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Effects of Nonleukemogenic and Wild-Type Moloney Murine Leukemia Virus on Lymphoid Cells In Vivo: Identification of a Preleukemic Shift in Thymocyte Subpopulations

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Infection of mice with Moloney murine leukemia virus (M-MuLV) as well as with a nonpathogenic variant, Mo+PyF101 M-MuLV, was studied. Mo+PyF101 M-MuLV differs from wild-type M-MuLV by the addition of enhancer sequences from polyomavirus in the long terminal repeat. Previous experiments indicated that Mo+PyF101 establishes infection in animals, even though it does not induce disease. *In vivo* infection studies with particular attention to the thymus were performed, since the thymus is the target organ for M-MuLV leukemogenesis. Mice inoculated at birth with wild-type M-MuLV developed maximal levels of thymic infection by 2 to 3 weeks. Animals inoculated with Mo+PyF101 M-MuLV showed considerably less thymic infection at early times (2 to 4 weeks); nevertheless, by 5 to 6 weeks infection equivalent to wild-type M-MuLV-inoculated animals developed. Therefore the nonpathogenicity of Mo+PyF101 M-MuLV did not simply reflect a lack of thymotropism. Furthermore, thymic infection by itself may not be sufficient to induce leukemia. The relative deficit of Mo+PyF101 M-MuLV thymic infection at early versus late times did not reflect a change in the nature of the cells in the thymus, since *in vitro* infection of primary thymocytes from 2- and 6-week-old animals was equally efficient. One possible explanation is that infected thymocytes normally arise from progenitor cells which were infected in the bone marrow or spleen, and the cells restricted for Mo+PyF101 M-MuLV are located in those organs. Comparison of wild-type and Mo+PyF101 M-MuLV also allowed identification of important preleukemic changes in the thymus of wild-type M-MuLV-inoculated mice. Flow cytometry with monoclonal antibodies specific for thymocyte subpopulations was used. Staining of cells for Thy-1 or Thy-1.2 antigens indicated a shift toward low or negative cells. A concomitant increase in cells positive for antigen Pgp-1 was also observed. This is consistent with an increase in the relative frequency of immature blastlike cells. Importantly, thymuses from mice inoculated with Mo+PyF101 M-MuLV did not show these shifts in thymocyte subpopulations.

Moloney murine leukemia virus (M-MuLV) is a classical, slowly transforming retrovirus. It is replication competent, lacks a viral oncogene, and induces thymus-derived lymphoblastic lymphoma when inoculated into neonatal mice (20). Animals typically develop disease in 3 to 4 months, with enlarged thymuses, spleens, or lymph nodes or all three. The resultant tumors are thymus dependent, in that surgical or chemical thymectomy inhibits the development of disease (20). Furthermore, the tumor cells generally show some phenotypic properties of T-lymphoid cells (14).

M-MuLV and other slowly transforming retroviruses induce disease with a relatively long latency. In particular, inoculated animals show evidence of viral infection well before the end-stage tumors appear (19). Thus, it is likely that a number of events occur in the inoculated animal which are necessary for eventual appearance of the malignant tumor. We are interested in identifying these preleukemic events and understanding how particular sequences in the viral genome are involved.

Previous studies have identified several features of leukemogenesis by slowly transforming MuLVs. First, recombinant (mink cell focus-inducing) derivatives of the initially infecting virus are generally present in the tumors (20, 22, 23). These mink cell focus-inducing derivatives arise by recombination with endogenous retroviral information present in normal mouse cells. In addition, insertion of MuLV or mink cell focus-inducing proviruses next to specific cellular sequences (e.g., c-*myc* and c-*pim* for MuLV-induced lymphomas) is observed in end-stage tumors (3, 12, 18). This insertion results in increased transcription of the adjacent cellular sequences. More recently, the U3 region of the MuLV long terminal repeats (LTRs) have been shown to carry an important determinant (2, 5, 10, 15). In particular, the transcriptional enhancer sequences in the LTRs of different strains of MuLV have been shown to control both their ultimate pathogenicity and their relative ability to replicate in different differentiated hematopoietic cells.

We recently described a variant of an M-MuLV, Mo+PyF101, which contains enhancer sequences from polyomavirus inserted into the U3 region of the wild-type M-MuLV LTR (4) (Fig. 1). Mo+PyF101 and wild-type M-MuLV particles are identical since the polyoma sequences were inserted into a region which does not encode viral protein. Interestingly, although animals inoculated with Mo+PyF101 M-MuLV sustain multiple rounds of infection, they do not develop leukemia (4). This suggests that insertion of the PyF101 sequences into the M-MuLV LTR altered the tissue specificity of expression such that Mo+PyF101 M-MuLV is unable to productively infect some cell type which wild-type M-MuLV must infect to develop leukemia. Thus, comparison of animals and cell types infected with wild-type versus Mo+PyF101 M-MuLV provides an important method for identifying critical preleukemic cells. In this report, infection of neonatal mice with the two M-MuLVs

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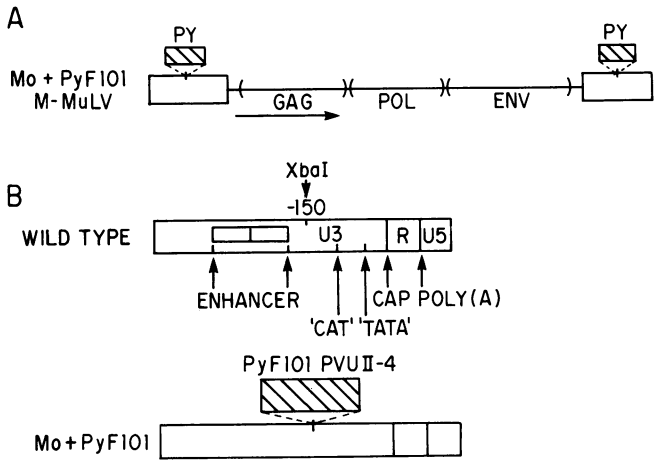

