To the Graduate Council :

I am submitting herewith a dissertation written by Jane F. Gallager entitled "Restriction Enzyme Analysis and Characterization of B10FV, an Infectious Ecotropic Retrovirus, and Its Effects on Histopathology, Hematology and Cellular Immunity of H-2$^{a/a}$ Mice." 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.

Raymond A. Popp, Major Professor

We have read this dissertation
and recommend its acceptance :

Accepted for the Council :

Vice Provost
and Dean of the Graduate School

RESTRICTION ENZYME ANALYSIS AND CHARACTERIZATION OF B10FV, AN INFECTIOUS ECOTROPIC RETROVIRUS, AND ITS EFFECTS ON HISTOPATHOLOGY, HEMATOLOGY AND CELLULAR IMMUNITY OF H-2$^{n/n}$ MICE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

Jane F. Gallager

August 1991

DEDICATION

This dissertation is dedicated to my father,

Donald J. Gallager,

who always knew that I would be a doctor.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Raymond A. Popp, for his patience and guidance during my research. I would also like to thank the other members of my committee, Dr. Frank Larimer, Dr. Wen Yang and Dr. Gene Rinchik, for their helpful comments and their expertise in cloning and retrovirus research.

Numerous members of the Biology Division staff at the Oak Ridge National Laboratories have provided lab space or technical assistance during my research: Diana Popp (immunology, hematology and FACS analysis); Sarah Shinpock (tissue culture techniques and immunology); Den Mei Yang (tissue culture techniques and retrovirus assays); Lynn Veach (computer and laser printer use); Greg Horesovsky (photography assistance); and Jim Wesley (tissue preparation for histology).

Finally, I would like to thank my mother, brothers and sisters, for the network of support they provided, and my husband, Richard Mural and all our animals, for their endless patience, love and Rick's killer red beans.

ABSTRACT

Retroviruses are associated with a number of diseases in mice; lymphomas, leukemias and immunodeficiencies can result from retrovirus infections. The mouse strains C57BL/10 (B10) and C57BL/10.F-H-$2^{a/a}$ (B10.F) are congenic strains that differ only at the H-2 locus. They share a defective endogenous ecotropic retrovirus, emv-2, on chromosome 8. B10.F, however, also has an infectious ecotropic retrovirus called B10FV that is passed via maternal transmission. Few B10.F mice (<10%) die as a result of neoplastic diseases before 6 months of age. B10.F mice are deficient in cellular immune responses when compared to B10 mice. Responses to sheep red blood cells, T cell mitogens and B cell mitogens are lower in B10.F mice. FACS analysis of MoAB-treated B10.F spleen cells indicate a lower frequency of B cells and $CD8^+$ T cells, but the $CD4^+$ T cells frequency is unchanged in B10.F mice. A unique set of B cells that also carry T cell antigens on their surface, Ly-1 B cells, are increased in B10.F mice. To study whether the differences observed in B10.F mice are the result of the retrovirus infection or some H-2-linked gene(s), retrovirus-free H-$2^{a/a}$ mice (BXF2NN) mice were bred and infected with biologically cloned B10FV. BXF2NN mice infected with B10FV were anemic, with low hematocrits and abnormal RBC sizes. Infected BXF2NN mice likewise exhibited lower responses to T and B cell mitogens and to sheep red blood cells when compared to uninfected BXF2NN mice. The frequency of T cells, of both the $CD4^+$ and $CD8^+$ cell subsets, were reduced in infected BXF2NN mice, but the B cell frequency was unchanged in comparison to uninfected BXF2NN mice. The frequency of Ly1-B cells was also similar in the uninfected and infected BXF2NN mice, and was at levels equivalent to B10.F mice; this indicated that high Ly-1 B cell frequencies may be an H-2-related trait. Therefore, T

and B cell responses, as well as $CD4^+$ cell frequencies, are reduced in B10FV-infected mice. Also, important immune cell frequency differences exist between B10 ($H\text{-}2^b$) and B10.F ($H\text{-}2^a$) mice that must be considered when these strains are compared. Frequencies of B cells were lower in uninjected BXF2NN mice. The CD4 and CD8 T-cell subsets were significantly altered infrequency, with uninjected BXF2NN mice having a greater frequency of $CD4^+$ T cells and a lower frequency of $CD8^+$ T cells than B10 mice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Retroviruses: Introduction and History

Retroviruses have a unique genome and lifecycle. Instead of having a genome composed of DNA, the retrovirus genome consists of two strands of single-stranded RNA. In the retrovirus lifecycle, the RNA genome is converted to a DNA form using a virally encoded enzyme, reverse transcriptase. The DNA form of the retrovirus is called a provirus when it becomes integrated into the host chromosomal DNA. As a part of the host chromosomal DNA, the integrated provirus is replicated and inherited in the infected somatic cell during cell division. Retroviruses can also be passed to new hosts through a process of external infection, called horizontal transmission, or through vertical transmission, i.e., when the provirus integrated in the host's germline DNA is inherited in typical Mendelian fashion in the progeny.

Retroviruses have been found in all vertebrates. Fish, chickens, cows, horses, goats, mice and rats, as well as many primates, have been found to be infected by retroviruses (Lazo and Tsichlis, 1990). Retrovirus-like sequences have also been found in invertebrates (Aeby et al., 1986; Link et al., 1987). In addition to true retroviruses, numerous species,

from fungi to Drosophila, contain retrovirus-like sequences that encode reverse transcriptase-like enzymes (Dolittle et al., 1989).

Retroviruses were among the earliest described viruses. They were originally found as filterable particles that could cause lymphomas in chickens (Rous, 1911), and later as the cause of mammary carcinomas (Bittner, 1934) and spontaneous lymphomas (Gross, 1951) in mice. C3H and C57BL mice receiving x-radiation developed lymphomas (Gross, 1958; Lieberman and Kaplan, 1959) that were caused by the activation of viruses (Laterjet and Duplan, 1962). The demonstration that AKR mice have genes that could induce murine leukemia virus (MuLV) infection (Rowe and Hartley, 1972; Rowe, 1973), and that the structure transmitted to the offspring was an integrated DNA provirus, Akv-1 (Chattopadhyay et al., 1974), confirmed Temin's hypothesis (1964) that a DNA form of the retrovirus provided chromosomal inheritance of the retrovirus genome. This concept was further supported by the discovery of the enzyme that allows the synthesis of DNA from a RNA template, reverse transcriptase (Baltimore, 1970).

The Retrovirus Genome and Lifecycle

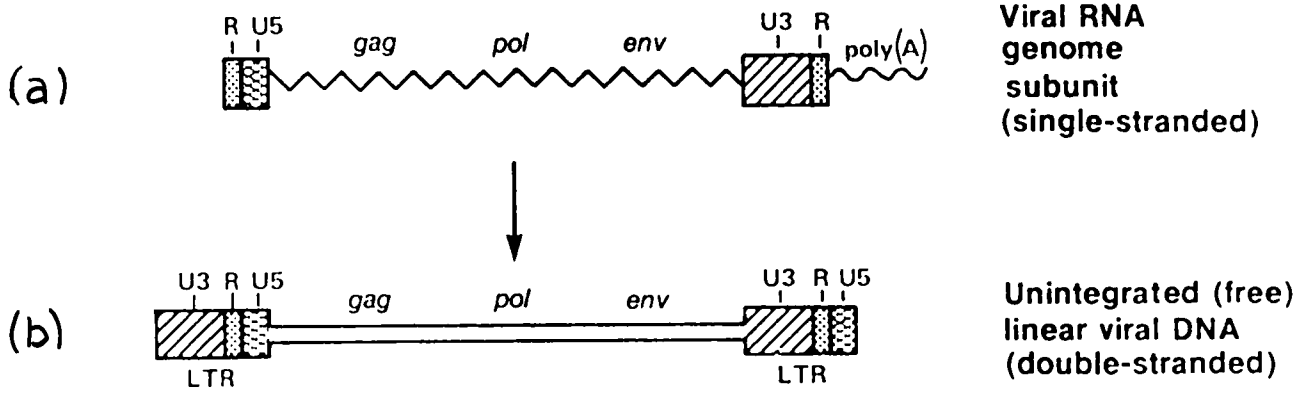
The retrovirus virion genome

The murine C-type retrovirus genome consists of two copies of RNA approximately 8.3 kilobases (kb) in size. A single strand of viral mRNA is depicted in Figure 1.1a. A 5' cap structure and a 3' polyadenylated (poly A) ribonucleotide tail enclose a structure flanked by short unique 5' and unique 3' sequences and short direct 5' and 3' repeats. Each direct repeat (R) consists of a repeated terminal sequence of 45 to 66 bases. A

Figure 1.1 Structure of MuLV (Lowy, 1984).

a. Structure of a single strand of viral mRNA.

b. Structure of the unintegrated linear viral DNA, depicting organization of the LTRs.


(a)
R U5
gag
pol
env
U3 R
poly(A)
Viral RNA genome subunit (single-stranded)
(b)
U3 R U5
gag
pol
env
U3 R U5
LTR
LTR
Unintegrated (free) linear viral DNA (double-stranded)

unique 5' sequence of 150 nucleotides (U5), and a unique 3' stretch of 300 to 500 nucleotides (U3) enclose the internal coding sequences (Coffin, 1979). In the integrated provirus, the U3-R-U5 sequences form the long terminal repeats (LTR) that flank the internal coding sequences of the provirus as direct repeats and provide transcriptional regulatory elements such as promoters, enhancers and polyadenylation signals (Figure 1.1b).

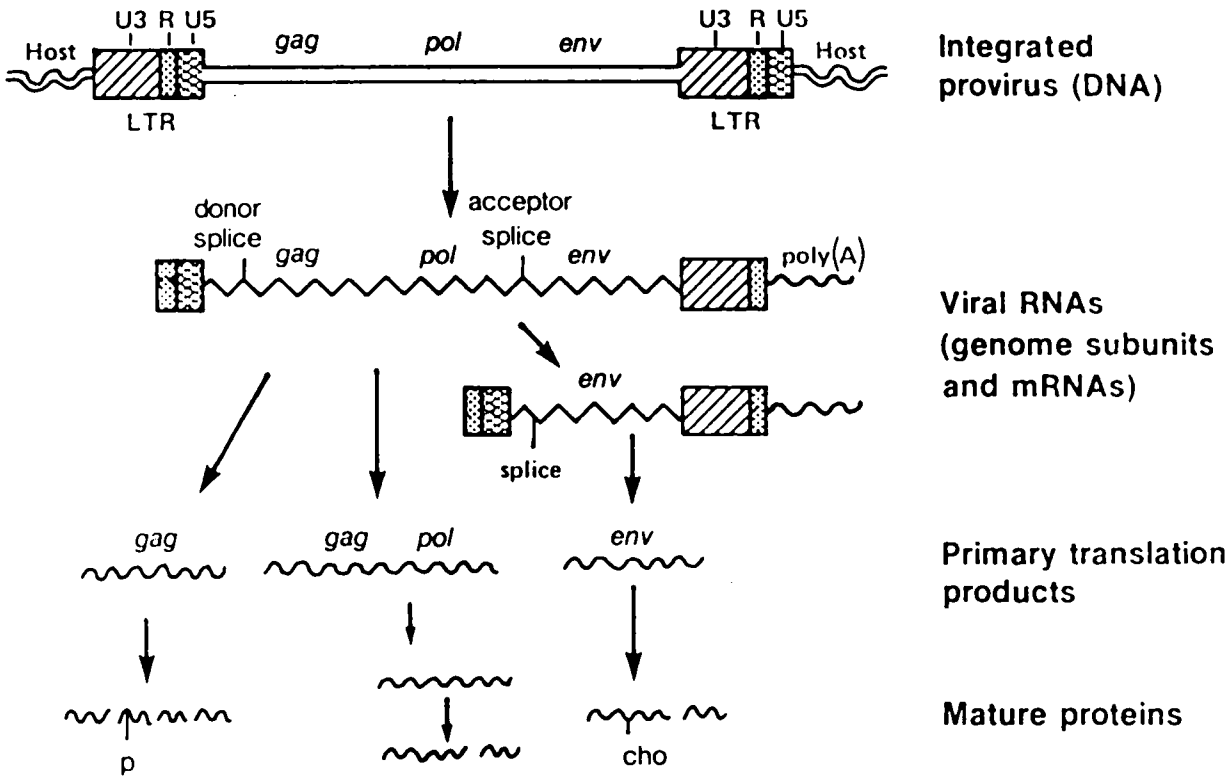
The internal coding regions of the retrovirus consist of the gag, pol and env genes (Figure 1.2). Virus core proteins are produced by gag, with four protein products ranging in size from 10,000 to 30,000 daltons produced from this region of the provirus (Strand and August, 1974). A RNA-dependent DNA polymerase (Baltimore, 1970), a RNA-DNA duplex-degrading enzyme, RNase H (Moelling et al., 1971) and an endonuclease vital to integration, integrase (Gerard and Grandgenett, 1980) are produced from the pol gene. The env gene produces a glycoprotein of 70,000 daltons, gp70, and an envelope protein of 15,000 daltons, p15E (Pinter and Fleissner, 1977).

Infection and integration

Retrovirus virions are generally 80 to 130 nm in diameter. The virion is composed of an inner nucleocapsid core surrounded by an outer envelope made from the host cell's lipid bilayer membrane. The core of the virion is the RNA genome of the retrovirus found as a ribonucleoprotein surrounded by an icosohedral protein capsid. An inner core protein separates the capsid structure from the outer envelope. The external surface of the lipid bilayer envelope, which forms as the retrovirus virion buds at the plasma membrane of the host cell, has glycoprotein projections formed from virion proteins.

The retrovirus begins the process of infection and integration by entering the cell

Figure 1.2 Internal coding regions of MuLV with RNA and protein products (Lowy, 1984). Please see text for description of the figure.

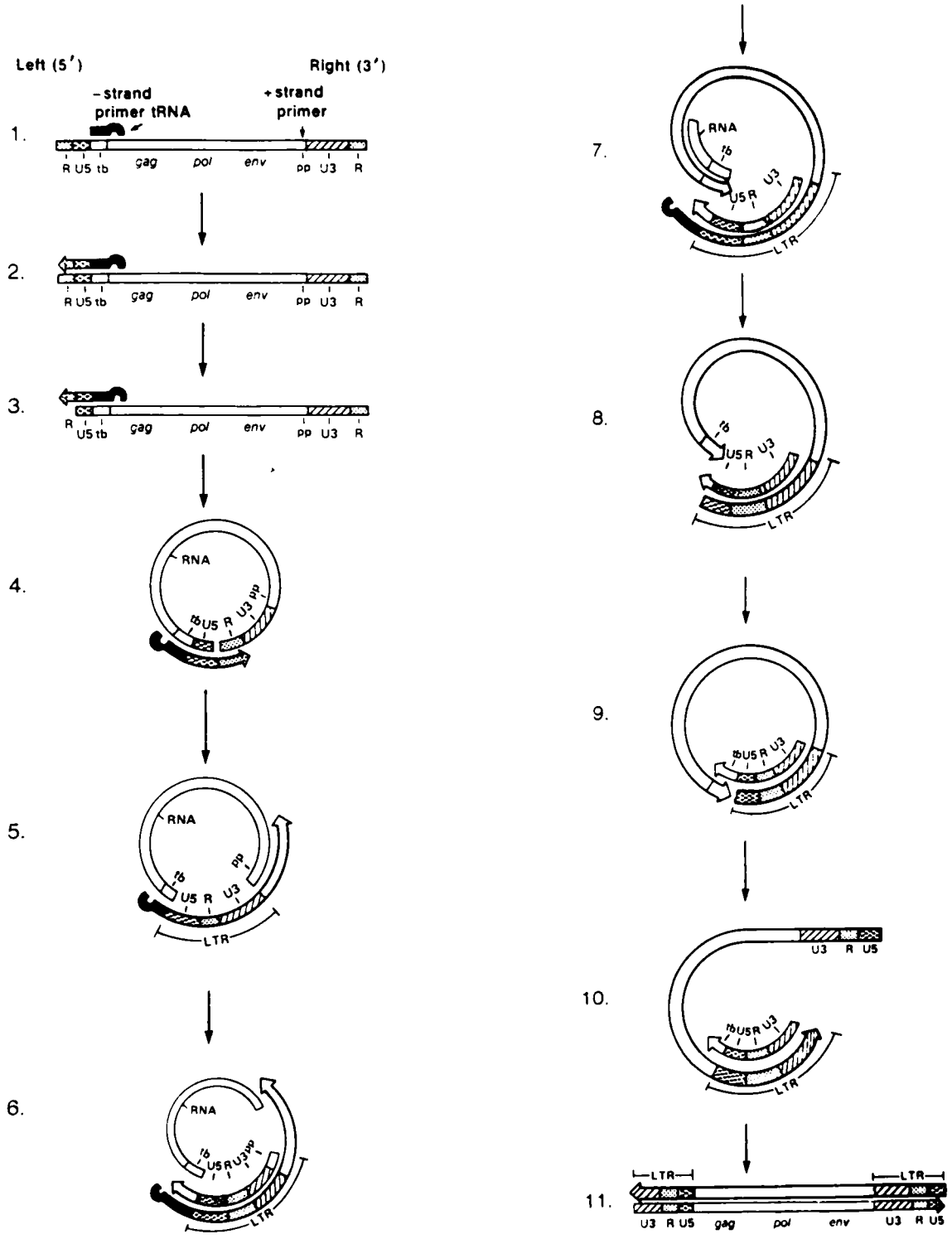

U3 R U5
Host
gag
pol
env
U3 R U5
Host
LTR
LTR
Integrated provirus (DNA)
donor splice
acceptor splice
gag
pol
env
poly(A)
Viral RNAs (genome subunits and mRNAs)
env
splice
gag
gag pol
env
Primary translation products
p
cho
Mature proteins

through a process of receptor-mediated endocytosis using normal cell-surface proteins specifically recognized by products of viral env genes (Varmus, 1988). The putative receptor for ecotropic MuLv, a previously unknown transmembrane protein, has recently been characterized (Albritton et al., 1989). The putative ecotropic retrovirus receptor has no similarities to any known protein. The protein sequence is extremely hydrophobic, with fourteen potential membrane-spanning domains. Albritton et al. (1989) suggested that this receptor protein may permit infection of ecotropic retroviruses either as a receptor that specifically binds to ecotropic gp70, or the receptor protein may mediate virus fusion with the target cell membrane.

Once in the cytoplasm, the virion envelope is removed, and the RNA genome is present as an enzymatically active nucleoprotein complex. The RNA genome is made into a colinear DNA genome through the action of the pol gene product, reverse transcriptase (Figure 1.3). To initiate DNA synthesis, a tRNA primer with sequences homologous to a primer binding site in the 5' portion of gag (Harada et al., 1979), is used to begin synthesis of a short stretch (100 to 200 bases) of minus-strand (-) DNA (Haseltine et al., 1976). The 3' end of the newly synthesized DNA initially hybridizes to the RNA template. The RNA/DNA hybrid duplex is substrate for another pol gene product, RNase H. The RNase H degrades most of the RNA from the RNA/DNA hybrid structure and the remaining DNA is free to hybridize with the R sequences at the 3' end of the RNA genome. Essentially, the (-) strand DNA "jumps" from the left (5') to the right (3') end of the viral RNA, using the R sequences that are duplicated at both ends of the viral RNA and the RNase H activity of the reverse transcriptase. From this 3' R sequence the remainder of the (-) strand is synthesized.

