

QUANTITATIVE STUDIES OF NATURALLY OCCURRING MURINE LEUKEMIA VIRUS INFECTION OF AKR MICE*

BY WALLACE P. ROWE AND THEODORE PINCUS

(From the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014)

(Received for publication 5 October 1971)

Since its original derivation by Furth (1), the mouse strain AK has been of central importance to the study of spontaneous murine leukemia. Mice of this strain, and its derivative, AKR (2), show high rates of spontaneous thymic lymphoma and are chronically infected with murine leukemia virus (MLV)¹ (3-5).

In attempting to elucidate the complex pathogenesis of spontaneous murine leukemia, it is important to know the general characteristics of MLV infection in a high leukemic mouse strain. Because of the previous lack of quantitative procedures for assay of naturally occurring MLV, little is known of the sites of virus replication in AKR mice, or of the relationship of the course of the infection to the development of lymphoma. With the development of a rapid, sensitive, and quantitative procedure for detection of MLV infectivity (6), precise studies of the virology of AKR mice have become feasible. This report describes studies of the quantity of infectious MLV in various tissues of AKR mice as a function of age and of the presence or absence of leukemia.

Materials and Methods

Mice.—AKR mice were obtained from the Jackson Laboratories, Bar Harbor, Maine, and from the Animal Production Section of the National Institutes of Health (NIH). Also, sera were obtained from a number of AKR mice in the colony of Dr. Lloyd W. Law. Studies of weanling and older mice were confined to females. Embryos were from plug-dated pregnancies in AKR/J mice.

Mice were killed by cervical dislocation or exsanguination, and tissues were removed aseptically using a separate set of instruments for each tissue; a cautery was used to incise the uterus for removal of embryos. The tissues were ground in a TenBroeck grinder or with a mortar and pestle, and 2-10% extracts were prepared, using a diluent consisting of Eagle's basal medium with 15% veal infusion broth. The extracts were clarified and held at -60°C until tested.

Virus Assays.—Tests for MLV infectivity were done in secondary cultures of NIH strain Swiss mouse embryo (NIH-ME), using the UV-XC procedure to develop focal areas of MLV

* This work was supported in part by the Etiology Area of the National Cancer Institute.

¹ Abbreviations used in this paper: MLV, murine leukemia virus; PFU, plaque-forming units.

infection as plaques (6). Naturally occurring AKR virus has been found to be N-tropic, i.e., to initiate infection most efficiently in N-type (NIH) cells (7, 8).

NIH-ME cells were seeded into 60-mm Falcon plastic petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) at 3.5×10^6 cells/dish. The next day the cultures were treated for 1 hr with 25 $\mu\text{g}/\text{ml}$ diethylaminoethyl (DEAE)-dextran (Sigma Chemical Co., St. Louis, Mo.), rinsed, refed with 4 ml of medium, and inoculated with 0.1 ml of serial dilutions of the tissue extracts. The dishes were held at 36°–37°C in 5% CO_2 atmosphere. Medium was Eagle's minimal essential medium with 10% unheated fetal calf serum, 2 mM glutamine, 250 units/ml penicillin, and 250 $\mu\text{g}/\text{ml}$ streptomycin. Culture fluids were generally changed at 2-day intervals. 6 or 7 days after infection, the fluids were removed and the cells were exposed to 1500–1800 ergs/ mm^2 of ultraviolet light. Medium containing 10^6 XC cells was then added. The cultures were fixed and stained with hematoxylin 3 or 4 days later, and the number of plaque areas was counted under a dissecting microscope. Titers are presented as the number of plaque-forming units (PFU) per 0.1 g of tissue.

Since production of plaques in this system is a complex, multiple-step process, the possibility was considered that naturally occurring AKR virus might not be efficient in triggering the syncytial response of XC cells which occurs when these cells are in contact with ME cells infected with MLV (9). To evaluate this hypothesis, comparative titrations were done on several AKR tissue extracts using the UV-XC plaque test and the induction of immunofluorescent-stainable foci of MLV infection (10) as end points. The latter technique can be considered as a baseline quantitative procedure for determining the maximum number of MLV particles which can initiate detectable foci of infection. Tissue culture passaged virus strains generally show equal titers by the two tests. With unadapted virus in AKR tissue extracts, the number of plaques detected by the UV-XC test was generally about threefold lower than the number of immunofluorescent-stainable foci. Thus, the titers presented here can be considered as being about 0.5 \log_{10} lower than those detectable by the most sensitive infectivity test currently available.

