Transplacental Transmission of a Leukemogenic Murine Leukemia Virus

H. G. BEDIGIAN,1* L. A. SHEPEL,1,2 AND P. C. HOPPE1
The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, 1 and Department of Human Oncology, University of Wisconsin, Madison, Wisconsin 537922

Received 22 March 1993/Accepted 16 July 1993

Recombinant inbred BXH-2 mice spontaneously produce a B-tropic murine leukemia virus (MuLV) beginning early in life and have a high incidence of spontaneous myeloid leukemia. These traits are not characteristic of the progenitor strains (C57BL/6J and C3H/HeJ) or of 11 other recombinant inbred BXH strains. Genetic analysis has shown that the virus is not transmitted through the germ line, suggesting that the virus is passed from one generation to the next by horizontal transmission. An additional ecotropic proviral locus was detected in some mice of this strain after several generations of inbreeding. We show that BXH ecotropic virus was transmitted to other strains when fostered on viremic BXH-2 mice and that these mice go on to develop tumors of hematopoietic origin. Our earlier finding that virus is expressed early in gestation suggested that the ecotropic MuLV is also transmitted in utero. In order to determine the stage at which the ecotropic MuLV is transmitted in utero, preimplantation stage embryos were transferred to the uterus of recipient ecotropic virus-negative mice. These mice were found to be negative for the presence of the ecotropic MuLV, suggesting that transplacental transmission of the ecotropic virus readily occurs in BXH-2 mice. Although other viruses, including human lentiviruses, are transmitted across the placental barrier, transplacental transmission of MuLV is a rare event. Thus, the BXH-2 mouse strain may contribute to our understanding of the mechanism of transplacental transmission and pathogenesis and offers a potential new model for use in drug therapy of exogenously transmitted viruses related to lentiviruses.

The transmission of retroviruses from parent to offspring can occur via the germ line or by congenital transmission. Congenital transmission of infectious ecotropic murine leukemia virus (MuLV) has been shown to occur in the birth canal, through mammary secretions, and possibly by sperm (10, 13, 16-18). Although it is possible for a viremic mother to infect her offspring prior to implantation of the embryo, the frequency of transplacental transmission of MuLV is reportedly rare and may not occur at all (10).

Of 12 recombinant inbred (RI) BXH strains derived from crossing C57BL/6J and C3H/HeJ mice, one RI strain, BXH-2, expresses ecotropic MuLV early in life. Viremic BXH-2 mice (>90%) develop leukemias of myeloid origin by 1 year of age. In contrast to the RI BXH-2 strain, the progenitor strains express low or undetectable levels of ecotropic virus and have a low incidence of leukemia (3). Genetic analysis and Southern blot hybridizations have shown that the BXH-2 ecotropic virus is not transmitted from parent to offspring through the germ line (12). Thus, the spread of virus from one generation to the next in BXH-2 mice occurs in utero, by milk transmission, or both.

Our earlier finding that BXH-2 embryos expressed virus as early as 10 to 12 days of gestation suggested that BXH-2 mice acquire the B-ecotropic virus in utero (2, 3). To determine the stage at which viremic BXH-2 mice infect their offspring, preimplantation stage BXH-2 embryos were transferred to the uterus of recipient pseudopregnant ecotropic virus-negative BXH-7 mice. If virus infection occurs prior to implantation of the embryo, then a new infectious virus would be present in the germ line. Conversely, if the transferred embryos were negative for ecotropic virus, then the B-ecotropic virus can be transmitted transplacentally in the viremic BXH-2 strain. The transmission of the BXH-2 B-ecotropic virus through mothers’ milk was also investigated. The data presented show that the BXH-2 ecotropic virus is transmitted via infection of postimplantation embryos and by milk transmission and that by either route the viremic mice go on to develop leukemia.

