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THE Eμ-*myc* TRANSGENIC MOUSE

A Model for High-incidence Spontaneous Lymphoma and Leukemia of Early B Cells

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Translocation of the c-*myc* protooncogene into or near one of the Ig loci is found in almost every case of Burkitt's B cell lymphoma in man and experimentally induced plasma cell tumor in the mouse (reviewed in references 1, 2). This accidental rearrangement of the cellular genome appears to free the c-*myc* gene from its normal susceptibility to negative regulation and to render it constitutively active in cells that express Ig genes (reviewed in references 1–4). The contribution of the rearranged gene to the neoplastic phenotype may stem from the ability of the *myc* polypeptide to drive cellular proliferation, perhaps by promoting DNA replication (5, 6). While its biochemical function is not yet defined, c-*myc* expression normally correlates closely with cell-cycle activity in a broad range of cell types, increasing greatly when resting cells are mitogenically stimulated and declining when they cease to proliferate (3, 4, 7).

The effects of altered oncogenes on cells in their natural environment can be assessed through the use of transgenic mice (8, 9). A transgenic strain is initiated by inserting a gene into the mouse genome and is perpetuated thereafter by normal breeding. By this means, a c-*myc* gene coupled to regulatory sequences from the mouse mammary tumor virus was shown to cause mammary carcinomas, and sometimes other tumors, after a substantial latent period (10, 11). To mimic the translocated *myc* genes found in lymphoid tumors, we introduced into mice a DNA sequence, designated Eμ-*myc*, isolated from a mouse plasmacytoma in which a normal *myc* gene had become coupled to the Ig heavy chain enhancer (12). In the resultant transgenic mice, the Eμ-*myc* transgene appears to be expressed exclusively in B-lymphoid cells (13) and causes them to proliferate more than normal (14). Starting before birth, the pre-B cell population expands progressively up to about five times its normal size, while B cells are somewhat reduced in number despite their increased cell-cycle activity (14). The immune

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responsiveness of Eμ-*myc* animals is not greatly affected by these perturbations (15). Thus, the excessive proliferative activity induced by the Eμ-*myc* gene is limited and benign. However, these mice also develop lymphomas of B-lineage cells (16).

To establish the Eμ-*myc* mouse as a model of lymphomagenesis, we here quantify the incidence, pathology, and cell surface phenotypes of the tumors. Despite some variation in the pathology, all of the tumors have proven to be malignant lymphomas of B-lineage cells, usually accompanied by lymphoid leukemia. We compare them with other mouse lymphomas and with the tumors that characterize the SJL strain, a contributor to the genetic background of the transgenic line. We also describe a characteristic of the blood of young Eμ-*myc* mice that provides a rapid means of distinguishing them from normal mice.

A notable feature of these mice is that lymphomagenesis has proven to be virtually inevitable. Nevertheless, individual tumors appear to initiate at random with age. Analysis of the kinetics of tumor onset allows us to estimate the rate of malignant conversion within the susceptible cell population.

Materials and Methods

Mice. Primary transgenic mice and initial breeding lines were produced by the techniques of Brinster et al. (17, 18) using the Eμ-*myc* DNA construct specified by Adams et al. (16), and (C57BL/6J × SJL/J)F_2 embryos descended from inbred mice purchased from The Jackson Laboratory (Bar Harbor, ME). For subsequent detailed analysis, the Eμ-*myc* 292-1 transgenic line was propagated by breeding (heterozygous) transgenic males with normal (C57BL/6JWehi × SJL/JWehi)F_1 hybrid (BSJF1)[1] females. As expected, ~50% of the progeny were transgenic. Transgenic females were not generally used for breeding because of their high risk of developing lymphomas before their pups reached weaning age. Animals used for assessing tumor onset kinetics were produced by mating multiple BSJF1 females with 4 third-generation transgenic males to randomize the contributions of C57BL and SJL traits. At 3 wk of age, the progeny of BSJF1 × transgenic matings were segregated by gender and numbered by ear punching. To identify transgenic pups, DNA extracted from tail tips (18) was hybridized with ^{32}P-labeled pUC12 plasmid DNA, sequences of which were present in the introduced Eμ-*myc* construct (16). An alternative test using blood cell nuclei is described below. When it was necessary to retest older animals for transgene carriage, DNA was extracted either from a skin biopsy or from the liver after sacrifice.

The mice were maintained under clean conventional conditions with free access to 2.5 Mrad-irradiated mouse breeder ration cubes (Barastoc Stock Feeds, Victoria, Australia) and acidified filtered tap water (3 mM HCl).

Monitoring Tumor Development. At weekly intervals after weaning, each mouse was closely inspected and its inguinal lymph nodes were palpated to detect enlargement. Any animal with an overt tumor was examined more frequently so that it could be autopsied when it became ill. For histology, soft tissues were fixed in Bouin's solution, bony tissues in neutral buffered 10% formalin. Paraffin-embedded fixed tissues were sectioned 4 μm thick and stained with hematoxylin and eosin.

Electronic Counting of Cells and Sizing of Nuclei. The equipment comprised a Zm Counter and C1000 Channelizer (Coulter Electronics Ltd., Luton, United Kingdom) connected to a microcomputer. These units were interfaced and programmed by Dr. F. Battye of the Walter and Eliza Hall Institute Flow Systems Laboratory. The system was calibrated with 9.7 μm polydivinylbenzene latex (Coulter Electronics Ltd.) and verified electronically for linearity of response.

Leukocytes were counted and sized as nuclei, obtained by adding the cell lysing agent, Zaponin (Coulter Electronics, Ltd.), to diluted blood. The diluent was PBS containing 5 mM KCl and adjusted to mouse tonicity (305–310 mosmol/kg). Blood was obtained from

TABLE I
Time Parameters of Tumor Development

Pathology onset	Median age	9 wk
	Age range	3–81 wk
Illness or death	Median age	12 wk
	Age range	6–83 wk
Progression from first signs to illness or death	Mean time ± SD	25 ± 19 d
	Range	0–94 d

134 mice from the fourth generation of the Eμ-*myc* 292-1 line were monitored for tumor development as described in Materials and Methods.

the retroorbital sinus using heparinized capillaries, or by cardiac puncture from animals anesthetized intraperitoneally with tribromoethanol. For routine typing of weanling mice for enlarged leukocyte nuclei (see Results), 5 μl of retroorbital blood was diluted into 20 ml of diluent and 6 drops of Zaponin were added just before the Coulter Counter analysis. The particle volume range used for counting and sizing was 22–110 fl.

Cell Surface Antigen Expression. The presence of Thy-1, Ly-5(B220), and Ig antigens on cells dispersed mechanically from tumors was detected by flow cytometric analysis of immunofluorescence as described (14).