RESULTS

Initial Appearance of Virus during the Perinatal Period.—We have reported previously that infectious MLV is regularly found in weanling and older AKR mice, but is not detectable in extracts of 15–17-day AKR embryos (5); however, virus is produced by AKR embryo cells when grown in tissue culture (5, 11). To determine the time-course of appearance of infectious MLV during development, tissue extracts were tested from embryos and infant mice. Pools of tissues from three mice were tested, consisting of the thoracoabdominal viscera or decapitated, eviscerated carcasses. In a number of instances the mice were dipped in diethyl ether before dissection to inactivate possible contaminating virus; the results were comparable to those without the ether rinse. The results are shown in Fig. 1.

Virus was not detected in 16- or 17-day embryos, was present in trace amounts in two of six carcass extracts of 18–19-day embryos, and was found with increasing consistency and gradually increasing titer thereafter. Thus the initial appearance of detectable virus tended to occur within a few days before birth or shortly thereafter.

Distribution of Virus in Organs of Weanling Mice.—In order to gain insight into which organs are the chief sites of virus replication, a broad sampling of

tissues of weanling female AKR mice was tested (Table I). While virus was present in considerable amounts in all tissues, there was a 30- to 100-fold difference between organs. Highest titers were found in bone, including the tail and the cortex and marrow of the femur, and in the uterus and spleen. The infectivity titer of the weanling thymuses was not exceptional.

Titers of Selected Organs as a Function of Age and Leukemic Status.—Temporal studies of virus distribution were concentrated on those organs involved in leukemogenesis and those with highest titers as determined in the organ

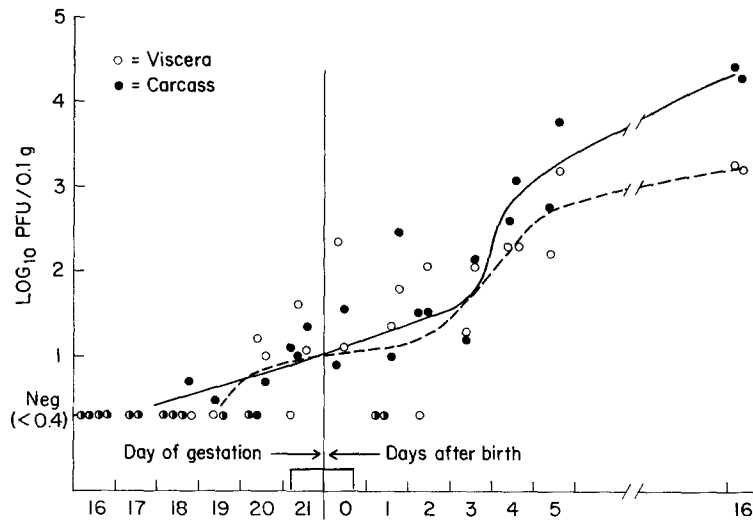


FIG. 1. MLV infectivity titers of extracts of thoracoabdominal viscera (○) and carcass (●) of AKR mice during the perinatal period. The two pools from the same group of mice are on the same vertical axis. The lines are drawn to show the general trend for the viscera (dashed line) and carcass (solid line) extracts.

testing described above. Fig. 2 shows the results. The data are primarily tests of individual mice, but a few pools of tissues of two-five mice are included. All specimens from leukemic mice were from individual mice, and all of the mice in the 3-4 month age group were pregnant. In the 5-10 month age group, there were no notable differences in titers between the younger and older animals, and the data were pooled for presentation.

Except for a small number of sera, all specimens contained virus; of 45 mice tested as individuals for virus in tissue extracts, all were virus positive. In nonleukemic animals, the uterus contained the highest infectivity titers, with bone and spleen slightly lower. Lymph nodes, thymus, and kidney were about 10-fold lower in titer, and serum was lowest.