Following the (-) strand synthesis, the plus strand (+) RNA strand is displaced and

Figure 1.3 Reverse transcription of viral mRNA into double stranded DNA provirus (Lowy, 1984). See text for further detail. Abbreviations used in the figure: *pp*, site of plus-strand primer and sequences complementary to it, and *tb*, tRNA binding site and sequences complementary to it.


Left (5′)
Right (3′)
−strand primer tRNA
+strand primer
1.
R U5 tb
gag
pol
env
pp U3 R
2.
3.
4.
RNA
5.
LTR
6.
7.
8.
9.
10.
U3 R U5
11.

DNA synthesis of the (+) strand begins, using the 3' (-) strand LTR as a template. The (+) strand DNA 'jumps' to pair with the R-U5 sequences in the 5' LTR, and the (+) strand synthesis is completed using the (-) strand DNA as a template. The synthesis of the 5'(-) strand LTR is completed using the (+) strand LTR DNA as a template.

The linear provirus DNA is transported to the nucleus of the infected cell where it will be integrated into the host chromosomal DNA. In the infected cell, three forms of proviral DNA can be found. Linear, one- and two-LTR circular DNA proviruses can be isolated from the nuclei of infected cells (Hsu et al., 1978). It is the linear form of the provirus that integrates into DNA in vitro (Brown et al., 1987; Fujiwara and Mizuuchi, 1988; Brown et al., 1989). The binding of integrase, an endonuclease encoded by the viral pol gene, to the LTR is necessary for integration to occur (Brown et al., 1989). The integrase removes two bases from the 3' hydroxyl termini of each linear strand and the 3' ends are covalently attached to the complementary 5' phosphoryl ends of the host DNA. When integration is completed, an invariant structure is formed in the host chromosomal DNA. The provirus has joined to the host DNA at sites two base pairs inside the ends of the retroviral DNA. The provirus is also flanked by short duplications of host chromosomal DNA caused by the excision of four to six bases of host chromosomal DNA, possibly through the action of the integrase protein (Fujiwara and Craigie, 1988).

Transcription and expression of proviral DNA

In chronically infected cells, several factors influence the expression of the proviral genome. The integrated proviral DNA is expressed using the host cell's transcriptional factors, and availability of these factors can impact retrovirus expression. Host RNA polymerase II is essential for the transcription of the provirus genome. The site into which

a provirus integrates also influences the expression of the provirus. When provirus sites are located near hypomethylated regions of the DNA, they are more often transcriptionally active proviruses; alternately, proviruses in hypermethylated sites of the genome are often unexpressed (McGeady et al., 1983).

The regulation of provirus transcription is through the LTR enhancers, promoters and initiation sites. Transcription begins in the 5' LTR in the R sequence, and each full-length viral transcript continues through the provirus to the 3' LTR and terminates in the R sequence following the addition of a poly A tail. Some of the full-length transcripts become RNA virion genomes. The majority of the transcripts are used as mRNA for the gag, pol and env products.

The gag and pol genes are translated from the full-length viral mRNA as both single gag translation products and as a gag/pol polyprotein (Dickson et al., 1982). To regulate the expression of gag and pol, suppression of the termination codons separating the two coding regions creates a gag/pol fusion protein. The polyprotein is cleaved into its separate components by a product of the pol gene, a viral protease (pro). This strategy of translation favors the formation of gag products, which are needed in more abundance by the virion for the formation of the structural virion than are pol products, which are primarily used at reverse transcription and integration (Lazo and Tsichlis, 1990)

After the synthesis of viral structural proteins the virion is assembled. The pol genes products are not present in final, proteolytically processed form in the virion; a $Pr180^{gag/pol}$ that contains no enzymatic activity in its fused state is present as the precursor for the pol product reverse transcriptase. The four proteins encoded by gag account for most of the viral core and capsid proteins. The gag proteins are processed from a larger precursor protein, $Pr65^{gag}$. From the $Pr65^{gag}$ precursor, p15, pp12, p30 and p10 are

proteolytically cleaved. The p15 is probably located between the envelope of the virion and the capsid proteins; its hydrophobic nature may allow it to direct trafficking of the ribonucleoprotein complex to the site on the cell plasma membrane where encapsidation of the virion will occur prior to virus budding (Rein et al., 1986; Shultz and Rein, 1989). The pp12 has an unknown position and function in the virion, but it can bind to RNA in vitro, suggesting some association with the viral genome. The p30 is the major capsid protein, and p10 forms the protein that binds to the RNA genome to form the ribonucleoprotein complex in the virion (Risser et al., 1983; Lazo and Tsichlis, 1990)

The env proteins are expressed from a spliced message as a larger, glycosylated precursor protein, $Pr90^{env}$, that is cleaved into two disulfide-linked products: gp70 and p15E. The p15E is the transmembrane protein of the virion and is associated with the host range-determining gp70 atop the N-terminus of p15E. These proteins, which are known to cause the host range specificity of MuLV, become inserted into the plasma membrane of the infected cell and may also direct the location where virion encapsidation and budding occur (Lazo and Tsichlis, 1990).

Retroviruses of Mice

One of the unique features of retroviruses is their ability to be inserted into host germline DNA and become a part of their host's heritable genetic material. Numerous proviruses have been identified in the inbred mouse strains and are transmitted from parent to offspring as a result of a germline insertion in an ancestor of that mouse strain.

Four types of endogenous retrovirus and retrovirus-like sequences are found in the

genomes of inbred strains of mice. They are classified by their unique nucleic acid sequences:

1. C-type retroviruses are the typical murine leukemia viruses (MuLV) and contain the internal gag, pol and env genes surrounded by LTRs;

2. B-type retroviruses, such as mouse mammary tumor virus (MMTV), have an internal EcoRI restriction endonuclease site that distinguish them from other mouse retroviruses by producing two junction fragments per provirus (Cohen and Varmus, 1979) and an open reading frame (orf) from the 3' end of env through most of the LTR sequence;

3. Retrovirus-like intracisternal A particle (IAP) genes are found in many embryo and tumor cell lines (Dalton et al., 1961); and

4. Virus-like 30S RNA (VL30 sequences) bear little genome resemblance to C-type MuLV (Scolnick et al., 1979), but can be encapsidated with the presence of helper C-type MuLV (Besmer et al., 1979).

C-type MuLV

Within the endogenous C-type MuLV group there are several different groups of retroviruses that are distinguishable from each other by their host ranges and their antigenic relationships as characterized by anti-virus antibodies. The determinants of their host ranges are found in their gp70 env protein (Hartley and Rowe, 1976). While in most cases serologically distinct, the nucleotide sequences of endogenous C-type MuLV have extensive homology (Chattopadhyay et al., 1974; Chattopadhyay et al., 1981). The four

groups of endogenous C-type MuLV found in inbred mouse strains are the ecotropic, xenotropic, polytropic and modified polytropic MuLV, and each is discussed below.

Ecotropic retroviruses

Ecotropic retroviruses replicate only in mouse cells and usually do not infect cells of other species. Most ecotropic retroviruses induce the formation of syncytia in XC cells, a transformed rat epithelial cell line, when the XC cells come in contact with ecotropic retroviruses (Rowe et al., 1970); The use of XC cells cultured with mouse cells infected by ecotropic retroviruses provides a way to quantitate ecotropic virus produced by the mouse cells. Ecotropic retroviruses share a unique 5' region of the env gene that has been molecularly cloned from the Akv-1 provirus (Chattopadhyay et al., 1980). The cloned 400 base pair (bp) env fragment provides an ecotropic-specific probe for molecular analyses of other ecotropic provirus genomes (Chan et al., 1980).

Ecotropic retroviruses have been subdivided into three different host range groups based upon the control of the Fv-1 gene products, which control the ability of certain types of retroviruses to mount an infection in inbred mice (Pincus et al., 1971a; Pincus et al., 1971b). N-tropic ecotropic MuLV can replicate only in the cells of hosts having the $Fv\text{-}1^{n/n}$ genotype i.e., BALB/c. B-tropic ecotropic MuLV replicate only in hosts having the genotype $Fv\text{-}1^{b/b}$, such as C57BL-related mouse strains. NB-tropic ecotropic MuLV replicate equally well in cells having either genotype. Cells that are heterozygous for the Fv-1 locus ($Fv\text{-}1^{n/b}$) restrict the growth of both N-tropic and B-tropic ecotropic retroviruses. The $Fv\text{-}1^{nr/nr}$ genotype partially inhibits N-tropic virus replication and completely inhibits B-tropic retrovirus replication.

Most endogenous ecotropic retroviruses are N-tropic (Rowe, 1973; McCubrey and

Risser, 1982). While only N-tropic virus is recovered from Fv-$1^{n/n}$ mice (Peters et al., 1973), mouse strains carrying the Fv-$1^{b/b}$ genotype and N-tropic endogenous ecotropic MuLV produce only B-tropic ecotropic retroviruses (Odaka, 1975). Some of the B-tropic retroviruses appear to have arisen from recombination between the endogenous N-tropic ecotropic MuLV and endogenous nonecotropic sequences because they have some serological characteristics of retroviruses (Boone et al., 1988). The endogenous nonecotropic MuLV may be either a subset of xenotropic retroviruses carrying the B-tropism determinants, or polytropic or modified polytropic MuLV that have the B-tropism determinant region.

Strains containing endogenous ecotropic MuLV sequences can be divided into categories based on the number and positions of endogenous ecotropic proviruses, called emv loci, found when the germline DNA of individual strains is hybridized with the ecotropic-specific env probe (Jenkins et al., 1982). The emv loci were given number designations to identify the ecotropic proviruses common to a given mouse strain or related groups of mouse strains. The presence or absence of a particular emv locus correlates with the pedigree of the strain.

Inbred mouse strains can be described as ecotropic retrovirus-negative, low-virus and high-virus strains based on the number of endogenous ecotropic proviruses found in their genomes. Examples of ecotropic virus-negative strains, such as 129, NZB, NFS and SWR, contain no endogenous ecotropic proviruses (Jenkins et al., 1982). Low-virus strains BALB/c and related strains (A/J, C3H/He, and CBA/J), C57BL-related strains (C57BL/10, C57BL/6 and C57BR) and DBA/2 all contain a single emv locus. BALB/c and its related strains carry emv-1 on chromosome 5, while C57BL-related strains carry a separate provirus, emv-2, on chromosome 8 (Jenkins et al, 1982). High-virus strains, such as AKR,

C58 and C3H/Fg, contain three to six full-length proviruses at different chromosomal locations and are the strains most often associated with a high incidence of lymphoma (Rowe, 1978; Chattopadhyay et al., 1980)

Xenotropic retroviruses

Xenotropic MuLV are retroviruses only able to infect and grow in cells of species other than the mouse. Their xenotropic host range specificity is determined by the env genes, because pseudotyping xenotropic retroviruses with ecotropic env genes allows their replication in mouse cells (Ishimoto et al., 1977). Xenotropic viruses do not induce the formation of syncytia in XC cells (Hartley and Rowe, 1976), so these retroviruses are detected using fluorescent antibodies to xenotropic cell-surface glycoproteins, through reverse transcriptase activity or through their ability to rescue replication-defective transforming viruses (Peebles, 1975). When the inbred mouse genome is molecularly analyzed for xenotropic retrovirus-related sequences with gag/pol-related sequence probes, thirty to fifty copies of xenotropic-like retrovirus sequences are found. Further analysis of these sequences with a xenotropic env-specific probe shows that only eighteen to twenty-eight of the thirty to fifty sequences are xenotropic proviral sequences (Hoggan et al., 1983); the remainder of these sequences that did not have the xenotropic env region are polytropic MuLV.

Polytropic and modified polytropic retroviruses

Polytropic and modified polytropic MuLV (also known as dualotropic or recombinant mink cell focus-forming [MCF] viruses) differ from ecotropic and xenotropic MuLV by having the ability to grow in cells of both mouse and non-mouse origin.

Structurally, the polytropic precursor proviral sequences have different LTR sizes (Khan and Martin, 1983; Ou et al., 1983), a unique tRNA binding site (Nikbakht et al., 1985), different env sequences (Khan, 1984) and Fv-1 B-tropism determinents (Boone et al., 1988). Molecular analysis of non-ecotropic, non-xenotropic endogenous MuLV sequences suggests that the polytropic MuLV are divided into two classes based on slightly different restriction endonuclease cleavage patterns (Steffan et al., 1982) and RNA foot print analyses (Stoye and Coffin, 1987). Polytropic MuLV appear to have been generated by the recombination between endogenous ecotropic proviruses and one or more endogenous non-ecotropic proviruses, causing a substitution of the gp70 and p15E coding regions of env (Chattopadhyay et al., 1982; Khan et al., 1982; Lung et al., 1983) and the B-tropism determinant region (Boone et al., 1988).

Retroviruses of wild mice

In addition to the above classes of endogenous C-type MuLV, two additional classes of C-type MuLV have been found in wild mouse populations. Amphotropic MuLV, found in wild mice isolated in California, possess both ecotropic and xenotropic host ranges but cannot be neutralized by anti-ecotropic or anti-xenotropic MuLV antibodies (Hartley and Rowe, 1976; Rasheed et al., 1976). The env-region DNA clone that is specific for ecotropic MuLV does not hybridize with amphotropic env sequences; xenotropic env probes weakly cross hybridize with amphotropic env sequences, indicating that there are some env region differences between xenotropic and amphotropic MuLV.

Feral mouse ecotropic retroviruses do not induce lymphomas in inbred mice; instead, a hind-limb paralysis develops in infected mice (Lai et al., 1982). While the feral mouse ecotropic retroviruses have a host range similar to endogenous ecotropic

retroviruses of inbred mice, wild mouse ecotropic retroviruses do not induce XC cell syncytia formation (Hartley and Rowe, 1976).

The Pathogenic Effect of Retroviruses

The majority of endogenous C-type MuLV examined have proven to be nonpathogenic (Hartley et al., 1969), and in fact, MuLV can be isolated from most inbred strains of mice and from feral mouse populations where no detrimental effects had been observed. Through the act of integrating and replicating their genomes, however, MuLV can accumulate mutations due to errors in reverse transcriptase and RNA polymerase II functions. Both of these enzymes lack proof reading properties, and mutation rates as high as 10^{-4} to 2×10^{-5} per generation have been estimated from experimental data (Dougherty and Temin, 1988). Recombination between retroviruses or between retroviruses and cellular genes can also lead to greater heterogeneity in the retrovirus pool.

Occasionally, retroviruses with enhanced pathogenic potential are produced through a mutation in a nonpathogenic retrovirus population. Both mutant and recombinant (MCF/polytropic) retroviruses have been generated in high leukemic mouse strains during the preleukemic period (Kawashima et al., 1976). Variants of feline leukemia virus (FeLV) that have enhanced immunosuppressive potential have been found following FeLV infection in cats (Mullins et al., 1986).

Retrovirus infections are responsible for a wide range of diseases in animals. Leukemia/lymphoma, carcinoma, wasting disease, immune deficiency, anemia and neurological disorders have all been induced by retroviruses in multiple animal species

(Lazo and Tsichlis, 1990).

Murine C-type retroviruses are responsible for the induction of hematopoietic neoplasms. Lymphoid, myeloid and erythroid neoplasms are induced by MuLV through a complex, multi-step process. Polyclonal proliferation, clonal expansion, the appearance of the neoplasm and the progression of the tumor to metastasis can describe the course of most neoplastic events induced by MuLV (Lazo and Tsichlis, 1990). Embedded within this multi-step process, the retrovirus must infect a cell, such as a lymphocyte, and persist in that cell. As an important part of their persistence in the host and to insure their own survival, the retrovirus must be able to replicate without causing massive cell death. The host's immune response, while trying to destroy retrovirus-infected cells, may inadvertently impair the immune system through the resulting inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ). Immune system impairment is observed in many retrovirus infections leading to tumors in experimental animals and in man (Lazo and Tsichlis, 1990).

Some MuLV-induced events never proceed to a state of true neoplasm. For example, in spleen focus-forming virus (SFFV) infections and in animals infected with the murine acquired immune deficiency syndrome (MAIDS) virus, the spleen cell proliferation that occurs in infected animals overwhelms them. The infected animals do not survive the initial proliferative event so neoplasia is rarely observed (Teich et al., 1982; Morse et al., 1989).

In addition to the carcinogenic or lymphomagenic potential of retroviruses, the act of proviral integration necessary to the lifecycle of the retrovirus could have detrimental consequences to the host. The major function of proviral integration is the production of a stable, efficient template for the production of viral RNA (Varmus and Swanstrom,

1985). In most cases, the randomly integrating provirus incurs no harmful effects to the cell; in other instances the provirus may cause an insertional mutation in or near a gene. A provirus may integrate directly in coding regions of a gene, physically disrupting the gene and inactivating it. Classic gene inactivations by proviral insertions include the dilute (d) mutation (Jenkins et al., 1981) and the inactivation of a pre-existing Rous sarcoma provirus by the insertion of a new provirus (Varmus et al., 1981).

The insertion of a provirus next to a cellular coding region can alter the regulation of the nearby gene. The long terminal repeats (LTRs) of a provirus contain strong promoters and enhancer sequences that can provide transcriptional signals to the nearby gene and augment expression of the gene (Varmus, 1982). Normally silent genes, such as cellular oncogenes, can be activated by a provirus through insertional mutagenesis. Proviral insertions next to the murine oncogenes c-myc (O'Donnell et al., 1985) and pim-1 (Selton et al., 1985) are associated with increased mRNA production from these loci and ultimately lead to early T-cell lymphoma in mice.

Proviruses can not only affect the functions of genes adjacent to the proviral integration site but can also affect genes located long distances away from the site of integration through the effects of the LTR enhancer regions. Lazo et al. (1990) provide evidence for cis long-distance activation of myc oncogenes by Moloney MuLV proviruses inserted at Mlvi-1 and Mlvi-4, located on the same chromosome but separated by 24 kilobases (Lazo et al., 1990). Inappropriate production of the Evi-1 zinc finger protein in mouse myeloid leukemias has been shown to be caused by a provirus integration at Cb-1/fim-3, a common retroviral integration site located 90 kilobases from Evi-1 (Bartholomew and Ihle, 1991). Results from Evi-1 activation studies suggest that the aberrant expression of the zinc finger protein in the myeloid leukemias is driven by an

enhancer located in the LTR of the provirus at Cb-1/fim-3.

Inbred and Inbred Congenic Mice

The inbred laboratory mouse, Mus musculus, is an ideal experimental animal. Its size, short gestation period and ease of care have made it indispensable as an experimental system. The ability of inbred mouse strains to be infected by many human pathogens, or their ability to develop diseases similar to human genetic diseases has allowed the inbred mouse to be used as an in vivo testing system for many scientists.

Inbred mice were first developed for cancer research, beginning in 1909 with DBA, the first inbred strain developed by C. C. Little (Morse, 1981). Mouse strains are designated "inbred" when a lineage of mice has been brother-sister mated for at least twenty generations so that genetic variability among siblings is minimized. Inbred mice exhibit great uniformity in physical and biochemical traits, which makes inbred mice useful in experiments where it is necessary to establish that the observed results have a genetic basis.