Results

Lymphomas in Primary Transgenic Mice and their Descendants. The Eμ-*myc* construct was highly tumorigenic in independent transgenic lines. Of 15 primary transgenic mice that developed from eggs microinjected with the Eμ-*myc* construct, 12 became ill with lymphomas at 6 to 14 wk of age. Of the other 3, one was found cannibalized at 6 wk, another died at 34 wk without gross evidence of lymphoid pathology, while the third, which remained healthy for more than a year, was suspected of mosaicism from the low rate (2/13) at which the transgene was transmitted to its progeny. From five of the primary animals, breeding lines were initiated through matings with normal BSJF1 mice. All their transgene-bearing progeny developed lymphoid tumors, almost all becoming ill at 6–18 wk of age. The median onset of illness was 11 wk for the whole pool of 12 primary and 41 second- and third-generation animals. It varied only between 10 and 13 wk in the five separate lines. Consequently, one of the lines (denoted 292-1) was arbitrarily selected for long-term breeding and detailed investigation.

Tumor Onset and Progression. Tumor development was assessed in the entire fourth generation of the Eμ-*myc* 292-1 line (134 animals). In 83% of animals the first sign was enlargement of one or both inguinal lymph nodes, detected by palpation. A few mice died unexpectedly, but the others were killed for autopsy when they became ill, as manifest by hunched posture, slow movement, ruffled fur and, often, tachypnea.

The time course of disease varied markedly between individuals, in both the age at which the first signs appeared and the rate of progression to illness or death (Table I). The male and female incidence was equivalent (not shown). The onset of first externally detectable signs and of terminal illness as functions of age are plotted as semi-log "survival" curves in Fig. 1. The curves appear to have three components (see Discussion). A major portion of the population (~70%) produced a plot closely approximating a simple exponential over the age range from 6 to ~16 wk, so that the frequency of appearance of new tumor cases

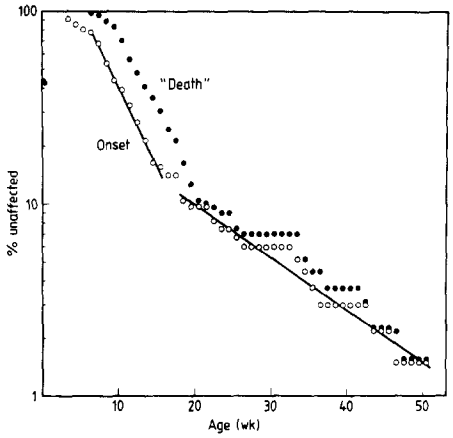

FIGURE 1. Onset of tumors and appearance of terminal illness with age in Eμ-*myc* mice. The first sign of lymphoma was designated as "onset" and ill health (or actual death) as "death." Regression lines were calculated for the ranges of "onset" data points indicated. Data from 70 females and 64 males (as in Table I) were pooled.

TABLE II

Autopsy Findings in Mice with Advanced Disease

Feature	Incidence
	%
Enlarged lymph nodes and spleen	84*
Tumor (or swelling) on thoracic vertebral column	68
Enlarged thymus (threefold or more)	54
Bowel obstruction	13
Hind limb paralysis	1.5

134 Eμ-*myc* mice (as in Table I) were autopsied when ill or after natural death.

* The 16% without general enlargement of lymph nodes comprised nine cases of thymoma, five of intussusception, four with a single large lymph node, and four with no grossly visible pathology.

among hitherto healthy animals (the hazard rate) was constant at 17% per week. The "death" curve was displaced by about 3 wk and was parallel. Below 5 wk of age, the hazard rate was lower and progression slower, terminal illness requiring an average of 5 wk to develop. Animals that remained free of disease for more than ~17 wk were subject to a reduced hazard rate of ~6% per week. Once initiated, their tumors progressed rapidly to terminal illness in 1–3 wk.

Lymphomatosis is an essentially inevitable fate for Eμ-*myc* mice. >90% had succumbed within 6 mo. By 1 yr only two of the original 134 animals remained healthy. One was found cannibalized at 65 wk, and the other developed tumors at 81 wk of age.

Gross Pathology. Dissections revealed gross evidence of disseminated lymphoma in all but 4 of the 134 animals. Typically, most or all lymph nodes were enlarged 5–50-fold and the spleen 2–6-fold (Fig. 2, *A* and *B*). Enlargement was usually similar in bilaterally paired lymph nodes. Frequencies of particular autopsy findings are given in Table II.

A majority of animals showed substantial enlargement of the thymus, in at least some cases clearly distinct from the three enlarged mediastinal (parathymic) lymph nodes adjacent to it. In nine of the animals, a thymoma was the dominant abnormal tissue mass (Fig. 2*C*). Another common finding in the thorax was a