Several age patterns of virus titer were seen. Nonleukemic spleen, lymph

nodes, thymus, and kidney showed essentially constant titers throughout life. Titers in the uterus increased during early life, and remained constant there-

TABLE I
MLV Infectivity Titers in Various Tissues of 5-6-Wk Old Female AKR Mice

Tissue*	MLV titer†		Tissue	MLV titer	
	AKR/J	AKR/N		AKR/J	AKR/N
Leg bone—cortex	5.8	NT	Intestine	4.6	3.6
Tail	5.7	NT	Adrenals	4.4	4.1
Uterus	5.4	4.9	Kidneys	4.2	4.0
Spleen	5.0	4.8	Pancreas	4.2	3.8
Leg bone—marrow	4.9	5.6	Salivary glands	4.1	3.4
Heart	4.9	4.3	Thymus	3.9	3.9
Ovaries	4.7	NT	Lung	3.8	3.7
Lymph nodes	NT	4.3	Brain	3.6	3.8
Liver	4.7	3.8	Muscle	3.6	3.5
Serum	NT	4.1	Blood clot	NT	3.6

* Pooled tissues from five mice.

† \log_{10} PFU/0.1 g. NT indicates no test.

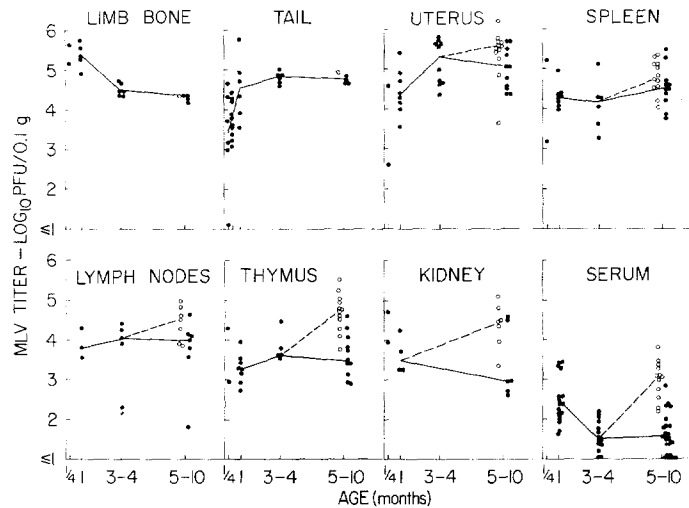


FIG. 2. MLV infectivity titers of selected tissues of nonleukemic (●) and leukemic (○) AKR mice. The lines connect the median titers, solid lines = nonleukemic, and dashed lines = leukemic animals.

after. The two types of bone tested, limb bone and tail, showed contrasting patterns. Limb bone was highest in titer in the young mice, while the titer of tail extracts increased during the first few months of life. The consistency of titer in the bone extracts in later life was striking, particularly in view of the

great variations seen with the other tissues. Serum titers showed a sharp drop after the 1st month of life.

Tissues of leukemic animals tended to show higher virus titers than tissues of nonleukemic mice of comparable age. The difference was highly significant ($P < 0.002$) with the thymus and serum specimens, and was minimal with the uterus and spleen extracts. The higher titers in kidneys of leukemic mice were probably a reflection of infiltration of the organ by leukemic cells.

Virus Titers in Pregnant Mice.—Results of titrations of uterus, ovaries, and the products of conception of AKR mice 15-17 days pregnant are shown in Table II. Uterus and ovary showed high titers, placenta was 10- to 30-fold lower in titer, while the embryos were again negative or positive in only trace amounts.

TABLE II
MLV Infectivity Titers in Tissues of Pregnant AKR/J Mice

Mouse No.	MLV titer (\log_{10} PFU/0.1 g)*			
	Uterus	Ovaries	Placenta	Embryos
1	5.6	4.6	3.8	neg*
2	5.8	5.0	4.1	neg
3	5.7	5.4	4.0	neg
4	5.5	NT	3.8	neg
5	5.6	NT	3.9	1.3
6	5.0	NT	3.8	1.0

* Neg = $< 10^1$. NT = not tested.

DISCUSSION

We have recently presented evidence that all AKR mouse embryo cells have the capacity to initiate production of murine leukemia virus, but that in the 15-18 day embryo only a very rare cell is doing so (11). Despite the presence of the viral genome, the cells which are not producing virus are susceptible to infection (11, 12). Thus, AKR cells can become virus producers by either of two mechanisms: activation of indigenous viral genome, or infection from without by virus released from other cells of the same animal.