The effect of one genetic locus on the development of a measurable trait or phenotype is best studied if alleles of that locus are placed onto a common genetic background. Congenic pairs are those inbred strains that are genetically identical except for alleles at one known area of a chromosome (Snell, 1948; Flaherty, 1981). Congenic inbred strains are particularly useful in examining the effects of a single compound locus, such as the major histocompatibility complex (MHC), or H-2 locus, located on mouse chromosome 17. Numerous H-2 congenic strains were generated and used by George Snell

to identify and describe the many H-2 haplotypes and the unique H-2 antigens in inbred mice (Snell, 1948; Klein, 1986).

Comparisons between inbred congenic strains become less clearly defined if the congenic strains differ at more than a single chromosomal region. Any exogenous stimulus, such as a bacterial or parasitic infection that is restricted to one of the congenic strains, creates additional variables when interpreting data from congenic pairs; the congenic pairs not only differ genetically at one region of a chromosome but also may differ as a result of the exogenous stimulus. An example of this situation would be where two congenic strains differ at the H-2 locus but one strain also expresses infectious retroviruses. Any differences observed between the congenic inbred strains might not be attributable entirely to genetic differences between the congenic inbred strains. The presence of the infectious retrovirus and its influence must also be considered in analyses of the experimental data from the congenic strains.

The Congenic Pair: C57BL/10 and C57BL/10.F

Strain C57BL/10.F (B10.F) was derived from (F/St x C57BL/10)F_2 H-$2^{a/a}$ homozygotes produced by Dr. D.B. Amos at Duke University, Durham, North Carolina. The H-$2^{a/a}$ homozygous female was backcrossed to C57BL/10 (B10) male by Dr. Jack Stimpfling at the McLaughlin Research Institute, Great Falls, Montana. H-$2^{a/b}$ heterozygous females were selected at ten successive generations of backcrossing to B10 males. When mating pairs were received from Dr. Stimpfling, B10.F mice had been brother-sister intercrossed for twelve generations, and since that time an additional 35 to

40 generations have been raised.

B10.F mice were received at the Biology Division by D.M. Popp in 1974. Originally, B10.F mice were used as a model to understand the genetic effects of aging and degenerative diseases (Popp, 1978). The congenic B10.F differs from C57BL/10 (B10), its inbred partner strain, at the H-2 region on chromosome 17 with B10.F carrying the $H\text{-}2^a$ allele from the donor strain F/St and B10 carrying the $H\text{-}2^b$ allele. Since B10.F is congenic to B10, this strain can be used to study the effects of H-2 on the differences between the two strains. B10.F mice grey early, suffer severe weight loss and develop dermatopathies (Popp, 1978). B10.F mice that survive to 18 months of age resemble B10 mice that are 30 to 36 months of age. Fifty percent survival time for B10.F mice is 455 days, whereas B10 mice have a 50% survival time of 730 days. The congenic strains have similar growth rates as infants and juveniles (Popp, 1982).

Since B10 and B10.F differ at a defined region of chromosome 17, backcross and F_2 mice were used to determine if H-2 alone controls the differences in their lifespans. Early death was found to be associated with the $H\text{-}2^a$ allele of B10.F, and the lifespan in the $H\text{-}2^{a/b}$ group was similar to or slightly below the lifespan of the $H\text{-}2^{b/b}$ F_2 mice. These data suggested that the lifespans of these mice are influenced by their H-2 genotypes (Popp, 1982). Later studies showed that only those mice with low serum IgA levels were compromised in lifespan (Popp, 1986).

The first suggestion that B10.F mice were immunocompromised was the determination that B10.F mice had a severely reduced capacity to form plaques to sheep red blood cells at twelve months of age (Popp, 1978; unpublished data). This was in sharp contrast to the results from B10.F at four months of age, at which time the plaque-forming capacity was equal to, if not greater than, the response in young B10 mice.

Basal serum IgA levels also are decreased in B10.F mice (Popp, 1979b; Popp, 1982). The serum IgA levels are one-third that in B10 mice. Analysis of serum IgA levels in progeny from reciprocal F_1 crosses with the two strains indicated that when B10.F was the maternal parent of the F_1 animals the IgA levels were low, but when B10 was the maternal parent the IgA levels were high. Segregation of the high and low serum IgA phenotypes in (B10 x B10.F)F_2 progeny was 3:1 (hi:lo), and this suggested that a single pair of genes controlled the expression of the high and low serum IgA phenotypes in B10 and B10.F mice, respectively. However, when (B10 x B10.F) F_1 mice were backcrossed to the low serum IgA strain (B10.F), only 20% of the offspring expressed the low serum IgA phenotype. Backcrossing (B10 x B10.F)F_1 mice to the high strain (B10) produced 25% progeny expressing the low IgA phenotype. (B10.F x B10)F_2 and backcross progeny raised from (B10.F x B10)F_1 animals showed a different segregation of high and low serum IgA phenotypes; the numbers of high serum IgA and low serum IgA animals were equal. When (B10 x B10.F)F_1 females were backcrossed to the high serum IgA strain, 75% of the offspring expressed the high serum IgA phenotype; crossing (B10.F x B10)F_1 females to the low serum IgA strain produced 75% progeny that expressed the low serum IgA phenotype (Popp, 1979a).

Association of the serum IgA phenotype with H-2 was analyzed. No evidence for linkage was observed in (B10 x B10.F)F_2 or the backcross progeny of (B10 x B10.F)F_1 animals. The reciprocal F_2 population and backcross progeny did exhibit significant association between H-2 haplotype and serum IgA phenotype. The numbers observed did not follow the rules of simple Mendelian inheritance, so it was postulated that some maternal factor(s) caused the low serum IgA phenotype when B10.F was the maternal parent to the F_1 progeny (Popp, 1979b).

The maternal influence was examined through foster nursing experiments. Foster nursing of B10 and (B10 x B10.F)F_1 neonates on B10.F females produced animals that had serum IgA levels lower than expected for B10 and (B10 x B10.F)F_1 mice at four months of age, and the serum IgA levels remained low in all the B10 and nearly all the F_1 animals throughout their lives. B10.F neonates foster nursed on B10 females retained their low IgA levels. This suggested that some maternal factor was secreted in the milk of B10.F females that influenced the full expression of serum IgA production in fostered B10 neonates, and that the B10.F serum IgA phenotype was fixed at birth (Popp et al., 1986).

Maternally derived immune cells were postulated to explain the low serum IgA phenotype and its maternal transmission. (B10.F x B10)F_1 spleen cells injected into (B10 x B10.F)F_1 neonates resulted in the expression of low serum IgA levels in the recipient animals. Bone marrow from adult (B10.F x B10)F_1 mice transplanted into young (B10 x B10.F)F_1 mice was also capable of suppressing the serum IgA levels (Popp et al., 1986). Cell-free supernatants from B10.F spleen cell extracts injected into young B10 mice could also induce the B10.F phenotype in the injected animals.

Strain F/St, the maternal parent in the original mating to produce B10.F, is unique in that it expresses high levels of both ecotropic and xenotropic MuLV (Morse et al., 1982). Other strains, such as AKR, C58, PL, SL and C3H/Sg produce high levels of ecotropic MuLVs from early in life (Chused and Morse, 1978), while NZB expresses high titers of only xenotropic viruses (Rowe, 1978). High virus titers are associated with one or more virus-inducing loci that segregate with Mendelian ratios and are dominant in crosses with permissive strains (Chused and Morse, 1978; Rowe, 1978). The same has been found for F/St, with three or more loci allowing ecotropic MuLV expression and one locus allowing xenotropic expression. The xenotropic virus phenotype behaves as a recessive trait

in all F_1 crosses, whether to permissive or nonpermissive strains, and the gene controlling this expression is located on chromosome 1. Bxv-1, also on chromosome 1, has been shown to govern the expression of xenotropic virus inducibility in several other strains; crosses with F/St and SEA, a xenotropic virus induction-negative strain, indicate that the gene allowing expression in F/St may not be allelic to Bxv-1. The locations of the genes allowing expression of the ecotropic viruses have not been located in F/St. Furthermore, F/St mice also show a high frequency of somatic cell integrations of ecotropic viruses as judged by Southern blot hybridization with an ecotropic MuLV-specific probe to somatic cell DNA (Morse et al., 1982).

B10.F, along with F/St and B10, was also being raised and analyzed in the laboratory of Herbert C. Morse III at NIH. Along with the previously described V loci and Fv-1, another locus was described in F/St that had a major effect on the expression of xenotropic viruses and xenotropic cell-surface antigens. A locus located on chromosome 17 and tightly linked to H-2 appeared to regulate the levels of xenotropic viruses and xenotropic cell surface antigens that were expressed from loci on chromosome 1 (Yetter et al., 1983). F_1 mice from various strains crossed to F/St had low spontaneous virus expression; high xenotropic virus expression was recessive, while high ecotropic virus expression was dominant. Crosses between AKR and F/St demonstrated that a single locus, Cxv-1, governed the high versus low expression of infectious xenotropic virus and xenotropic cell-surface antigens. F/St carried a recessive, permissive allele at this locus, and AKR carried the dominant, restrictive allele. Only mice that carried the $H\text{-}2^{s/s}$ from F/St were able to produce infectious xenotropic virus. From their experiments, Yetter et al. showed that the locus had no effect on the expression of ecotropic virus. Since B10.F mice carry the same H-2 allele as F/St mice, it was expected that if Cxv-1 was closely linked to

H-2 then B10.F mice would also have high levels of xenotropic virus. When spleen and thymus cells were analyzed, B10.F were found to produce levels of xenotropic virus equivalent to F/St. B10.F mice also produced high levels of B-tropic infectious ecotropic virus due to maternal transmission of the virus, and dualotropic MCF virus. There was no correlation between the Cxv-1 locus and the expression of ecotropic or MCF viruses in crosses tested (Yetter et al., 1983).

Yetter and his colleagues found no indication that ecotropic retrovirus expression involved Cxv-1. This result may be due to the differences between examining endogenous expression without spread of virus (as is observed with xenotropic MuLVs), the amplification by exogenous infection characteristic of the highly inducible multiple V loci of F/St and AKR (as in ecotropic MuLVs), or maternally transmitted virus as in B10.F (Yetter et al., 1983). They suggested that an $H\text{-}2^a$ strain carrying a single V locus on a restrictive $Fv\text{-}1^b$ background would provide an opportunity to determine whether Cxv-1 affects the expression of both ecotropic and xenotropic MuLVs. Caesarian derivation of B10.F mice could answer that question. B10.F mice have the single xenotropic locus at the H-2 region, Cxv-1, and the B10 $Fv\text{-}1^{b/b}$ genotype, and any ecotropic viremia that develops in Caesarian-derived progeny would be the result of either the xenotropic locus or in utero infection of the B10.F mice prior to Caesarian derivation.

Since there was a maternal effect on the level of serum IgA in B10 mice foster nursed on B10.F females and since retroviruses are known to be passed to offspring of infected animals through milk-borne transmission (Melief et al., 1975), it was postulated that the maternal effect observed with serum IgA levels may be caused by the passage of viruses from B10.F females to offspring foster nursed on them. The expression of retroviruses from B10 and B10.F spleens was tested using immunofluorescent antibodies

to viral epitopes and the XC plaque assay (Popp et al., 1986). Both B10 and B10.F mice contained detectable virus, but the percentages of cells expressing virus in the two strains were different. B10.F had more virus-positive cells (94%) than did B10 (20%). The large amount of infectious virus in B10.F mice may be responsible for the maternal effect on IgA expression. The viruses isolated were B-tropic and ecotropic.

The spleens of B10.F were analyzed by Southern blotting techniques to identify if integrated proviral DNA was present in genomic DNA digests. A 400-base-pair probe specific to the 5' end of the env region (Chattopadhyay et al., 1980) of ecotropic retroviruses recognizes a single 5.2 kb PvuII restriction fragment in C57BL/10, the congenic, nonviremic partner of B10.F. This 5.2 kb fragment contains a portion of the endogenous murine leukemia provirus emv-2, a proviral element that is replication deficient due to a mutation in the pol region of the retrovirus (King et al., 1987). DNA from B10.F spleens, in addition to carrying the 5.2 kb fragment, often carries additional integrations of ecotropic proviruses ranging in size from 3.0 to 15 kb when the genomic DNA is digested with PvuII. The integrated proviruses are most often found in DNA isolated from lymphoid organs; when DNA from nonlymphoid organs exhibit additional proviruses, the additional proviruses are usually associated with metastasizing lymphomas or other neoplastic events. A few PvuII restriction fragment sizes (4.8, 4.2 and 6.8 kb) were often found in B10.F splenic DNA that contained new proviral integrations. DNA from different tissues of a single mouse can have different restriction endonuclease fragment patterns.

B10 and B10.F mice were also analyzed for the induction of provirus integration by X-radiation. DNA analyses of B10 and B10.F spleens with the ecotropic env-specific probe showed multiple copies of ecotropic proviruses integrated in the somatic DNA of

B10.F mice. The integrated proviral sequence produced a characteristic 5.5 kb fragment size when the DNA was digested with the restriction endonuclease PstI. No proviruses were detected in B10 splenic DNA other than the endogenous ecotropic virus, emv-2. The new proviruses in B10.F splenic DNA were integrated at random sites in the DNA. B10.F mice treated with a single dose of 2 Gy of X-rays at two months of age and examined at six months of age had acquired more new proviruses than untreated B10.F mice and all B10 mice (Sozer, 1989).

Research Objectives

C57BL/10.F (B10.F) mice have a spontaneous, uninduced retrovirus infection that changes the immune system responses of the mice without causing their early death by lymphoma, leukemia or solid tumor. Its inbred partner, the non-viremic C57BL/10 (B10) strain, is available to compare the immune responses of B10.F mice. Since B10 and B10.F mice also differ at the H-2 locus on chromosome 17, any differences observed between B10 and B10.F mice might not be directly attributable to the retrovirus infection.

The objectives of this dissertation are:

1. To characterize the histologic, hematologic and immunologic differences of congenic B10.F mice when compared to their inbred partner, B10;
2. To quantitate the viremia present in B10.F mice using the XC plaque assay;
3. To map restriction endonuclease sites in the most common ecotropic retrovirus in B10.F mice, B10FV, using free viral DNA obtained from Hirt supernatant extracts;
4. To raise and characterize a population of virus-free H-$2^{n/n}$ mice, called BXF2NN mice;

and

5. To infect the virus-free BXF2NN H-2$^{n/n}$ mice with B10FV derived from infected tissue culture cell supernatants and study the effects of B10FV viremia on the histology, hematologic parameters and immune system of BXF2NN mice.

CHAPTER II

A DESCRIPTION OF HISTOLOGIC, HEMATOLOGIC AND IMMUNOLOGIC PARAMETERS OF C57BL/10 AND C57BL/10.F MICE

Introduction

As described in Chapter 1, C57BL/10.F (B10.F) mice are congenic with C57BL/10 (B10) except at the H-2 locus. Previous data indicated that B10.F may be immunodeficient in comparison to B10 (Popp, 1990; Popp, 1979b; Popp, unpublished data). When compared to B10 mice, B10.F mice had a lower level of serum IgA (Popp, 1979a). Serum IgA levels in B10.F mice were 25 to 35% the levels of B10 mice. The low serum IgA phenotype was also found when B10.F mice were the maternal parents of F_1 and F_2 populations. Because the strain of the maternal parent affected the serum IgA phenotype of offspring, foster nursing experiments were used to elucidate the nature of the maternal effect on IgA. Newborn B10, (B10 x B10.F)F_1 and (B10.F x B10)F_1 mice foster nursed on B10.F mice exhibited low serum IgA levels at four months of age, and the serum IgA level remained suppressed through twelve months of age. Yetter et al. (1983) found that their colony of B10.F mice were highly viremic, passing a B-tropic, ecotropic retrovirus through the milk to progeny. The B10.F mice in

our laboratory were also tested and were found to possess B-tropic, ecotropic retrovirus (Popp, 1986). It was hypothesized by Popp that the maternal effect on serum IgA may be caused by the maternal transmission of retrovirus, either causing a deficiency of IgA secreting cells or a higher rate of degradation of serum IgA by B10.F mice.

In addition to the low IgA phenotype, animals homozygous for the H-$2^{a/a}$ genotype of B10.F mice showed a reduced survival (Popp, 1982). The mean age at death for B10 was 706±14 days; for B10.F the mean age at death was 467±15 days, and heterozygous H-$2^{a/b}$ animals had an intermediate lifespan of 666±16 days.

Experiments were performed to assess the ability of B10 and B10.F mice to respond to exogenous antigen. B10 and B10.F mice were immunized with sheep red blood cells (SRBC) and the spleen cells of these mice were assayed for the number of cells producing hemolytic antibody to SRBC. The hemolytic plaque-forming capacity of SRBC-primed spleen cells from four-month-old B10.F mice was equal to, if not greater than, the response of young B10 mice. By ten months of age, however, fewer spleen cells from immunized B10.F mice were able to respond to SRBC (Popp, 1982, Popp, 1990; Popp, unpublished data).

Collectively, the low serum IgA levels, shortened lifespan and lower number of primed spleen cells to form hemolytic plaques to SRBC of B10.F mice suggests that B10.F mice may be immunocompromised. Also, data from other laboratories have shown that other mouse strains can develop immunodeficiencies following infection with retroviruses (Mosier et al., 1985). Since B10.F mice had been shown to harbor an infectious retrovirus not found in B10 mice, it was of interest to compare the pathobiology and immune competence of B10 and B10.F mice.

This chapter describes: 1) the histology and 2) hematology of B10 and B10.F

mice; 3) the response of B10 and B10.F splenic lymphocytes to the T-cell mitogens Concanavalin A and Phytohemagglutinin, and the B-cell mitogen Lipopolysaccharide A; 4) the ability of SRBC-immunized B10 and B10.F spleen cells to secrete hemolytic antibodies in culture; and 5) the subsets of B10 and B10.F splenic lymphocytes using fluorescently-labeled antibodies to lymphocyte cell-surface antigens.

Materials and Methods

Animals

Two-month-old and four- to six-month-old C57BL/10 (B10) and C57BL/10.F (B10.F) mice were obtained from the colony of Raymond and Diana Popp, Oak Ridge National Laboratory, Oak Ridge, Tennessee. The B10.F mice were originally obtained from Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Montana.

Unless otherwise noted, both male and female B10 and B10.F mice were used for these experiments.

Hematology

Prior to sacrifice, blood was removed from the intraorbital sinus and 200 to 300 µl of blood were placed into microcontainers containing 5 µl 1.5% EDTA. Blood samples were analyzed using an Ortho ELT-8/ds laser-based hematology analyzer (Ortho Diagnostics) previously calibrated with standardized control blood samples, or hand calibrated in the case of hematocrit values. White blood cell counts (WBC), red blood cell counts (RBC) and mean corpuscular volume (MCV) were measured from

laser light-scattered whole blood samples. Hemoglobin (HGB) concentrations were measured by a modified cyanomethemoglobin colorometric method. Hematocrit (HCT), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from the directly measured indices. White blood cell differentials, expressed as the percent lymphocytes (LYM%), monocytes (MON%) and granulocytes (GRAN%) were calculated from data generated by the ELT-8/ds analyzer using an Ortho Model 2140 data handling system.

Blood films were made on #1 glass coverslips from the EDTA-treated blood samples. Blood films were stained with Wright's stain, air dried, mounted on glass slides and examined by light microscopy.

Histology

Small portions of spleen, liver, lymph node, kidney, intestine with Peyer's patch, thymus and sternum were removed at sacrifice and placed in 10% buffered formalin (10% formaldehyde in distilled water buffered to pH 7.0 with monobasic and dibasic sodium phosphate). Other tissues were also removed when gross examination indicated abnormalities. The fixed tissues were paraffin-embedded, cut into 5-10 μ thick sections, mounted on glass slides and stained with hematoxylin and eosin (H&E). The stained tissues were examined by light microscopy.

