Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 789–792, February 1977
Microbiology

A new class of murine leukemia virus associated with development of spontaneous lymphomas

(ecotropic and xenotropic viruses/mink cell cultures/AKR and C58 strains/*Akv-1* and *Akv-2* congenic mice)

JANET W. HARTLEY*, NANCY K. WOLFORD*, LLOYD J. OLD†, AND WALLACE P. ROWE*

* Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014; and †Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021

Contributed by Wallace P. Rowe, December 9, 1976

ABSTRACT **A new type of murine leukemia virus has been detected in thymuses of leukemic and late preleukemic AKR mice, in lymphomas developing in NIH Swiss mice carrying the AKR ecotropic virus-inducing loci *Akv-1* or *Akv-2*, and in the thymus of a preleukemic C58 mouse. The viruses induce focal areas of morphologic alteration in a mink lung cell line and are tentatively referred to as "mink cell focus-inducing" (MCF) strains. They have the host range of both xenotropic and N-tropic ecotropic murine leukemia viruses, are neutralized by antisera to both ecotropic and xenotropic viruses, and are interfered with by both viruses. They may represent a particular type of genetic recombinant which emerges during the preleukemic period in high-ecotropic-virus mouse strains, and they may play a significant role in the etiology of spontaneous lymphomas.**

At 5–6 months of age, mice of the high-ecotropic-virus, high-lymphoma strain AKR undergo marked increases in the level of expression of murine leukemia virus (MuLV) antigens in thymocytes (1). Grafts of these preleukemic thymuses into young AKR mice induce a similar increase in MuLV antigen expression in the recipients' thymocytes (2). The age-related and graft-induced increases in antigen expression are not accompanied by any detectable change in the already high expression of ecotropic MuLV, but they correlate closely with emergence in thymus of MuLVs that have the capacity to infect mink cell cultures (2). Because this capacity is the hallmark of the xenotropic class of MuLV (3–5), we initially assumed that these isolates were xenotropic strains. On further characterization, however, it became clear that many of the isolates are highly unique viruses in many respects and may represent recombinants between endogenous ecotropic and xenotropic MuLVs.

MATERIALS AND METHODS

As described in detail previously (2), MuLV strains capable of infecting heterotypic cells were tested for by plating mitomycin C-treated lymphoid cells as infectious centers on a mink lung cell line (ATCC no. CCL-64) (5). The mink cell cultures so inoculated were maintained as cell lines and, at each passage, were tested for MuLV infection by the fluorescent antibody (FA) procedure. Culture conditions and details of the FA procedure were as described previously (2). Generally, if a line gave no FA staining after one or two transfers, it was discontinued. If FA reaction to MuLV antigen was seen, attempts were made at various passage levels to transmit the virus to other mink cell cultures, or to mouse cells, by passage of cell-free culture extracts or, if these attempts were negative, by plating the mink cells as mitomycin C-treated infectious centers.

Our standard procedures for isolation, propagation, and quantitation of ecotropic and xenotropic MuLV strains have been described (6–8).

Abbreviations: MuLV, murine leukemia virus; FA, fluorescent antibody; CPE, cytopathic effects; ME, mouse embryo; MCF, mink cell focus-inducing.

RESULTS

Recovery of two types of "mink infectious" virus from AKR thymus and other lymphoid tissues

The mink cell lines that became FA-positive as a result of infection with lymphoid cells of AKR mice were of two distinct types—that is, some remained morphologically normal, and others developed, after one or two transfers, focal areas resembling cytopathic effects (CPE), as described below.

The lines that showed no morphologic alterations generally yielded classical xenotropic viruses, with the properties of those recovered from other mouse strains (3–5). That is, the isolates did not induce morphological changes on passage to mink cells, and, of several representative strains tested, none gave XC-cell syncytium formation, all induced transformed cell foci in the mink S^+L^- cell line ($MiCl_1$) of Peebles (9), and none induced FA foci in NIH Swiss- or BALB/c-mouse embryo (ME) cell cultures. Also, none of these strains could be established in serial passage in the SC-1 mouse cell line, although, like other xenotropic strains, they occasionally induced a few small FA foci in the initially infected SC-1 cultures (8). The two strains studied by neutralization testing were serologically like other xenotropic isolates (see Table 1).