FIG. 1. Organization of Mo+PyF101 M-MuLV (A) and an expanded view of the wild-type and Mo+PyF101 LTRs (B). (A) The proviral DNA structure of Mo+PyF101 M-MuLV is shown (4). The virus is replication competent and contains the standard *gag*, *pol*, and *env* genes. The horizontal arrow indicates the direction of RNA transcription relative to the proviral DNA template; Mo+PyF101 M-MuLV differs from wild-type M-MuLV only by the insertion of polyomavirus enhancers within the wild-type LTRs. (B) The U3 region contains the enhancer, "CAT," and "TATA" elements. The cap site and poly(A) site at either end of the R region are also indicated. The *Pvu*II-4 enhancer-containing fragment from PyF101 was inserted into the M-MuLV LTR at the *Xba*I site (4).

was studied. The goals were to identify the cell (or cells) which was restricted for Mo+PyF101 M-MuLV infection and to identify important preleukemic changes induced by wild-type M-MuLV.

MATERIALS AND METHODS

Viruses and cell lines. The wild-type M-MuLV used in this study was obtained as the supernatant from the A9 cell line, a clone of NIH 3T3 mouse fibroblasts infected with wild-type M-MuLV (6). Mo+PyF101 M-MuLV was obtained as the supernatant from the 25-3 cell line which is confluently infected with Mo+PyF101 M-MuLV. The 25-3 cell line was derived by transfection of a recombinant Mo+PyF101 M-MuLV provirus onto NIH 3T3 cells (4). The Ti-6 cell line, a non-virus-producing lymphoid line derived from a primary radiation-induced thymoma in C57BL/6 mice (13), was a generous gift of Paul Jolicoeur (University of Montreal, Montreal, Que., Canada). Ti-6 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum.

Inoculation of mice. For investigations of the infection of lymphoid organs in vivo, newborn (1 to 4 days after birth) NIH Swiss mice were inoculated intraperitoneally with 2.5 $\times$ 10^4 XC PFU of either wild-type or Mo+PyF101 M-MuLV. For studies of preleukemic changes by antibody staining and flow cytometric analysis, newborn (1 to 2 days after birth) NIH Swiss mice were inoculated intraperitoneally with 7.5 $\times$ 10^4 or 1.2 $\times$ 10^5 XC PFU of virus.

Infectious-center plating of thymocytes and splenocytes from inoculated mice. The procedure used for infectious-center plating of thymocytes and splenocytes was essentially as described by desGrossiellers et al. (5). Briefly, thymuses and spleens were removed from sacrificed mice at different times after inoculation with wild-type or Mo+PyF101 M-MuLV, and cell suspensions were obtained from each organ by puncturing and extruding with syringe needles. Thymic or splenic cell suspensions (10^2 to 10^6 cells) were seeded onto 5 $\times$ 10^4 NIH 3T3 cells in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum and 1.5 μg of Polybrene per ml for 24 h. After this time, the nonadherent lymphoid cells were removed by aspiration and two washes with phosphate-buffered saline (PBS). The remaining NIH 3T3 cells were grown until confluent (approximately 5 days) in DMEM plus 10% calf serum. Infectious centers, observable as macroscopic plaques, were identified by the UV-XC syncytial plaque assay (17).

In vitro infection of thymocytes. Uninoculated NIH Swiss mice were sacrificed at either 2 or 6 weeks of age, the thymuses were removed, and single-cell suspensions were prepared. A total of 10^7 thymocytes, maintained in 4 ml of RPMI 1640 medium plus 10% defined fetal bovine serum (Hyclone) Logan, Utah and 5 μg of Polybrene per ml, were incubated together with 2.5 $\times$ 10^5 XC PFU of either wild-type or Mo+PyF101 M-MuLV for 48 h. The viability of the thymocytes for this length of time depended greatly upon the source of serum; however, Hyclone serum yielded satisfactory results, with 25 to 50% of the thymocytes still viable according to trypan blue staining after 48 h. The infected cells were harvested by centrifugation (1,200 $\times$ *g*, 10 min), washed once in 2 ml of Hanks buffer (Irvine Scientific, Irvine, Calif.) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and counted. Cells (2.5 $\times$ 10^2 to 2.5 $\times$ 10^6) were seeded onto 5 $\times$ 10^4 NIH 3T3 cells in DMEM plus 10% calf serum and 1.5 μg of Polybrene per ml for 24 h. Virus-producing cells were then scored in the infectious-center assay.

Infection of Ti-6 and NIH 3T3 cell lines. A total of 10^6 Ti-6 cells were incubated together with 2.5 $\times$ 10^4 XC PFU of wild-type or Mo+PyF101 M-MuLV for 48 h in 4 ml of RPMI 1640 medium plus 10% fetal bovine serum and 5 μg of Polybrene per ml. Infectious-center plating onto NIH 3T3 cells was performed as described above. The NIH 3T3 cell infection was performed by incubating 10^6 adherent cells with 2.5 $\times$ 10^4 XC PFU of virus for 48 h in DMEM plus 10% calf serum and 5 μg of Polybrene per ml. After this time, the NIH 3T3 cells were trypsinized and harvested, and 10^1 to 10^4 cells were plated together with 5 $\times$ 10^4 uninfected NIH 3T3 cells in DMEM plus 10% calf serum and 5 μg of Polybrene per ml. Cells were grown to confluency, and infectious centers were counted by using the UV-XC assay.

Antibodies. Rat monoclonal antibodies directed against surface markers found on murine T cells and other cells were obtained from the following sources: Thy-1 (M5/49; Hybritech, San Diego, Calif.), Pgp-1 (IM7.8.1; kindly provided by Jayne Lesley and Robert Hyman, The Salk Institute, La Jolla, Calif.). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulin [$F(ab)_2$ affinity-purified fragment; Cappel Laboratories, Worthington, Pa.] was used as a second antibody. A directly FITC-conjugated rat anti-mouse Thy-1.2 monoclonal antibody (MAb) (30H12; Becton Dickson and Co., Mountain View, Calif.) was used in some experiments.

