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The Leukemogenic Potential of an Enhancer Variant of Moloney Murine Leukemia Virus Varies with the Route of Inoculation

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We previously showed that the Mo+PyF101 variant of Moloney murine leukemia virus (M-MuLV) is poorly leukemogenic when inoculated subcutaneously (s.c.) into neonatal mice. We recently found that intraperitoneal (i.p.) inoculation of neonatal mice with the same virus significantly enhanced its leukemogenicity. In this study, infections of neonatal mice by the two different routes of inoculation were compared. We studied replication of the virus in vivo to identify critical preleukemic events. These would be observed in mice inoculated i.p. by Mo+PyF101 M-MuLV but not when inoculation was s.c. Infectious center assays indicated that regardless of the route of inoculation, Mo+PyF101 M-MuLV showed delayed infection of the thymus compared with wild-type M-MuLV. On the other hand, i.p.-inoculated mice showed more rapid appearance of infectious centers in the bone marrow than did s.c.-inoculated animals. Thus, the enhanced leukemogenicity of i.p. inoculation correlated with efficient early infection of the bone marrow and not with early infection of the thymus. These results suggest a role for bone marrow infection for efficient leukemogenesis in Mo+PyF101 M-MuLV-infected mice. Consistent with this notion, if bone marrow infection was decreased by injecting 10- to 12-day-old animals i.p., leukemogenicity resembled that of s.c. inoculation. Thus, two cell types that are critical for the induction of efficient leukemia were implicated. One cell delivers virus from the site of s.c. inoculation (the skin) to the bone marrow and is apparently restricted for Mo+PyF101 M-MuLV replication. The second cell is in the bone marrow, and its early infection is required for efficient leukemogenesis.

Moloney murine leukemia virus (M-MuLV) is a replication-competent, nonacute retrovirus. Neonatally infected mice typically develop T-cell lymphoblastic lymphoma with a mean latency of 3 to 4 months. The relatively long latency of disease development likely reflects the multistage nature of the disease process, including insertional activation of proto-oncogenes and also virus-induced preleukemic events.

We have described a variant of M-MuLV, Mo+PyF101 M-MuLV, that contains enhancer sequences from the F101 strain of polyomavirus (Py) inserted into the U3 region of the wild-type M-MuLV long terminal repeat (LTR) (5). This LTR variant is poorly leukemogenic when inoculated subcutaneously (s.c.) into newborn NIH Swiss mice, even though the virus replicates efficiently in vivo (6, 7). The weak leukemogenic potential of Mo+PyF101 M-MuLV may result from an alteration in the tissue tropism of this variant virus such that it cannot replicate in some critical cell type whose infection is necessary for M-MuLV leukemogenesis. The leukemogenic defect of Mo+PyF101 M-MuLV has also been associated with inability of this virus to induce a preleukemic state characterized by hematopoietic hyperplasia in the spleen (6, 17). Additionally, the preleukemic defect may result from the inability of this virus to form *env* gene (mink cell focus-forming [MCF]) (1a) recombinants in vivo. MCF recombinants have been implicated in the generation of preleukemic splenic hyperplasia induced by wild type M-MuLV (13, 14).

Recently, we have observed that intraperitoneal (i.p.) inoculation of Mo+PyF101 M-MuLV leads to a dramatic increase in leukemogenicity. In this report, infections of neonatal mice by the two different routes of inoculation were compared. The goals of the study were to characterize the replication of the virus in vivo in order to identify critical preleukemic infection events which could be carried out by Mo+PyF101 M-MuLV when inoculated i.p. but not when inoculated s.c. This analysis might provide further insight into M-MuLV leukemogenesis. The results suggested that early infection of the bone marrow compartment was important in leukemogenesis at early stages of the infection process.

MATERIALS AND METHODS

Virus and inoculation of mice. The generation of Mo+PyF101 M-MuLV (6) and the molecular clone of wild-type M-MuLV (clone 43D) have been described previously (13). Viral stocks were cell culture supernatants derived from productively infected NIH 3T3 cells. Virus titers were determined by the UV/XC plaque assay (16). NIH Swiss mice were inoculated i.p. or s.c. with 0.15 ml of virus stock (approximately 10^5 XC PFU) within 48 h of birth.

Southern blot analysis. DNA was obtained from tumors as previously described (9). Southern blot analyses were performed as previously described (9), using GeneScreen Plus (New England Nuclear). The T-cell receptor β chain (TCRβ) hybridization probe has been previously described (12). The Py enhancer probe was prepared from the Py F101 enhancer-containing *Pvu*II-4 fragment isolated from a pGEM construct containing the complete Py F101 genome. Radioactive probes were prepared by the random primer method, following standard procedures (18).

Assays for infectious virus. The presence of infectious virus in hematopoietic organs was determined by infectious center assays on NIH 3T3 cells as previously described (4). Briefly,

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animals were sacrificed at the times indicated, and single cell suspensions were prepared from the spleen, thymus, and bone marrow (obtained by femur flush). Cell suspensions were serially diluted and cocultivated for 24 h with NIH 3T3 cells (5×10^4 cells per 5-cm-diameter dish) in Dulbecco modified Eagle's medium containing 10% calf serum and 2 μg of Polybrene per ml. After cocultivation, the nonadherent hematopoietic cells were aspirated, and the NIH 3T3 monolayers were washed twice with phosphate-buffered saline and allowed to grow to confluency. Upon reaching confluency, the cultures were assayed for infectious centers by UV/XC overlay and counting XC syncytial plaques (7).

