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Tissue-Specific Replication of Friend and Moloney Murine Leukemia Viruses in Infected Mice

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We have studied the replication of ecotropic murine leukemia viruses (MuLV) in the spleens and thymuses of mice infected with the lymphocytic leukemia-inducing virus Moloney MuLV (M-MuLV), with the erythroleukemia-inducing virus Friend MuLV (F-MuLV), or with in vitro-constructed recombinants between these viruses in which the long terminal repeat (LTR) sequences have been exchanged. At 1 week after infection both the parents and the LTR recombinants replicated predominantly in the spleens with only low levels of replication in the thymus. At 2 weeks after infection, the patterns of replication in the spleens and thymuses were strongly influenced by the type of LTR. Viruses containing the M-MuLV LTR exhibited a remarkable elevation in thymus titers which frequently exceeded the spleen titers, whereas viruses containing the F-MuLV LTR replicated predominantly in the spleen. In older preleukemic mice (5 to 8 weeks of age) the structural genes of M-MuLV or F-MuLV predominantly influenced the patterns of replication. Viruses containing the structural genes of M-MuLV replicated efficiently in both the spleen and thymus, whereas viruses containing the structural genes of F-MuLV replicated predominantly in the spleen. In leukemic mice infected with the recombinant containing F-MuLV structural genes and the M-MuLV LTR, high levels of virus replication were observed in splenic tumors but not in thymic tumors. This phenotypic difference suggested that tumors of the spleen and thymus may have orginated by the independent transformation of different cell types. Quantification of polytropic MuLVs in late-preleukemic mice infected with each of the ecotropic MuLVs indicated that the level of polytropic MuLV replication closely paralleled the level of replication of the ecotropic MuLVs in all instances. These studies indicated that determinants of tissue tropism are contained in both the LTR and structural gene sequences of F-MuLV and M-MuLV and that high levels of ecotropic or polytropic MuLV replication, per se, are not sufficient for leukemia induction. Our results further suggested that leukemia induction requires a high level of virus replication in the target organ only transiently during an early preleukemic stage of disease.

Murine leukemia viruses (MuLVs) are a diverse group of retroviruses originating in mice. The viruses can be grouped into several distinct host ranges defined by their ability to infect various cell lines or cultures in vitro. In vitro host ranges of MuLVs are thought to be conferred by their respective envelope proteins, which specifically interact with distinct cellular receptors (18, 19, 25). In addition to selective replication in different in vitro cell lines, MuLVs frequently exhibit selective tropism for different tissues in vivo (7, 24, 27). Thus, the replication-competent Friend erythroleukemia-inducing virus (F-MuLV) replicates more efficiently in the spleen compared to the thymus in infected mice, whereas the SL3-3 MuLV and some polytropic MuLVs originating from AKR mice replicate preferentially in the thymus and induce thymic neoplasms (7, 8, 24, 27). Very little is known about the factors that influence the tissue-specific replication of MuLVs or the relevance of such specificity to the disease process.

Until recently little was known concerning the MuLV genetic elements which influence disease specificity; however recent studies utilizing in vitro-constructed recombinants between viruses which differ in their oncogenicity have defined the viral long terminal repeat (LTR) as an important determinant of disease specificity for several MuLVs (1, 2, 11–13, 21). The ecotropic AKV MuLV exhibits low in vivo infectivity and is not oncogenic, whereas the SL3-3 MuLV, as noted above, is thymotropic and induces thymomas. Substitution of the AKV LTR with the LTR of SL3-3 yielded an MuLV which readily induced thymomas, similar to SL3-3 (21). Another striking example of the involvement of the LTR in disease specificity was demonstrated by using in vitro-constructed recombinant MuLVs between F-MuLV and Moloney MuLV (M-MuLV) (1, 2). F-MuLV induces predominantly erythroleukemias in susceptible mice, whereas M-MuLV induces predominantly lymphocytic leukemias. In vitro-constructed recombinants containing the structural genes of F-MuLV and the LTR of M-MuLV induce lymphocytic leukemia rather than erythroleukemia. Conversely, recombinants containing the structural genes of M-MuLV and the LTR of F-MuLV induce erythroleukemia rather than lymphocytic leukemia.

Several of the studies cited above have postulated that a critical element determining the specificity of disease may be an enhanced replication of the MuLV in a particular target tissue as a result of transcriptional enhancement by the LTR (1, 12, 13, 21). In this regard, a recent study reported that substitution of the AKV LTR with the LTR of the thymotropic SL3-3 MuLV or the splenotropic F-MuLV resulted in recombinant viruses which were thymotropic or splenotropic, respectively (27). Although this result demonstrated that the LTRs of SL3-3 and F-MuLV contain elements which influence tissue-specific replication, it did not exclude the possibility that the structural gene sequences of SL3-3 or F-MuLV may also exert an influence on virus replication in specific tissues.