In contrast, many of the virus strains established from infected mink cell culture lines that showed the CPE-like focal changes have sufficiently unique properties to warrant consideration as a new class of C-type virus, as described in the next section.

As expected from the large number of AKR thymic cells producing ecotropic virus (2), some of the mink cell lines yielded small amounts of XC-positive ecotropic virus, which had entered the mink cells as phenotypically mixed particles. This did not correlate with presence or absence of the CPE-type changes, and, once the mink-infectious viruses were established by cell-free passage, ecotropic virus was no longer detected.

Characteristics of mink cell CPE-inducing MuLV strains

The following is a listing of the distinctive properties of this class, based primarily on tests of the prototype strain no. 247, which is an isolate from the thymus of a 6-month-old AKR mouse.

1. They induce foci of morphologic alteration in the mink lung cell line like those seen in the originally infected cultures. The foci bear no resemblance to foci of murine sarcoma virus transformation. The mink cell foci develop at 4–7 days after inoculation and appear as somewhat elongated, thickened areas of the cell sheet, slightly darker than the surrounding monolayer and containing pycnotic cells. There is often increased vacuolization of the cells at the edge of these foci, particularly if the foci are in cell cultures that were serially transferred after infection. The vacuolar membranes stain brilliantly for MuLV

Table 1. Comparative neutralization tests of mink cell focus-inducing (MCF) and xenotropic and ecotropic MuLVs

Virus		Serum neutralizing antibody titer*				
		Antiserum to xenotropic virus	Antiserum to ecotropic virus		Normal serum	
Group	Strain†	Rat anti-NZB	Rat anti-AKR	Rabbit anti-AKR	Rat	NZB mouse
MCF	247	80	160	160	—	320
	1659	≥160	160	≥160	—	≥100
	V2-34	160	80	20–40	—	≥100
Xenotropic	BALB-IU-1	640	—	—	—	1280
	AKR-Th-6	320	—	—	—	320
	AKR-Th-40	640	—	—	—	
Ecotropic	AKR L1	—	160	80	—	—

* Reciprocal of highest dilution giving 67% or greater reduction in virus infectivity. — = <20; blank = not tested. Neutralization tests were performed as described in ref. 7. The focus-inducing strains were assayed by CPE-focus induction in mink lung cells; xenotropic strains were tested by focus induction in S^+L^- mink cells; and ecotropic virus was assayed by the XC plaque test in SC-1 cells. Anti-NZB and anti-AKR rat sera were pooled sera from tumor-bearing Fischer rats and were supplied by R. Wilsnack, Huntingdon Research Laboratories. Anti-AKR rabbit serum was prepared by immunization with purified AKR ecotropic virus gp71 and was supplied by J. Ihle, Frederick Cancer Research Center.

† The no. 247 and no. 1659 strains are isolates from normal 6-month-old AKR mice; the V2-34 strain was isolated from a spontaneous lymphoma in an NIH Swiss mouse partially congenic for the AKR virus-inducing locus *Akv-2*. BALB-IU-1 is a xenotropic strain recovered from iododeoxyuridine-induced BALB/c ME cells; AKR-Th-6 and AKR-Th-40 are xenotropic isolates from thymuses of 2- and 6-month-old AKR mice, respectively.

antigen in the FA test, and in thin-section electron micrographs the vacuoles are seen to contain virus-like particles, some of which appear to be budding and free C-type viruses. Large numbers of C-type particles budding from the plasma membranes and in the extracellular space are also seen. In stained preparations, the foci appear as densely staining areas of cells that are smaller than in the surrounding cell sheet. No inclusion bodies have been seen.

In initially infected cultures, the CPE-like changes generally remain localized, presumably because spread of virus is inefficient in confluent cultures. When cultures with large numbers of foci are trypsinized and transferred, infection may progress to involve essentially all cells with eventual destruction of the culture.

The foci are sufficiently distinct to permit counting with a dissecting microscope under oblique lighting as used for murine sarcoma virus foci. Dose-response relationships are consistently one-hit, and the titer estimate is generally within 2- to 3-fold of that obtained by counting MuLV FA foci. The altered cell foci are consistently FA-positive.