**MATERIALS AND METHODS**

**Mice.** The RI BXH mouse strains were derived by systematic inbreeding, beginning with randomly chosen pairs of mice from the F2 generation of a cross of C57BL/6J mice and C3H/HeJ inbred strains (3). The C57BL6-H-2fla and B10.D2/SJvEg strains were obtained from I. Egorov of The Jackson Laboratory. The other inbred strains were from The Jackson Laboratory Animal Resources, Bar Harbor, Maine. The various strains used for the milk transmission studies were hysterectomy derived and fostered on viremic BXH-2 mice.

All mice were reared under conventional conditions and given acidified water and food pellets ad libitum.

**DNA isolation, restriction enzyme analysis, DNA transfers, and hybridizations.** High-molecular-weight DNA was extracted from frozen tissues or from tail biopsies (5, 12). DNA (5 μg per lane) was digested to completion with excess restriction enzymes (PvuII and EcoRI) under the reaction conditions recommended by the manufacturers (Bethesda Research Laboratories, Gaithersburg, Md., and Promega, Madison, Wis.). The digested DNAs were subjected to electrophoresis in 0.8% agarose gels. Southern blot transfers, hybridizations, and washes were performed as previously described (2, 12). The ecotropic MuLV-specific probe used (pEco) is a 400-bp SmaI fragment derived from the env gene of pAKV623 (4).

* Corresponding author. Electronic mail address: hgb@aretha.jax.org.
**Virus assays.** Expression of ecotropic MuLV was determined by the XC plaque assay, using tail biopsies or embryo cultures, and reverse transcriptase assay as described previously (2, 3).

**Embryo transfer.** Adult BXH-2 females were injected with 5 IU of pregnant mare serum at 4 p.m., and 48 h later, they were injected intraperitoneally with 5 IU of human chronic gonadotropin before being paired with fertile males. The following morning, females were checked for vaginal plugs and those that had mated were euthanized. Zygotes were collected from the oviducts and cultured for 4 days (8). Embryos were transferred to the uterus of pseudopregnant BXH-7 females who had mated with vasectomized BALB/cJ males 3 days prior to the embryo transfer. Recipient females were housed individually and were allowed to give birth to their litters, or the pups were delivered by cesarian section. The hysterectomy-derived mice (derived mice) were designated BXH-2 V− to distinguish them from the normal BXH-2 mice (nonderived mice).

**RESULTS**

Absence of B-ecotropic virus expression in BXH-2 mice derived by embryo transfer. Previous results have indicated that BXH-2 mice are infected at an early stage of gestation (2). In order to determine the stage of development (post- or preimplantation) at which BXH-2 mice become infected, 4-day-old BXH-2 embryos were transferred to recipient pseudopregnant ecotropic virus-negative BXH-7 mice (12). Embryo cultures were prepared at 15 to 17 days of gestation from five recipient BXH-7 mice. After the cultures had undergone three passages in vitro, they were centrifuged and the supernatant fluids were assayed for ecotropic virus expression by the XC plaque and reverse transcriptase assays. All embryo cultures established from transferred BXH-2 fetuses were negative for ecotropic virus expression, indicating that the BXH-2 fetuses assayed were negative for ecotropic virus (Table 1). As expected, embryo cultures prepared from normally reared BXH-2 mice were positive for ecotropic virus, whereas embryo cultures established from BXH-7 mice were negative for ecotropic virus (Table 1).

Twenty-five BXH-2 mice derived by embryo transfer to BXH-7 mice were assayed for ecotropic virus expression at 1 to 4 months of age (Table 1). Tail biopsies of these mice showed that 22 of 25 mice were negative for ecotropic virus expression, as determined by the XC plaque assay. The three ecotropic virus-positive mice were from one litter and had plaque titers that ranged from one to nine plaques per ml of tail suspension. The parents of the virus-positive mice were examined and also found to be positive for ecotropic virus expression. All ecotropic virus-positive BXH-2 mice developed tumors of myeloid origin by 9 months of age.