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In this report we have examined the replication of F-MuLV and M-MuLV and of reciprocal LTR recombinants between the two viruses in the spleens and thymuses of infected mice. We have found that the relative levels of virus replication in the spleens and thymuses of infected mice did not strictly correlate with the type of disease induced (erythroleukemia versus lymphocytic leukemia) but were strikingly dependent upon the time after inoculation. At early times after infection (1 week) all of the viruses replicated to high levels in the spleens but not the thymuses of infected mice and thus appeared splenotropic. Early in the preleukemic stage (2 weeks after infection) a predominant influence on tissue-specific replication was exerted by the LTR: those MuLVs containing the M-MuLV LTR replicated to high levels in both the spleen and thymus, whereas those containing the F-MuLV LTR replicated to high levels only in the spleen. At later times (5 to 8 weeks after infection) the MuLV structural genes predominantly influenced tissue-specific replication: MuLVs containing the structural genes of M-MuLV replicated to high levels in both the spleen and thymus, whereas those containing the structural genes of F-MuLV replicated to high levels in the spleen but to low levels in thymus. Our results indicated that both the LTRs and structural gene sequences of F-MuLV and M-MuLV exerted an influence on the tissue-specific replication, that a high level of virus replication in a tissue was not sufficient for transformation, and that high levels of replication in the target tissue may be required only transiently during an early preleukemic stage of disease.

MATERIALS AND METHODS

Viruses and mice. The sources of molecularly cloned isolates of F-MuLV and M-MuLV were described previously (16). The in vitro-constructed recombinant viruses FM-I2 (1) and MF-3 (2) were kindly provided by N. Hopkins (Massachusetts Institute of Technology) and J. Hartley (National Institute of Allergy and Infectious Diseases) as chronically infected SC-1 cells. FM-I2 contains the *gag*, *pol*, and almost all of the *env* genes of F-MuLV. A 3′ portion of the FM-I2 genome encompassing the 3′ end of the p15E gene, the U3 sequences containing transcriptional enhancer sequences, and part of the R region are derived from M-MuLV. For purposes of discussion, we have referred to this 3′ sequence of M-MuLV or the allelic sequence of F-MuLV as the M-MuLV or F-MuLV LTR, respectively. The remainders of the genomes are referred to as the M-MuLV or F-MuLV structural genes. MF-3 has a reciprocal structure to FM-I2 and contains the structural genes of M-MuLV and the LTR of F-MuLV. For logistic reasons these studies were performed with inbred IRW mice derived at the Rocky Mountain Laboratories from an outbred colony of Carworth Farms White mice as previously described (4). The oncogenic specificities of the viruses examined in this study have been extensively investigated in IRW mice (M. Sitbon, Hôpital Cochin, Paris, France, personal communication) and were found to be essentially identical to those previously reported with NFS/N mice (1, 2). Mice were inoculated intraperitoneally at 1 to 2 days of age with 5×10^3 to 5×10^4 focus-forming units. Titers of virus stocks were determined as described below.

Monoclonal antibodies and virus assays. Three monoclonal antibodies, 48, 500, and 514, were employed to detect ecotropic or polytropic MuLVs in a focal immunofluorescence assay (FIA) on live SC-1 cells as previously described (31). Briefly, the thymuses and spleens of infected animals were dispersed, and serial dilutions of the cells were cocultivated as infectious centers (IC) with SC-1 cells seeded the previous day. The following day the spleen or thymus cells were removed, and the SC-1 cells were allowed to grow to confluency. The confluent monolayers were incubated with the appropriate antibody (48 for assays of F-MuLV or FM-I2, 500 for assays of M-MuLV or MF-3, and 514 for all polytropic MuLVs), rinsed with tissue culture medium, and subsequently incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin to develop fluorescent foci. Antibody 48 (5) reacts specifically with the F-MuLV envelope protein and is unreactive with any other MuLVs tested, including ecotropic, polytropic, xenotropic, and amphotropic MuLVs. Antibody 500 (3) reacts with M-MuLV and F-MuLV envelope proteins but is unreactive with xenotropic and amphotropic MuLVs and nearly all polytropic MuLVs tested. Examination of many virus isolates from IRW mice indicated that all of the antibody 48- or 500-reactive viruses tested corresponded to ecotropic MuLVs which induced syncytia on XC cells (unpublished observations). Antibody 514 (3) is specific for polytropic MuLV envelope proteins and is reactive with virtually all polytropic MuLVs we have examined (3, 15–17). Previous studies have demonstrated that detection of ecotropic MuLVs, including F-MuLV or M-MuLV by the FIA is at least as efficient and frequently more efficient than the XC assay (31). Assays of polytropic MuLVs by the FIA on SC-1 cells detect both unpseudotyped and pseudotyped polytropic MuLVs and have been shown to be much more efficient than the detection of polytropic MuLVs on mink cells (30).

Statistical analyses. The average IC titers from spleen and thymus cells were expressed as the geometric mean (the mean of the $\log_{10}$ values of the titers) plus or minus the standard error of the geometric mean or, alternatively, the antilogarithm of the geometric mean multiplied or divided by the relative standard error (the antilogarithm of the standard error). Average titers were compared by using the two-sided t test, and P values less than 0.05 were considered significant.

RESULTS

Replication of ecotropic MuLVs in spleens and thymuses of mice 1 week after infection. Previous studies of tissue-specific replication of MuLVs have examined virus replication several weeks (>3 weeks) after infection. The infection of mice by MuLVs precipitates a complex series of events which frequently includes hyperplasia, malignant proliferation of cells, and recombination of the inoculated MuLV with genetic sequences of the mouse to generate host-range variants (38). Some or all of these events could conceivably influence the tissue-specific replication of MuLVs. Because of these considerations, we elected to examine ecotropic MuLV replication at early times as well as at later times after infection. Preliminary experiments indicated that the earliest time after inoculation that we could consistently detect higher than trace levels of virus (quantifiable without in vitro passage of indicator SC-1 cells) in the spleens or thymuses of infected animals was approximately 1 week. At 1 week after infection the spleens of all animals, irrespective of the inoculated MuLV, exhibited substantial titers of virus, whereas MuLV replication in the thymus was low or, with some mice, undetectable (Fig. 1). Few differences were observed among the spleen or thymus titers of mice 7 days after inoculation with any of the MuLVs. Among the spleen titers, a significant difference ($P < 0.02$) was observed