MAb staining and analysis by flow cytometry. Thymuses were removed from uninoculated mice or mice inoculated with wild-type or Mo+PyF101 M-MuLV at different times after birth. Single-cell suspensions of thymocytes were obtained by grinding the organ through a fine wire mesh, which retains the reticular network. In indirect immunofluorescence experiments, 10^6 cells suspended in PBS (pH 7.2) were incubated with the appropriate dilutions of MAbs for 30 min on ice, washed three times with PBS, incubated for 30 min on ice with the labeled second antibody, washed three

times with PBS, and suspended in 0.5 ml of PBS. In direct immunofluorescence experiments with FITC-conjugated anti-mouse Thy-1.2, a second antibody was not used.

Cells (10^4 per sample) were analyzed by the fluorescent activated cell analyzer (Becton Dickinson), and the results were displayed as both a "dot plot" with fluorescence intensity versus cell volume and a fluorescence histogram. The fluorescence gates were set to exclude 99% of unstained thymocytes to eliminate autofluorescence.

RESULTS

Infection of lymphoid cells by wild-type and Mo+PyF101 M-MuLV. We previously showed that mice inoculated with Mo+PyF101 M-MuLV developed very significant levels of viral infection in comparison to wild-type M-MuLV-inoculated animals, as assessed by levels of viremia in the blood serum (4). Since wild-type M-MuLV predominantly infects lymphoid cells in vivo (9) and induces T-lymphoid disease, it was important to test if the nonleukemogenicity of Mo+PyF101 M-MuLV was due to an inability to infect lymphoid (and particularly thymic) cells. In Fig. 2, the levels of infection in thymocytes or splenocytes from animals inoculated with wild-type and Mo+PyF101 M-MuLV were determined. Single-cell suspensions from thymuses or spleens of animals inoculated with the two viruses were prepared at different times postinjection and cocultivated with uninfected NIH 3T3 cells in an infectious-center assay. Wild-type M-MuLV rapidly established infection in the thymus, and maximal levels of infection occurred by 2.5 to 3 weeks postinoculation (Fig. 2A). In contrast, the pattern of thymocyte infection with Mo+PyF101 M-MuLV was different. At early times (2.5 to 3 weeks), thymocytes from Mo+PyF101 M-MuLV-inoculated animals showed much less (3 to 4 logs) viral infection in comparison to wild-type M-MuLV. On the other hand, similar levels of thymocyte infection were achieved by 4 to 5 weeks postinoculation, with most Mo+PyF101 M-MuLV-inoculated animals showing as high levels of thymocyte infection as wild-type inoculated animals. These data indicate that the nonpathogenicity of Mo+PyF101 M-MuLV did not result from a failure of the virus to infect thymocytes. These results also suggest that M-MuLV infection of the thymus is not in itself sufficient to induce leukemia, since nonpathogenic Mo+PyF101 M-MuLV was able to establish high levels of thymic infection by 4 to 5 weeks.

The levels of viral infection in the spleens of wild-type and Mo+PyF101 M-MuLVs are shown in Fig. 2B. Mo+PyF101 M-MuLV was also able to establish infection in the spleen, although at reduced levels in comparison to wild-type M-MuLV. Interestingly, at early times, the relative deficit of Mo+PyF101 M-MuLV infection was not as great for the spleen as for the thymus.

The marked deficit in thymocyte infection of Mo+PyF101 M-MuLV-inoculated animals at early times was investigated further. This deficit was particularly noteworthy, since the levels of infectious virus in the blood serum of wild type- and mutant-inoculated animals are nearly equal at this time (4). One possible explanation was that different cell types were present in thymuses of different ages and that cells of the early thymus were specifically resistant to Mo+PyF101 M-MuLV infection. To test this, in vitro infection of thymocytes was performed (Table 1). Primary thymocyte suspensions were prepared from uninfected NIH mice and infected with wild-type or mutant M-MuLV in vitro (multiplicity of

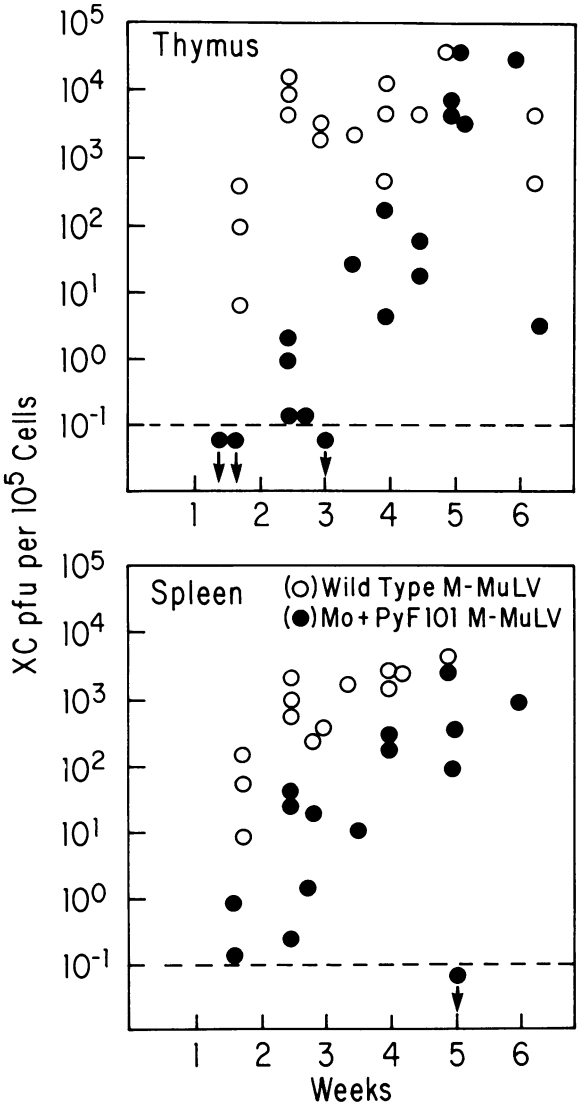

FIG. 2. Thymic and splenic infection in inoculated mice. The levels of infection in the thymus (A) and spleen (B) in NIH Swiss mice inoculated with wild-type and Mo+PyF101 M-MuLV are shown. Neonatal animals were inoculated with 2.5×10^4 XC PFU of wild-type or Mo+PyF101 M-MuLV. Thymocytes and splenocytes were plated as infectious centers onto NIH 3T3 cells (5), and the amount of infectious virus was quantified by using the UV-XC assay (17). The results of these experiments are plotted as number of XC PFU per 10^5 cells plated. All data points represent individual animals. Data points with downward arrows indicate animals having virus levels undetectable in this assay (<0.1 XC PFU/10^5 cells).