Formation of stromal/fibroblastoid colonies. Bone marrow obtained from the femur was plated at 5×10^5 cells in Dulbecco modified Eagle's medium supplemented with 20% fetal calf serum and 0.8% (wt/vol) methylcellulose. Cultures were plated into 30-mm-diameter petri dishes and incubated for 9 days (19).

Modified XC plaque assay. Adherent colonies from methylcellulose cultures were washed three times with fresh medium to remove the methylcellulose. New medium containing 5×10^5 XC cells was added to the cultures, and the cultures were incubated for an additional 2 days. Colonies containing XC syncytia with more than six nuclei were scored as positive.

RESULTS

Effect of route of inoculation on the pathogenicity of Mo+PyF101 M-MuLV. Neonatal NIH Swiss mice were inoculated either i.p. or s.c. with the same stock of Mo+PyF101 M-MuLV. As controls, animals were similarly inoculated with wild-type M-MuLV. The mortality plot shown in Fig. 1A demonstrates that the time course of disease development in wild-type M-MuLV-inoculated mice was essentially unaffected by the route of inoculation. Mean time to disease (T-cell lymphoblastic lymphoma) was 3 to 4 months. In contrast, there was a dramatic difference in the rate of disease development in Mo+PyF101-inoculated animals, depending on the route of inoculation (Fig. 1B). Consistent with our previous studies, animals inoculated s.c. showed a greatly decreased rate of disease development (1a, 5, 6). However, animals inoculated i.p. developed disease with kinetics more similar to that of wild-type M-MuLV. Mean time to disease induction was approximately 4 months, and a much higher percentage of animals died. Thus, the previously characterized leukemogenic defect of Mo+PyF101 M-MuLV could largely be overcome by changing the route of inoculation.

At necropsy, animals infected by Mo+PyF101 showed greatly enlarged thymuses with frequent spleen and lymph node involvement, typical of the pathology observed in wild-type M-MuLV-infected mice. Molecular analyses of tumor DNAs for gene rearrangements were carried out to confirm the gross pathological diagnosis of T-lymphoblastic lymphoma. Rearrangements of the T-cell receptor genes are characteristic of T-lymphoid tumors. Tumor DNAs were digested with *Hpa*I and analyzed by Southern blot hybridization using a probe specific to a TCRβ constant region as previously described (12). All of the tumors analyzed showed rearrangement of the TCRβ locus, indicating that they were T lymphoid in origin (data not shown).

Detection of Mo+PyF101 M-MuLV in tumors. It was important to test the resulting tumors for the presence of Py-containing viruses. One explanation for the increased leukemogenicity of Mo+PyF101 M-MuLV when inoculated i.p. was that i.p. inoculation allowed for rapid loss of the Py enhancer sequences from the Mo+PyF101 LTR and subsequent replication of an effectively wild-type M-MuLV. Schematic diagrams of wild-type M-MuLV and Mo+PyF101 M-MuLV proviral DNAs are shown in Fig. 2A. Tumor DNAs were digested with *Pvu*II and analyzed by Southern blot hybridization with a Py-specific probe. The Mo+PyF101 M-MuLV producer cell line (Fig. 2B, lane a) contained the predicted 1.9-kb Py-containing fragment. The additional hybridizing bands in lane a resulted from junction fragments between the proviral DNA and adjacent cellular sequences.

