JOURNAL OF VIROLOGY, Sept. 1984, p. 586–594
0022-538X/84/090586-09$02.00/0

Vol. 51, No. 3

# Spontaneous and Induced Leukemias of Myeloid Origin in Recombinant Inbred BXH Mice

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Received 1 March 1984/Accepted 29 May 1984

**BXH-2 recombinant inbred (RI) mice produce high titers of B-ecotropic murine leukemia virus beginning early in life and have a high incidence of non-T-cell leukemias that occur before 1 year of age. The leukemias that develop are in some cases associated with hind limb paralysis. In addition, a dualtropic mink cell focus-forming virus has been isolated from leukemic cells of BXH-2 mice. Immunological and cytochemical characterization of the BXH-2 leukemias showed that they are of the myeloid lineage. To assess the oncogenicity of the BXH-2 viruses, newborn mice of several BXH RI strains were inoculated at birth with biologically cloned B-ecotropic or mink cell focus-forming murine leukemia virus. These studies demonstrated that the B-ecotropic virus can induce myeloid leukemias in other BXH RI strains, whereas the dualtropic mink cell focus-forming isolates were nononcogenic in the strains tested. DNA-DNA reassociation analysis indicated that the organotropism of the B-ecotropic murine leukemia virus is confined to lymphoid tissues. Southern analysis of tumor DNAs showed that there was amplification of ecotropic virus-specific sequences in BXH-2 myeloid tumors and in all leukemias induced in other BXH RI strains by inoculation of the BXH-2 B-ecotropic virus. Although B-ecotropic virus is expressed in central nervous tissues of paralyzed BXH-2 mice, we were unable to induce the disorder in several BXH RI strains inoculated intracranially at birth with either the B-ecotropic or dualtropic virus. These results suggest that the paralysis that occurs in BXH-2 mice is due to the infiltration of leukemic cells into the central nervous system.**

A vast amount of evidence has accumulated to indicate that the expression of type C murine leukemia viruses (MuLVs) is causally associated with lymphomagenesis. The majority of high-tumor-incidence mouse strains, such as AKR, C58, HRS, and others, spontaneously release an N-ecotropic MuLV throughout life, and the lymphatic lymphomas that occur in these mice are primarily of T-cell origin. Although these strains continuously express N-ecotropic virus throughout life, these viruses do not appear to accelerate lymphoma development directly when injected into newborn mice of these strains (28). The discovery of recombinant or dualtropic mink cell focus-forming (MCF) viruses in the preleukemic and leukemic tissues of these high lymphoma strains suggested an etiological role for this class of virus in T-cell lymphomagenesis (11, 12). Furthermore, these MCF viruses have in some cases been shown to accelerate lymphoma development when injected into newborn animals (12), supporting an etiological role for these viruses in the disease process.

In contrast to the early spontaneous expression of N-ecotropic viruses, B-ecotropic MuLVs in many high-leukemic strains are usually observed only in aged *Fv-1*$^b$ mice. These viruses are thought to be generated by a somatic recombination event between N-ecotropic and xenotropic MuLVs within the *gag* region (5, 17). Since *Fv-1*$^b$ strains are relatively resistant to N-ecotropic virus expression, the recombination event leads to the formation of a virus that is permissive for growth in *Fv-1*$^b$ cells.

Among 12 recombinant inbred (RI) strains of mice derived from crossing C57BL/6J and C3H/HeJ mice, one RI strain, BXH-2, has a high leukemia incidence (89% by 1 year of age). The tumors that begin to develop around 7 months of age are of non-T-cell origin and in some cases are associated with hind limb paralysis (4). BXH-2 mice carry the *Fv-1*$^b$ allele and spontaneously express a B-tropic MuLV beginning early in gestation and continuing throughout life. Southern analysis and hybridization with an ecotropic MuLV DNA-specific probe demonstrated that BXH-2 mice carry two N-ecotropic proviruses that were inherited from the parental strains but lacked endogenous B-ecotropic MuLV DNA sequences (24), indicating that the B-ecotropic virus is maternally transmitted in BXH-2 mice. The high incidence of leukemia and the spontaneous production of B-ecotropic MuLV were traits acquired simultaneously during inbreeding of the BXH-2 RI strain, suggesting that they are causally related. In addition, spontaneous leukemias from some BXH-2 mice also express an MCF-like virus; the role of these viruses in BXH-2 leukemogenesis is uncertain.

Since spontaneous leukemias of non-T-cell origin are rare in mice, it was of interest to characterize further the type of leukemias that occur in BXH-2 mice and to define the role of both the B-ecotropic and dualtropic MCF MuLVs in BXH-2 disease. We now present evidence indicating that the leukemias that develop in BXH-2 mice are primarily of the myeloid lineage and that the BXH-2 B-ecotropic virus, but not the dualtropic MCF virus, has a causative role in the disease process.

## MATERIALS AND METHODS

**Mice.** The BXH RI mouse strains were derived by systematic inbreedings, beginning with randomly chosen pairs of mice from the $F_2$ generation of the cross of C57BL/6J and C3H/HeJ inbred strains (4). C57BL/6J mice used in these studies were obtained from Jackson Laboratory Animal Resources, Bar Harbor, Maine.

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**Virus transmission studies.** Newborn BXH-7, BXH-14, and BXH-6 mice were inoculated by intraperitoneal or intracranial injection of $1 \times 10^5$ to $5 \times 10^5$ PFU of B-ecotropic MuLV or $5 \times 10^4$ focus-forming units of the BXH-2 dualtropic isolate. Virus stocks used for inoculation were from 24-h culture fluids that were filtered through a 0.45-μm filter before use. The mice were observed for signs of tumor development, paralysis, or general illness. Moribund mice were killed for autopsy, and peripheral blood and other tissues were examined histochemically.

**Virus assays.** Expression of ecotropic and dualtropic MuLVs was determined by the XC plaque assay, using tail biopsies or spleen suspension, and by the reverse transcriptase assay as previously described (4).