Whether these areas should be considered as cytopathic areas or transformed cell foci is not yet clear.

2. They are "N-tropic amphotropic" in host range. They replicate in the mink lung cells, in the SIRC rabbit cell line ATCC no. CCL 60, in the SC-1 mouse cell line, and in ME cells of NIH Swiss and C57BR (*Fv-1^n* mouse strains). They do not replicate in ME cultures of BALB/c, A/J (*Fv-1^b* strains), or (BALB/c × DBA/2)F_1 (*Fv-1^{nb}*) or in the RD line of human cells (10). The N-tropic amphotropic host range pattern was seen with strain no. 247 both before and after limiting-dilution purification, and with all eight other strains tested, including five from preleukemic and leukemic AKR mice and three from lymphomatous NIH Swiss mice carrying the *Akv-2* locus from AKR (see below).

The focal alterations have been seen only in the mink cell cultures, and evidence for replication of the virus in the other cell types was obtained by demonstrating that the cells became FA-positive and produced virus that caused the CPE-like changes in mink cells. Quantitation of the virus in cells in which it does not produce CPE can be achieved by an *in situ* infectious center test in which the infected cultures are killed with UV light (as described in ref. 6) and overlayed with 2×10^5 mink cells; 6 or 7 days later the number of CPE-like foci are counted ("UV-mink procedure"). In comparative titrations, the mink lung and SC-1 cells are of equivalent sensitivity, the NIH-ME cells are 10-fold less sensitive, and the SIRC cells are a further 10-fold less sensitive.

It should be noted that the mink S^+L^- cell assay (9), which is generally quite sensitive for detection of xenotropic MuLV, is inefficient for detection of mink infectious viruses in AKR lymphoid cells. When passed in tissue culture, AKR xenotropic strains become efficient in inducing mink S^+L^- foci, giving equal titers by FA-positive antigen induction in mink lung cells and by S^+L^- focus induction. In contrast, the CPE-inducing strains give 10- to 100-fold lower titers in S^+L^- cells and induce little or no CPE in them.

3. They appear to be genetically stable, nondefective MuLV strains. As mentioned, titrations in mink cells show one-hit patterns; the same is seen in SC-1 and NIH-ME cell cultures, in which the quantitation is done by the UV-mink procedure.

Limiting-dilution purification did not alter the characteristics of the virus. The no. 247 virus was carried through two cycles of limiting-dilution purification in mink lung cells by the procedure previously described (7) and then through a third cycle in which 10 dishes were inoculated per dilution. Of the 10 cultures inoculated with 0.2 ml of a 10^{-5} dilution, four were FA-positive; harvests from all four induced the mink cell focal alterations as well as MuLV FA antigen. Harvests from the six others did not induce FA antigen or CPE-like foci on blind passage to mink cell cultures.

4. They are "XC-negative"; i.e., neither infected mink cell cultures nor infected mouse cell cultures induce syncytia when cocultivated with XC cells.

5. They demonstrate envelope properties of both xenotropic and ecotropic viruses. As shown in Table 1, the viruses are doubly neutralizable, being inhibited by type-specific antisera to both ecotropic and xenotropic viruses; this was true of the no.

Table 2. Detection of MCF-type MuLV in lymphoid cells from normal and lymphomatous mice*

Source [age, month]		Cells tested	Specimens studied	No. cell lines with CPE-like changes/ no. antigen-positive lines obtained
Normal	AKR [2–3]	Thymus	17	0/7
Normal	AKR [6]	Thymus	12	5/11
Grafted†	AKR [3]	Thymus	7	6/7
Normal	AKR [6]	Spleen, LN‡	4	0/4
Normal	C58/Lw [6]	Thymus	2	1/2
		Spleen, LN	2	0/2
Leukemic§	AKR	Lymphoma	3	2/3
Leukemic	*Akv-1* congenic	Lymphoma	3	2/3
Leukemic	*Akv-2* congenic	Lymphoma	4	4/4
Leukemic	C57BL/6	Lymphoma	1	0/1

* Mink cell cultures exposed to mitomycin C-treated lymphoid cells were serially transferred and examined for MuLV FA antigen and CPE-like changes at each subculture. Cell lines in which FA-positive cells were seen were maintained in serial subculture until essentially all cells were FA-positive and usually for several passages thereafter; almost all were carried at least four or five passages. The CPE-like changes developed within the first two passages or not at all.