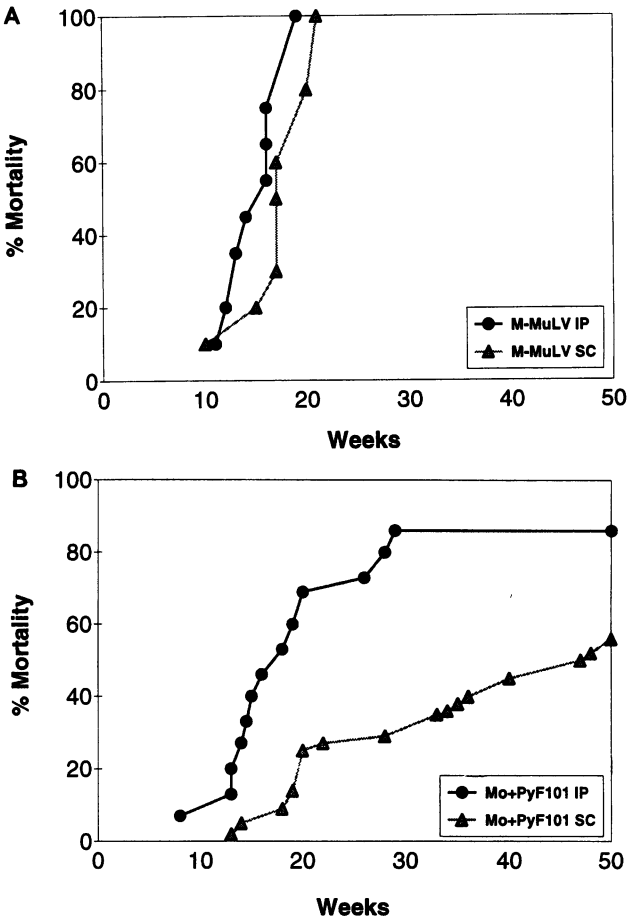

FIG. 1. Pathogenicity of wild-type (A) or Mo+PyF101 (B) M-MuLV. Neonatal Swiss mice were inoculated either s.c. or i.p. with 1×10^5 to 2×10^5 XC PFU. The time course for the appearance of moribund animals is shown. (A) Wild-type M-MuLV; $n = 9$ i.p., $n = 11$ s.c. (B) Mo+PyF101 M-MuLV; $n = 20$ i.p., $n = 18$ s.c.

All of the tumors contained the diagnostic 1.9-kb fragment which hybridized with the Py-specific probe, as shown in Fig. 2B, lanes c to j. Thus, the tumors resulting from i.p. inoculation of Mo+PyF101 M-MuLV retained the Py sequences from the input Mo+PyF101 M-MuLV LTR. The majority of the tumors induced by Mo+PyF101 M-MuLV contained, in addition to the 1.9-kb fragment, a hybridizing species that had a slightly slower mobility. The source of this fragment is under further investigation.

Virus infection in preleukemic mice. We previously showed that s.c. infection of Mo+PyF101 M-MuLV resulted in delayed kinetics of infection in the thymus (7). Little thymic infection was detected at 2 to 3 weeks postinfection relative to the levels in M-MuLV-infected animals, although high levels equivalent

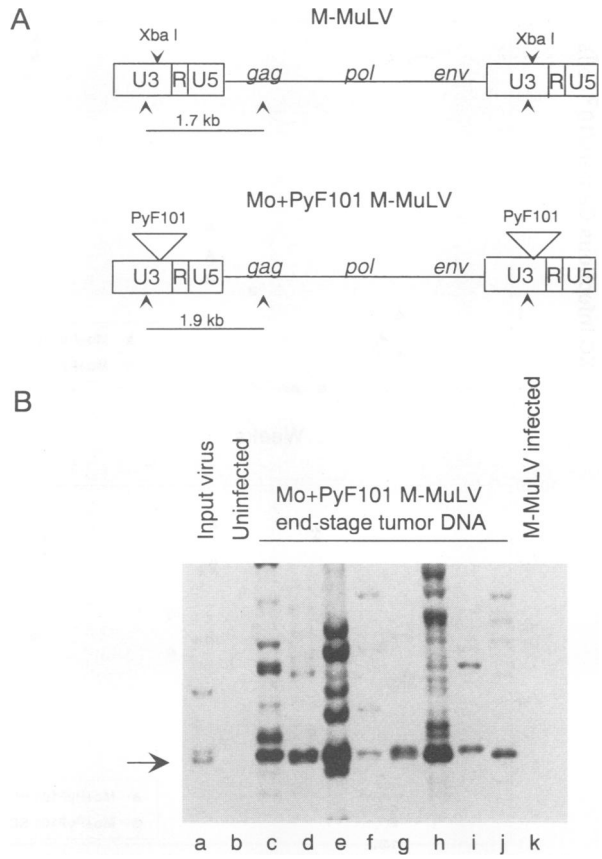

FIG. 2. Detection of provirus by Southern blot analysis. (A) Restriction maps of wild-type M-MuLV (top) and Mo+PyF101 M-MuLV (bottom). Restriction enzyme digestion of high-molecular-weight DNA with *Pvu*II yields a unique 1.9-kb fragment containing the 5′ LTR and the 5′ end of *gag* which hybridizes with the Py enhancer probe. (B) High-molecular-weight DNA (5 μg) was digested with *Pvu*II and analyzed by gel electrophoresis and Southern blot hybridization using a probe specific for the inserted Py enhancer sequences. Lanes: a, producer cell line DNA; b, uninfected control thymus; c to j, Mo+PyF101 M-MuLV-induced tumor DNAs; k, M-MuLV-induced tumor DNA. The Py-specific fragments larger than 1.9 kb represent junction fragments involving the downstream LTR and host cellular DNA.

to wild-type M-MuLV levels were established by 6 weeks postinfection. We tested the hypothesis that the enhanced leukemogenicity of Mo+PyF101 M-MuLV when inoculated i.p. was due to more rapid infection in the thymus. Infectious center assays were performed on thymocytes from infected animals at various times postinfection. As shown in Fig. 3A, wild-type M-MuLV established early high-level infection in the thymus regardless of the route of inoculation. In contrast, Mo+PyF101 M-MuLV showed significantly delayed infection of the thymus, as shown in Fig. 3B. However, the initial low level of thymocyte infection for Mo+PyF101 M-MuLV was unaffected by the route of inoculation. The fact that i.p. inoculation of Mo+PyF101 M-MuLV did not give early high-level thymocyte infection indicated that this was not required for efficient leukemogenesis. This observation was reinforced by the fact that the kinetics of thymocyte infection were equivalent for i.p. and s.c. inoculation by Mo+PyF101 M-MuLV, procedures with very different leukemogenic efficiencies.

