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I am submitting herewith a dissertation written by Harold I. Saavedra entitled "Characterization of pBOR-Il-3 transgenic mice: Effects of the pBOR-Il-3 transgene on normal hematopoiesis and growth of azacytidine-induced thymic lymphomas." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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Characterization of pBOR-Il-3 transgenic mice: Effects of the pBOR-Il-3 transgene on normal hematopoiesis and growth of azacytidine-induced thymic lymphomas.

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Harold I. Saavedra

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To my parents, Honorio and Milagros, to my wife Fanny and my daughter Carla

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Abstract

The pBOR-Il-3 transgenic mice were developed by the pronuclear microinjection method to study how activation of transcriptional expression driven by a retroviral long terminal repeat occurs and especially how insults by both chemical and physical agents modulate transcription driven from the LTR, and to study the biological functions of interleukin-3. Our studies showed that pBOR-Il-3 mice that were not treated with any chemical or physical agents were completely normal, and did not develop any particular spontaneous tumors, relative to the FVB/N control group. Different hematological/cellular immunological techniques revealed no major differences between the hematopoietic systems of pBOR-Il-3 and the FVB/N mice. Only minor differences were observed, such as a higher number of monocytes in 2-months-old pBOR-Il-3 mice, and a higher number of neutrophils in 5-month-old pBOR-Il-3 mice, relative to FVB/N control mice. The fact that the hematopoietic subpopulations in the pBOR-Il-3 mice were not different from the ones from FVB/N controls, suggested that Il-3 was not being expressed in transgenic mice at levels high enough to disturb hematopoiesis. Two methods were used to perturb the hematopoietic system of the mice: the first was the use of a physical agent, x rays. Both pBOR-Il-3 mice, and FVB/N mice were irradiated with 2 Gy of x ray, and the recovery of the different hematopoietic subpopulations was asessed on days 1 and 8 after irradiation. There were no differences in the radiation recovery of the different hematopoietic subpopulations between Il-3 and FVB/N mice. The other method used to perturb the hematopoietic system of the mice was the injection of azacytidine, a demethylating agent traditionally used to activate the LTR-driven expression of retroviral genomes. Upon azacytidine treatment, both the pBOR-Il-3 mice and the FVB/N controls developed thymic lymphomas, the transgenic mice developed the tumors at a higher frequency and relatively faster than the FVB/N controls. This result suggested that Il-3 expression is not a necessary step to the development of thymic lymphomas, but that Il-3 accelerates the

growth of the thymic lymphoma cells. The thymic lymphoma cells were of a T-cell origin, as determined by the T-cell receptor gene rearrangement analysis, and, in most cases, were of a monoclonal origin. According to the flow cytometric analysis of thymic lymphoma cells, labelled with florescet-labelled T-cell-specific antibodies, the thymic lymphoma cells did not lose the ability to differentiate, but the differentiation process was aberrant. Studies on the CD4/CD8 T-cell surface markers revealed that in pBOR-Il-3 thymic lymphomas the predominant differentiation pathway is one in which the $CD4^+CD8^+$ subpopulation differentiates toward the $CD8^+CD4^-$ subpopulation; conversely, in the FVB/N group, the predominant differentiation pathway is the $CD4^+CD8^+$ subpopulation differentiating toward the $CD4^+CD8^-$ subpopulation.

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CHAPTER I

General Introduction

A. General overview

The working hypothesis in our laboratory has been that chromosomal-related gene elements play important roles in gene rearrangement and gene activation events induced by environmental agents (Yang et al., 1989). We have developed several mouse transgenic lines to study LTR-containing gene elements and their relationship to carcinogenesis and environmental mutagenesis (Yang et al., 1989; Wang et al., 1993). Those mouse transgenic lines contain an LTR sequence modulating the transcription of a reporter gene; one of those transgenic lines is pBOR-Il-3. The pBOR-Il-3 transgenic mice were produced in our laboratory to: a) elucidate the properties of specific regulatory sequences carried by the LTR in the context of the mouse genome; b) study the potential relationship between environmental insults and transcriptional activation of a transcriptionally silent LTR; c) study the effects of Interleukin-3 on normal hematopoiesis; and d) to study the potential role of Il-3 in the growth of thymic lymphoma cells.

This dissertation deals with the way in which the pBOR-Il-3 transgenic mouse line was produced; with the effects of Il-3 on different hematopoietic subpopulations, assessed by the use of immunological and histological techniques; it also deals with the effects of Il-3 in hematopoietic subpopulations recovering from irradiation; and with the possible role played by Il-3 in the growth of azacytidine-induced thymic lymphomas.

B. Biological Functions and Structure of Interleukin-3

Interleukin-3 (Il-3) is a 28 kDa glycoprotein produced by a Lyt-$1^{+}2^{-}$ subpopulation of T-lymphocytes after antigen or mitogen stimulation (Ihle et al., 1981). Il-3 stimulates the in vitro growth of cell lines from long-term bone marrow cell cultures and induces the proliferation of mast cell cultures. Il-3 also induces histamine synthesis by normal spleen or bone marrow cells in vivo, an event which is required for mast cell differentiation. Additionally, Il-3 promotes the proliferation and differentiation of bone marrow stem cells in vivo and in vitro to granulocytes, macrophages, basophil/mast cells (Ihle et al., 1983), megakaryocytes (Metcalf et al., 1975), erythroid cells (Johnson and Metcalf, 1977), and neutrophils (Hara, 1978).

The interleukin-3 cDNA was isolated using mRNA from the mouse T-cell clone Lyt 1+2-/9, which produces Il-3 upon Con A stimulation. The full length cDNA clone is 1000 bp in length, and contains a single open reading frame. The amino terminal segment of the predicted Il-3 sequence is hydrophobic, as it would be expected for a signal peptide (Yokota et al., 1984). The genomic *Il-3* gene is 8 kb in length, and is present as a single copy in the mouse genome. The gene consists of 5 exons and 4 introns. The consensus sequences for splicing junctions, G-T, and A-G, were found at the boundaries of all four introns. The lengths of introns 1, 3, and 4 are 93 bp, 136 bp, and 123 bp, respectively; intron 2, the longest, is 994 bp. Exon 1 could encode 55 amino acids, and contains the untranslated region; exons 2, 3, and 4 encode 14, 32, and 14 amino acids, respectively. Exon 5 is 480 bp long, and it encodes 51 amino acids, and contains the 3' untranslated region.

C. Interleukin-3 and T-cells

Interleukin-3 was initially described as a factor that induced the expression of the enzyme 20-α hydroxysteroid dehydrogenase (20αSDH) in cultures of splenic lymphocytes from athymic mice (Ihle et al., 1981). The ability of Il-3 to induce the above-mentioned enzyme, and the Thy-1 cell-surface antigen, (both markers thought at the time to be specific for mature T- cells), indicated that Il-3 might be involved in T-cell differentiation. However, the subject became controversial when it was discovered that other hematopoietic cells also expressed these two markers (Basch and Berman, 1982; Garland and Dexter, 1982; and Schaffar-Deshayes et al., 1983). It was later suggested that Il-3 induced the proliferation of an immature subpopulation of cells capable of expressing 20-αSDH and Thy-1 that was present in the bone marrow of both normal and athymic mice; it was suggested by the authors that this cell subpopulation could be either an early hematopoietic/lymphocyte stem cell or the precursor to thymocytes (Keller et al., 1985).

Several groups have given support to the hypothesis that Il-3 induces the proliferation of pre-T lymphocytes. Pre-T cells in the bone marrow of nude mice of the cell surface phenotype $TCR^{+}\delta\gamma^{+}$ carried Il-3 receptors (Sideras and Palacios, 1987) and proliferated in response to Il-3 (Palacios et al., 1987). Other groups have been able to maintain pre-T cell cultures only in the presence of Interleukin-3. Chervernak and Altazan (1990) were able to grow bone marrow resident pre-T cells from AKR/C mice in cultures containing Il-3. These cells, when reintroduced into mice, developed into normal T-cells; that is, they expressed Il-2 receptors in early stages of development and subsequently differentiated into $CD3^{+}CD4^{+}$ single positive or $CD3^{+}CD8^{+}$ single positive developmental stages (Chevernak et al., 1991, Soloff et al., 1991). Ueno et al. (1985) showed that Il-3 increased the proliferation of $CD3^{+}$ human thymocytes, but when the

cultures were depleted of the $CD3^+$ population, Il-3 continued to maintain the proliferation and survival of the remaining thymocytes, suggesting that an immature $CD3^-$ subpopulation of thymocytes can respond to Il-3. Interleukin-3 can also support the growth and survival of T-cell leukemias in culture. O'Connor et al. (1991) derived cell lines from T-acute lymphoblastic leukemias, and several of the cell lines could grow in vitro only in the presence of Il-3. Those cells were early pluripotent stem cells that could differentiate into either the myeloid or the lymphoid lineages.

The subject of whether Il-3 induces proliferation and/or differentiation of mature T-cells still remains controversial. Several groups have not found any effect of Il-3 on the proliferation of mature T-cells (Kimoto et al., 1988; Ueno et al., 1989; Palacios et al., 1987; Sideras and Palacios, 1987; Santoli et al., 1988; and Keller et al., 1985). Other groups, however, propose Il-3 as a differentiation and growth factor for mature T-cells. Londei et al., (1989), reported that Il-3 stimulates the proliferation of a rare subpopulation of T-cells of the $CD4^-CD8^-\alpha\beta^+$ surface phenotype. In a C57BL/6 mouse thymus culture system, Wood et al. (1990) observed that Il-3 induced halving of the $CD4^+CD8^+$ T-cell population and doubling of the $CD4^+CD8^-$ mature T-cells. Frasca et al. (1992) irradiated (C57BL/10 x DBA/2)F1 mice with x ray doses ranging from 1 to 4 Gy, and then injected Il-3 subcutaneously for 5 days. The irradiation caused the percentage of $CD4^-CD8^-$ immature T-cell subpopulation to double, the percentage of $CD4^+CD8^+$ to be markedly reduced and both the percentage of mature CD4 and CD8 single positive subpopulations of thymocytes to reduce in numbers. When Il-3 was injected, it caused the double negative subpopulation of thymocytes to be reduced to normal levels, and it caused both the CD8 and the CD4 single positive subpopulations to increase to normal levels. They suggested that the mature T-cell recovery was accelerated by the Il-3 stimulation of immature, double negative subpopulations of thymocytes, which subsequently differentiated into mature CD4 and CD8 single

positive cell populations. Although Santoli et al. (1987) did not find any effect of Il-3 on normal, peripheral human T-cells, Il-3 did potentiate the Il-2-driven proliferation and long-term growth of mature T-lymphocytes; Il-2 and Il-3 supported predominantly the growth and viability of $CD4^+$ T-cells; these results suggested a synergism between Il-2 and Il-3 in the proliferation and survival of mature T-cells. Since it was found that Il-3 binds endothelial cells in the thymus, but not thymocytes (Metcalf and Nicola, 1988), it has been suggested that the effect of Il-3 on thymocyte differentiation is a consequence of Il-3 modulation of accessory cell types within the thymus (Frasca et al., 1992).

D. Interleukin-3 and Leukemogenesis

Myeloid leukemia is characterized in the mouse by the enlargement of the spleen and lymph nodes, and by the presence of undifferentiated myeloid cells in the peripheral blood. In these tissues, a collection of immature granulocytic cells is found, which are responsible for the invasion of the capsule of lymph nodes and fatty tissues (Green, 1966).

Spontaneous myeloid neoplasias are uncommon in mice. Myeloid leukemias can be induced by viruses (e.g. Ableson MuLV) (Furmaski and Rich, 1982), by chemical carcinogens like polycyclic hydrocarbons, nitrosoureas and nitrosamines (Furmaski and Rich, 1982); and by physical agents such as x rays (Jenkins and Upton, 1963). Both chemical agents (Furmaski and Rich, 1982) and physical agents (Jenkins and Upton, 1963; Parsons et al., 1962) have been shown to induce the expression of endogenous retroviral elements related to murine leukemia viruses. Jenkins and Upton (1963) reported that 40% of male RFM/Un mice that received 150-450 rads of x rays developed myeloid leukemia, and that males developed the disease more frequently than females. These leukemias could be induced in other mice of the same strain by

injecting supernatants from a cell-free spleen cell extract or blood plasma of leukemic animals; those extracts were previously shown to contain type-C retroviruses (Parsons et al., 1962). Tennant et al. (1983) isolated a similar retrovirus particle (RFV) from RFM mice, and demonstrated that this retrovirus was expressed at low levels in the hemopoietic tissues of RFM mice throughout their life span, and the retrovirus was also expressed in thymoma and myeloid leukemia cell cultures. This virus from the single ecotropic type-C proviral locus of RFM/Un mouse was isolated and characterized by Liou et al. (1983), and it was later demonstrated that additional ecotropic proviral copies were present in hematopoietic neoplasms induced by radiation (Boone et al., 1986).

Several observations have suggested that Il-3 may play a role in the genesis of leukemia. Lee and Ihle (1981) observed that lymphocytes from preleukemic mice inoculated with Moloney murine leukemia virus showed an increased response to partially purified Il-3; they suggested that this lymphokine-dependent proliferation of lymphocytes is one of the premalignant steps in leukemogenesis. Later, Shrader and Crapper (1983) developed cell lines from bone marrow requiring Il-3 for growth, and had mast cell morphology. These cells were non-leukemogenic when transplanted into mice. However, cell clones were derived from those cells that grew without the presence of Il-3 in the medium, which produced Il-3 constitutively. This is supported by Northern gel blot analyses which showed that the Il-3 gene was transcriptionally active in those Il-3 independent cell lines (Shrader et al., 1985). These cells were tumorigenic in vivo, suggesting that the autogenous production of Il-3 is one of the steps for the malignant transformation of bone-marrow derived cells. In human acute myeloid leukemia, there is a deletion in the long arm of chromosome 5, at a region that contains the genes for Il-3, Il-4, Il-5, and GM-CSF; this deletion is associated with a preleukemic state, called myelodysplastic syndrome; 30% of the patients ultimately develop acute myelogenous leukemia (Le Beau et al.,

1989); whether any of these genes are directly associated with the myelodysplastic syndrome is still not known (Clark, 1991). Some leukemic cell lines, such as cell lines derived from patients suffering from AML and from patients suffering from chronic myelogenous leukemia require Il-3 for growth and survival (Clark, 1991; Delwel et al., 1988).

Another observation made by Shrader's group was that when frozen stocks of two different myeloid leukemias from mice infected with Ableson-MuLV grew in culture, they were Il-3 independent and gave rise to myeloid leukemias when transplanted back into mice. The cells also became Il-3 independent by the autogenous production of Il-3. These experiments were the first to suggest that in vivo, the aberrant activation or overexpression of the *Il-3* gene may be a frequent and common pathway in the genesis of myeloid leukemia in the mouse (Shrader et al., 1985).

Based on the previous observations, Wong et al. (1987) derived non-tumorigenic, bone marrow-derived, Il-3 dependent cell lines and transfected them with an *Il-3* recombinant retrovirus. As expected, the cells became Il-3 independent. When injected into nude (nu/nu) mice they gave rise to tumors. However, when normal bone marrow stem cells were transfected with the same recombinant retrovirus and then transplanted into nu/nu mice, they did not give rise to tumors. These observations demonstrate that Il-3 dependency is one step for the development of tumorigenesis, but that previous steps are needed for the malignant state to occur. In other words, Il-3 independence is not the sole step to convert a normal cell into a malignant one.

One of the mechanisms by which hemopoietic cells become Il-3 independent is by the insertion of a retrovirus. The constitutive production of Il-3 in the myelomonocytic leukemia cell line WEHI-3H was due to the insertion of a retrovirus-like Intracysternal-A particle in the 5' region of the *Il-3* gene. The LTR of the retrovirus caused the transcription of the *Il-3* gene resulting in its constitutive synthesis (Ymer et al., 1984). In this laboratory Yang et al.

(unpublished results) it was found that in a RFM/Un myeloid leukemia cell line, an element of approximately 6.1 kb, possibly a retrovirus-like element was inserted in a 0.7 kb region containing the fourth and fifth exons at the 3' end of the *Il-3* gene.

Based on the observations that acquisition of Il-3 dependency was the final stage to the conversion from a preleukemic state of leukemic cell lines that needed Il-3 to grow, to the fully leukemic state, Oliff et al. (1984) proposed the autocrine hypothesis for leukemogenesis, which involves three steps: 1) The immortalization of a bone-marrow cell to give rise of cells dependent on Il-3 for growth. 2) The autonomy and tumorigenesis through the autogenous production of the growth factor, and 3) The progression of the tumor.

E. Specific Aims

The general objective of this work was to determine the effect of the pBOR-Il-3 transgene on the hematopoietic cell populations of the pBOR-Il-3 line of mice. Another objective of this dissertation was to determine whether chemical and physical agents agents can modulate the expression of the Il-3 transgene, by acting on the long terminal repeat (LTR) which controls the expression of the transgene; two agents, one chemical (azacytidine), and one physical (x ray), were used to activate the transcription of the Il-3 transgene. Particular attention was placed on the effects of the Il-3 transgene on the growth and differentiation of the azacytidine-induced thymic lymphomas. Several molecular techniques were used to determine a) whether the Il-3 transgene was expressed in the azacytidine-induced thymic lymphomas; b) the clonality of the thymic lymphomas; and c) the state of differentiation of the thymic lymphoma cells.

CHAPTER II

Development of the pBOR-Il-3 Transgenic Mouse Lines

A. Introduction

1. Microinjection of FVB Embryos with the Il-3 Transgene

A transgenic line was produced in our laboratory by Dr. P. Hoyt and Dr. T. Savin by the pronuclear microinjection technique (Hogan et al., 1986). Briefly, females FVB mice were superovulated and then mated; their fertilized eggs still showing separate male and female pronuclei were collected from the ampulae of the oviducts; then 1 to 2 pL containing 200-400 DNA molecules/pl (1-2μg/μL) of the pBOR-Il-3 DNA construct were microinjected into the male pronucleus of each fertilized egg; and, after overnight incubation and development into the 2-cell stage, these eggs were implanted into the uterus of a pseudopregnant $B_6C_3F_1$ female mouse (a female mated to a vasectomized sterile male, so implantation of the fertilized embryo could occur), for subsequent development into mice that contain germline integrations of the pBOR-Il-3 DNA construct.

2. Structure of the Transgene

The pBOR-Il-3 recombinant plasmid DNA used for microinjection was constructed by Dr. L.Y. Ch'ang as described in the following. The mouse interleukin-3 cDNA was originally isolated by Yokota et al., (1984). The cDNA was restricted using the *XhoI/NcoI* restriction enzymes, and was inserted by ligation into the *NcoI/XhoI* sites of the pBOR retroviral vector. The product of this ligation contains the following elements in the order of 5' to 3' position: the B5 5' LTR; primer tRNA binding site (PBS); splice donor (SD) site-retroviral packaging signal (psi);

splice acceptor (SA) sequences, all derived from the pWNB5 clone of a BALB/c mouse spleen-derived retrovirus, WN1802B (Boone et al., 1983); the *Il-3* cDNA; the 3' LTR that is a composite of the B5 LTR enhancer sequence and the LTR promoter sequence derived from AL10, a polytropic endogenous proviral clone of BALB/c mouse liver chromatin origin (Ou et al., 1983); and the a 3' cellular flanking sequence of the AL10 clone, all of which are inserted in the pUC19 plasmid. For microinjection, pBOR-Il-3 plasmid DNA was linearized by cutting the *EcoR1* site within the polycloning site of pUC19 and purified extensively.

B. Materials and methods

1. Screening for Positive Mice and Establishment of the Il-3 Transgenic Line

a) Tail DNA extraction method

The albino FVB/N mice born from dark brown haired $B_6C_3F_1$ mothers implanted with pBOR-Il-3 microinjected 2-cell embryos were examined after weaning for the presence of the transgene by a procedure described by Hogan et al., (1986) and modified by P. Hoyt. Briefly, about 2 cm of tail removed from each mouse was minced and digested at 50°C overnight in a solution containing 500 ng/mL pronase, 50μg/μL proteinase K, 0.5% SDS, 5mM EDTA and 10mM Tris-Cl, pH 7.6; the digest was extracted, first in an equal volume of Tris-saturated phenol, pH8, and centrifuged in a Beckman model 12 centrifuge at 12,000 rpm for 15 minutes; the supernatant was re-extracted with an equal volume of a 50:50 mixture of phenol/chloroform and centrifuged at 12,000 rpm for 10 minutes; after a final extraction with an equal volume of chloroform, the mixture was centrifuged at 10,000 rpm for 5 minutes, and the DNA was precipitated from the aqueous phase by adding 2 volumes of 100% ethanol; after remaining at room temperature for >40 minutes, the precipitated DNA was spun down at 12,000 rpm for 15

min, washed with 70% ethyl alcohol, and vacuum dried; and the pellets were dissolved in 100 uL Tris/EDTA buffer (10 mM Tris-Cl/1 mM EDTA).

b) Dot blot hybridization for the initial screening of the mice

Dot blot hybridizations were performed to detect the presence of the *Il-3* transgene sequence in the DNA tail preparation. Five uL of the tail DNA was mixed in a well of a microtiter dish with 5 uL of a denaturing solution (0.4M NaOH, 1M NaCl); 5 uL of the mixture was spotted onto a Zetabind membrane (Cuno, Ohio). The membrane was then dried in a vacuum oven for 45 minutes at 80°C.; it was washed for 1 hour at 65°C in 0.2X SSC/0.5% SDS in a Robbins Scientific hybridization rolling machine/incubator at constant rolling; and prehybridized in a buffer containing 0.5 M $NaPO_4$ (Church and Gilbert, 1984). Then, radioactive [^{32}P] *Il-3* cDNA (Yokota et al., 1984), labeled using either the nick translation (Maniatis et al., 1982) or the random priming method (Manheim-Boehringer protocol) to a specific activity ranging from $1x10^8$ to $1x10^9$ cpm/μg; and denatured by heating at 100°C for 10 minutes; was mixed with 10 ml of prehybridization solution and hybridized with the membrane overnight. The membrane was then washed to remove free unhybridized probe as the following: first, 4 times for 15 minutes each with 2X SSC/0.1% SDS at room temperature, and then 3 times for 1 hour each at 63°C with 0.2 X SSC/0.5% SDS. The washed membrane was dried and exposed overnight to X-ray film.

c) Southern gel blot hybridization for the screening of the Il-3 transgene

The animals that tested positive for the transgene by the dot blot technique were called founders and were mated to FVB wild type mice to produce F1 progeny. The F1 progeny mice that tested positive (again, by using the Dot Blot technique) were brother/sister mated to produce F2 mice homozygous for the *Il-3* transgene. The homozygous state of Il-3 transgenic mouse lines was maintained by brother/sister mating of the positive F2 mice.