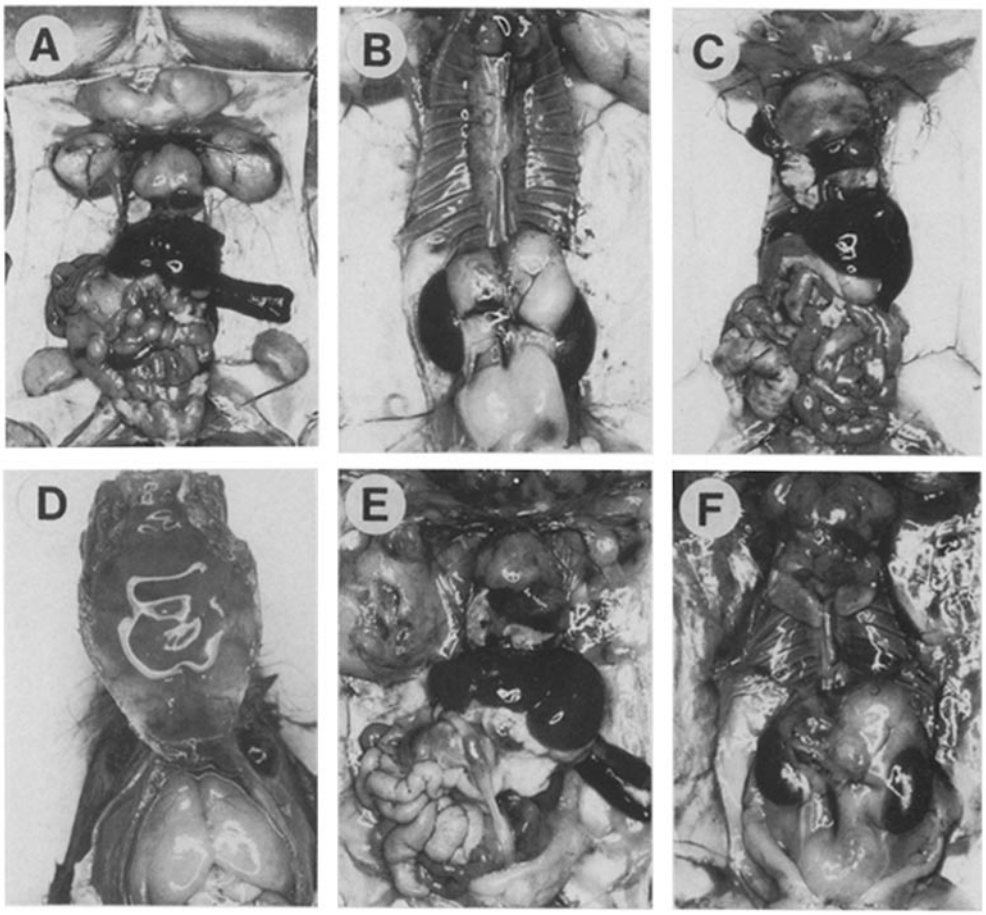

FIGURE 2. Dissections of Eμ-*myc* mice with advanced tumors and of BSJF1 mice bearing transplanted Eμ-*myc* tumors. (*A*) 13-wk-old Eμ-*myc* mouse with typical enlargement of lymphoid organs including the Peyer's patches and the thymus (× 0.7); (*B*) same animal after removal of loose abdominal and thoracic organs to reveal greatly enlarged renal and lumbar lymph nodes and thickening of thoracic vertebral column (× 1.1); (*C*) 9-wk-old Eμ-*myc* mouse with large thymoma but near-normal lymph nodes (× 0.9); (*D*) 11-wk-old Eμ-*myc* mouse with top of skull hinged open to show layer of tumor adhering to the parietal and interparietal bones (× 2.2); (*E*) BSJF1 animal, 35 d after injection intraperitoneally and subcutaneously of 100 mesenteric lymph node cells from a lymphomatous Eμ-*myc* mouse, showing a large tumor mass at the site of injection (*left*), a flat subcutaneous mass on the contralateral side, and enlarged lymph nodes, thymus and spleen (× 0.9); (*F*) BSJF1, recipient of 10^4 femoral bone marrow cells from another primary tumor mouse, showing flat subcutaneous masses near the axillae, enlarged lymph nodes and thymus, tumor tissue around the heart, and some thickening of the thoracic vertebral column (× 1).

visible swelling along the vertebral column (Fig. 2*B*), sometimes in the form of a discrete tumor terminating anterior to the diaphragm. In some animals, the Peyer's patches were enlarged (Fig. 2*A*). Tumor growth within the wall of the small bowel presumably caused the bowel obstruction found in 13% of the animals. This usually took the form of intussusception, which rapidly caused terminal illness. About one-quarter of all the animals had tumor masses inside the skull adherent to the parietal and interparietal bones (Fig. 2*D*), occasionally

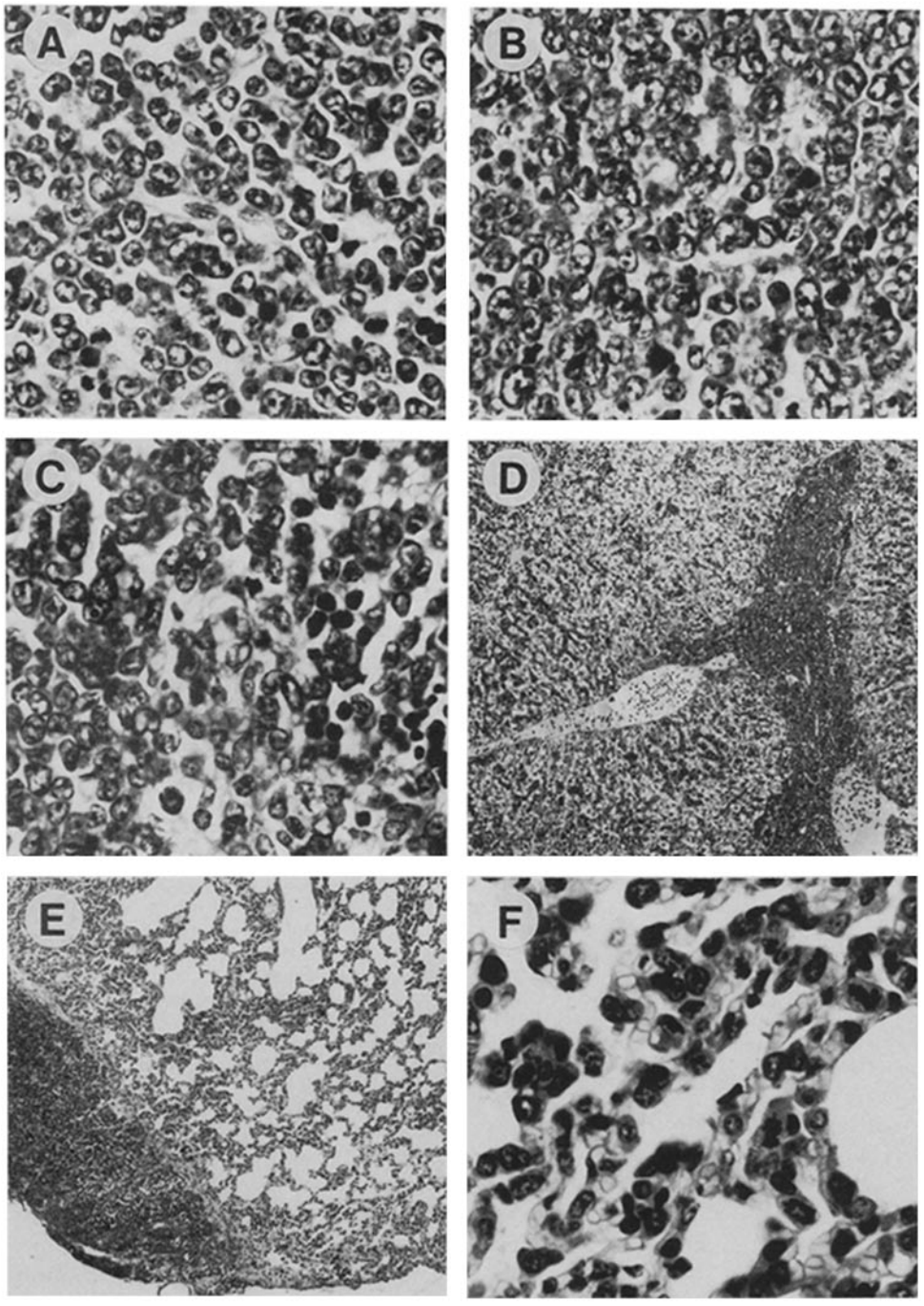

FIGURE 3 A–F.