TABLE 1. In vitro infection of primary thymocytes or tissue culture cells with wild-type and Mo+PyF101 M-MuLVs

M-MuLV	XC PFU per 10^6 cells			
	Thymocytes (2 wk)[a]	Thymocytes (6 wk)[a]	Ti-6[b]	NIH 3T3[b]
Wild type	1.7×10^2	2.3×10^2	2.8×10^4	2.1×10^5
Mo+PyF101	3.6×10^1	7.2×10^1	6.1×10^3	4.1×10^5

[a] Thymocytes (10^7) from uninoculated NIH Swiss mice of either 2 or 6 weeks of age were incubated for 48 h with equal infectious units (multiplicity of infection = 2.5×10^{-2}), of either virus, and plated as infectious centers onto NIH 3T3 cells.

[b] Ti-6 lymphoid cells and NIH 3T3 mouse fibroblasts were infected at the same multiplicity of infection (2.5×10^{-2}).

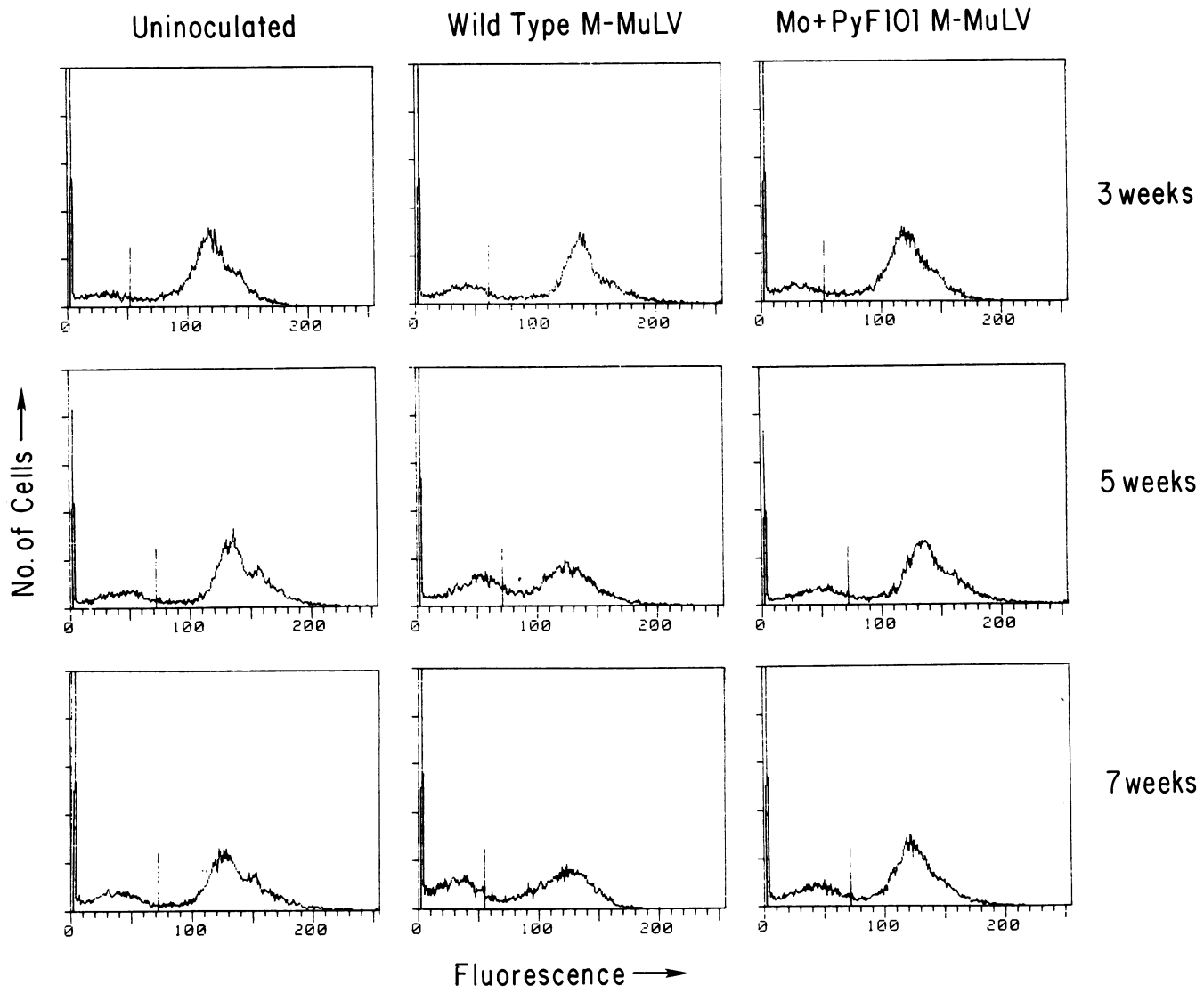

FIG. 3. Distribution of Thy-1 antigen on thymocytes. The distribution of Thy-1 surface marker on thymocytes from uninoculated mice or from mice at different times after inoculation with wild-type or Mo+PyF101 M-MuLV (7.5×10^4 or 1.2×10^5 XC PFU) is shown. In each experiment, 10^6 cells were stained with anti-Thy-1 Mab and an FITC-conjugated second antibody, and 10^4 cells were analyzed by flow cytometry. Fluorescence intensity is shown on the horizontal axis (logarithmic scale), and the vertical scale shows the number of cells. The fluorescence gates were set to eliminate autofluorescence corresponding to unstained cells. Beginning at 5 weeks postinoculation, the thymuses from wild-type M-MuLV-inoculated mice show a shift in distribution toward low Thy-1 cells (preleukemic stage). This is observable as an increase in the number of cells with fluorescence intensity less than the fluorescence gate. In contrast, Mo+PyF101 M-MuLV-inoculated animals, as late as 9 weeks postinoculation, show a pattern indistinguishable from that of uninoculated mice.

