GENETIC STUDY OF LYMPHOMA INDUCTION
BY AKR MINK CELL FOCUS-INDUCING VIRUS IN AKR X NFS CROSSES

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The spontaneous thymic lymphoma that is the hallmark of AKR mice is unique in representing a genetically transmitted virus-induced neoplasm. So far, four genetic loci have been identified that play a major role in leading to development of the disease (1-4), Akv-1 (chromosome 7) and Akv-2 (chromosome 16), which are chromosomal loci containing sequences of ecotropic murine leukemia viruses (MuLV), with inheritance of either locus leading to high frequency of spontaneous expression of virus; Fv-1a (chromosome 4), which is permissive for cell-to-cell spread of the endogenous ecotropic AKR MuLV; and the H-2k haplotype (chromosome 17), which favors leukemogenesis, presumably by determining immunologic unresponsiveness to the virus and/or transformed cells.

An apparently crucial step in the pathogenesis of the disease in AKR is the generation, in the preleukemic thymuses, of a novel class of MuLV, termed mink cell focus-inducing (MCF) viruses. The viruses are so designated because of their ability to induce cytopathic foci in monolayers of a tissue culture line of mink cells, and are recombinants between the endogenous ecotropic virus and endogenous genetic information related to xenotropic viruses (5-8). When inoculated into young AKR mice, the thymotropic AKR-type MCF viruses (referred to as class I strains [9]) are highly lymphomagenic as judged by the marked acceleration of AKR thymomagenesis (10). In contrast, inoculation of these MCF viruses into several other mouse strains, particularly the NFS mouse, has produced no disease. NFS mice carrying either Akv-1 or Akv-2 from AKR mice were partially susceptible (10), suggesting that ecotropic virus may provide a complementary function for development of lymphoma by MCF viruses.

The finding that AKR mice were uniformly susceptible to lymphoma induction by MCF virus, whereas NFS mice were uniformly resistant, provided an opportunity to study further the genetic basis of the AKR disease.

Materials and Methods

Mice. AKR/J and NFS/N mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and from the National Institutes of Health Small Animal Colony, respectively. Their

Abbreviations used in this paper: MiLu, mink lung; MCF, mink cell focus-inducing; MuLV, murine leukemia virus.
relevant genotypes are as follows: AKR: Aka-1+, Aka-2+, Fa-1", H-2k, Gpi-1°, and Thy-1.1; NFS: no ecotropic virus-inducing locus, Fa-1", Gpi-1°, and Thy-1.2. NFS mice have been tentatively typed as H-2K9 and H-2Dq (J. S. Crowell, personal communication). Hybrid mice were bred in our laboratory. In designating crosses, the convention is followed that the strain of the mother is given first.

Mice, either as babies (2-5 d old) or weanlings (21-35 d old), were injected in the region of the thymus with 0.02 ml of AKR-247 MCF virus (5). The virus was grown in mink lung (MiLu) cells (ATCC CCL64) and harvested as described previously (10); the virus had been cloned previously by three limiting dilution titrations in MiLu cells. Virus pools used for inoculations were XC negative and had titers in MiLu cells of 10³.1-10³.° focus-forming units per inoculum. This amount of virus was well above the dose (10¹) previously determined to accelerate lymphoma in half of the AKR mice inoculated (10). Uninoculated mice were kept as controls; observations were made for up to 1 yr.

Criteria for Diagnosis of Lymphoma. Mice were checked weekly. Mice that showed one or more signs (sickness, palpable lymph nodes or spleen, or labored breathing) were killed and autopsied. Only mice with grossly enlarged lymphoid organs were considered to have lymphoma. The overwhelming majority of lymphomatous animals had a thymoma as well as general involvement of lymphoid organs. Histological examinations were performed on many cases and they always supported the gross diagnosis of lymphoma. Surviving NFS backcross mice were killed at 6.5-7 mo after inoculation and the thymus was examined grossly.

Virological Assays. The majority of NFS backcross mice were tested for the presence of ecotropic and MCF viruses in the thymus. Thymus cells were plated onto the SC-1 wild mouse cell line (11) and the cultures were scored for replication of ecotropic virus by the XC procedure (12). Replication of the MCF virus was quantitated by plating serial dilutions of the thymus cell suspension onto MiLu and SC-1 cells; the SC-1 cells were killed with ultraviolet light (UV) 3 d later and overlaid with MiLu cells (2 × 10⁵ cells/60-mm dish); both sets of plates were subsequently scored for foci of MCF cytopathic changes in the confluent MiLu monolayers. Mink cultures negative for characteristic MCF foci were subcultured for one to three transfers and monitored for development of cytopathic effect. These procedures detect MCF virus particles that are phenotypically masked by ecotropic virus-host range determinants (13) as well as those that are directly infectious for MiLu cells.

