the XC test. It was shown without exception that such specimens always contained large plaques. In contrast, fluids from cultures which contained no altered cell foci did not produce large plaques. SC-1 cells producing altered cell foci were directly examined by the UV-XC procedure. Hematoxylin staining was carried out 24 h later. XC cell syncytia appeared

FIG. 2. Mink cell focus, induced by MCF-type virus isolated from G-MuLV complex on MLC (unstained; $\times 100$).

TABLE 2. Correlation of MuLV titers and focus producibility

Animal Viruses ^a	Animal No.	Titer of infectious virus (log ₁₀ PFU/0.04g)	Focus production on SC-1 cell layer	Large XC plaque formation by culture fluids ^b
		SC-1 cell UV XC cell		
NIH/Swiss	1145 ♂	3.42	+	+
G-MuLV	1146 ♂	3.96	+	+
	1151 ♂	4.01	+	+
	1156 ♀	3.77	+	+
	1176 ♀	3.88	N.T. ^c	N.T.
NIH/Swiss	808 ♀	2.30	N.T.	N.T.
G-MuLV-AR	829 ♂	2.90	—	—
	830 ♂	4.05	+	+
	831 ♀	2.82	—	—
	836 ♀	2.38	—	—
W/Fu	3372 ♀	3.14	—	—
G-MuLV-AR	3374 ♀	2.56	—	—
	3377 ♀	2.58	—	—
	3417 ♀	2.08	N.T.	N.T.
	3515 ♀	3.27	+	+

^a Viruses were inoculated at birth.

^b SC-1 plates were inoculated with 0.5 ml of culture medium in which foci have been produced on SC-1 cell layers.

^c N.T., Not tested.

in association with the altered cell foci; XC cell syncytia were formed on exactly the same spots as altered cell foci.

Focus production on SC-1 cell layers by collaboration of a cloned MCF-type virus with an ecotropic virus. SC-1 cell cultures were infected with G-MCF 1302 corresponding to $>3 \log_{10}$ focus-forming units on MLC. Medium changes were carried out at 2-day intervals. When the infected SC-1 cell culture became almost confluent, large-plaque-forming SC-positive ecotropic G-MuLV 1302, corresponding to about $2 \log_{10}$ PFU by the XC test, was doubly infected. Altered cell foci (Fig. 3) similar to those induced by thymic CFEs appeared at 2 to 3 days after ecotropic virus inoculation. Both infected SC-1 cells and culture fluids produced similar foci on the next passage. XC cell syncytia were observed at the same spot as cell foci (Fig. 4). A similar focus induction was also observed with a combination of G-MCF-AR 3341 and large-plaque-forming XC-positive ecotropic G-MuLV-AR 3341. Infection of either MCF-type virus or ecotropic virus alone never produced cell foci on SC-1 cells.

No transformed cell foci appeared on MLC when MLC were infected with viruses released from SC-1 cells (culture fluids) containing altered cell foci. However, formation of transformed cell foci readily occurred if MLC were infected with SC-1 cell culture fluids and a xenotropic virus.

In vivo tumorigenicity of SC-1 cells containing altered cell foci. To judge whether

morphologically altered cell foci on SC-1 cell layers were transformed cell foci or not, virus-infected SC-1 cells were inoculated intramuscularly or subcutaneously into nude mice. No tumor formation was observed in the recipient mice if the mice were inoculated with SC-1 cells harvested soon after a few altered cell foci appeared after double infection of G-MCF 1302 (or G-MCF-AR 3341) and G-MuLV 1302 (or G-MuLV-AR 3341). On the other hand, all recipients developed tumorous nodules at the site of inoculation within 30 days if the mice were given doubly infected SC-1 cells which had been yielding many foci for approximately 30 days. The tumors were grayish-white and elastic to hard in consistency, and they were histologically diagnosed as fibrosarcomas (Fig. 5). These tumors were transplantable in nude mice.

The fibrosarcoma cells grew readily in vitro. The recultured cell density of the fibrosarcoma cells was higher than that of uninfected normal SC-1 cells. SC-1 cells, NIH/Swiss mouse embryo cells, and MLC were infected with viruses produced by the fibrosarcoma cells in vitro. Transformed cell foci appeared on both SC-1 cells and NIH/Swiss mouse embryo cells, whereas no such foci appeared on MLC.