This finding provides a possible explanation of the pattern of virus appearance in the perinatal period described here. The rare spontaneous induction of virus in the embryo or newborn animal would lead to local spread and eventual dissemination of infection. Since tissue culture studies indicate that productively infected cells show no cytotoxicity and do not stop producing MLV, an established infection in vivo would have maximal opportunity to continue throughout life. The efficiency of the two initial events, that is, spontaneous induction and subsequent infection of uninduced cells, may undergo marked changes with the state of cell differentiation and the physiologic changes

occurring during the perinatal period. It is conceivable that the frequency of spontaneous induction is relatively constant after a certain stage of embryonic development, but that spread of infection occurs efficiently only under the physiologic conditions of immediate postnatal life. This would explain the relatively rapid rise in infectivity titer occurring during the 1st wk after birth. On the other hand, it is possible that the increase in titer results from a marked increase in frequency of spontaneous activation, but this seems less likely.

Several observations indicate that bone tissue may be the major source of virus in the early stages of the infection. Limb bone was by far the highest titer tissue in 1-4-wk old mice. Also, the relatively high virus titers of extracts of infant mouse carcass, which consists chiefly of muscle and bone, are probably attributable to bone, since muscle of weanling mice was a very poor source of virus (Table I). As shown in Table I, and confirmed in other studies not presented here, the bone cortex contained as much or more virus than the marrow. These findings are in agreement with the electron microscope observations of Schofield et al. (12), who observed large numbers of C-type particles in osteocytes, osteoblasts, and osteocyte lacunae in the bone of young AKR mice.

The finding of high virus titers in extracts of the tail confirms the observation of Huebner (R. J. Huebner, personal communication) that AKR tail extracts contain high levels of MLV complement-fixing antigen. This provides a highly convenient tissue for repeated virologic testing of individual animals.

The high titers of virus in the uterus of adult AKR mice raised the possibility that transplacental transfer of virus could be playing a role in the vertical transfer of MLV infection in AKR mice. However, we have recently found that C3H/Bi mice born after transplantation of fertilized ova into AKR mice did not appear to acquire MLV infection, and that the virologic pattern of AKR mice was not affected by their being carried in BALB/c uteri (T. Pincus, K. B. Bechtol, and W. P. Rowe, manuscript in preparation).

With two exceptions, the virus titers of the various tissues remained relatively constant from weaning age on. One exception is the serum, which showed a marked decrease in titer between 1 and 3 months; this probably indicates a neutralizing antibody response. The occurrence of an antibody response to MLV in AKR mice has been suggested by the finding of immune complexes in glomeruli of older AKR mice (13).

The other exception was tissues of animals which had developed lymphoma. The titers in their sera and thymuses were markedly increased over those of nonlymphomatous mice, while the titers in other tissues also tended to be higher. These changes probably followed, rather than preceded, development of lymphoma, since the majority of the normal animals in the 5-10 month age group were destined to develop lymphoma in the near future. The most likely explanation for the increase in serum virus titer is a breakdown in the immune system resulting from the leukemia, although increased numbers of virus-

producing cells in the circulation could also play a role. The increase in virus content of the lymphomatous thymus requires additional explanations. Virus titer is only an indirect reflection of the extent of virus infection of an organ. It is possible that high proportions of cells are infected in both the normal and lymphomatous thymus, but that the undifferentiated malignant cells have a greater capacity to produce infectious virus than the highly differentiated thymocytes which they replace.

It is striking that the highest titers of virus were found in a tissue that is only rarely a site of spontaneous tumors, i.e., the uterus. In preliminary studies of the number of virus-producing cells in various organs as determined by infectious center tests, we have again found the uterus to be the most heavily infected tissue. Thus, it is clear that there is no simple, direct correlation between the extent of virus activity and the propensity of the organ to develop spontaneous tumors. On the other hand, as pointed out by Schofield et al. (12), the high virus titers in AKR bone are accompanied by a high risk of developing bone tumors.

SUMMARY

Quantitative studies were made of the organ distribution of murine leukemia virus in AKR mice of various ages. Infectious virus first appeared shortly before or after birth and was continuously present in all mice thereafter. Highest infectivity titers were found in uterus and bone, with spleen slightly lower. Virus titers in normal thymus were relatively low, but increased significantly with the development of thymic lymphoma. The level of viremia decreased after the 1st month of life, but increased sharply in lymphomatous mice.

We are greatly indebted to Mr. James B. Humphrey for invaluable technical assistance in the animal dissections, and to Mrs. Joan B. Austin and Mr. Wendell E. Pugh for the tissue culture assays.

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