The positive animals have been screened for homozygosity, using the Southern Blot technique (Southern, 1975). The genomic DNA was cut with *BamH1* using the Biolab or BRL protocols: 20 units of the enzyme per 10 ug of the tail DNA preparation, in buffer containing 10mM Tris-HCl, 10mM MgCl, 50mM NaCl, and 1mM DTT; the reaction was incubated overnight at 37°C, and electrophoresed in a 0.8-1% agarose/Tris-Borate-EDTA gel at 40-50 volts overnight; the gel was stained with ethidium bromide, photographed, destained, treated with 0.4M NaOH-1M NaCl solution to depurinate the DNA and facilitate transfer, and transferred by the Southern blot technique (Southern, 1975) to a Zetabind nylon membrane overnight. The membrane was then washed in 6X SSC for 20 min., U.V. irradiated for 2.5 min., dried in a vacuum oven for 30-60 min., prehybridized, hybridized, and washed using the method described above for the Dot Blot technique. Homozygosity or heterozygosity was judged by the intensity of the 5.2 Kb band in the Southern Blot (homozygous animals show double the intensity of the heterozygous counterparts) and then tested for homozygosity by backcrossing to an FVB wild-type mouse; if 100% of the progeny were positive for the transgene, then the F2 or F3 parent was judged to be homozygous for the transgene.

d) Quantitative dot blots for the screening of homozygous lines

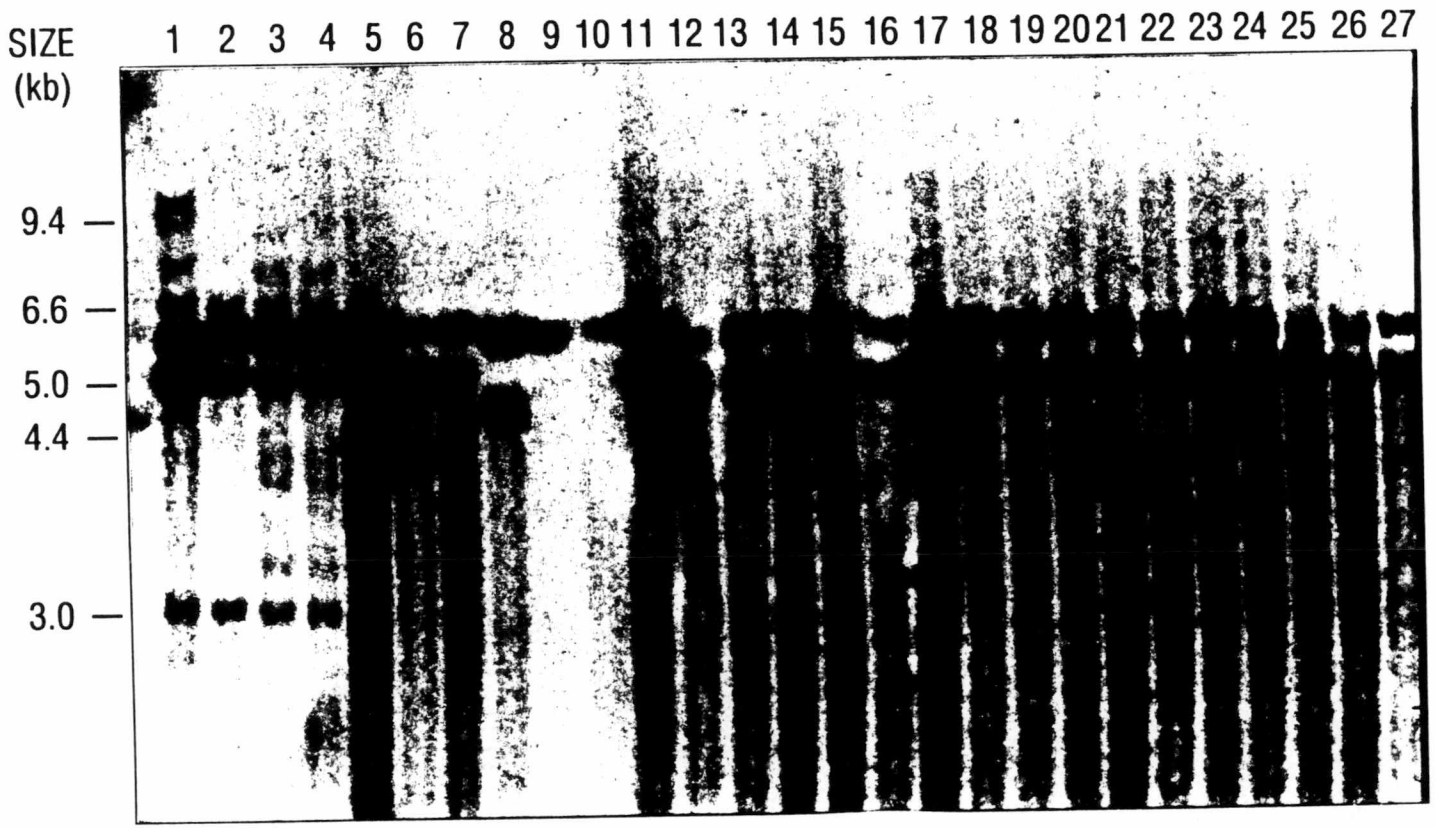
This technique is similar to the Dot Blot technique, except that the A_{260} of the tail DNA was determined, and 10 μg of DNA was diluted in 10 μL of denaturing solution. Then, 5 μL of the solution (5 μg of DNA) was spotted into the nylon membrane; the rest of the procedure followed as described in the Dot Blot technique.

C. Results

Of a total of 284 FVB/N mouse eggs microinjected with the pBOR-Il-3 DNA vector, a total of 136 reached the two-cell embryo stage following overnight incubation. Of the 90 2-cell embryos transplanted into surrogate mother mice, 8 newborn mice were obtained, of which 4 tested positive for Il-3 sequences. Of these 4 founder mice one showed runted growth, one gave no progeny with FVB/N mates; only 2 (Il-3-12 and Il-3-15) proved to be fertile; the Il-3-15 line was subsequently lost, probably due to chromosomal instability at the integration site (Wang et al., 1993). Figure 2.1 shows one of the autoradiograms for determining whether the mice contained the *Il-3* transgene and whether the mice were homozygous or heterozygous with respect to the *Il-3* transgene locus. Only DNAs from *Il-3* positive mice, detected by the dot blot hybridization procedure were loaded into this gel. Lanes 1-4 represent the founders' DNA samples. The figure also shows the DNA samples of F1 progeny from matings between founder (*Il-3* positive animals) and non-transgenic FVB mice (lanes 5-8 and 11-13 for the progeny of the *Il-3* founder). Mating pairs were setup between F1 males and females positive for the 5.2 kb band; in this case, Il3-12 # 10 (a female, lane 11) and Il3-12 # 18 (a male, lane 7) were mated. The F2 (lanes 14 to 27) should contain 25% homozygous mice, if the transgene represents a single autosomal Mendelian locus. The F2 mice whose DNAs showed a 5.2 kb band with double intensity as compared to DNAs of the other positive litter mates were selected and bred. The possible homozygous mice according to this autoradiogram were Il-3-12 F2 # 11 (lane 15), Il-3-12 F2 # 13 (lane 17), Il-3-12 # 16 (lane 20), and Il-3-12 # 18 (lane 22), the first two being females, and the two others being males.

Il-3-12 F2 # 11 and 18 were chosen for the mating and their F3 progeny were screened by the quantitative dot blot technique. It was found this particular mating was that only the female

Figure 2.1. Southern Blot analysis for the screening of pBOR-Il-3 mice. Genomic DNA was restricted using the *Bam Hl* restriction enzyme, electrophoresed, and hybridized to an Il-3 cDNA, as described in Materials and Methods. Lanes 1-4 represent the founder DNAs, (pBOR-Il-3 F0 # 12, 13, 14, and 15), lanes 5 to 8 represent the pBOR-Il-3 F1 generation (pBOR-Il-3 F-1 # 10, 14, 18, and 20), lane 9, pBOR-Il-3-15 F1 # 10, lane 10, pBOR-Il-3-13 F1 # 31, lanes 11-13, pBOR-Il-3-12 F1 # 24, lanes 14-23, pBOR-Il-3 F2 (10x18) # 10-19, and lanes 24-27, pBOR-Il-3-12 F2 (14x20) # 20, 21, 22, and 26.


SIZE
(kb)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
9.4
6.6
5.0
4.4
3.0

progeny of the F3 generation could be homozygous; while all positive F3 male progeny remained heterozygous. Therefore, all of the positive F3 males were back-crossed with wild type FVB/N female mice. Of 47 progeny mice obtained by such back-crossings, all 35 females were positive, whereas all 12 males were negative, suggesting that the transgene of this Il-3-12 transgenic mouse line was not following an autosomal segregation, but rather a sex-linked segregation (i.e. integration of the pBOR-Il-3 transgene in the X-chromosome). To further test this point, we mated homozygous F3 Il-3 females with the heterozygous F3 Il-3 males, and screened the progeny using the quantitative dot blot technique. Of the 26 progeny mice obtained and screened, all 16 females were found to be homozygous, with all 10 males hemizygous, thus establishing the fact that the particular Il-3 transgene of the Il-3-12 line had been integrated in the X-chromosome. Thereafter, the Il-3-12 transgenic line was maintained by mating of the homozygous females and the hemizygous males. The animals were observed for a period of 4 to 6 months, and they did not show any obvious signs of sickness, being physically similar to the FVB/N controls. Neither the FVB/N nor the pBOR-Il-3 mice developed any spontaneous tumors during the observation period; the fact that the transgene was expressed at low levels in the Il-3-12 mouse thymus was later confirmed by the use of the RNA/PCR technique (Chapter V).

D. Discussion

A pBOR-Il-3 # 12 transgenic mouse line has been obtained by the pronuclear microinjection method, and subsequent breeding for several generations to establish the homozygosity of a single locus. The progeny mice of this line that carried the transgene in their germline were viable and fertile. This particular line of transgenic mice is interesting because of the following features: it contains an ecotropic murine leukemia virus long terminal repeat, which

has the propensity of becoming largely silent following integration into the mouse germline; and hence activation and/or enhancement of the expression of the transgene (driven by the LTR) may be used as a test for genetic effects of chemical or physical agents. Another aspect that can be studied by using this transgenic mice line is how the activation of the LTR and the expression of the Il-3 transgene affects the hemopoietic system of the mice, and how expression of the Il-3 transgene affects the behavior of tumors that are induced in the mouse. The observation that the non-treated pBOR-Il-3 # 12 mice raised under normal laboratory conditions did not show any predisposition any spontaneous tumors is in contrast to results obtained by two groups that used retroviral vectors to produce somatic Il-3 transgenic mice (Wong et al., 1989; Chang et al., 1989). Chang et al. (1989) lethally irradiated C57BL/6JWEHI and DBA/6J using 9 to 9.5 Gy of γ rays; the animals were injected intravenously with fetal liver cells or with bone marrow cells from syngeneic animals 4 days following fluorouracil treatment; these cells had previously been infected with Mo-MuLV-Il-3-containing retroviral particles. Of the 25 animals treated using the above-mentioned protocol, 20 became moribund within 5 weeks, and the remaining animals died within 5 months after the treatment; the major symptoms of the moribund animals included tremor, partial paralysis of the hind limbs, and infected ears. The spleens of most of the animals were pale and enlarged. In contrast, we did not observe any of the above-mentioned symptoms in our line 12 Il-3 transgenic mice. Given the observation that neither the FVB/N nor the particular Il-3 transgenic mice developed spontaneous tumors or the myeloproliferative syndrome during the observation period, this would suggest that the Il-3 transgene in these mice was not being expressed or was being expressed at low levels. Even though there were no obvious physiological or pathological differences between FVB/N and Il-3 mice, different techniques were used, as will be described in chapter III of this dissertation, to determine whether there are any difference between subpopulations of the hematopoietic systems of the Il-3 transgenic mice and

FVB/N mice. Studies on the response of transgenic Il-3 mice to physical agents such as x rays, or to chemical agents, such as 5-azacytidine, are presented in chapters IV and V. Of special interest is whether any particular type of tumor, e.g. myeloid leukemia, a cancer in which Il-3 overexpression is thought to be one of the transformation steps is induced, and whether Il-3 transgene expression either leads to the formation of the tumor or influences its growth after genetic changes leading to transformation are activated.

CHAPTER III

Effects of the pBOR-Il-3 transgene on hematopoietic subpopulations

A. Effects of the pBOR-Il-3 transgene in hematopoietic stem cells

Following the discovery and purification of Il-3, it was observed that Il-3 was required for the growth of a series of cell lines derived from long-term bone marrow cultures (Ihle et al., 1982). Experimental evidence implied that Il-3 promotes the differentiation of pluripotent stem cells (CFU-S) that exist in spleens of nu/nu mice, in normal bone marrow (Ihle et al., 1983), and in suspension cell cultures of fetal liver cells (Hapel et al., 1985). Il-3 stimulates the clonal proliferation of early (BFU-E) and late (CFU-E) erythroid progenitors, granulocyte, macrophage/eosinophil (CFU-GM or -C), megakaryocyte and natural killer cell progenitors (Hapel et al., 1985). In recent years, the cell surface antigens of hematopoietic stem cells have been elucidated (Ikuta et al. 1992). The cell surface phenotype of the hematopoietic stem cells was determined to be Thy-1^{lo}, Lin^{-}: (lineage minus means that the cells do not contain the following antigens: TER-119 [erythroid]; B220, [B-lymphoid]; Mac-1 [monocyte/macrophage/granulocytic]; Gr-1, [granulocyte], CD4 and CD8, [T-cells]) and Sca-1^{+} (van de Rijn et al., 1989). Another group, however, has discovered a new pluripotent stem cell population with the following cell surface phenotype: Thy-1^{lo}, Lin^{-}, Sca-1^{-}, Fall-3^{+} (Muller, 1991). It has become possible to utilize specific antibodies to various cell-surface antigens for measurement of these hematopoietic cell populations in pBOR-Il-3 line 12 transgenic mice.

1. Materials and methods

a) Cell-surface antigens of hematopoietic cell lineages

Fall-3. Fall-3 is a novel stem cell antigen expressed in a bone marrow cell population which includes most (if not all) pluripotent stem cells. The Fall-3$^+$ subpopulation is able to rescue and repopulate lethally irradiated mice. A subpopulation of the Fall-3$^+$Lin$^-$ cells also carries low levels of the Thy-1 cell surface antigen present on hematopoietic stem cells (Muller-Sieburg, 1991).

Thy-1.1. The Thy-1 antigen is a glycoprotein found on the cell surface of rodent thymocytes (Campbell et al., 1981). It was later found that this antigen is also detected in bone marrow cells, such as multipotent stem cells that give rise to myelocytes, lymphocytes, and erythrocytes (Basch and Berman, 1982). The Thy-1^{lo} Lin$^-$ cells comprise 0.1-0.3% of the bone marrow cell population, which has the capacity to completely repopulate the hematolymphoid system of the mouse after lethal irradiation (Spangrude et al., 1988).

Pgp-1 (CD44). Pgp-1 is expressed by the thymic progenitors of T-lymphocytes (Hyman et al., 1986) and by bone marrow stem cells (Trowbridge et al., 1982). The Thy-1^{lo} Lin$^-$ Sca-1$^+$ fraction of bone marrow cells (the pluripotent stem cells) is of a Pgp-1^{int} phenotype (Spangrude et al., 1989).

F4/80. F4/80 antibody reacts with tissue macrophages and blood monocytes. The F4/80 antigen is not present on the surface of neutrophils or lymphocytes (Lee et al., 1985, Austyn and Gordon, 1981). CFU-C and CFC progenitors in bone marrow lack antigen F4/80, but give rise to F4/80 positive macrophages in culture. In vivo, F4/80 antibody binds to 6% of bone marrow cells and expression of F4/80 increases as the monocyte matures into a macrophage (Hirsch et al., 1981).

Gr-1. Antibody to Gr-1 reacts with an antigen expressed on mature granulocytes

(Holmes, 1986).

Mac-1 (CD11b). Mac-1 antibody recognizes the M1/70 antigen found at low levels on mature granulocytes, in moderate amounts on spleen and blood monocytes, and at high levels on macrophages; thus, M1/70 antigen defines a differentiation antigen on mononuclear phagocytes and granulocytes, the expression of which is increased during monocyte maturation (Springer et al., 1979).

b) Three-color flow cytometric analysis of bone marrow stem cells

Six 3-months-old FVB/N and six Il-3 mice living in a conventional mouse facility were selected for the study. The animals were killed by cervical dislocation; the two tibias and two femurs were removed and the bone marrow was flushed out (using 1 ml of PBS^- [without calcium and magnesium] in a 1 cc, 27 1/2 needle) into a Falcon tissue culture dish; one more mL of PBS^- was used to reflush the bone marrow and to make sure all the bone marrow cells were collected. The cell suspension was centrifuged at 1000 rpm for 10 min. at 4◦C and resuspended in 2 mL of SPBS (calcium-magnesium-free PBS containing 0.1% BSA, and 0.1% sodium azide). A 1:100 dilution of resuspended cells was made for cell counting; then, 10^6 bone marrow cells were collected in a microfuge tube.

The antibody reactions were carried out as follows: tube number one contained bone marrow cells with no antibody; tube two contained cells stained with Pgp-1-FITC (fluorescein isocyanate); tube three contained bone marrow cells stained with Thy-1-PE (R-phycoerythrin); cells in tube 4 were stained with Fall-3-(IgM-biotin-streptavidin-cychrome), and tube 5 contained a cocktail of antibodies to isolate the stem cells from the mature bone marrow cells; [it contained Mac-1 (Ig_{L+H}-PE) or Mac-1-FITC, Thy-1.1-PE, Pgp-1-FITC, and Fall-3 (Igm-Biotin-streptavidin-cychrome)].

All antibodies were purchased from Pharmingen (San Diego, California), except Thy 1.1

and F4/80, purchased from Bio Source International, Camarillo, California). The reactions were carried out in the following order: the antibody against Mac-1 (5 μL) was added first and the cell suspension was incubated for 15 min. and the cells were then washed once; 2 μL of the $Ig_{(L+H)}$-PE antibody were added and the cells washed three times; the Fc receptor antibody was added (0.25 $\mu g/10^6$ cells) and the cells were incubated for 3 min. (in later experiments instead of the indirect labelling procedure Mac-1 antibody directly conjugated to FITC was bought from Pharmingen and used for direct labelling); 100 $ng/10^6$ cells of Fall-3 antibody was added, the cells were incubated for 15 min. and washed once; 2 μL of IgM-Biotin were added, the cells was incubated for 15 min. and washed once; and finally Thy-1.1-PE (4 μL), Pgp-1-FITC (10 μL), and streptavidin-cychrome (8 μL) were added. All the reactions were then washed with 1 mL of SPBS each wash followed by centrifugation at 1,000 rpm, 4°C, for 10 min. The antibody-labeled cell preparations were analyzed in a FACSTAR-Plus flow cytometer.

c) <u>In vitro</u> colony assays

Hematopoietic colony forming assays were done using a semisolid methylcellulose medium supplemented with 3 units of erythropoietin/mL and PWM-CM (pokeweed mitogen spleen cell conditioned medium) at a final concentration of 1%. This medium was purchased from Terry Fox Laboratories (Vancouver, Canada). All cells were maintained at 37°C in a 5% CO_2 humidified incubator. For CFU-E (colony-forming units-erythroid) determination, cells were plated in 0.1 mL aliquots containing $5x10^4$ cells, and were scored on day 2 using an inverted microscope at a 100X magnification; only small colonies which contained hemoglobin and at least 8 cells, were counted. For measuring BFU-E (burst-forming units-erythroid) and CFU-C (colony-forming units-granulocyte and macrophage), bone marrow cells were plated at $5x10^4$ cells/mL in 1 mL aliquots, and colonies were scored on days 7-9. Data are presented as the mean of the number of colonies in $5x10^4$ cells$\pm$standard deviation.

2. Results

Table 3.1 presents the results obtained from the flow cytometric analysis of bone marrow cells with the following cell-surface phenotype: Thy-1^{low}Pgp-1^{int}Fall-3^{high}. According to the results, there is no difference between Il-3 and FVB/N group in this population. Also shown in the table are the results of the methylcellulose assay, done to determine whether there were differences in progenitor populations more differentiated than the stem cell, such as the CFU-E, the BFU-E, and the CFU-C; and again, there are no apparent differences in these cells.

B. Effects of the pBOR-Il-3 transgene on differentiated hematopoietic cells

1. Introduction

Besides the in vitro biological activities of Il-3, Il-3 also has multiple in vivo effects. When 3-month old C57BL mice were injected with recombinant Il-3 (Metcalf, 1986), the blood eosinophil levels increased, minor but less constant rises were observed in the neutrophil levels and no significant changes were observed in lymphocyte, reticulocyte, or hematocrit levels in the blood. In the spleen, increases in immature granulocytes, eosinophils, nucleated RBCs, megakaryocyte and immature mast cells were observed. In view of these results, studies were done to determine whether the presence of an Il-3 had any effect on the mature hematopoietic cells in the spleen, bone marrow and blood of the line 12 pBOR-Il-3 transgenic mice. The same studies were performed with the FVB/N control mice.

2. Materials and methods

a) Total white blood cell counts and blood smears. Two- and 3-month-old Il-3 and FVB/N mice were bled from the orbital sinus with the use of glass Pasteur Pipettes (5 3/4 inches) and

Table 3.1

Comparison between the primary hematopoietic cells of 5-month-old FVB/N and pBOR-Il-3 mice.

Line	Thy-1^{low}Pgp^{int}Fall-3^{high} [a]	CFU-E[b]	BFU-E	CFU-C
FVB/N	0.34±0.4[c1]	179.4±79[c2]	14.9±5.8[c3]	73.9±17.6[c4]
pBOR-Il-3 [1]	0.21±0.1	172.0±63.3	11.3±3.6	57.1±6.9

[1]

a. The numbers represented in the Thy-1^{lo}Pgp-1^{int}Fall-3^{+} column are expressed in number of cells times a million.

b. The numbers represented in the CFU-E, BFU-E, and in the CFU-C columns represent the number of colonies counted per sample (in $5x10^4$ bone marrow cells)±standard deviation.