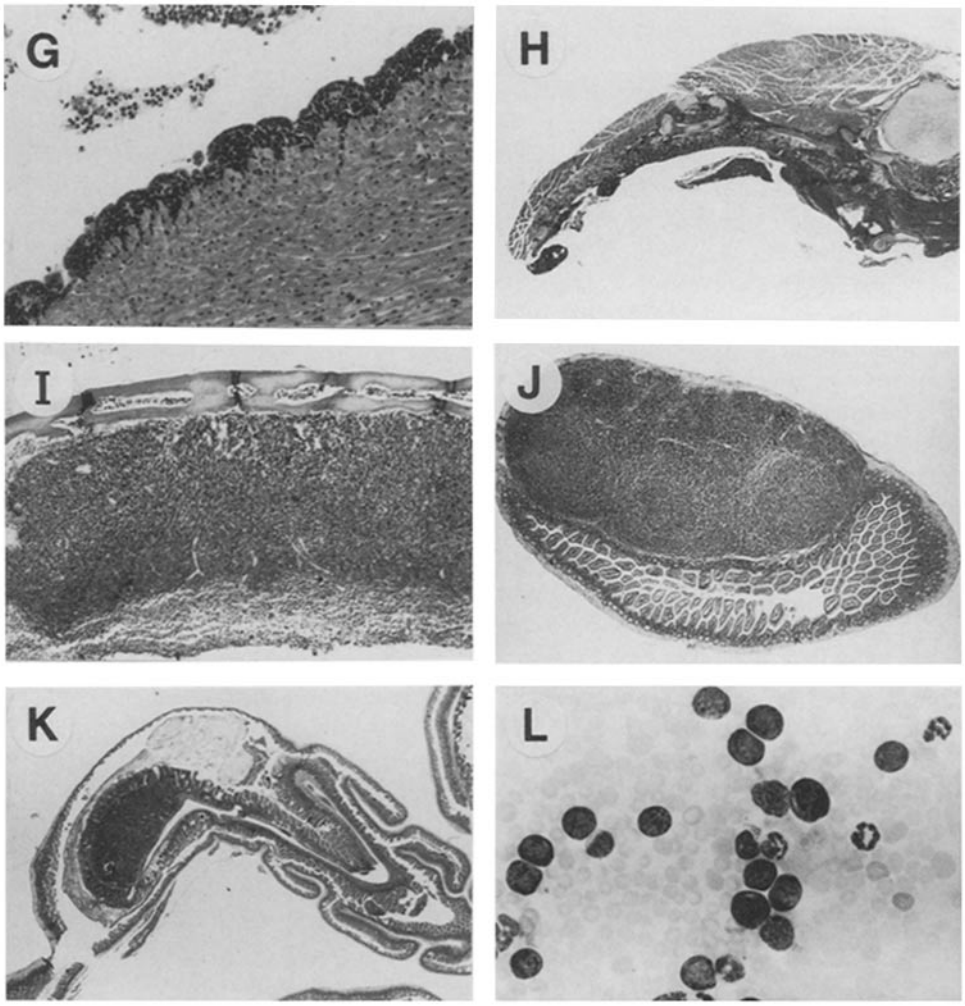

FIGURE 3. Histology of Eμ-*myc* tumors. Sheets of lymphoblasts replacing the lymphocytes in (*A*) the brachial lymph node (× 600) and (*B*) the thymus (× 600); (*C*) lymphoblasts in the splenic white pulp (*left*) and lymphoblasts among nucleated erythroid and other cells in the red pulp (*right*) (× 600); (*D*) liver with large deposits of lymphoblasts and lobular infiltration (× 60); (*E*) lung with a large pleural mass and hypercellularity in the parenchyma (× 60), revealed at higher power as *F*, infiltration of alveolar septa causing collapse of air spaces (× 600); (*G*) a layer of tumor cells on the surface of the heart with limited penetration of cardiac muscle (× 90); (*H*) transverse section of thoracic cage showing tumor on the ventral surface and infiltrating neighboring muscle (× 14); (*I*) tumor mass attached to parietal bone of the skull (× 37); (*J*) Peyer's patch enlarged by lymphoblasts and intruding into the lumen of the small bowel (× 18); (*K*) intussusception, with a lymphoblast-enlarged Peyer's patch everted and drawn into adjacent bowel (× 9); (*L*) blood smear showing lymphoblasts and granulocytes (× 600).

large enough to deform the skull and depress the cerebrum and cerebellum. The other major organs with visible abnormalities were the liver, which was often enlarged (up to twofold); the lungs, which occasionally had a few presumptive tumor deposits; and the skin, which sometimes showed flat masses of tumor

spreading posteriorly from the axillary region, presumably through mammary tissue.

Histopathology. Histologic examination of 60 animals with advanced tumors revealed tumor cells within many tissues. In the enlarged lymph nodes, the normal architecture was largely obliterated. Normal cells were almost completely replaced by medium-sized cells with little cytoplasm and open-looking, often indented, nuclei containing large nucleoli and fine reticular chromatin, i.e., lymphoblasts (Fig. 3*A*). The thymus appeared similar (Fig. 3*B*), even in some of the mice in which it was not grossly enlarged. The cortex tended to be more densely packed with lymphoblasts than the medulla. In one case the thymus was normal except for patches of abnormal lymphoblasts in the cortex, presumably representing an early stage of thymic invasion by tumor cells.

The spleen and bone marrow were altered even in young healthy Eμ-*myc* mice. The pretumor spleen was up to twice normal size, and its red pulp showed greatly increased extramedullary hematopoiesis, with many more megakaryocytes and nucleated erythroid cells, albeit less erythrocytes than normal. Many lymphoblasts were scattered through the red pulp (but were not apparent in the white pulp). In terminally ill animals, the red pulp sometimes contained more abundant lymphoblasts, which largely or completely replaced normal lymphocytes in the white pulp (Fig. 3*C*). Pretumor bone marrow was dominated by pre-B lymphoblasts (14). In the femoral marrow of animals with advanced lymphoma, there was often a further increase in the number, and sometimes the size, of lymphoblasts, although general hematopoietic activity was never completely displaced (not shown).

In tumorous animals, the liver was routinely invaded by lymphoblasts, which formed variable-sized masses in portal areas, and sometimes infiltrated the liver parenchyma, occupying the sinusoids of the lobules (Fig. 3*D*). While none of 8 kidneys examined histologically showed invasion, lungs from 17 diseased animals all showed widespread diffuse infiltration of the alveolar septa by lymphoblasts. The resulting partial collapse of the alveolar air spaces (Fig. 3, *E* and *F*) may have caused the tachypnea commonly suffered by animals with advanced tumors. Lung lymphatic vessels were sometimes strikingly distended by a glut of lymphoblasts, and major blood vessels commonly contained large numbers, indicative of lymphoblastic leukemia (see below). In some cases the pleura contained discrete masses of lymphoblasts (Fig. 3*E*).

Muscle and bowel were sometimes involved. Cardiac muscle was occasionally penetrated (Fig. 3*G*), while intercostal muscle was extensively infiltrated by the lymphoblasts accumulated along the thoracic vertebral column (Fig. 3*H*). However, neither these swellings nor the tumor deposits under the roof of the skull (Fig. 3*I*) ever reflected any infiltration of the spinal cord or the brain. In the digestive tract, apart from a single cecal lymphoma, lymphoblasts were prevalent only in Peyer's patches. When gross enlargement occurred, there was intrusion into the lumen (Fig. 3 *J*) and, in cases of intussusception, a lymphoblast-packed Peyer's patch could often be seen at the head of an everted portion of bowel, drawn by peristalsis into the adjacent bowel (Fig. 3*K*).

Transplantability. To verify that the masses of lymphoblasts seen in the enlarged lymphoid tissues of Eμ-*myc* mice were malignant, up to 10^6 cells from

TABLE III
Transplantability of Eμ-myc Tumor Cells

Donor animal	Donor tissue*	Days‡ until first sign of tumor growth at cell numbers per recipient of:				
		10^5	10^4	10^3	10^2	10^1
F-527	Mesenteric lymph node	18	18	21	26	21
		21	21	21	26	26
		21	21	21	26	32
F-527	Femoral bone marrow	18	18	21	21	21
		18	26	26	26	26
		26	26	26	30	26
F-546	Mesenteric lymph node	10	13	13	17	17
		10	17	17	17	17

* Tissues were taken from seventh generation E*μ-myc* mice with advanced lymphoma.
‡ BSJF1 recipients were examined at 3–5-d intervals after subcutaneous and intraperitoneal injection of cells in 0.2 ml balanced salt solution containing 5% FCS.

each of 22 primary tumor animals were transplanted into normal BSJF1 mice. Only two failed to generate tumors in the recipients within the 3-mo observation period, and one other yielded tumors in only a few recipients. From the remaining 19 donors, 10^5–10^6 cells initiated tumors in all recipients, tumors being apparent after 10–35 d and terminal illness by 20–50 d. Transplantation titrations, in which cells from four donors were reduced in 10-fold steps from 10^5 to 10^1 cells per recipient, yielded tumors at all cell doses. Examples are shown in Table III. Irrespective of dose, all tumors arose within 10–30 d.