† Thymuses from 6-month-old AKR mice grafted into 2-month-old AKR mice (2).

‡ LN = lymph nodes, generally pooled axillary and mesenteric.

§ AKR and *Akv-1* and *Akv-2* congenic mice had spontaneous lymphomas, involving thymus and/or spleen and lymph nodes; the C57BL/6 tumor was a primary x-irradiation-induced lymphoma. The *Akv-1* and *Akv-2* partially congenic mouse strains are NIH Swiss carrying one or the other of the two ecotropic virus-inducing loci of AKR/J.

247 virus both before and after limiting-dilution purification. Like xenotropic MuLV strains (11, 12), the mink cell focus-inducing (MCF) viruses are neutralized by normal NZB mouse serum (Table 1).

Interference tests also indicated a relationship to both ecotropic and xenotropic viruses. Mink lung cell cultures preinfected with xenotropic virus [BALB-IU-1 and NZB-IU-1 strains (7)] were completely refractory to focus induction by strain no. 247, and harvests from the challenged cultures did not contain detectable focus-inducing virus. Also, SC-1 cells preinfected with ecotropic virus (AKR L1 strain) were 10- to 80-fold less sensitive to strain no. 247 than were control SC-1 cells, as determined by the UV-mink procedure. Preinfection of mink and SC-1 cultures with a feral mouse amphotropic MuLV (7) gave no inhibition. The interference between no. 247 and ecotropic MuLV does not appear to be reciprocal, however, since XC plaque production by ecotropic viruses in SC-1 cells preinfected with the no. 247 strain was not significantly affected, the reduction being no more than 2-fold. Interference against xenotropic MuLV has not yet been studied.

Nomenclature

In view of the many uncertainties about the nature of this type of virus and the variety of unique properties that it demonstrates, it is important to select one or more defining criteria by which other isolates can be classified. We consider the production of the characteristic altered cell foci in mink cell cultures to be the most distinctive and readily scored trait, and provisionally we will refer to MuLV isolates having this property as MCF (from "mink cell focus-inducing") strains. Whether all such strains will have the "N-tropic amphotropic" host range and the other properties of both ecotropic and xenotropic viruses remains to be determined.

Detection of MCF strains of MuLV in normal and lymphomatous mice

Our experience with detecting virus strains that have the capacity to induce the characteristic mink cell foci strongly suggests a relationship to leukemogenesis (Table 2). They were not encountered among seven mink-infectious virus isolates from thymus of 2-month-old AKR mice; in contrast, of 21 mink-infectious MuLV strains isolated from thymus tissue of 6-month-old AKR mice, 3-month-old AKR mice that had been grafted with 6-month-old AKR thymus (2), or lymphomatous AKR tissue, 13 (62%) induced the CPE-like foci (Table 2). Further, we have detected this type of virus in the thymus of a 6-month-old C58/Lw mouse and in spontaneous lymphomas developing in NIH Swiss mice made partially congenic for the *Akv-1* or *Akv-2* ecotropic virus-inducing loci of AKR (13). It is perhaps significant that none of the six mink-infectious isolates from nonthymic lymphoid tissues of preleukemic mice gave the CPE-like foci, even though four of these were from mice whose thymus was positive for MCF virus. We presume that the MCF-positive specimens also contained xenotropic virus; in addition, all specimens contained large amounts of ecotropic virus, as expected from the known high-virus phenotype of the tested strains.

MCF virus has not been encountered in infectious center tests of thymic cells from small numbers of normal NZB, NZW, BALB/c, or DBA/2 mice or in one primary radiation-induced C57BL/6 thymoma.

A cell-free tissue extract preparation of Gross passage A virus, provided by F. Lilly, did not induce either altered foci or FA foci in the mink lung cells. However, because inoculation of cultures with viable cells, rather than with extracts, has been the procedure by which almost all the MCF isolates have been obtained to date, this negative result cannot be taken to mean that MCF virus is not in some way involved in leukemogenesis by the Gross passage A virus.