The following numbers represent probability values resulting from the ANOVA comparison between FVB/N and pBOR-Il-3 mice.

c1=0.12

c2=0.89

c3=0.3

c4=0.4

the blood was immediately placed in BD microtainer capillary blood collector tubes containing 5 uL of 1.5% EDTA to avoid coagulation of the blood. Total white blood cells (WBC) and red blood cells (RBC) were counted in an Ortho ELT-8WS hematology analyzer and the differential counts of lymphocytes, granulocytes and monocytes were done with the use of an Ortho Diagnostics system 2140 analyzer, designed specifically to analyze mouse blood. In addition, further analyses were made from blood smears prepared from six 5-month-old FVB/N and eight 5-month-old Il-3 mice. Each mouse was bled from the eye, a drop of blood was placed on a # 1 coverslip, and the blood was spread with another coverslip. The blood on the coverslip was then air-dried, and then stained with Wright's stain. The Wright's stain (Fisher) was added to the coverslip (the number of drops necessary to cover the surface of the coverslip was recorded) and left for 2 minutes to fix (by the alcohol in the staining solution), as well as to stain the blood cells. Wright's stain buffer (6.63 g monobasic potassium phosphate, 3.2 g dibasic sodium phosphate, pH 6.4) was subsequently added (2 drops of buffer per each drop of Wright's stain), and left for 4 minutes. After that, the coverslips were washed with water and air dried. For each coverslip 500 white blood cells were counted, under a light microscope at a 400X magnification, to determine the relative numbers of basophils, eosinophils, neutrophils, monocytes and lymphocytes in the blood.

b) Flow cytometric analysis of the mature granulocytes and macrophages in the spleen and bone marrow of FVB/N and pBOR-Il-3 mice

Single cell suspensions were prepared from bone marrows and spleens of six 5-month-old FVB/N and six 5-month-old pBOR-Il-3 mice; the bone marrow cell suspensions were done as already described previously. Single cell suspensions of the spleen made by injecting 1 mL of PBS through the middle of the spleen and then teasing the tissue; the spleen cells were then passed through a 27 1/2 gauge needle to break up the remaining pieces of tissue. One million

cells of bone marrow or spleen were placed in 1.7 mL Eppendorff tubes, and the following cell surface marker reactions were performed: tube no.1 contained non-stained cells, tube no. 2 contained 0.25 ug FcR gama II plus F4/80 (10 uL per 10^6 cells), tube no. 3 contained FcR plus Gr-1 antibody (1 ug), an antibody specific for mature granulocytes (Holmes et al., 1986), tube no. 4 contained FcR plus Mac-1 (5 uL), an antibody specific for granulocytes and macrophages (Springer et al., 1979) [after this reaction cells had to be washed before incubation with anti-rat Ig light and heavy chain PE-conjugated antibody (2 uL); in later experiments, Mac-1 antibody directly conjugated to FITC was used to circumvent the indirect conjugation reaction]; All labelled cells were washed and analyzed in the FACSTAR-Plus flow cytometer, as previously described. Tube no. 5 contained cells doubly stained with GR-1-FITC and Mac-1-Ig-PE.

3. Results

Table 3.2 presents the results of the comparison of the blood granulocytes, lymphocytes, and monocytes between 2- and 3-month-old Il-3 and FVB/N mice. There were no statistically significant differences in the total cell, lymphocyte, or granulocyte counts; however, in 2-month-old mice, the monocyte count was significantly higher in the Il-3 transgenic mice than in FVB/N mice. No differences were found in 3-month old mice. Table 3.3 presents the results from the analysis of the blood smears from 5-month-old Il-3 and FVB/N mice. The eosinophil and monocyte levels were identical in FVB/N and Il-3 mice; the number of neutrophils was higher in Il-3 mice than in FVB/N mice, and the number of lymphocytes was slightly higher in FVB/N mice than in Il-3 mice. Figures 3.1 and 3.2 present the results from the flow cytometric analysis of bone marrow and spleen cells, respectively. In panel A, the unstained cell distribution pattern is shown; it shows the different sizes and subpopulations of the red blood cells (lower left), the dead cells (upper left), and the live cells (lower right). Before doing the analyses of cells stained

Table 3.2

Comparison of the white blood cell counts and populations between pBOR-Il-3 and FVB/N mice.

Line	WBC count (cells/mm^3)	Lymphocytes	Monocytes	Granulocytes	No.	Age (months)
FVB	6476±1011[a1]	5658±785[a2]	190±37[a3]	531±199[a4]	4	2
IL3	6154±2550	5073±2302	312±96.4	644±213	9	2
FVB	4650±216[b1]	3408±204[b2]	231±49.3[b3]	822±159[b4]	9	3
IL3	5567±1764	4233±1533	263.0±83.5	740±292	15	3

This numbers represent the p-values from the ANOVA comparison of the differentblood cell populations of pBOR-Il-3 and FVB/N mice.
a1=0.81 b1=0.23
a2=0.6 b2=0.211
a3=0.03 b3=0.38
a4=0.3 b4=0.50

Table 3.3

Comparison between cell subpopulations in the blood smears of 5-month-old FVB/N and pBOR-Il-3 mice.

Line	Eosinophils	Monocytes	Neutrophils	Lymphocytes
FVB/N	3.4±1.6[a1]	2.4±1.7[a2]	7.6±4.8[a3]	87.0±7.0[a4]
pBOR-Il-3	3.2±1.5	4.0±1.9	14.2±4.2	78.6±5.4

[1]

The numbers represent the percentage of each kind of cell in the blood.
The following numbers represent the probability values (p-values) resulting from the ANOVA comparison between the different blood subpopulations in FVB/N and pBOR-Il-3 mice.
a1 = 0.8
a2 = 0.2
a3 = 0.025
a4 = 0.03

Figure 3.1. Dot plot diagrams resulting from the flow cytometric analysis of bone marrow cells from pBOR-Il-3 mice. Panel A, FL-2/Forward scatter; panel B, F4/80-FITC/Forward scatter; panel C, Gr-1-FITC/Forward scatter; and panel D, Mac-1-FITC/Forward scatter.

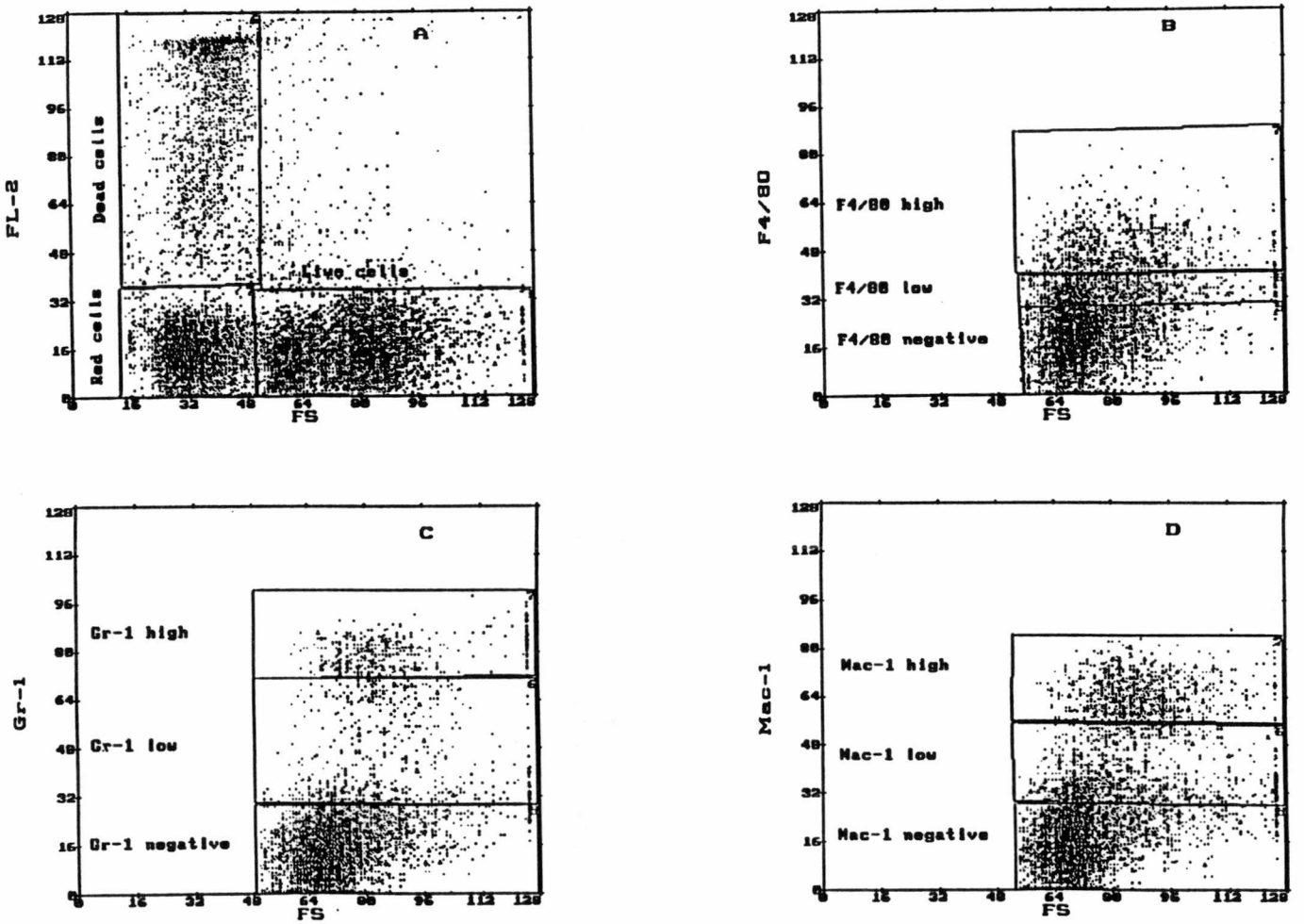
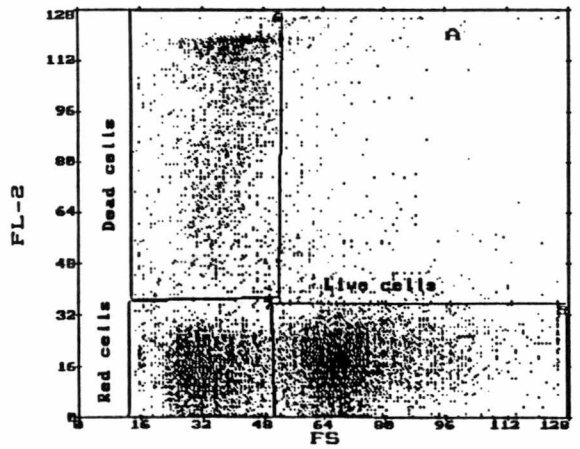
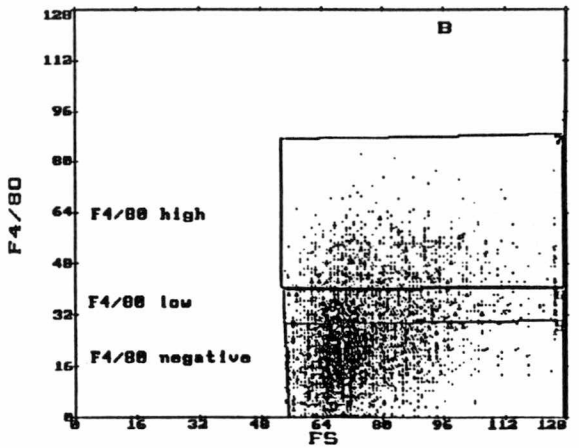
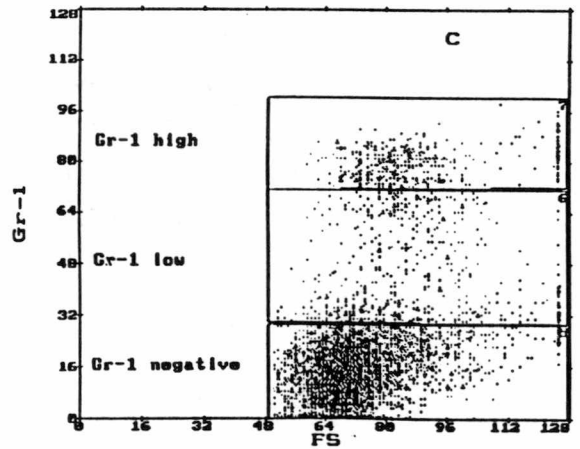
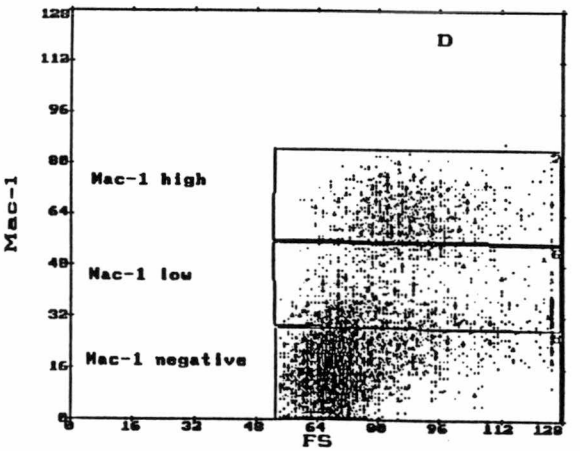

A
Dead cells
Live cells
Red cells
FL-2
FS
B
F4/80 high
F4/80 low
F4/80 negative
F4/80
FS
C
Gr-1 high
Gr-1 low
Gr-1 negative
Gr-1
FS
D
Mac-1 high
Mac-1 low
Mac-1 negative
Mac-1
FS

Figure 3.2. Dot plot diagrams resulting from the flow cytometric analysis of spleen cells fron pBOR-Il-3 mice. Panel A, FL-2/Forward scatter; panel B, F4/80-FITC/Forward scatter; panel C, Gr-1-FITC/Forward scatter; and panel D, Mac-1-FITC/Forward scatter.


A
FL-2
FS
Dead cells
Live cells
Red cells


B
F4/80
FS
F4/80 high
F4/80 low
F4/80 negative


C
Gr-1
FS
Gr-1 high
Gr-1 low
Gr-1 negative


D
Mac-1
FS
Mac-1 high
Mac-1 low
Mac-1 negative

with a particular antibody (such as the plots shown in panels B to C), the red blood cells as well as the dead cells are excluded from the analysis. Panel B is a dot plot showing the F4/80 negative, low, and high populations; panel C is the dot plot revealing the Gr-1 negative, low, and high populations; and panel D is the dot plot diagram of cells showing the Mac-1 negative, low, and high populations. Tables 3.4 and 3.6 show the results of the flow cytometric analysis of phagocytes and granulocytes in the bone marrow and spleen, respectively, of 5-month-old pBOR-Il-3 and FVB/N mice.; it also shows the total cell numbers in the bone marrow and spleen, respectively. Tables 3.5 and 3.7 show the results of the bone marrow and spleen macrophage analysis, respectively. No significant differences were noted in total cell numbers, or numbers of phagocytes, granulocytes or macrophages in the bone marrow and spleen; histological analysis showed no major differences in the mast cell or megakaryocyte levels between Il-3 transgenic mice and non-transgenic FVB/N mice; there were differences, however, in the number of macrophages producing haemosiderin [a compound stored in macrophages; it results from the degradation of ferritin, J. H. Brock (1989)] in the spleen of Il-3 transgenic mice.

C. Flow cytometric analysis of normal thymi and spleens of the Il-3 transgenic mice and the FVB control mice

1. Introduction

As already discussed in the introduction, the effect of interleukin-3 on T-lymphocyte subpopulation is still a controversial subject, and more studies should be done to clarify the subject. These Il-3 transgenic mice provided an opportunity to investigate whether the normal T-cell composition was different in the transgenic Il-3 and the control FVB/N mice. Analyses were done to determine the percentage of the different T-cell subpopulations in the thymus, such as

Table 3.4

Bone marrow phagocytes and granulocytes in 5-month-old FVB/N and pBOR-Il-3 mice

Line	Total cell no.	Mac-1$^{neg.\ a}$	Mac-1^{low}	Mac-1^{high}	Gr-1$^{neg\ b}$	Gr-1^{low}	Gr-1^{high}
FVB/N	26.7±7.4^{c1}	18.1±26.3^{c2}	6.0±5^{c3}	13.0±4.7^{c4}	10.8±5.2^{c5}	5.9±2.5^{c6}	17.6±20.7^{c7}
pBOR-Il-3	25.7±10.3	9.6±3.7	5.7±2.9	8.9±6.4	9.8±5.5	5.7±3.5	11.3±4.2

[4]

[4]

a. Mac-1 is an antigen present on the surface of phagocytes.
b. Gr-1 is an antigen present on the surface of granulocytes.
The numbers shown in the columns represent the means ± the standard deviations; they represent the number times a million cells.
The following numbers represent the probability values (p-values) resulting from the ANOVA comparison between the different bone marrow granulocyte and macrophage subpopulations in FVB/N and pBOR-Il-3 mice.
c1=0.8
c2=0.4 c5=0.7
c3=0.9 c6=0.9
c4=0.2 c7=0.4

Table 3.5

Bone marrow macrophages in 5 month-old FVB/N and pBOR-Il-3 mice

Line	F4/80$^{neg.\ a}$	F4/80^{low}	F4/80^{high}
FVB/N	12±3.9^{b1}	5.7±3.0^{b2}	2.8±1.8^{b3}
pBOR-Il-3	11.6±5.7	3.7±3.4	2.2±2.1

[4]

a. F4/80 is an antigen present on the surface of macrophages.
The numbers shown in the columns represent the means ± the standard deviations; they represent the numbers times a million cells.
The following numbers represent the probability values (p-values) resulting from the ANOVA comparison between the different splenic granulocyte and macrophage subpopulations in FVB/N and pBOR-Il-3 mice.
b1=0.9
b2=0.3
b3=0.6

Table 3.6

Splenic phagocytes and granulocytes in 5-month-old FVB/N and pBOR-Il-3 mice

Line	Total cell no.	Mac-1$^{neg.\ a}$	Mac-1^{low}	Mac-1^{high}	Gr-1^{neg}	Gr-1^{low}	Gr-1^{high}
FVB/N	109.3±37.3^{b1}	84.2±33.4^{b2}	18.7±7.8^{b3}	4.4±4.8^{b4}	96.2±32.5^{b5}	7.2±2.8^{b6}	4.9±2.8^{b7}
pBOR-Il-3	106.1±51.9	81.8±42.3	20.0±5.9	4.0±2.4	93.6±47.1	8.0±4.2	4.4±2.4

a. Mac-1 is an antigen present on the surface of phagocytes.
The numbers shown in the columns represent the means ± the standard deviations; they represent the numbers times a million cells.
The following numbers represent the probability values (p-values) resulting from the ANOVA comparison between the different splenic granulocyte and macrophage subpopulations in FVB/N and pBOR-Il-3 mice.
b1=0.9
b2=0.9 b5=0.9
b3=0.7 b6=0.7
b4=0.8 b7=0.8

Table 3.7

Splenic macrophages in 5-month-old FVB/N and pBOR-Il-3 mice

Line	F4/80$^{neg.\ b}$	F4/80^{low}	F4/80^{high}
FVB/N	80.0±23.1^{a1}	16.8±5.6^{a2}	4.3±1.4^{a3}
pBOR-Il-3	79.1±32.6	12.5±8.2	3.0±1.5

a. F4/80 is an antigen present on the surface of macrophages.
The numbers shown in the columns represent the means ± the standard deviations; they represent the numbers times a million cells.
The following numbers represent the probability values (p-values) resulting from the ANOVA comparison between the different splenic granulocyte and macrophage subpopulations in FVB/N and pBOR-Il-3 mice.
b1=0.96
b2=0.3
b3=0.1

the double negative, CD8 and CD4 double positive, CD4 single positive, and the CD8 single positive subpopulations, and the percentage of the mature CD4 single positive and CD8 single positive cells in the spleen of both Il-3 and FVB mice. After studying the T-cell composition in the normal thymus and spleen, analyses were done to determine the T-cell composition in thymomas, looking for differences between thymomas from the Il-3 transgenic and control FVB/N mice.

2. Materials and methods

a) Animals

Il-3 transgenic and FVB mice were sacrificed at the following time points: 5 days, 9 days, 15 days, and 26 days. Three to 5 animals were sacrificed in each time point, and, when possible, an equal number of males and females was chosen. After being sacrificed the animals were weighed, the thymi and spleens were extirpated, weighed and single-cell suspensions were prepared for the flow cytometry analysis.

b) Flow cytometric analysis

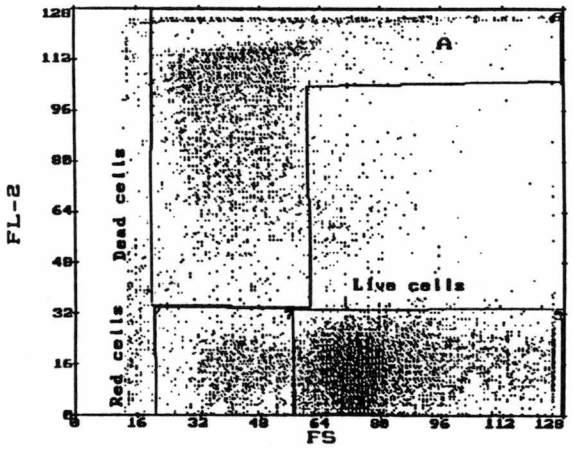
Flow cytometric analyses were performed as described in Wang et al. (manuscript in preparation). Single-cell suspensions of thymi, or spleens were prepared by gently grinding fresh tissue between the frosted ends of two glass slides in SPBS. Large tissue fragments were removed by filtering the suspension through Miracloth, a glass-fiber fabric. Approximately 10^6 cells in 100 μL were stained in suspension with fluorescein isothiocyanate (FITC) conjugated hamster anti-mouse CD3, R-phycoerythrin conjugated rat anti-mouse L3T4 (CD4) and FITC-conjugated rat anti-mouse Ly-2 (CD8a) monoclonal antibodies on ice. After staining for 30 min., cells were washed twice with 1 mL SPBS, each wash followed by centrifugation at 1,000 rpm, 4°C, for 10 min. The antibody-labeled cell preparations were analyzed in a FACSTAR-Plus flow

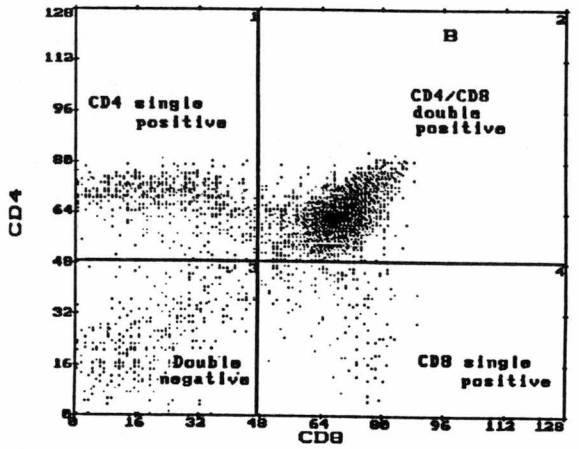
cytometer.