With a few exceptions, the gross pathology (Fig. 2, *E* and *F*) and histopathology (not shown) in transplant recipients was similar to that in the primary tumor animals. Depending on the donor, tumor growth at the site of subcutaneous injection varied from undetectable to very large masses, but was almost always accompanied by enlargement of the lymph nodes, the spleen, and the thoracic tissue around the vertebral column, and by infiltration of the thymus, the bone marrow, the liver, and the lungs. The blood was leukemic and granulocytotic. Transplant recipients did not, however, develop subparietal tumors or intussusception, and enlarged Peyer's patches were rare.

Selective homing patterns were not evident. For example, cells from primary thymomas did not predominantly cause thymic enlargement in recipients, and cells from two subparietal tumors did not produce visible growth inside the skull. On the other hand, one large primary subparietal tumor that showed little spread to other organs produced massive tumor growth only at the subcutaneous injection site, presumably reflecting its heritable inability to metastasize.

Transplantation provides a sensitive test for the presence of malignant cells before overt tumors. We examined 149 E*μ-myc* mice, between 4 and 10 wk of age, that appeared free of disease. Dissection revealed that 23 (15%) had enlarged lymph nodes and/or an enlarged thymus. 79 of the others were tested for malignant cells by transplanting $1–5 \times 10^6$ bone marrow cells from both femurs and, from some of these donors, $5–20 \times 10^6$ spleen and mesenteric lymph node

TABLE IV

Leukocytes of Lymphomatous and Prelymphomatous Eμ-myc Mice and Nontransgenic Littermates

Parameter	Nontransgenic	Eμ-*myc*		
		Pretumor	Early tumor	Terminal tumor
Nucleated cells (× 10^6/ml)*				
Range (number of samples)	1.5–8.1 (22)	2.3–11 (10)	3.0–41 (17)	3.7–130 (17)
Geometric mean $\overset{\times}{+}$ SD	4.2 $\overset{\times}{+}$ 1.6	5.7 $\overset{\times}{+}$ 1.7	11 $\overset{\times}{+}$ 2.3	29 $\overset{\times}{+}$ 4.0
Mean nuclear volume (fl)*				
Range (number of samples)	35–39 (18)	37–41 (10)	40–57 (8)	41–70 (17)
Mean ± SD	37.4 ± 1.0	39.6 ± 1.4	49.2 ± 6.6	52.9 ± 6.5
Polymorphs (Percent of nucleated cells)‡				
Range (number of samples)	6–17 (15)	5–20 (10)	4–34 (16)	5–45 (13)
Mean ± SD	11.4 ± 3.3	10.1 ± 5.2	14.2 ± 7.4	19.5 ± 15.2
Polymorphs (× 10^5/ml)§				
Range (number of samples)	2–8 (15)	2–14 (10)	5–49 (16)	4–180 (13)
Geometric mean $\overset{\times}{+}$ SD	4.2 $\overset{\times}{+}$ 1.7	5.2 $\overset{\times}{+}$ 1.8	14 $\overset{\times}{+}$ 2.1	28 $\overset{\times}{+}$ 3.1

Animals of both sexes aged 6–21 wk in the terminal tumor group and 5–11 wk in the other three groups were bled from the heart or the retroorbital sinus.

* Diluted heparinized blood was treated with Zaponin (Coulter Electronics) and the lysed-cell nuclei were counted and sized using a Coulter Counter and Channelizer (see Materials and Methods).

‡ Giemsa-stained blood smears were scored for polymorphs as a percentage of 200–400 nucleated cells.

§ Concentrations were calculated from each percentage and the corresponding Coulter count of nuclei.

cells, by subcutaneous and intraperitoneal injection into normal BSJF1 mice. Within 2 mo, eight had generated disseminated lymphomas in the recipients, all of which were observed for at least 3 mo. Thus, overall, 24% of ostensibly healthy transgenic animals were actually in early stages of lymphoma development.

Blood Leukocytes. The lymphoblasts in sections of blood vessels from animals with advanced lymphoma indicated that the disease had a leukemic component. The development of leukemia was therefore investigated by microscopic examination of blood smears and by counting and sizing blood cell nuclei in a Coulter Counter. Terminally ill animals and those at an early stage of tumor development were compared with pretumor young adult Eμ-*myc* animals and their nontransgenic littermates.

Table IV shows that, although white-cell parameters varied greatly within each group, there were clear differences between the four groups. On average, pretumor animals had somewhat more cells and slightly larger nuclei than the nontransgenic controls. The blood of most lymphomatous animals, however, contained markedly more cells and larger cell nuclei (Table IV), and stained smears revealed large lymphoid cells (Fig. 3*L*), even when the total leukocyte count was within the normal range. Clearly, though, the extent to which these lymphoblasts invaded the circulation varied greatly in both early and late stages of tumor growth in different animals. Fig. 4 depicts examples of nuclear size distributions from which the mean volumes were computed. The profiles from lymphomatous animals (Fig. 4, *c–f*) were all distinguishable from those from healthy animals (Fig. 4, *a* and *b*), but appeared to vary in the relative proportions of nuclei of two sizes, centered on ~35 and ~60 fl.

Leukemic blood frequently had more abundant granulocytes (Table IV and Fig.3*L*). 10 of 13 advanced cases scored for these cells had levels higher than

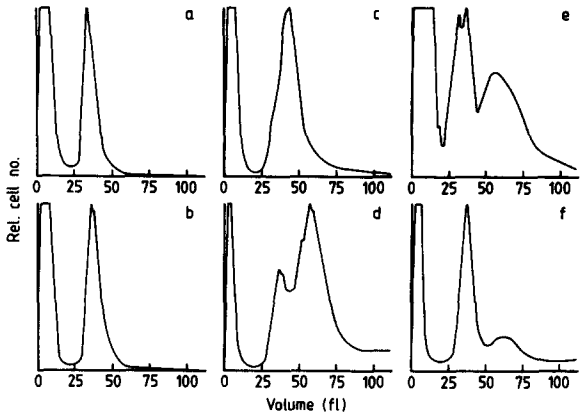

FIGURE 4. Size distributions of leukocyte nuclei from normal and from Eμ-*myc* mice. The animals, the mean volumes of their leukocyte nuclei, and their leukocyte counts were; (*a*) 42-d nontransgenic, 37 fl, 8.1 × 10^6/ml; (*b*) 42-d Eμ-*myc* pretumor, 40 fl, 11 × 10^6/ml; (*c*, *d*, *e*, and *f*) Eμ-*myc* mice with advanced lymphoma, 48, 59, 52, and 51 fl, 88, 43, 4.5, and 130 × 10^6/ml, respectively. Counts and volumes were derived from Zaponin-lysed blood with a Coulter Counter and Channelizer.