DISCUSSION

The properties of the new class of MuLV described here are distinct in several respects from those of the three presently recognized classes of naturally occurring MuLV. The MCF strains are distinct from the AKR ecotropic viruses in host range (mink and rabbit cells), in reactivity in neutralization and interference tests, and in being XC-negative; also, when mink cells were infected with ecotropic viruses, by phenotypic mixing with xenotropic viruses, they did not show any morphologic alterations (A. Ishimoto, J. W. Hartley, and W. P. Rowe. un-

published data). The MCF viruses differ from xenotropic viruses in host range (mouse cells), focus formation in mink cells, reduced efficiency of transformation of mink S^+L^- cells, and reactivity in neutralization and interference tests. They differ from the amphotropic class of MuLV isolated from wild mice (7, 14) in interference specificity and mink cell focus formation; also, the wild mouse amphotropic viruses are not neutralized by antisera to either ecotropic or xenotropic MuLVs (7), whereas the MCF viruses are neutralized by both.

Because the MCF strains have type-specific envelope components of both ecotropic and xenotropic MuLV, as indicated by virus neutralization tests, and apparently are interfered with by both types of virus, our working hypothesis is that they represent a genetic recombinant between the two. Although the possibility of a phenotypically mixed virus population must always be kept in mind, the failure to dissociate the unique phenotypic elements of strain no. 247 by three cycles of limiting-dilution purification appears to rule this out as the basis of the patterns observed here. Support for the recombinational origin of the MCF virus comes from comparison of its properties with those of HIX virus, described by Fischinger *et al.* (15). This virus was detected during passage of a Moloney MuLV stock in feline cells; it is an "NB-tropic amphotropic" virus and is neutralized by antisera to both Moloney MuLV and xenotropic MuLV. The fact that both the MCF and HIX isolates possess the *Fv-1*- determined host range and serological specificity of the postulated parental ecotropic virus argues against an origin independent of the ecotropic MuLV component.

If the MCF viruses do in fact represent genetic recombinants between ecotropic and xenotropic MuLVs, the question arises whether (*i*) the recombination occurred in some previous generation and is preserved as a genetic locus distinct from the known ecotropic virus-inducing loci, or (*ii*) the recombinants are generated independently within the lifetime of the mouse. The detection of MCF strains in the *Akv-1* and *Akv-2* partially congenic mice favors the latter hypothesis; if the AKR MCF virus were the product of a distinctive chromosomal locus in AKR, it is most unlikely that it would be present as a linked or contaminating locus in both of these strains. Other models are certainly possible.

Although it is clear that high expression of ecotropic virus is a necessary factor in the pathogenesis of the high lymphoma incidence of AKR mice (16), a number of unique observations have been reported which indicate that additional virologic factors must be involved. It has long been known that thymus extracts from older AKR mice are more leukemogenic than those from young animals (17, 18), even though more recent studies (19) showed that the amount of ecotropic virus in the thymus does not change with age. Likewise, thymus extracts are more oncogenic than spleen extracts (18), although the latter contain higher titers of ecotropic virus (19). Instances of marked dissociations between leukemogenicity and ecotropic virus titer as determined in fibroblast-type tissue culture have been presented by Vredevoe and Hays (20), Haas and Hilgers (21), and Lieberman *et al.* (22). The MCF virus could be the basis for these discrepancies. It is infectious for mouse cells *in vitro* and thus presumably *in vivo;* it appears in the late preleukemic period, is found in thymus and not in spleen, and would not be detected in XC assays in tissue culture. Other observations that may be explained by the MCF virus are the recently reported ability of thymic epithelium of 6-month-old, but not of 2-month-old, AKR mice to induce malignant transformation of AKR thymocytes *in vitro* (23) and, as noted above, the ability of grafted thymuses of 6-month-old AKR mice to induce the amplified expression of MuLV antigens in young AKR mice (2).