Mitogenesis

Mitogen-induced proliferation of B10 and B10.F spleen cells was quantified using the method of Strong et al. (1973). Spleen cells from B10 and B10.F mice were dissociated using a loose-fitting Teflon pestle in a smooth-wall glass homogenizer

containing unsupplemented RPMI 1640. Cell suspensions were washed two times and resuspended after the final wash in HEPES-buffered RPMI 1640 supplemented with 100 U penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 100 µM nonessential amino acids, and 1 mM sodium pyruvate. Spleen cells were kept on ice until they were dispensed into microtiter plates.

The numbers of spleen cells per milliliter in the cell suspensions were counted using a hemacytometer. Cells were placed in 96-well microtiter plates at a concentration of $1x10^6$ cells per well in 100 µl of media. The $CD4^+$-cell-specific mitogen Concanavalin A (Con A, 1 µg/ml final concentration), the $CD8^+$-cell-specific mitogen Phytohemagglutinin (PHA, 2 µg/ml final concentration) or the B-cell-specific mitogen Lipopolysaccharide A (LPS, 25 µg/ml final concentration) was added to the appropriate wells in 100 µl volumes. All cultures were made in triplicate. Spleen cells were cultured for 72 hours at 37°C and 7% CO_2. ^{3}H-thymidine incorporation was measured following the addition of 1 µCi per well ^{3}H-thymidine (initial specific activity 1 mCi/ml) during the final 24 hours of culture. Cells were harvested onto glass filters using a Titertek automatic 12-well vacuum harvester. Filters were placed in scintillation fluid and ^{3}H-thymidine incorporation was measured in a Packard Model 3255 scintillation counter. Stimulation indices (SI) for each triplicate set of cultures used to determine mitogen responses were calculated as follows:

$$SI = \frac{\sum_{n=1}^{n} x_n - \bar{y}}{\frac{n}{\bar{y}}}$$

where x = counts per minute (cpm) mitogen well

y = mean cpm unstimulated cells

n = number of mitogen wells

Plaque-forming cells to SRBC

The number of plaque-forming cells (PFC) present in the spleens of B10 and B10.F mice after immunization to sheep red blood cells (SRBC-PFC) was counted using the procedure of Mishell and Dutton (1967). B10 and B10.F mice received an i.v. injection of 0.5 ml of a 2% suspension of SRBC. Five days later, the animals were sacrificed and the spleens were removed aseptically. Single-cell suspensions were prepared by homogenizing the spleens in 1X balanced salt solution (BSS) using a Teflon pestle. The spleen cell suspensions were pelleted by centrifugation at 600 g one time and the cells were resuspended in a volume of 1X BSS that, with the appropriate dilution, was expected to yield 100-200 plaques in culture. Immediately before dispensing the in vitro cultures, the diluted spleen cells in 100 µl and 50 µl volumes were mixed with 50 µl 6.6% SRBC and 0.5 ml 0.6% agarose in 1X BSS held at 45°C. The mixture was poured evenly onto a previously prepared 0.1% agarose-coated microscope slide. Triplicate cultures were prepared for each cell dilution. The cultures were incubated for two hours at 37°C in the presence of SRBC-absorbed guinea pig complement diluted 1:15 in 1X BSS. During the incubation period, the number of cells in the diluted spleen cell preparations were counted using a hemacytometer and the total number of cells in the spleen from which the cell suspensions were made was calculated. Hemolytic plaques were scored using a dissecting microscope, and the numbers of SRBC-PFC per spleen and per 10^6 spleen cells were calculated.

Fluorescence-activated cell sorter (FACS) analysis

B10 and B10.F spleen cells ($1x10^6$) in 100 µl PBS supplemented with 0.1% sodium azide and 0.1% bovine serum albumin (PBS+NaAz+BSA) were incubated on ice for 30 minutes with 1 µg anti-mouse monoclonal antibodies to Thy 1.2, Lyt1, B220, L3T4 (CD4) and Lyt2.2 (CD8). The antibodies B220 and L3T4 (CD4) were labeled by the manufacturer with phycoerythrin (PE); all other antibodies were labeled by the manufacturer with fluoroisothiocyanate (FITC). The antibody-labeled lymphocytes were washed three times in PBS+Az+BSA at 5°C to remove unbound antibody and then fixed in 0.5% paraformaldehyde in PBS. The number of lymphocytes expressing specific cell-surface markers was analyzed using a FACStar Plus (Becton Dickinson) fluorescence-activated cell sorter equipped with a 5 watt argon laser, with the excitation wavelength set at 488 nm. Ten thousand cells were analyzed for each sample. The level of auto fluorescence for each spleen cell preparation was measured on fixed, unstained cells, i.e. cells not treated with monoclonal antibodies. The level of auto fluorescence was determined to correct for small percentages (0.1-3%) of cells that fluoresce without prior binding of fluorescently labeled antibodies. In addition to auto fluorescence and the specific fluorescence of the samples, data for forward scatter, a measurement of cell size, and side scatter, an indicator of nuclear complexity and size, were also collected for each sample.

Results

Histology

Histological sections of B10 and B10.F mice have been examined over a four year period (Gallager et al., 1987); sections of more than 30 B10 and 50 B10.F mice have been examined. The results of 12 representative B10.F mice used in assays described in this chapter are presented in Table 2.1 and Figures 2.1 through 2.3. When compared to B10 (Figure 2.1a), the red pulp areas of B10.F spleens had increased hematopoietic activity (Figure 2.1b). Cells of the myeloid lineages which were increased in B10.F red pulp included megakaryocyte, granulocyte and erythroid precursor cells. The lymphatic follicles in the white pulp of B10.F spleens (Figure 2.1c) were larger than those of B10 spleens (Figure 2.1a), with generalized hyperplasia of all cell types. The germinal centers of the lymphoid follicles of the spleen were larger in B10.F mice and had increased mitotic activity, indicating that some stimulus had caused an increased proliferation of lymphocytes. Follicular atrophy, plasmacytosis and amyloidosis were less often observed changes in B10.F spleens.

Sections of lymph nodes of B10 and B10.F mice are shown in Figure 2.2. The lymph nodes of B10.F mice were characterized by a large number of B cells at varying states of differentiation; immunoblasts and plasma cells were often located between highly active and expanded lymphoid follicles (Figure 2.2b). Amyloid was often located in the paracortical regions of B10.F lymph nodes. In contrast to the lymphoid follicles, the medullary cords of the B10.F lymph nodes were sometimes depleted of mature plasma cells, but contained large numbers of fibrocytes and macrophages.

When compared to B10, most of the nonlymphatic tissues in B10.F mice were

TABLE 2.1

SUMMARY OF HISTOLOGY OF B10.F MICE

Organ and Description	Number of Animals[a]
SPLEEN	
Increased EMH[b]	11
Follicular hyperplasia	7
Follicular atrophy	1
Amyloid	3
Plasmacytoid cell infiltration[c]	1
LYMPH NODE	
Follicular hyperplasia	11
Amyloid	4
Plasmacytoid cell infiltration	2
Cellular depletion of medullary cords	1
LIVER	
Hydropic change[d]	6
Increased EMH	1
Periarteriolar lymphoid cell aggregates	6
OTHER TISSUES	
Lung - lymphocyte accumulation	1
Kidney - periarteriolar lymphocyte aggregates	1
Ovary/uterus - lymphocyte infiltration	1
Lymphoma/leukemia with nonlymphoid organ involvement	1

[a] Number of animals out of total of 12 B10.F examined

[b] EMH: Extramedullary hematopoiesis

[c] Plasmacytosis composed of immunoblastic B cells, plasma cells and Russell's bodies

[d] Hydropic change as characterized by centralized nuclei with clear, non-vacuolated, stringy cytoplasm

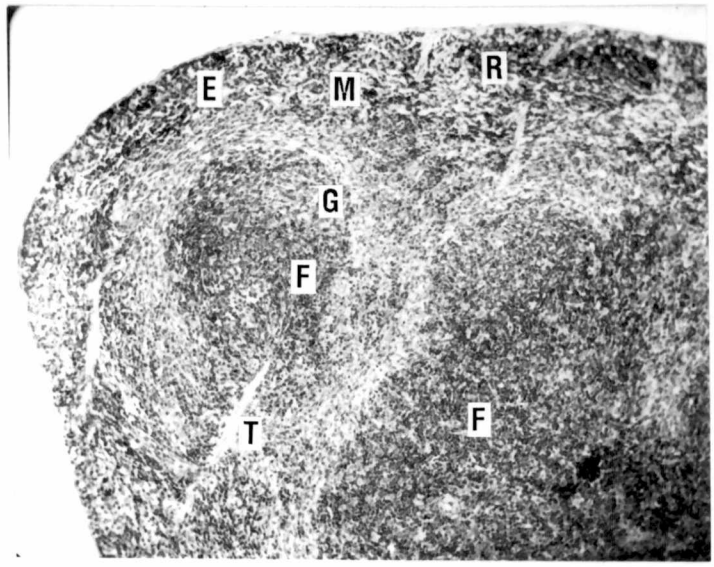
Figure 2.1 Histological sections of spleens from B10 and B10.F mice (H&E, 288X).

Fig. 2.1a The normal B10 spleen consists of a moderate zone of red pulp, composed of erythrocytes, erythrocytic precursor cells, granulocytic precursor cells and megakaryocytes. The splenic follicles, or white pulp, are nodules of lymphocytes containing a small area of developing B cells, the germinal center.

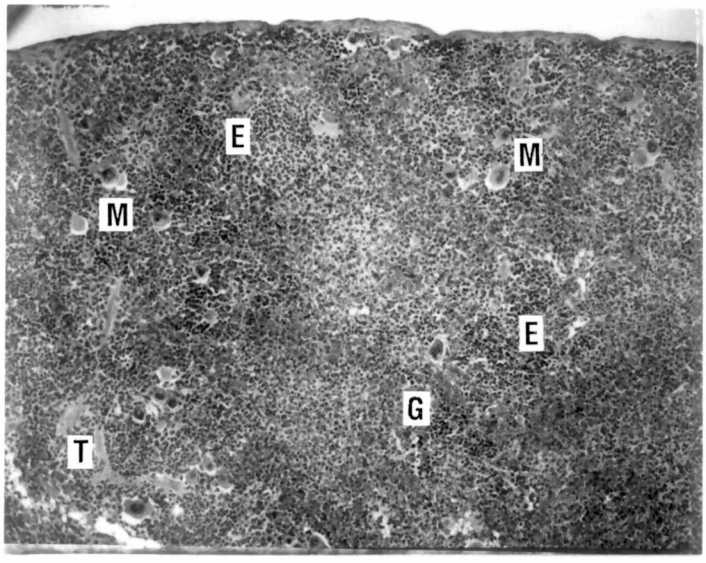
Fig. 2.1b Compared to B10, the red pulp of the B10.F spleen is highly cellular, with larger numbers of megakaryocytes and erythrocytic and granulocytic precursor cells.

Fig. 2.1c The splenic follicles of B10.F mice have large germinal centers surrounded by an expanded area of lymphocytes.

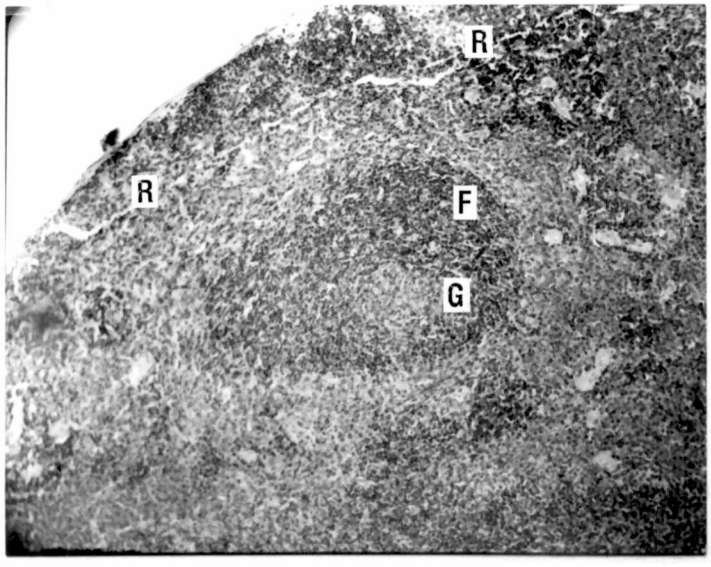
RP - Red pulp
F - Splenic follicle (white pulp)
A - Central arteriole
GC - Germinal center
T - Trabeculum
M - Megakaryocyte
G - Granulocyte precursor cells
E - Erythrocytic precursor cells



(a)



(b)



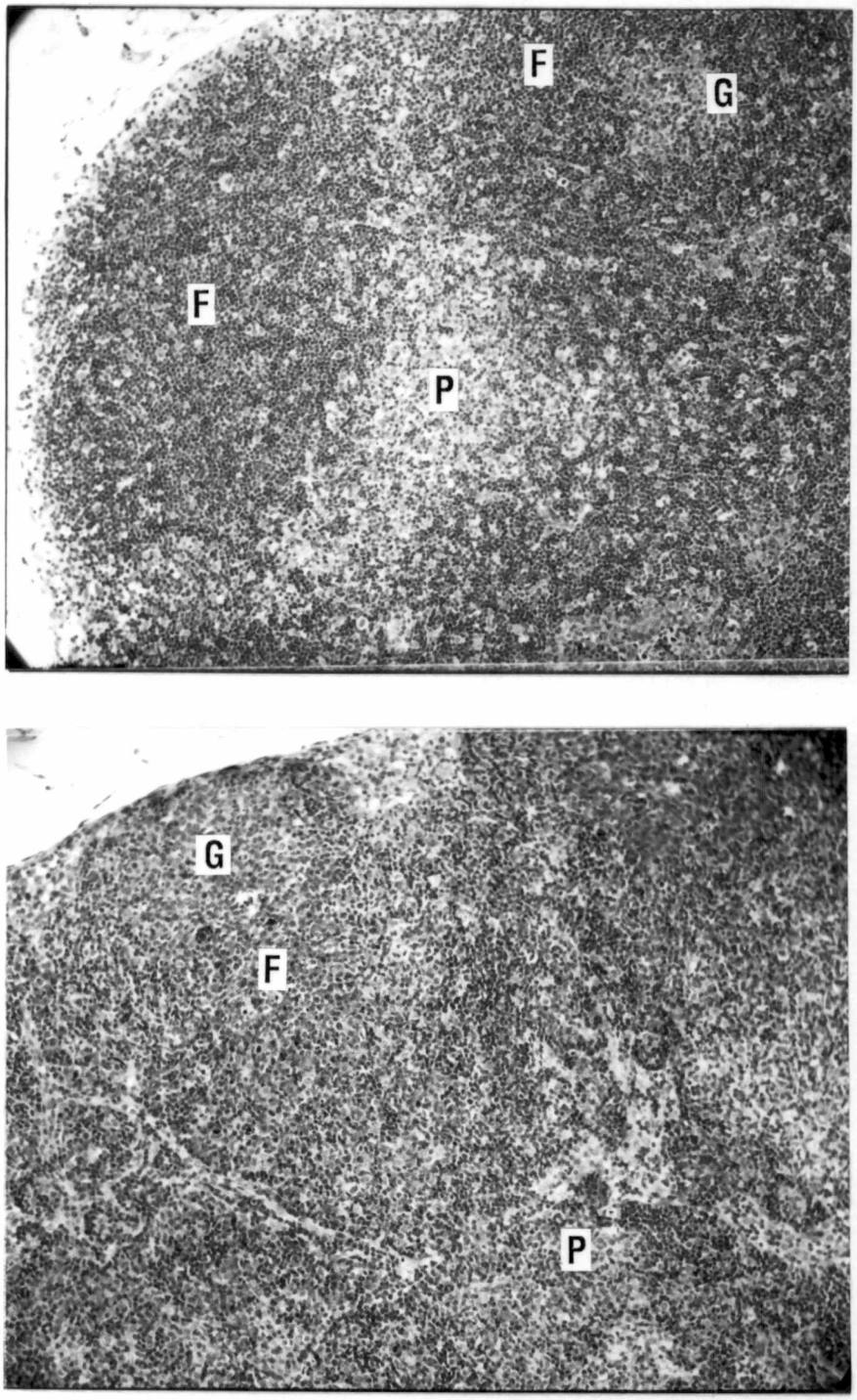
(c)

Figure 2.2 Histological sections of mesenteric lymph nodes from B10 and B10.F mice (H&E, 288X).

Fig. 2.2a A small germinal center is visible among an area of lymphocytes in the B10 mesenteric lymph node. A small paracortical region surrounds the follicle.

Fig. 2.2b The B10.F mesenteric lymph node lymphatic follicle is expanded by a large germinal center. A disorganized paracortical region is combined with small, dark lymphocytes with plasmacytoid features.

F - Lymphatic follicle
GC - Germinal center
PC - Paracortical region



(a)

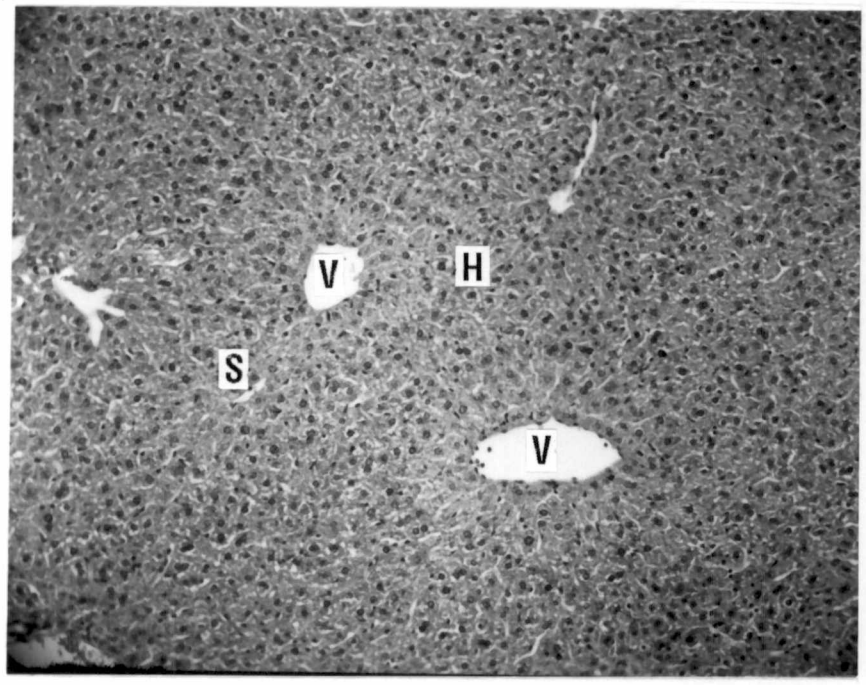
(b)

Figure 2.3 Histological sections of livers from B10 and B10.F mice (H&E, 288X).