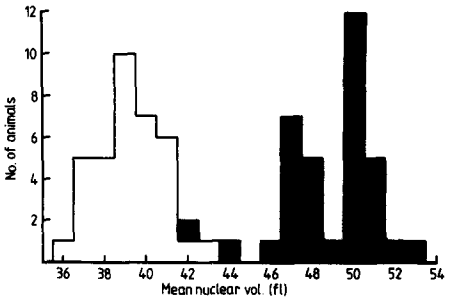

FIGURE 5. Leukocyte nuclear size in weanling Eμ-*myc* mice and nontransgenic littermates. 10 litters (71 mice), born of normal females mated to Eμ-*myc* males, were tested for transgene carriage by tail DNA hybridization and were bled for determination of mean leukocyte nuclear volume at 21–27 d of age. Filled areas indicate transgene-positive animals, open areas transgene-negative animals. Nuclear volumes were measured on Zaponin-lysed blood from the retroorbital sinus with a Coulter Counter and Channelizer.

the maximum in the 15 control animals tested. The granulocytosis presumably is a response to tumor cells, since it developed also in BSJF1 animals carrying transplanted lymphomas (not shown).

Rapid Identification of Eμ-myc Mice. In producing large numbers of transgenic animals, it becomes very time-consuming to identify the transgenic progeny of heterozygous parents by DNA hybridization. The slightly enlarged leukocyte nuclei in young adult Eμ-*myc* mice (Table IV), together with our earlier finding that B-lymphoid cells in these animals cycle more than normal (14), led us to test whether they could be identified at weaning (3 wk) by size measurements on blood leukocyte nuclei. Fig. 5 compares the frequency distribution of mean nuclear size with results of tail DNA hybridization tests on 21- to 27-d pups. Fortunately, the nuclear size difference was accentuated at this age. Despite the spread of values in the normal group (Fig. 5, *open bars*) and the apparent bimodality in the transgene-positive group (Fig. 5, *solid bars*), >90% could be confidently classified by this rapid test, which requires only 5 μl of blood (see Materials and Methods). Subsequent routine tests on several hundred weanlings have confirmed that mean nuclear volumes above 43 fl are reliably diagnostic of transgene carriage and below 42 fl of normality. Weanlings with values of 42 or 43 fl are either retested by DNA hybridization or discarded.

The concentration of leukocytes was also elevated in most weanling Eμ-*myc* mice. While 90% of normal weanlings had from 4 to 10 × 10^6/ml, their transgenic littermates had 8.5–50 × 10^6/ml with a median of 19 × 10^6/ml. Therefore, cell counts identify most transgenic animals, but nuclear volume has proven more

discriminating. Comparison of the data from young adults (Table IV) and from weanlings (Fig. 5) reveals that early in the life of an Eμ-*myc* mouse there is a transient flood of enlarged leukocytes into the circulation. These cells have lymphoid morphology in stained smears (not shown).

Immunologic Classification of Tumors. Since the tumors consisted of lymphoblasts, they would be classified as lymphoblastic lymphomas, but tumors of this morphology can derive from either the T or the B lineage (19). Therefore, cells from primary lymphomas were tested for staining with fluorescent antibodies against the T cell marker Thy-1 and the B-lineage markers Ly5(B220) and Ig. None of 31 tumors tested bore Thy-1, not even the thymic cells from six animals with dominant thymoma. 6 of the 31 showed surface expression of Ig on essentially all cells, while another 9 contained mixed populations of tumor cells ranging from 5% to 87% surface Ig^+. Of 16 Thy-1^- tumors tested for the Ly-5(B220) surface antigen, 15 were positive. In addition, primary tumors and cultured tumor cell lines all carry rearranged Ig genes (16; Cory, S., A. W. Harris, W. Y. Langdon, and J. M. Adams, manuscript in preparation). Thus all Eμ-*myc* tumors appear to derive from the B-lineage, pre-B lymphomas and mixed pre-B/B lymphomas predominating.

Tumors in Nontransgenic Mice. Middle-aged SJL mice develop a tumor known as reticulum cell neoplasm, type B (20). Because the Eμ-*myc* animals contained a variable assortment of genes of SJL (and of C57BL) origin, we assessed the incidence and nature of tumors arising in a group of >100 of their nontransgenic littermates. No tumors were found until 10 mo, when one mouse was killed with a disseminated lymphoma, and two others began to develop slowly progressing tumors. 10 of the 105 animals surviving the first year were killed during the following 6 mo with advanced tumors in lymphoid organs, and 21 others were killed (sick, or with fight wounds) without evidence of neoplasia. When the 75 survivors were autopsied at 18 mo of age, 25 had one or more enlarged lymphoid organs. The estimated tumor incidence in the 12- to 18-mo age range was 37% (35/95).

The gross pathology varied, partly because a third of the tumorous animals were killed while apparently healthy, but the most frequent findings were greatly enlarged mesenteric lymph nodes (83%) and large spleens (69%) (Fig. 6*A*). In 16 of the 36 tumor-bearing animals (44%), one or more Peyer's patches were enlarged (Fig. 6*A*). Widespread lymph node enlargement was found only in sick animals and probably is a late event in tumor progression.

None of the 31 tumors from nontransgenic animals examined histologically were lymphoblastic lymphomas. A few comprised fairly uniform masses of large lymphoid cells with moderate amounts of faintly staining cytoplasm and probably were follicular center cell lymphomas. Most tumors, however, were highly varied mixtures of lymphoid and nonlymphoid cells, like those described as reticulum cell neoplasm, type B (21). The mesenteric lymph node was replete with such cells, often including distinctive giant multinucleate cells (Fig. 6*B*). The large nodular spleen (Fig. 6*C*) contained greatly enlarged white pulp areas filled with tumor cells, and increased hematopoiesis in the red pulp. The lungs contained discrete tumor nodules (Fig. 6*C*).