The 22 ecotropic virus-negative mice that were hysterectomy derived (designated BXH-2 V− for the absence of MuLV expression) were monitored for 18 months and showed no evidence of tumor development. Tissues from nontumor- and tumor-bearing animals were analyzed by Southern blot hybridization for ecotropic MuLV. DNAs prepared from the normal spleens and tumors of the three viremic BXH-2 mice derived by embryo transfer were digested with PvuII and EcoRI. PvuII cleaves once within the viral genome, generating virus-cell junction fragments (12). EcoRI does not cleave within the BXH-2 ecotropic provirus, thus generating fragments larger than the 8.6-kb viral genome. The Southern blots were probed with the 400-bp ecotropic virus-specific (pEco) probe (4) or the pAKV5 probe (detects the presence of both ecotropic and dual-tropic MuLVs) (7). Southern blots of BXH-2 DNA showed the presence of the two endogenous N-ecotropic proviruses (Emv-1 and Emv-2) acquired from the parental strains, C3H/HeJ and C57BL/6J, respectively (12). Additional proviral sequences that were not present in nontumor tissue were detected in tumor DNA (Fig. 1A). When DNAs from unaffected brain tissue in the tumor-bearing mice were analyzed after PvuII digestion, an additional provirus fragment of 11.5 kb was detected (Fig. 1A, lane 5 and 6). Further Southern blot analysis on tail DNAs from young BXH-2 mice showed that some BXH-2 mice had acquired the additional 11.5-kb provirus in the germ line (Fig. 1). That the 11.5-kb fragment represents a new endogenous provirus in this strain was supported by genetic crosses and Southern analysis of tail DNAs of F1 mice. All F1 mice showed the presence of the additional 11.5-kb provirus (Fig. 2). Additional mice (six mice) that carried the additional provirus (confirmed by Southern blotting) were also tested to determine whether they expressed ecotropic virus. Splenic tissue samples from all six mice were found to express high titers of ecotropic virus (greater than $5 \times 10^5$) when tested at 3 weeks of age by the XC plaque assay.

**Congenital transmission of B-ecotropic virus via mothers’ milk.** To further determine the efficiency by which the BXH-2 B-ecotropic virus can be transmitted and the influence of the host genetic background on tumor occurrence, various inbred and congenic strains were hysterectomy derived and fostered on both BXH-2 (Fv-1b-H-2β) or C57BL/6J (Fv-1b-H-2β) mice. After the mice were weaned and when they were 3 to 4 months of age, tail biopsies were taken and assayed for the presence of ecotropic virus by the XC plaque assay. Milk samples from BXH-2 lactating mice were positive for B-ecotropic virus when assayed on suscep-

---

**TABLE 1. Ecotropic virus expression in derived and nonderived BXH-2 mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of XC plaques</th>
<th>No. of mice with tumors* (no. positive/no tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo culturesa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BXH-2 V−</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>BXH-2</td>
<td>83</td>
<td>ND</td>
</tr>
<tr>
<td>BXH-7</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Derived miceb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BXH-2 V−</td>
<td>5d</td>
<td>3/25</td>
</tr>
<tr>
<td>Nonderived mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BXH-7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BXH-2</td>
<td>TNTC*</td>
<td>18/20 (90%)</td>
</tr>
</tbody>
</table>

a Lympoma incidence in nonderived and derived BXH-2 mice was determined by histological analysis. The mice were monitored for 18 months, ND, not done.

b Embryo cultures were prepared from five mice at 15 to 17 days of gestation. Culture fluid was used to infect mouse embryo fibroblasts. After 5 days, the cultures were UV irradiated and overlaid with XC cells. Four days later, syncytial plaques were counted as described previously (3).

c BXH-2 embryos were transferred to ecotropic virus-negative BXH-7 recipients, and tail extracts from individual mice (25 mice from 8 litters) were assayed for ecotropic virus by the XC plaque assay (3). The derived mice were designated BXH-2 V− to distinguish them from normal BXH-2 mice. Tail extracts from nonderomed BXH-2 mice were positive for ecotropic virus, indicating that the virus detected in nonderomed BXH-2 mice was an exogenous ecotropic virus.

d The number of plaques shown represents the average number of plaques counted in three ecotropic virus-positive mice (three mice all from one litter).