Serological Assays. Segregation of H-2k was followed in backcross mice by the hemagglutination technique of Gorer and Mikulska (14), using anti-H-2k sera provided by Dr. Frank Lilly, Albert Einstein College of Medicine, Bronx, N. Y., and the Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. Briefly, ~10⁹ washed erythrocytes were incubated in microtiter plates at 4°C with antiserum diluted in saline containing 25% fetal bovine serum, 0.9% dextran, and 0.5% glucose. Hemagglutination was scored the following day.

Thy-1.1 was determined on live thymocytes of individual mice using an indirect microimmunofluorescence assay described elsewhere (15). Basically, 1 × 10⁶ cells were incubated in 50 µl of diluted mouse anti-Thy-1.1 serum (obtained from Dr. Herbert Morse, National Institute of Allergy and Infectious Diseases), rinsed, and secondarily reacted with fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories, Inc., Springfield, Va.). The cells were examined under UV epi-illumination at × 300 magnification. In tests of NFS X (AKR X NFS) backcross mice, either 100% of the thymocytes stained (Thy-1.1) or they were completely negative (Thy-1.2).

Antibody response to the injected MCF virus was also assayed by the indirect immunofluorescence test. Mice were bled from the tail 1–3 mo after infection, and serial dilutions of heat-inactivated serum were tested on suspensions of fresh, unfixed AKR-247-infected MiLu cells. The percentage of positive cells at each dilution was determined by counting cells on an epifluorescence microscope, and the titer was calculated as the reciprocal dilution giving 50% of the cells staining. A serum giving a titer estimate of <20 was considered negative.

Gpi-1 Typing. The isozymes of Gpi-1 (which is linked within 15 cM to Aka-1) were determined using standard vertical starch gel electrophoresis of kidney extracts (16).
Results

Susceptibility of AKR, NFS, and Various Hybrids to Lymphoma Induction by AKR-247 MCF Virus. Susceptibility to lymphoma induction by inoculated AKR-247 MCF virus was determined for AKR and NFS mice, as well as for F₁, F₂, and various backcrosses between them (Fig. 1; Table I). AKR mice, either inoculated as babies (2–5 d old) or as weanlings (females 21–35 d old) were completely susceptible, as reported earlier (10). All developed lymphoma within 2–5 mo after inoculation, whereas uninoculated

![Image](https://example.com/image)

**Table I**

| Strain or cross | Inoculated suckling mice | Inoculated weanling mice |
|-----------------|--------------------------|--------------------------|
|                 | Number leukemic/total    | With lymphoma/total      | Median latent period (range) | Number leukemic/total | With lymphoma/total | Median latent period (range) |
|                 | %                        | wk                       |                          | %                        | wk                       |
| Parental        |                          |                          |                          |                          |                          |
| AKR/J           | 26/26                    | 100                      | 15 (9–23)                | 31/31                    | 100                      | 13 (8–18)                |
| NFS/N           | 0/33                     | 0                        |                          |                          |                          |
| F₁              | 13/13                    | 100                      | 12 (7–17)                | 2/10                     | 20                        | 11 (9–13)                |
| AKR × NFS       | 18/18                    | 100                      | 11 (7–16)                | 12/40                    | 30                        | 15 (10–20)               |
| NFS × AKR       |                          |                          |                          |                          |                          |
| Backcross to NFS|                          |                          |                          |                          |                          |
| NFS × (NFS × AKR) | 21/35                    | 60                       | 11 (7–17)                |                          |                          |
| NFS × (AKR × NFS) | 20/42                    | 48                       | 12 (7–20)                |                          |                          |
| (NFS × AKR) × NFS | 32/61                    | 52                       | 12 (7–18)                |                          |                          |
| (AKR × NFS) × NFS | 15/30                    | 50                       | 10 (6–17)                |                          |                          |
| Total           | 88/168                   | 52                       |                          |                          |                          |
| F₂              |                          |                          |                          |                          |                          |
| (AKR × NFS)F₂   | 29/38                    | 76                       | 11 (7–16)                |                          |                          |
| Backcross to AKR|                          |                          |                          |                          |                          |
| AKR × (NFS × AKR) | 39/45                    | 87                       | 10 (7–19)                |                          |                          |