In contrast to the results for thymocytes, analysis of infectious centers did demonstrate a significant difference in the rate of infection in the bone marrow. As shown in Fig. 3D, mice inoculated i.p. with Mo+PyF101 M-MuLV showed a more rapid appearance of bone marrow infectious centers. By 2 weeks postinfection, levels of infection were equivalent to those found in bone marrow from mice infected with wild-type M-MuLV (Fig. 3C). However, at these times and earlier, the levels of infection in bone marrow from mice inoculated s.c. with Mo+PyF101 M-MuLV were 2 to 3 log units lower than levels observed in animals inoculated i.p. by the same virus. In addition, at later times there was a prolonged difference (1 to 2 log units) between the levels of infection in bone marrow from mice infected i.p. versus s.c. with Mo+PyF101 M-MuLV. In contrast, just as the pathogenicity of M-MuLV was unaffected by the route of inoculation, similarly the rates of bone marrow infection were observed in mice inoculated i.p. or s.c. with wild-type M-MuLV (Fig. 3C). Thus, the enhanced leukemogenicity of i.p. inoculation by Mo+PyF101 M-MuLV correlated both with early and continued high-level infection of the bone marrow.

Effect of decreasing bone marrow infection on leukemogenesis. The results of Fig. 3 suggested that early infection of bone marrow cells was necessary for efficient leukemogenesis by M-MuLV. One possible way to test this was to inoculate progressively older animals in an attempt to introduce the virus at a time when the critical bone marrow compartment would be unavailable for infection. Initially, mice were inoculated with wild-type M-MuLV at different days after birth. As shown in Fig. 4A, mice inoculated up to 10 days of age became moribund with kinetics parallel to those of animals inoculated at 1 day of age. All animals developed typical T-lymphoblastic lymphoma with rearrangements at the TCRβ locus (data not shown). Thus, neither the kinetics nor the disease specificity was altered as a function of the age of inoculation within this time frame for wild-type M-MuLV.

This experiment was repeated with Mo+PyF101 M-MuLV. As shown in Fig. 4B, mice inoculated i.p. with Mo+PyF101 M-MuLV developed disease as rapidly as neonatally inoculated animals when infected at 5 and 7 days of age. However, i.p. infection with Mo+PyF101 M-MuLV at 11 days of age resulted in markedly reduced leukemogenicity reminiscent of s.c. inoculation of newborns. We then tested if efficient early infection of the bone marrow was correlated with efficient leukemia induction. As shown in Table 1, animals inoculated up to 7 days of age showed high levels of infection in the bone marrow approximately 2 weeks later, whereas animals inoculated at 11 days of age did not. Thus, there was a good correlation between efficient early infection of the bone marrow and efficient leukemogenesis. The mice inoculated i.p. with Mo+PyF101 M-MuLV at 7 days of age were particularly informative since they showed high-level infection in the bone marrow but not in spleen or thymus (not shown). The fact that they showed efficient leukemogenesis further emphasized that early infection of the thymus or spleen was not necessary for efficient leukemogenesis.

Animals inoculated i.p. with Mo+PyF101 M-MuLV at 5 and 7 days of age died of typical T-cell lymphoma, as diagnosed by rearrangement at the TCRβ locus (data not shown). Additionally, all tumors contained Mo+PyF101 M-MuLV provirus that retained the Py sequences, as shown in Fig. 5A. Thus, the tumors arising in animals inoculated i.p. with Mo+PyF101 at 5 and 7 of days of age were indistinguishable by molecular criteria from tumors arising in neonatally inoculated animals. However, animals which were inoculated at 11 days of age showed an interesting phenomenon. Although they developed