3. Results

Figures 3.3 and 3.4 show the flow cytometric patterns resulting from the flow cytometric analysis of thymus and spleen, respectively. Panel A shows how the dead cells, and the red cells were excluded, and how the live cells were gated for analysis. Panel B shows the CD4/CD8 pattern; the upper left quadrant represents the CD4 single positive cells, the upper right quadrant the CD4/CD8 double positive population, the lower right, the CD8 single positive population, and the lower left quadrant represents the CD4/CD8 double negative T-lymphocyte subpopulation. Panel C shows the dot plot resulting from the CD3 determination; it shows the CD3 negative, low, and high T-lymphocyte subpopulations. Tables 3.8 and 3.9 show the results of the flow cytometric analyses of CD4/CD8 T-lymphocyte cell surface markers in the spleens and thymi of the FVB and the Il-3 mice. The values of the two way ANOVA test are shown in the bottom part of the tables. The results show that there are no significant differences between the FVB or the Il-3-12 mice in the composition of their T-lymphocyte subpopulation. Significant differences are apparent in the following groups: in the 26-day-old group the CD8 single positive subpopulation of the spleen was higher in the FVB/N mice than in the Il-3 mice; in the 15-day-old group, in which the CD8 single positive subpopulation of the thymus was higher in the Il-3 mice, and in the 26-day-old mice, in which the CD4/CD8 double positive subpopulation of the thymus is higher in Il-3 mice. Despite these minor differences in certain T cell populations between the age-matched individual groups, there does not appear to be any general differences between the pBOR-Il-3 # 12 transgenic mice and the congenic FVB/N mice.

Figure 3.3. Dot plot diagrams resulting from the flow cytometric analysis of thymus cells from pBOR-Il-3 mice. Panel A, Fl-2/Forward scatter plot; panel B, CD4/CD8 plot; and panel C, FL-2/CD3 plot.


A
Dead cells
Red cells
Live cells
FL-2
FS
0
16
32
48
64
80
96
112
128


B
CD4 single positive
CD4/CD8 double positive
Double negative
CD8 single positive
CD4
CD8
1
2
3
4

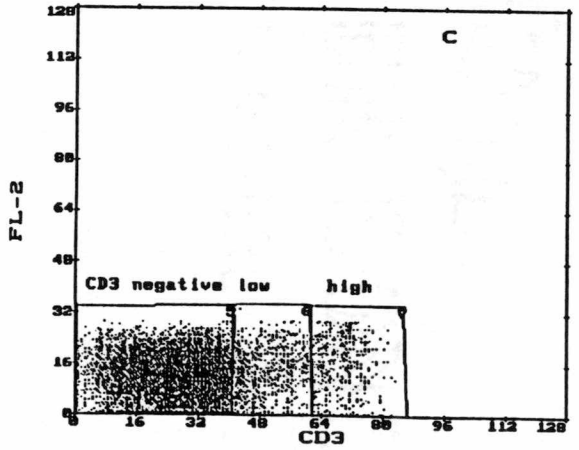
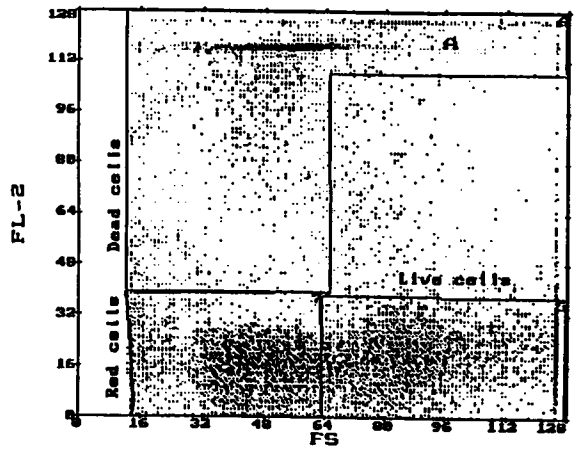
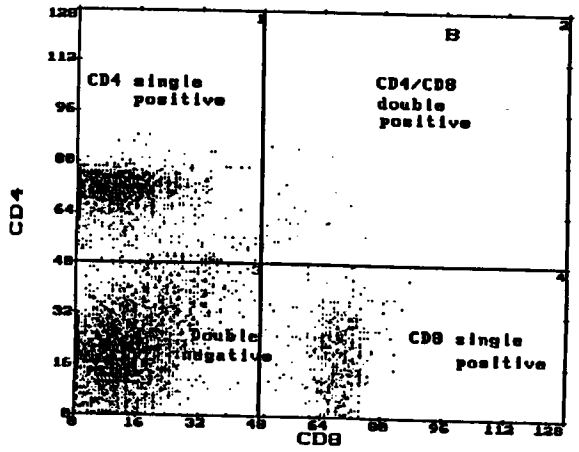

C
CD3 negative low
high
FL-2
CD3

Figure 3.4. Dot plot diagrams resulting from the flow cytometric analysis of spleen cells from pBOR-Il-3 mice. Panel A, Fl-2/Forward scatter plot; panel B, CD4/CD8 plot; and panel C, FL-2/CD3 plot.


A
Dead cells
Live cells
Red cells
FL-2
FS


B
CD4 single positive
CD4/CD8 double positive
Double negative
CD8 single positive
CD4
CD8

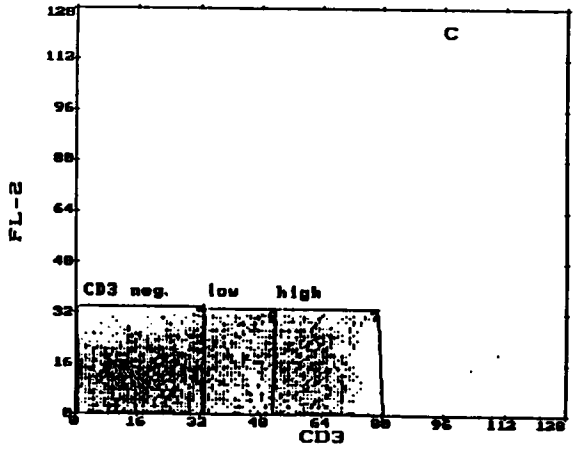

C
CD3 neg.
low
high
FL-2
CD3

Table 3.8

Flow cytometric analysis of the CD4 and CD8 cell surface antigens of the FVB/N and pBOR-Il-3 thymic T-lymphocytes

Age (days)	Line	Total cell no. ($x10^6$)	$CD4^a$ sp ($x10^6$)	$CD4^+CD8^+$ $x10^6$)	$CD4^-CD8^-$ ($x10^6$)	CD8 sp ($x10^6$)	No.
5	FVB	35 ± 24.7^{b1}	3.3 ± 2.4^{c1}	16.6 ± 15.2^{d1}	3.4 ± 2.34^{e1}	0.74 ± 0.6^{f1}	4
9		134 ± 33.0^{b2}	10.9 ± 3.2^{c2}	109.4 ± 27.0^{d2}	10.4 ± 2.5^{e2}	2.8 ± 1.04^{f2}	4
15		272.5 ± 87.0^{b3}	23.7 ± 7.5^{c3}	151.4 ± 106.8^{d3}	12.6 ± 4.7^{e3}	4.8 ± 1.6^{f3}	4
26		332 ± 40.9^{b4}	23.3 ± 3.2^{c4}	264.6 ± 35^{d4}	18.12 ± 3.2^{e4}	10.4 ± 0.9^{f4}	5
5	Il-3	27 ± 11.6	2.3 ± 1.2	21.3 ± 11.2	2.9 ± 1.09	0.5 ± 0.2	5
9		58 ± 15.5	4.6 ± 0.5	$48.\pm14.17$	4.35 ± 0.6	1.1 ± 0.3	4
15		242.2 ± 35	20.7 ± 2.8	203 ± 30.4	13.1 ± 3.7	4.3 ± 0.4	5
26		340 ± 73.2	29.8 ± 16.9	277.2 ± 60	17.2 ± 1.8	8.6 ± 2.2	5

a. CD4 and CD8 are antigens present on the surface of T-cells.

This numbers represent the probability values from the comparison between the different thymic T-lymphocyte subpopulations in FVB/N and pBOR-Il-3 mice.

b1=0.54	c1=0.47	d1=0.60	e1=0.66	f1=0.50
b2=0.005	c2=0.008	d2=0.007	e2=0.003	f2=0.02
b3=0.49	c3=0.4	d3=0.30	e3=0.86	f3=0.40
b4=0.83	c4=0.25	d4=0.70	e4=0.56	f4=0.12

Table 3.9

Flow cytometric analysis of the cell surface CD4 and CD8 antigens of the FVB and Il-3 splenic T-lymphocytes

Age (days)	Line	Total cell No. ($x10^6$)	$CD4^+$ sp ($x10^6$)	$CD4^+CD8^+$ ($x10^6$)	$CD4^-CD8^-$ ($x10^6$)	CD8 sp ($x10^6$)	No.
5	FVB	8.95 ± 4.8^{b1}	0.35 ± 0.17^{c1}	0.14 ± 0.08^{d1}	8.22 ± 4.6^{e1}	0.12 ± 0.05^{f1}	4
9		44.5 ± 18.1^{b2}	4.6 ± 2.8^{c2}	0.36 ± 0.16^{d2}	37.87 ± 14.6^{e2}	1.6 ± 0.8^{f2}	4
15		64.5 ± 9.9^{b3}	7.9 ± 2.4^{c3}	0.34 ± 0.05^{d3}	53.5 ± 8.3^{e3}	3.3 ± 0.4^{f3}	4
26		77.6 ± 18.4^{b4}	12.7 ± 1.3^{c4}	0.32 ± 0.05^{d4}	60.4 ± 20.1^{e4}	4.1 ± 0.5^{f4}	5
5	Il-3	11.06 ± 4.1	0.45 ± 0.22	0.10 ± 0.02	10.4 ± 3.8	0.16 ± 0.09	6
9		25.5 ± 9.0	2.1 ± 0.8	0.14 ± 0.07	22.2 ± 7.9	0.8 ± 0.3	4
15		49.4 ± 13.4	7.4 ± 2.3	0.14 ± 0.05	39.3 ± 10.5	2.5 ± 0.73	5
26		80.5 ± 19.8	13.9 ± 4.2	0.64 ± 0.25	$62.\pm14.6$	4.1 ± 1.0	4

a. CD4 and CD8 are antigens present on the surface of T-lymphocytes.

This numbers represent the probability values from the comparison between the different splenic T-lymphocyte populations in FVB/N and pBOR-Il-3 mice.

b1=0.47 c1=0.48 d1=0.38 e1=0.44 f1=0.37
b2=0.11 c2=0.15 d2=0.07 e2=0.11 f2=0.12
b3=0.10 c3=0.76 d3=0.006 e3=0.06 f3=0.11
b4=0.80 c4=0.56 d4=0.03 e4=0.90 f4=0.99

D. Discussion

An extensive study was performed to investigate whether or not, under normal conditions an Il-3 transgene has any influence on various hematopoietic cell subpulations, which range from the population that includes the pluripotent stem cells (the Thy-1^{low}Pgp-1^{int}Fall-3^{high} population) to more differentiated precursors (the CFU-C, BFU-E, and CFU-E populations). These cell populations are the ones reported to be influenced by Il-3 and hence are expected to be increased in numbers in the Il-3 transgenic mice. The late stage mature leukocytes were also studied, which included blood leukocyte populations such as monocytes, eosinophils, neutrophils, and lymphocytes, and also the granulocytes as well as and macrophages of the bone marrow and spleen. In addition, T-lymphocytes from 5- to 26-day-old mice were studied. The comparison between FVB/N and pBOR-Il-3 mice revealed no major differences except that minor alterations were found, such as an increased level in monocytes in two-months-old pBOR-Il-3 mice, an increased number of blood neutrophils in 5-month-old pBOR-Il-3 mice, and an increase in haemosiderin-containing macrophages in the spleen of Il-3 transgenic mice. These results are consistent with the idea that the Il-3 transgene is being expressed at too low levels to exert a signifficant influence on the growth and/or differentiation of the above-mentioned leukocytes. These results are different from those of Metcalf (1986); Chang et al. (1989); and Wong et al. (1989). The latter two groups used a retroviral vector (a Moloney MuLV-Il-3 construct) to infect and somatically transduce bone marrow stem cells which were used to reconstitute lethally irradiated mice. These investigators found that although recovery from lethal irradiation was achieved by the transplantation of the transduced bone marrow cells, the mice developed a non-neoplastic hematopoietic syndrome characterized by enlargement of the spleen and liver, a higher spleen and blood leukocyte cellularity, a decreased bone marrow cellularity, a striking elevation

of mast cells, eosinophils, neutrophils, erythroblasts, monocytes/macrophages and blast cells, and a decrease in lymphocyte numbers in the blood, bone marrow, spleen and peritoneum. None of these changes, however, have been observed in the pBOR-Il-3 line 12 mice. This could be due to the following possibilities: First, the methods of obtaining the transgenic line were different; the pronuclear microinjection of fertilized eggs was used to produce pBOR-Il-3 mice in our study whereas a retroviral vector was used to transfer the gene into bone marrow cells. Second, the long terminal repeat used in their experiments was of a Moloney-MuLV type, whereas an ecotropic LTR, derived from a BALB/c retrovirus, WN1802B, was used to make the pBOR-Il-3 construct in this study. The difference in the LTR sequences could account for the differences in Il-3 expression between our experiments and theirs. Third, differences in the chromosomal DNA sites of integration of the Il-3 transgene could affect their expression. The fourth reason could be that the pBOR-Il-3 construct still contains plasmid sequences which might severely inhibit the expression of the associated eukaryotic genes following their incorporation into the mouse germ line; Similar to the observations of Chada, (1985) and Townes et al., (1985), that transgenic beta globin was expressed only if the plasmid sequences in the transgene were removed. However, other genes, such as the alpha-fetoprotein, the metaloprotein-growth hormone, and the elastase-growth hormone genes, have been shown to express in an appropriate tissue-specific pattern in transgenic mice, although higher levels of transcription were observed when plasmid sequences were removed prior to microinjection. It is thus not known whether a specific sequence is responsible for this inhibition, or whether it is a general property of prokaryotic DNA (Hogan et al., 1982). Since under normal conditions, expression of the Il-3 transgene pBOR-Il-3 mice might not be high enough to disturb hematopoiesis, two experimental approaches have been used in attemps to activate the function of LTRs. X rays and azacytidine, were used in an attempt to perturb the system and eventually to increase the transcription of the

Il-3 gene, which is controlled by a LTR.

CHAPTER IV

X irradiation of FVB/N and pBOR-Il-3 mice

A. Introduction

Frasca et al. (1992) sublethally irradiated (C57BL/10 x DBA/2) F1 mice with x ray at doses ranging from 1 to 4 Gy and subsequently injected 0.5 to 5 ug of purified Il-3 into the animals to test whether Il-3 had any effect on the growth and differentiation of thymocytes. By flow cytometric analysis, they found that after irradiation the $CD4^+CD8^-$ subpopulation was halved, and the $CD4^-CD8^-$ cells increased; injection of 5μg Il-3 into the mice irradiated with 2 Gy resulted in the recovery of the $CD4^+CD8^-$ subpopulation, and in the reduction in the $CD4^-CD8^-$ thymic T-lymphocytes eight days after irradiation. Their findings suggest that Il-3 induces differentiation of immature T-cell precursors in the thymus, and selectively stimulates the proliferation of mature $CD4^+CD8^-$ T-cells. Since it has been demonstrated that Il-3 binds to endothelial cells in the thymus, but not to T-lymphocytes (Metcalf and Nicola, 1988), it has been proposed that, rather than a direct influence on T-lymphocyte differentiation, Il-3 modulates other cell types within the thymus, which in turn secrete lymphokines, such as the release of Il-6 by intrathymic macrophages/dendritic cells (King and Katz, 1990) that influence $CD4^+CD8^-$ differentiation. In order to test whether the same phenomenon could be observed in the pBOR-Il-3 mice, the same experimental approaches of irradiation and flow cytometric analysis were followed. Besides the study of T-cell recovery after irradiation, the recovery of other leukocytes, such as blood neutrophils, eosinophils, monocytes, lymphocytes, and bone marrow and spleen tissue macrophages, monocytes, and granulocytes were also studied.

B. Materials and methods

1. Irradiation of mice

FVB/N and transgenic Il-3 mice were kept in a conventional mouse facility in the Biology Division of the Oak Ridge National Laboratory. At 40 days of age they were irradiated with 2 Gy of x rays, using a G.E. Maxitron 250 x ray machine with a 0.5 mm copper filter, at a dose rate of 0.958 Gy/min. Mice were then euthanized on days 1 and 8 after irradiation. On day one, two nonirradiated FVB/N control, six irradiated FVB/N mice, and five irradiated pBOR-Il-3 mice were chosen; on day 8, one FVB/N control, six irradiated FVB/N mice, and six irradiated pBOR-Il-3 were selected.

2. Blood smears, histological preparations and single cell suspensions

The mice were bled from the retroocular vein plexus using a Pasteur pipette; a small drop of blood was spread on a microscope coverslip, and the blood was stained using the Wright's staining procedure. After staining, the slides were mounted onto a microscope slide, and differential counts of 500 white blood cells were made using a light microscope. After euthanizing the mice by cervical dislocation, the two femurs and two tibias, spleen and thymus from each mouse were removed and placed at 4°C in a culture dish containing PBS^-. The thymi and the spleens were weighed. A small piece of thymus and spleen, and the sternum were placed in a vial containing 3 mL of buffered 10% formalin for preparation of histological sections stained with Hematoxylin/Eosin. Single cell suspensions were prepared, either by gently mincing the thymi and spleens with a loose homogenizer, or by releasing the cells out of the tissues by first injecting the PBS^- into the tissue, and then teasing with a needle. The cells from the bone marrow were collected by inserting a 27 1/2 gauge needle into the marrow of the bones, and then flushing out

with 1L of the solution; this procedure was repeated until no bone marrow was left in the bone. After the cells were collected, they were centrifuged for 10 min. at 1000 rpm, and then, the bone marrow cells were resuspended in 2 mL of SPBS; the thymic and splenic cells similarly centrifuged, but resuspended in 3 mL of SPBS.

3. Flow cytometric analysis

The cells were diluted 1/100, counted in a hemocytometer, the cells were diluted to a final concentration of $10x10^6$ cells/mL, and 100 μL of the cell suspension was used for the flow cytometric analysis. Antibody against Fc receptor was used to block the binding of the Fc sites of the antibodies to the Fc receptor sites on phagocytic cells. For this study, F4/80-FITC, Mac-1-FITC, and Gr-1-FITC antibodies were used to identify splenic and bone marrow macrophages, phagocytic cells, and granulocytes, respectively. Anti-CD4-PE, anti-CD8-FITC and anti-CD3-FITC were used with thymus and spleen cells, to identify different T-lymphocyte subpopulations. After the cells were analyzed in the FACS machine, the dot plots results were obtained from the M2D computer software.

C. Results

Table 4.1 presents the results of differential counts of the white blood cells in the blood smears. The numbers in the columns represent the percentage of each population in the blood. One day after irradiation, the neutrophils in FVB/N and Il-3 increased [as observed in acute whole-body irradiation of humans, Andrews, (1980)] and remained relatively constant up to day eight after irradiation; the eosinophil, monocyte and lymphocyte levels remained constant, even in the experimental animals; there are no major differences between FVB/N and pBOR-Il-3 in the levels of any of the studied leukocyte subpopulations. Table 4.2 presents the results of the

Table 4.1

White blood cell levels in 2 Gy x irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days After Treatment	Neutrophils	Eosinophils	Monocytes	Lymphocytes
FVB/N	control	0	11.0±1.9	1.3±0.8	7.7±11.1	86.2±1.7
FVB/N	x ray	1	22.1±5.6[a1]	2.6±2.0[a2]	1.1±0.9[a3]	74.4±6.8[a4]
FVB/N	x ray	8	19.7±6.2[b1]	0.9±0.4[b2]	2.1±0.9[b3]	77.3±6.3[b4]
pBOR-Il-3	x ray	1	24.7±7.9	2.5±2.9	1.8±1.0	71.3±7.7
pBOR-Il-3	x ray	8	17.3±3.7	0.8±0.4	2.4±1.2	79.5±4.5

[1]

This numbers represent the p-values from the ANOVA comparison between leukocyte subpopulations of the FVB/N and pBOR-Il-3 mice.
a1=0.6 b1=0.4
a2=0.9 b2=0.9
a3=0.3 b3=0.6
a4=0.5 b4=0.5

Table 4.2

Total cell numbers in the bone marrows, spleens and thymi from 2 Gy x-irradiated pBOR-Il-3 and FVB/N mice.