**Identification of cell surface phenotypes.** Indirect and direct immunofluorescence assays were used to detect the T-cell surface antigen *Thy-1* and surface immunoglobulins, respectively. Anti-immunoglobulin antibody was a polyvalent affinity-purified goat antibody against mouse immunoglobulin (heavy and light chains). Anti-*Thy-1* was a monoclonal antibody from hybridoma clone HO-22-1. The presence of *Lyt-1* and *Lyt-2* was measured by flow cytometry with an Ortho 50-H Cytoflurograf, as described previously (26). The rat monoclonal antibodies used were clones 53-73 (anti-*Lyt-1*, all alleles) and 53-6.72 (anti-*Lyt-2*, all alleles). All antibodies used were obtained from the Salk Cell Science Center, La Jolla, Calif.

Leukemia cells were analyzed cytochemically, using published techniques for nonspecific esterase (NSE), chloroacetate esterase (CAE), and the Kaplow technique for myeloperoxidase (MP), (44). NSE, CAE, and MP are conventional cytoplasmic markers for monocytes/macrophages, granulocytes, and granulocytes/monocytes, respectively. Cells to be analyzed were sedimented onto glass slides with a Sharon Cytospin 2 and air dried before staining procedures were initiated. Normal peritoneal macrophages served as positive controls for NSE staining, and thioglycolate-elicited peritoneal granulocytes, harvested 6 to 8 h after injection, were used as positive controls for CAE and MP staining.

**Analysis of DNA.** The preparation of total cellular DNA, restriction enzyme digestion, gel electrophoresis, and transfer to nitrocellulose paper were all as previously described (24). Filters were backed, prehybridized, hybridized ($5 \times 10^6$ cpm/ml) with $^{32}$P-labeled MuLV probes ($>10^9$ cpm/μg of DNA by the T4 polymerase labeling system supplied by Bethesda Research Laboratories, Bethesda, Md.), washed, air dried, and autoradiographed at −70°C, using Kodak XAR-5 X-ray film and Dupont Lightning-plus intensifying screens. The ecotropic virus-specific hybridization probes used were a 400-base pair (bp) fragment from the *env* (envelope) gene of AKR ecotropic MuLV (9) or a probe representing 167 bp from the N-aminoterminal end of the p15E region of the AKR ecotropic virus *env* gene (20). The 400-bp probe hybridizes only to ecotropic viral DNA sequences and not to xenotropic, amphotropic, or MCF MuLV DNAs (9) and was kindly provided by D. R. Lowy (National Institutes of Health, Bethesda, Md.). The p15E probe spans the *Xba*I restriction site of the ecotropic virus *env* gene that is retained when oncogenic MCF viruses are generated via recombination in AKR mice (20). Therefore, this probe detects both ecotropic and certain oncogenic MCF MuLV proviruses but has very little cross-reactivity with endogenous xenotropic MuLV sequences (20); this probe was provided by Winship Herr (Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.).

Ecotropic MuLV DNA sequences were quantitated in target and nontarget tissues of inoculated BXH-7 and BXH-14 mice by DNA-DNA hybridization, using the ecotropic virus-specific probes. High-molecular-weight DNA was prepared from tissues of BXH-2 mice, as described by Steffen et al. (38), and sheared by sonication to an average size of 400 nucleotides for use in hybridizations. DNA-DNA hybridization and S1 nuclease treatment was performed as described by Jaenisch and Mintz (22). $^{32}$P-labeled ecotropic virus-specific (400 bp) viral DNA ($3 \times 10^{-4}$ to $10 \times 10^{-4}$ μg/ml) was added to a reaction containing 300 to 1,000 μg of mouse or calf thymus DNA per ml in 0.01 M Tris-hydrochloride (pH 7.4)–1 mM EDTA. DNA was denatured by boiling for 10 min and then chilled on ice. NaCl was added to a final concentration of 1 M, and the solution was overlaid with paraffin oil and incubated at 68°C. Samples of 30 to 100 μl were removed at different times and diluted into 1 ml of cold S1 digestion buffer (0.1 M sodium acetate, pH 4.5, 0.01 M NaCl, 600 μM $ZnCl_2$) containing 40 μg of denatured and 5 μg of native calf thymus DNA per ml. The samples were digested for 30 min at 37°C, precipitated with 10% trichloroacetic acid, and counted for S1-resistant radioactivity.

The reciprocal of the fraction of labeled DNA remaining single stranded is plotted as a function of time of hybridization (22).

**Analysis of RNA.** Total cellular RNA from fresh tissues and cytoplasmic RNA from cells in culture were prepared by phenol extraction in the presence of the RNase inhibitor vanadyl adenosine (Bethesda Research Laboratories) at a final concentration of 10 mM as described before (3). Polyadenylated [poly ($A^+$)] RNA was selected by oligodeoxythymidylate-cellulose and analyzed by Northern analysis on 1% agarose gels containing 2.2 M formaldehyde (29). Restriction fragments of $^{32}$P-labeled DNA (*Hin*dIII-digested λ phage) were run as molecular weight markers. The RNA was transferred to nitrocellulose filters in 20× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) as described by Thomas (41). Filters were baked and hybridized as described above.

## RESULTS

**Characterization of BXH-2 leukemias.** Histological analysis of tissues and blood smears prepared from moribund BXH-2 mice suggested that approximately 90% of the affected mice had a leukemia of myeloid origin. The remaining neoplasias were characterized histologically as mixed-cell follicular lymphomas (B-cell lymphomas) and occasionally (<1% of all BXH-2 leukemias) as lymphocytic leukemia.

Leukemic animals were first detected by enlargement of spleen and lymph nodes as determined by palpation. Peripheral blood taken from moribund BXH-2 mice contained predominantly immature myeloid or monocytic cells; nucleated cells in peripheral blood ranged from 15,000 to 70,000 cells per mm$^3$. The leukemic cells infiltrated into most tissues, including in some cases tissues of the central nervous system (CNS). As reported earlier (4), a hind limb paralysis was associated with some leukemias but was never observed independently. Histological examination of brain and spinal cord of paralyzed BXH-2 mice indicated the presence of leukemic infiltrates, resulting in associated necrosis within brain substance.