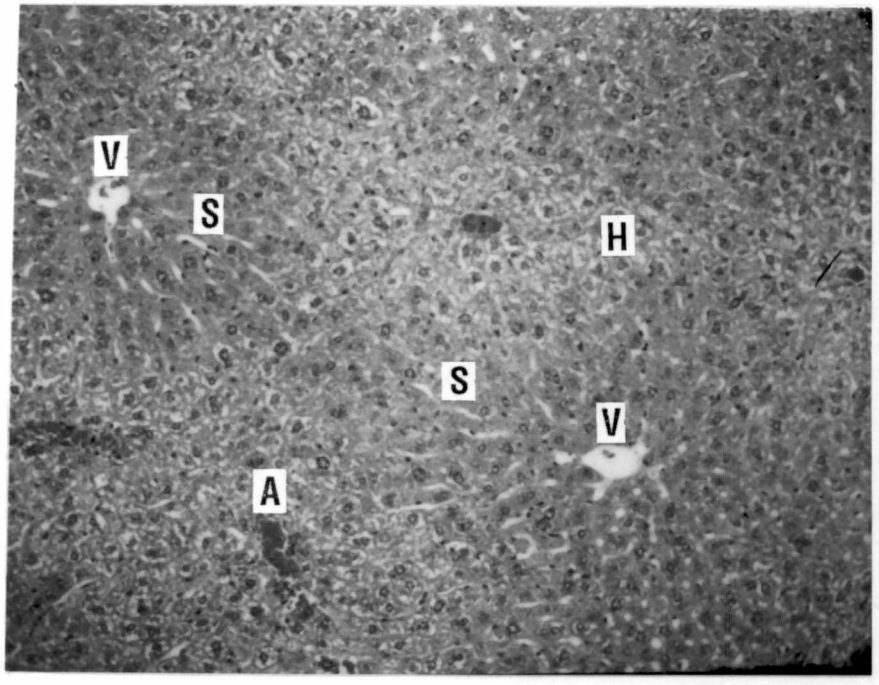
Fig. 2.3a Hepatocytes surrounding a portal vein and radiant sinusoids draining into the central vein are shown in this section of B10 liver. Note that the hepatocytes are uniformly shaded both around the central vein and extending out from the central vein.

Fig. 2.3b In the liver of B10.F mice, hepatocytes surrounding the hepatic artery are larger in size and the cells have centralized nuclei with stringy cytoplasm. Hepatocytes closer to the central vein appear more normal in cytoplasm density but remain larger in size, and the sinusoids near the central veins are dilated.

V - Central vein
S - Sinusoid
H - Hepatocytes
A - Hepatic artery



(a)



(b)

normal in appearance. The livers of B10.F mice often had histological changes associated with either the deposition of glycogen or the retention of fluids within the hepatocytes; cells with centralized nuclei surrounded by stringy, scant cytoplasm containing vacuoles were found clustered around the hepatic portal triad areas (Figure 2.3a and b). Periarteriolar lymphoid cell aggregates were often observed surrounding the hepatic arteries in the hepatic portal areas. Cell types observed at these areas were primarily small lymphocytes.

Other changes, less often observed in B10.F tissues, included the aggregation of lymphocytes in the lung bronchiole areas or at the arteriolar regions of the kidney hilus. One animal in this group of 12 B10.F mice was frankly lymphomatous; all organs contained metastasizing cells. The morphology of the metastasizing cells distinguished them as B-cell immunoblasts.

Hematology

The hematologic parameters of blood from four- to six-month-old B10 and B10.F mice are presented in Table 2.2. Blood samples from B10 and B10.F did not differ significantly in RBC counts, hemoglobin concentrations nor the calculated RBC indices MCV and MCHC. The only apparent difference between B10 and B10.F in RBC parameters was the HCT. B10.F mice had a lower mean HCT when compared to B10 (49.5% for B10 and 45.7% for B10.F), but these values were not statistically significantly different ($p>0.05$).

While the total WBC count did not differ between B10 and B10.F mice, the distribution of the cells did differ. B10.F mice had a lower frequency of lymphocytes (78.98% vs. 61.37%) and larger frequencies of monocytes (3.69% vs. 7.31%) and

TABLE 2.2

HEMATOLOGY OF FOUR- TO SIX-MONTH-OLD B10 AND B10.F MICE

Strain	WBC ($x10^6$/ml)	RBC ($x10^9$/ml)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg/RBC)	MCHC (g/dl)	LYM (%)[a]	MON (%)	GRAN (%)
B10 (12)[b]	7.14 (0.31)[c]	10.17 (0.13)	14.84 (0.29)	49.04 (1.35)	48.17 (0.99)	14.6 (0.85)	30.45 (0.79)	78.98 (2.58)	3.69 (0.72)	13.26 (2.19)
B10.F (25)	9.80 (2.42)	9.73 (0.24)	14.13 (0.38)	45.06 (1.20)	46.4. (0.64)	14.52 (1.08)	31.45 (0.48)	61.37 (4.19)	7.31 (0.77)	27.93 (3.79)
p value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	<0.025	<0.001	<0.025

[a] Total of LYM, MON and GRAN does not equal 100% because a few cells fall outside preset boundaries used to identify these cell types.

[b] Number of animals

[c] Standard error of the mean

granulocytes (13.26% vs. 27.93%) in the WBC population when compared to B10 mice. As with the other parameters there was a greater variability in the WBC differentials among the B10.F mice, which may be associated with different disease states or sequelae.

Mitogenesis

The responses of spleen cells from two-month-old B10 and B10.F mice to mitogens are presented in Table 2.3. Responses of the B10 and B10.F spleen cells to the mitogens Con A, PHA and LPS were compared. The response of B10.F spleen cells to Con A and LPS were slightly increased, while the PHA response was slightly decreased in two-month-old animals. The responses of B10 and B10.F spleen cells to all three mitogens were not statistically, significantly different at two months of age.

The response of unstimulated cultures of spleen cells from four- to six-month-old B10.F mice (7391 cpm ^{3}H-thymidine incorporation) was higher than the unstimulated spleen cell responses from B10 mice (1704 cpm). Although the difference was not statistically significant, the difference between the two mouse groups was substantial.

The response of spleen cells from four- to six-month-old B10.F mice to the mitogens Con A, PHA and LPS were greatly reduced when compared to the response of the B10 spleen cells to these mitogens (Table 2.4). The T-cell mitogen Con A induced a response only one-third as great as that of B10 spleen cells to the same mitogen, with B10 spleen cells having a stimulation index of 37.80 and B10.F spleen cells having a stimulation index of 13.81. PHA responses showed comparable differences; B10.F spleen cells gave PHA stimulation indices one-third (9.10) that of

TABLE 2.3

MITOGEN RESPONSE OF SPLEEN CELLS FROM TWO-MONTH-OLD B10 AND B10.F MICE

Strain	^{3}H-thymidine uptake[a]	Mitogen Dose		
		Con A 1 µg/ml	PHA 2 µg/ml	LPS 25 µg/ml
B10 (11)[b]	5515 (547)[c]	35.96[d] (4.77)	22.68 (2.38)	10.10 (1.87)
B10.F (13)	4724 (456)	41.76 (4.69)	19.96 (1.69)	11.57 (0.86)
p value	>0.05	>0.05	>0.05	>0.05

[a] Counts per minute in unstimulated culture

[b] Number of animals used

[c] Standard error of the mean (sem)

[d] Stimulation index (SI) as calculated in "Materials and Methods"

TABLE 2.4

MITOGEN RESPONSE OF SPLEEN CELLS FROM FOUR- TO SIX-MONTH-OLD B10 AND B10.F MICE

Strain	^{3}H-thymidine uptake[a]	Mitogen Dose		
		Con A 1 µg/ml	PHA 2 µg/ML	LPS 25 µg/ml
B10 (13)[b]	1704 (95)[c]	37.80[d] (4.99)	30.22 (3.86)	12.55 (1.21)
B10.F (24)	7391 (1748)	13.81 (2.57)	9.10 (1.45)	6.67 (0.88)
p value	>0.05	<0.005	<0.001	<0.025

[a] Counts per minute in unstimulated culture

[b] Number of animals used

[c] Standard error of the mean (sem)

[d] Stimulation index (SI) as calculated in "Materials and Methods"

B10 spleen cells (30.22). B10.F spleen cells had about half the proliferative response to the B-cell mitogen LPS as B10 spleen cells. The stimulation index was 12.55 for B10 spleen cells, while the B10.F spleen cell stimulation index was 6.67.

SRBC-PFC

Table 2.5 cummulates the responses of spleen cells from age-matched B10 and B10.F mice to the T-cell-dependent antigens of SRBC. At two months of age, the responses of B10 and B10.F spleen cells to SRBC were not significantly different ($p>0.05$) whether the SRBC-PFC were expressed as the number of SRBC-PFC per spleen (135790 vs. 107518 for B10 and B10.F, respectively) or the number of SRBC-PFC per 10^6 spleen cells (852 vs. 824). At four months of age, the numbers of SRBC-PFC per 10^6 spleen cells in B10 and B10.F mice were not significantly different. However, the number of SRBC-PFC per spleen differed significantly ($p<0.05$) between B10 and B10.F mice; 141167 SRBC-PFC per spleen were found for B10 spleen cells, while B10.F spleen cells only had 42000 SRBC-PFC per spleen. The lower responsiveness of B10.F spleen cells to SRBC became even more evident at six months of age, where the number of SRBC-PFC per 10^6 spleen cells in B10.F mice was significantly lower than in B10 mice. Both the number of SRBC-PFC per spleen and per 10^6 spleen cells continued to be significantly different through 12 months of age for B10 and B10.F mice.

TABLE 2.5

NUMBER OF SRBC-PFC IN SPLEENS OF B10 AND B10.F MICE AT TWO TO TWELVE MONTHS OF AGE

Age (Months)	Strain	PFC/ Spleen	p Value[a]	PFC/10^6 Spleen Cells	p Value[b]
2	B10	135790 (18329)[c]	>0.05	852 (116)	>0.05
	B10.F	107518 (5554)		824 (129)	
4	B10	141167 (24576)	<0.025	599 (70)	>0.05
	B10.F	42000 (10742)		299 (89)	
6	B10	129950 (17072)	<0.001	588 (45)	<0.001
	B10.F	44828 (8168)		151 (33)	
8	B10	101313 (23584)	<0.05	400 (67)	<0.001
	B10.F	29837 (3368)		129 (15)	
10	B10	72667 (14455)	<0.01	337 (28)	<0.001
	B10.F	2250 (1045)		18 (8)	
12	B10	83209 (11431)	<0.001	331 (38)	<0.001
	B10.F	7400 (4802)		22 (15)	

[a] The value given is for the PFC/spleen for B10 and B10.F mice at the indicated age.

[b] The value given is for the PFC/10^6 B10 or B10.F spleen cells at the indicated age.

[c] Standard error of the mean

Flow cytometric analysis of B10.F spleen cells

The flow cytometric analyses of spleen cells from two-month-old and four- to six-month-old B10 and B10.F mice are presented in Tables 2.6 and 2.7. At two months of age, the percentage of $Thy1^+$ lymphocytes in the spleen was lower in B10.F mice (26.22) than in B10 mice (31.59) (Table 2.6). $Lyt1^+$ spleen cells, delineating primarily T cells, and $Lyt1^+/B220^+$ spleen cells, a population of cells expressing both T- and B-cell antigens, were present in similar percentages in B10 and B10.F mice. The percentage of the L3T4 ($CD4^+$) T-cell subset was also found in similar percentages in spleens of B10 and B10.F mice, but the percentage of the Lyt2.2 ($CD8^+$) T-cell subset was reduced in B10.F mice. B10.F spleens contained fewer $CD8^+$ cells (2.78%) than was found in B10 spleens (4.03%). As a result of the decreased percentage of $CD8^+$ cells in the spleens of B10.F mice, the $CD4^+/CD8^+$ ratio was significantly higher for B10.F mice.

When compared to B10, the percentage of B10.F spleen cells from four- to six-month-old expressing the T-cell antigens $Thy1^+$ and $Lyt1^+$ were slightly lower than found in the two-month-old animals, but the differences between these two subsets were not significant between the two age groups (Table 2.7). At four to six months of age, $Thy1^+$ spleen cells frequencies were reduced to 19.35% in B10.F mice, down from the 26.33% found in the spleens of two-month-old B10.F mice. This is compared to the 21.62% $Thy1^+$ spleen cells found in four- to six-month-old B10 mice, and the 31,59% $Thy1^+$ spleen cells found in the two-month-old B10 mice. The incidence of $Lyt1^+$ T cells reflected these $Thy1^+$ T cell perturbations. The frequencies of cells in the $CD4^+$ and $CD8^+$ T-cell subsets were also perturbed. The percentage of $CD4^+$ T cells were equivalent, or only slightly elevated, in B10.F mice; however, the percentage of spleen

TABLE 2.6

FACS ANALYSIS OF SPLEEN CELLS FROM
TWO-MONTH-OLD B10 AND B10.F MICE

Strain	Monoclonal Antibodies						
	Thy1	B220	Lyt1	Ly-1 B	L3T4 (CD4)	Lyt 2.2 (CD8)	CD4/CD8
B10 (11)[a]	31.59 (0.77)[b]	44.99 (2.39)	9.67 (0.77)	2.92 (0.25)	7.12 (0.61)	4.03 (0.34)	1.80 (0.12)
B10.F (13)	26.33 (1.33)	39.98 (1.80)	9.22 (0.56)	3.34 (0.24)	7.90 (0.49)	2.78 (0.17)	2.88 (0.12)
p value	<0.005	>0.05	>0.05	>0.05	>0.05	<0.005	<0.001

[a] Number of animals

[b] Standard error of the mean

TABLE 2.7

FACS ANALYSIS OF SPLEEN CELLS FROM FOUR- TO SIX-MONTH-OLD B10 AND B10.F MICE

Strain	Monoclonal Antibodies						
	Thy1	B220	Lyt1	Ly-1 B	L3T4 (CD4)	Lyt 2.2 (CD8)	CD4/CD8
B10 (5)[a]	21.62 (1.77)[b]	63.94 (0.93)	18.80 (1.02)	4.88 (0.41)	11.37 (1.15)	8.20 (0.74)	1.39 (0.06)
B10.F (6)	19.35 (0.28)	47.87 (3.67)	19.80 (0.62)	11.63 (0.99)	13.98 (0.82)	4.91 (0.39)	2.88 (0.14)
p value	>0.05	<0.005	>0.05	<0.005	>0.05	<0.005	<0.001

[a] Number of animals

[b] Standard error of the mean

cells expressing the $CD8^+$ T-cell antigen was much lower in B10.F mice (4.91%) than in B10 mice (8.20%). When compared to the two-month-old values, the CD4/CD8 ratio dropped slightly in B10 mice to 1.39, but remained the same in B10.F mice, at 2.88.

The percentage of splenic B cells labeled by the antibody B220 was significantly lower in B10.F mice (47.87%) when compared to B10 mice (63.94%). A subset of B cells that are characterized by both the B-cell antigen, B220, and the T-cell antigen, Lyt1, called Ly-1 B cells, was significantly increased in the B10.F spleen. B10.F spleens contained nearly three times the number of Ly-1 B cells (11.63%) found in B10 spleens (4.88%).

Discussion

Histology

Examination of histological sections reveals that the lymphatic tissues of B10 and B10.F mice were different in several ways. Increased extramedullary hematopoiesis was observed in the spleen of B10.F mice; increased numbers of myeloid cells were observed beneath the splenic capsule in the red pulp.

Extramedullary hematopoiesis in the spleen is a common, low-level event in adult mice but is more common in the spleen and liver of fetal mice. Generally, increased extramedullary hematopoiesis occurs in mice with anemias, some leukemias and chronic bleeding problems. In most cases of anemia, immature hematopoietic cells, i.e. reticulocytes and lymphoblasts, are observed in the peripheral blood (Wintrobe et al., 1974). However, as indicated in Table 2.2, B10.F mice are not anemic according to

hematologic data.

The lymphatic follicles of the spleen and lymph nodes of B10.F mice were strikingly different from those found in B10 mice. Increased germinal center size and increased mitotic activity in the lymphoid follicles suggested a response to some stimulus resulting in hyperplasia. Although the spleens of B10.F mice occasionally had infiltrates of immunoblasts and/or plasma cells in the peritrabecular regions of the spleen, the most commonly observed tissue with large numbers of these cells was the paracortical regions of the lymph nodes. In the areas where mature plasma cells are expected to exist, i.e. the medullary cord areas of the lymph nodes, often few cells with plasmacytoid features were found.

The changes in lymphoid follicles observed in the spleen and lymph nodes of B10.F mice may be the result of a proliferative response caused by some viral or cellular product. Increased germinal center size and, correspondingly, lymphoid follicle size have been reported for several types of viral diseases. Using the RCN-BM5 MuLV virus mixture injected into adult C57BL/6 mice, Pattengale et al. (1982) were able to induce a nonthymic, polyclonal lymphoproliferation comprised of immunoblasts and plasmacytoid cells. Immunoblastic lymphomas were found to arise from B-cell follicular center areas of the lymph nodes and later move to the follicular areas of the splenic white pulp. Moribund animals were found through histological examinations to have complete involvement of spleen and lymph nodes with a nonuniform population of immunoblasts, plasmacytoid lymphocytes and plasma cells (Pattengale et al., 1982). Moderate involvement of the thymus and of nonlymphatic organs was also observed in moribund animals. Minimal or no involvement of bone marrow, peripheral blood, central nervous system or gut was observed in even the most seriously affected mice.

Hematology

The RBC indices in blood of B10.F mice did not differ from those in B10 mice. RBC counts and hemoglobin concentrations were similar, as were the RBC indices that described the hemoglobin per unit volume of packed RBC (MCHC), and the RBC size (MCV) and number (RBC). Only one RBC index, the hematocrit (HCT) did appear to differ. Blood from B10.F mice had a lower HCT value than B10 mice, with a great deal of variability between individual B10.F mice. The HCT values in blood from B10 and B10.F mice did not differ significantly at the $p>0.05$ level, but the values were significantly different at the $p<0.10$ level.

The total WBC count in the peripheral blood of B10.F mice did not differ between B10 and B10.F mice, but the distribution of the cell types within the WBC count was perturbed. In B10.F mice, lymphocyte percentages were decreased, while the percentages of monocytes and granulocytes were increased. Lymphopenia in humans can be caused by bacterial, viral, protozoal and all types of overwhelming infectious processes, especially in individuals with poor immune resistance (Wintrobe et al., 1974).

Monocytes and granulocytes, as they appear in the blood, are considered transitory cells. Monocytes transit the blood to settle in tissues, where they function as phagocytic cells or interact with lymphocytes in immune responses (Wintrobe et al., 1974). Granulocytes, a category of cells including neutrophils, eosinophils and basophils, function as a first line of defense against foreign organisms, especially bacteria.

The decreased lymphocyte frequencies, monocytosis and granulocytosis in B10.F mice may have a common origin. The retrovirus infection in B10.F mice is an overwhelming, chronic infection that apparently leads to decreased lymphocyte

numbers, while elevating the monocyte and granulocyte compartments. Decreased lymphocyte frequencies occur in other retrovirus infections. A combination of low WBC counts and a decreased frequency of lymphocytes occurs in HIV-infected humans (Zon et al., 1987). Experimentally induced FeLV-FAIDS causes no change in WBC numbers but does cause a reduced percentage of peripheral blood lymphocytes in infected cats (Shelton et al., 1990).