Line	Treatment	Days after treatment	Tissue	Total live cells ($x10^6$)
FVB/N	control	0	bone marrow	31.3±31.4
			spleen	54.9±46.6
			thymus	104.3±62.8
FVB/N	x ray	1	bone marrow	7.5±5.6[a1]
			spleen	18.7±4.8[a2]
			thymus	33.8±26.8[a3]
FVB/N	x ray	8	bone marrow	20.8±8.6[b1]
			spleen	29.3±14.4[b2]
			thymus	84.8±19.7[b3]
pBOR-Il-3	x ray	1	bone marrow	10.2±6.3
			spleen	30.6±14.8
			thymus	27.4±10.0
pBOR-Il-3	x ray	8	bone marrow	21.2±4.4
			spleen	32.2±21.6
			thymus	75.3±37.1

[9]

[9]

This numbers represent the probability values resulting from the ANOVA comparison between the different cell numbers.
a1=0.5 b1=0.9
a2=0.09 b2=0.8
a3=0.6 b3=0.6

effect of irradiation on the total cell numbers in the Il-3 and FVB/N bone marrows, spleens, and thymi. As indicated in the table, the cell numbers in all of the three tissues were reduced on day one after irradiation, and recover to the initial levels by day eight; the response in Il-3 and FVB/N animals was identical. Table 4.3 presents the effects of the irradiation on the bone marrow macrophages; the F4/80 negative, low, and high subpopulations decreased after irradiation, and then recovered to the original levels by day eight after irradiation; the number of phagocytes (macrophages and granulocytes) also decreased after irradiation, and recovered rapidly to their original levels (Table 4.4); the Mac-low subpopulation (presumably immature phagocytes) remained lower than the control group 8 days after irradiation. The granulocyte negative, low, intermediate, and high subpopulations were reduced after irradiation, and all of them recovered to normal levels by day eight (Table 4.5). Table 4.6 presents the results of the irradiation effects on the spleen phagocytic subpopulations. As in the bone marrow, the phagocyte populations (Mac-low and high) were reduced by day one after irradiation, and recovered to normal levels by day 8; the only difference in this study was in the number of immature phagocytes on day one after irradiation, where Il-3 animals have double the numbers of cells than FVB/N animals, this cell population eventually returns to normal levels in the FVB/N animals. Table 4.7 presents the results of the radiation effects on the macrophage subpopulations in the spleens of FVB/N and Il-3 animals. Again, the different macrophage subpopulations were decreased after irradiation, and returned to normal levels after day eight, the response was identical in Il-3 and FVB/N animals. The effects of irradiation on the granulocyte subpopulations is presented in table 4.8. The Gr-1-FITC intermediate and high populations recover 8 days after irradiation; the Gr-1-FITC intermediate population in the Il-3 animals was slightly higher in Il-3, relative to the FVB/N animals. Tables 4.9 and 4.10 present the results of the effect of radiation on the T-lymphocyte subpopulations in the spleen and thymus of Il-3 and FVB/N mice. In the

Table 4.3

Macrophage subpopulations in the bone marrow of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	F4/80$^{neg.}$ [a] ($x10^6$)	F4/80^{low} ($x10^6$)	F4/80^{high} ($x10^6$)
FVB/N	control	0	23.6±5.9	5.9±4.1	3.9±2.2
FVB/N	x ray	1	6.6±4.1[a1]	1.03±0.7[a2]	0.52±0.5[a3]
FVB/N	x ray	8	14±5.3[b1]	5.4±3.4[b2]	1.6±0.8[b3]
Il-3	x ray	1	8.3±5.2	1.2±0.8	0.67±0.5
Il-3	x ray	8	15.2±3.3	5.0±1.7	1.5±0.8

9

a. F4/80 is a surface antigen present on the surface of macrophages.
This numbers represent the probability values from the ANOVA comparison between cell populations of pBOR-Il-3 and FVB/N mice.
b1=0.5 c1=0.6
b2=0.7 c2=0.8
b3=0.6 c3=0.8

Table 4.4

Phagocyte subpopulations in the bone marrow of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	Mac-1$^{neg.\ a}$	Mac-1^{low}	Mac-1^{high}
FVB/N	control	0	16.0±11.5	5.1±1	12.9±16.3
FVB/N	x ray	1	4.7±2.6^{b1}	0.43±0.25^{b2}	3.0±2.5^{b3}
FVB/N	x ray	8	9.5±3.3^{c1}	1.7±1.0^{c2}	9.9±6.0^{c3}
Il-3	x ray	1	6.4±3.4	0.74±0.88	3.1±2.4
Il-3	x ray	8	10.3±4.6	1.8±0.8	12.6±11.9

9

a. Mac-1 is an antigen present on the surface of phagocytes.

These numbers represent the p-values from the comparison of cell populations between pBOR-Il-3 and FVB/N mice.

b1=0.4 c1=0.7

b2=0.4 c2=0.8

b3=0.9 c3=0.6

Table 4.5

Granulocyte subpopulations in the bone marrow of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	Gr-1$^{neg.\ a}$	Gr-1^{low}	Gr-1$^{int.}$	Gr-1^{high}
FVB/N	control	0	17.2±15.5	2.4±1.8	3.9±4.5	7.9±10.8
FVB/N	x ray	1	3.8±1.9^{b1}	0.3±0.2^{b2}	0.52±0.3^{b3}	2.1±2.6^{b4}
FVB/N	x ray	8	10.2±2.8^{c1}	1.7±1.0^{c2}	2.0±1.5^{c3}	7.4±6.3^{c4}
pBOR-Il-3	x ray	1	5.9±2.8	0.2±0.1	0.35±0.3	1.2±0.6
pBOR-Il-3	x ray	8	11.4±6.3	1.2±0.6	3.9±5.8	5.4±5.4

a. Gr-1 is an antigen present on the surface of granulocytes.

These numbers represent the p-values from the ANOVA comparison of different cell populations between pBOR-Il-3 and FVB/N mice.

b1=0.2 c1=0.7

b2=0.5 c2=0.3

b3=0.4 c3=0.5

b4=0.5 c4=0.6

Table 4.6

Phagocyte subpopulations in the spleen of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	Mac-1$^{neg.}$ [a]	Mac-1^{low}	Mac-1^{high}
FVB/N	control	0	47±9.9	7±5.9	2±0.9
FVB/N	x ray	1	15.3±4.2[b1]	2.6±0.9[b2]	1.0±0.4[b3]
FVB/N	x ray	8	23.6±12.5[c1]	4.1±2.0[c2]	2.1±1.0[c3]
pBOR-Il-3	x ray	1	23.7±10.9	5.0±2.4	1.8±0.9
pBOR-Il-3	x ray	8	26.2±18.7	4.3±2.3	2.0±1.3

9

a. Mac-1 is an antigen present on the surface of phagocytes.
These numbers represent the p-values resulting from the ANOVA comparison between cell populations of pBOR-Il-3 and FVB/N mice.
b1=0.1 c1=0.8
b2=0.04c2=0.9
b3=0.09c3=0.8

Table 4.7

Macrophage subpopulations in the spleen of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	F4/80$^{neg.}$ [a]	F4/80^{low}	F4/80^{high}
FVB/N	control	0	46.0±10	8.3±5.6	1.6±1.2
FVB/N	x ray	1	16.3±3.4[b1]	2.0±1.3[b2]	0.53±0.23[b3]
FVB/N	x ray	8	26.5±20[c1]	4.9±2.1[c2]	1.2±0.7[c3]
pBOR-Il-3	x ray	1	27.4±13.4	2.7±1.5	0.48±0.24
pBOR-Il-3	x ray	8	26.5±20	4.9±2.1	1.2±0.7

9

a. F4/80 is an antigen present on the surface of macrophages.

These numbers represent the p-values from an ANOVA comparison between cell subpopulations of pBOR-Il-3 and FVB/N mice.

b1 = 0.08 c1 = 0.8
b2 = 0.5 c2 = 0.8
b3 = 0.7 c3 = 0.6

Table 4.8

Granulocyte subpopulations in the spleen of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	Gr-1$^{neg.\ a}$	Gr-1^{low}	Gr-1$^{int.}$	Gr-1^{high}
FVB/N	control	0	49.2±40.3	2.7±2.7	2.1±1.3	1.7±2.2
FVB/N	x ray	1	16.4±4.3^{b1}	1.4±0.6^{b2}	0.38±0.11^{b3}	0.68±0.3^{b4}
FVB/N	x ray	8	24.2±12.6^{c1}	3.3±2.1^{c2}	1.0±1.0^{c3}	0.9±0.6^{c4}
pBOR-Il-3	x ray	1	26.6±13.5	2.1±1.2	0.66±0.29	1.3±0.9
pBOR-Il-3	x ray	8	28.0±18.6	2.7±2.0	0.7±0.5	0.9±0.8

9

a. Gr-1 is an antigen present on the surface of granulocytes.
These numbers represent the p-values resulting from the ANOVA comparison between cells populations of pBOR-Il-3 and FVB/N mice.
b1=0.1 c1=0.7
b2=0.2 c2=0.6
b3=0.07 c3=0.6
b4=0.2 c4=0.97

Table 4.9

Lymphocyte subpopulations in the spleen of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	$CD4^{sp}$ [a]	$CD4^+CD8^+$	$CD4^-CD8^-$	$CD8^{sp}$	$CD3^-$	$CD3^{low}$	$CD3^{high}$
FVB/N	control	0	11.6±9.9	0.4±0.4	39.5±33.4	3.4±3.2	30.7±23.7	12.3±13.7	12±9.6
FVB/N	x ray	1	4.4±1.5[b1]	0.2±0.04[b2]	13.2±3.4[b3]	0.8±0.2[b4]	7.2±2.1[b5]	5.8±2.6[b6]	6±3.2[b7]
FVB/N	x ray	8	5.6±3.3[c1]	0.2±0.07[c2]	20.2±10.6[c3]	1.7±0.8[c4]	16±9[c5]	5.7±3.1[c6]	7±3.6[c7]
pBOR-Il-3	x ray	1	8.2±3.7	0.3±0.2	20.4±10	1.7±1.0	11.2±8.7	10.2±5.6	9.9±4.0
pBOR-Il-3	x ray	8	10.2±7.6	0.2±0.1	20±13	2.1±1.2	13±8	8.1±4.7	12±11

9

a. CD4, CD8, and CD3 are antigens present on the surface of T-lymphocytes.

These numbers represent the p-values from the ANOVA comparison between cell populations of FVB/N and pBOR-Il-3 mice.

b1=0.045 c1=0.2
b2=0.3 c2=0.5
b3=0.13 c3=0.9
b4=0.053 c4=0.5
b5=0.3 c5=0.5
b6=0.11 c6=0.3
b7=0.1 c7=0.3

Table 4.10

Lymphocyte subpopulations in the thymus of 2 Gy x-irradiated FVB/N and pBOR-Il-3 mice.

Line	Treatment	Days after treatment	$CD4^{sp}$ [a]	$CD4^+CD8^+$	$CD4^-CD8^-$	$CD8^{sp}$	$CD3^-$	$CD3^{low}$	$CD3^{high}$
FVB/N	control	0	15.5±8.2	81±50	3.8±2.8	3.8±1.9	46.3±25.7	42.4±39.3	18±13
FVB/N	x ray	1	7.9±4.8[b1]	20.1±19.5[b2]	3.4±2.9[b3]	2.4±1.5[b4]	17.5±19[b5]	11.3±12.3[b6]	10±5[b7]
FVB/N	x ray	8	8.1±2[c1]	67.3±20[c2]	6±2.5[c3]	4±2.2[c4]	43±33[c5]	31±29[c6]	9±2.2[c7]
pBOR-Il-3	x ray	1	8.7±2.5	13±6.8	3.2±1.9	2.8±0.8	6.2±3	11.7±5.8	10±2.2
pBOR-Il-3	x ray	8	8.2±3.9	49.5±36.5	4.9±3.4	2.6±1.3	50.8±22.5	17.5±22	7.7±4.8

9

a. CD4, CD8, and CD3 are antigens present on the surface of T-lymphocytes.

These numbers represent the p-values from the ANOVA comparison between cell populations of FVB/N and pBOR-Il-3 mice.

b1=0.8 c1=0.96
b2=0.5 c2=0.3
b3=0.9 c3=0.6
b4=0.6 c4=0.2
b5=0.2 c5=0.7
b6=0.9 c6=0.4
b7=0.9 c7=0.6

Il-3 spleen, the CD4 single positive and CD8 single positive subpopulations remained at normal levels one day after irradiation while the population was dramatically decreased in the FVB/N mice; then, 8 days after irradiation, the FVB/N levels recovered to the same level as the Il-3 animals. In the thymus, the CD4 single positive population decreased one day after the irradiation; and then it remained relatively constant through day 8; the double positive subpopulation decreased after treatment, but recovered to normal levels after 8 days; the double negative and the CD8 single positive populations remained unchanged throughout the experiment. The CD3 negative and the CD3 low populations were reduced after irradiation, but they also recovered to normal levels by day 8 post-irradiation. The CD3 high subset showed a slight decrease after irradiation, and it remained relatively constant throughout the experiment. Other results not shown were that no apparent histological differences were observed between Il-3 and FVB/N in any of the tissues studied and there were no differences in the numbers of mast cells or megakaryocytes in the spleen and bone marrow of the mice.

D. Discussion:

These experiments were designed to test the hypothesis that Il-3 would accelerate the recovery of some leukocyte subpopulation after irradiation of the mice. The idea was to disturb the hematopoietic system by the killing of leukocyte subpopulation, so that the effect of Il-3 on the system could be more easily studied. The observation that there were no differences in the radiation recovery between FVB/N and pBOR-Il-3 mice suggests that Il-3 produced from the transgene did not have any obvious effect on radiation recovery of cells of the hematopoietic system, probably because it is being expressed at low levels. The negative results are in contrast to the ones by Frasca et al. (1992), who found that thymic CD4 single positive cells recovered

after 9 days of irradiation and injection of Il-3. A reason for these differences could be that in Frasca's experiments, the irradiated mice were injected with high amounts of purified Il-3, whereas in our case, the Il-3 transgene is being expressed at low levels. Our x ray experiments were designed to determine whether irradiation could increase the expression of the Il-3 transgene by activating the LTR; apparently, that was not the case, since no differences were observed in the response to radiation, or the recovery of the hematopoietic system after irradiation.

CHAPTER V

Molecular analysis of azacytidine-induced thymic lymphomas

A. Azacytidine treatment of the FVB and the Il-3 mice

1. Introduction

Retroviral LTRs are methylated during mouse embryogenesis, and this usually renders those genomes silent, that is, there are usually not expressed (Jahner et al., 1982). In order to demethylate the LTR of the transgene, and thus activate transcription, the animals were injected subcutaneously with azacytidine, an agent that has been used to demethylate ecotropic LTRs (Jaenish, 1985). This compound, a deoxynucleotide analog, is incorporated into DNA, inhibits DNA synthesis and blocks cytosine methylation by inhibition of DNA methyltransferase (Glover and Leyland, 1987). Azacytidine accelerates the development of leukemias in mice (Vesely and Cihak, 1973) and increases the incidence of lymphoid neoplasms, lung adenomas, liver tumors, skin tumors, and lung tumors in mice (IARC Monographs, 1990; National Cancer Institute, 1978; Carr et al., 1984; Stoner et al., 1973; Cavaliere et al., 1987; Carr et al., 1988; and Schmahl et al., 1985). Since the results from Chapter III suggested that the Il-3 transgene did not perturb the hematopoietic system of the Il-3 transgenic mice, FVB/N and Il-3 mice were injected with different doses of azacytidine in order to increase the transcriptional expression of the Il-3 transgene. The following studies were designed to determine the effects of azacytidine injection to FVB/N and Il-3 mice and to study the relationship between the transcriptional activation of the Il-3 transgene and thymic lymphoma incidence.

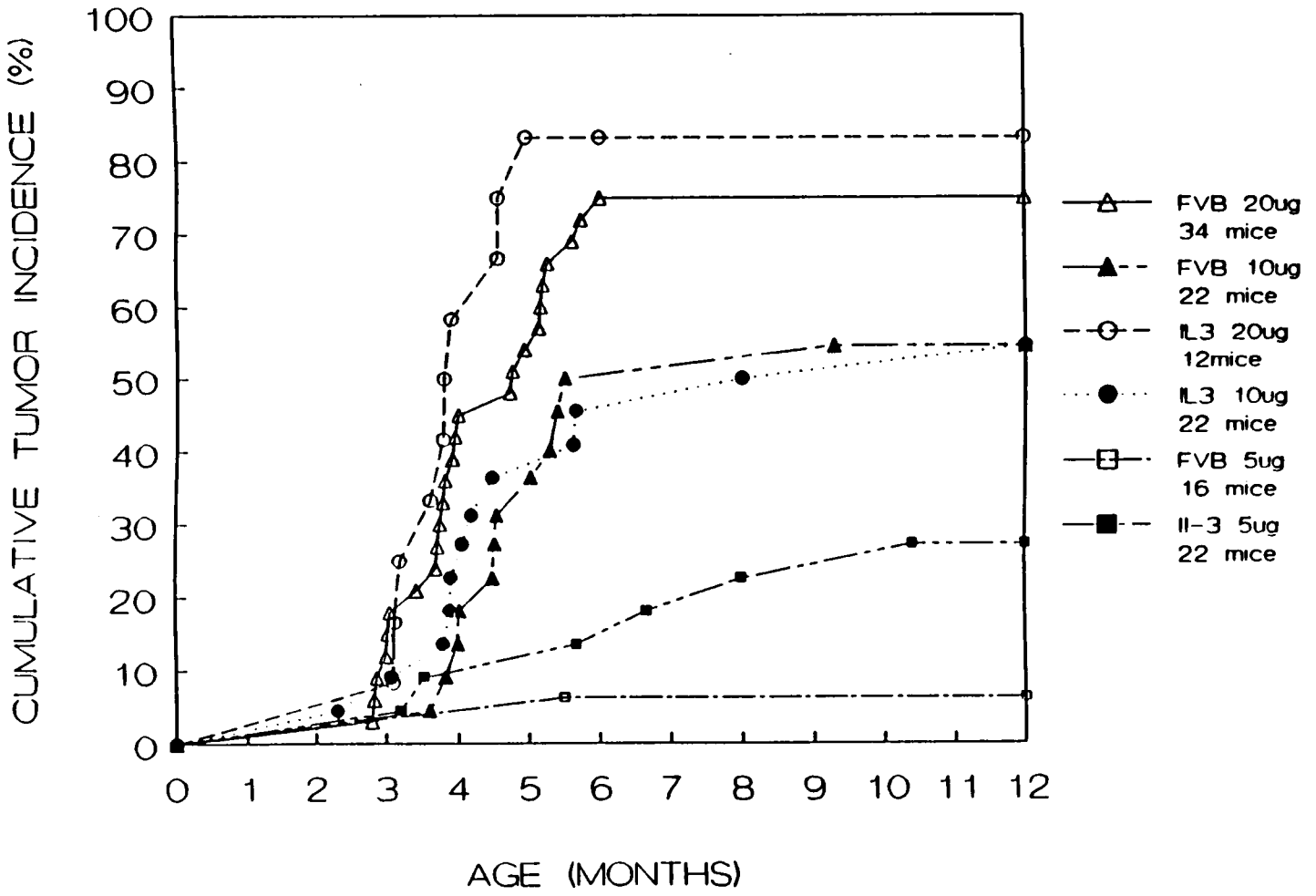
2. Materials and methods

Mice were treated as follows: FVB and Il-3 homozygous and heterozygous mice were injected subcutaneously with 5, 10 or 20 μg of azacytidine (dissolved in sterile 0.85% NaCl solution), on days 3, 5, and 7 after birth. After the injections, the animals were maintained in observation in a clean barrier mouse facility for 12 months.

3. Results

After azacytidine treatment, the mice developed solid tumors in the thymus area, and the time of detection of the tumors was dependent on the Aza-C dose administered (figure 5.1). Mice injected with 20 μg Aza-C developed the thymic lymphomas between 2 to 5 months the average time of detection being 4.6 months; at the 20 μg dose, the pBOR-Il-3 mice had a cumulative tumor incidence of 85%, whereas the FVB/N group had a cumulative incidence of 73%; in the 20 μg group, the two curves are similar up to the 4 month-period, however, between 4 and 6 months, the pBOR-Il-3 mice developed the thymic lymphomas earlier than the FVB/N group. The 10 μg group developed the thymic lymphomas between 2 to 12 months, the average detection time being 5 months for both pBOR-Il-3 and FVB/N mice. At this azacytidine dose, the cumulative tumor incidence was 54% for both pBOR-Il-3 and FVB/N mice. In the 5 μg group, the thymic lymphoma detection time ranged between 3 to more than 10 months, the average time of detection being 6.2 months. At the 5μg dose, in the pBOR-Il-3 mice the cumulative tumor incidence reached 25%, whereas in the FVB/N mice the incidence reached 5%; at that dose, the curves of the pBOR-Il-3 and of FVB/N mice look very different; the Il-3 group developed the tumors earlier than the FVB/N group.

Figure 5.1. Cumulative thymic lymphoma incidence (%) vs. age (months) of the mice. The graph shows FVB animals treated with 20 μg azacytidine (△), 10 μg (▲), and 5 μg (□), and pBOR-Il-3 animals treated with 20 μg (○), 10 μg (●), and 5 μg (■) of azacytidine.


CUMULATIVE TUMOR INCIDENCE (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
AGE (MONTHS)
FVB 20ug
34 mice
FVB 10ug
22 mice
IL3 20ug
12mice
IL3 10ug
22 mice
FVB 5ug
16 mice
Il-3 5ug
22 mice

4. Discussion

The injection of azacytidine to pBOR-Il-3 and FVB/N newborn mice resulted in the development of thymic lymphomas at a high frequency and in a relatively short period of time. As already discussed on Chapter II, the nontreated FVB/N or pBOR-Il-3 mice did not develop spontaneously any particular type of tumor, including thymic lymphomas. Thymic lymphomas were the only tumor observed in the azacytidine-injected mice. The fact that azacytidine injection induced thymic lymphomas in Il-3 and FVB/N mice was not surprising, since thymic lymphomas is the most common type of tumor induced by chemical or physical agents in other strains of mice (Fumarski and Rich, 1982). One possibility contemplated before the azacytidine injections was that the pBOR-Il-3 mice could develop myeloid leukemia; this was possible, since, as already discussed in chapters I and III, overexpression of Il-3 in mice results in a myelodysplasic syndrome, and also, Il-3 has been associated with the development of myeloid leukemias in mice; the thinking behind this possibility was that Il-3 could increase the target myeloid progenitor cells, and that upon treatment with azacytidine, genetic changes would occur, transforming the myeloid progenitor cells. None of our mice developed myeloid leukemia, a cancer induced in other strains, such as RFM, CBA/H and SJL/J mice. There are two main reasons why the Il-3 mice did not develop myeloid leukemia: first, the genetic background is very important in the succeptibility to myeloid leukemias; it has bee recently suggested that one of the genetic changes leading to the development of myeloid leukemia is a chromosome fragile site in mouse chromosome 2 (Hayata et al., 1983; Breckton et al., 1988; Trakhtenbrot et al., 1988). Upon irradiation, the region is deleted, and this event is thought to be one of the initiation steps in myeloid leukemia. The deleted mouse chromosome 2 region contains the homeobox gene *Hox 4.1* and the interleukin-1 beta gene (Blatts and Sachs, 1988; Silver et al., 1989). The translocation causes the deregulation of the Interleukin-1 beta gene, due to hypomethylation of the promoter

(Silver et al., unpublished results, Breckton et al., unpublished results). The mechanism by which thymic lymphomas are is different than for the myeloid leukemias. First, one of the initiation events leading to the devewlopment of thymic lymphomas is the translocation of a region from mouse chromosome 1 to 5, and later the appearance of a trisomy in mouse chromosome 15. Other changes associated with the development of thymic lymphomas is the activation of cellular oncogenes; the oncogenes that are involved depend on the method that is used for the induction of the thymic lymphoma; in chemical induction of thymic lymphomas, the *N-* or *Ki-ras* proteins are mutated (Guerrero et al., 1984 a, b); the two most common integration sites in Mo-MuLV or AKR-derived MuLV induced thymic lymphomas are the *myc* and the *pim-1* protooncogenes activating these oncogenes' transcriptional expression (Steffen et al., 1984; Selten et al., 1985; Cuypers et al., 1986). An alternative explanation why the mice did not develop myeloid leukemias could be that even after azacytidine treatment, the pBOR-Il-3 transgene was not expressed at levels sufficiently high to trigger the myeloid disorders described by Chang et al., (1989). The finding that pBOR-Il-3 mice had a higher cumulative incidence of thymic lymphomas and a faster rate of development (in the 20 and 5 μg doses) of the lymphomas than the FVB/N mice, may suggest a role of Il-3 in the stimulation of the growth of thymic lymphoma cells; it can be either be acting directly on the thymoma cells, or it could be stimulating the production of other cytokines by thymic accessory cells. A model to explain the previous results is the following: azacytidine could be activating the expression of some protooncogenes, possibly *myc* and *pim-1* (the protooncogenes most commonly involved in the induction of thymic lymphomas) or some other protooncogene, such as *N-* or *Ki-ras*; after a particular T-cell is transformed, it starts to take over the normal thymic T-cells; this process occurs rather quickly, depending on the dose of azacytidine that was injected. The fact that the tumors occur rather quickly in FVB/N and suggests that all the genetic events leading to the development of thymic lymphomas occurred

even in the control animals, and that the activation of Il-3 is not a necessary event in the development of the thymic lymphomas (RNA/PCR analysis demonstrated that the endogenous Il-3 was not being expressed in the thymic lymphomas). However, although Il-3 expression is not a necessary event for the induction of thymic lymphomas, the tumors occurred at a higher frequency and faster in the pBOR-Il-3 line, relative to the FVB/N line. That may suggest that Il-3 may be targeting the transformed T-cell and accelerating its growth either by the direct effect on the thymic lymphoma cell, or by the induction of other cytokines by thymic accessory cells.