Primary BXH-2 leukemic cells as well as leukemic cells established in culture were negative for the expression of B-cell (surface immunoglobulin) and T-cell (*Thy-1*, *Lyt-1*, and *Lyt-2*) cell surface antigens as determined by immunofluorescence and cytofluorimetry, using monoclonal antibodies directed against these cellular differentiation antigens. The

specificities of these antibodies for the detection of cell surface antigens on T- and B-cell lymphomas as well as plasmacytomas have been presented by Lanier et al. (26).

Leukemic cells were further characterized by cytochemical staining (Table 1). The tumor cell suspensions were all positive for NSE and CAE. However, staining levels were variable in different individual tumors. The absence of MP staining suggested that the leukemic cells did not differentiate beyond the myeloblastic stage, after which azurophilic granules with associated MP develop (13). This pattern of cytochemical staining taken together with the absence of T- and B-cell surface antigens and histological examination further suggests that the BXH-2 leukemias are of myeloid origin.

DNAs of 25 independent spontaneous BXH-2 tumors were analyzed by Southern blotting to determine whether amplification of viral sequences occurred in BXH-2 leukemias. The hybridization probe used in these studies (N-p15E) spans the *Xba*I site within the ecotropic virus *env* gene that is retained when oncogenic MCF viruses are generated via recombination in AKR mice (20). Therefore, this probe detects both ecotropic and certain oncogenic MCF MuLV proviruses. Representative results, after *Pvu*II, *Eco*RI, or *Pst*I/*Eco*RI double digestions, are shown in Fig. 1. *Pvu*II cleaves twice within the ecotropic viral genome and should generate a 3′ viral DNA junction fragment larger than 3 kilobases (kb) detectable with the p15E probe (24, 32). *Eco*RI does not cleave the DNA of most ecotropic MuLVs; however, nonecotropic proviral sequences are sensitive to *Eco*RI digestion (8). *Pst*I only cleaves ecotropic proviral DNA within the long terminal repeat sequences, generating an 8.2-kb viral DNA fragment (32). As expected, all tumor DNAs digested with *Pvu*II contained the two endogenous N-ecotropic MuLV genomes (4.3- and 5.2-kb fragments) carried by BXH-2 mice (24). In addition, all tumors contained one to four somatically acquired proviral sequences, ranging in size from approximately 4 to 8 kb after *Pvu*II digestion. DNA prepared from brain tissue that was not infiltrated by leukemic cells did not contain these somatically acquired ecotropic MuLV genomes (Fig. 1, tumor 14). Eleven of the 25 tumors appeared to contain a single somatically acquired provirus. Digestion with a second enzyme, *Eco*RI, confirmed the presence of a single tumor-specific provirus in each tumor (data not shown). The proviruses appear to be integrated in many sites in DNA of different BXH-2 mice, but DNAs prepared from different tumor tissues of individual BXH-2 mice showed the same pattern of somatically acquired proviral insertions, indicating that the tumors were monoclonal (Fig. 1, tumor 15).

*Pst*I/*Eco*RI double digestion of the 25 BXH-2 tumors showed that three tumor DNAs (tumors 1, 2, and 8) contained proviruses that were smaller than genome size (not shown). However, hybridization of these and other tumor DNAs with the 400-bp ecotropic virus-specific probe which does not hybridize to most dualtropic MCF proviruses (9) also hybridized to the same restriction fragments (Fig. 1B). Together, these results suggest that the somatically acquired proviruses present in BXH-2 tumor DNAs represent either nondefective or defective ecotropic proviruses rather than MCF proviruses.

**Experimental transmission of BXH-2 leukemic cells.** Tumor cells obtained from a BXH-2 spleen retained their neoplastic properties on transplantation into isogenic as well as (C3H/HeJ × C57BL/6J)$F_1$ mice. Donor cells injected intraperitoneally at a concentration of $10^6$ cells per animal grew as a solid tumor and in some instances produced an ascites-type tumor when passaged in $F_1$ mice. In all instances, host tissues became infiltrated with tumor cells and death occurred by 3 to 4 weeks from disseminated neoplastic cells.

The ascites tumor, after several in vivo passages in (C3H/HeJ × C57BL/6J)$F_1$, mice, was placed in culture where the cells grew as rounded, highly refractile cells in association with adherent fibroblastic-like cells (Fig. 2). Upon subsequent passages the adherent cell population continuously gave rise to the rounded refractile cells that were continuously being released into the culture medium. These floating cells remained viable and upon transfer would adhere and grow as above. This cell line, designated bh2-1, was positive for both NSE and CAE (Table 1), suggesting that these cells were of the myeloid lineage. The bh2-1 cell line expressed a B-ecotropic virus; no dualtropic MCF-like virus has been isolated from this cell line to date. Cleavage of DNA from the bh2-1 cell line with the restriction enzyme *Pvu*II and hybridization with the p15E probe demonstrated the presence of the parental N-ecotropic endogenous proviruses characteristic of BXH-2 mice (24). In addition, other virus-specific fragments were observed; these most likely represent somatically acquired proviruses. Restriction analysis of tumor DNAs induced by the bh2-1 cell line shows the same virus-specific fragments observed in the cell line, indicating that the tumors were due to infiltration and outgrowth of bh2-1 cells.