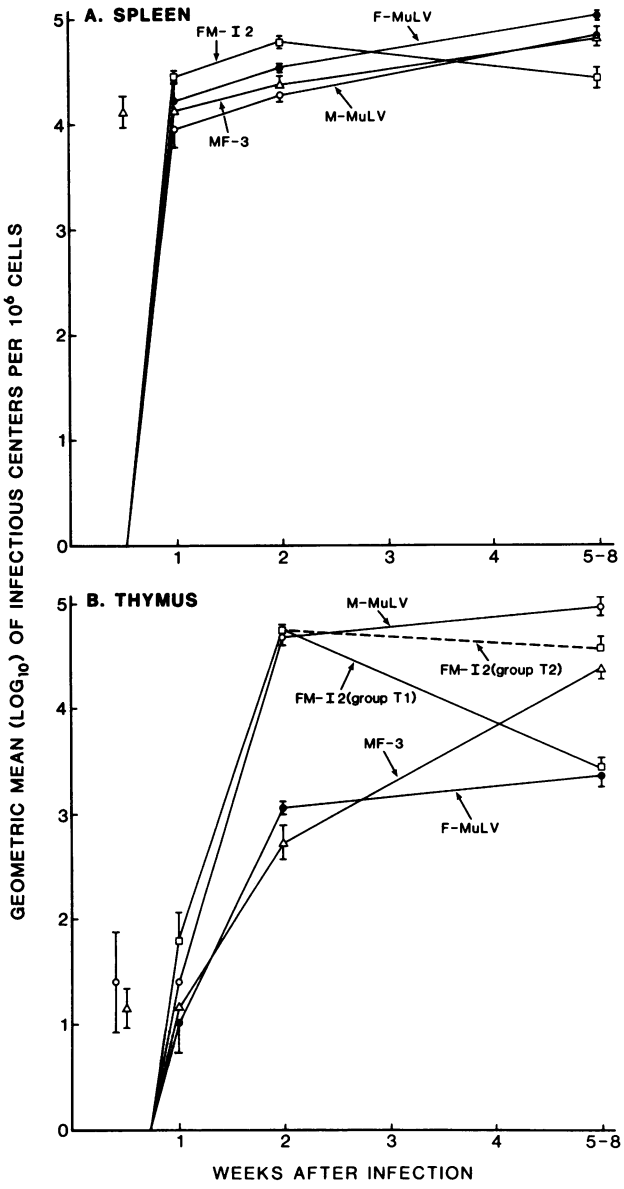

FIG. 1. IC titers of spleen (A) and thymus cells (B) at different times after infection with F-MuLV, M-MuLV, MF-3, or FM-I2. Serial dilutions of spleen or thymus cells from mice infected 1, 2, or 5 to 8 weeks earlier, were plated as ICs on SC-1 cells and scored for fluorescent foci by using the FIA (25). IC titers (number of foci per 10^6 cells) were calculated for the spleen and thymus cells of each mouse, and the average titers for groups of mice infected with F-MuLV (●), M-MuLV (○), MF-3 (△), or FM-I2 (□) at each time were calculated as geometric means (the mean of the $\log_{10}$ values of the titers). The error bars indicate plus or minus the standard errors of the geometric means. At 5 to 8 weeks the geometric means were calculated separately for two groups (T1 and T2) of FM-I2-infected mice. Group T1 refers to a subset comprised of most (11 of 15) FM-I2-infected mice, which exhibited low thymus titers, whereas group T2 (dashed line) refers to a subset (4 of 15) of FM-I2-infected mice that exhibited high thymus titers. When standard error bars overlap, the bar is shown only in the nonoverlapping direction. In those cases where standard error bars overlap in both directions, the mean and the standard error bars are offset to the left of the plot. For all MuLVs, ICs could initially be detected with spleens cells of some animals at 4 days and with thymus cells at 5 days after infection. Thus, we have extrapolated the geometric means of the titers to zero (1 focus per 10^6 cells) at 4 days for spleen cells and at 5 days for thymus cells. The number of mice assayed for each virus at 1, 2, and 5 to 8 weeks was, respectively, as follows: F-MuLV (8, 17, and 8) M-MuLV (8, 11, and 14) MF-3 (10, 10, and 14) FM-I2 (9, 12, and 15).

between the MuLVs exhibiting the highest (FM-I2) and the lowest (M-MuLV) average titers. Similarly, among the thymus titers, only the highest (FM-I2) and lowest (F-MuLV) average titers were significantly different ($P < 0.05$). The finding that all of the MuLVs were initially splenotropic when inoculated into newborn mice was unexpected, considering the different oncogenic activities of the ecotropic MuLVs examined. As was reported with NFS/N mice (1, 2), IRW mice inoculated with M-MuLV or the in vitro-constructed recombinant containing the M-MuLV LTR (FM-I2), develop almost exclusively thymomas, whereas mice inoculated with F-MuLV or the recombinant containing the F-MuLV LTR (MF-3) develop almost exclusively erythroleukemias (Sitbon, personal communication).

