

STAGES IN DEVELOPMENT OF MINK CELL FOCUS-INDUCING (MCF) VIRUS-ACCELERATED LEUKEMIA IN AKR MICE

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Analysis of murine leukemia virus (MuLV)¹-induced leukemogenesis has demonstrated a strong correlation between *in vivo* expression of virus with recombinant *env* genes and disease (for a review, see reference 1). In mouse strains with a high incidence of spontaneous thymic lymphoma, such as AKR, the generation of *env* gene recombinants is an age-dependent, preleukemic event (2-4) apparently resulting from recombination between replicating ecotropic virus and endogenous xenotropic virus-related sequences (4-10). Such recombinant viruses, also termed mink cell focus-inducing (MCF) viruses (3), appear to play a direct role in the disease process. Leukemia development can be accelerated by the injection of neonatal or young adult AKR mice with cloned isolates of MCF virus (11-13). MCF viruses are thymotropic (12-15) and rapidly establish infection of thymocytes, resulting in the expression of high levels of MCF virus-coded gp70 on the cell surface (13, 14). Thus, infection by newly generated MCF viruses accounts for amplified expression of MuLV gp70 on thymocytes, one of the critical changes observed in the late preleukemic period (5-7 mo of age) of spontaneous leukemogenesis in AKR mice (2, 16). A second change ascribed to the late preleukemic period in AKR mice is a shift in quantitative expression of Thy-1 and H-2 alloantigens on thymocytes from high Thy-1/low H-2 to low Thy-1/high H-2 (16, 17). These findings have been interpreted as a shift to a more mature thymocyte phenotype during leukemogenesis. However, the thymus is an organ in which a complex and incompletely understood program of T cell differentiation is occurring. The thymus includes a number of cell populations that express Thy-1 and H-2 as well as other T lymphocyte markers such as Lyt-1 and Lyt-2 in varying amounts (for a review, see 18). Analysis of AKR leukemogenesis properly should take changes in these subpopulations into account.

In this report we describe a sequential series of cellular changes in thymus of AKR mice during development of MCF virus-accelerated leukemia. This study differs in certain critical respects from previous studies of spontaneous leukemia in this strain. First, in spontaneous leukemogenesis there is considerable asyn-

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¹Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MCF, mink cell focus-inducing; MuLV, murine leukemia virus; PNA, peanut agglutinin.

chrony in the kinetics of appearance of MCF viruses and initiation of disease development, which may account for the extended range of latent period in this system (12, 13). It is also unclear to what extent independent recombination events in individual AKR mice generate MCF viruses that differ in biological activity. Thus, one cannot assume that all AKR mice of a certain age will be infected by the same MCF virus or will be at the same stage of leukemogenesis. We have attempted to overcome such uncertainties by studying MCF virus-accelerated disease in young mice where leukemia development was rapid and synchronous after intrathymic injection of cloned MCF virus isolates. Second, we used the flow cytometric technique of correlated dual parameter analysis of forward angle light scatter and fluorescence to measure changes in cell size and in the expression of differentiation alloantigens or viral gp70 on specifically resolved thymocyte subpopulations. Previous studies of spontaneous AKR leukemia used cytotoxicity assays (16, 17) or single parameter flow cytometric analysis of immunofluorescence (21) to measure changes in antigen expression in the bulk thymocyte population but could not discriminate age-dependent from virus-induced changes nor relate such changes to specific thymocyte subpopulations.

Three stages of leukemogenesis were defined before the appearance of frankly leukemic mice. Stage I represented steady-state infection of thymocytes by MCF virus without apparent changes in light scatter properties of the cells or in the expression of several differentiation alloantigens on the major thymocyte subpopulations. Stage II was observed as early as 42 d postinjection and represented the emergence of a clonal population of cells with restricted transplantation properties. These cells could be resolved from normal thymocytes by flow cytometry. Stage III was observed when considerable enlargement of thymus had occurred and represented the outgrowth of fully transformed cells that replaced the normal thymocyte subpopulations. Our results are consistent with MCF virus transformation of immature thymocytes, i.e., cells in the lineage to the small cortisone-sensitive thymocyte subpopulation which normally express Thy-1, Lyt-1, Lyt-2, L3T4a, and H-2K antigens, receptors for peanut agglutinin, and an antigen recognized by monoclonal antibody B2A2 (22).

Materials and Methods

Virus Inoculation. Female AKR/J mice (40–45 d old) were anaesthetized by intraperitoneal injection of avertine (aqueous solution of 290 mg 2,2,2-tribromoethanol-160 mg isoamyl alcohol per kilogram body weight). The thymus was then exposed surgically as described by Kaplan (23) and the left lobe injected with 0.05 ml of MCF 69L1 virus (13) or tissue culture medium as control. Virus inocula contained $\sim 5 \times 10^4$ infectious units as determined by immunofluorescent focus assay on mink cells (13).

Antibodies and Fluorescent Reagents. Monoclonal mouse IgG2a antibody (19-E12) to Thy-1.1 (24) was provided by Dr. R. Nowinski, Genetic Systems, Seattle. Monoclonal mouse IgG2b antibody (2-2.1) to Lyt-1.2, mouse IgG2 antibody (116-13.1) to Lyt-2.1, and mouse IgG2a antibody (TL.m4) to TL.2 were provided by Dr. F.-W. Shen and Dr. E. A. Boyse of this institution (25, 26). Monoclonal mouse IgM antibody (6/68) to Thy-1.2, mouse IgG2 antibody (100-5-R18) to H-2K^k, and mouse IgG2a antibody (17/227) to I-A^k were provided by Dr. U. Hammerling of this institution (27, 28). Monoclonal rat IgG2b antibody (GK-1.5) to L3T4a (29) was provided by Dr. F. Fitch, University of Chicago. Monoclonal rat IgG2a antibody (35/56) to the gp70^f epitope has been described

previously (14, 30). Monoclonal mouse IgM antibody (514), which recognizes MCF virus gp70 specifically (31), was provided by Dr. M. Cloyd, National Institutes of Health. Monoclonal rat antibody B2A2, which recognizes predominantly small cortical thymocytes (22), was provided by Dr. P. Bartlett and Dr. R. Scollay, Walter and Eliza Hall Institute, Melbourne, Australia. Antibodies present in sera of hybridoma-bearing nude mice (Thy-1.1, 35/56), ascites fluids of hybridoma-bearing mice (Thy-1.2, Lyt-1.2, Lyt-2.1, H-2K, I-A, TL.m4), or concentrated hybridoma culture fluids (514, B2A2) were diluted in medium and used at saturating concentrations.

Fluorescein isothiocyanate-conjugated anti-mouse and anti-rat immunoglobulin sera were obtained from the Viral Oncology Program, National Cancer Institute. Fluoresceinated peanut agglutinin (PNA) was obtained from Vector Laboratories, Inc., Burlingame, CA and used at a dilution of 1:800.