* Mice that developed lymphoma before 6 mo of age were considered susceptible to the oncogenic effects of the virus. In none of the crosses did uninoculated control mice develop lymphoma before 6.5 mo of age. Suckling mice were 2–5 d old when inoculated; weanlings were 21–35 d old.
control mice developed lymphoma spontaneously from 6.5 mo to ~1 yr of age (Fig. 1). The longer median latency observed with inoculated babies, as compared with the inoculated female weanlings, was due to generally longer latencies in male mice. NFS mice were completely resistant, with no lymphomas or other types of neoplasms being seen during 1 yr of observation.

F₁ progeny were completely susceptible when inoculated as babies, but were only partially susceptible when inoculated as weanlings (Table I; Fig. 1). It made no difference whether the mother was AKR or NFS, therefore excluding any maternal effect. Studies of F₂ and backcross progeny were done only with babies in order to circumvent this age-related resistance.

There was clear segregation for lymphoma susceptibility in F₂ and NFS backcross mice. The mice either developed lymphoma ~2-5 mo after inoculation, or, like uninoculated controls, remained free of disease for the 7-mo period of observation (Fig. 1). In all, 52% of the progeny of backcrosses to NSF were susceptible to AKR-247 MCF lymphomagenesis; all directions of the cross gave similar results (Fig. 1; Table I). Similarly, a suggestive one gene segregation ratio (~75% susceptibility) was observed in F₂ mice. Backcrossing to AKR produced nearly all susceptible progeny, as expected (Table I).

Association among Presence of Endogenous Ecotropic Virus, Replication of AKR-247 MCF Virus, and Susceptibility to Lymphomagenesis. Although the phenotypic ratios for susceptibility observed above were suggestive that a single gene regulated lymphoma induction by AKR MCF virus in crosses between AKR and NFS, when various factors associated with virus infection were examined, the genotype involved in conferring susceptibility was clearly more complex.

When the interrelationships between the virologic attributes of the NFS backcross mice were studied, several striking correlations were observed. First, the replication of the inoculated AKR-247 MCF virus was completely dependent on the expression of endogenous AKR ecotropic virus (Table II). Among 123 inoculated mice initially characterized, there was complete concordance between presence or absence of MCF and ecotropic viruses in the thymus (Table II, lines 1 and 2). Because it was conceivable that some of the MCF virus detected in these mice (especially those tested

| Category                          | Age when tested | Number of backcross mice with MuLV phenotypes | Total tested |
|-----------------------------------|-----------------|---------------------------------------------|--------------|
|                                   |                 | ECO+  | ECO-  | ECO+  | ECO-  | Total |
| Susceptible to MCF lymphoma       | 2-4 mo (when    | 58    | 0     | 0     | 0     | 58    |
|                                   | moribund)      |       |       |       |       |       |
| Resistant to MCF lymphoma         | 6-7 mo          | 34    | 0     | 0     | 31    | 65    |
| In latent period                  | 6-7 wk          | 19    | 0     | 0     | 6     | 25    |
| Total                             |                 | 111   | 0     | 0     | 37 (25%) | 148  |

* ECO, ecotropic virus.
TABLE III
Interrelation of Virus Replication, Inheritance of Akv-1, and Susceptibility to Lymphomagenesis in 123 NFS Backcross Mice Challenged with MCF-247

| Virus (MCF and ecotropic) | Gpi-1<sup>a,b</sup> (≥ Akv-1) | Number in category | Number developing lymphoma (%) |
|--------------------------|-------------------------------|-------------------|-------------------------------|
| +                        | +                             | 52                | 38 (74%)                      |
| +                        | −                             | 29                | 12 (41%)                      |
| +                        | NT<sup>*</sup>                   | 11                | 6                             |
| Subtotal                 |                               | 92                | 58 (63%)                      |
| −                        | NT                             | 31                | 0                             |
| Total                    |                               | 123               | 58 (47%)                      |

<sup>*</sup> Not tested.

Fig. 2. Number of MCF-positive thymus or thymoma cells titrated as infectious centers from individual virus-positive resistant or susceptible NFS backcross mice. Arrows indicate median values.