Replication of ecotropic MuLVs in spleens and thymuses of mice 2 weeks after infection. The results above indicated that extensive fluctuations in the tissue-specific replication of the ecotropic MuLVs must occur at later times after inoculation. We therefore examined virus production in the spleens and thymuses of mice 2 weeks after infection with each of the MuLVs. At this age we found a striking correlation between the pattern of MuLV replication in the spleens and thymuses and the presence of the F-MuLV or M-MuLV LTR, respectively. The average titers for spleen cells were slightly higher (ca. twofold) than spleen titers for mice 1 week after infection with all viruses (Fig. 1A); however this difference was found to be statistically significant only with FM-I2-infected mice ($P < 0.005$). The thymus titers of mice infected with MuLVs containing the F-MuLV LTR (F-MuLV and MF-3) were elevated compared with mice at 7 days ($P < 0.001$) but were still quite low compared with the spleen titers (Fig. 1). In contrast, titers from thymus cells of mice infected with MuLVs containing the M-MuLV LTR (M-MuLV and FM-I2) were remarkably elevated compared with the levels found with these MuLVs at 1 week and were much higher than the thymus titers observed at 2 weeks after inoculation with either F-MuLV or MF-3 (Fig. 1B). Thus the M-MuLV LTR very likely facilitated a more rapid infection of the thymus between 1 and 2 weeks of age than did the F-MuLV LTR.

Replication of ecotropic MuLVs in spleens and thymuses of mice 5 to 8 weeks after inoculation. Ecotropic virus replication in the spleens and thymuses of F-MuLV- or M-MuLV-infected mice 5 to 8 weeks after inoculation was similar to the replication observed at 2 weeks postinoculation. Both viruses exhibited moderate (ca. twofold) increases in the spleen and thymus IC titers compared with 2-week-old mice (Fig. 1). However, the levels of replication of the recombinant viruses (MF-3 and FM-I2) at 5 to 8 weeks were quite different from the levels observed at earlier times after inoculation. Between 2 and 5 to 8 weeks after infection, MF-3-infected mice exhibited an increase in the average spleen titer of approximately the same magnitude as was observed with F-MuLV- and M-MuLV-infected mice (Fig. 1A). However, the thymus titers of MF-3-infected mice, which were low at 2 weeks after infection, had increased dramatically by 5 to 8 weeks (Fig. 1B). This was in contrast to F-MuLV, which maintained comparatively low thymus titers during the same interval of time. Since MF-3 and F-MuLV do not differ in their LTRs, the increase in thymus

TABLE 1. Bimodal distribution of IC titers of ecotropic MuLVs from spleen and thymus cells of late-preleukemic and leukemic mice inoculated with FM-I2

Disease state	Age (wk)	Group[a]	Tissue	Avg IC/10^6 cells,[b] geometric mean ×/÷ RSE[c] (no. of mice)
Preleukemic	5–8	T1	Spleen	23,500 ×/÷ 1.20 (11)
			Thymus	2,700 ×/÷ 1.24 (11)
		T2	Spleen	33,000 ×/÷ 1.35 (4)
			Thymus	36,700 ×/÷ 1.32 (4)
Leukemic	9–16	T_L1	Spleen	50,900 ×/÷ 1.39 (7)
			Thymus	2,300 ×/÷ 1.26 (7)
		T_L2	Spleen	61,600 ×/÷ 1.62 (3)
			Thymus	58,300 ×/÷ 1.38 (3)

[a] Groups T1 and T2 refer to subsets of preleukemic mice at 5 to 8 weeks after infection which exhibited low or high FM-I2 titers in the thymus, respectively. Groups T_L1 and T_L2 refer to subsets of leukemic mice which exhibited low or high FM-I2 titers in the thymus, respectively.

[b] FM-I2 IC titers were determined on SC-1 cells by using the FIA with antibody 48.

[c] RSE, Relative standard error.

replication of MF-3 must have been mediated by elements contained within the M-MuLV structural gene sequences.

The analyses of ecotropic MuLV replication in the spleens and thymuses of FM-I2-infected mice at 5 to 8 weeks after infection was more complex than analyses with mice infected with F-MuLV, M-MuLV, or the recombinant MF-3 MuLV. In contrast to other MuLVs, which displayed increases in their average spleen titers between 2 weeks and 5 to 8 weeks after infection, FM-I2-infected mice exhibited a moderate but significant reduction (ca. 50%, $P < 0.001$) in the average spleen titer during this interval of time (Fig. 1A). The thymus titers of FM-I2-infected mice varied over a wide range (1×10^3 to 6×10^4 foci in 10^6 cells), and examination of the thymus titers from individual mice revealed a clear bimodal distribution (Table 1). A group comprising nearly three-fourths of the mice (11 of 15) (group T1, Table 1, Fig. 1B) exhibited average thymus titers which were not significantly different than the low thymus titers observed with F-MuLV-infected mice. The remaining four mice (group T2, Table 1, Fig. 1B) exhibited thymus titers which averaged much higher (ca. 14-fold) and were more similar to thymus titers observed with M-MuLV. Considering that all of the FM-I2-infected mice tested had relatively high thymus titers at 2 weeks after infection (data not shown), it is very likely that the low thymus titers observed with the majority of FM-I2-infected mice at 5 to 8 weeks were the result of decreases in the thymus titers rather than a delayed onset of thymus infection in these mice.

The results of the determinations of the spleen and thymus titers of the MuLVs at 5 to 8 weeks after infection indicated that tissue-specific replication at this time correlated more closely with the structural genes of the MuLVs than with the LTRs. Viruses containing the structural genes of M-MuLV (M-MuLV and MF-3) replicated to high titers in both the spleen and thymus, whereas mice infected with viruses containing the structural genes of F-MuLV (F-MuLV- and most FM-I2-infected mice) exhibited high titers in the spleen but relatively low thymus titers.