Immunofluorescence Assays. Thymus or thymoma tissue was dispersed in a No. 22 tissue grinder (Kontes Co., Vineland, NJ) in the presence of Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS). Cell clumps were removed by pipetting through Nitex nylon cloth (Tetko Inc., Elmsford, NY). In some instances, especially for thymomas, the cell suspension was centrifuged for 10 min at 1,000 *g* on a 3 ml cushion of lymphocyte separation medium (Litton Bionetics, Inc., Kensington, MD) to remove nonviable cells and erythrocytes. Viability of thymocytes prepared in this way was consistently >90%. Cells were added to 3-ml conical centrifuge tubes (Bellco Glass, Inc., Vineland, NJ) (3×10^6 cells per reaction) which were prewet with DME containing 5% FCS and 0.1% sodium azide and centrifuged at 1,000 *g* for 10 min at 4°C. Cells were resuspended in 0.3 ml of medium containing primary antibody at the appropriate dilution for saturating conditions and incubated on ice for 30 min with periodic shaking. After incubation, the cell suspension was centrifuged as above through a 0.5 ml FCS cushion, resuspended in 0.3 ml of medium containing a 1:25 dilution of appropriate fluoresceinated antiimmunoglobulin, and incubated on ice for 30 min. To control for nonspecific fluorescence, cells also were incubated with normal mouse serum or normal rat serum as appropriate, followed by incubation with the specific fluoresceinated antiimmunoglobulin reagent. After a final centrifugation of the cells through a 0.5 ml FCS cushion, 1 ml of medium was added per tube. Before analysis by flow cytometry, the cells were resuspended and pipetted through Nitex nylon cloth to remove clumps.

Flow Cytometric Analysis. A multiparameter cell sorter (EPICS V; Coulter Electronics Inc., Hialeah, FL) was used to analyze forward or low angle light scatter and green fluorescence from fluorescein isothiocyanate-conjugated antibodies bound per cell. Green fluorescent light excited by an argon laser (164-05; Spectra-Physics, Inc., Mountain View, CA) tuned to 488 nm was collected using 515-nm long-pass and 530-nm short-pass filters. For single parameter analysis, light scatter or fluorescence intensity was divided into 256 channels; for dual parameter analysis, the division was 64 channels. Fluorescence intensity was determined by both a three decade logarithmic amplifier and a linear amplifier. Linear amplification is useful in analyzing the fluorescence of cell populations that show broad or skewed distribution patterns such as Lyt-1 or gp70, while logarithmic amplification provides a powerful means of resolving discrete populations of cells that differ markedly in fluorescence intensity, especially positive and negative populations. Daily calibration was performed using both 10- μ m fluorescent microspheres (Coulter Electronics, Inc.) and immunofluorescent staining of control thymocytes with anti-Thy-1 antibody. For linear amplification, the major peak fluorescence intensity of Thy-1 was set at channel 100 at a gain of 2. Fluorescence reactions of all other antibodies were then measured relative to Thy-1: Lyt-1.2, gain of 20; Lyt-2.1, gain of 5; L3T4a, gain of 20; B2A2, gain of 20; H-2K^k, gain of 20; gp70, gain of 20. For light scatter analysis, the peak scatter of the predominant thymocyte subpopulation was set at channel 100 by adjusting the laser power. Under these conditions, dead cells and erythrocytes that scatter light less intensely are scored in channels <80 (32) and were excluded from analysis of fluorescence by appropriate gating techniques (33). The results of the analysis of 10,000 viable cells are presented as 64 \times 64 channel isocontour displays of fluorescence intensity per cell (*y* axis) as a function of forward angle light scatter (*x* axis). The contour intervals, shown by three

intensities of shading, were chosen arbitrarily to indicate the peak areas of the corresponding three-dimensional histograms. Depending on antibody used, the contour intervals represent 2, 10, and 20–80 cells per channel. As a result of such data reduction, it was then possible using available software to integrate different areas of a contour plot and to determine the percent of total cells per area as well as to determine modal fluorescence and light scatter coordinates.

Transplantation Bioassay. Since AKR/J and AKR/Cu mice express different alleles of Thy-1 antigen on thymocytes, an assay for transformed phenotype of MCF virus-infected AKR/J thymocytes was performed by transplanting 5×10^6 cells intravenously or intraperitoneally into 2–4-mo-old AKR/Cu recipients. Resultant leukemias were typed as donor AKR/J or recipient AKR/Cu by membrane immunofluorescence assays of Thy-1.1 or Thy-1.2, respectively (34). The incidence of spontaneous leukemia in a population of 112 AKR/Cu mice was determined as a control for these studies. The median latent period of leukemia development (303 d of age) was significantly longer than that observed for AKR/J mice (232 d of age). In some experiments, virus-infected AKR/J thymocytes were transplanted into AKR/J mice by intravenous, intraperitoneal, or intramuscular routes. In these instances, donor and recipient leukemias could not be established unequivocally but successful transplantation was assumed if multicentric leukemia resulted in a latent period of ~30 d or less or if a large tumor mass grew in thigh muscle at the site of injection.

Results

Kinetics of Virus-accelerated Leukemia. Fig. 1 shows that leukemia development occurs rather synchronously in a population of AKR mice injected intrathymically with a cloned isolate of MCF virus, in contrast to that observed for spontaneous development of leukemia in this strain of mice. After injection of MCF 69L1 virus, mice showing signs of frank leukemia (ruffled fur, hunched appearance with chest enlargement, labored breathing, lymph node enlargement) were observed until ~60 d postinjection. Incidence of disease then rose sharply to >90% within 100 d postinjection (median latent period, 76 d). Thus, a study of the time course of changes in thymus of virus-injected mice during the preleukemic period can be considered a feasible undertaking.

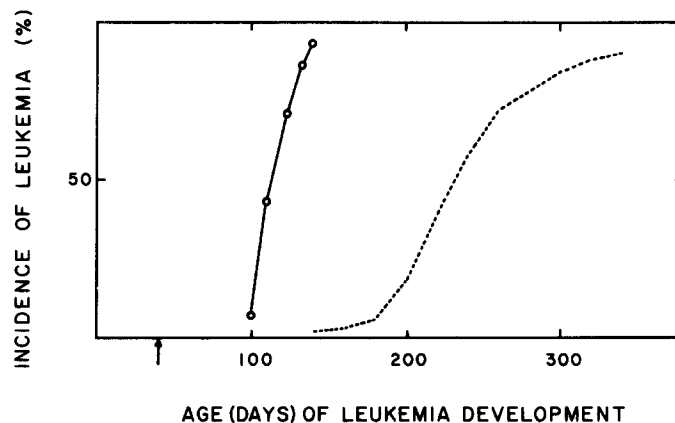


FIGURE 1. Development of virus-accelerated leukemia in AKR/J mice. Cumulative incidence of frank thymic lymphoma in female AKR/J mice injected intrathymically at 40–45 d of age (arrow) with 5×10^4 infectious units of MCF 69L1 virus (O) or with tissue culture medium as control (---). Incidence of spontaneous leukemia in control and unmanipulated mice was identical (13).

Stages in Development of Virus-accelerated Leukemia. Thymocyte subpopulations were characterized by established surface markers at different time intervals from the time of virus injection to the appearance of frankly leukemic mice. Immunofluorescence reactions of thymocytes with monoclonal antibodies directed against differentiation alloantigens (Thy-1, Lyt-1, Lyt-2, L3T4a, B2A2, H-2K), viral gp70, or with fluoresceinated PNA were quantitated by flow microfluorometry. Correlated two-parameter analysis of forward angle light scatter and fluorescence intensity was determined for each surface marker (Figs. 2-5).