The presence of the MCF type virus in leukemic tissue of the *Akv-1* and *Akv-2* partially congenic mice and in the thymus of a preleukemic C58 mouse further supports the concept that this virus may play a key role in the genesis of lymphoma. It is tempting to speculate that the role of the high-ecotropic-virus phenotype of high-lymphoma mouse strains is to provide large numbers of cells at risk for generating a recombinant that is able to transform thymocytes. Another possibility is that a flurry of recombinational events occurs during the late preleukemic period, and the MCF virus is only one of a number of novel phenotypes that emerge; if this is the case, the MCF may not be the leukemogenic variant but rather a correlate of its presence. The production of the altered cell foci in the mink cells could be a manifestation of an ability to transform. Also, it may be relevant that the vacuolization of mink cells by MCF virus is somewhat similar to that produced in chicken cells by the BH-RSV strain of Rous sarcoma virus (24). Tests of the ability of exogenous MCF virus infection to induce MuLV antigens and lymphomas will be of much interest.

We wish to thank Dr. C. F. T. Mattern, who carried out the electron microscopy; Ms. Sara L. Hourihan, Microbiological Associates, Inc., who has maintained the *Akv-1* and *Akv-2* congenic mouse lines; Dr. James N. Ihle, NCI Frederick Cancer Research Center, who provided the AKR MuLV gp 71 antiserum and the C57BL radiation-induced lymphoma; and Julia Chandler, Marilyn R. Lander, Joan B. Austin, and James M. Toliver, who provided expert technical assistance. This work was supported in part by the Viral Oncology Program of the National Cancer Institute.

1. Kawashima, K., Ikeda, H., Stockert, E., Takahashi, T. & Old, L. J. (1976) *J. Exp. Med.* **144,** 193–208.
2. Kawashima, K., Ikeda, H., Hartley, J. W., Stockert, E., Rowe, W. P. & Old, L. J. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4680–4684.
3. Levy, J. A. (1973) *Science* **182,** 1151–1153.
4. Todaro, G. J., Arnstein, P., Parks, W. P., Lennette, E. H. & Huebner, R. J. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 859–862.
5. Henderson, I. C., Lieber, M. M. & Todaro, G. J. (1974) *Virology* **60,** 282–287.
6. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42,** 1136–1139.
7. Hartley, J. W. & Rowe, W. P. (1976) *J. Virol.* **19,** 19–25.
8. Hartley, J. W. & Rowe, W. P. (1975) *Virology* **65,** 128–134.
9. Peebles, P. T. (1975) *Virology* **67,** 288–291.
10. McAllister, R. M., Melnyk, J., Finkelstein, J. Z., Adams, E. C. & Gardner, M. B. (1969) *Cancer* **24,** 520–526.
11. Aaronson, S. A. & Stephenson, J. R. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1957–1961.
12. Levy, J. A., Ihle, J. N., Oleszko, O. & Barnes, R. D. (1975) *Proc. Natl. Acad. Sci. USA* **71,** 5071–5075.
13. Rowe, W. P. (1972) *J. Exp. Med.* **136,** 1272–1285.
14. Rasheed, S., Gardner, M. B. & Chan, E. (1976) *J. Virol.* **19,** 13–18.
15. Fischinger, P. J., Nomura, S. & Bolognesi, D. P. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 5150–5155.
16. Lilly, F., Duran-Reynals, M. L. & Rowe, W. P. (1975) *J. Exp. Med.* **141,** 882–889.
17. Kaplan, H. S. (1967) *Cancer Res.* **27,** 1325–1340.
18. Nishizuka, Y. & Nakakuki, K. (1968) *Int. J. Cancer* **3,** 203–210.
19. Rowe, W. P. & Pincus, T. (1972) *J. Exp. Med.* **135,** 429–436.
20. Vredevoe, D. L. & Hays, E. F. (1973) *J. Natl. Cancer Inst.* **51,** 1619–1626.
21. Haas, M. & Hilgers, J. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3546–3550.
22. Lieberman, M., Niwa, O., Decleve, A. & Kaplan, H. S. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1250–1253.
23. Waksal, S. D., Smolinsky, S., Cohen, I. R. & Feldman, M. (1976) *Nature* **263,** 512–514.
24. Bader, J. P. (1972) *J. Virol.* **10,** 267–276.