This histologic picture held for only two of the 134 Eμ-*myc* animals, one of

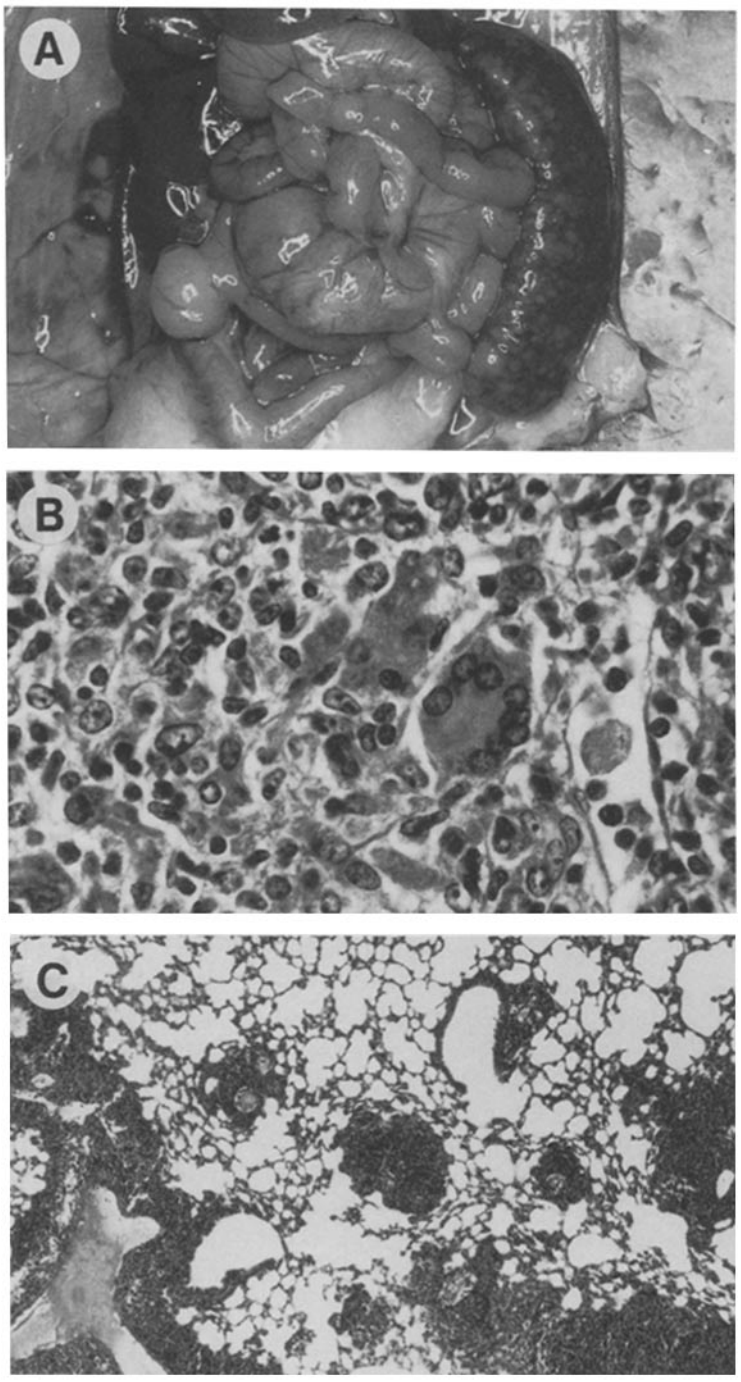

FIGURE 6. Gross pathology and histopathology of tumors in nontransgenic BSJF1-backcrossed mice. The animals commonly exhibited (*A*) enlarged mesenteric lymph node and Peyer's patch(es) and an enlarged, nodular spleen (× 1.9); (*B*) mesenteric lymph node containing an abnormal mixture of cells including a giant multinucleate cell (× 600); and (*C*) lung containing dense nodular deposits of tumor cells (× 60).

which succumbed at 42 wk of age with both reticulum cell neoplasm, type B and lymphoblastic lymphoma. The other, the longest surviving confirmed transgene carrier, became ill at 83 wk of age with reticulum cell neoplasm, including nodular lung deposits. This unfortunate animal had won a Pyrrhic victory against the high odds of developing a lymphoblastic lymphoma!

Discussion

We have characterized the Eμ-*myc* mouse in some detail as a model for lymphoid tumorigenesis. After a variable period during which there is a benign, limited overgrowth of pre-B cells (14), essentially every mouse develops tumors. Although the disease manifestations are somewhat variable in detail (Table II and Fig. 2), the dominant picture is disseminated lymphoma. The morphology of the tumor cells, and the almost invariant infiltration of the lungs and liver (Fig. 3, *D*, *E*, and *F*) fit the classification of lymphoblastic lymphoma in the Pattengale-Taylor (19) scheme for mouse lymphoid tumors.

Lymphoblastic lymphomas of mice can derive from either the T or the B lineage (19). An enlarged thymus is usually indicative of a T lymphoma, such as those that arise in AKR and C58 mice, or in other strains given Moloney leukemia virus or x rays (e.g., reference 22). Thymic enlargement was common in Eμ-*myc* mice with advanced tumors (Table II), and in 9 of 134 it was the dominant abnormality at autopsy. Nevertheless, all these proved to be B-lineage tumors, mostly pre-B lymphomas. Since pre-B cells are not found in the prelymphomatous thymus (14), and since the lymphoma cells commonly metastasized to the thymus after transplantation, we assume that the primary thymomas were caused by invasion from outside the thymus. The same argument applies to the lymph nodes and leads us to suggest that acquisition by pre-B cells of the ability to invade the thymus and lymph nodes is a critical step in tumor development. Some other B-lineage tumors are known to metastasize to the thymus to a limited extent, including B-immunoblastic lymphomas (23) and the B-lymphocytic leukemia line BCL_1 (24). The pre-B lymphoblastic lymphomas induced by Abelson virus, on the other hand, never appear to involve the thymus (25, 26).

Features common to Abelson and Eμ-*myc* tumors, apart from the enlarged lymph nodes and spleen common to most mouse lymphomas, are the deposits inside the skull and along the vertebral column. It has been postulated that these prominent features of Abelson disease are formed by cells emerging from the marrow of adjacent bones (26). With advanced Eμ-*myc* tumors, vertebral column swelling by lymphoma cells was common; tumors inside the skull much less so. Recipients of transplanted cells did not develop skull tumours, but usually did show paravertebral tumor and bone marrow invasion. Thus, pre-B lymphoma cells can readily metastasize from the periphery to the tissues along the vertebral column, although this could involve an intermediate sojourn in the regional bone marrow.

The rate of appearance of tumors in Eμ-*myc* mice is not constant throughout life (Fig. 1). In the first few weeks it averages ~5% per week, later increasing to 17% per week and finally falling again to 6% per week. The low initial rate may reflect a smaller number of cells at risk of malignant transformation in the young growing animal. It is also possible that the differing rates manifest the presence

of genetically determined subpopulations of animals, since the genetic background is a segregating mixture of C57BL and SJL alleles. From ~6 wk of age, when adult size is neared, the tumor onset rate is constant at 17% per week for ~70% of the animals. This corresponds to a mean time of 38 d for a malignancy-initiating event in mice of 6–16 wk of age. Animals remaining free of tumor for longer periods appear to represent a relatively resistant subpopulation with an onset rate of only 6% per week and a mean time of 108 d to initiation of malignancy. This reduced rate is associated with a reduced expansion of the pre-B cell population in the pretumorous animals (14), and appears to be genetically determined. Preliminary results from serial backcrossing with inbred C57BL/6J mice indicate that one or more C57BL genes confer a relative resistance to transgene-associated lymphomagenesis (Harris, A. W., unpublished observations).