Control Mice. The identity of thymocyte subpopulations defined by flow cytometric analysis has been well-described (reviewed in 18). Briefly, four subpopulations were resolved by Thy-1 staining (Fig. 2). Population 1, with intermediate Thy-1 fluorescence and modal scatter of 25, represents small, cortisone-sensitive, cortical thymocytes; population 2, with the lowest Thy-1 fluorescence and modal scatter of 28, represents small, cortisone-resistant, medullary thymocytes which are also immunocompetent; population 3, with high Thy-1 fluorescence and light scatter of 32-44, represents medium-sized thymic lymphoblasts; population 4 is a mixture (~12:1), consisting of population 1 doublets and a second population of cells with the highest Thy-1 fluorescence and light scatter of 40-59, which represents large thymic lymphoblasts. The composition of population 4 was determined by sorting cells onto slides and quantitating doublets and large cells. Doublet formation is an artifact of the indirect immunofluorescence procedure used in these studies, which results from cross-linking of antigens present in very high density, like Thy-1. Thymic lymphoblasts were characterized as cycling cells by acridine orange staining and by Thy-1 fluorescence after separation from total thymocytes by centrifugal elutriation (F. Traganos and P. O'Donnell, unpublished results).

Fig. 2 also shows the expected patterns of expression of Lyt-1, Lyt-2, and H-2K antigens on control cells, based on published data (18). Lyt-1 fluorescence varied from predominantly dull to very bright. Because of the light scatter overlap of small cortical and medullary cells, it was difficult to resolve the staining patterns of individual populations. However, analysis of cortisone-resistant cells (data not shown) revealed that the fluorescence intensity of medullary cells was much higher than that of cortisone-sensitive cortical cells as reported by Scollay and Shortman (18). Thus, the phenotype of small cortical thymocytes was Lyt-1 low, Lyt-2 high, and H-2K low. Medullary cells were Lyt-1 high, H-2K high but were also resolved into two populations of Lyt-2⁻ and Lyt-2⁺ cells that were present in a ratio of ~2:1, respectively. The fluorescence intensity of thymic lymphoblasts (light scatter, >32) appeared to be relatively low for Lyt-1 and H-2K and high for Lyt-2.

Fig. 3, panel 1 shows the pattern of expression of L3T4a antigen on control AKR thymocytes. L3T4a was defined recently by monoclonal antibody GK-1.5 and appears to be an antigenic determinant of the murine homologue of the human Leu 3/T4 molecule on T cells that is involved in class II major histocompatibility complex reactivity (29, 35). Small cortical thymocytes express a high level of L3T4a while medullary thymocytes are divided into L3T4a⁺ and L3T4a⁻ subpopulations (35).

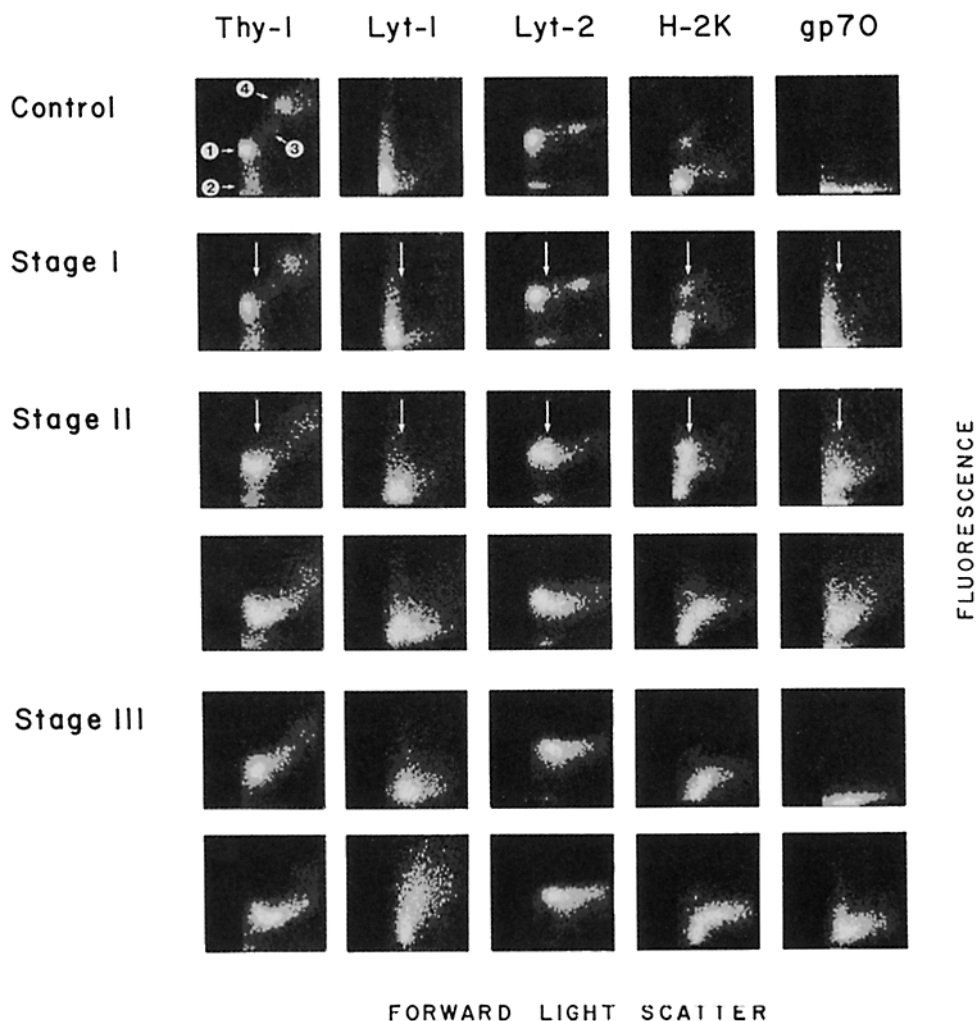
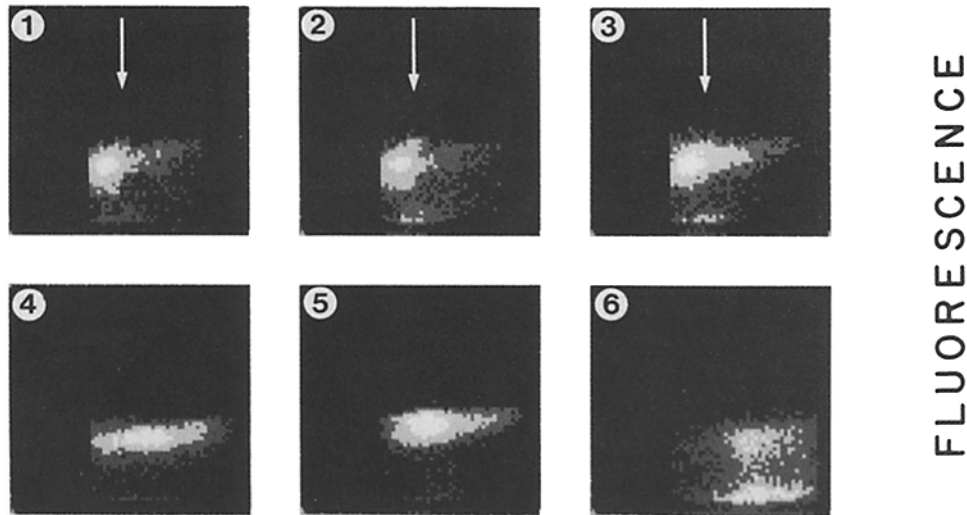


FIGURE 2. Fluorescence distribution patterns of preleukemic AKR/J thymocytes stained with monoclonal antibodies to differentiation alloantigens or to MuLV gp70. Mice (40–45 d of age) were injected intrathymically with MCF 69L1 virus or tissue culture medium as control. Mice were thymectomized at different time intervals postinjection and assayed for expression of alloantigens Thy-1, Lyt-1, Lyt-2, H-2K, or MuLV gp70 by immunofluorescence assay of viable cells. Membrane fluorescence was quantitated by flow cytometry as described in Materials and Methods: Thy-1, linear amplification at a gain of 2; Lyt-1, linear amplification at a gain of 20; Lyt-2, logarithmic amplification; H-2K, logarithmic amplification; gp70, linear amplification at a gain of 20. Thymus weights of individual mice: control, 122 mg; stage I, 120 mg; stage II (a) 124 mg, (b) 189 mg; stage III (a) 309 mg, (b) 397 mg. Four major subpopulations of thymocytes were resolved by Thy-1 staining as indicated in the contour map of control mice. Arrows indicate the modal light scatter of the novel cell population observed at stage II.