Bacterial infections, caused by the low immune response in B10.F mice, may increase the numbers of monocytes and, especially, granulocytes. An impaired immune response could result in the increase in the number of opportunistic infections in B10.F mice, further debilitating the animals and their ability to fight infections. Although certain B10.F T- and B-cell immune responses appear to be impaired, the defense against exogenous organisms provided by the monocytes and granulocytes may still be active. The higher numbers of monocytes and granulocytes may provide B10.F mice their only defense against exogenous bacterial infections.

Mitogenesis, SRBC-PFC and FACS analysis

The responses of B10 and B10.F spleen cells to T- and B-cell mitogens and to SRBC antigens were compared to assess the immune system capabilities of B10.F mice. Con A and PHA are T-cell-specific mitogens, with Con A stimulating primarily the helper (T_H) T-cell ($CD4^+$) subset at the concentrations used in these experiments (Persson et al., 1978). PHA acts primarily as an inducer of the cytotoxic/suppressor T-cell ($CD8^+$) subset (Greaves and Janossy, 1972). LPS is a B-cell mitogen (Greaves and Janossy, 1972). SRBC elicit a T-cell-dependent response in SRBC-primed animals. T_H cells are required in this immune process; antigen-presenting cells must interact with

T_H cells before the T_H cells will produce B-cell-signaling lymphokines. Lymphokines and T_H-B cell interactions stimulate B cells to produce the hemolytic antibodies which lyse SRBC in the plaque-forming cell assay.

At two months of age, the immune competence of B10 and B10.F appeared to be similar. Spleen cells from two-month-old B10 and B10.F mice responded equally well to Con A, PHA and LPS (Table 2.3). The equivalent hemolytic responses of spleen cells from two-month-old B10 and B10.F mice primed to SRBC also supported the hypothesis that young B10 and B10.F mice initially have a similar capacity to respond immunologically to antigen stimulation (Table 2.4).

Even though spleen cells from two-month-old B10 and B10.F mice showed similar mitogen responses and similar ability to form hemolytic plaques to SRBC, the percentage of T-cell subsets that comprise their immune systems were different. The incidences of both $Thy1^+$ and $CD8^+$ T cells were significantly lower in B10.F mice ($p<0.05$) (Table 2.6). The mitogenic responses of B10 and B10.F spleen cells to the $CD8^+$ T-cell-specific mitogen PHA were similar, which suggested that the $CD8^+$ spleen cells present in B10.F mice were hyperresponsive to PHA and were able to compensate for the reduced $CD8^+$ numbers, or some growth factor induced by the PHA from B10.F spleen cells caused higher proliferation of $CD8^+$ cells in vitro.

Compared to the two-month-old B10 and B10.F mitogen data, both the background proliferative responses and the mitogen-induced proliferative responses of spleen cells from four- to six-month old B10.F mice were different (Table 2.4). The uninduced ^{3}H-thymidine incorporation in B10.F spleen cells was substantially higher than that found in B10 spleen cells. While these differences were not statistically significant, the background ^{3}H-thymidine incorporation of B10.F spleen cells reflects

the hyperplasia observed histologically in the spleens of the B10.F mice. Due to the hyperplasia, B10.F mice have a higher mitotic index and therefore a higher unstimulated ^{3}H-thymidine incorporation than B10 mice.

The response of spleen cells from four- to six-month-old B10.F mice to the mitogens Con A, PHA and LPS was less than one-half the response of spleen cells from four- to six-month-old B10 mice to the same mitogens. In addition, the response of primed B10.F spleen cells to the T cell-dependent SRBC antigen was decreased at four to six months of age, and continued to be lower than B10 SRBC responses through twelve months of age. FACS data for B10.F spleen cells showed a slight but not significant decrease in the number of Thy1$^+$ T cells, a significant decrease in the number of CD8$^+$ T cells and a significant decrease in the number of B220$^+$-labeled B cells in the spleen.

The number of cells stimulated by Con A or SRBC, the T_H cells, might be decreased in B10.F spleens if replicating viruses cause a reduction in T_H cell frequency in the spleen. However, FACS analysis data indicate no loss of Con A-sensitive, T_H (CD4$^+$) cells in B10.F spleen; the frequencies of CD4$^+$ cells were similar for B10 and B10.F. CD4$^+$ cells are involved in the response to SRBC by providing the T cell help necessary for B cell antibody production against a specific antigen target, by providing important B-cell-signalling lymphokines that stimulate the production of antigen-specific antibodies by B cells. The reduction in Con A and SRBC-PFC responses by B10.F spleen cells would indicate that some mechanism other than responder cell loss is involved in the lower mitogen and SRBC responses. CD4$^+$ cells may not be producing the necessary amount of lymphokines to stimulate the production of antibodies in B cells, or the CD4$^+$ cells may not be able to react to antigen presented

to them by monocytes to produce the B-cell stimulating lymphokines.

The retrovirus infection in B10.F mice may indirectly affect the immune system. T-cell activation by Con A requires the presence of functional antigen-presenting cells. The binding of Con A to the responding cell surface is not sufficient to cause the cells to proliferate; direct interaction of the lectin with antigen presenting cells in the culture causes the release of a growth factor, IL-2 (Ahmann et al., 1978; Andersson et al., 1979). Interaction of Con A with the responding lymphocytes causes an increase in IL-2 receptors; the binding of the IL-2 with its receptors on lymphocytes activates the lymphocyte and causes it to proliferate (Larsson and Coutinho, 1979). The presence of replicating virus may render T cells in B10.F less sensitive to Con A or IL-2 through some part of the virion or some soluble substance from the virus.

Alternately, the cells that secrete the IL-2 necessary for sensitization to Con A may in some way be altered by the presence of replicating virus or its products. Soluble substances, primarily proteins necessary for the retrovirus' lifecycle, have been implicated in the immunosuppression of other organisms. In cats, feline leukemia virus (FeLV) induces an immunosuppression characterized by generalized immune hyporeactivity (Cotter et al., 1975). An envelope protein, p15E, along with UV-attenuated FeLV (FeLV-UV), contributes to immunosuppression by direct T-cell inhibition of T-cell function (Coplan et al., 1983). Proliferation of purified T cells in the presence of FeLV-UV or p15E was examined in Con A-proliferation assays. Suppression of immune response by FeLV-UV or p15E was not caused by antigen-presenting-cell dysfunction, since monocytes preincubated with FeLV-UV or p15E were able to provide accessory cell activity in Con A-proliferation assays equal to accessory cell activity of unmanipulated monocytes. Immune suppression by FeLV-UV or its

p15E product was directly linked to the inhibition of response to and secretion of IL-2, normally produced by activated T lymphocytes, by the addition of UV-attenuated FeLV or purified p15E to Con A mitogen cultures. However, the defect in lymphocyte proliferation induced by FeLV-UV was not limited to the impaired secretion of IL-2, since exogenous IL-2 added to cultures could not overcome the suppression of proliferation (Orosz et al., 1985).

FACS data indicated that, at least for PHA-responsive $CD8^+$ cells in B10.F, the decreased frequency of these cells may be a partial cause of their reduced proliferative response. In B10.F, the incidence of $CD8^+$ cells was reduced to approximately half that observed in B10 spleens. The PHA mitogen response by B10.F spleen cells was reduced to one-third the response of B10 spleen cells. The difference in the observed frequency of $CD8^+$ spleen cells and the response of B10.F spleen cells to PHA indicated that the $CD8^+$ spleen cells remaining in B10.F spleens were altered in their responses to PHA or PHA-induced cytokines. The remaining PHA-sensitive cells may not be as sensitive to lymphokines as normal spleen cells; alternately, another cell type, affected by the presence of the replicating retroviruses, may not be producing the necessary lymphokines for $CD8^+$ cells to respond to the mitogen PHA.

Correlating with the decreased response of B10.F spleen cells to the mitogen LPS, the frequency of $B220^+$ B cells was significantly decreased in B10.F spleen. The lower frequency of B cells in B10.F may also be affecting the response of B10.F spleen cells to SRBC by decreasing the number of cells available to produce antibodies against SRBC. However, the decreased function of T_H cells in B10.F could also hinder the production of hemolytic antibodies to SRBC. Whether the remaining B cells in B10.F mice are normal is unknown; mixing of normal, uninfected $\underline{H\text{-}2}^{a/a}$ T_H cells with

separated B cells from B10.F mice in in vitro SRBC-PFC experiments would be necessary to determine the functional ability of the remaining B cells in the spleens of B10.F mice.

As indicated in Table 2.7, Ly-1 B cells, a population of cells having on their surfaces the T-cell antigen recognized by the Lyt1 monoclonal antibody and the B-cell antigen recognized by monoclonal antibody B220, were significantly increased in four- to six-month-old B10.F mouse spleens but not in two-month-old B10.F mouse spleens. Ly-1 B cells were originally identified in the autoimmune mouse, NZB (Hayakawa et al., 1983). The NZB mouse, which has a genetically determined polyclonal activation of B cells (Davidson et al., 1981), has an expanded Ly-1 B cell population. The human homologue of Lyt1, CD5, has also been found to be a marker for activated B cells (Werner-Favre et al., 1989). Some chronically malignant B cells in human neoplasms also express Lyt1 (Martin et al., 1981). Murine B cell tissue culture lines transformed with oncogenic retroviruses also express Lyt1, suggesting a preferential target for retrovirus transformation (Pierce and Aaronson, 1982).

The Ly-1 B cell population, a normal component of the B-lymphocyte compartment because of its ability to secrete IgM (Hayakawa et al., 1984), constitutes a higher percentage of spleen cells in young mice (>30%) and a lower percentage in adult mice (1 to 4%). Ly-1 B cells are numerous in the peritoneum of normal mice (Hayakawa et al., 1983). The higher percentage of Ly-1 B cells in B10.F spleens suggests that B10.F mice possibly exhibit an autoimmune disorder, but in fact the higher percentage of Ly-1 B cells may be an indicator of increased cellular proliferation in the B cell compartment.

Conclusions

B10.F mice differ from their congenic partner B10 in several ways. Genetically, they are identical except for the H-2 region on chromosome 17. Only B10.F mice have an ecotropic retrovirus that infects neonates through milk-borne transmission. Histologically, distinct hyperplastic changes were observed in the lymphoid organs of B10.F mice suggesting that the cells have a chronic immune stimulation. Hematologic changes in RBC parameters were not observed in B10.F mice, but there were alterations in the WBC differential. A lower frequency of lymphocytes and an increased frequency of monocytes and granulocytes were found in the blood of four- to six-month-old B10.F mice. No differences were observed in the responses of spleen cells from two-month-old B10 and B10.F mice to Con A, PHA and LPS or PFC responses to SRBC, even though the incidence of T cells, and in particular the $CD8^+$ T-cell subset, was lower in two-month-old B10.F mice. By four to six months of age, responses to all three mitogens and the ability to form hemolytic plaques to SRBC became significantly lower in B10.F mice than in B10 mice and the ability to respond to SRBC remained low through twelve months of age. FACS analyses indicated that B10.F mice had only a slightly lower frequency of T cells but a statistically significant decreased frequency of $CD8^+$ T cells was found in the spleen of B10.F mice when compared to B10 mice. The number of splenic B cells was lower in four- to six-month-old B10.F mice, but a B-cell subset found in autoimmune mice, the Ly-1 B cell subset, was significantly increased in these mice. While many of these differences may be attributable to the infectious ecotropic retrovirus present in B10.F mice, the different H-2 regions carried by B10 and B10.F mice may also influence some of the changes

observed between the congenic pair. To compare the effects of retrovirus infection and H-2 in B10.F mice, H-2$^{a/a}$, ecotropic retrovirus-free animals had to be raised to find which traits are associated with ecotropic retrovirus infection and which traits are associated with the H-2 region of B10.F mice. These data are presented in Chapter IV.

CHAPTER III

QUANTITATION AND CHARACTERIZATION OF INFECTIOUS ECOTROPIC RETROVIRUSES IN C57BL/10.F MICE

Introduction

The C57BL/10.F mouse strain (B10.F), congenic with C57BL/10 (B10) except at the H-2 locus, has previously been described to have a decreased lifespan and early greying of the fur in comparison to B10 (Popp, 1978; Popp, 1979a.; Popp, 1979b). The B10.F phenotype can be induced in B10 mice through the injection of B10.F spleen cells into neonatal recipients or the transplantation of B10.F bone marrow into lethally irradiated B10 mice (Popp et al., 1986). Cell-free procedures can also be used to induce the B10.F phenotype, including foster nursing of B10 pups on B10.F females and injection of B10 neonates with cell-free splenic supernatants (Popp et al., 1986).

Association of the decreased lifespan with the H-2^{a} genotype of B10.F has been studied using F_2 and backcross progeny from (B10.F x B10)F_1 parents. The decreased lifespan segregates with the animals of H-$2^{a/a}$ genotype when B10.F is the female parent (Popp, 1982; Popp et al., 1986). Animals with the H-$2^{b/a}$ genotype derived from either of the reciprocal F_1 matings have similar mean ages at death, and their lifespans

are intermediate between those of the longer-lived H-$2^{b/b}$ B10 mice and the shorter-lived H-$2^{a/a}$ B10.F mice (Popp et al., 1986).

The decreased lifespan and greying of the fur in B10.F are attributable to the presence of infectious ecotropic retroviruses (Popp et al., 1986; Morse et al., 1985). In a separate colony of B10.F mice, Yetter et al. (1983) found that large numbers of ecotropic, xenotropic and recombinant dualotropic MCF viruses were expressed by B10.F spleen cells. All ecotropic viruses present in B10.F spleens are B-tropic, unlike viruses present in its parental strain, F/St, which are N-tropic. The primary means of transmission of the ecotropic viruses is through milk-borne transfer.

Restriction enzyme analyses of the DNA from the tissues of B10.F mice were done using an ecotropic-specific probe derived from the env region of the AKV proviral genome (Chattopadhyay, 1980). These experiments indicated that ecotropic retroviruses other than emv-2 were present in the lymphoid tissue DNA of many B10.F mice (Popp et al., 1986; Gallager et al., 1987; Popp et al., 1989; Sozer et al., 1989). Occasionally DNA from non-lymphoid tissue was found to contain integrated proviruses, but in most cases these integrated proviruses were associated with lymphoid cell metastasis. The restriction fragments generated from these experiments provided little information as to the structure of the viruses present because in most cases only a single restriction enzyme, PvuII, was used, creating a junction fragment between the 3' end of the proviral sequence and the adjoining 3' flanking host DNA.

Limited mapping of the integrated proviruses in B10.F was done using lymphoid tissue DNA from B10 and B10.F mice and the ecotropic-specific probe, env. When B10 and B10.F splenic DNA were digested with PstI, a restriction enzyme that cuts internally in the retrovirus genome on the LTRs, two fragments hybridized with the

ecotropic-specific probe. An 8.3 kilobase (kb) PstI-PstI restriction fragment corresponding to the endogenous ecotropic provirus emv-2 was found in both B10 and B10.F mice. A second 5.5 kb PstI-PstI fragment corresponding to a different proviral genome was found only in B10.F mice. The 5.5 kb fragment was found in the splenic DNA of all B10.F mice examined. It was not present in the germline DNA but was integrated randomly in the cellular DNA of B10.F mice. This new retrovirus was called B10FV (Sozer, 1989).

Restriction mapping of the B10FV provirus in lymphocyte cellular DNA was limited by the presence of homologous sequences in the endogenous ecotropic provirus emv-2 . The presence of many other MuLV proviral sequences in the B10.F mouse genome did not allow the use of DNA probes to other segments of the retrovirus genome to study the structure of B10FV in the somatic cell DNA of B10.F mice because the 5' sequences and LTRs of the many endogenous xenotropic viruses in B10.F share many similarities to the sequences of ecotropic retroviruses. The hybridization of a DNA probe from the 5' portion of a provirus to the numerous endogenous xenotropic viruses would create a situation where further mapping of the B10FV provirus would be virtually impossible. The infectious proviral genome of B10FV needed to be isolated to allow restriction enzyme site mapping of the proviral genome using several different probes.

This chapter describes 1) the titer of infectious ecotropic retroviruses in B10.F mice, 2) the isolation of B10FV proviral DNA from Hirt supernatants, and 3) the restriction enzyme site mapping of the B10FV proviral DNA. DNA probes to the 5', 3' and env regions of ecotropic retroviruses were used to locate restriction fragments of B10FV via Southern blotting to generate a restriction map. The map of B10FV is

compared to other well characterized ecotropic retroviruses.

Materials and Methods

Cell lines

SC_1 cells, a feral mouse embryo cell line (Hartley, 1970), and XC cells, a rat tumor cell line induced by Rous sarcoma virus (Rowe, 1970), for early studies were kindly provided by Mrs. Den-Mei Yang (Oak Ridge National Laboratory, Oak Ridge, Tennessee). Later cell stocks were obtained through the American Type Tissue Collection (Rockville, Maryland). All cells were maintained in Modified McCoy's 5A Medium supplemented with 6% heat-inactivated fetal bovine serum (HI-FBS), 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin and 100 µM non-essential amino acids.

XC plaque assay

The XC plaque assay on infected SC_1 cells was performed as described by Rowe et al. (1970). Eighteen hours before assay, SC_1 cells ($1x10^5$ cells per well) were plated in 6-well plates in Modified McCoy's Medium supplemented with 2% HI-FBS, 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin and 100 µM non-essential amino acids (hereafter referred to as "McCoy's complete medium"). The next day, the SC_1 cells were treated with 2 µg/ml Polybrene (Sigma) in unsupplemented McCoy's medium for one hour at 37°C.

Animals were sacrificed and spleens were removed aseptically into unsupplemented McCoy's medium. The spleen cells were dissociated with a Teflon

pestle, washed one time and counted. Spleen cells were diluted in unsupplemented McCoy's medium to initial dilutions of 1 to $2x10^6$ cells per milliliter for B10 samples and 2 to $4x10^5$ cells per milliliter for B10.F samples. The diluted spleen cell preparations were placed on the Polybrene-treated SC_1 cells. Cells were added to wells in duplicates, and two 1:2 dilutions of the original B10 and B10.F dilution preparations were also made in duplicate. The spleen cells were incubated with the SC_1 cells for 2 hours at 37°C. At the end of the incubation, the spleen cells were washed off the SC_1 cells with unsupplemented McCoy's medium and the SC_1 cells were cultured in complete McCoy's medium supplemented with 2 μg/ml Polybrene.

The infected SC_1 cells were cultured for two to three days with daily medium changes. When nearly confluent, the SC_1 cells were irradiated with 1800 erg/mm^2 UV and 3 x 10^5 XC cells were placed on top of the UV-treated SC_1 cells in complete McCoy's medium supplemented with 2 μg/ml Polybrene and 1 μM hydrocortisone. The SC_1-XC cell cultures were cultured for three to four days with daily medium changes, until the SC_1-XC uninfected control wells appeared nearly confluent. At the end of the culture period the medium was removed from the wells and the cells were stained in 3 parts 1% methylene blue in methanol, 1 part carbol fuchsin (8 g basic fuchsin, 40 ml phenol and 800 ml methanol) and 2 parts methanol for 20 minutes. The wells were rinsed with water, inverted and air dried.

Syncytia were scored with the aid of a dissecting microscope. The number of background syncytia were scored on the SC_1-XC uninfected control wells and subtracted from each experimental count. The syncytia counts were adjusted for infecting cell number and the number of ecotropic retrovirus infecting cells was expressed as the $\log_{10}$ plaque forming cells (PFC) per 10^7 B10 or B10.F spleen cells

used to infect the SC_1 cells.