The leukemogenicity of the BXH-2 B-ecotropic and dualtropic viruses was examined by inoculation into newborn mice. The viruses were biologically cloned by endpoint dilution, and $5 \times 10^5$ PFU of the B-ecotropic MuLV or $10^4$ focus-forming units of the dualtropic MCF virus were injected intraperitoneally into BXH-7 (*Fv-1*$^b$), BXH-14 (*Fv-1*$^b$),

TABLE 1. Characterization of BXH-2 leukemia for cell surface antigens and cytochemical reactions

| Tissue | Immunofluorescence[a] | | % Positive cells | | | | |
|---|---|---|---|---|---|---|---|
| | | | Cell sorting[b] | | Cytochemistry[c] | | |
| | SIg | *Thy-1* | *Lyt-1* | *Lyt-2* | NSE | CAE | MP |
| BXH-2 leukemic spleen | – | – | <2[d] | <2 | 90 | 95 | 5 |
| BXH-2 bone marrow | – | – | | | 95 | 95 | 2 |
| bh2-1 myeloid cell line | – | – | <1 | <1 | 100 | 100 | 1 |

[a] Identification of *Thy-1* and surface immunoglobulin (SIg) was by indirect and direct immunofluorescence, respectively. Anti-*Thy-1* antibody was a monoclonal antibody from hybridoma clone HO-22-1. Anti-immunoglobulin antibody was a polyvalent affinity-purified goat antibody against mouse immunoglobulin (heavy and light chains).

[b] The presence of *Lyt-1* and *Lyt-2* was measured by flow cytometry as described previously (26). Rat monoclonal antibodies used were clones 53-7.3 (anti-*Lyt-1*, all alleles) and 53-6.72 (anti-*Lyt-2*, all alleles) obtained from L. Shultz of the Jackson Laboratory.

[c] Cells were analyzed cytochemically, using published techniques for NSE, CAE, and the Kaplow technique for MP.

[d] Values represent the average of 10 independent mice.

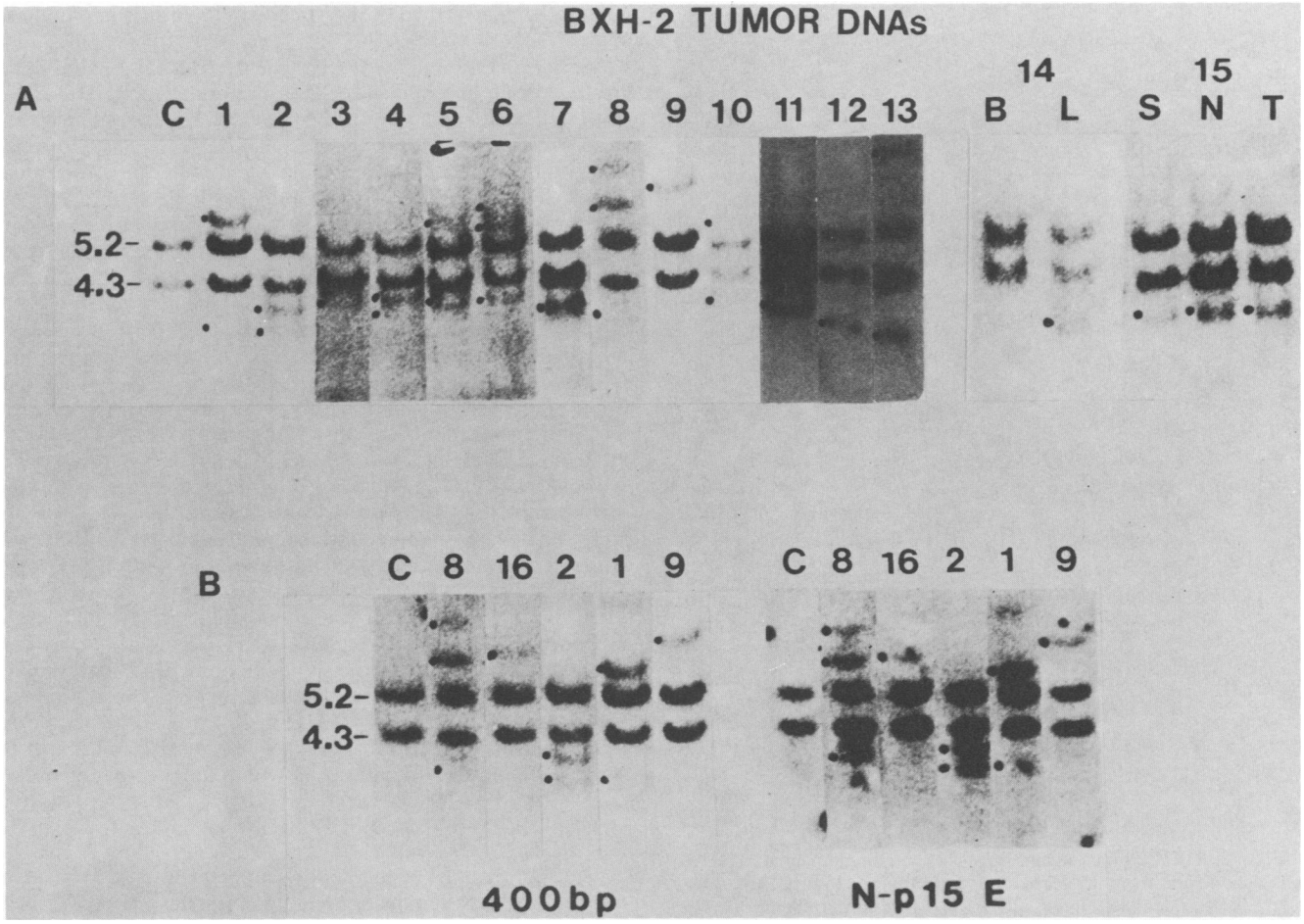


FIG. 1. Characterization of ecotropic MuLV sequences in DNAs of leukemic BXH-2 mice. (A) DNAs were prepared from BXH-2 normal (C) and leukemic spleens that in all cases were shown by histological examination to be infiltrated with leukemic cells. DNAs were digested with *Pvu*II. Fragments were detected after electrophoresis through 0.8% agarose gels, Southern blotting, and hybridization with the p15E probe ($10^9$ cpm/μg). The numerical lane notation (1 to 15) represents individual BXH-2 tumors. Some of the somatically acquired proviruses are difficult to see and are therefore noted by (●). In tumors 14 and 15 the letters designate DNAs from various tissues (B, brain; L, liver; S, spleen; N, node; T, thymus). The molecular weight in kilobases of viral DNA-containing fragments was determined from *Hin*dIII-digested λ DNA electrophoresed in parallel lanes of the same gels. (B) The presence of subgenomic fragments was observed in some BXH-2 tumors. To determine whether these fragments represent dualtropic virus or defective ecotropic virus, the tumor DNA was digested with *Pvu*II and hybridized in parallel with the 400-bp ecotropic virus-specific probe and the N-p15E probe. The 400-bp probe does not hybridize to most dualtropic viruses, whereas the N-p15E probe detects both ecotropic and certain oncogenic dualtropic MCF MuLV sequences. The results suggest that the subgenomic fragments represent defective ecotropic genomes.