TNTC, too numerous to count.
viremic were by BXH-2 All infectious and brain tissue not infiltrated with leukemic cells (lanes 5 and 6) are shown. The 11.5-kb fragment is readily observable in brain and tumor DNAs. (B) DNA from BXH-2 V− mice (lanes 1 and 2), DNA from BXH-2 V− mice fostered on viremic BXH-2 mice (lane 3), and normal DNA from C57BL/6J and C57BL/10J mice (lanes 4 and 6, respectively) and from C57BL/6J and C57BL/10J mice fostered on viremic BXH-2 mice (lanes 5 and 7, respectively) are shown.

FIG. 1. DNAs (5 µg per lane) from normal and tumor tissue were digested to completion with restriction enzyme (PstII) and analyzed for the presence of ecotropic provirus by Southern blot analysis with the ecotropic virus-specific probe pEco. (A) Normal DNA from a BXH-2 V− mouse (lane 1), BXH-2 tumor DNA (lanes 2 to 4), and DNA from brain tissue not infiltrated with leukemic cells (lanes 5 and 6) are shown. The 11.5-kb fragment is readily observable in brain and tumor DNAs. (B) DNA from BXH-2 V− mice (lanes 1 and 2), DNA from BXH-2 V− mice fostered on viremic BXH-2 mice (lane 3), and normal DNA from C57BL/6J and C57BL/10J mice (lanes 4 and 6, respectively) and from C57BL/6J and C57BL/10J mice fostered on viremic BXH-2 mice (lanes 5 and 7, respectively) are shown.

tible Fv-1b embryo fibroblast derived from BALB/cJ mice. Milk samples obtained from C57BL/6J or BXH-2 V− mice were negative for ecotropic virus expression when assayed on embryo fibroblasts established from SWR/J (Fv-1b) or BALB/cJ mice. The BXH-2 mice used in these studies did not carry the third endogenous provirus, as determined by Southern blot analysis of tail DNAs (Fig. 1B, lanes 1 and 2). All BXH-2 mice (a total of 10 mice) fostered on C57BL/6J females were positive for ecotropic virus, supporting our notion that in the BXH-2 strain exogenous ecotropic virus is acquired in utero.

When BXH-7 (Fv-1b H-2b) mice were fostered on BXH-2 viremic mothers, the majority of mice were negative for ecotropic virus expression at 4 months of age, as determined by tail biopsies or XC infectious center assay. The BXH-7 strain has been shown to be resistant to leukemia induction by the BXH-2 B-tropic MuLV (2). The results presented in Table 2 show that the level of virus expression detected by the infectious center assay varied in the strains used (32 to 10,000 plaques per 10⁷ splenocytes). In addition, 85% of the

C57BL/10SnJ and 80% of the C57BL/6J (both Fv-1b H-2b) mice fostered on BXH-2 viremous virus when assayed at 3 months of age (Table 2). C57BL/6J and C57BL/10SnJ mice reared normally do not express virus at an early age. The viremic mice began showing signs of neoplastic disease as early as 10 months of age. Of the viremic C57BL/6J and C57BL/10SnJ mice, 60 and 73% of the mice, respectively, developed tumors by 1 year of age. Histologically the majority of tumors were classified as being of B-cell origin (Table 2). When the H-2 congenic C57BL−H-2b FlaeEg (Fv-1b H-2b) strain was fostered on BXH-2 mice, tumor onset was delayed (mean age of mice at tumor onset was 14.5 months) and the incidence of disease was lower (Table 2). These results would indicate that the H-2b haplotype has some effect on tumor susceptibility. However, BXH-2 V− H-2b mice fostered on BXH-2 viremic mothers developed leukemias of myeloid origin by 1 year of age, implying that in the BXH-2 strain other factors override the modifying effect of H-2b on virus expression and tumor occurrence. When tumor DNAs were analyzed by Southern blot hybridization, additional proviral sequences that were not present in uninfected mice were detected (Fig. 1B, lanes 3, 5, and 7). These results support the notion that the virus has a causal role in the disease process.
TABLE 2. Susceptibility to virus infection and tumorigenesis by milk transmission