infection, 2.5×10^{-2}). After 48 h of incubation, the infected thymocytes were then cocultivated with uninfected NIH 3T3 cells in an infectious-center assay. The data indicated that Mo+PyF101 M-MuLV was slightly less infectious (three- to fivefold) than wild-type M-MuLV in the primary thymocytes. However, more importantly, thymocytes from 2-week-old animals did not show a greater deficit for Mo+PyF101 M-MuLV infection than did thymocytes from 6-week-old animals. These results suggest that the deficit in Mo+PyF101 M-MuLV infection of early thymocytes was not the result of a different and noninfectable population of cells being present in the thymus. One possible explanation for these results could be that the major sites of viral infection may be the bone marrow or spleen in young animals and that infected thymocytes result from differentiation of lymphoid precursors which were infected elsewhere (see Discussion).

In the in vitro thymocyte infections, one concern was the fact that the thymocyte cultures have rather limited potential for growth. This might influence the results, since retroviral infection and replication are dependent on cell division. Therefore, infection of the two viruses was also performed in a thymocyte cell line, Ti-6 (13). Cell surface marker analyses indicate that Ti-6 cells have some properties of rather primitive thymocytes (high in Pgp-1 and low in Thy-1 surface markers; see below). Mo+PyF101 M-MuLV showed the same lowered infectability in Ti-6 cells as observed in the primary thymocytes (Table 1). This was reassuring since in the primary thymocyte infections the low levels of infection might have resulted from adsorbed virus which survived the

washing and initial infection. However, the levels of infection for the Ti-6 cells were clearly much higher, yet the same relative infectivies for the two viruses were observed.

Induction of preleukemic changes in thymuses of mice inoculated with wild-type but not Mo+PyF101 M-MuLV. Thymuses from mice inoculated with the two viruses were also studied from the perspective of identifying important preleukemic changes induced by wild-type M-MuLV. One easily identifiable preleukemic change induced by wild-type M-MuLV was thymic atrophy (19). In comparison to uninoculated animals, M-MuLV-inoculated animals show marked thymic atrophy beginning at 5 to 8 weeks, resulting in a pronounced decrease in thymus size, and a three- to fivefold decrease in cell count. Importantly, Mo+PyF101 M-MuLV-inoculated animals did not show thymic atrophy, even at times when high-level infection of the thymus should have taken place according to Fig. 2A. This is consistent with the M-MuLV-induced thymic changes resulting in atrophy being a necessary event in leukemogenesis.

More detailed analysis of preleukemic changes in the cell populations of the thymus was performed by using MAbs directed against T-cell differentiation antigens. The first surface marker analyzed was Thy-1 antigen, which is present on most thymocytes and mature T cells. Thymocytes from mice inoculated with wild-type or Mo+PyF101 M-MuLV were stained with an anti-Thy-1 MAb plus fluorescein-conjugated second antibody and analyzed by flow cytometry. Data from typical analyses at different times after inoculation are shown in Fig. 3. During the first 3 to 4 weeks postinoculation, the majority of thymocytes were Thy-1 positive, and no differences were observed in M-MuLV-inoculated animals versus control uninoculated animals. Between 5 and 9 weeks postinoculation, a shift in thymocyte subpopulations was observed for wild-type M-MuLV-inoculated animals, at about the same time that thymic atrophy occurred. There was a decrease in the percentage of high Thy-1 cells and an increase in the percentage of low or Thy-1-negative cells. This shift was reproducible and, using this Thy-1 MAb, indicated a change in Thy-1 status of approximately 10 to 20% of the thymocytes (Table 2). Importantly, thymocytes from Mo+PyF101 M-MuLV-inoculated animals did not show the shift toward low Thy-1 cells. From 10 to 16 weeks, thymus size in the wild-type M-MuLV-inoculated animals began to increase and the percentage of low Thy-1 cells in the thymus decreased, probably reflecting the appearance of tumor cells. End-stage tumor cells themselves are generally highly Thy-1 positive (14; K. G. Chandy, B. R. Davis, and B. K. Brightman, unpublished data).

The results shown in Fig. 3 indicated a somewhat higher percentage of control cells negative or low for Thy-1 than reported by several other groups (11, 16). This was probably due to the particular combination of anti-Thy-1 MAb and second antibody used. When a directly conjugated MAb specific for the Thy-1.2 allele was used instead, 97% of control thymocytes stained brightly and 3% were Thy-1.2 negative. The percentage of Thy-1.2-negative thymocytes from an M-MuLV-inoculated animal was 8%. Furthermore, the mean fluorescence of the Thy-1.2-positive cells from the M-MuLV-inoculated animal was markedly lower than that of control thymocytes, indicating that the shift in thymocyte subpopulations involved a significantly greater percentage of cells than evident from the Thy-1 staining.