Bimodal distribution of thymus IC titers in leukemic FM-I2-infected mice. The finding that some of the preleukemic FM-I2-infected mice at 5 to 8 weeks after infection exhibited high ecotropic MuLV titers in the thymus suggested the possibility that those preleukemic mice may have corresponded to mice at a later stage of disease progression than mice with low thymus titers. To examine this possibility, assays of spleen and thymus cells were carried out on FM-I2-infected mice which had progressed to the leukemic stage. The leukemic FM-I2-infected mice selected for these analyses exhibited grossly enlarged thymuses (0.1 to 1.2 g) or spleens (0.4 to 2.1 g) or both. All spleen and thymus tumors were found by histochemical examination to be of lymphoid origin (data not shown). As was the case with preleukemic mice, the ecotropic MuLV titers from thymus cells of leukemic mice exhibited a clear bimodal distribution; 7 of 10 mice exhibited low ecotropic MuLV titers in the thymus (group T_L1, Table 1), whereas three mice exhibited much higher (ca. 20-fold) thymus titers (group T_L2, Table 1).

The data with FM-I2-infected mice in the late preleukemic or leukemic stage of disease indicated that the level of virus replication in the target organ at these times did not closely correlate with disease progression. It is of interest that the severely depressed levels of ecotropic MuLV replication observed with thymus cells of most FM-I2-infected mice (groups T1 and T_L1) were not apparent in the ecotropic MuLV titers observed with spleen cells from the same animals (Table 1). This result was surprising, considering that spleen tumors of lymphoid origin were observed in all but one of the leukemic mice. This finding suggested that lymphoid cells from splenic tumors of FM-I2-infected mice were phenotypically distinct from cells of the thymic tumors. Thus, the splenic tumors may have resulted from transfor-

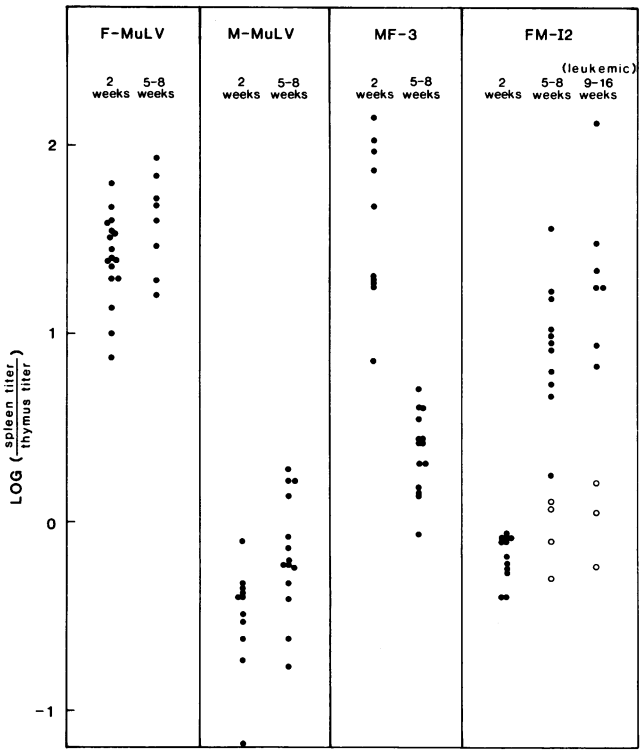

FIG. 2. Spleen/thymus ratios of IC titers from individual mice infected with F-MuLV, M-MuLV, MF-3, or FM-I2. The ratio of the IC titers from the spleen and thymus was calculated for each mouse assayed at 2 weeks or at longer times after infection. The logarithms of the arithmetic quotients were plotted for each group of mice inoculated with each of the MuLVs for the times indicated. Each circle represents the spleen/thymus titer ratio from a single mouse. Open circles represent FM-I2-infected mice which exhibited high thymus titers (groups T2 and T_L2, Table 1).

TABLE 2. IC titers of ecotropic and polytropic MuLVs from spleen and thymus cells of late preleukemic mice inoculated with FM-I2, F-MuLV, M-MuLV, or MF-3

Inoculated MuLV	Age (wk)	Group[a]	Tissue	Avg IC/10^6 cells, geometric mean ×/÷ RSE[b] (no. of mice)	
				Ecotropic[c]	Polytropic[d]
FM-I2	5–8	T1	Spleen	23,500 ×/÷ 1.20 (11)	19,300 ×/÷ 1.18 (5)
			Thymus	2,700 ×/÷ 1.24 (11)	4,800 ×/÷ 1.44 (5)
		T2	Spleen	33,000 ×/÷ 1.35 (4)	37,900 ×/÷ 1.22 (2)
			Thymus	36,700 ×/÷ 1.32 (4)	53,500 ×/÷ 1.23 (2)
F-MuLV	5–8	NA[e]	Spleen	102,100 ×/÷ 1.09 (8)	74,100 ×/÷ 1.22 (8)
			Thymus	2,400 ×/÷ 1.29 (8)	1,600 ×/÷ 1.29 (8)
M-MuLV	5–8	NA	Spleen	65,000 ×/÷ 1.20 (14)	35,400 ×/÷ 1.16 (9)
			Thymus	93,100 ×/÷ 1.23 (14)	93,300 ×/÷ 1.23 (9)
MF-3	5–8	NA	Spleen	60,400 ×/÷ 1.14 (14)	35,200 ×/÷ 1.23 (11)
			Thymus	24,100 ×/÷ 1.25 (14)	15,000 ×/÷ 1.25 (11)