<table>
<thead>
<tr>
<th>Strain</th>
<th>Virus titer/10^3 splenocytes</th>
<th>No. of virus-positive tumors/total no. (%)</th>
<th>H-2 allele</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>1,584</td>
<td>12/20 (60)</td>
<td>b</td>
<td>B-cell</td>
</tr>
<tr>
<td>C57BL6-H-2{k}/FlaEg</td>
<td>316</td>
<td>2/10 (20)</td>
<td>k</td>
<td>B-cell</td>
</tr>
<tr>
<td>B10.D2/nSnEg</td>
<td>10,000</td>
<td>8/10 (80)</td>
<td>d</td>
<td>B-cell</td>
</tr>
<tr>
<td>C57BL/10J</td>
<td>6,300</td>
<td>11/15 (73)</td>
<td>b</td>
<td>B-cell</td>
</tr>
<tr>
<td>BXH-7</td>
<td>32</td>
<td>0/20 (0)</td>
<td>k</td>
<td>ND</td>
</tr>
<tr>
<td>BXH-14</td>
<td>100</td>
<td>8/20 (40)</td>
<td>k</td>
<td>B-cell</td>
</tr>
<tr>
<td>BXH-2 V^-</td>
<td>6,542</td>
<td>9/10 (90)</td>
<td>k</td>
<td>Myeloid</td>
</tr>
</tbody>
</table>

* Mice from various strains were hysterectomy derived, and the progeny were fostered on BXH-2 mothers.
* Virus expression was determined by the infectious center assay when the mice were 3 months old. The number of mice fostered and tested ranged from 10 to 20 mice per strain, and the mice were from two to five litters. Mouse embryo fibroblasts derived from SWR/J (FV-1') and BALB/c (FV-1') mice were infected with serial dilutions of mitomycin-treated splenocytes. After 5 days, the cells were UV irradiated and overlaid with XC cells. Four days later, syncytial plaques were counted as described previously (3).

DISCUSSION

Although some viruses that infect domestic animals and humans readily cross the placental barrier, it is an uncommon route of transmission for murine retroviruses. Studies of retrovirus transmission in mice have shown that the most efficient mode of retrovirus spread appears to be by milk transmission (10, 13, 16, 18), whereas transplacental infection via viremic mothers to the embryo is unlikely (10).

To obtain a better understanding at which stage in development (pre- or postimplantation) viremic BXH-2 mothers infect their offspring, we derived BXH-2 ecotropic virus-negative mice by embryo transfer. Of the 25 BXH-2 mice derived by embryo transfer, 22 did not express ecotropic virus nor did they develop leukemia. The three positive BXH-2 mice were either contaminated at the time of embryo transfer or were expressing an endogenous virus. Southern blot analysis of tumor DNA and unaffected tissue showed the presence of a new common ecotropic proviral band, suggesting that virus expression may be related to the presence of this newly acquired ecotropic provirus. By virus typing, we determined that the expressed virus was B-ecotropic. It is unlikely that the virus being expressed is one of the endogenous proviruses acquired from the parental strains because these endogenous ecotropic proviruses were classified as N-ecotropic (12).