Focus production on mink S⁺L⁻ cell layers by CFEs of thymic lymphoma tissue. The cell-transforming ability of CFEs prepared from G-MuLV complex- and G-MuLV-AR complex-induced thymic lymphomas was tested on mink S⁺L⁻ cell layers. All CFEs tested produced transformed cell foci (Table 3). There were no

FIG. 3. Altered cell focus, induced by MCF-type virus with an ecotropic virus on SC-1 cells (unstained; $\times 100$).

FIG. 4. XC cell syncytium formation at the same spot as cell focus (stained with hematoxylin; $\times 40$).

apparent differences in focus productivity among CFEs, regardless of their origin.

Focus production on MLC layers by CFEs of thymic lymphoma tissue or by double infection of a cloned MCF and a xenotropic virus. We examined the transformability of the thymus CFEs on MLC. Petri dishes (60 mm)

were seeded with 3×10^5 MLC. On the next day, the cultures were infected with 0.2 ml of 20% (wt/vol) CFE of the thymic lymphomas. The medium was changed at 2- to 3-day intervals. There were no appreciable changes in morphology of MLC up to 10 days after inoculation. However, cell transformation occurred if CFE-

FIG. 5. Histology of a tumor which developed in a nude mouse inoculated with SC-1 cells containing altered cell foci (hematoxylin and eosin staining; $\times 400$). The picture shows the typical appearance of a fibrosarcoma.

infected cultures were infected with a xenotropic virus (BALB-IU-1) corresponding to about 70 focus-forming units on mink S^+L^- cells (Table 3). The transformed cell focus could be recognized as a gathering of round refractile cells surrounded by an intact cell monolayer. The features of the foci resemble those produced in MLC by T8 virus, originally isolated from an *in vitro* thymoma cell line of a lymphomatous AKR/J mouse (29).

The transformed cell foci increased in number and became clearer as the culture generation increased. MLC cultures were also infected with G-MCF 1302 or G-MCF-AR 3341, corresponding to $>3 \log_{10}$ focus-forming units in MLC. The BALB-IU-1 virus, corresponding to about $2 \log_{10}$ focus-forming units on mink S^+L^- cells, was doubly infected 3 to 4 days later. A few transformed cell foci, similar to those induced by double infection of thymic CFE and BALB-IU-1, appeared 3 to 5 days after xenotropic virus inoculation. Again, repeated transfer of infected cells produced clearer and larger numbers of transformed cell foci (Fig. 6).

No altered cell foci were formed on SC-1 cells when SC-1 cells were infected with viruses released from MLC (culture fluids) containing transformed cell foci. However, altered cell foci developed readily if SC-1 cells were coinfecting with MLC culture fluids and an ecotropic virus.

DISCUSSION

We have studied the correlation of MuLV infectivity and the development of leukemia by XC assay. In G-MuLV-AR complex-inoculated W/Fu rats, the transition of virus infectivity correlated well with the development of thymic lymphomas (14). In contrast, in G-MuLV complex-inoculated NIH/Swiss mice, high virus infectivity titers were detected in every tissue tested, whether it was involved in leukemogenesis or not (19). The major part of this study was motivated by the finding that, despite the fact that both G-MuLV complex and G-MuLV-AR complex are highly leukemogenic in animals, their infectivity patterns during leukemogenesis are distinctly different. We suppose that both viruses consist of plural viral subpopulations.

Among endogenous RNA viruses, the dual-tropic virus has been suspected to be the one responsible for the induction of leukemia (3, 5, 21, 23). In an effort to characterize the viral subclass responsible for leukemogenic activity of G-MuLV complex and G-MuLV-AR complex, we took advantage of the sharp difference in the size of the XC plaques formed by the two viruses. Particularly noteworthy was that an overwhelming number of plaques observed in thymic CFEs during G-MuLV-AR complex-induced leukemogenesis in NIH/Swiss mice and W/Fu rats were less than 1.0 mm in diameter (Fig. 2). It

TABLE 3. Focus production on mink S^+L^- cell and MLC layer

Animal Viruses ^a	Animal No.	Focus production on mink S^+L^- cell layer (log ₁₀ FFU/0.04g) ^b	Focus production on mink lung cell layer ^c
NIH/Swiss G-MuLV	1157 ♂	2.10	+
	1163 ♀	1.70	—
	1164 ♀	1.48	+
	1177 ♀	2.16	N.T. ^d
NIH/Swiss G-MuLV-AR	818 ♂	1.08	N.T.
	1202 ♂	1.52	—
	1204 ♂	1.88	—
	1208 ♂	1.38	+
W/Fu G-MuLV-AR	3418 ♀	1.88	+
	3515 ♀	2.11	+
	3524 ♀	2.14	+
	3526 ♀	2.07	+

^a Viruses were inoculated at birth.^b FFU, Focus-forming units.^c Xenotropic virus (BALB-IU-1) was added at 24 h after inoculation of CFEs on MLC layer.^d N.T., Not tested. FIG. 6. Transformed cell focus, induced by collaboration of a cloned MCF-type virus with a xenotropic virus on MLC (unstained; $\times 100$).

was inferred that the viral subclass which produces small plaques might be most closely associated with the leukemogenic activity of G-MuLV or G-MuLV-AR complexes.