B. Expression of the Il-3 transgene in the thymic lymphomas and normal thymi of pBOR-Il-3 mice

1. Materials and methods

a) RNA extraction

In order to determine whether the Il-3 transgene was expressed in the thymic lymphomas, RNA was extracted usin the method of Berger and Chirgwin (1989) and then RNA-PCR followed by Southern blotting were be carried out. The guanidinium thiocyanate method procedure for extraction of RNA from either normal pBOR-Il-3 or FVB/N thymi, and of FVB/N or pBOR-Il-3 thymomas was the following: tissues were homogenized at full speed for one min. using a Wheaton 2MO78 homogenizer, in a solution containing guanidinium thiocyanate (4M guanidinium thiocyanate, 0.1 M Tris-Cl, pH 7.5, 1% mercaptoethanol) per 0.7 g of tissue; after homogenization, sodium lauryl sarcosinate was added to a final concentration of 0.5 %, then, the DNA and RNA were precipitated from the rest of the homogenate by adding potassium acetate (pH 5.5) to a final concentration of 0.1 M acetic acid to a final concentration of 0.08 M, and 3/4 volumes of absolute ethanol (the ethanol was added drop by drop while the tube containing the

homogenate was been vortexed). The solution was placed at -20°C for at least two hours and centrifuged in a Sorval RC2 centrifuge at 10,000 rpm for 10 min.; the pellet was then dissolved at room temperature in 5 mL of unbuffered 7.5 M guanidine-HCl (pH 7)/10 mM dithiothreitol. Then, in order to separate the RNA from the remaining DNA, 0.25 mL of 2.2 M potassium acetate (pH 5.5), and 1/2 vol of absolute ethanol (drop-by-drop) were added and the solution was placed at -20°C for at least two hours; this step was repeated twice. The RNA was then recovered by centrifugation at 10,000rpm for 10 min, then, the excess guanidine-HCl was extracted by dispersing the pellet in absolute ethanol at room temperature and centrifuging for 5 min at 10,000 rpm in the Sorval centrifuge. The pellet was dried and dissolved in 1 mL of 20 mM EDTA (pH 7), then chloroform:n-butanol (4:1 v/v) was added and the mixture vortexed for 5 min; after centrifuging briefly to separate the phases, the organic phase was reextracted with 1mL of 20 mM EDTA until the interface decreased. The aqueous phases were combined and precipitated with 2 volumes of 4.5 M sodium acetate (pH 7) at -20°C overnight; if residual DNA was not a problem, the RNA was precipitated by the addition of 0.1 vol. of 2 M sodium acetate (pH 7) and 2.5 vol. of absolute ethanol at -20°C for at least 2 hours. The RNA was then pelleted by centrifugation at 10,000 rpm for 30 min. The RNA was dissolved in DEPC-treated water and the absorbancy read at 260 nm (1 A_{260}=40 μg/mL of RNA).

b) Reverse transcription-PCR of thymic lymphoma RNA

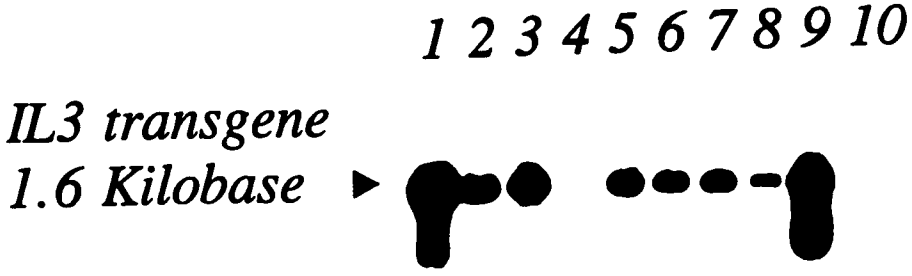
To detect expression of the Il-3 transgene, primers were designed to amplify the transgenic Il-3 mRNA by the polymerase chain reaction, according to the Cetus gene amplification RNA PCR kit instruction manual. The primer specific for the coding strand (the upstream primer) was the proline-tRNA primer binding site of retroviruses ($_pTGGGGGCTCGTCCGGGAT_{OH}$), and the minus strand primer was an anticoding sequence of the 3' end of the Il-3 cDNA ($_pACATTCCACGGTTCCACGGTT_{OH}$). The reverse transcription protocol was done as follows:

a master mix was prepared using reagents at the following final concentrations: $MnCl_2$, 1 mM, 1x rTth reverse transcriptase buffer, dGTP, 200 μM, dATP, 200 μM, dTTP, 200 μM, dCTP, 200 μM, rTth DNA polymerase, 5U/20 μL, downstream primer, 0.75 μM, thymoma or thymus total RNA, $\leq$250 ng, and sterile water to a final volume of 20 μL. The reactions were overlayed with 100 μL of mineral oil and incubated at 70°C for 15 min. The PCR protocol was performed as follows: a master mix was prepared by adding this reagents at the following final concentrations: 0.8 X chelating buffer,1.5 to 2.5 mM $MgCl_2$, 0.15 μM upstream primer, and distilled water to a final volume of 80 μL. The 80 μL of the master mix was dispensed into each reverse transcription reaction tube. The samples were placed in a Perkin Elmer thermocycler, denatured at 94°C for 2 min., annealed at 55°C for 2 min, and amplified at 72°C for 2 min., these three steps were repeated 25 times, and after the final cycle, the samples were incubated at 72°C for 30 min., so the DNA strands in which the synthesis was not finished, could be then completed; the samples were then left at 4°C until the next day. After PCR, the samples were loaded into a 1% agarose/TAE gel and analyzed using the Southern blot procedure and the IL-3 cDNA probe.

2. Results

Figure 5.2 shows the results of the Southern blot analysis of reverse transcribed and PCR amplified thymoma DNA. Lanes 1-3 and 5-8 are thymic lymphomas of Il-3 transgenic mice. Lane 4 represents thymic lymphoma of control FVB/N mice. Lanes 9 and 10 are Il-3-12 thymus (2 months, 26 days-old) and WEHI cell line DNAs, respectively. According to this figure, the pBOR-Il3 transgene is being expressed in both normal thymi and thymomas of Il-3 transgenic

Figure 5.2. Southern blot analysis of RNA/PCR amplified DNA. The RNA was reverse transcribed using the RNA/PCR method, ran on an agarose gel, Southern-transferred, and probed using an Il-3 cDNA, as described in materials and methods. Lanes 1-3, and 5-8, represent thymic lymphoma DNA from pBOR-Il-3-12 mice, lane 4, thymic lymphoma DNA from and FVB/N mouse, lanes 9, pBOR-Il-3 normal thymus, and lane 10, WEHI cell line DNA.


1 2 3 4 5 6 7 8 9 10
IL3 transgene
1.6 Kilobase

mice, given the presence of the expected 1.6 kb band in the autoradiogram. Previous results using the RNA/PCR technique (data not shown), demonstrated that the endogenous Il-3 was not expressed in the FVB/N thymic lymphomas.

3. Discussion

RNA/PCR analysis of the thymic lymphomas demonstrated that the transgene is being expressed in both the azacytidine-induced thymic lymphomas, and in normal, 2 months, 26 day-old Il-3 thymus. This results may explain the higher cumulative tumor incidence and faster development of the thymic lymphomas reached in the pBOR-Il-3 mice, relative to the FVB/N mice. The injections of azacytidine in the pBOR-Il-3 mice might increase the expression of the Il-3 transgene; however, the transgene is expressed at approximately the same, but low levels in the thymic lymphomas, and the normal thymi of Il-3 mice. Expression of the Il-3 transgene was too low to be observed in Northern blots or in the agarose gels resulting from the RNA/PCR amplification (data not shown), but expression of the Il-3 transgene could be detected following Southern transfers of the RNA/PCR reaction and hybridizations with the Il-3 cDNA (figure 6). Even though the transgene is being expressed at low levels, these experiments suggest that this low expression accounts for the differences in tumor incidence and the time of development of the azacytidine-induced thymic lymphomas between pBOR-Il-3 and FVB/N mice. Results of any RNA/PCR reaction have to be analyzed with caution, because even minute amounts of DNA contamination could lead to amplification of the DNA sequence. In this study, to reduce contaminating DNA in these RNA preparations., the last step of the RNA extraction procedure (a step done to eliminate contaminating DNA) was repeated at least three times.

C. T-cell receptor analysis of the thymic lymphomas

1. Introduction

T-cells recognize and eliminate antigens that are presented to the T-cell by the major histocompatibility complex present in antigen-presenting cells, such as macrophages. T-cells are produced in the bone marrow and migrate to the thymus to differentiate into either cytotoxic effector cells ($CD8^+TCR^+$), inducer or helper cells ($CD4^+TCR^+$), or suppressor cells; the T-cell receptors genes rearrange in a similar fashion as the immunoglobulin genes in order to assemble a functional T-cell antigen receptor and to carry out the diverse functions of a T-cell in the immunological system (Hood et al., 1985). Flug et al. (1985), Minden et al. (1985), and Vasmel et al. (1987) applied the analysis of TCR β gene rearrangements to study the lineage, clonality and differentiation of T-cells. When they used the constant region of the β subunit gene and probed the Hind III-cut genomic DNA, two bands of 3 kb and 9 kb appeared in tissues that do not contain T-cells that have rearranged T-cell receptor genes (the genes were in germline configuration); in tissues such as the thymus, which has a high content of T-cells with rearranged T-cell receptor genes, a 3 kb band will always appear in the Southern blot, and a smear will appear in the region in the region on top of the 3 kb band, due to the heterogeneity of the T-cell population in the thymus. The clonality of a thymic lymphoma can be tested using its TCR gene rearrangements, since a particular TCR rearrangement is specific to a particular T-cell population. If a thymic lymphoma is of a monoclonal origin, it will contain the control, 3 kb band, plus a band rearranged from the 9 kb region, to another size; also, a monoclonal tumor could be one in which two different rearrangements occur in one cell, one in one chromosome and one in the other (if this is the case, the intensities of the bands in the Southern blot will be the same); another type of single rearrangement could be the deletion of the 9 kb band. If a thymic

lymphoma is of oligoclonal origin, it will show more than one rearrangement of the T-cell receptor genes.

2. Materials and methods

a) Thymoma DNA extraction

DNA was extracted from thymomas and thymi using the Hirt's method (1967), modified by Dr. W. Yang. Briefly, 1 mL of cold solution H (0.5 M sucrose, 15 mM Tris-Cl, pH 7.4, 25 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA) was added to 0.03-0.05 g of tissue, and then it was homogenized. Then 1 more mL of solution H and 0.18 mL of 10 % Triton were added. The homogenized tissue was then placed in a 10 mL Oak Ridge centrifuge tube, and was spun at 2000 rpm for 10 min in a refrigerated International centrifuge. The supernatant was then discarded. The nuclear pellet was then suspended in 1 mL of $T_{10}E_{10}N_{150}$(10 mM TrisCl, pH 7.6, 10 mM EDTA, 150 mM NaCl), resuspended by vortexing the tube gently and then, the solution was dropped in 3 mL Hirt solution (10 mM TrisCl, pH 7.6, 10mM EDTA, 1% SDS) at 65°C. Then, 200 μL of proteinase K and 20 μL of 20 mg/mL pronase were added, and the tubes containing the solution was placed at 55°C and incubated for at least 4 hours. Then, 0.42 mL of 5 M NaCl was added, and extracted with an equal volume of phenol:chloroform (1:1 v/v). Then 0.8 mL aliquots were transferred into 2 mL microfuge tubes. After this step, the DNA can be precipitated by adding 0.8 mL isopropanol, 1 mL of 95% ETOH, centrifuged at 10,000 rpm for 15 min; the supernatant was discarded, the pellet was washed with 70% ETOH, and the pellet dried in a Savant speed dried concentrator. The pellet was then dissolved in 100 μL TE (10 mM TrisCl, pH 7.4, 1 mM EDTA, pH 8).

b) Electrophoresis and Southern Blots of thymoma DNAs

Restriction digestion was performed by cutting 10 to 20 μg of the thymoma DNA with the *Hind III* restriction enzyme, and the digestion electrophoresed through 0.8% gels at 50 to 60 volts overnight. The Southern blots were performed as described in Chapter II.

c) DNA probe

The probe used in this study was the 86T5 cDNA containing the Cβ2 fragment of the mouse TCR β chain gene, that also hybridizes to the homologous Cβ1 gene.

3. Results

Figure 5.3 show the autoradiogram from the T-cell receptor rearrangement experiments. The faint 5 kb band present in the Il-3 samples is the result of hybridization of contaminating plasmid sequences in the isolated TCR cDNA. Lanes 1 to 6 show FVB thymoma samples, lane 7 is FVB liver DNA and lanes 9 to 16 are Il-3 thymoma DNAs, lane 17 is DNA from Il-3 thymus, and lane 18 is DNA from Il-3 liver. Samples in lanes 1, 3, 4, 10, and 13 show deletion of the 9 kb band; samples in lanes 2, 6, 9, 14 and 15 show single rearrangements; samples in lanes 5, and 12 show two rearrangement patterns, and the sample in lane 5, show three rearrangements.

The results, as shown in figure 5.3, are summarized in table 5.1. From 28 thymic lymphomas analyzed for TCR gene rearrangements, 28 showed T-cell receptor gene rearrangements. Of the 28 lymphomas analyzed, 15 were induced by azacytidine in the Il-3 line and 13 in the control group. In the Il-3 thymomas, five showed deletions of the 9 kb band, four had an 8kb band, one had an 11 kb band, one had a 10 kb, two samples showed two different rearrangements, and two showed three different rearrangements. In the FVB group, four samples showed deletion of the 9kb band, one had a 4.4 kb band, another had a 7.5 kb band, one had a 10 kb and another had an 8.5 kb band; there were two samples showing two rearrangements, and

Figure 5.3. T-cell receptor rearrangements in thymic lymphomas. The genomic DNA was restricted with the *Hind* III restriction enzyme, electrophoresed through an agarose gel, Southern transferred, and hybridized to the 86T5 probe. Lanes 1-6 represent FVB/N thymoma DNAs; lane 7, FVB thymus DNA; lane 9, FVB/N liver DNA; lanes 10-16, pBOR-Il-3 thymoma DNA; lane 17, pBOR-Il-3 thymus DNA; and lane 18, pBOR-Il-3 liver DNA.

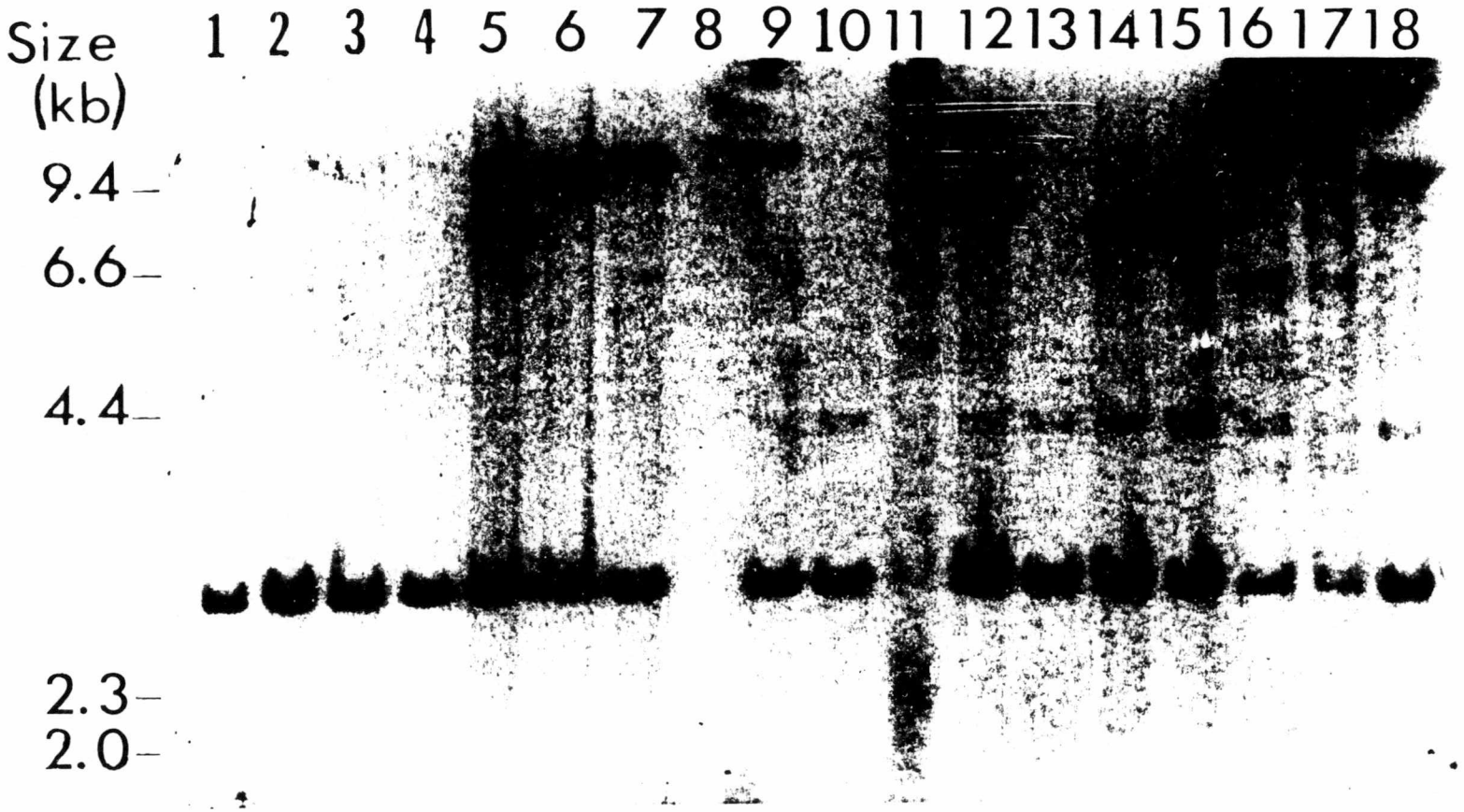

Size
(kb)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
9.4
6.6
4.4
2.3
2.0

Table 5.1

T-cell receptor gene rearrangements in FVB and pBOR-Il-3 mice.

	FVB[a]	pBOR-Il3[a]
I. Single rearrangements	8	11
Size of the rearranged band (kb)		
4.4	1	0
8.5	1	0
8.0	0	4
7.5	1	0
10	1	1
11	0	1
Deletions of the 9 kb band	4	5
II. Two rearrangements	2	2
III. Multiple rearrangements	2	2

[a] The numbers in each column represent the type of rearrangement and the size of the rearranged band in the individual thymic lymphomas.

three showing three rearrangements.

4. Discussion

In order to assess whether the thymic lymphomas were of a T-cell origin, and whether the tumor had originated from a single or multiple cells, the T-cell receptor gene rearrangement analysis was performed. The analysis revealed that all of the thymomas in FVB/N and pBOR-Il-3 had rearranged T-cell receptor genes, demonstrating that the thymic lymphomas were of a T-cell origin. Although the current hypothesis is that cancer occurs through the genetic transformation of a single cell that expands and takes over the normal cells (Fialkow, 1972), thymic lymphoma is one of the exceptions (Cuypers et al., 1986). It was frequently claimed that MuLV-induced thymic lymphomas in AKR and BALB/Mo mice were monoclonal in origin (Chattopadhyay et al., 1982; Quint et al., 1981; Collins et al., 1982) Later on, it was demonstrated that thymic lymphomas that were induced by the intrathymic injection of the MCF69L1 virus to AKR/J mice could be of an oligoclonal nature (O'Donell et al., 1985) In our experiments about 80% of the thymic lymphomas showed single rearrangements of the T-cell receptor genes, suggesting that most of the thymic lymphomas induced by azacytidine were of monoclonal origin. The other 20% of tumors showed more than one rearrangement of the T-cell receptor genes, meaning that more than one cell became transformed; the maximum number of T-cell receptor gene rearrangements in a thymoma was three. These results, along with the tumor incidence observations, suggest that in most of the azacytidine-induced thymic lymphomas, a single cell was transformed by the azacytidine treatment, and that transformed cells grew rapidly and replaced the normal cells in the thymus of the mice; the fact we also obtained tumors arising from the transformation of different cells has been observed by other laboratories (Cuypers et al., 1986).