Hirt supernatant production

Free viral DNA was produced using the method of Hirt (1967) with modifications by Jolicoeur and Rassart (1980). SC_1 cells were infected with mouse spleen cells, as for the XC plaque assay (Rowe et al., 1970), and the SC_1 cells were grown through several passages prior to use in the Hirt supernatant assay

Infected SC_1 cells were cocultivated with uninfected SC_1 cells at a ratio of 1 infected cell to 5 uninfected cells in Modified McCoy's 5A medium supplemented with 10% HI-FBS, 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin, 100 µM non-essential amino acids and 8 µg/ml Polybrene. The cells were cocultivated 17 hours at 37°C in 5% CO_2 with humidity.

At the end of the cocultivation, the cells were washed in calcium- and magnesium-free phosphate buffered saline (CMF-PBS) and then lysed in the flasks with a solution of 10 mM Tris pH 7.4, 10 mM EDTA pH 8 and 0.5% SDS. The viscous supernatant was decanted into a polypropylene tube and the SC_1 cell genomic DNA and cellular proteins were precipitated with the addition of 5 M NaCl until a 1 M NaCl solution was reached. The flocculent lysate was placed at 4°C for 6 hours to precipitate the cellular proteins and genomic DNA, which were removed by centrifugation at 10,000 x g. The supernatant was saved and treated with 50 µg/ml RNase A and 50 µg/ml Proteinase K at 50°C for 3 hours. Following the RNA and protein digestion, the supernatant was extracted with PCA (phenol:chloroform: isoamyl alcohol in a 25:24:1 ratio) and the DNA in the aqueous phase was precipitated in 67% cold ethanol. The DNA solution was centrifuged at 25,000 x g at -20°C to pellet the DNA. The pellet was

resuspended in a small volume of TE (10 mM Tris pH 7.4, 1 mM EDTA pH 8), reprecipitated with 70% ethanol in an Eppendorf tube, pelleted by centrifugation and the precipitated DNA was dissolved in 50 µl of TE.

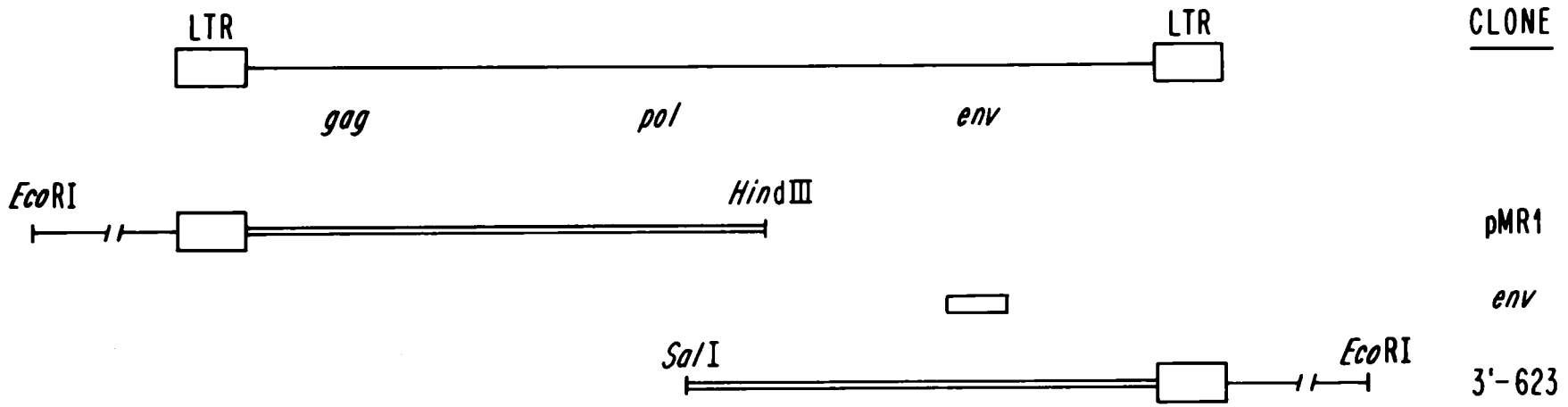
DNA probes

pMR1, a plasmid containing the 5' portion of Moloney MuLV from the 5' LTR to the internal HindIII site with 5.0 kb of flanking 5' mouse genomic DNA in pBR322, was obtained from R. Mural (Oak Ridge National Laboratory, Oak Ridge, Tennessee). 3'-623, a portion of AKV from the internal SalI site through the 3' LTR with 0.5 kb of 3' flanking mouse DNA cloned in pBR322, was also obtained from R. Mural. The ecotropic retrovirus-specific probe, env, was a 400 bp SmaI-SmaI fragment derived from the 5' end of the env gene from AKV and EcoRI linkers were added and cloned into pBR322 (Chattopadhyay, 1980). The relationships of these DNA probes to each other and to the MuLV genome are depicted in Figure 3.1.

Restriction enzyme analysis, agarose gel electrophoresis, Southern transfer and hybridization

Hirt supernatant viral DNA was digested to completion with restriction endonucleases using the methods, enzyme concentrations and buffers provided by the supplier (BRL) . The digested DNA was electrophoresed in 0.8% agarose gels in 1X TBE (0.089 M Tris-borate, 0.089 M boric acid and 0.02 M EDTA). The electrophoresed DNA was denatured in 0.5 M NaOH and 1.5 M NaCl then neutralized in 1.0 M Tris pH 7.4 and 1.5 M NaCl. The DNA was transferred to Nytran hybridization membrane (Schliecher and Schuell) using either a modified blotting

Figure 3.1 Relationship of DNA probes to ecotropic MuLV.
Please see "Materials and Methods" section for complete description of the figure.


LTR
LTR
CLONE
gag
pol
env
EcoRI
HindIII
pMR1
env
SalI
EcoRI
3'-623

protocol described by Maniatis et al. (1982) and Sozer (1988), or a pressure blotting apparatus (Stratagene). The filters were UV irradiated for 6 minutes to bind the DNA to the filters. The Nytran filters were prehybridized for 1 to 3 hours at 42°C in a hybridization buffer consisting of 45% formamide, 5X SSPE, 0.1% SDS, 10% dextran sulfate, 5X Denhardt's solution and 100 μg/ml denatured salmon sperm DNA. Random-primed dCT(^{32}P)-labeled probe made using the methods and solutions provided by the supplier (BRL) was added to give a concentration of 10-20 ng of probe per milliliter of hybridization fluid. The filters were hybridized for 18 hours at 42°C, washed extensively with 2X SSC and 0.1% SDS, and autoradiographed as previously described (Popp, 1981).

Results

Quantitation of infectious ecotropic retrovirus in B10.F Mice

Spleen cells from 2-, 4-, 6- and 8-month-old B10 and B10.F mice were used to infect SC_1 cells for quantifying the titer of the infectious ecotropic retroviruses. The results from these experiments are given in Table 3.1 . At two months of age no measurable titer of infectious ecotropic retrovirus was found in the spleen cells of B10 mice; this lack of ecotropic virus in B10 continued through 8 months of age. In B10.F spleens at two months of age, there was a high titer (3.03 $\log_{10}$ PFC per 10^7 spleen cells) of ecotropic retrovirus, and the titer did not change significantly through 8 months of age.

TABLE 3.1

TITERS OF ECOTROPIC RETROVIRUS IN B10 AND B10.F MICE

Strain	Age (months)	Titer Log_{10} (PFC/10^7 Spleen Cells)
B10 (5)[a]	2	0
B10.F (4)	2	3.03
B10 (5)	4	0
B10.F (19)	4	3.90
B10 (6)	6	0
B10.F (9)	6	3.82
B10 (1)	8	0
B10.F (8)	8	3.47
B10 (1)	12	0
B10.F (1)	12	4.46

[a] Number of animals

Isolation of B10FV viral DNA from Hirt supernatants

The spleen cells from two B10 and fifteen B10.F mice were used to infect SC_1 cells for Hirt supernatant DNA analyses. The free viral DNA was analyzed with the 5' MuLV probe, pMR1, and the data are presented in Table 3.2. In uninfected SC_1 cell uncut Hirt supernatant control DNA preparations, a small amount of a retroviral DNA of approximately 8.8 kilobases (kb) in size hybridized with the pMR1 probe. In B10-infected SC_1 cell uncut Hirt supernatants, an 8.8 kb retrovirus was also found, and an additional 7.6 kb species was found in one B10 Hirt preparation. Digestion of both B10 and SC_1 cell control Hirt supernatant DNA with PstI created several fragments, with sizes of 8.6, 8.5, 4.9 and 3.0 kb. When the same uncut and PstI-digested samples were hybridized with the ecotropic-specific probe, env, no hybridization occurred with either the B10 or SC_1 cell control Hirt supernatant DNA preparations. These data show that ecotropic retroviruses were not obtained from either B10-infected SC_1 cells or uninfected SC_1 cell control Hirt supernatants.

Like the B10 and SC_1 cell control Hirt supernatant samples, the uncut B10.F Hirt supernatant viral DNA yielded an 8.8 kb fragment as the primary retrovirus species, with one of fifteen animals having an additional 7.6 kb species. Five B10.F samples had an undeterminable uncut fragment size, as these Hirt supernatant DNA samples migrated as an indistinct band above the 23.1 kb λ-HindIII DNA molecular weight marker. Digestion of the B10.F Hirt supernatant DNA samples with PstI caused a range of fragment sizes when hybridized with the 5' MuLV probe, pMR1. Four samples in particular had more than fifteen minor fragments, with sizes ranging from 8.6 to 3.0 kb. Two major fragments in common with all samples, with two exceptions, were 4.9 and 5.2 kb in size. The distribution of the band intensities of the 4.9 and 5.2

TABLE 3.2

ANALYSIS OF HIRT SUPERNATANT DNA FROM B10 AND B10.F MICE

Spleen Cell Origin[a]	Treatment	Major Linear Species[b] (kb)	No.[c]	ENV-Specific Species[d] (kb)
B10 (2)	Uncut	8.8	1	-
		8.8, 7.6	1	-
	PstI	8.6, 8.5, 4.9, 3.0	1	-
		8.8, 7.6[e]	1	-
SC_1[f]	Uncut	ND[g]	2	-
	PstI	8.6, 4.9, 3.0	1	-
		4.9	1	-
B10.F (15)	Uncut	8.8	9	8.8
		8.8, 7.6	1	8.8
		ND[g]	5	8.8
	PstI	8.6, 8.5, 5.2, 4.9, 3.0	3	5.2
		8.6, 5.2, 4.9, 4.4, 3.0	3	5.2
		8.6, 5.2, 4.9, 3.0	3	5.2
		5.2, 4.9, 3.0	1	5.2
		8.6, 4.9, 3.0	2	5.2[h]
		5.2, 3.0	2	5.2

[a] Spleen cells were used to infect SC_1 cells for Hirt supernatant production

[b] Major linear species visualized with the pMR1 probe

[c] Number of animals

[d] Size, in kilobases, of linear species visualized with the ecotropic env probe

[e] PstI did not cut the sample

[f] Uninfected SC_1 cell control Hirt supernatant DNA

[g] ND: Not determinable in the sample

[h] The 5.2 kb species was not visualized in the pMR1-probed sample but was visualized with the env probe

kb fragments among the B10.F Hirt supernatant samples showed that equal numbers of B10.F Hirt preparations had one or the other fragment as the more intense band when hybridized with pMR1; one Hirt preparation had both fragments present in equal intensity. When the same Southern transfers were hybridized with the ecotropic-specific probe only the 5.2 kb fragment was visualized. The two B10.F Hirt supernatants in which a 5.2 kb fragment was not detected by hybridization with pMR1 did show a 5.2 kb fragment when hybridized with env.

Restriction enzyme mapping of B10FV

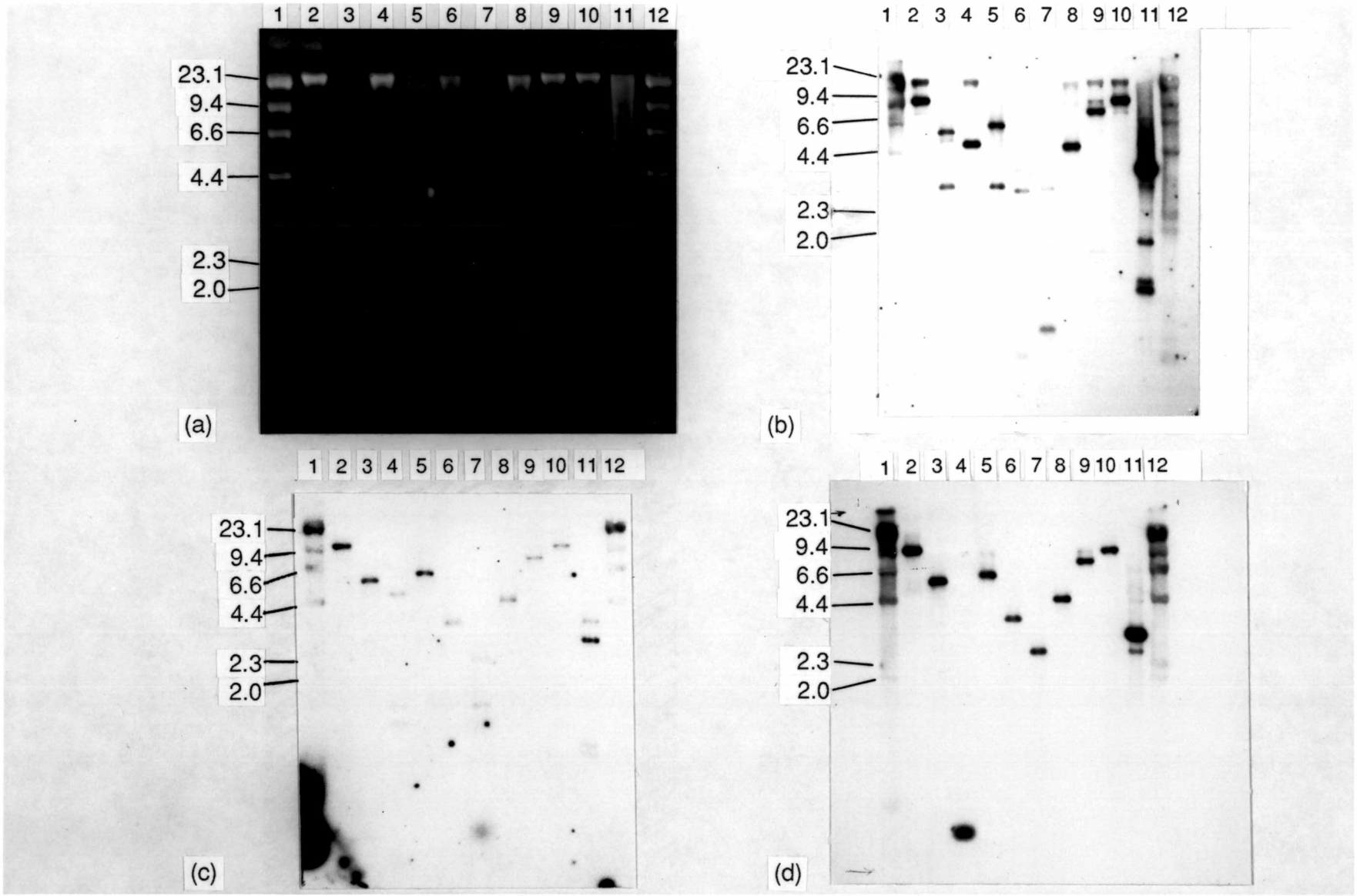
One retrovirus-infected SC_1 cell line, 513900, was derived from a seven-month-old B10.F male, ear mark number 900. The animal had a greatly enlarged liver, spleen and lymph nodes. Histological examination of paraffin-embedded tissues revealed that the spleen and lymph nodes were homogeneously composed of the large, undifferentiated cells, probably of follicular center origin. An infiltration of the same type of large, basophilic cells was also present in the liver, kidneys, heart and lungs of the animal.

Spleen cells from B10.F #900 were prepared for infection of SC_1 cells for permanent cultures from which Hirt supernatant DNA was prepared. Hirt supernatant DNA from this cell line contained virtually a single proviral form. Initial restriction enzyme analysis of this provirus revealed that the virus present in 513900 was ecotropic and resembled B10FV as described by Sozer (1989). Digestion of the Hirt supernatant DNA with PstI and probed with the 5' MuLV probe pMR1 (Figure 3.2b, lane 3) created three visible fragments of 3.1, 5.2 and approximately 0.5 kb in length. The 3.1 kb fragment hybridized most intensely with pMR1, indicating that this fragment

Figure 3.2 Restriction enzyme analysis of B10FV Hirt supernatant DNA.

Figure 3.2a Ethidium bromide-stained agarose gel
Figure 3.2b Southern blot hybridized with pMR1
Figure 3.2c Southern blot hybridized with 3'-623
Figure 3.2d Southern blot hybridized with env

Lane 1: λ-HindIII
2: Uncut 513900 Hirt supernatant DNA
3: PstI
4: SmaI
5: SstI
6: KpnI
7: HincII
8: XhoI
9: SstII
10: PvuI
11: BstEII
12: λ-HindIII

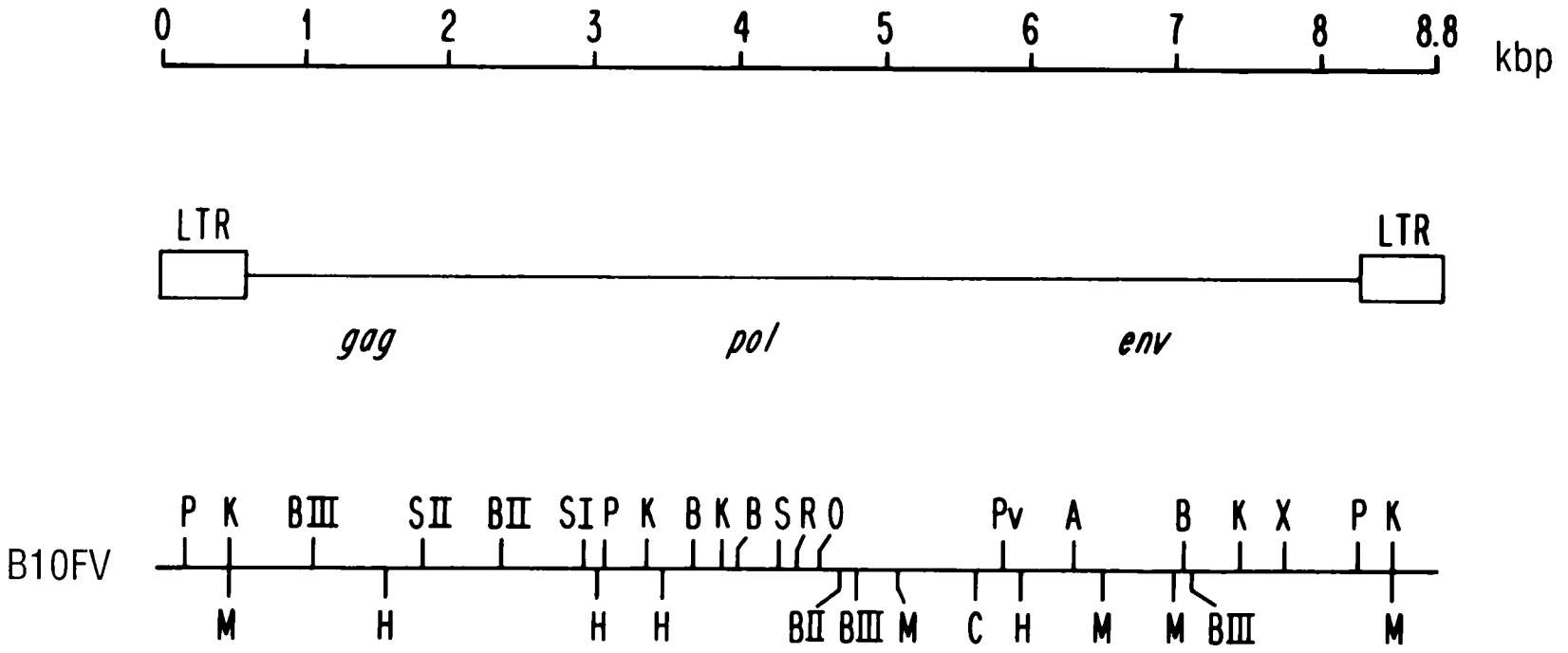

1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.4
6.6
4.4
2.3
2.0
(a)
1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.4
6.6
4.4
2.3
2.0
(b)
1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.4
6.6
4.4
2.3
2.0
(c)
1 2 3 4 5 6 7 8 9 10 11 12
23.1
9.4
6.6
4.4
2.3
2.0
(d)

corresponds with the 5' end of the provirus. The 5.2 kb fragment hybridized minimally, due to some overlap of pMR1 with the 5' portion of this fragment. The 0.5 kb fragment corresponded actually to fragments containing LTR sequences cleaved at their PstI sites. The plasmid pMR1 contains one LTR, allowing hybridization of the probe to LTR sequences. Hybridization of PstI-cut 513900 with 3'-623 (Figure 3.2c, lane 3) revealed only two fragments. Probe 3'-623 hybridized more intensely with the 5.2 kb fragment, the 3' proviral fragment. A LTR band at 0.5 kb was also visible. The env probe hybridized only with the 5.2 kb fragment, as this fragment contained the ecotropic envelope region to which env is specific (Figure 3.2d, lane 3). Other restriction enzymes were used to digest the Hirt supernatant DNA from 513900, probed with pMR1, 3'-623 and env (Figure 3.2 a,b,c and d, lanes 4 through 11).