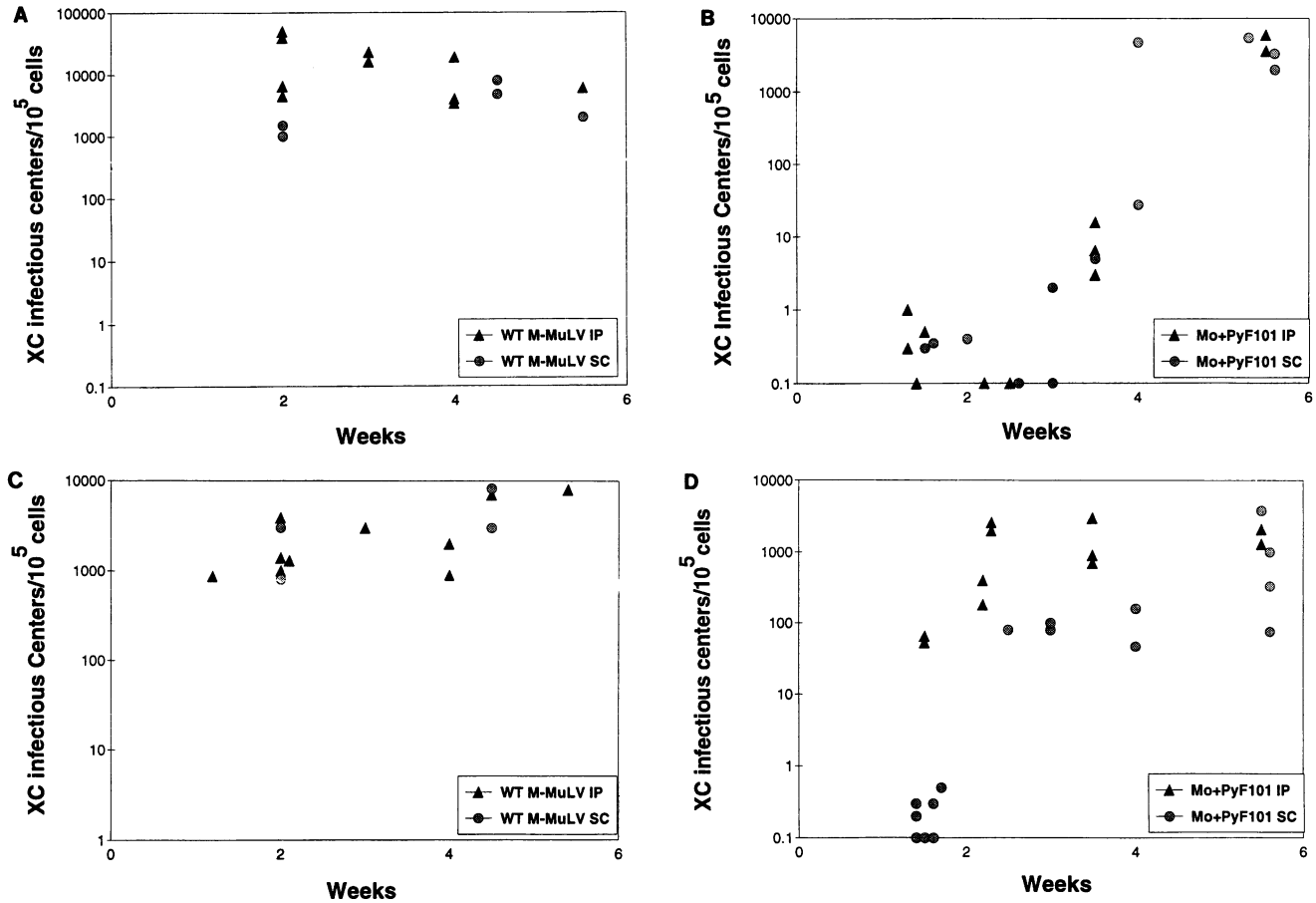

FIG. 3. Levels of infection in the thymus (A and B) and bone marrow (C and D) in NIH Swiss mice inoculated either s.c. or i.p. (see legend to Fig. 1). Thymocytes and bone marrow cells were plated as infectious centers onto NIH 3T3 fibroblasts, and the number of infectious centers was quantified by using the UV/XC plaque assay as described in Materials and Methods. Results are plotted as the number of XC infectious centers per 10^5 cells plated. All datum points represent individual animals. Datum points resting on the x axis indicate animals having virus levels undetectable in this assay (<0.1 XC infectious centers per 10^5 cells plated). WT, wild type.

tumors of T-cell origin (data not shown), many of the tumors contained proviruses that contained significantly less Py-containing provirus when analyzed by Southern blot hybridization using a Py-specific probe (Fig. 5B). To quantify the relationship between provirus which contained or had deleted the Py sequences and the presence of virus in these tumors, the same blot was rehybridized with a probe specific for M-MuLV sequences in the LTR (Fig. 5C). All of the tumors contained M-MuLV DNA by this test (lanes c to i). Moreover, over half of them contained a diagnostic M-MuLV-specific band that comigrated with the LTR fragment found in a tumor induced by wild-type M-MuLV (lane j). These results indicated that the tumors obtained from animals inoculated at 11 days of age with Mo+PyF101 M-MuLV were virus induced and that they often contained proviruses with variant LTRs. More detailed studies of variant LTRs in Mo+PyF101 M-MuLV induced tumors will be described elsewhere.

Characterization of infected cells in the bone marrow. These results indicated that early infection of the bone marrow was essential for leukemogenesis by wild-type or Mo+PyF101 M-MuLV. However, the critical infection could potentially involve either stromal or hematopoietic cells or both. Bone marrow stroma is generally defined functionally as the population of adherent cells capable of supporting hematopoiesis. Early work by other researchers has suggested that the stromal cell compartment is a major source of ecotropic virus in the early stages of infection by Soule MuLV (19). If stromal cells were also a primary site of bone marrow infection by M-MuLV, it seemed possible that differential stromal infection might be observed in s.c. versus i.p. inoculation of Mo+PyF101 M-MuLV. To test this, an in vitro culture system for bone marrow stromal cells developed by Zipori and van Bekkum (19) was used. Bone marrow from animals infected either s.c. or i.p. at birth with Mo+PyF101 M-MuLV was grown in methylcellulose culture without added growth factors for 9 days. Stromal colonies were defined as adherent cell colonies that grew out under these conditions. The stromal cell colonies were assayed for infection by XC overlay and looking for syncytia. The results showed that when animals were inoculated i.p. with Mo+PyF101 M-MuLV or were inoculated s.c. with wild-type M-MuLV and assayed 2 weeks postinfection, the resulting stromal cell colonies were highly infected (73 and 100% infected, respectively). In contrast, stromal cell colonies obtained 2 weeks postinfection from animals infected s.c. with Mo+PyF101 M-MuLV had very low levels of virus infection (ca. 5% infected). These results supported the hypothesis that the bone marrow stromal cell compartment may be an important site of early infection by M-MuLV.