TABLE IV
Lack of Relationship of H-<sup>2</sup>k or Thy-1.1 to Susceptibility to Lymphomagenesis by AKR-247 MCF Virus in NFS Backcross Mice

| Category | Number of mice with attribute |
|----------|------------------------------|
|          | Number tested |
| H-<sup>2</sup>k     | Thy-1.1       |
| Susceptible | 15/27 (56%) | 8/15 (53%) |
| Resistant    | 37/70 (53%)  | 9/20 (45%)  |

at 6 mo of age) represented spontaneously arising MCF viruses rather than the inoculated strain, an additional group of 25 inoculated backcross animals was tested at 6–7 wk after inoculation, before occurrence of lymphomas (Table II, line 3). Again, there was complete concordance between the two types of virus; all mice with ecotropic (XC positive) virus yielded MCF virus, and all animals that were negative for ecotropic virus were also negative for MCF virus. Thus, expression of ecotropic virus appeared to convey permissiveness for replication of the inoculated MCF virus.
In accord with previous studies (17), three-fourths of the animals were in the virus-positive category, representing segregation of the two unlinked ecotropic virus-inducing loci \( Akv-1 \) and \( Akv-2 \).

Second, susceptibility to lymphomagenesis was entirely confined to virus-positive animals (Tables II and III). All mice not inheriting ecotropic virus loci and consequently not replicating the MCF virus were resistant to lymphoma induction. However, the virus-positive animals were not uniformly susceptible; 37% were resistant to MCF lymphomagenesis.

It was not clear whether there was a significant difference in the amount of virus between the susceptible and resistant animals within the virus-positive group. Infectious center titrations were done at time of killing on 38 mice with thymoma and on 31 virus-positive mice that remained free of tumor (Fig. 2). Although the number of virus-producing cells was about fivefold higher in the susceptible group, this could reflect differences between tumor and nontumor tissue, or could be because the animals with thymoma had generally much lower levels of anti-MCF antibody at the time of killing than did the resistant animals, as described below.

Third, among the virus-positive mice there was a correlation between susceptibility and the inheritance of the \( Akv-1 \) locus from AKR, as measured by the linked marker \( Gpi-1 \) (Table III). However, this correlation was not absolute, indicating the \( Akv-1 \) was not singularly responsible for susceptibility to the exclusion of \( Akv-2 \). In the group of backcross mice inheriting \( Akv-2 \) without \( Akv-1 \) (virus-positive mice not possessing \( Gpi-1^{ab} \)), nearly half were susceptible to lymphomagenesis.

Antibody Response to MCF Virus. We attempted to determine whether the ability to mount a humoral antiviral immune response was a determinant of susceptibility to the neonatal MCF virus infection. This seemed possible because with the immunofluorescent assay for MCF antibody described in Materials and Methods, NFS mice injected as 2-5-d-old sucklings uniformly developed antibody, in titers of 1:200-1:300, within 1 mo after inoculation, whereas similarly inoculated AKR mice did not. However, F1 mice also developed comparable antibody responses, as did all of 28 NFS backcross mice tested at that interval; AKR backcross animals were not tested. When F1 and NFS backcross animals were tested at later times after inoculation, the antibody levels showed a marked decline, often to undetectable levels, in animals that were lymphomatosus or that soon afterwards developed lymphoma. This led to a strong correlation between presence of antibody in later tests and resistance to lymphoma, but we consider the absence of antibody a consequence of the disease, rather than a contributing factor.

Thus, antibody response to MCF-specified cell surface antigens did not appear to play a major role, if any, in resistance to MCF lymphomagenesis.

Typing of Backcross Mice for H-2 and Thy-1. Table IV shows the results of testing NFS backcross mice for inheritance of \( H-2^k \) and \( Thy-1.1 \) from AKR in relation to susceptibility to MCF lymphomagenesis. There was no indication that either of these genes was a determinant of susceptibility in this cross.

Discussion

On the basis of the segregation ratios, the susceptibility of AKR mice to MCF 247 lymphomagenesis, as compared with the resistance of NFS, initially appeared to be determined by a single dominant gene. That is, in the tests with suckling mice, all F1
were susceptible, as were 52% of the NFS backcross mice and 76% of the F2. However, it is clear from the virologic studies on the backcross segregants that the results cannot be attributed to a single gene. Rather, they represent the interaction between inheritance of the two genes for endogenous ecotropic MuLV (Akv-1 and Akv-2) and a third factor, possibly genetic, which influences lymphoma development.