Another useful thymocyte cell surface marker was Pgp-1 antigen. Pgp-1 is a surface molecule on T-lymphoid precursor cells in the bone marrow, and Pgp-1-positive cells in the thymus are enriched for thymocyte progenitors (11, 16, 21). Pgp-1 is absent from most mature thymocytes (11, 21). The preleukemic (6- to 10-week) thymuses from M-MuLV-inoculated animals showed a specific increase in Pgp-1-positive cells (Fig. 4), in comparison to either uninoculated or Mo+PyF101 M-MuLV-inoculated animals. The mean size of the emerging Pgp-1-positive population was also somewhat larger than average for thymocytes. Thymocytes from preleukemic wild-type M-MuLV-inoculated mice also showed a shift toward lower levels of L3T4 and Lyt-2 (data not shown). All of these properties were consistent with a shift toward more immature blastlike cells in the preleukemic thymuses of MuLV-inoculated mice (16). The preleukemic shifts in thymocyte subpopulations could result from an increase in the number of immature thymocytes, selective depletion of mature (medullary or cortical or both) thymocytes, or both. However, it was not possible to distinguish between these two possibilities due to the thymic atrophy.

TABLE 2. Percentage of low or negative Thy-1 thymocytes in inoculated mice[a]

Mice	% of low or negative Thy-1 thymocytes				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Uninoculated	27.9	30.4	30.8	31.1	46.7
Wild type M-MuLV	38.9	49.0	51.2		54.8, 76.7
Mo+PyF101 M-MuLV	28.7			32.9	

[a] Neonatal NIH Swiss mice were inoculated with wild-type or Mo+PyF101 M-MuLV and sacrificed 6 to 7 weeks postinoculation. Thymocytes were stained and analyzed by flow cytometry for Thy-1 expression as in Fig. 3. The percentage of low/negative Thy-1 cells was determined from percentage of cells which had fluorescence less than the indicated vertical gates in Fig. 3 (corresponding to 99% of unstained thymocytes). While the percentage of low/negative Thy-1 cells was generally the same for uninoculated and Mo+PyF101 M-MuLV-inoculated mice, wild-type M-MuLV preleukemic mice showed increased levels of low/negative Thy-1 cells.

The Thy-1 and Pgp-1 analyses indicated that the preleukemic thymuses showed coincident shifts toward low/negative Thy-1 and high Pgp-1. It is thus possible that the shifting populations included an increased concentration of low Thy-1, high Pgp-1 cells, which would be characteristic of immature thymocytes. Since the flow cytometry was not performed with simultaneous (two-color) analysis of both markers, it was not possible to directly test the hypothesis. However, cells from one M-MuLV-inoculated mouse were very interesting from this standpoint. This animal was analyzed at 13 weeks postinoculation and showed an atrophied thymus but enlarged spleen. Thus it appeared to represent an extreme example of the preleukemic stage even at a very late time. Flow cytometry indicated that the thymocytes consisted of a nearly homogeneous population which was low in Thy-1 and intermediate to high for Pgp-1 (Fig. 5). The percentages of low Thy-1 and high Pgp-1 cells were such that it could be concluded that most of the thymocytes were both low Thy-1 and high Pgp-1. This supports the suggestion that similar cells are among those detected in the less extreme preleukemic thymuses. It cannot be ruled out that the thymocytes for this particular animal represented an unusual evolving tumor. However, it should be noted that M-MuLV-induced tumors are generally Thy-1 positive and low to intermediate for Pgp-1 (not shown).

DISCUSSION

In these experiments the infection of animals with M-MuLV and its nonpathogenic variant Mo+PyF101 M-