and BXH-6 (*Fv-1*$^n$) mice. None of the MCF-infected mice showed any signs of leukemia development by 1 year of age. These findings confirm our earlier assumption, based on tryptic peptide analysis of the dualtropic MCF viral envelope glycoprotein, that the BXH-2 virus is a member of the nonleukemogenic MCF virus family (4). In contrast, the B-ecotropic BXH-2 virus induced neoplasias in 100% (10 of 10) of the inoculated BXH-14 mice before 1 year of age and in 14% (2 of 14) of the BXH-7 mice by 14 months of age. Inoculated mice were assayed for XC-positive virus by tail biopsies 3 to 4 months after inoculation. All but the 12 BXH-7 mice that did not develop leukemia were positive for B-ecotropic virus. As expected, the *Fv-1*$^n$ BXH-6 mice which are nonpermissive for B-ecotropic MuLV growth did not produce B-ecotropic MuLV and were refractory to disease induction. The range of neoplasias induced by the B-ecotropic virus paralleled that occurring spontaneously in BXH-2 mice. Based on histochemical and cytochemical analyses, 11 of the 12 affected BXH-7 and BXH-14 mice were diagnosed as having myeloid leukemia, with the remaining tumor diagnosed as a mixed-cell follicular lymphoma. B-ecotropic virus was isolated from all tumor tissues of virally induced leukemias. We were not able to isolate a dualtropic MCF virus from any of the B-ecotropic inoculated mice.

Restriction enzyme analysis of DNAs from tumor tissues of these leukemic mice again demonstrated the presence of somatically acquired viral DNA sequences (Fig. 3). DNAs were digested with *Pvu*II, *Eco*RI, or *Eco*RI in combination with *Pst*I and analyzed by Southern blotting, using the p15E probe. Figure 3 shows that both BXH-7 and BXH-14 leukemic mice have acquired multiple copies of proviral DNA sequences in tumor DNA. These viral sequences were not present in unaffected tissue of the same or uninoculated mice. The BXH-14 RI strain carries a single N-ecotropic provirus inherited from the C57BL/6J parental strain, whereas the BXH-7 RI strain did not inherit any endogenous N-

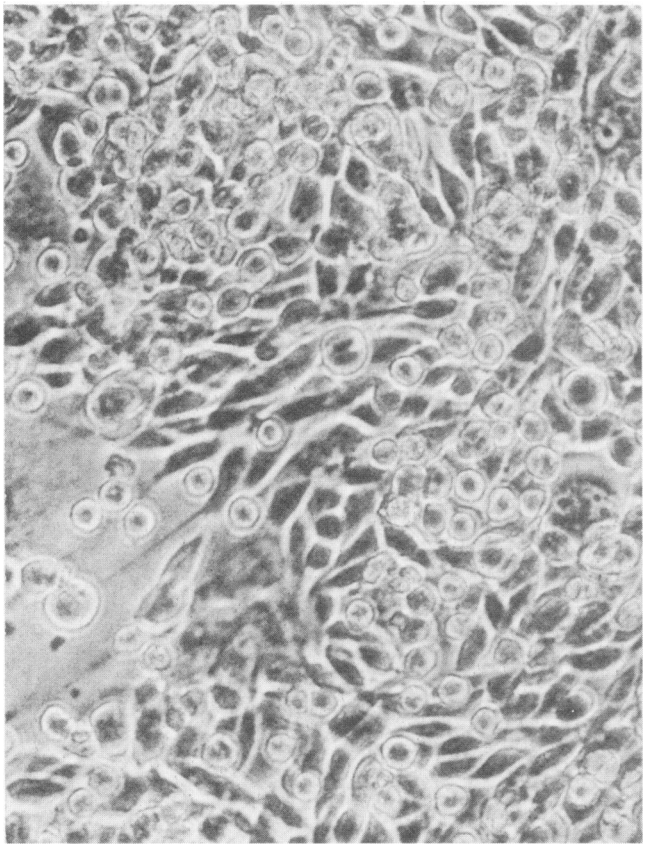

FIG. 2. BXH-2 myeloid cell line. This cell line, designated bh2-1, was established from an ascites tumor obtained from (C57BL/6J × C3H/HeJ)$F_1$ mice inoculated with BXH-2 leukemic spleen cells. The cells were characterized cytochemically as belonging to the myeloid lineage and induced tumors when inoculated into BXH RI mice (BXH-2, -7 and -14 RI strains). Phase contrast (×10).

ecotropic proviruses (24) (Fig. 3, lanes a and d, respectively). With the exception of one virally induced tumor (Fig. 3B, lane e'), subgenomic fragments were not observed in tumor DNAs after digestion with *Eco*RI or *Pst*I and *Eco*RI, indicating that most of these proviruses represent nondefective ecotropic proviruses.

Molecular hybridization experiments aimed at identifying the target organ for B-ecotropic MuLV replication in newborn BXH-7 and BXH-14 mice were determined by DNA-DNA reassociation kinetics. Amplification of virus-specific DNA sequences was detected in DNA from spleen and bone marrow of 3-month-old BXH-7 and BXH-14 mice (Table 2), whereas in the other organs tested few or no ecotropic MuLV sequences were detected. Ecotropic MuLV expression in a second group of infected BXH mice was determined by RNA blot analysis. Poly($A^+$) RNA from spleen and bone marrow of inoculated BXH-14 mice was positive for virus expression, whereas other tissues from the same mice were virus negative (data not shown). In addition, virus replication appears to be higher in bone marrow cells than in splenic cells. With the exception of one BXH-7 mouse in which low levels of virus RNA expression was detected in bone marrow cells, tissues from inoculated BXH-7 mice were negative for virus expression as were tissues from uninoculated BXH-7 and BXH-14 mice. These results suggest that the organotropism of the BXH-2 B-ecotropic virus is hemopoietic.