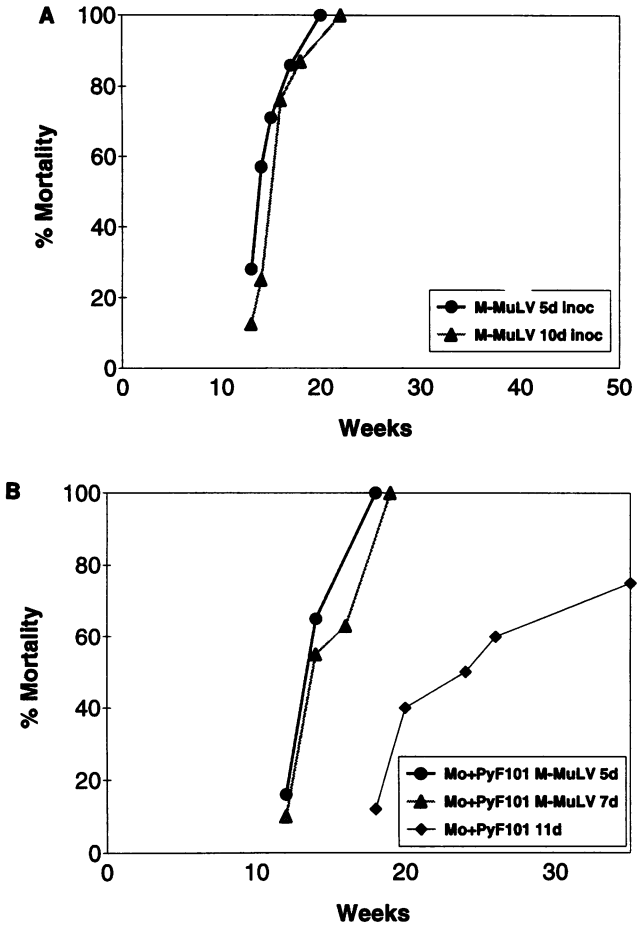

FIG. 4. Pathogenicity as a function of age. (A) NIH Swiss mice were inoculated s.c. with wild-type M-MuLV at 5 days (5d; $n = 7$) or 10 days ($n = 8$) after birth with 1×10^5 to 2×10^5 XC PFU. The time course for the appearance of moribund animals is shown. (B) NIH Swiss mice were inoculated s.c. with Mo+PyF101 M-MuLV at either 5 day ($n = 6$), 7 days ($n = 8$), or 11 days ($n = 8$) after birth with 1×10^5 to 2×10^5 XC PFU. The time course for the appearance of moribund animals is shown.

In preliminary double immunofluorescence experiments on bone marrow stromal colonies from animals infected i.p. by Mo+PyF101 M-MuLV, some of the infected (M-MuLV *gag*-positive) stromal colonies also stained with a monoclonal antibody specific for Mac-1 antigen, while other infected

TABLE 1. Infectious virus in hematopoietic organs of infected animals[a]

No. of XC infectious centers/10^6 cells plated			
Mo+PyF101			M-MuLV, 11 days, assayed 12 days p.i.
5 days, assayed 14 days p.i.	7 days, assayed 12 days p.i.	11 days, assayed 12 days p.i.	
2,500; 1,200	900; 1,200	210; 0; 0	1,400; 3,000

[a] NIH Swiss mice were inoculated i.p. at 5, 7, or 11 days of age with 1×10^5 to 2×10^5 XC PFU of Mo+PyF101 M-MuLV or wild-type M-MuLV. At the indicated number of days postinfection, infectious centers were assayed as described in the legend to Fig. 3. Results of multiple assays at each time point are presented.