The experiments described here show that the BXH-2 ecotropic virus is transmitted after implantation of the BXH-2 embryo. This study suggests that the BXH-2 ecotropic virus is able to cross the placental barrier and is causally associated with leukemia in this strain. It appears that the factors that inhibit transplacental infection in other strains are absent from BXH-2 mice. The high efficiency of retroviral transmission in BXH-2 mice may be related to the local production of retrovirus within the female reproductive system coupled with the absence of an immune response to the ecotropic virus (2, 3). It is also possible that one of the endogenous ecotropic viruses is being expressed or that the BXH-2 B-ecotropic virus has integrated into the germ line and is expressed. Previous studies have demonstrated that in highly viremic strains the provirus copy number can increase after several generations (18, 19). An estimate of the rate at which new endogenous ecotropic viruses are fixed in the germ line of these strains is one new provirus every 15 to 30 years (37 to 75 generations of inbreeding). An unusually high frequency of germ line integration of endogenous ecotropic viruses has been reported for SWR/J × RF/J hybrid mice (11). Approximately 20% of the animals obtained from this cross acquired new proviruses. BXH-2 mice, now in their inbreeding generation 76, have acquired an additional provirus that was not present when tested at an earlier generation of inbreeding. However, while we have detected ecotropic virus integration into the germ line of some BXH-2 mice, our hybridization data show that the BXH 2V^- mice harbor only the Env-1 and Env-2 proviruses (Fig. 1B, lanes 1 and 2). These results show that at the time of embryo transfer the BXH-2 V^- mice had not acquired additional endogenous ecotropic proviruses in the germ line and that in the BXH-2 strain the exogenous B-ecotropic virus is able to traverse the placental barrier.

Our data also show that the B-ecotropic virus is readily transmitted through mothers' milk. BXH-2 V^- mice, obtained by hysterectomy derivation, fostered on noninfected BXH-2 mice develop tumors of myeloid origin by 1 year of age. Interestingly, the majority of tumors that developed in other strains fostered on BXH-2 mice transmitting the B-ecotropic virus were of B-cell origin. Also, not all strains that would be permissible to virus infection showed the same degree of susceptibility to tumor induction. The overall resistance of an animal to an infectious agent is generally the result of the cumulative effects of genes at multiple loci either alone or in combination with other host and environmental factors. In spite of the fact that disease resistance to virus infection is a multigenic trait, some of these genes have effects that are large enough to be detected. The H-2 complex has been shown to have an effect on virus titer, immunological responsiveness, and tumor incidence (1, 16).

The studies described here indicate that mice carrying the H-2^b allele fostered on BXH-2 viremic mice have a lower incidence of virus expression and tumor incidence than mice carrying the H-2^a or H-2^d haplotype. However, BXH-2 V^- mice (H-2^d) fostered on viremic BXH-2 mice are as prone to leukemogenesis as the original stock and do not show the delayed response observed in the other H-2^k strains tested. Although the mechanism of H-2 influence on lymphoma susceptibility is poorly understood, it is most likely mediated through its effect on the immune response to virus-induced tumor cells. We have analyzed peripheral blood samples of BXH-2 mice for the presence of T- and B-cell lymphocytes by fluorescence-activated cell sorting. Our preliminary information shows a normal distribution of T and B cells but an elevated number of myeloid cells in young mice. However, there appears to be a progressive decline of T-cell subsets with age. Recently, minor stimulating lymphocyte genes (Mls) or superantigens encoded by mouse mammary tumor
viruses (14), and possibly other retroviruses, initiate the deletion of specific T-cell subsets that in association with the major histocompatibility complex induce immune suppression and enhance virus spread (6, 9). Whether the development of myeloid leukemia in BXH-2 mice is related to an altered expression of viral antigens remains to be determined.

In conclusion, this study demonstrates that in the RI BXH-2 strain the B-ecotropic virus is transmitted across the placental barrier and that this exogenously transmitted virus has a causative role in myeloid leukemogenesis. Although not discussed here, some mice develop a hind limb paralysis associated with leukemogenesis (3). The transplacental transmission of this exogenously transmitted MuLV and the occurrence of a neurological disorder in some mice are characteristics of the pathogenesis of infection in human beings by human T-cell leukemia virus type I and human immunodeficiency virus lentiviruses (15). Therefore, this animal model may be useful for investigating the mechanism of transplacental transmission of exogenous retroviruses as well as preventive therapies of virus-associated leukemia and immunodeficiencies.

ACKNOWLEDGMENTS

We thank Sandra Rodick for technical assistance and B. Taylor and W. Frankel for critical review of the manuscript.

This research was supported in part by the Cancer Center Grant (CORE CA34196) (P.C.H.) and by Public Health Service grant CA-31102 (H.G.B.) from the National Cancer Institute.

REFERENCES