[a] Groups T1 and T2 refer to subsets of preleukemic mice at 5 to 8 weeks after infection with FM-I2, which exhibited low or high ecotropic MuLV titers in the thymus, respectively.
[b] RSE, Relative standard error.
[c] Ecotropic MuLV titers were determined on SC-1 cells by using the FIA with antibody 48 for F-MuLV and FM-I2 or antibody 500 for M-MuLV and MF-3. FM-I2 ecotropic titers presented in Table 1 are reiterated here to facilitate comparisons to polytropic MuLV titers.
[d] Polytropic MuLV titers were determined on SC-1 cells by using the FIA with antibody 514.
[e] NA, Not applicable

mation of a different cell type, rather than from infiltration of the spleens with cells from the thymic tumors.

Spleen/thymus ratios of IC titers of ecotropic MuLVs from individual mice infected with F-MuLV, M-MuLV, MF-3, and FM-I2. To compare the status of individual mice infected with each of the MuLVs, the ecotropic MuLV titers from each mouse at 2 weeks or at 5 to 8 weeks postinfection were expressed as the ratio of the spleen titer to the thymus titer (Fig. 2). Treatment of the data in this manner revealed a number of features not readily apparent from the averaged values.

At 2 weeks and at 5 to 8 weeks the spleen/thymus titer ratios of F-MuLV-infected mice were essentially the same and were clearly discernible from the ratios of M-MuLV-infected mice at these times (Fig. 2). No overlapping ratios were observed between F-MuLV and M-MuLV-infected mice, indicating that the distinction in tissue-specific replication between the two MuLVs was characteristic of all mice examined. Similarly, at 2 weeks after infection the ratios of MF-3- and FM-I2-infected mice were distinct from each other, and their ranges approximately coincided with those of F-MuLV- or M-MuLV-infected mice, respectively. Thus, at 2 weeks a complete correspondence of tissue-specific replication with the presence of the F-MuLV or M-MuLV LTR was observed.

At 5 to 8 weeks after inoculation, the ratios of MF-3 mice shifted to a lower range which overlapped, but was not completely coincident with, ratios observed with M-MuLV-infected mice (Fig. 2). The reduction in the spleen/thymus titer ratios, compared with those of mice 2 weeks after infection, was characteristic of all MF-3-infected mice tested at 5 to 8 weeks. The thymus titers of several MF-3-infected mice at 5 to 8 weeks were lower than most thymus titers of M-MuLV-infected mice. This was reflected in intermediate ratios for several MF-3-infected mice and also in a lower average thymus titer (ca. fourfold) for MF-3-infected mice compared with M-MuLV-infected mice at 5 to 8 weeks (Fig. 1).

The ratios observed with FM-I2-infected mice at 5 to 8 weeks after inoculation exhibited a broad range which overlapped with ratios observed with both F-MuLV- and M-MuLV-infected mice (Fig. 2). Mice that had low thymus titers (group T1, Table 1) did not exhibit a corresponding decrease in their spleen titers and consequently exhibited high spleen/thymus titer ratios. Thus, the broad range of ratios observed with FM-I2-infected mice was largely a result of the bimodal distribution of the thymus titers found at 5 to 8 weeks after infection (Table 1, Fig. 2). Almost all of the ratios observed with the remaining FM-I2-infected mice had shifted to a higher range which overlapped, but was not completely coincident with, ratios observed with F-MuLV-infected mice. This shift reflected the low thymus titers of these mice compared with the titers observed at 2 weeks after infection (Fig. 1). Some FM-I2-infected mice had spleen titers which were lower than those of most F-MuLV-infected mice. This resulted in intermediate ratios for those mice and in a moderately decreased (ca. fourfold) average spleen titer compared with F-MuLV-infected mice at 5 to 8 weeks after inoculation (Fig. 1). One exceptional mouse exhibited low titers in both the spleen and thymus, which resulted in a low spleen/thymus titer ratio. The analyses of the ratios with leukemic mice infected with FM-I2 closely paralleled the analyses of FM-I2-infected mice at 5 to 8 weeks (Fig. 2).

From these analyses, it was apparent that the patterns of ecotropic MuLV replication in the spleens and thymuses of MF-3- and FM-I2-infected mice at 2 weeks after infection were characteristic of the parental MuLV LTR donor. However by 5 to 8 weeks after infection the patterns of replication in MF-3-infected mice and most FM-I2-infected mice had shifted to patterns more similar to the parental MuLV from which the structural genes were derived.

Polytropic MuLV replication in late-preleukemic mice. Inoculation of F-MuLV or M-MuLV in mice results in the generation of host range variants, termed polytropic or mink cell focus-inducing viruses, which are recombinants between the inoculated ecotropic MuLV and endogenous retroviral gene sequences of the mouse (16–19, 26). Such recombinant viruses have been suggested to be involved in the induction of leukemia by F-MuLV or M-MuLV (6, 28, 33, 36, 37). It was of interest to examine the level of polytropic MuLV production in infected mice to determine whether a correla-

tion was evident between ecotropic and polytropic MuLV replication. This was of particular interest in the case of FM-I2-inoculated mice, which exhibited a marked decrease of ecotropic MuLV replication in the thymuses of most late-preleukemic mice as well as in the thymuses of mice with advanced lymphocytic leukemia (Fig. 1, Table 1). Conversely, MF3-infected mice exhibited an increase in ecotropic MuLV titers in thymuses of late-preleukemic mice but do not develop lymphocytic leukemia (Fig. 1). It is possible that the level of polytropic MuLV replication, rather than the replication of the ecotropic MuLVs, correlates with the presence or absence of lymphocytic leukemia in FM-I2- or MF-3-infected mice, respectively.