Stage I. We have shown previously that steady-state infection of AKR thymocytes is reached by 28 d after injection of $\sim 5 \times 10^4$ infectious units of MCF 69L1 virus (13, 14). At this time postinjection, >60% of thymocytes were infected by MCF virus and expressed levels of MuLV gp70 on their cell surface above



FORWARD LIGHT SCATTER

FIGURE 3. Fluorescence distribution patterns of preleukemic and leukemic AKR/J thymocytes stained with monoclonal antibody to L3T4a antigen. Control thymocytes (panel 1) or thymocytes of MCF 69L1-injected mice at stage I (panel 2), stage II (panel 3), and stage III (panel 4) of leukemogenesis were analyzed at 69 d postinjection. Staging was confirmed by parallel analysis of Thy-1, B2A2, and gp70 expression (data not shown). Primary leukemias were analyzed at 106 d (panel 5) and 103 d (panel 6) postinjection. Logarithmic amplification of fluorescence intensity was used. The pattern of L3T4a expression in panel 6 in which 50% of the cells were L3T4a⁻ was observed in 1/9 leukemias examined.

those of control mice injected with tissue culture medium (Fig. 2). Increased gp70 expression is encoded specifically by MCF virus and not other endogenous MuLV (14). This observation was confirmed in the present studies by use of an MCF-specific gp70 monoclonal antibody 514 (31) which gave results identical to those shown in Fig. 2 with antibody to the gp70^f epitope. It can be seen that the gp70 fluorescence intensity is skewed from dull to very bright, possibly a gene dosage effect of the infection of thymocytes in the population at varying multiplicities. Also, at this stage of leukemogenesis, the frequency of thymocytes producing MCF viruses as measured by infectious center assay on S⁺L⁻ mink cells is maximum (0.1–0.3%) and constant for the duration of the preleukemic period. The expression of high levels of MCF viral gene products intracellularly and on the cell surface but low levels of mature, infectious virus is characteristic of virus-infected thymocytes and leukemia cells (14, 36).

We attempted to determine the relative contribution of small cortical and medullary thymocytes to the observed gp70 fluorescence and to MCF and ecotropic virus infectious centers measured in total thymocyte suspensions. Mice injected with MCF 69L1 virus were administered cortisone at 42 d postinjection and analyzed for gp70 expression or infectious centers 72 h later. As shown in Table I, cortisone-resistant (medullary) thymocytes express low levels of MCF

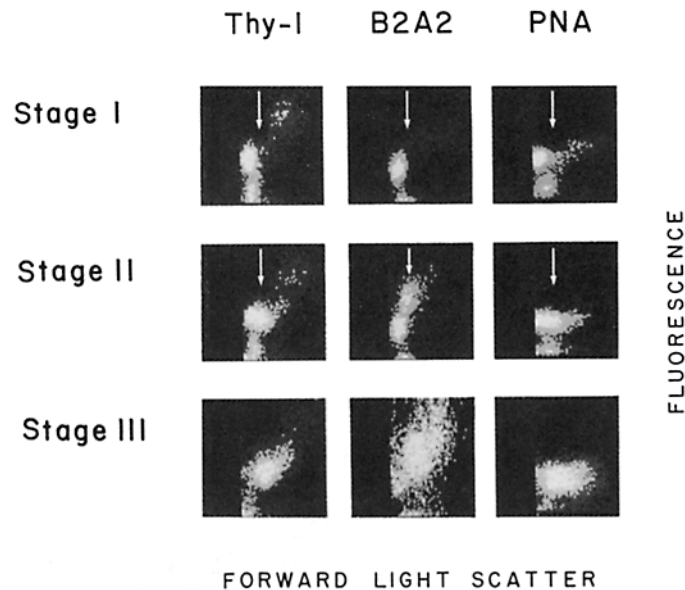


FIGURE 4. Fluorescence distribution patterns of preleukemic AKR/J thymocytes stained with Thy-1 or B2A2 monoclonal antibodies or with fluoresceinated PNA. Thymocytes of MCF 69L1-injected mice were analyzed at 69 d postinjection. Cells were characterized as stage I, II, or III on the basis of Thy-1 staining as shown in Fig. 2 and of gp70 staining (data not shown). Thymus weights: stage I, 80 mg; stage II, 225 mg; stage III, 411 mg. Linear amplification: Thy-1 at gain of 2, B2A2 at gain of 20. Logarithmic amplification: PNA. By logarithmic amplification the fluorescence intensity of medullary thymocytes compared with normal rat serum controls was identical for B2A2 but significantly higher for PNA.

virus as judged by a threefold decrease in fluorescence intensity almost to the level of endogenous MuLV gp70 expression observed for controls. Also, we observed a marked reduction (up to 200-fold) in the frequency of MCF 69L1 infectious centers. The infectious centers observed in cortisone-resistant cells may be due, in fact, to residual contaminating cortical thymocytes because, in separate experiments, we observed that at least 80% of total MCF infectious centers were recovered in the major cortical thymocyte subpopulation that was sorted on the basis of Thy-1 fluorescence (cf., Fig. 2). This result with sorted cells also indicated that the low frequency of MCF 69L1 infectious centers observed in the bulk thymocyte population was characteristic of lymphoid cells and not a minority cell population in the thymus such as stromal cells.

As shown in Figs. 2 and 3, no differences were detected in the patterns of expression of alloantigens between control and MCF virus-infected thymocytes at stage I of leukemogenesis. Fig. 4 shows the pattern of expression of PNA receptors on thymocytes at stage I, which was identical to that of control mice (data not shown). As described previously by others (18, 37, 38), small cortical thymocytes and thymic lymphoblasts express high levels of receptor while medullary cells express low but significant levels. The relative frequency of virus-injected mice whose thymocytes displayed stage I phenotype was maximum at 40 d and declined thereafter (Fig. 6).