D. Flow cytometric analysis of thymic lymphomas derived from Il-3 transgenic mice and FVB mice

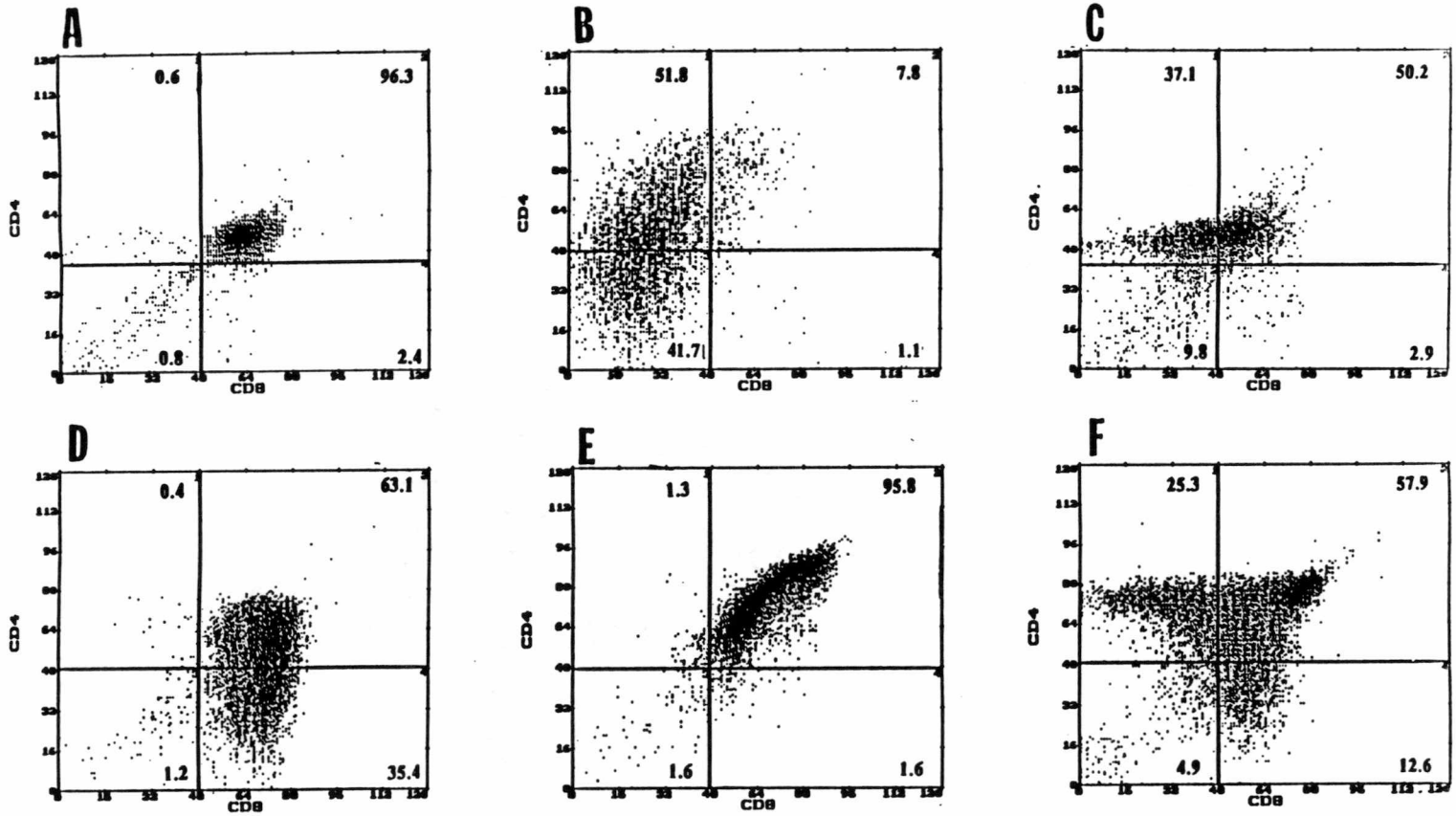
1. Materials and methods

Flow cytometry was used to analyze the T-lymphocyte composition in the thymomas, to determine whether or not there were differences in the composition of the T-cell subpopulations between Il-3 and FVB, and to analyze the stage of differentiation of the T-cell lymphomas. After a thymic lymphoma was detected in the animal, the animal was sacrificed, and a small piece of the thymoma (about 0.5 cm in length) was used to obtain the single cell suspension. The monoclonal antibodies used were the CD3-FITC or CD3-PE, the CD4-PE and the CD8-FITC. The T-cell preparations from the thymomas and the flow cytometric analysis was identical to the methods described in chapter III section C.

2. Results

Figure 5.4 shows dot plot analyses of the six different patterns of thymic lymphomas found in this study. In pattern A, the predominant population of thymocytes was CD4/CD8 double positive; in type B, there were two major populations, a CD4 single positive and a CD4/CD8 double negative subpopulation; in pattern C, the predominant subpopulation was the CD4/CD8 double positive and is continuous with the CD4 single positive subpopulation; in pattern D, the predominant subpopulation was the CD4/CD8 double positive and was continuous with the CD8 single positive subpopulation; in pattern E, the CD4 low/CD8 low subpopulation was gaining the CD8 and CD4 antigens to become CD4/CD8 double positive; and in pattern F, the predominant subpopulation was double positive and was continuous with the CD4 single positive and the CD8 single positive subpopulations. After determining the percentage of each

Figure 5.4. Dot plot diagrams resulting from the flow cytometric analyses of thymic lymphoma cells. The six different patterns of azacytidine-induced thymic lymphomas obtained are shown. Panel A, the CD4/CD8 population is predominant, panel B, the CD4/CD8 double negative, and the CD4 single positive populations are predominant, panel C, the CD4/CD8 double positive, and the CD4 single positive populations are predominant, panel D, the CD4/CD8 double positive, and the CD8 single positive subpopulations are predominant, and in panel F, the CD4/CD8 double positive, the CD4 and CD8 subpopulations are predominant.


A
0.6
96.3
0.8
2.4
B
51.8
7.8
41.7
1.1
C
37.1
50.2
9.8
2.9
D
0.4
63.1
1.2
35.4
E
1.3
95.8
1.6
1.6
F
25.3
57.9
4.9
12.6
CD4
CD8

subpopulation according to the CD4/CD8 cell surface antigens, CD3 analysis was done to determine the percentage of CD3 negative, CD3 low, CD3 intermediate, and CD3 high T-cells; the results are summarized in Table 5.2. The first column shows the mouse line (FVB/N or pBOR-Il-3) from which the thymic lymphoma was obtained; the second column describes the name of each sample; the third column shows the sex of the animal, the fourth column shows the amount of azacytidine injected, the fifth column shows the age at which the thymic lymphoma was obtained and the following columns show the T-cell subpopulations in the thymic lymphomas: $CD3^-$, $CD3^{low}$, $CD3^{int}$, $CD3^{high}$, CD4 single positive, CD4/CD8 double positive, CD4/CD8 double negative, and the CD8 single positive. In the normal animal, the distribution of the different subpopulations is the following: the CD4 single positive-$CD3^{high}$ subpopulation accounts for 8% of the thymocytes in the thymus, the CD4/CD8 double positive-$CD3^-$ accounts for 40%, the CD4/CD8 double positive-$CD3^{low}$ for 40%, the CD4/CD8 double negative-$CD3^-$ for 5%, and the CD8 single positive-$CD3^{high}$ subpopulation accounts for 5% of the thymic T-cells (Crispe et al., 1987, von Boehmer, 1988). The summary of these experiments is shown in Table 5.3. This table shows the pattern of each thymoma, as described in Figure 5.4 and the main differentiation pathway of each thymoma, according to Figure 5.4 and Table 5.2. In FVB/N, one pattern A thymoma was observed, as in pBOR-Il-3 mice. There was one mouse with thymoma pattern B in FVB/N and none in pBOR-Il-3. There were 5 cases of thymoma pattern C in FVB/N and 2 cases in pBOR-Il-3. There were three cases of thymoma pattern D in FVB/N and 6 cases in pBOR-Il-3. There were two cases of thymoma pattern E in FVB/N versus one in pBOR-Il-3 and one case of thymoma pattern F in FVB/N versus 3 in pBOR-Il-3. Table 5.4 shows the thymic lymphomas and the clonality of those for which the T-cell receptor gene rearrangement analysis and the flow cytometric analysis were performed. This table is useful to analyze the clonality of each individual thymic lymphoma and if the clonality correlates with one or more population

Table 5.2

Flow cytometric analysis of T-cell-specific surface markers in thymic lymphomas of the FVB/N and the pBOR-Il-3 mice.

Line	Sample no.	Sex	Dose (μg)	Age (days)	$CD3^{-}$ [3]	$CD3^{low}$	$CD3^{int}$	$CD3^{high}$	$CD4^{+}CD8^{-}$	$CD4^{+}CD8^{+}$	$CD4^{-}CD8^{-}$	$CD4^{-}CD8^{+}$
FVB	TH91219	F	20	158	1.3	3.3	38.4	62.6	3.1	95.1	1.1	0.7
	TH92013	F	20	142	15.8	60.7	24.0	5.2	51.8	7.8	41.7	1.1
	TH92023	F	20	142	20.8	57.5	16.4	8.3	4.9	75.8	15.6	3.8
	TH92038	M	20	137	1.9	25.6	52.9	21.5	35.6	57.9	4.0	2.7
	TH92043	M	20	137	31.0	58.4	10.8	8.8	3.1	81.9	5.9	9.8
	TH100215	F	20	151	7.3	27.0	21.5	47.0	72.0	18.9	7.6	1.8
	TH100223	M	20	150	8.5	53.6	35.2	4.5	1.3	95.8	1.6	1.6
	TH100231	M	20	150	8.2	8.6	11.2	72.2	34.0	45.8	15.6	4.6
	TH100239	M	20	150	5.1	54.1	37.7	7.5	1.1	90.2	3.4	5.4
	TH109153	M	20	244	1.1	67.3	30.0	7.0	37.1	50.2	9.8	2.9
	TH04293	F	10	151	3.3	66.7	27.0	7.8	6.9	51.4	2.9	38.8
	TH042930	M	10	165	1.0	3.2	57.9	38.8	0.4	63.1	1.2	35.4
	TH042945	F	10	165	11.1	0.0	33.0	59.6	25.3	57.3	4.9	12.6
Il3	920C4828	F	20	137	10.7	65.5	27.3	4.5	0.9	67.4	2.0	31.7
	920C4833	F	20	137	11.6	60.7	28.3	4.2	0.0	96.4	1.9	1.7
	1002C487	F	20	150	7.2	62.9	26.6	9.0	3.4	95.2	0.9	0.5
	312C4839	F	20	150	2.7	13.7	53.0	36.0	80.5	2.3	16.4	0.8
	317C483	F	10	114	14.9	78.1	8.4	3.9	26.3	43.1	16.2	14.0
	317C4830	M	5	106	17.1	68.3	9.3	5.7	1.1	67.3	3.5	28.2
	317C4833	F	10	117	19.0	77.0	6.9	1.7	2.6	16.3	25.0	57.5
	403C4818	M	10	147	17.5	81.3	3.0	0.9	0.6	96.3	2.4	0.8
	403C4821	M	10	127	9.3	76.4	12.3	4.5	1.2	53.4	6.6	41.0
	403C4842	F	10	135	15.4	83.1	3.3	1.2	18.8	79.3	1.6	0.6
	429C4812	M	10	122	9.1	0.0	62.4	34.2	18.2	11.8	57.3	13.1
	429C4821	F	10	164	46.8	0.0	54.8	2.6	0.3	61.0	1.3	41.4

[3] The numbers in the columns represent the percentage of the population in the gated live thymoma cells.

Table 5.3

Differentiation states and clonality of azacytidine-induced thymic lymphomas.

Line	Sample no.	Predominant differentiation pathway	Clonality
FVB	TH91219	$CD4^+CD8^+CD3^{-/Low}$	N/D[a2]
	TH92013	$CD4^+CD8^-CD3^{High}$ to $CD4^-CD8^-CD3^{Low}$	N/D
	TH92023	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^+CD8^{Low}CD3^{Int}$	N/D
	TH92038	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^+CD8^-CD3^{High}$	N/D
	TH92043	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	N/D
	TH100215	$CD4^+CD8^{Low}CD3^{Low}$ to $CD4^+CD8^-CD3^{High}$	Oligoclonal
	TH100223	$CD4^+CD8^+CD3^{Int}$ to $CD4^{Low}CD8^{Low}CD3^{Low}$	Monoclonal(2 bands, same intensity)
	TH100231	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^+CD8^-CD3^{High}$	N/D
	TH100239	$CD4^+CD8^+CD3^{Int}$ to $CD4^{Low}CD8^{Low}CD3^{Low}$	Monoclonal
	TH109153	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^+CD8^-CD3^{High}$	Monoclonal
	TH04293	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	Monoclonal
	TH042930	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	Monoclonal
	TH042945	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^+CD8^-CD3^{High}$ and $CD4^-CD8^+CD3^{High}$	Monoclonal
113	920C4828	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$ and $CD4^+CD8^{Low}CD3^{Int}$	N/D
	920C4833	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	N/D
	1002C487	$CD4^+CD8^+CD3^{Int}$ to $CD4^{Low}CD8^{Low}CD3^{Low}$	N/D
	312C4839	$CD4^+CD8^{Low}CD3^{Int}$ to $CD4^+CD8^-CD3^{High}$	N/D
	317C483	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^+CD8^{Int}CD3^{High}$ and $CD4^{Int}CD8^+CD3^{Int}$	N/D
	317C4830	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	Oligoclonal
	317C4833	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	Monoclonal
	403C4818	$CD4^+CD8^+CD3^{Low}$	Monoclonal
	403C4821	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^-CD8^+CD3^{High}$	N/D
	403C4842	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^{Low}CD8^{Low}CD3^{Int}$	Monoclonal
	429C4812	$CD4^-CD8^-CD3^-$ to $CD4^+CD8^+CD3^{Int}$ to $CD4^+CD8^-CD3^{High}$ and $CD4^-CD8^+CD3^{High}$	Biclonal
	429C4821	$CD4^+CD8^+CD3^{-/Low}$ to $CD4^{Low}CD8^+CD3^{Int}$	N/D

2

a. The clonality of the tumor was not determined (N/D)

Table 5.4

Differentiation state of the different types of azacytidine-induced thymic lymphomas in FVB/N and pBOR-Il-3 mice

Type of thymoma	Main differentiation pathway	Number of cases	
		Line	
		FVB/N	pBOR-Il3
A	$CD4^{+}CD8^{+}CD3^{-/Low}$	1	1
B	$CD4^{+}CD8^{-}CD3^{High}$ and $CD4^{-}CD8^{-}CD3^{-/Low}$	1	0
C	$CD4^{+}CD8^{+}CD3^{-/Low}$ to $CD4^{+}CD8^{-}CD3^{High}$	5	2
D	$CD4^{+}CD8^{+}CD3^{-/Low}$ to $CD4^{-}CD8^{+}CD3^{High}$	3	6
E	$CD4^{-/Low}CD8^{-/Low}CD3^{-/Low}$ to $CD4^{+}CD8^{+}CD3^{Low/Int}$	2	1
F	$CD4^{+}CD8^{+}CD3^{-/Low}$ to $CD4^{+}CD8^{-}CD3^{High}$ and $CD4^{-}CD8^{+}CD3^{High}$	1	2

present on the dot plot. Of 7 FVB/N thymic lymphomas analyzed, 6 were of a monoclonal origin (1 showing two bands in the Southern blot, both bands being of the same intensity) and one of the tumors was of an oligoclonal origin. Of 5 pBOR-Il-3 analyzed, 4 were monoclonal (one showing two bands with the same intensity) and one was of an oligoclonal origin.

3. Discussion

Since it was determined that Il-3 affects the growth of the thymic lymphomas, the flow cytometric analysis was performed in order to determine whether the T-cell composition in the Il-3 thymic lymphomas was different from the FVB/N control group. There were no significant differences in any of the T-cell subpopulations in the thymic lymphomas, although in pBOR-Il-3, there were a higher number of cases of the thymoma type in which the $CD4^+CD8^+$ is differentiating towards the $CD8^+CD4^-$ subpopulation, relative to FVB/N. On the other hand, in FVB/N, there were more cases of thymic lymphomas in which the $CD4^+CD8^+$ subpopulation is differentiating towards the $CD4^+CD8^-$ subpopulation, relative to the pBOR-Il-3 mice. The possibility that Il-3 promotes the differentiation of the thymic lymphoma cells towards the $CD8^+CD4^-$ subpopulation, needs to be tested by doing the flow cytometric analysis of more FVB/N and pBOR-Il-3 thymic lymphomas. If this phenomenon is true, it suggests that Il-3 is acting in the Il-3 thymomas, by stimulating the growth of the CD8 single positive subpopulation. This result would suggest that Il-3 may act directly or indirectly (through other cytokines) to increase the CD8 single positive subpopulation. The only report in the literature suggesting that Il-3 may help recover the CD8 single positive subpopulation after radiation damage, was reported by Frasca et al., (1992), in which Il-3, after the irradiation of mice, helped the CD4 and the CD8 single positive subpopulations to recover after their numbers were diminished by the x-irradiation. Given that the predominant type of T-cell subpopulation in the thymic lymphoma is of a

$CD4^+CD8^+CD3^{-/low}$ phenotype, which can differentiate either to a $CD4^+CD8^-CD3^{high}$ or $CD8^+CD4^-CD3^{high}$ subpopulation, and given the fact that all of the thymic lymphomas had rearranged T-cell receptor genes, it is clear that thymocytes treated with azacytidine do not lost the ability to undergo differentiation, although their differentiation process is aberrant. The presence. The presence of thymic lymphoma cells at different stages of differentiation, given the presence of a smear from the $CD4^+CD8^+$ population to the $CD4^+CD8^-$ cells in thymomas that followed pattern C or to the $CD8^+CD4^-$ in thymomas following pattern D has been observed in other laboratories (Davis et al., 1993; Chan et al., 1993); Chan et al, (1993) reported the existence of a $CD4^+CD8^{lo}$ thymocyte subset, which was exaggerated in class-II MHC deficient mice; an equivalent phenomenom is observed in class-I MHC deficient mice, in which the $CD4^{lo}CD8^+$ subset is exaggerated; those cells are intermediate in maturity between the double positive and either the CD4 or CD8 T-lymphocytes. The fact that the differentiation of azacytine-induced thymic lymphomas is gradual (the presence of stages intermediate in differentiation) and, in most cases, committed to either the CD4 single positive, or the CD8 single positive subpopulations, makes the azacytidine-induced thymic lymphomas a good model to study the molecular events that determine how T-cells are committed from a double positive cell into either a CD8 single positive or a CD4 single positive cell.

Summary

The pBOR-Il-3 transgenic mice were developed by the pronuclear microinjection method to study how activation of the transcriptional expression driven by a retroviral long terminal repeat occurs and especially how do insults by both chemical and physical agents modulate transcription driven from the LTR, and to study the biological functions of interleukin-3, a cytokine involved in the proliferation and differentiation of bone marrow precursor cells to mature granulocytes, macrophages, megakaryocytes, and erythroid cells. Since interleukin-3 has been implicated as one of the steps involved in the development of myeloid leukemias in mice, particular attention was placed on whether the pBOR-Il-3 transgene in the cells of mice could predispose them to myeloid leukemias. Our studies showed that pBOR-Il-3 mice that were not treated with any chemical or physical agents were completely normal, and they did not develop any particular spontaneous tumors, relative to the FVB/N control group. Different hematological/cellular immunological techniques, such as flow cytometry, in vitro colony assays, and differential cell counting of blood cells, and the use of histological techniques, revealed no differences between pBOR-Il-3 and the FVB/N mice for the following hematopoietic subpopulations: a) the number of Thy-$1^{low}Pgp^{int}Fall\text{-}3^{+}$ cells (a population that contains the pluripotent stem cell), b) the number of hematopoietic precursor cells (CFU-C, BFU-E, CFU-E), c) the number of blood leukocyte subpopulations, such as eosinophils, neutrophils, and lymphocytes, d) in spleen and bone marrow megakaryocytes, mast cells, granulocytes, monocytes, or macrophages, or e) in spleen and thymus T-lymphocytes. Only minor differences were observed, such as a higher number of monocytes in 2-months-old pBOR-Il-3 mice, and a higher number of neutrophils in 5-months-old pBOR-Il-3 mice, relative to FVB/N control mice. The fact that the hematopoietic subpopulations in the pBOR-Il-3 mice were not different from the

ones from FVB/N controls, suggested that Il-3 was not being expressed in transgenic mice at levels high enough to perturb hematopoiesis. This could be due to a silenced long-terminal repeat; this was finally confirmed by the use of the Reverse Transcription/Polymerase Chain Reaction technique, which revealed that the Il-3 transgene was being expressed at low levels in normal Il-3 thymi. Therefore, the hematopoietic system of the pBOR-Il-3 mice would need to be perturbed or transcription from the Il-3 transgene would have to be increased to detect any differences in the hematopoietic system of the pBOR-Il-3 mice, relative to FVB/N controls. Two methods were used to perturb the hematopoietic system of the mice: the first was the use of a physical agent, x ray. Both pBOR-Il-3 mice, and FVB/N mice were irradiated with 2 Gy of x rays, and the recovery of the different hematopoietic subpopulations was asessed on days 1 and 8 after irradiation. As it turned out, there were no differences in the radiation recovery of the different hematopoietic subpopulations in the blood, bone marrow, spleen, or thymus; this could be due to the fact that the radiation dose used in this experiments was not sufficient to signifficantly increase the LTR-driven Il-3 transcription. The other method used to perturb the hematopoietic system of the mice was the injection of azacytidine, a demethylating agent traditionally used to activate the LTR-driven expression of retroviral genomes. Upon azacytidine treatment, both the pBOR-Il-3 mice and the FVB/N controls developed thymic lymphomas, the transgenic mice developed the tumors at a higher frequency and relatively faster than FVB/N controls. This result suggested that Il-3 expression is not a necessary step to the development of thymic lymphomas, but that Il-3 accelerates the growth of thymic lymphoma cells. The thymic lymphoma cells were of a T-cell origin, as determined by the T-cell receptor gene rearrangement analysis, and, in most cases, were of a monoclonal origin. According to the flow cytometric analysis of thymic lymphoma cells, labelled with florescent-labelled T-cell-specific antibodies, the thymic lymphoma cells did not lose the ability to to differentiate, but the differentiation process is

aberrant. Studies on the CD4/CD8 T-cell surface markers revealed that in pBOR-Il-3 thymic lymphomas the predominant differentiation pathway is one in which the $CD4^+CD8^+$ subpopulation differentiates towards the $CD8^+CD4^-$ subpopulation; conversely, in the FVB/N group, the predominant differentiation pathway is the $CD4^+CD8^+$ subpopulation differentiating towards the $CD4^+CD8^-$ subpopulation; additional flow cytometric analyses must be done on the thymic lymphoma cells, to confirm this hypothesis.

Bibliography

Bibliography

Andrews, G. A., (1980). Medical management of accidental total-body irradiation. In: Medical Basis of Radiation Accident Preparedness, K. F. Hubner, S. A. Fry, eds. Elsevier/North Holland, 279-310.

Austyn, J.M., and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. 11:805-815.

Basch, R.S., and Berman,J.W. (1982). Thy-1 determinants are present on many murine hematopoietic cells other than T-cells. Eur. J. Immunol. 12:359.