**Virus expression in the CNS of paralyzed BXH-2 mice.** CNS tissues (brain and spinal cord) from paralyzed BXH-2 mice express B-ecotropic virus, whereas CNS tissues of apparently normal BXH-2 mice were generally negative for B-ecotropic virus expression as determined by the XC plaque assay. To assay further for viral expression in CNS of normal and paralyzed mice, we analyzed poly($A^+$) mRNA by Northern blot hybridization with the 400-bp and p15E ecotropic virus-specific probes. Figure 4 shows the pattern of viral RNA expression observed by analyzing poly(A) RNA from brain tissue and tumor tissue of the same as well as leukemic tissue of other BXH-2 mice. RNA from the diseased mouse contained two virus-specific RNAs of 8 and 3.6 kb detected by hybridization with the p15E probe (Fig. 4). Neither fragment was observed in poly(A) RNA extracted from normal or brain tissue of leukemic BXH-2 mice without paralysis. Similar results were obtained with the 400-bp ecotropic virus probe (not shown).

We also were unable to induce paralysis in mice of several BXH RI strains inoculated as newborns with BXH-2 B-ecotropic virus. Biologically cloned BXH-2 B-ecotropic MuLV ($10^5$ PFU/0.1 ml) was inoculated intracranially into newborn BXH-2, BXH-14, and BXH-7 mice (15 mice per strain). We did not observe the development of paralysis in any of these mice up to 15 months of age. These findings suggest that in paralyzed BXH-2 mice the B-ecotropic virus infiltrates the CNS via infected leukemic cells and that it is the infiltration of leukemic cells which induces paralysis.

## DISCUSSION

The spontaneous occurrence of myelogenous leukemias in mice is rare. Leukemias of myeloid origin have been induced in mice by irradiation or by passage of cell-free tumor filtrates (42). Although a virus etiology has been implicated in the induction of myeloid leukemias (25, 39, 42), the presence of a myeloid leukemia virus remained speculative. The Friend MuLV which primarily induces lymphatic lymphomas in mice is occasionally associated with myeloid leukemia development (10, 36, 37) several months after viral infection. In radiation-induced myeloid leukemias of RFM/Un mice, there is no apparent difference in virus expression between normal and leukemic mice nor do N-ecotropic viruses isolated from these mice induce neoplastic disease (40). Thus, the association between virus expression and myeloid leukemias remains unclear.

TABLE 2. Number of ecotropic MuLV sequences in normal and infected BXH mice[a]

| BXH RI strain | No. tested | No. of virus-specific sequences | | | | |
|---|---|---|---|---|---|---|
| | | Spleen | Bone marrow | Liver | Thymus | Brain |
| BXH-7 | 2 | 0.2 | 0.1 | ND[b] | 0.1 | ND |
| BXH-7, infected | 4 | 1.3 | 1.8 | 0.1 | 0.1 | 0.1 |
| BXH-14 | 3 | 1.4 | 1.3 | 1.3 | 1.4 | 1.3 |
| BXH-14, infected | 4 | 2.3 | 2.8 | 1.4 | 1.5 | 1.4 |

[a] Number of ecotropic virus-specific sequences in normal and B-ecotropic infected BXH-7 and BXH-14 mice per haploid mouse genome equivalent. The BXH-7 and BXH-14 mice were inoculated with $5 \times 10^5$ PFU of BXH-2 biologically cloned B-ecotropic MuLV at 1 day of age. Hybridization conditions were as described before (22). Calculations were based on a $C_0t$ for unique mouse DNA of 3,200. All values represent the average of three independent kinetic analyses.

[b] ND, Not done.