The determinations of polytropic MuLV titers in late-preleukemic mice infected with FM-I2 revealed a close correspondance of ecotropic and polytropic MuLV replication (Table 2). Mice that exhibited low ecotropic MuLV titers in the thymus (group T1, Table 2) also exhibited low polytropic MuLV thymus titers, whereas mice that had high ecotropic MuLV titers in the thymus (group T2, Table 2) had correspondingly high polytropic MuLV titers. Likewise, the polytropic MuLV titers in spleens of FM-I2-infected mice correlated with the ecotropic MuLV titers. This bimodal distribution of polytropic MuLV replication, in parallel with ecotropic MuLV replication, was also very clear for the leukemic mice infected with FM-I2 (data not shown).

Mice infected with F-MuLV, M-MuLV, or MF-3 exhibited correlations between polytropic and ecotropic MuLV replication similar to that observed with FM-I2-infected mice (Table 2). High polytropic MuLV titers in the spleens and low polytropic titers in the thymuses of F-MuLV-infected mice paralleled the corresponding ecotropic MuLV titers. Similarly, the polytropic MuLV titers in both the spleens and thymuses of M-MuLV- and MF-3-infected mice corresponded approximately to the ecotropic MuLV titers. The ecotropic and polytropic MuLV titers in the thymuses of MF-3-infected mice were approximately 10-fold higher than the corresponding titers in F-MuLV-infected mice, but lower than the ecotropic and polytropic MuLV titers observed in the thymuses of mice infected with M-MuLV. The lower thymus titers in MF-3-infected mice, compared with M-MuLV-infected mice, reflected the delayed infection of the thymus by MF-3. We have not yet determined whether the ecotropic or polytropic MuLV titers in the thymuses of older MF-3-infected mice reach the high levels observed in the thymuses of mice infected with M-MuLV.

The results of these experiments, in particular those with FM-I2 and MF-3, suggested that a high level of polytropic or ecotropic MuLV replication in older preleukemic mice was not sufficient or necessary for disease progression.

DISCUSSION

The results of this study demonstrated striking fluctuations in virus replication in the spleens and thymuses of infected mice at different times after inoculation. Our initial observation, that the spleen was the major target for replication at the earliest times after inoculation, was unexpected. This result suggested that the viruses did not initially exhibit different tropisms, and that the tissue-specific replication observed at later times may have been the result of events that occurred after the initial infection. These studies did not address the possibility that the viruses may infect different subsets of spleen cells; however in other ongoing studies electron microscopic examination of peripheral blood cells identified erythroid cells with budding C-type particles from mice 2 to 3 weeks after inoculation with each of these MuLVs (Sitbon, personal communication). Therefore, any difference in the major types of spleen cells infected by the MuLVs is likely to be quantitative rather than qualitative.

A second striking observation of this study was the elevation of thymus titers observed at 2 weeks after infection with M-MuLV and FM-I2 compared with F-MuLV and MF-3. A clear correlation of tissue-specific virus replication with the identity of the LTR was apparent only during this early preleukemic phase of the disease. The rapid infection of the thymus, which correlated with the presence of the M-MuLV LTR, occurred well after virus infection was established in the mouse, as evidenced by substantial spleen titers observed 1 week earlier (Fig. 1A). There are several possible mechanisms that may explain the rapid infection of thymus cells by M-MuLV and FM-I2 after a delay of 1 to 2 weeks. Among these are the secondary infection of the thymus by migration of infected cells from the spleen (or elsewhere), the generation of thymotropic recombinant viruses that facilitate infection of the thymus by pseudotyping the ecotropic virus, and the direct alteration of the ecotropic virus to yield a thymotropic variant. Each of these mechanisms would require a process secondary to the initial infection which could account for the delayed infection of the thymus. The possibility of direct genetic alteration of M-MuLV to yield a new ecotropic which was thymotropic appears unlikely, in that M-MuLVs reisolated from the thymuses of infected mice also appeared splenotropic at 1 week after infection (unpublished observations). An alternative explanation for the abrupt rise in thymus titers in M-MuLV- and FM-I2-infected mice is that the lag in virus replication by the thymus is largely a kinetic phenomenon associated with a change in the mode of thymus infection after inoculation. Thymus cells may be much more refractory to infection by cell-free virus than spleen cells and may require a high multiplicity of virus for efficient infection. Initially, a small number of thymus cells may be infected by all of the viruses; however only those MuLVs containing the M-MuLV LTR (M-MuLV and FM-I2) may produce sufficiently high levels of virus to readily infect neighboring cells. Virus spread by cell-to-cell contact would result in a geometric increase of infected thymus cells, which in turn could result in an abrupt rise of the IC titer between 1 and 2 weeks after infection.