Stage II. As early as 42 d after injection of MCF 69L1 virus, occurring

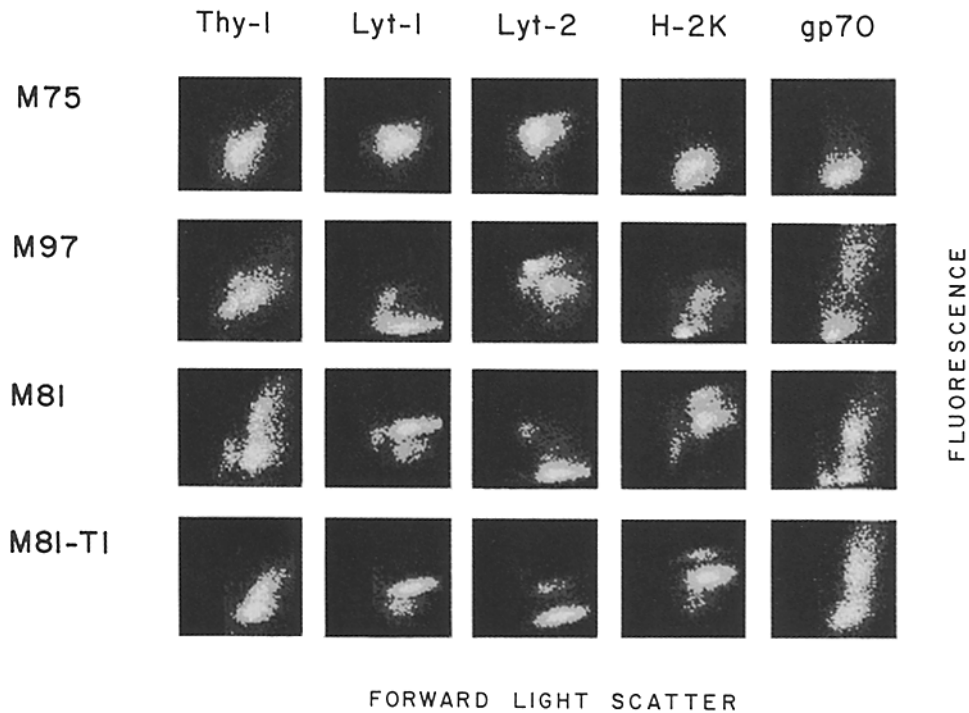


FIGURE 5. Fluorescence distribution patterns of MCF 69L1 virus-accelerated AKR/J leukemias stained with monoclonal antibodies to differentiation alloantigens or to MuLV gp70. Primary AKR/J thymomas M75, M97, M81 were observed at 77 d postinjection of MCF 69L1 virus and analyzed as described in Fig. 2 legend and in Materials and Methods. Thymoma weights: M75, 1128 mg; M97, 692 mg; M81, 650 mg. M81-T1 was a primary transplant to an AKR/Cu recipient and was derived from a metastatic deposit in the mesenteric lymph node at 21 d after intraperitoneal inoculation.

maximally at 50–60 d postinjection (Fig. 6), a novel population of cells could be identified in the presence of normal thymocyte subpopulations on the basis of its characteristic light scatter and its unique pattern of staining with antibodies to alloantigens and MuLV gp70. Representative examples are shown in Figs. 2 and 3. As indicated by the arrows, the new cell population was distinguished by (a) light scatter that was higher than that of the predominant population of small cortical thymocytes, (b) high gp70 fluorescence, (c) Thy-1 fluorescence comparable to or slightly less than that of cortical thymocytes, and (d) H-2K fluorescence that was intermediate between cortical and medullary thymocytes. Lyt-1, Lyt-2, and L3T4a fluorescence was comparable to that of small cortical thymocytes. Fig. 4 shows that the PNA fluorescence of stage II cells also was comparable to that of small cortical thymocytes.

In Fig. 2, the thymus weight of one of the stage II mice was comparable to that of controls while the other was slightly enlarged. It is clear that the percent of the stage II cell population is increased in the mouse with the enlarged thymus but in neither case is the population well-resolved from other subpopulations by the monoclonal antibodies used. More recently, we obtained a monoclonal antibody B2A2 isolated by Bartlett et al. (22) which recognizes antigen present

TABLE I
Effect of Cortisone Treatment on Expression of gp70 and Mature Infectious Ecotropic Virus or MCF Virus by AKR/J Thymocytes

Injection	MuLV gp70*		Infectious centers [‡] per 10 ⁶ cells		Percent residual cortical thymocytes [§]
	Percent positive	MFLR	ECO	MCF 69L1	
Control + saline	47	15	1,187	<0.02	
	52	14	437	<0.02	
Control + cortisone	40	14	232	<0.02	7
	36	13	1,050	<0.02	11
69L1 + saline	82	79	800	1,025	
	79	108			
	78	102			
	80	103			
	80	115			
69L1 + cortisone	53	21	345	15	13
	55	23	800	5	5
	54	39			<5
	59	40			12
	42	19			<5
	61	25			12
	62	52			13

Mice injected intrathymically with MCF 69L1 virus or tissue culture medium (control) were administered cortisone acetate (125 mg per kg body weight) or saline intraperitoneally at 42 d postinjection. Such cortisone treatment resulted in a maximum fivefold decrease in thymus weight within 72 h, the time chosen for immunofluorescence assay and infectious center assay of virus-infected thymocytes.

* Immunofluorescent assay of gp70^f epitope expression. Data are given as median fluorescence intensity (MFLR) of cells with fluorescence greater than normal rat serum controls determined by subtraction of single-parameter fluorescence histograms (fluorescence intensity range, 0–255).

[‡] Ecotropic MuLV infectious centers are given as XC plaque-forming units. MCF 69L1 infectious centers are given as S⁺L⁻ mink focus-forming units.

[§] Residual cortical thymocytes not lysed by cortisone treatment were quantitated by integrating the appropriate area of the two-parameter Thy-1 contour map.

almost exclusively on the small cortical thymocyte subpopulation. Fig. 4 shows the characteristic staining pattern of B2A2 antibody compared with Thy-1 antibody for virus-infected stage I thymocytes. This pattern was identical to control thymocytes (data not shown). Cortical thymocytes stained positively while medullary thymocytes were negative or very low. Fig. 4 also shows that B2A2 staining of thymocytes resolved the novel stage II cell population from cortical thymocytes unambiguously. The modal light scatter of this new cell population was 30 compared with 25 for cortical thymocytes and 28 for medullary thymocytes. Stage II cells represented 41% of the total thymocyte population in this particular mouse.

It appears that the novel population of cells observed at what we have defined as stage II has a distinct phenotype because 43 virus-injected mice showed the same characteristic patterns of forward light scatter and fluorescent staining as shown in Figs. 2–4. Stage II also was observed in AKR mice injected with MCF 247 and MCF 13 viruses and in C3Hf/Bi mice injected with AKR SL3-2 virus (39), indicating that this is not a phenotype unique to MCF 69L1 virus or to

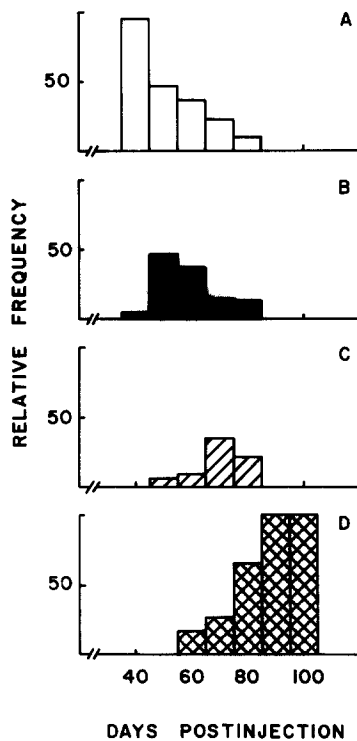


FIGURE 6. Relative frequency of stages of leukemogenesis as a function of time after injection of MCF virus. AKR/J mice (40–45 d old) were injected intrathymically with MCF 69L1 virus. Mice were sacrificed at appropriate intervals of time postinjection and characterized as stage I (A), II (B), or III (C) of leukemogenesis during the preleukemic period or as primary leukemias (D) as described in the text. Data represents a total sample of 137 mice (stage I, $n = 51$; stage II, $n = 33$; stage III, $n = 18$; primary leukemia, $n = 35$).