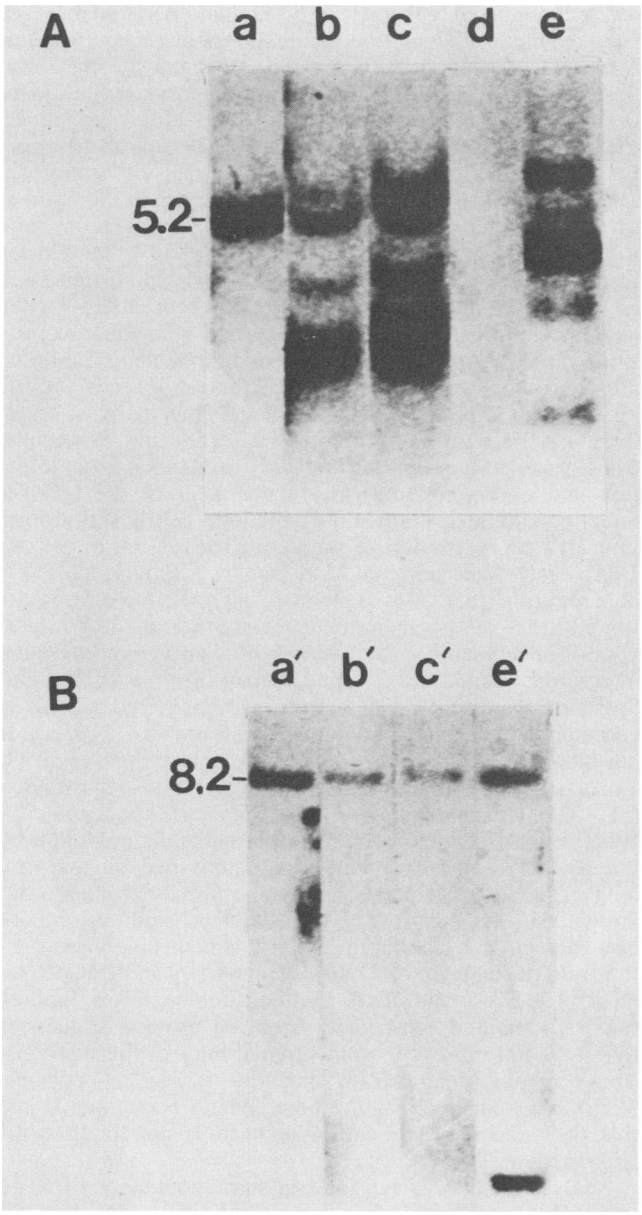


FIG. 3. Characterization of ecotropic MuLV sequences in DNAs of control and inoculated BXH-7 and BXH-14 mice. (A) High-molecular-weight DNAs prepared from spleens of normal and leukemic BXH-7 and BXH-14 mice were digested to completion with *Pvu*II. Fragments were detected after electrophoresis through 0.6% agarose gels, Southern blotting, and hybridization with the p15E probe. The parental endogenous N-ecotropic provirus inherited by BXH-14 mice is indicated (5.2 kb) in lane a; BXH-7 mice lack endogenous ecotropic MuLV DNA sequences (lane d). Representative results of two different virally induced tumors of BXH-14 mice (lanes b and c) as well as a virally induced myeloid leukemia in a BXH-7 mouse (lane e) are shown. (B) *Pst*I/*Eco*RI double digestion of these same tumor DNAs revealed the presence of an 8.2-kb fragment in all of the tumors (lanes b', c', and e'). One tumor (lane e') shows the presence of an additional tumor-specific provirus smaller than genome size. *Pst*I cleaves only with the viral long terminal repeat sequences of ecotropic proviruses and yields a single detectable fragment of 8.2 kb. Since *Eco*RI does not cleave within the ecotropic MuLV genome, but does cleave with the genome of many MCF viruses, it is possible that the subgenomic viral DNA fragment seen in lane e' represents a dualtropic or a defective ecotropic MuLV genome.

We have previously shown that the BXH-2 RI mouse strain develops tumors of non-T-cell origin before 1 year of age (4). These tumors have now been characterized and found to be negative for the expression of T-cell (*Thy-1*, *Lyt-1*, and *Lyt-2*) and B-cell (surface immunoglobulin) surface markers as determined by either immunofluorescence or cytofluorimetry. The tumor cell suspensions examined by cytochemical staining were all positive for NSE and CAE, although individual tumors showed varying intensities of staining; all cells were consistently negative for peroxidase staining. This pattern, when interpreted in reference to the known developmental stages of hematopoiesis, suggests that the BXH-2 leukemias are of myeloid origin showing different degrees of differentiation.

Specific consistent chromosome translocations and other abnormalities have been reported for murine myeloid leukemias induced by irradiation and in human promyelocytic leukemias (19, 33, 35). Also, cellular oncogenes have been shown to be involved in specific chromosomal translocations characteristic of particular classes of myelocytic neoplasia (34, 35). In the studies of Bartram et al. (2), it was shown that the c-*abl* oncogene is translocated to the Philadelphia chromosome in many chronic myelocytic leukemias but not in Philadelphia chromosome-negative chronic myelocytic leukemia patients. The chronic myelocytic leukemias in patients without the translocation reportedly represent a distinct subclass of myelocytic leukemias. Whether these consistent translocations are causally related to the transformation event is not known. Cytogenetic studies in collaboration with F. Wiener (Karolinska Institute, Stockholm, Sweden) indicate that the BXH-2 tumors are diploid without any specific chromosome aberrations. The possibility exists,

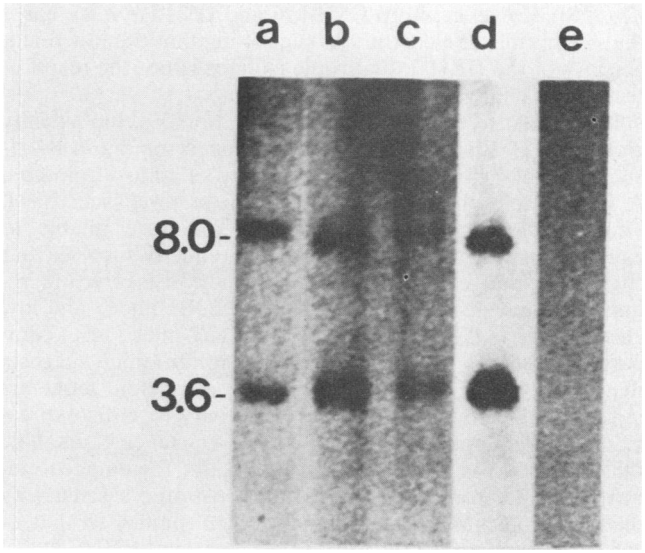


FIG. 4. Hybridization of the p15E ecotropic MuLV probe to RNA from BXH-2 tissues of leukemic and paralyzed mice. Poly(A$^+$) RNA was isolated as described in the text, and 5 μg was subjected to electrophoresis in 1% agarose gels containing formaldehyde, transferred to nitrocellulose paper, and hybridized with the $^{32}$P-labeled p15E probe as described before (41). The autoradiograph shows RNA from leukemic tissue (lane a), brain tissue of three paralyzed mice (lanes b to d), and normal brain tissue (lane e). Restriction fragments of $^{32}$P-labeled DNA (*Hin*dIII-digested λ phage) were electrophoresed in a parallel lane and served as molecular weight markers.

however, that chromosomal aberrations might be present but are too small to be detected by this analysis or that chromosonal loss and reduplication may occur as demonstrated in retinoblastoma tumors (6). Specifically, we have not observed chromosomal rearrangements involving the v-*abl*, v-*ras*, or v-*myc* oncogenes in 12 independent BXH-2 tumors examined by Southern blotting and hybridization with molecularly cloned viral oncogene probes (data not shown). Whether chromosomal rearrangements involving other oncogenes occur in BXH-2 myeloid leukemias is being investigated.