Berger, S.L., and Chirgwin, J.M. (1989). Isolaton of RNA. Methods in Enzymology 180: 3-7.

Blatt, C., and Sachs, L., (1988). Deletion of a homeobox gene in myeloid leukemias with a deletion of chromosome 2. Biochemical and Biophysical Research Communications, 156:1265-1270.

Boone, L. R., Myer, F. E., Yang, D. M., Kiggans, J. O., Koh, C., Tennant, R. W., and Yang, W. K. (1983). Analysis of recombinant DNA clones of the endogenous BALB/c murine leukemia virus WN1802N: variation in long terminal repeat length. Journal of Virology, 45:484-488.

Breckton, G., Silver, A. R. J., and Cox, R., (1988). Consistent chromosome changes in radiation-induced murine leukaemias. Kew Chromosome Conference III, edited by P. E. Brandham and M. D. Bennet (London:HMSO), p. 179-184.

Breckton, G., Papworth, D., and Cox, R. (in press). Murine radiation myeloid leukaemogenesis: A possible role for radiation sensitive sites on chromosome 2. Genes, Chromosomes and Cancer.

Brock, J. H. (1989). The biology of iron. In: Iron in Immunity. Edited by M. de Sousa and J. H. Brock; John Wiley and Sons, publishers, 35-53.

Campbell, D.G., Gagnon, J., Reid, K.M.B., and Williams, A.F., (1981). Rat brain Thy-1 glycoprotein. Biochem. J. 195:15-30.

Carr, B.I., Reilly, G., Smith, S.S., Winberg, C., and Riggs, A. (1984). The tumorigenicity of 5-azacytidine in the male Fischer rat. Carcinogenesis, 5:1583-1590.

Carr, B.I., Rahbar, S., Asmeron, Y., Riggs, A., and Winberg, C.D. (1988). Carcinogenicity and haemoglobin synthesis induction by cytidine analogues. Br. J. Cancer, 57:395.

Cavaliere, A., Bufalari, A., and Vitali, R. (1987). 5-azacytidine carcinogenesis in BALB/c mice. Cancer lett., 37:51-58.

Chada, K.J., Magram, J., Raphael, K., Radice, G., Lacy, E., and Constantini, F. (1985). Specific expression of a foreign beta-globin gene in erythroid cells of transgenic mice. Nature, 314:377-380.

Chan, S. H., Cosgrove, D., Waltzinger, C., Benoist, C., and Mathis, D. (1993). Another view of the selective model of thymocyte selection. Cell, 73:225-236.

Chang, J.M., Metcalf,D., Lang,R., Gonda,T.J., Johnson,G.R. (1989). Nonneoplastic hematopoietic myeloproliferative syndrome induced by disregulated Multi-CSF (Il3) expression. Blood 73(6):1487-97.

Chattopadhyay, S. K., Cloyd, M. W., Linemeyer, D. L., Lander, M. R., Rands, E., and Lowry, D. R. (1982). Cellular origin and role of mink cell focus-forming viruses in murine thymic lymphomas. Nature, 295:25-31.

Chervenak, R., and Altazan, J. (1990). In vitro maintenance of bone marrow-residents T-cell precursors in cultures supplemented with exogenous Il-3. Cellular immunology, 130:150-159.

Chevernak, R., Soloff, R., Dempsey, D., Jennings, S., and Wolcott, R. (1991). Characterization of the progeny of pre-T cells maintained in vitro by Il-3: expression of the Il-2 receptor and CD3 during thymic development. Cellular Immunology 134: 349-358.

Church,G.M., Gilbert,W. (1984). Genomic sequencing. PNAS USA. 81:1991-95.

Clark, S. C. (1991). Hematopoietic growth factors and the myeloid leukemias. In "Origins of Human Cancer: A Comprehensive Review. Cold Spring Harbor Laboratory Press, 453-462.

Collins, S. J., and Fialkow, (1982). Clonal nature of spontaneous AKR leukemia: studies utilizing the X-linked enzyme phosphoglycerate kinase. Int. J. Cancer, 29:673-676.

Cuypers, H., Selten, G., Zijlstra, M., De Goede, R., Melief, C., and Berns, A. (1986). Tumor progression in murine leukemia virus-induced T-cell lymphomas: monitoring clonal selection with viral and cellular probes. J. Virol. 60(1):230-241.

Davis, C. B., Killeen, N., Casey-Crooks, M. E., Raulet, D., and Littman D. R. (1993). Evidence for a stochastic mechanism in the differentiation of mature subsets of T-lymphocytes. Cell, 73:237-247.

Delwel, R., Salem, M., Pellens, C., Doessers, L., Wagemaker, G., Clark, S., and Lowenberg, B., (1988). Growth regulation of human acute myeloid myeloid leukemia: Effects of five recombinant hematopoietic factors in a serum-free culture system. Blood, 72:1944.

Enjuanes,L., Lee,J.C., and Ihle,J.N., (1981). T-cell recognition of Moloney Sarcoma virus proteins during tumor regression. The Journal of Immunology. 126(4):1478-84.

Fialkow, P. J. (1972). Use of genetic markers to study cellular origin and development of tumors in human females. Advances in Cancer Research, 15:191-226.

Flug, F., Pelicci, P., Bonetti, F., Knowles, D., and Dalla-Favera, R. (1985). T-cell receptor gene rearrangements as markers of lineage and clonality in T-cell neoplasms. PNAS. 82:3460-3464.

Frasca, D., Zingoni, A., Leter, G., Spano, M., and Doria, G. (1992). Il-3 induces differentiation and growth of thymocytes and splenic T and B lymphocytes in irradiated mice. J. Immunol. Res. 4(2):61-66.

Fumarski,P., Rich, M.A., (1982). The mouse in biomedical research:experimental biology and oncology. Academic press. 352-71.

Garland, J.M., and Dexter, T.M. (1982). 20-α dehydrogenase expression in hemopoietic cell cultures and its relationship to Interleukin-3. Eur. J. Immunol. 12:998.

Glover,A.B., and Leyland-Jones,B. (1987). Biochemistry of Azacytidine:a review. Cancer treatment reports. 71(10):959-64.

Green,E.L. (1966) Biology of the Laboratory Mouse. MacGraw Hill Co. Second Edition. 521-34.

Guerrero, I., Calzada, P., Mayer, A., and Pellicer, A., (1984a). A molecular approach to leukemogenesis:mouse lymphomas contain an activated *c-ras* oncogene. Proceedings of the National Academy of Sciences, USA, 81:202-205.

Guerrero, I., Villasante, A., Corces, V., and Pellicer, A., (1984b). Activation of a *c-Ki-ras* oncogene by somatic mutation in mouse lymphomas induced by gamma radiation. Science, 225:1159-1162.

Hapel, A.J., Fung, M.C., Johnson, R.M., Young, I.G., Johnson, G., and Metcalf, D. (1985). Biologic properties of molecularly cloned and expressed murine Interleukin-3. Blood, 65(6):1453-1459.

Hara,H., Ogawa,M. (1978). Murine hemopoietic colonies in culture containing normoblasts, macrophages, and megakaryocytes. Am.J.Hematol. 4:23.

Hayata, I., Seki, M., Yoshida, K., Hirashima, K., Sado, T., Yamagiwa, J., and Ishihara, T. (1983). Chromosomal aberrations observed in 52 myeloid leukemias. Cancer Research, 43:367-373.

Hirsch, S., Austyn, J.M., and Gordon, S.G. (1981). Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. J. Exp. Med. 154:713-725.

Hirt, B. (1967). Selective extraction of polyomavirus DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.

Hogan, B., Constantini, F., and Lacy, E.,(1982). Manipulating the mouse embryo: A laboratory manual. Cold Spring Harbor Laboratory, New York.

Holmes, K.L., Langdon, W.Y., Fredrickson, T.N., Coffman, R.L., Hoffman, P.M., Hartley, J.W., and Morse, H.C. (1986). Analysis of neoplasms induced by CAS-BR-M MuLV tumor extracts. Journal of Immunology. 137(2):679-688.

Hood,L.H., Kronenberg, M., and Hunkapiller, T. (1985). T-cell antigen receptors and the immunoglobulin supergene family. Cell 40:225-229.

Hyman , R.J., Lesley, J., Schulte, R., and Trotter, J. (1986). Progenitor cells in the thymus:most thymus-homing progenitor cells in the adult mouse thymus bear Pgp-1 glycoprotein but not Interleukin-2 receptors on their cell surface. Cell. Immunol. 101, 320-327.

Ihle, J.N., Lee,J.C., and Rebar,L. (1981). T-cell recognition of Moloney Leukemia virus proteins. Journal of Immunology. 12(6):2565-70.

Ihle,J.N., Keller,J., Lee,J.C., Farrar.W.L., and Hapel,A.J. (1981). Purification and biological properties of Interleukin 3. Lymphokines and Thymic Hormones: Their Potential Utilization in Cancer Therapeutics; edited by L. Golstein and M. Chirigos. Raven Press. 77-92.

Ihle,J.S., Keller,J., Greenberger,J.S.,Henderson,L., Yetter,R.A., and Morse,H.C. (1982). Phenotypic characteristics of cell lines requiring Interleukin-3 for growth. The Journal of Immunology. 129(4):1377-83.

Ihle,J.N., Keller,J., Oroszlan,S., Henderson,L.E., Copeland,T.D., Fitch,F., Prystowsky,M.B., Goldwasser,E., Schrader,J.W., Palaszynski,E., Dy,M., Lebel,B. (1983). Biologic properties of homogeneous Interleukin-3. The Journal of Immunology. 131(1):282-87.

Ihle,J.N., Pepersack,L., and Rebar,L. (1981). Regulation of T cell differentiation: in vitro induction of 20 -Hydrosteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. The Journal of Immunology. 126(6):2184-89.

Ikuta, K., Uchida, N., Friedman, J., and Weissman, I.L. (1992). Lymphocyte development from stem cells. Annu. Rev. Immunol, 10:759-783.

International Agency for Research on Cancer (1990). Monographs on the evaluation of carcinogenic risks to humans. Pharmaceutical drugs, 50:47-64.

Jaenisch,R. Schnieke,A., and Harbers,K. (1985). Treatment of mice with 5-azacytidine efficiently activates silent retroviral genomes in different tissues. Proc. Natl. Acad. USA. 82:1451-55.

Jahner,D., Stulhmann,H., Steward,C.L., Harbers,K., Lohrer,J., Simon,I., and Jaenisch,R. (1982). De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature 298:623-28.

Jenkins, V.K, and Upton,A.C. (1963). Cell-free transmission of radiogenic myeloid leukemia in the mouse. Cancer Research. 23:1748-55.

Johnson,G.R., Metcalf,D. (1977). Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. Proc. Natl. Acad. Sci. USA. 74:3879.

Keller, J., Weinstein, Y., Hursey, M., and Ihle, J. (1985). Interleukins 2 and 3 regulate the in vitro proliferation of two distinguishable populations of 20-α hydrosteroid dehydrogenase-positive cells. J. Immunol. 135 (3): 1864-1871.

Kimoto, M., Kindler, V., Higaki, M., Ody, C., Izni, S., and Vassali, P. (1988). Recombinant murine Il-3 fails to stimulate T or B lymphopoiesis in vivo, but enhances immune responses to T-cell dependent antigens. J. Immunol. 140:1899-1894.

King, P.D., and Katz, D.R., (1990). Mechanisms of dendritic cell function. Immunol. Today, 11:206.

Le Beau, M., Lemons, R., Espinosa, R., Larsen, N., Arai, N., and Rowley, J., (1989). Il-4 and Il-5 map to human chromosome 5 in a region encoding growth factors and receptors and are deleted in myeloid leukemias with a del 5(q). Blood, 73:647.

Lee,J.C., and Ihle,J.N. (1981). Increased responses to lymphokines are correlated with preleukemia in mice inoculated with Moloney leukemia virus. Proc. Natl. Acad. Sci. USA. 78(12):7712-7716.

Lee, S., Starkey, P.M., and Gordon, S. (1985). Quantitative analysis of total macrophages in adult mouse tissues. Immunological studies with monoclonal antibody F4/80. J. Exp. Med. 161, 475-489.

Londei, M., Verhoef, A., De Berandinis, P., Kissonerghis, M., Grubeck-Loebernstein, B., and Feldman, M. (1989). Definition of a population of $CD4^{-}CD8^{-}$ T-cells that express the $\alpha\beta$ T-cell receptor and respond to interleukins 2, 3, and 4. PNAS 86:8502-8506.

Metcalf, D., MacDonald,H.R., Odartchenko,N., Sordat,B. (1975). Growth of mouse megakaryocyte colonies in vitro. Proc. Natl. Acad. Sci. USA. 72:1744.

Metcalf, D., and Nicola, N., (1988). Tissue localization and fate in mice in mice of injected multipotential colony- stimulating factor. PNAS. 85:3160.

Minden, M., Toyonaga, B., Ha, K., Yanagi, Y., Chin, B., Gelfand, E., and Mak, T. (1985). Somatic rearrangements of T-cell antigen receptor gene in human T-cell malignancies. PNAS. 82:1224-1227.

Muller, C.E. (1991). Separation of pluripotent stem cells and early B lymphocyte precursors with antibody Fall-3. J. Exp. Med., 174:161-168.

National Cancer Institute (1978). Bioassay of 5-azacytidine for possible carcinogenicity (NCI

Publ. No. 279-526), Bethesda, MD.

O'Connor, R., Cesano, A., Lange, B., Finan, J., Nowel, P., Clark, S., Raimendi, S., Rovera, and Santoli, D. (1991). Growth factor requirements of childhood acute lymphoblastic leukemia: correlation between presence of chromosomal abnormalities and ability to grow permanently in vivo. Blood 77(7):1534-1545.

O'Donnell, P. V., Fleissner, E., Lonial, H., Koehne, C. F., and Reicin, A. (1985). Early clonality and high-frequency proviral integration into the *c-myc* locus in AKR leukemias. J. Virol., 55:500-503.

Oliff,A., Oliff,I., Schmidt,B., and Famulary,N. (1984). Isolation of immortal cell lines from the first stage of murine leukemia virus-induced leukemia. Proc. Natl. Acad. Sci. USA. 81:5464-67.

Ou,C.Y., Boone,L.R., and Yang,W.K. (1983). A novel sequence segment and other nucleotide structural features in the long terminal repeat of a Balb/C Mouse Genomic Leukemia Virus-Related DNA clone. Nucleic Acids Research 11(18):5603-20.

Palacios, R., Kieffer, M, Brockhaus, M., Karjalainer, K., Dembic, Z., Kisielow, P., and von Boehmer, H. (1987). Molecular, cellular and functional properties of bone marrow T-lymphocyte progenitor clones. J. Exp. Med. 166:12-32.

Parsons,D.F., Upton,A.C., Bender,M.A., Jenkins,V.K., Nelson,E.S., and Johnson,R.R. (1962). Electron microscopic observations on primary and serially passaged radiation-induced myeloid leukemias of the RF mouse. Cancer Research. 22:728-36.

Quint, W., Quax, W., van der Putten, H., and Berns, A. (1981). Characterization of AKR murine leukemia virus sequences in AKR mouse substrains and structure of integrated recombinant genomes in tumor tissues. J. Virol., 39:1-10.

Sambrook, J., Fritsch,E., and Maniatis, T. (1989). Molecular Cloning. Cold Spring Harbor Laboratory Press. Second edition.

Santoli, D., Clark, S., Kreider, B., Maslin, P., and Rovera, G. (1988). Amplification of Il-2-driven T-cell proliferation by recombinant human Il-3 and Granulocyte-macrophage colony-stimulating factor. J. Immunol. 141:519-526.

Schmal, W., Geber, E., and Lehmacher, (1985). Diaplacental carcinogenic effects of 5-azacytidine in NMRI mice. Cancer Lett., 27:81-90.

Schrader,J.W. (1986). The panspecific hemopoietin of activated T-lymphocytes (Interleukin-3). Ann. Rev. Immunol. 4:205-30.

Schrader,J.W., and Crapper,R.M. (1983). Autogenous production of a hemopoietic growth factor, persisting cell-stimulated factor, as a mechanism for transformation of bone marrow-derived cells. Proc. Natl. Acad. Sci. USA. 80:6892-96.

Schrader,J.W., Schrader,S., Leslie,K., and Dunn,A. (1985). Role of autogenous production of a hematopoietic growth factor in myeloid leukemia. Leukemic Recent Advances, Biology and Treatment. Allan Liss, Inc. 293-302.

Selten, G., Cuypers, H., and Berns, A. (1985). Proviral activation of the putative oncogene pim-1 in MuLV induced thymic lymphomas. EMBO 4(7):1793-1798.

Shaffar-Deshayes, L.J., Heard, J.M., Sola, B., Fichelson, S., and Levy, J. (1983). Exp. Hematol. 11:899.

Silver, A. R. J., Boultwood, J., Breckton, G., Masson, W. K., Adam, J., Shaw, A.R., and Cox, R., (in press). Interleukin-1β deregulation associated with chromosomal rearrangement-an initiating event for radiation induced leukemogenesis?. Molecular Carcinogenesis (in press).

Silver, A., George, A., Masson, W., Breckton, G., Adam, J., and Cox, R. (in press). DNA methylation changes in the Il-1 (2F) chromosomal region of some radiation-induced acute myeloid leukaemias carrying chromosome 2 rearrangements. Genes and Development.

Sideras, P., and Palacios, R. (1987). Bone marrow pro-T and B lymphocyte clones express functional receptors for Interleukin(Il)3 and Il-4/BSF1 and non-functional receptors for Il-2. Eu. J. Immunol. 17:217-221.

Soloff, R., Dempsey, D., Jenning, S., Wolcott, R., and Chervenak, R. (1992). Characterization of the progeny of precursor-T (pre-T) cells maintained in vitro by interleukin-3 (Il-3). Development of T-cell function in vivo. Immunology 76:279-285.

Southern,E.M., (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J.Mol.Biol. 98:503-17.

Spangrude, G.J., Muller-Sieburg, C.E., Heimfeld, S., and Weissman, I.L., (1988). Two rare populations of mouse Thy-1^{lo} bone marrow cells repopulate the thymus. J. Exp. Med.167:1671-1683.

Spangrude, G.J., Klein, J., Heimfeld, S., Aihara, Y., and Weissman, I.L. (1989). Two monoclonal antibodies identify thymic-repopulating cells in mouse bone marrow. J. Immunol. 142, 425-430.

Springer, T., Galfre, G., Secher, D.S., and Milstein, C. (1979). Mac-1: a macrophage differentiation antigen identified by a monoclonal antibody. Eur. J. Immunol. 9:301-306.

Stephen, D. (1984). Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas. PNAS 81: 2097-2101.

Stoner, G.D., Shimkin, M.B., Kniazeff, A.J., Weisburger, J.H., Weisburger, E.K., and Gori, G.B., (1973). Test for carcinogenicity of of food additives and chemoterapeutic agents by the pulmonary tumor response in strain A mice. Cancer Res., 33:3069-3085.

Tennant,R.W., Otten,J.A., Wang,T.-W., Liou,R.S., Brown,A., and Yang,W.K., (1983). Control of RFM mouse strains endogenous retrovirus in theRFM mouse cells. J. Virol. 45:47-54.

Townes, T.M., Lingrel, J.B., Brinster, R.L., and Palmiter, (1985). Erythroid specific expression of human beta-globin genes in transgenic mice. EMBO J., 4:1715-1723.

Trakhtenbrot, L., Krauthgamer, R., Resnitzky, P., and Haran-Ghera, N. (1988). Deletion of chromosome 2 is an early event in the development of radiation-induced myeloid leukemia leukemia in SLJ/J mice. Leukemia, 2:545-550.

Trowbridge, I.S., Lesley, J., Schulte, R., Hyman, R., and Trotter, J. (1982). Biochemical characterization and cellular distribution of a polymorphic murine cell-surface glycoprotein expressed on lymphoid tissues. Immunogenetics. 15, 299-312.

Ueno, Y., Boone, T., and Uitterbogaart, C. (1989). Selective stimulation of human thymocyte subpopulations by recombinant Il-4 and Il-3. Cell. Immunol. 118:382-393.

van de Rijn, M., Heimpheld, S., Spangrude, G.J., Weissman, I.L., (1989). Mouse hematopoietic stem cell antigen is a member of the Ly-6 antigen family. Proc. Natl. Acad. Sci. USA 86:4634-4638.

Vasmel, W., Radaszkiewicz, T., Miltenburg, A., Zijistra, A., and Meliet, C. (1987). Refinement and presicion in the classification of murine lymphomas by genotyping with immunoglobulin and T cell receptor probes. Leukemia. 1(2):155-162.

Vesely, D., and Cihak, A., (1973). High frequency induction in vivo of mouse leukemia in AKR strain by 5-azacytidine and 5-iodo-2'-deoxyuridine. Experientia, 15:1132-1133.

Wang, T. H., Yang, W. K., Hoyt, P., Ch'ang, L. Y., and Savin, T. J., (1993). Effect of maternal aging on transgene heritability in transgenic founder mice derived from zygotes microinjected with retroviral long terminal repeat-containing recombinant deoxyribonucleic acid. Biology of Reproduction, 48:1104-1112.

Wong,P.M.C., Chung,S., and Nienhuis,A.W. (1987). Retroviral transfer and expression of the Interleukin-3 gene in hemopoietic cells. Genes and Development. 1:358-65.

Wong, P.M.C., Chung, S., Dunbar, C.E., Bodine, D.M., Ruscetti, S., and Nienhuis, A.W. (1989). Retrovirus-mediated transfer and expression of the Interleukin-3 gene in mouse hematopoietic cells result in a myeloproliferative disorder. Molecular and Cellular Biology. 9:798-808.

Wood, P., Givan, A., and Brooks, C., (1990). IL-3 and Il-4 affect thymocyte differentiation in organ culture. Immunology 71: 83-89.

Yang, W. K., Ch'ang, L. Y., Koh, C. K., Myer, F. E., and Yang, D. M. (1989). Mouse endogenous retroviral long-terminal-repeat (LTR) elements and environmental carcinogenesis. Progress in Nucleic Acid Research, 36:247-67.

Ymer,S., Tucker,W.Q.J., Sanderson,C.J., Hapel,A.J., Campbell,H.D., and Young,I.G. (1985). Constitutive synthesis of interleukin-3 by the leukaemia cell line WEHI-3B is due to retroviral insertion near the gene. Nature. 317:255-58.

Yokota,T., Lee,F., Rennick,D., Hall,C., Arai,N., Mossmann,T., Nabel,G., Cantor,H., Arai,K. (1984). Isolation of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells. Proc. Natl. Acad. Sci. USA. 81:1070-74.

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