Since early-onset tumors did not progress more rapidly than late-onset tumors (Fig. 1), the early tumors are not simply the more aggressive ones. Instead, the random onset must be related to the appearance of the first malignant cell within each animal. That cell must be generated by a low-frequency event, presumably a genetic accident, within the abnormally expanded pre-B population. We can estimate the frequency of malignant conversion. The young adult Eμ-*myc* mouse contains ~2.4×10^8 pre-B cells, most of which are in a proliferative state (14). If half these cells divide on average every 8 h, like the cycling subset of pre-B cells in a normal mouse (27), then in the 38 d required for each tumor-initiating event to occur, the total pre-B pool will have undergone about 10^{10} cell divisions. Thus, the rate of occurrence of malignancy-inducing events is about 10^{-10} per cell per generation. This is an exceptionally low rate for a single mutation. Perhaps only certain extremely specific mutations suffice, or perhaps even a dysregulated *myc* gene–expressing pre-B cell requires two mutations to become a malignant cell. Another possibility is that most newly mutated potentially malignant cells are eliminated by the mechanisms responsible for the rapid turnover of pre-B cells before their mutant phenotype can be effectively expressed.

The high transplantability of the primary lymphomas (Table III) implied that transplantation might reveal malignant cells in transgenic animals not yet exhibiting signs of lymphoma. When many such animals were examined internally for lymphoid organ enlargement and tested for transplantable tumor cells, 24% proved to be in early stages of lymphoma development. By relating this figure to the tumor onset rate of 17% per week, we conclude that lymphomas actually initiate in Eμ-*myc* mice an average of 10 d before externally recognizable signs become apparent.

We found that the Eμ-*myc* mice develop leukemia as well as lymphoma. Although other mouse lymphoid malignancies are commonly called leukemias, it is difficult to find published quantitative data on the blood cells, and qualitative statements (e.g., references 19, 25, 26, 28) suggest that those tumor models do not develop the high concentrations of circulating tumor cells that can occur in human leukemias. An exception is the mouse chronic B-lymphocytic leukemia BCL_1, which yields blood concentrations 10–30 times above normal leukocyte levels (24). Abelson pre-B lymphomas are associated with a granulocytosis, but

no detectable lymphoid leukemia (25, 26). Lymphomatous Eμ-*myc* animals, on the other hand, commonly show both a myelocytic leukemoid reaction and a substantial lymphoblastic leukemia. The granulocytosis might be provoked by factors released from the lymphoma cells.

Because there are many more large pre-B and B cells than normal in the spleen and bone marrow of the prelymphomatous Eμ-*myc* mouse (14), it seemed likely that the B cells in the blood would also be larger. The average size of total leukocyte nuclei in young adult transgenic animals did tend to be higher than normal (Table IV and Fig. 4), but larger differences were seen with younger animals, most weanling transgenic and nontransgenic animals falling into two distinct groups (Fig. 5). We have subsequently used this rapid test to identify transgenic progeny in the Eμ-*myc* line, with an error rate of <5% in several hundred mice. The apparent bimodal distribution of mean nuclear volumes among the transgenic weanlings (Fig. 5) may reflect their genetic heterogeneity and is under investigation. The blood cell assays revealed that, early in life, elevated numbers of enlarged lymphoid (presumably B or pre-B) cells circulate in the blood of the Eμ-*myc* mouse. This phenomenon is present at 21 d through 33 d of age, but has almost disappeared by 42 d (Fig. 4). In a few animals, of course, the early leukocytosis reflects early-onset leukemia, but in the majority, whose tumors initiate later, there is an intervening period in which the size and number of blood white cells are near normal.

All Eμ-*myc* tumors seem to derive from the B-lymphoid lineage, but they are not all uniform in phenotype; we found pre-B lymphomas, mixed pre-B and B lymphomas, and B lymphomas. The random initiation and the demonstrated clonality of Eμ-*myc* tumors (16) indicate that the mixed tumors, and perhaps even the "pure" B lymphomas, are descendants of malignant pre-B cells that can differentiate. In this respect, they differ from Abelson tumors that rarely make the step from pre-B cell to surface Ig-expressing B cell (29, 30). We have seen no evidence of spontaneous plasmacytoma, the third main class of B-lineage tumor, by electrophoretic screening of many Eμ-*myc* sera for paraproteins (Harris, A. W. and M. Crawford, unpublished observations).

We looked for possible relationships with the tumors that arise in some nontransgenic littermate animals possessing the same mixture of C57BL and SJL genes. None of more than 100 nontransgenic animals developed tumors within the first 9 mo of life, a period that saw 98% of the Eμ-*myc* group succumb; but when monitoring was concluded at 18 mo, the incidence was 37%. The tumors, which enlarged some lymphoid organs, varied histologically from uniformly large lymphoid cells, through mixed collections of lymphoid and nonlymphoid cells, to sarcoma-like masses of fusiform cells. They were all readily distinguishable from the lymphoblastic lymphomas in the transgenic animals. Also, the lung infiltration was nodular rather than diffuse. Thus, the Eμ-*myc* transgene did not simply hasten the onset of the tumors that would have occurred later in its absence.

The tumors in the nontransgenic animals resembled those characteristic of middle-aged SJL mice. SJL tumors vary in cell composition, and their classification, nature, and malignant character is uncertain (19, 21, 31–33). Some are said to contain neoplastic pre-B cells (31) and many are associated with clonal

proliferations of Ig-secreting cells (34). Therefore, despite the morphological differences between SJL and Eμ-*myc* tumors, SJL genes (such as *Rcs*-1 [35]) that predispose to the so-called reticulum cell neoplasm may accelerate lymphomagenesis in Eμ-*myc* animals. Our preliminary results with C57BL-backcross animals support this idea and suggest that the Eμ-*myc* strain might be useful in exploring other inbred mouse strains for genes which can modulate B-lymphoid tumorigenesis.

Because the initiation of malignancy occurs at random in Eμ-*myc* mice, we believe these mice constitute a model of spontaneous onset lymphoma and leukemia. In its high incidence early in life, rapid progression, mixed lymphoma–leukemia pathology and predominantly pre-B cell origin, the disease can be considered as a murine analog of the common form of childhood acute lymphoblastic leukemia (36–38). The nature of the low-frequency events, possibly spontaneous mutations in other oncogenes, that generate the first neoplastic lymphoid cell is under study. Such animals should provide a highly sensitive assay for carcinogens. Moreover, by introducing other known oncogenes into these animals, or into their pre-B cells in vitro, it should be feasible to delineate which of them can cooperate with a dysregulated *myc* gene to precipitate B-lymphoid malignancy.

Summary

Mice transgenic for a c-*myc* gene driven by the IgH enhancer (Eμ-*myc*) were shown to almost invariably develop lymphomas, 90% succumbing in the first 5 mo of life. The tumors typically presented as rapidly progressive lymphadenopathy with thymic involvement and were highly malignant by transplantation assay. Morphologically, they were lymphoblastic lymphomas, usually accompanied by lymphoid leukemia and granulocytosis, and were distinct from the tumors that arose much later in 37% of nontransgenic mice of the same (C57BL/6 × SJL)F_2 genetic background. Cell-surface markers on 31 Eμ-*myc* tumors identified 52% as pre-B lymphomas, 29% as mixed pre-B and B lymphomas, and 19% as B lymphomas. The tumors appeared to arise at random from a population of pre-B cells expanded by constitutive expression of the *myc* transgene. A majority of the animals initiated malignancy at the rate of 17% per week. The rate at which the cycling, benign pre-B cells spontaneously convert to malignancy was estimated to about 10^{-10} per cell per generation.

A transient leukocytosis identified in young Eμ-*myc* mice was developed into a rapid assay for inheritance of the transgene.

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