Although MuLV's cause malignant hematopoietic tumors in vivo, they generally do not

transform cells in vitro. Scher and Siegler (28) reported the ability of the Abelson MuLV to transform NIH/3T3 and BALB/c-3T3 cells in vitro. We were able to demonstrate the focus-forming ability of both G-MuLV complex and G-MuLV-AR complex on SC-1 cells, and we

proved that the induction of the cell focus is associated with the large-plaque-forming XC-positive ecotropic virus. More surprisingly, the similar focus induction on SC-1 cells was achieved by collaboration with a cloned MCF-type virus and an ecotropic virus, and such focus-containing SC-1 cells were shown to be tumorigenic upon inoculation into nude mice. Staal et al. (29) have isolated MuLV's capable of malignant transformation of MLC from an AKR thymoma cell line and from a spontaneous reticulum cell sarcoma developed in an NIH/Swiss mouse partly congenic for the AKR ecotropic virus-inducing locus *Akv-2*. They also found that at least one of the two transforming strains is replication defective, and they interpreted their findings to indicate that the strain might have been rescued by a helper virus, resulting in the generation of pseudotype transforming viruses.

We obtained a negative result in attempting to produce cell foci on MLC by using G-MuLV complex or G-MuLV-AR complex alone. Although these viruses produced cell foci on mink S⁺L⁻ cells, we considered that this did not necessarily imply the presence of the xenotropic virus component because the transforming gene could also be rescued by the dual-tropic virus (8). The transformed cell foci on MLC could be produced after double infection with thymus CFE (G-MuLV complex or G-MuLV-AR complex) and a xenotropic virus. The similar transformed cell foci were also induced by the collaboration of a cloned MCF-type virus with a xenotropic virus.

From these findings it can be inferred that virus capable of replication can also acquire cell transformability with the assistance of a helper virus. It has recently been shown that oncogenic viruses can be obtained by infecting chemical- or radiation-induced transformed cell lines with nononcogenic viruses, suggesting the cellular origin of the transforming gene (17, 25). If we assume that the dual-tropic virus, one of the components of G-MuLV complex or G-MuLV-AR complex, possesses the transforming gene, a question arises as to why cell transformation does not take place by the single infection of these vigorously replicating viruses.

Cell transformation in vitro by the double infection of dual-tropic MCF-type virus and a helper virus (ecotropic or xenotropic virus) may represent the mechanism of initiation of leukemogenesis in vivo. We have set up experiments in which newborn NIH/Swiss mice were inoculated with either a cloned MCF virus (G-MCF 1302) alone, a cloned ecotropic virus (G-MuLV 1302) alone, or both viruses together. Up to now, one of six mice inoculated with both viruses developed thymic lymphoma, with a latency of

62 days after inoculation, whereas none of the mice inoculated with either virus alone developed any lesions (unpublished data). This study suggests that a considerable number of MCF-type viruses and a relatively small number of helper viruses are required for cell transformation in vitro. This phenomenon coincides with the fact that it takes 2 to 3 months to develop lymphomas after neonatal infection of G-MuLV complex or G-MuLV-AR complex, despite the vigorous replication of infected virus. These findings, both in vitro and in vivo, may imply that MCF-type virus infrequently incorporates the transforming gene which resides in the host cells.

We offer the following hypothesis of cell transformation by MuLV based on our observations. There may be two distinct pathways in dual-tropic virus infection: (i) continuous self-replication occurs in infected cells, which become producer cells; and (ii) the transforming gene, endogenous to the host cell, is recombined, and infected cells become nonproducers. In the latter case, cell transformation is brought about by concurrent infection with the helper virus. There might be other factors, as yet unknown, involved in either pathway.

Although we have not been able to demonstrate the cell transformability of nondefective MCF-type virus that was isolated from both G-MuLV complex and G-MuLV-AR complex, we are still not fully convinced of our results. Every effort is being made to get a definite answer to this important question.

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