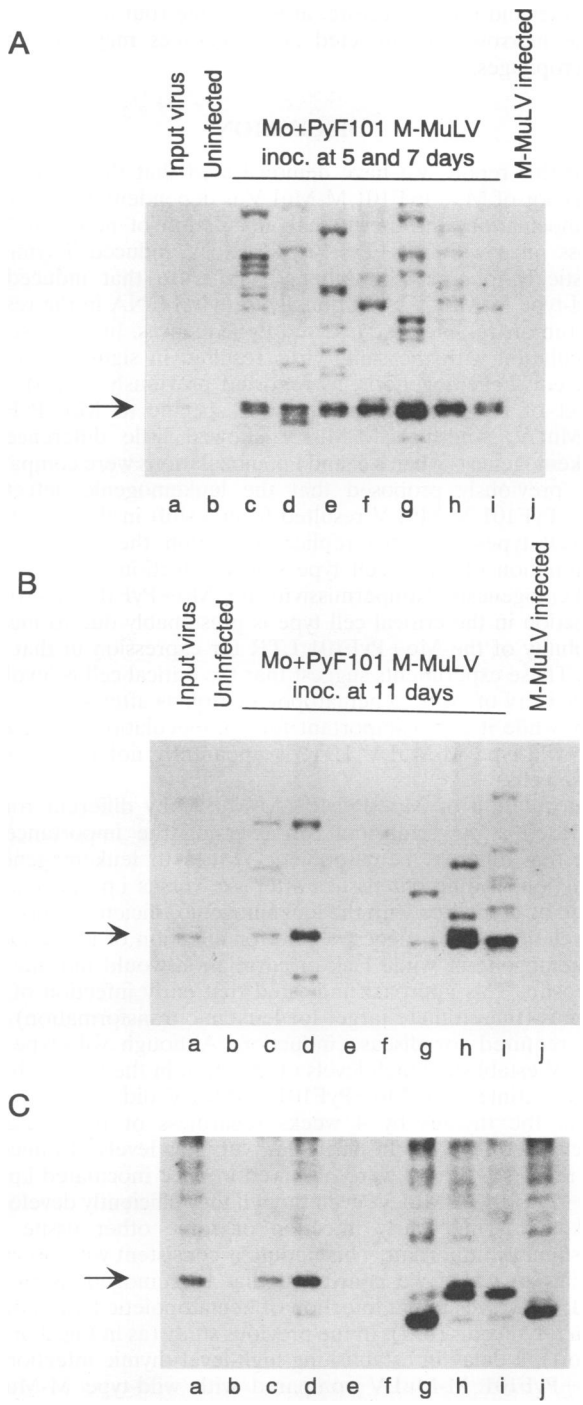

FIG. 5. Detection of provirus by Southern blot analysis. Restriction maps of wild-type M-MuLV and Mo+PyF101 M-MuLV are shown in Fig. 2A. Restriction enzyme digestion of high-molecular-weight DNA with *Pvu*II yields a unique 1.9-kb fragment containing the 5′ LTR which hybridizes with the Py enhancer probe. Rehybridization with a probe specific for M-MuLV sequences detects all LTR-specific fragments, both those that have retained and those that have eliminated Py-specific sequences. (A) High-molecular-weight DNA (5 μg) isolated from tumors obtained from animals inoculated at 5 or 7 days of age. (B) High-molecular-weight DNA (5 μg) isolated from tumors obtained from animals inoculated at 11 days of age. (C) The blot shown in panel B was stripped and rehybridized with a probe specific for the U3 region of the LTR. As in Fig. 2, the larger hybridizing fragments probably represent host-derived junction fragments.

colonies did not. Therefore, at least some (but not all) of the bone marrow cells infected at early times may have been macrophages.

DISCUSSION

In this report, we have demonstrated that the pathogenic behavior of Mo+PyF101 M-MuLV is dependent on the route of inoculation. Intraperitoneal inoculation of newborn NIH Swiss mice with Mo+PyF101 M-MuLV induced T-lymphoblastic lymphoma with kinetics similar to that induced by wild-type M-MuLV. Additionally, proviral DNA in the resulting tumors retained the inserted Py sequences. In contrast, s.c. inoculation with the same virus resulted in significantly less efficient leukemogenesis, as reported previously (1a, 5). The effect of route of inoculation was specific to Mo+PyF101 M-MuLV; wild-type M-MuLV showed little difference in leukemogenesis when s.c. and i.p. inoculations were compared. We previously proposed that the leukemogenic defect of Mo+PyF101 M-MuLV resulted from a shift in the repertoire of cell types for virus replication within the animal, with elimination of some cell type whose infection is critical for leukemogenesis. Nonpermissivity for Mo+PyF101 M-MuLV infection in the critical cell type is presumably due to incompatibility of the Mo+PyF101 LTR for expression in that cell (5). These experiments suggest that the critical cell is involved in delivery of virus to hematopoietic organs after s.c. inoculation, while it is not important for i.p. inoculation. Moreover, the wild-type M-MuLV LTR is apparently not restricted in these cells.

Inoculation of Mo+PyF101 M-MuLV by different routes provided a powerful tool for assessing the importance of infecting different hematopoietic tissues to leukemogenesis. Infection in different tissues after s.c. versus i.p. inoculation could be compared with the leukemogenic efficiencies: positive correlation would suggest a role for infection of the tissue in leukemogenesis, while lack of correlation would indicate the opposite. This approach indicated that early infection of the thymus (the ultimate target for leukemic transformation) was not required for disease induction. Although wild-type M-MuLV established high levels of infection in the thymus by 10 days postinfection, Mo+PyF101 M-MuLV did not efficiently infect the thymus by 4 weeks regardless of the route of inoculation (Fig. 3). In particular, very low levels of infection for the first 4 weeks were observed in mice inoculated i.p. by Mo+PyF101 M-MuLV, even though they efficiently developed leukemia. Thus, early infection of some other tissue was presumably important. This finding is consistent with previous studies in which we concluded that leukemogenesis by M-MuLV involves initial infection of hematopoietic tissues other than the thymus (6, 7). In the previous study (as in Fig. 3 of this report), a delay in establishing high-level thymic infection by Mo+PyF101 M-MuLV compared with wild-type M-MuLV was also noted (7), and it was possible that there was a requirement of early thymic infection for leukemogenesis. The experiments reported here show that this was not the case.