AKR mice. By integrating regions of the contour plots of different antigens that correspond to the new population of cells we estimated that stage II thymocytes represent 15–45% of total cells depending on thymus weight. Stage II changes also were observed to occur asymmetrically within a single thymus. In several mice each of whose thymus lobes were dissected and analyzed separately, there was evidence of stage I in one lobe and stage II in the other or stage II in one lobe and stage III in the other. The most advanced stage was observed invariably in the lobe that received the initial injection of virus.

Stage III. Figs. 2–4 show representative dual parameter analyses of thymocytes from four virus-injected mice in which considerable enlargement of the thymus had occurred (thymus weight, >300 mg). Mice at this stage of leukemogenesis did not display signs of frank leukemia, i.e., ruffled fur, hunched appearance with chest enlargement, labored breathing, or lymph node enlargement. At stage III, normal thymocyte subpopulations were replaced by a more homogeneous population defined by light scatter and fluorescence. Stage III cells showed increased light scatter compared with stage II cells. The light scatter of stage III cells from individual mice ($n = 18$) varied from 30 to 36 and in one mouse two cell populations that differed by light scatter were observed in the

same thymus (Fig. 2, *IIIb*). In general, the fluorescence intensities of thymocyte makers Thy-1, Lyt-1, Lyt-2, L3T4a, B2A2, H-2K, and PNA observed for stage III cells were those predicted on the basis of outgrowth of cells with stage II phenotype. However, gp70 fluorescence was lower and more variable than that observed on stage II cells. The frequency of mice at stage III of leukemogenesis was maximum at 70 d postinjection (Fig. 6). Stage III cells appear, therefore, to represent outgrowth of transformed cells in thymus.

Primary (Frank) Leukemias. We analyzed 22 primary leukemias induced by MCF 69L1 virus and 5 primary leukemias induced by MCF 13 virus that were observed at 62–99 d postinjection. The data confirmed an earlier report that the Lyt phenotype of spontaneous AKR leukemias is that of immature thymocytes, i.e., Lyt-1⁺2⁺ (21). This conclusion was strengthened by the finding that primary leukemias were also L3T4a⁺ (Fig. 3) and PNA⁺ (data not shown), two markers that are also found expressed at high levels on a subpopulation of thymic lymphoblasts and small cortical thymocytes (18, 29, 35). However, the resolving power of two-parameter flow cytometry revealed considerable heterogeneity among primary leukemias with respect to cell size and quantitative expression of antigens, which has not been reported previously. Light scatter of individual leukemias varied from 28 to 44 with a median of 35. A striking observation was that two to three distinct populations of leukemia cells were present in 13 of 22 leukemias induced by MCF 69L1 virus and in 1 of 5 leukemias induced by MCF 13 virus. Fig. 5 shows dual parameter analysis of three leukemias induced by MCF 69L1 virus that contained one, two, or three discrete subpopulations, respectively. In the MCF 13 leukemia and in 10 of 13 MCF 69L1 leukemias, multiple tumor cell populations were distinguished by both light scatter and Lyt-2 fluorescence intensity. Other alloantigens or gp70 also resolved distinct leukemic cell populations as illustrated by tumors M97 and M81 of Fig. 5 and by the tumor shown in Fig. 3, panel 6 but less frequently than did Lyt-2. The relative proportions of leukemia cell subpopulations also differed among the tumors analyzed. Four leukemias containing multiple cell populations were transplanted into AKR/Cu mice and the resultant leukemias were subjected to dual parameter analysis to assay for the persistence of subpopulations. In each instance, multiple cell populations were resolved among transplanted leukemia cells in the same relative proportions as in the inocula. Fig. 5 shows the analysis of one of these leukemias, M81, containing three distinct subpopulations, and the analysis of cells derived from the first transplant M81-T1. The two original cell populations with light scatter of 40 and 41 and characteristic staining patterns were resolved clearly in the transplanted leukemia. The third subpopulation comprising ~10% of cells in the primary leukemia did not transplant. It is possible that these cells may have represented residual normal thymocytes in the M81 tumor.

It has been reported that MCF virus-infected thymocytes and leukemia cells express increased levels of Ia antigens compared with young AKR mice (40). Accordingly, we assayed preleukemic thymocytes or leukemia cells from a total of 39 injected AKR/J mice with monoclonal antibody to I-A^k but were unable to confirm these data. Using logarithmic amplification, we observed that 43 ± 8% of control AKR thymocytes from medium-injected mice (3–4 mo old) showed Ia fluorescence just discernible above the background control. This level of

fluorescence was 60–100-fold lower than that of Thy-1. This same low fluorescent staining with Ia antibody also was observed for virus-infected thymocytes at stages I, II, and III as well as for primary leukemia cells and we conclude that it is not highly significant. Similarly, we were unable to detect any significant expression of TL antigen on 15 leukemias using monoclonal antibody to the TL.m4 epitope whose pattern of reactivity conforms to TL.2 by conventional antisera (26). However, the reported frequency of anomalous TL expression on spontaneous AKR leukemias is ~11% (41), the limit of detection in our sample.

Tissue Weights. Changes in the masses of thymus, spleen, and liver correlated with the stage of leukemogenesis defined by flow cytometry. A sample of 65 age-matched female AKR/J mice were injected with MCF 69L1 at 40 d of age. Leukemogenesis was staged at weekly intervals as described above between 35 and 123 d of age. There was no age-dependent change in thymus or spleen weight of control mice within the interval studied but liver weight increased linearly with age (1,600–2,400 mg). Thymus of stage I mice (106 ± 22 mg, $n = 25$) showed a slight but significantly lower weight than medium-injected control mice (135 ± 17 , $n = 8$, $P < 0.002$). This difference was confirmed by comparing thymus weights from a much larger sample obtained by pooling data from several experiments. In this instance thymus weight of stage I mice (101 ± 23 mg, $n = 36$) was significantly different from control mice (116 ± 26 , $n = 22$, $P < 0.05$). Thus, an early consequence of virus infection is a depressive effect on the mass of thymus tissue.

Stage II (168 ± 69 mg), stage III (424 ± 168 mg), and leukemic (930 ± 365 mg) thymuses showed a progressive increase in mean thymus weight consistent with emerging tumor growth but there was considerable variation at each of these stages as indicated by the large standard deviations. In the case of stage II mice, there was considerable overlap with stage I thymus weights, an observation demonstrating that it was possible to observe outgrowth of a new population of thymocytes in the absence of gross changes in thymus weight.

No significant differences were observed between spleen weights of control (81 ± 7 mg, $n = 8$), stage I (89 ± 13 mg, $n = 25$), or stage II mice (91 ± 12 mg, $n = 12$). However, spleen weights of 5 of 9 stage III mice and 10 of 11 leukemic mice exceeded 115 mg, the upper limit of normal spleen weight within the time interval studied, indicative of metastatic spread of tumor cells to this tissue. Metastatic spread to the liver indicated by a tissue weight $>2,400$ mg was less pronounced but was observed in 4 of 9 stage III mice and 6 of 11 leukemic mice.