The relevance of the high level of replication of M-MuLV and FM-I2 in the thymus at 2 weeks after infection to the induction of disease is not known. However, the finding that the level of replication of FM-I2 did not correspond to the disease state of leukemic mice suggested that a high level of replication may be required only transiently during the early preleukemic stage of disease. It is possible that the rate of infection of the target tissue is more relevant to disease induction than the level of virus replication per se. Several recent studies suggest that leukemogenesis by M-MuLV is mediated by integration of the provirus near cellular *onc* genes, resulting in the transcriptional activation of the cellular gene by the M-MuLV LTR (9, 20, 29, 32, 34, 35). A sufficiently rapid infection of target tissue may facilitate multiple infection of cells by the MuLV before the establishment of viral interference. Multiple infection of the target cell should increase the probability of integration in the vicinity of a c-*onc* gene. It has been demonstrated that M-MuLV-induced tumors exhibit multiple copies of the M-MuLV provirus (34). Alternatively, early infection of the target cell could lead to the establishment of a preleukemic

stage of disease which is prerequisite to the operation of subsequent events which lead to the development of frank leukemia. In this regard, O'Donnell et al. (23) have demonstrated in AKR mice the emergence of clonal populations of thymus cells at a stage of disease that is distinguishable from frank leukemia. Progression to the fully leukemic stage was accompanied by a much higher frequency of integration in the vicinity of the c-*myc* cellular *onc* gene, further suggesting a stepwise mechanism of tumor progression. A recent study utilizing in vitro-constructed LTR variants of M-MuLV has also demonstrated a high level of infection of the thymus during an early preleukemic stage of disease (10). The results of that study indicate that M-MuLV and M-MuLV LTR variants which retain their leukemogenicity exhibit a high level of thymus infection by 2 to 3 weeks after inoculation. However, a nonleukemogenic variant exhibits a delayed infection of the thymus which eventually reaches the levels of infection observed with leukemogenic MuLVs. In our study, the recombinant MF-3 MuLV exhibited a similar delayed infection of the thymus. Mice infected with MF-3 exhibit a high incidence of erytholeukemia but do not develop lymphocytic leukemia, further suggesting that events dependent upon early infection of the target organ may be necessary for the induction of disease.

The predominant influence of the LTR on tissue-specific replication of the recombinant MuLVs at 2 weeks was superseded at later times after infection by an influence of the MuLV structural genes. The influence of the structural genes appeared to be more direct in mice infected with MF-3 than in mice infected with FM-I2. With MF-3, high levels of thymus infection were established between 2 and 5 to 8 weeks after inoculation (Fig. 1), and the pattern of replication in the spleens and thymuses of MF-3-infected mice at 5 to 8 weeks resembled the pattern observed with M-MuLV-infected mice (Fig. 2). This was in contrast to F-MuLV, which did not establish high levels of thymus infection (Fig. 1). Since MF-3 and F-MuLV do not differ in their LTRs, other sequence elements of MF-3 must have exerted an influence on the infection of the thymus. In the case of FM-I2, a more complex series of events led to a pattern of replication which resembled that of F-MuLV. A high level of infection of the thymus was established in FM-I2-infected mice at 2 weeks after inoculation, but the IC titers of thymus cells from most mice were severely depressed by 5 to 8 weeks (Fig. 1, Table 1). The depressed titers may have been the result of decreased production of virus by infected cells rather than a decrease in the percentage of infected cells. If so, the influence of the structural genes of FM-I2 on its ultimate pattern of replication may have been indirect (e.g., selective pressure favoring cells producing low levels of virus) and fundamentally different than the influence of MF-3 structural genes, which appeared to exert a positive effect on thymus infection.

Another interesting aspect of this study was the bimodal distribution of thymus titers in leukemic FM-I2-infected mice. It is possible that two types of cells were transformed by FM-I2, one which exhibits a high level of replication of FM-I2 and another which exhibits a low level of replication. Since thymomas have been shown to be monoclonal or oligoclonal in origin (9, 22, 23, 29), they would express high or low levels of virus depending on the predominant clone(s). This may also explain the observation that leukemic spleens of mice infected with FM-I2 uniformly expressed high levels of virus, regardless of the level of replication in the thymus (Table 1). The predominant target cells in the spleen may correspond to cells that express high levels of virus, and those of the thymus may correspond to cells that express low levels. The exceptional thymus tumors that expressed high levels of virus may have originated in the spleen and then spread to the thymus. Alternatively, the thymus may contain both types of target cells, but an excess of cells which ultimately express low levels of virus, resulting in a higher frequency of tumors exhibiting that phenotype. A prediction of this hypothesis is that thymus and spleen tumors of most FM-I2-infected mice are of independent origin.

Examination of polytropic MuLV titers in the spleens and thymuses of late-preleukemic mice indicated that the replication of polytropic MuLVs approximately paralled the replication of the ecotropic MuLVs in every instance. Thus, like ecotropic MuLV replication, polytropic MuLV replication in mice of this age did not closely correlate with disease progression. The observation that both ecotropic and polytropic MuLV repliction was suppressed in most late preleukemic FM-I2-infected mice suggested that the low level of replication was a property of the tumor cells, rather than an alteration of the viruses. However, we cannot presently exclude the possibility that both ecotropic and polytropic MuLVs exhibit an alteration which suppresses their replication.

Previous studies addressing tissue-specific replication of viruses have examined mice several weeks after infection (7, 14, 27), and it has been concluded that sequences within the the viral LTR exert a predominant influence on tissue-specific MuLV replication (14, 27). Our studies also demonstrated a predominant influence of the LTR on tissue-specific replication, but only during a very early preleukemic stage of disease. Examination of replication at different times after infection indicated that important determinants of tissue-specific replication are contained within the structural gene elements of F-MuLV and M-MuLV as well as in the LTRs. It is apparent from the present studies that many factors may influence the tissue-specific replication of MuLVs and that different factors may exert a predominant influence at different times during the course of infection.

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