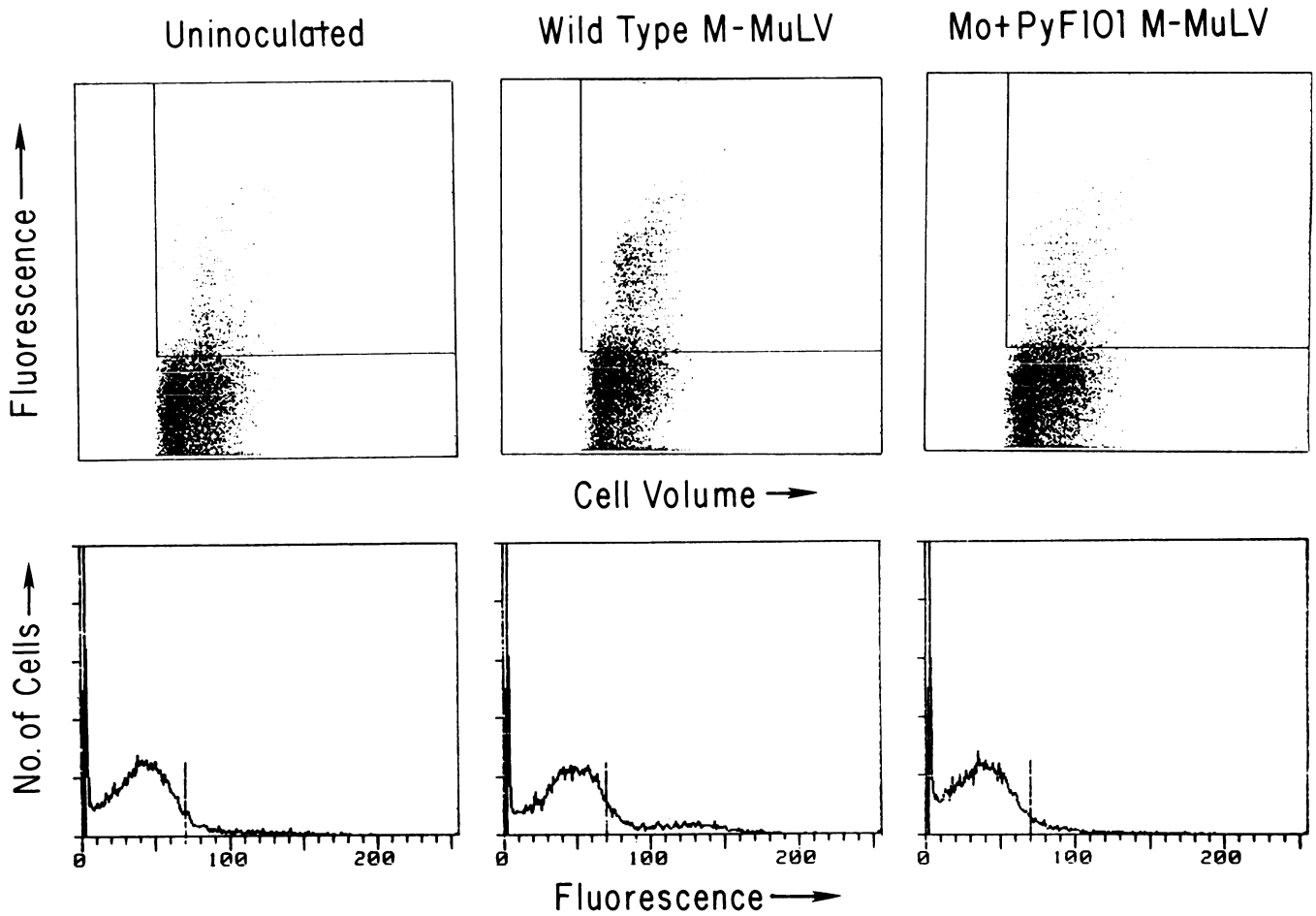

FIG. 4. Distribution of Pgp-1 on thymocytes. Shown is an example of the distribution of Pgp-1 surface marker on thymocytes obtained from uninoculated control mice or from wild-type or Mo+PyF101 M-MuLV-inoculated mice. The staining was performed by incubating thymocytes with a rat anti-mouse Pgp-1 MAb, followed by incubation with FITC-conjugated goat anti-rat immunoglobulin G. A dot plot of Pgp-1 fluorescence versus cell volume is displayed in the upper panels, and a fluorescence histogram showing number of cells versus Pgp-1 fluorescence is represented in the lower panels. In both, the fluorescence intensity is shown on a logarithmic scale. In contrast to the control thymus which only contained 6.5% Pgp-1-positive cells (those cells with fluorescence intensity greater than the fluorescence gate), the preleukemic wild-type M-MuLV thymus contained 17.5% Pgp-1-expressing cells. The thymus from the Mo+PyF101 M-MuLV-inoculated animal gave the same pattern and percentage as that from the uninoculated animal.

MuLV was studied. There were two goals: (i) to identify cells in which Mo+PyF101 M-MuLV could not replicate, and (ii) to use comparisons between wild-type and Mo+PyF101 M-MuLV infection to identify important steps in leukemogenesis. A major focus of these experiments was the thymus, since M-MuLV induces thymus-derived lymphoblastic lymphoma.

One possible explanation for the nonpathogenicity of Mo+PyF101 M-MuLV could have been a loss of thymotropism due to insertion of the PyF101 sequences. In comparison to wild-type M-MuLV, Mo+PyF101 developed similar levels of thymic infection by 5 to 6 weeks postinjection. Furthermore, **in vitro** infection of thymocytes indicated that Mo+PyF101 M-MuLV was able to infect these cells with near-wild-type efficiency. Therefore, by these criteria, Mo+PyF101 M-MuLV retained thymotropism. On the other hand, at 2 to 3 weeks postinjection, wild-type M-MuLV-infected animals had much higher levels of thymic infection (3 to 4 logs) than Mo+PyF101 M-MuLV-inoculated animals. Somewhat surprisingly, thymocytes from very young animals appeared to be as infectable in vitro with Mo+PyF101 M-MuLV as thymocytes from 6-week-old animals.

At first the in vivo and in vitro thymocyte infection data for Mo+PyF101 M-MuLV appear to be somewhat contradictory. However, it is important to consider the fact that the thymus is an organ in which cells are continually entering and exiting. Thymocyte precursor cells migrate from the bone marrow into the thymus, where maturation and differentiation to mature thymocytes occur. Cells with properties of prothymocytes have also been identified in the spleen, although the relationship of these cells to T-cell differentiation in the thymus remains to be elucidated (7). Most maturing thymocytes die in a short period of time without leaving the thymus, while a portion exit as mature T cells. It is therefore possible that some infected thymocytes may actually result from entry and differentiation of infected thymocyte progenitors. If wild-type M-MuLV is able to infect thymocyte progenitors in the bone marrow and Mo+PyF101 M-MuLV is restricted from infecting these cells, this would provide an explanation for the thymus infection results reported here. Wild-type M-MuLV-inoculated animals would develop high levels of thymocyte infection at very early times due to entry of infected thymocyte precursors. In contrast, Mo+PyF101-infected