Among the NFS backcross mice challenged with MCF-247 virus as sucklings, three distinct phenotypic populations were identified. One group, consisting of the 25% that did not inherit either of the two AKR virus-inducing loci, did not replicate the inoculated MCF virus and remained free of lymphoma. A second category, consisting of about one-third of the mice that carried ecotropic virus, replicated the MCF virus but remained free of lymphoma. The third group, composed of the remaining two-thirds of the ecotropic virus-positive segregants and thus constituting half of the total backcross population, replicated the MCF virus and developed accelerated lymphoma.

Thus, the major determinant of susceptibility to MCF lymphomagenesis in these crosses was the presence of endogenous ecotropic virus. Furthermore, the number of virus-inducing loci may affect susceptibility. This is suggested by the association between inheritance of Gpi-I (Akv-1) and susceptibility; because half of the mice with Akv-1 also carry Akv-2, the susceptible group contains an excess of mice carrying both loci.

It is possible that a third gene contributing to susceptibility is segregating in this cross; the backcross and F2 data are compatible with a model in which there are three genes, inheritance of any two of which produces susceptibility. However, further studies will be required to differentiate this model from one in which penetrance is a function of the number of copies of virus-inducing loci.

The basis for the failure of the inoculated AKR-247 MCF virus to replicate in mice not carrying ecotropic virus is not known. The MCF 247 virus is replication competent, replicating efficiently in fibroblast-type tissue cultures of either AKR or NFS origin. It is possible that it requires some helper function or phenotypic mixing with ecotropic virus to become established in the thymus. It is alternatively conceivable that ecotropic virus may stimulate division of thymic lymphocytes and thereby allow more extensive integration and replication of the introduced MCF virus. Another possibility is that in the absence of ecotropic virus the antibody response was able to eliminate the MCF infection; neutralization of the MCF virus might be particularly efficient in ecotropic-negative mice because there would not be phenotypic mixing with ecotropic gp70 molecules.

The lack of association of susceptibility with H-2 appeared to be at variance with the large body of data relating the H-2 complex to susceptibility to various MuLV and to immune responses to endogenous virus (2-4, 18). However, in the segregating cross studied here, the backcross was to the antibody responsive parent, so no effect of recessive H-2a-linked immune-response allele would be anticipated.

A number of studies have shown the importance of ecotropic virus and its presence in early life for later development of spontaneous leukemia (18, 19). Also, our previous studies of MCF lymphomagenesis showed that NFS mice partially congenic for Akv-1 or Akv-2 were more susceptible to MCF lymphomagenesis than were virus-negative NFS mice (10). The present study extends these findings by suggesting that in the case of AKR, the ecotropic virus not only serves to generate lymphomagenic MCF viruses, but also determines permissiveness for replication of the MCF virus. Our data
suggesting that the number of copies of virus-inducing loci may affect susceptibility would indicate that the multiplicity of virus-inducing loci seen in high leukemic mouse strains (20) may be a contributory factor for the high tumor incidence.

The etiologic importance of MCF viruses in spontaneous thymomas of AKR mice seems clear, but their role in other spontaneous hematopoietic neoplasms of high ecotropic virus strains is not yet defined. Although MCF viruses are generally present in such tumors, the MCF isolated from nonthymic neoplasms (class II MCF) have generally not induced any disease in mouse inoculation tests (10). The studies reported here indicate that caution must be exercised in interpreting negative mouse pathogenicity tests because induction of neoplasia by MCF viruses may require a complex balance of factors.

Summary

The mink cell focus-inducing (MCF)-247 virus, originally isolated from an AKR thymoma, is lymphomagenic in AKR mice but not in the ecotropic virus-negative NFS mouse strain. Analysis of sensitivity to lymphoma-induction by AKR-247 MCF virus in genetic hybrids between these two strains showed that F1 mice inoculated as sucklings were uniformly sensitive, whereas those inoculated as weanlings were generally resistant. In NFS backcross mice inoculated as sucklings, inheritance and expression of endogenous ecotropic virus from AKR was an essential correlate of replication of MCF virus and subsequent development of lymphoma. However, one-third of the mice expressing ecotropic virus and replicating the inoculated MCF virus did not develop lymphoma. The results suggested that an additional gene that influenced development of lymphoma may be involved, and that mice inheriting both virus-inducing loci from AKR were more susceptible than those inheriting only one.

These findings indicate that the causal role of ecotropic virus infection in spontaneous thymomagenesis in AKR mice involves not only the generation of leukemogenic MCF viruses but also the establishment of permissiveness for their growth.

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