Transformed Phenotype of Thymocytes at Different Stages of Leukemogenesis. Transplantation bioassays of cells at the various preleukemic stages defined by flow cytometric analysis or of primary leukemia cells were performed in AKR/Cu mice or AKR/J mice as recipients (Table II). AKR/Cu mice express the Thy-1.2 allele on thymocytes and thus allow donor AKR/J (Thy-1.1) cells and recipient cells to be distinguished (34). Stage I cells from 11 donors were transplanted to 37 AKR/Cu recipients. Leukemias with a mean latent period of 110 d were observed in 70% of recipients (over one leukemia per donor) but were Thy-1.2 (recipient) in origin, indicative of MCF virus transfer rather than transfer of virus-infected AKR/J cells that were “committed” to transformation. The latent

TABLE II
Transplantation Bioassay of MCF 69L1-infected AKR/J Thymocytes and Primary Leukemia Cells

Donor stage of leukemogenesis	Recipient							
	AKR/Cu*				AKR/J			
	No. donors	No. recipients	Percent leukemias	Latent period (days)	No. donors	No. recipients	Percent leukemias	Latent period (days)
I	11	37	70	110 ± 12	ND			
II	6	15	60	104 ± 9	8	20	85	24 ± 8
III	2	4	100	23 ± 4	1	2	100	27
°L	10	13	85	15 ± 9	12	18	100	17 ± 10

In separate experiments, 5×10^6 cells were injected either intravenously or intraperitoneally into 2–4-mo-old AKR/Cu or AKR/J recipient mice.

* Intrathymic injection of AKR/Cu mice (45–65-d-old) induced leukemia with a mean latent period of 87 d.

period observed was consistent with a mean latent period of 87 d for MCF 69L1 virus-accelerated leukemia in AKR/Cu mice injected intrathymically at 40–65 d of age. Similarly, stage II cells from six separate donors were transplanted into 15 AKR/Cu recipients. Leukemias were observed with a mean latent period of 104 d in 60% of recipients (one or more leukemia per donor), also of recipient Thy-1 type. Thus, stage II cells failed to transplant but rather transferred virus passively to AKR/Cu mice. In contrast, stage III cells (two donors) or primary leukemia cells (8 of 10 donors) transplanted in AKR/Cu mice with mean latent periods of 23 and 15 d, respectively. By transplantation bioassay in AKR/Cu mice, therefore, it appeared that transformed cells were first apparent at stage III. However, in separate experiments, transplantation to AKR/J recipients indicated that transformed cells could be detected as early as stage II of leukemogenesis. Although donor and recipient could not be distinguished in this instance, cells from 7 of 8 stage II donors injected intravenously or intraperitoneally resulted in multicentric leukemias with a mean latent period of 24 d, indicative of leukemic cell transfer. This conclusion was strengthened by the observation that cells of 3 of 7 of the same stage II donors also developed into a tumor in situ after intramuscular injection. Stage III cells and primary leukemic cells also transplanted in AKR/J mice by all three routes of injection.

It appears from the data of Table II that the limited transplantation phenotype of AKR/J stage II cells in AKR/Cu recipients might be Thy-1 related. If this were true we would expect that in the reciprocal experiment, stage II cells from AKR/Cu donors would fail to transplant into AKR/J recipients. The data in Table III supports this hypothesis. Transplantation of stage II cells from six separate AKR/Cu donors resulted in leukemia development in 39% AKR/J recipients, with a mean latent period of 88 d. These leukemias were recipient Thy-1 type, indicative of the passive transfer of virus. In contrast, leukemias were observed in 100% of AKR/Cu recipients injected with the same leukemia cells, with a mean latent period of 36 d. In accord with the data of Table II,

TABLE III
Transplantation Bioassay of MCF 69L1-infected AKR/Cu Thymocytes

Donor stage	No. donors	Recipient					
		AKR/J			AKR/Cu		
		No. recipients	Percent leukemias	Latent period (days)	No. recipients	Percent leukemias	Latent period (days)
II	6	18	39	88 ± 6	18	100	36 ± 6
III	1	3	100	40	3	100	28 ± 8

In the same experiment, $1-5 \times 10^6$ cells were injected intraperitoneally into 2-mo-old AKR/J or AKR/Cu recipient mice.

TABLE IV
Transplantation Bioassay of MCF 69L1-infected AKR/J Thymocytes and Primary Leukemia Cells in Irradiated and Unirradiated Recipients

Donor stage	No. donors	Recipients								
		AKR/Cu						AKR/J		
		Irradiated*			Unirradiated			Unirradiated		
		No. recipients	Percent leukemia	Latent period (days)	No. recipients	Percent leukemia	Latent period (days)	No. recipients	Percent leukemia	Latent period (days)
II	2	6	100	44 ± 11	6	100	99 ± 14	8	100	39 ± 8
^o L	2	6	100	18 ± 4	6	100	18 ± 5	8	100	18 ± 4

In the same experiment, $1-5 \times 10^6$ cells were injected intraperitoneally into 43-49-d-old AKR/Cu or AKR/J recipient mice.

* 400 rad administered 1 h before injection.

stage III cells from an AKR/Cu donor transplanted into both AKR/Cu and AKR/J recipients, with mean latent periods of 28 and 40 d, respectively.

We then tested the ability of stage II cells from AKR/J donors to transplant into irradiated AKR/Cu recipient mice. Data of Table IV shows that the transplantation resistance of AKR/Cu recipients was abrogated by a sublethal dose of irradiation administered 1 h before injection. Leukemias that developed in irradiated AKR/Cu recipients were of donor Thy-1 type and had a mean latent period of 44 d, comparable to that observed in AKR/J recipients injected with the same cells. In contrast, leukemias that developed in unirradiated AKR/Cu recipients were of recipient Thy-1 type and had a mean latent period of 99 d.

Differences in transplantation between AKR/J and AKR/Cu mice indicate that stage II cells display transformed properties that can be distinguished from stage III or primary leukemia cells, indicative, perhaps, of progressive changes in transformed phenotype during development of frank disease. The data suggest further that the transplantation resistance of stage II cells may involve an immune response to Thy-1 or Thy-1-associated antigens on the cell surface (42, 43).

Discussion

In these studies, development of leukemia in young AKR/J mice injected intrathymically with a cloned isolate of MCF virus was relatively synchronous with a latent period of frank disease ranging from 60 to 100 d postinjection.

During this time interval (40–140 d of age), there were no age-dependent changes in thymus of control mice observable by flow cytometric analysis of T lymphocyte markers on thymocyte subpopulations or by histologic examination (unpublished data).

Three stages of leukemogenesis were defined on the basis of flow cytometric analysis and transplantation bioassay of infected thymocytes during the preleukemic period before development of frank leukemia. By examining a population of 137 virus-injected mice, we were able to show that each stage occurred sequentially at ~10-d intervals from 40 d postinjection onward.

Stage I was characterized by steady-state infection of the major thymocyte subpopulation by MCF virus as described previously (13, 14). A careful analysis of MCF virus infection in the minority lymphoblast populations has not been carried out and will require fractionation procedures to enrich for these cells. However, studies of RadLV infection in thymus of C57BL/Ka mice (44, 45) indicated that lymphoblast cells were the first site of virus infection in that system. Infection then spreads throughout the thymus and in MCF 69L1-injected AKR/J mice was complete by ~28 d postinjection. Stage I persisted maximally until ~40 d postinjection. During this time, no changes were observed in expression of alloantigens Thy-1, Lyl-1, Lyl-2, L3T4a, B2A2, or H-2K on the major thymocyte subpopulations compared with controls. The expression of PNA receptors on thymocytes also was identical to controls. Expression of MCF virus appeared to be confined predominantly to the small, cortisone-sensitive thymocyte population that comprised 80% of total thymocytes, confirming Cloyd's recent studies (15). Thus, MCF virus expression was highest in the population of cells that appears to be destined for intrathymic cell death (18, 46).