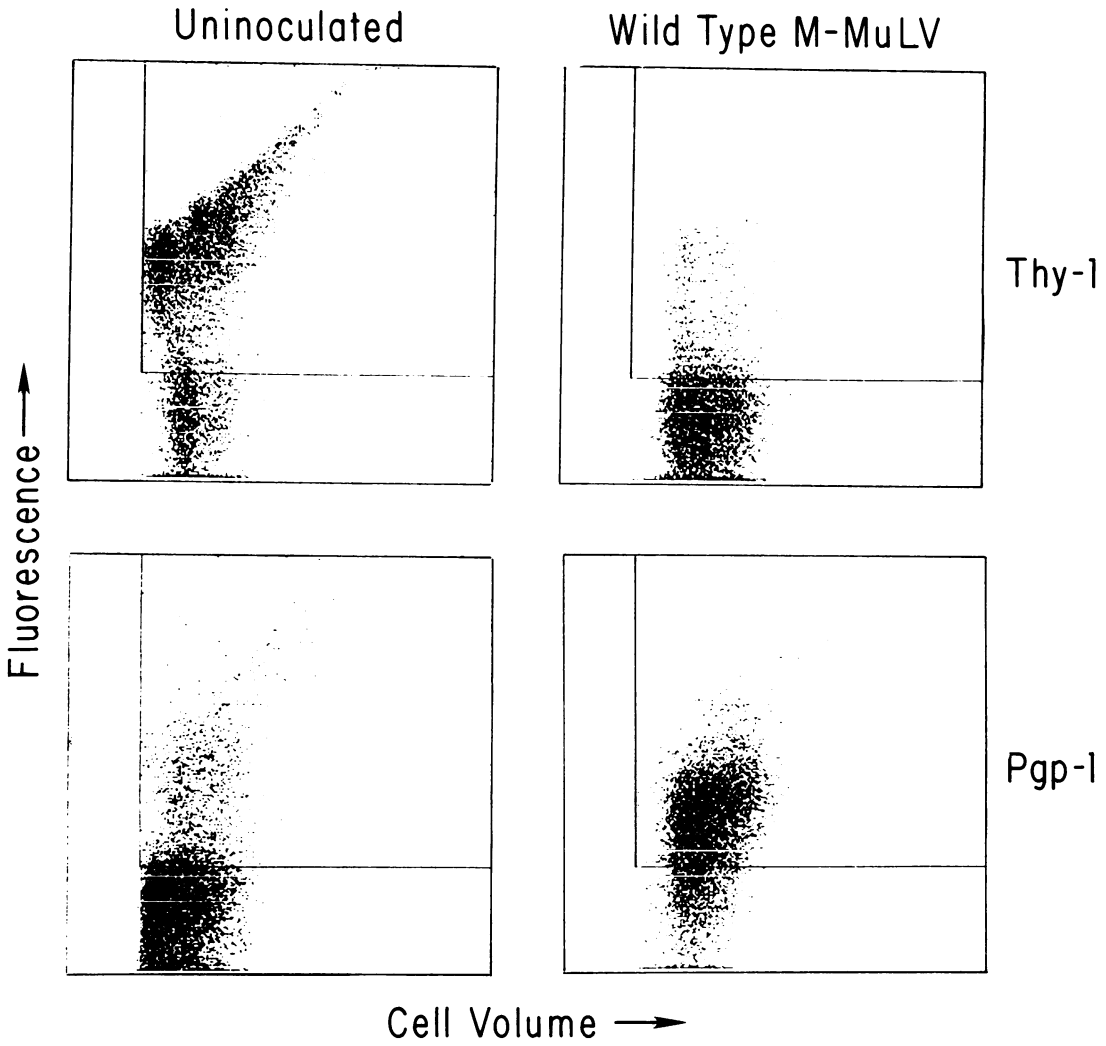

FIG. 5. Thymocytes from a potentially preleukemic mouse. Thymocytes from an M-MuLV-inoculated animal (13 weeks postinoculation) showing extreme thymic atrophy (described in the text) were stained with Thy-1- or Pgp-1-specific antibodies and compared with an uninoculated thymus. In contrast to a control animal whose thymus was primarily Thy-1 positive and Pgp-1 negative, this animal had a thymus which was almost exclusively low or negative Thy-1 and intermediate-to-high Pgp-1.

animals would not show equivalent thymocyte infection at these early times; however, eventual high-level infection in the thymus could result from direct infection of mature thymocytes within the thymus, or from eventual infection of thymocyte progenitors in the bone marrow or spleen. This suggests that attention should be focused on the bone marrow and spleen to identify cell types restricted for Mo+PyF101 M-MuLV infection.

An alternative explanation for the thymocyte infection results would be if nonlymphoid thymic epithelial cells are the normal source of infection for lymphoid thymocytes and if the thymic epithelial cells are specifically restricted for Mo+PyF101 M-MuLV infection.

The thymocyte infection data with Mo+PyF101 M-MuLV also suggest that infection of thymocytes is not in itself sufficient to induce leukemia. Mo+PyF101 M-MuLV establishes high levels of thymocyte infection at times well before appearance of leukemia in wild-type M-MuLV-inoculated animals and is thymotropic, but disease does not develop. This suggests that infection of other cells besides thymocytes may be necessary for M-MuLV leukemogenesis. Other investigators have reported preleukemic changes which occur in other hematopoietic organs. Storch et al. (19) reported that spleens from M-MuLV-inoculated mice show hyperplasia and increases in hematopoietic precursor cells for several lineages. Furthermore, these changes preceded or coincided with thymic changes. Asjo et al. (1) used reconstitution of irradiated mice to show that preleukemic cells can be detected in the bone marrow and spleen of wild-type M-MuLV inoculated mice before they can be detected in the thymus. These results suggest that M-MuLV must first induce hematopoietic hyperplasia to establish the preleukemic state. It remains to be demonstrated whether this hyperplasia results from direct infection of hematopoietic stem cells (19) or as an immunological response to infection (8). Migration of hyperplastic lymphoid progenitors to the thymus and subsequent reinfection (perhaps via mink cell focus-inducing derivatives) and integration next to c-*myc* or c-*pim* would result in development of the end-stage thymic tumor. The resulting thymic disease would reflect the

thymotropism of the M-MuLV enhancers and their ability to activate the cellular oncogenes in thymocytes. According to this model, Mo+PyF101 M-MuLV would be unable to participate in the first infection event leading to hematopoietic hyperplasia. This again directs attention toward the bone marrow or spleen or both. Experiments to investigate this are in progress.

In this report, preleukemic changes in the thymus were also characterized. A shift in thymocyte subpopulations occurred, which was consistent with an increase in the relative concentration of primitive thymocytes, although the exact nature of thymic precursors is not completely understood yet. The shift occurred at the same time as increased thymic atrophy. Both of these changes were specific preleukemic changes, and they did not simply result from M-MuLV infection; this could be concluded from the fact that animals inoculated with Mo+PyF101 M-MuLV developed high levels of infection without showing thymic atrophy or preleukemic thymocyte shifts. The causal relationship between thymic atrophy and shifts in thymic subpopulations has yet to be elucidated. It seems possible that the increased concentration of primitive thymocytes in preleukemic thymuses might reflect the hematopoietic hyperplasia in the bone marrow and spleen. In this light, it will be interesting to study animals inoculated with other strains of MuLV which cause tumors of other lineages (e.g., Friend MuLV, which causes erythroid disease). These other MuLVs might also induce generalized hematopoietic hyperplasia, but other tumors might result due to the different tissue tropisms for the second infection event. In this case, shifts in thymocyte subpopulations might also be observed, without eventual development of thymoma.

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