On the other hand, these same analyses suggested that early infection of the bone marrow may be important for efficient leukemogenesis. Intraperitoneal inoculation of Mo+PyF101 M-MuLV led to high levels of infectious centers in bone marrow, comparable to findings for animals infected with wild-type M-MuLV as early as 10 days postinfection. In contrast, animals inoculated s.c. with the same virus often had fewer than one infectious center per 10^6 cells assayed. Thus, early bone marrow infection was correlated with subsequent efficient development of leukemia. These results also suggest that a critical cell type that cannot support Mo+PyF101 M-MuLV replication is one that delivers virus from the skin to the bone marrow after s.c. inoculation.

Additional experiments involving infection of progressively older animals supported the proposed role for early bone marrow infection in leukemogenesis. When progressively older mice were inoculated i.p. with Mo+PyF101 M-MuLV, the leukemogenesis pattern eventually shifted to one resembling that of s.c.-inoculated animals. Mice equivalently inoculated i.p. with wild-type M-MuLV did not show this shift. Infectious center assays on these Mo+PyF101 M-MuLV-infected mice again demonstrated an excellent correlation between early bone marrow infection and efficient leukemogenesis. Moreover, early infection in both the spleen as well as the thymus was not correlated with leukemogenesis (not shown). It was also interesting that several animals inoculated i.p. with Mo+PyF101 M-MuLV at 11 days of age had tumors with little or no Py sequences in their proviruses (Fig. 5). This was also true for tumors arising at late times in neonatal mice inoculated s.c. with the same virus (to be described in more detail elsewhere). Thus, by this criterion, older mice inoculated i.p. with Mo+PyF101 M-MuLV also resembled mice inoculated s.c. at birth. The age-dependent decline in leukemogenicity for Mo+PyF101 M-MuLV probably did not reflect development of immune competence in older mice, since animals inoculated at equivalent ages with wild-type M-MuLV did not show this effect.

A role for early bone marrow infection has also been proposed for leukemogenesis in AKR mice. These mice develop T lymphoma due to activation and productive infection by the endogenously transmitted Akv MuLV beginning around birth (11). Passive immunization with an anti-MuLV antibody prevented leukemia, but only if the antibody was administered within the first few days after birth (2). Further studies indicated that prevention of leukemia was correlated with the elimination of productively infected cells from the bone marrow (3), but infection in other hematopoietic tissues was not affected. Thus, the results for AKR leukemogenesis and those described here are very consistent.

Other viruses have been shown to have altered pathogenic properties, depending on the route of inoculation. For instance, the Dearing strain of type 3 reovirus is pathogenic when inoculated intranasally, i.p., or intracranially but not when infected enterically (15). Virus infection is nonproductive by the enteric route as a result of destruction in the gastrointestinal tract of an essential virion protein.

These experiments implicate an important role for at least two cell types in early steps of M-MuLV infection and leukemogenesis. The most critical cell for leukemogenesis appears to be a bone marrow cell that must be infected at early times. This cell is accessible to infection by Mo+PyF101 M-MuLV when mice are inoculated i.p. but not s.c., and it is infectable by wild-type M-MuLV from either route. The infected bone marrow cell could be either stromal or hematopoietic. Assays of bone marrow stromal colonies supported the idea that the critical infected bone marrow cells are stromal and at least some may be stromal macrophages. However, the experiments did not rule out the possibility that hematopoietic cells in the bone marrow were also infected at early times.

The second cell type identified in this report is the cell that delivers infectious virus from the skin to the bone marrow after s.c. inoculation. This cell type apparently can be infected by wild-type but not by Mo+PyF101 M-MuLV. When Mo+PyF101 M-MuLV is inoculated i.p. into newborns, efficient infection of the bone marrow occurs. Thus, either different cells are used to deliver virus to the bone marrow

after i.p. inoculation or direct infection of bone marrow from the inoculated virus can occur under these conditions. It should also be noted that mice infected s.c. with Mo+PyF101 M-MuLV eventually develop levels of virus infection in many organs that are equivalent to wild-type M-MuLV levels (5, 7). Thus, it seems likely that other cell types can also propagate Mo+PyF101 M-MuLV in s.c.-infected animals.

We previously reported that another interesting feature of Mo+PyF101 M-MuLV is that it does not form MCF recombinants in the animal (1a). Moreover, this was apparently due to the inability of MCF recombinants driven by the Mo+PyF101 LTR to replicate in MCF propagator cells in the animal, although the inability to infect other cells involved in MCF generation was also possible. Regarding Mo+PyF101 M-MuLV, the relationship between the restrictive cells for MCF propagation and for virus delivery from the skin to bone marrow remains to be clarified. However, we have found that mice infected i.p. with Mo+PyF101 M-MuLV show MCF recombinants in their tumors, while those infected SC do not (1). However, MCF recombinants generally occurred at later times than those studied in this report.

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