Stage II was characterized by the emergence of a novel population of cells with a modal light scatter of 30 that could be resolved from normal thymocyte subpopulations by fluorescent staining with monoclonal antibodies against H-2K and gp70 and, in particular, by a monoclonal antibody B2A2 that is relatively specific for the population of small cortical thymocytes (22). The relative proportion of stage II cells varied from 10 to 15%, in AKR/J mice with thymus weight comparable to controls, to 45% in mice with obvious thymus enlargement (up to twice normal weight). Although stage II cells were not fractionated from the bulk thymocyte population, transplantation bioassays in AKR/J mice indicated the presence of cells with transformed phenotype. However, cells at this stage did not transplant to AKR/Cu mice, unlike cells at stage III or primary leukemia cells, possibly an important marker of stage II cells. Conversely, stage II cells from MCF 69L1-injected AKR/Cu mice transplanted to AKR/Cu mice but not to AKR/J mice. These findings suggest a possible role of Thy-1 in the transplantation resistance to stage II cells (42, 43). The involvement of an immune response in such resistance was indicated by the finding that the transplantation resistance could be abrogated by a sublethal dose of x irradiation.

Recently (47), we assessed the clonality of cells at stage II of leukemogenesis compared with stage I by Southern blotting using an ecotropic MuLV-specific p15(E) probe pAkv5, which recognizes all pathogenic MCF viruses but not endogenous xenotropic sequences (47). No newly integrated proviruses in excess of germline ecotropic proviruses were observed in stage I thymocytes, consistent

with the random integration in that population of infected cells. However, newly integrated proviruses with stoichiometry less than germline proviruses were observed in all stage II DNAs analyzed, consistent with clonal expansion of a subpopulation of cells (E. Fleissner and P. O'Donnell, unpublished data).

Stage III appears to represent clonal outgrowth of transformed cells with concomitant disruption of thymocyte homeostasis since the normal thymocyte subpopulations were no longer evident. There is considerable thymus enlargement at this stage but no overt signs of leukemia. Analysis of 27 primary leukemias revealed heterogeneity in cell size and quantitative expression of alloantigens that was not observed at earlier stages. The phenotype of each leukemia determined by dual parameter analysis appeared to be almost unique. Qualitatively, leukemia cells expressed all of the major thymocyte markers, Thy-1, Lyt-1, Lyt-2, H-2K, as well as the recently described markers L3T4a and B2A2. Using logarithmic amplification, no significant expression of Ia or TL antigens was detected above fluorescence of normal serum controls. All leukemias bound fluoresceinated PNA as did the novel population of stage II cells and stage III cells. These data, therefore, differ from previous reports of the phenotype of AKR spontaneous leukemias (16, 17, 21, 40). In attempting to use these thymocyte markers to identify the target cell for transformation, the phenotype of leukemia cells most closely resembles that of the small, cortisone-sensitive thymocytes (18), the predominant population in the thymus that is nonfunctional and is rapidly turned over *in situ* (46). Since this cell type is terminally differentiated and noncycling (18 and unpublished data), a likely candidate for transformation might be its immediate precursor, a lymphoblast subpopulation.

We were struck by the observation that the majority of virus-accelerated leukemias contained at least two populations of cells that could be resolved in dual parameter analysis using different markers, most commonly Lyt-2. These distinct cell populations were present in varying relative amounts in different leukemias and persisted upon transplantation. It is unclear at present whether multiple populations of leukemia cells in a single tumor represent the outgrowth of distinct clones or if they are related in some way. It will be necessary to separate these populations for analysis by Southern blotting and further transplantation studies to answer this question.

Our study indicates that the rate limiting step in MCF virus-induced leukemogenesis is the establishment of steady-state infection in the thymus, an event that took 30–40 d at the dose of virus administered. Cells with a transformed phenotype were then observed as early as 42 d postinjection by the techniques used. This represents a much shorter latent period for transformation by a nondefective leukemia virus than is generally assumed. Required, virus-induced events of transformation may occur at low frequency, for example, by activating an oncogene (48), but could occur relatively early in the latent period as soon as a sufficiently large pool of infected cells is achieved. The remainder of the latent period until development of frank disease might require additional mutational events at the cellular level, such as trisomy 15 (49), to achieve a state of autonomous growth. The data we have presented are consistent with such a notion of tumor progression in leukemogenesis. Stage II cells appear to represent

a clonal, proliferating cell population with light scatter and antigen phenotype distinct from normal thymocyte subpopulations and from transformed cells at later stages. Transplantation properties of these cells in AKR/Cu vs. AKR/J mice suggest that stage II cells may not yet express a fully transformed phenotype. At stage III there are further cellular changes, detectable by flow cytometry, and a fully transformed phenotype. Finally, primary leukemia cells show additional variation in cellular phenotype as evidenced by the relative uniqueness of antigen expression quantitatively and the appearance of multiple populations of leukemic blasts. There is also evidence to indicate that the phenotype of primary AKR leukemia cells does not remain static. Primary leukemia cells may undergo further selection upon serial transplantation *in vivo* or in tissue culture, leading to increased tumorigenicity (E. Stockert, personal communication) or the capacity for autonomous growth in the absence of feeder cells *in vitro* (14, 50). Thus, the tumor phenotype of leukemia cells is likely to be a composite of independent changes at the cellular level that must be analyzed separately at different stages for virus transformation to be understood.

Summary

Flow cytometric techniques involving correlated dual parameter analysis of fluorescence and light scatter and transplantation bioassays were used to describe a series of cellular changes in thymus of young (1–4 mo old) AKR mice during development of mink cell focus-inducing (MCF) virus-accelerated leukemia. Three stages of leukemogenesis were defined before appearance of frankly leukemic mice. Stage I, apparent 28–40 d after injection of MCF 69L1 virus, represented steady-state infection of thymocytes by MCF virus without apparent change in light scatter properties of the cells or in expression of alloantigens Thy-1, Lyt-1, Lyt-2, L3T4a, B2A2, or H-2K on the major thymocyte subpopulations. Expression of MCF virus was highest in the population of small cortical thymocytes. Stage II was observed at highest frequency 50–60 d postinjection and represented the emergence of a clonal population of cells with transformed properties which could be resolved from normal thymocytes by light scatter and expression of B2A2, H-2K, and gp70 antigens. Stage III was observed at highest frequency at 70 d postinjection, when considerable enlargement of thymus had occurred, and appeared to represent the outgrowth of fully transformed cells that replaced the normal thymocyte subpopulations. The alloantigen phenotype of blast cells from frankly leukemic mice did not differ qualitatively from that of stage II or stage III cells but displayed considerable heterogeneity with respect to quantitative expression of alloantigens and gp70. At least two populations of leukemic blasts could be resolved in the majority of primary thymomas analyzed. It is unclear whether these populations represent the outgrowth of independent clones of transformed cells or if they are related in some way. Our data are consistent with MCF virus-induced transformation of cells in the lineage to small peanut agglutinin-positive, cortisone-sensitive thymocytes, a subpopulation that predominates in the thymus and which is thought to be destined for cell death *in situ*.

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