That the BXH-2 B-ecotropic MuLV might be paralytogeneic was suggested by the spontaneous expression of this virus in CNS tissue of paralyzed mice but not in unaffected mice as determined by XC assay and Northern blot analysis. Thus, unlike the virus-associated paralysis of certain wild mouse populations (15), it appears from our histological and cytochemical data that the occasional regions of brain substance necrosis are associated with the infiltration of leukemic cells.

Several studies have implied that certain dualtropic MCF viruses play an important role in the induction of thymic lymphomas and erythroblastosis (2, 33). Although we have isolated dualtropic viruses from some spontaneous tumors in BXH-2 mice, our results suggest that these dualtropic viruses are not directly involved in the induction of BXH myeloid leukemias. The inconsistent association of dualtropic virus expression in mice with myeloid and lymphoid leukemias as well as in cell lines derived from these mice has been reported by others. It has been suggested by Chesebro et al. (10) that the heterogeneity of disease patterns observed with Friend MuLV may be due to a mechanism(s) that prevents slower ecotropic virus-mediated transformation events. Recently a dominant gene designated *Rmcf*$^r$, which restricts the replication of AKR dualtropic viruses, has been identified (18). However, both C57BL/6 and C3H/He mice carry the susceptible allele (*Rmcf*$^s$), suggesting that the low oncogenicity of the BXH-2 dualtropic isolates is not the result of restriction conferred by the *Rmcf* locus.

In contrast to the BXH-2 dualtropic MuLVs, biologically cloned BXH-2 B-ecotropic MuLV induced tumors in BXH-14 and BXH-7 mice with a latent period of 10 to 14 months. A B-ecotropic virus was isolated from tumor tissues of all virally induced myeloid leukemias. We were unable to isolate a dualtropic virus from any leukemic tissues, further suggesting that expression of dualtropic viruses is not required for myeloid leukemogenesis in BXH mice. The low efficiency of leukemia induction in BXH-7 mice (14%) compared with BXH-14 (100% disease induction) mice suggests that other host genes have an effect on myeloid leukemia induction. Whether the lowered sensitivity to virus expression in BXH-7 mice might be due to the presence of an allele that affects virus spread appears unlikely, for embryo cultures derived from BXH-7 mice demonstrated a sensitivity to B-ecotropic MuLV infection in vitro similar to that of other *Fv-1*$^b$ strains (BALB/cJ, C57BL/6J, and BXH-14).

The role of the immune response in leukemia induction in BXH-7 and BXH-14 mice is not known. That there may be a difference in immune responsiveness between BXH-7 and BXH-14 mice is suggested by the lower immunological response of BXH-14 mice to mitogenic stimulation by phytohemagglutinin and concanavalin A (unpublished data). An evaluation of the anti-MuLV response in BXH-7 and BXH-14 mice is being investigated to determine the role of such responses in preventing (21) or promoting (27) leukemia development.

Virally induced leukemias of BXH-7 and BXH-14 mice are accompanied by somatic amplification and reintegration of MuLV DNA sequences in new chromosomal sites of tumor tissues. Molecular hybridization experiments aimed at identifying the organs in which B-ecotropic MuLV genes are expressed have established the lymphoid organs as the target for B-ecotropic infection and replication. Results from reassociation kinetic analysis showed an increase in the number of viral genome equivalents in spleenocytes and in bone marrow cells of inoculated mice but not in other tissues tested. However, viral RNA expression was not observed in all inoculated mice. Only one of the four BXH-7 mice examined expressed viral RNA, whereas viral RNA expression was detected in all BXH-14 mice examined 2 months after virus inoculation. Therefore, the target cells for the virus appear to be within the spleen and bone marrow. These cells may or may not become virus producers or become transformed. Thus, within the host, exogenous virus infection and expression may be regulated at (i) the level of infection and integration and (ii) the level of transcription as indicated by repression of viral gene expression in cells of BXH-7 mice that acquired viral DNAs. Although our DNA data indicate that all exogenously infected mice have acquired the virus, the possibility exists that in the group of BXH-7 mice used for the RNA studies virus may not have integrated, accounting for the absence of virus expression. Specific organotropism of exogenously acquired viruses is common to both avian and murine retroviruses (1, 16, 23). It has also been shown that the virus long terminal repeat (7, 14) or structural differences within the viral coding region (31, 33) determine tissue specificity and oncogenicity. Whether virus targeting and myeloid leukemia induction by the BXH-2 B-ecotropic virus are determined by the viral long terminal repeat or are due to structural differences is not known. To investigate these and other possibilities, we are now molecularly cloning the BXH-2 B-ecotropic virus.

Southern analysis of DNAs from virally induced leukemias of BXH-7 and BXH-14 mice demonstrates that all tumors contained somatically acquired proviral sequences that were not present in uninoculated mice of these strains. These results demonstrate that the BXH-2 B-ecotropic MuLV is oncogenic in other host genetic backgrounds and that the endogenous N-ecotropic virus is not required for leukemogenesis.

Analysis of DNA from independent spontaneous BXH-2 tumors by Southern blotting and hybridization with the p15E probe showed that most tumors contained somatically acquired nondefective B-ecotropic MuLV genomes. The small number of newly acquired proviruses (one to four somatically acquired proviral sequences) in BXH-2 myeloid leukemias is in contrast to the large number of viruses detected in T-cell lymphomas (20). Thus, the BXH RI strains represent a unique and useful model system for the study of myeloid leukemia since they present both a disease model and 11 genotypically distinct but related controls to study host factors and genes affecting viral expression as well as biological and molecular mechanisms of myeloid leukemia induction by the BXH-2 B-ecotropic MuLV.

## ACKNOWLEDGMENTS

We thank Sandra Rodick and Kenneth Salvatore for technical assistance and George Carlson and Joe Angel for helpful discussions and critical reading of this manuscript.

This research was supported by Public Health Service grants CA 31102 (H.G.B.), CA 37283 (N.G.C.), CA 32630 (D.A.J.), and CA

27523 (R.E.) awarded by the National Cancer Institute, a grant from the Elsa U. Pardee Foundation (H.G.B.), and grant MV-124 from the American Cancer Society.

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