The results from restriction enzyme digestions and visualizations following hybridizations with the pMR1, 3'-623 and env probes were used to create a restriction map of B10FV (Figure 3.3). The probes located the fragments with which they had sequences in common, and the intensities of hybridization were used to place fragments in specific regions of the proviral genome. Fragments located in the 5' portion of B10FV hybridized more intensely with the pMR1 probe; those fragments in the 3' half of the retrovirus hybridized more intensely with 3'623. Restriction fragments carrying the ecotropic env sequences were easily placed in the restriction map using the env probe. Multiple restriction fragments produced by some enzymes were more difficult to place accurately, and restriction maps of similar retroviruses were used as a guide to place these restriction enzyme sites on the B10FV restriction map.

Figure 3.3 Restriction enzyme map of B10FV generated from 513900 Hirt supernatant DNA.

Restriction enzyme site abbreviations: A, HpaI; B, BamHI; BII, BglII; BIII, BstEII; C, ClaI; H, HincII; K, KpnI; M, SmaI; O, XhoI; S, SalI; SI, SstI; SII, SstII; P, PstI; Pv, PvuII; X, XbaI.


0
1
2
3
4
5
6
7
8
8.8
kbp
LTR
LTR
gag
pol
env
B10FV
P K BIII SII BII SI P K B K B S R O Pv A B K X P K
M H H H BII BIII M C H M M BIII M

Discussion

Measurements of infectious ecotropic retrovirus in B10.F mice

The XC plaque assay assesses the titer of infectious ecotropic retroviruses present in a sample of cells, culture supernatant or body fluid. To do the XC plaque assay, the SC_1 cells are infected with mouse cells or fluid and cultured for two to four days to establish the infection in the SC_1 cells. The infected SC_1 cells are then irradiated with enough UV to stop SC_1 cell growth but still allow a short period of virus expression. Immediately after UV irradiation, XC cells are placed on top of the UV-killed SC_1 cells. The XC cells detect the ecotropic viruses emitted from the SC_1 cells and express these viruses by forming cellular syncytia. The enumerated syncytia are expressed as the titer in the context of the number of infected cells present in 10^7 cells.

The large amounts of infectious ecotropic retrovirus in B10.F spleen may be, in part, attributable to the unique H-2 genotype present in B10.F. The H-2 region in B10.F was derived from F/St, a highly viremic strain of mice, and may contain certain genes that allow for the maintenance of a retroviral infection. In congenic mice differing at the H-2 locus, a closely linked gene (or genes) can determine either a high or a low virus titer (Tucker, 1977), and these genes are inherited as a dominant trait. Early experiments with the development of C57BL/10.A (B10.A) sublines that were virus-positive (B10.A V+) or virus-negative (B10.A V-) indicated that the $H\text{-}2^b$ haplotype conferred a dominant resistance to murine leukemia virus replication (Melief, 1980). The resistance to the viral infection appeared to be the result of an H-2-linked dominant immune response gene that regulated the antibody response to

murine leukemia viruses. The I-A region of H-2 controlled the immune response to murine leukemia viruses through I-A-regulated immunoselection against murine leukemia virus cell-surface antigens (Zijlstra, 1986).

Experiments using another mixture of ecotropic retroviruses in C57BL/6 mice have indicated that genes to the left or within the H-2D region allow the increased expression of ecotropic retroviruses (Makino et al., 1990). Several other viruses were influenced by genes within the H-2D region. A resistance gene to Gross leukemia virus, Rgv-1, was found in the H-2D region (Lilly, 1970). Recovery from Friend virus leukemia has been shown to be strongly influenced by Rfv-1, an H-2D-associated gene (Chesebro, 1974). This region was also found to be associated with the development of a Friend virus-specific T lymphocyte response (Britt, 1983).

The interaction of the $H\text{-}2^{a}$ genomic region from F/St on the C57BL/10 background may also enhance the activity of certain genes in the B10 background that would cause an increase in retrovirus expression. Other investigators have also observed an increased expression of ecotropic retroviruses in other C57BL/10 or C57BL/6 H-2 congenic strains of mice; however, further experiments established that the presence of infectious retroviruses was unrelated to the H-2 region (Datta, 1978).

Isolation and restriction enzyme mapping of B10FV

In the process of infecting SC_1 cells with infectious ecotropic retroviruses for XC plaque assays, permanent cultures of ecotropic retroviruses were made in SC_1 cells. The SC_1 cells were passage several times to remove exogenous mouse cells, because most normal mouse spleen cells do not attach to plastic culture dishes nor do they survive for more than two weeks in culture without special care. After several passages

the infected SC_1 cells were used for Hirt supernatant production. The supernatants were analyzed for the presence of proviral sequences using DNA probes to the 5', 3' and env regions of ecotropic murine leukemia viruses.

Hirt supernatant DNA derived from infectious retrovirus produced from B10.F spleen cells was molecularly screened using a restriction enzyme, PstI, and probes to the 5' portion of Moloney MuLV (pMR1) and the ecotropic envelope region (env). B10FV had been previously described to have a PstI site at approximately 3.0 kb. (Here, and in the remainder of this chapter, viral DNA restriction sites will be referred to by their distance from the 5' end of the viral DNA, unless stated otherwise). The infectious retroviruses obtained from B10.F Hirt supernatant DNA preparations were primarily mink-cell focus forming (MCF) viruses because they did not hybridize with the ecotropic env probe but they did grow in SC_1 cells. The 5' MuLV probe, pMR1, would detect MCF viruses because they are similar to Moloney in their 5' portions or their LTR structures. B10.F has previously been described as having high titers of xenotropic, ecotropic and MCF viruses (Morse et al., 1985). Since the SC_1 cells are of mouse origin and do not have the receptors for xenotropic retroviruses, the additional viral species in B10.F Hirt supernatant DNA that did not hybridize with the ecotropic-specific probe must be MCF viruses.

The only ecotropic fragment detectable in every B10.F Hirt supernatant preparation was the 5.2 kb fragment that corresponds to that portion of B10FV 3' to the PstI site at 3.0 kb. Even in Hirt supernatants where this fragment was not detectable with pMR1, the 5.2 kb fragment was visualized following hybridization with env. This suggests that B10FV was the only infectious ecotropic retrovirus present in the spleen cells of #900 mouse.

The Hirt supernatant DNA produced from culture 513900 created a fortuitous situation; whereas almost all Hirt supernatant DNA derived from B10.F mouse spleen cells contained both 4.9 and 5.2 kb species when hybridized with pMR1, the Hirt supernatant DNA produced from the SC_1 cell line 513900 contained only B10FV. Very long exposures of pMR1-hybridized 513900 Hirt supernatant DNA did allow visualization of several minor MCF forms, but during exposure times used to map B10FV these species were never observed.

The restriction map of B10FV viral DNA created with the use of the DNA probes to the 5' half, 3' half and env regions of MuLV agreed well with the map of the integrated provirus of B10FV created by Sozer (1988). Some differences, however, were found in the exact placement of restriction enzyme sites in B10FV between this dissertation and that by Sozer. The locations given in this dissertation are probably more accurate because they are based on the use of multiple probes to different regions of the retrovirus and B10FV free viral DNA rather than the genomic DNA analyses of B10FV proviral DNA using only the env probe.

Figures 3.4 through 3.6 and Table 3.3 compare the restriction endonuclease sites of the unintegrated provirus, B10FV, with four ecotropic proviruses: B10.F(ash), an ecotropic provirus obtained from B10.F mice (Langdon et al., 1984); emv-2, the endogenous ecotropic provirus on chromosome 8 of C57BL mice (Kozak and Rowe, 1980); LP-BM5eco, a radiation-induced ecotropic retrovirus (Mosier et al., 1985; Chattopadhyay et al., 1989); and Akv, the endogenous ecotropic retrovirus from AKR mice (Chattopadhyay et al., 1981).

These four proviruses have similar restriction endonuclease sites in the 3' half of the provirus as compared to B10FV. HincII sites located in the 3' half of B10FV at

Figure 3.4 Comparison of restriction enzyme maps of B10FV and B10.F(ash) (Langdon et al., 1984).

▼ : Restriction enzyme sites that differ between B10FV and B10.F(ash).
Restriction enzyme site abbreviations: B, BamHI; BII, BglII; H, HincII; K, KpnI; M, SmaI; P, PstI; PI, PvuI; Pv, PvuII; SI, SstI; X, XbaI.

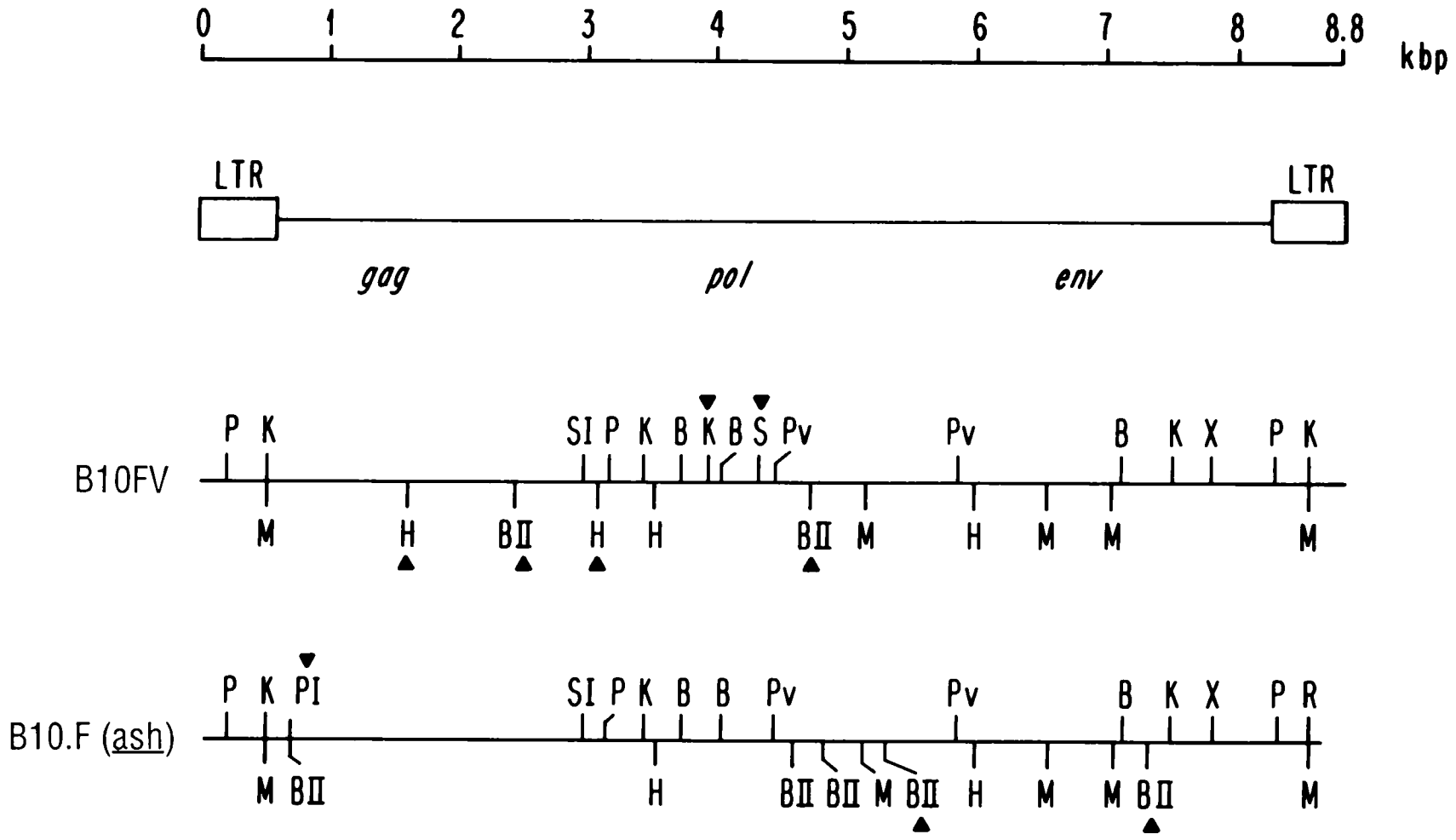

0 1 2 3 4 5 6 7 8 8.8
kbp
LTR
LTR
gag
pol
env
B10FV
P K SI P K B K B S Pv Pv B K X P K
M H BII H H BII M H M M M
B10.F (ash)
P K PI SI P K B B Pv Pv B K X P R
M BII H BII BII M BII H M M BII M

Figure 3.5 Comparison of restriction enzyme maps of B10FV and emv-2 (Kozak and Rowe, 1980; Sozer, 1989).

▼ : Restriction enzyme sites that differ between B10FV and emv-2.
Restriction enzyme site abbreviations: B, BamHI; HII, HindIII; P, PstI; Pv, PvuII; S, SalI; X, XbaI.

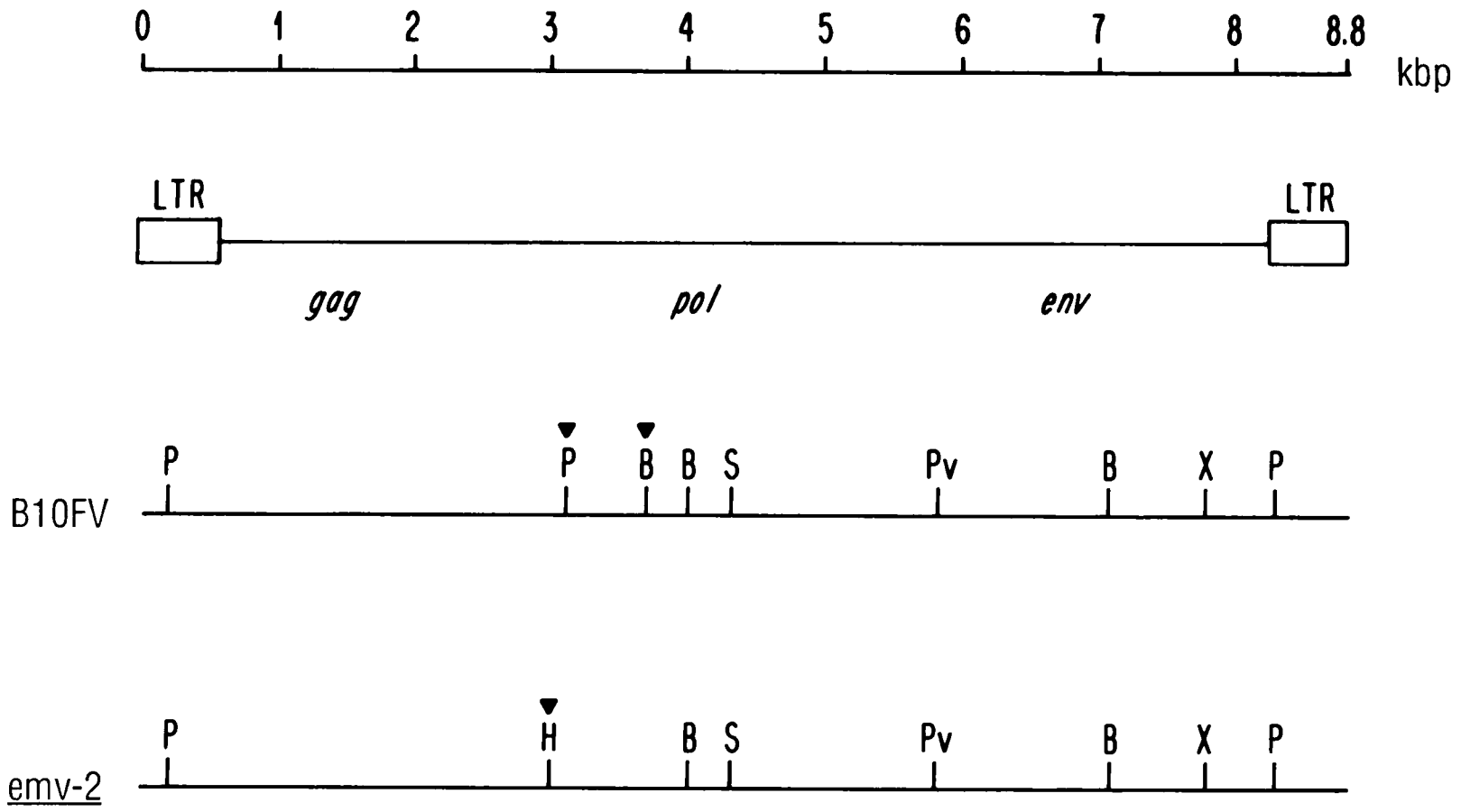

0
1
2
3
4
5
6
7
8
8.8
kbp
LTR
LTR
gag
pol
env
B10FV
P
P
B
B
S
Pv
B
X
P
emv-2
P
H
B
S
Pv
B
X
P

Figure 3.6 Comparison of restriction enzyme maps of B10FV, LP-BM5eco (Chattopadhyay et al., 1989) and Akv (Chattopadhyay et al., 1981).

▼ : Restriction enzyme sites that differ between B10FV, LP-BM5eco and Akv. Restriction enzyme site abbreviations: B, BamHI; C, ClaI; H, HincII; HIII, HindIII; K, KpnI; M, SmaI; O, XhoI; S, SalI; SI, SstI; SII, SstII; P, PstI; Pv, PvuII; PI, PvuI; X, XbaI.

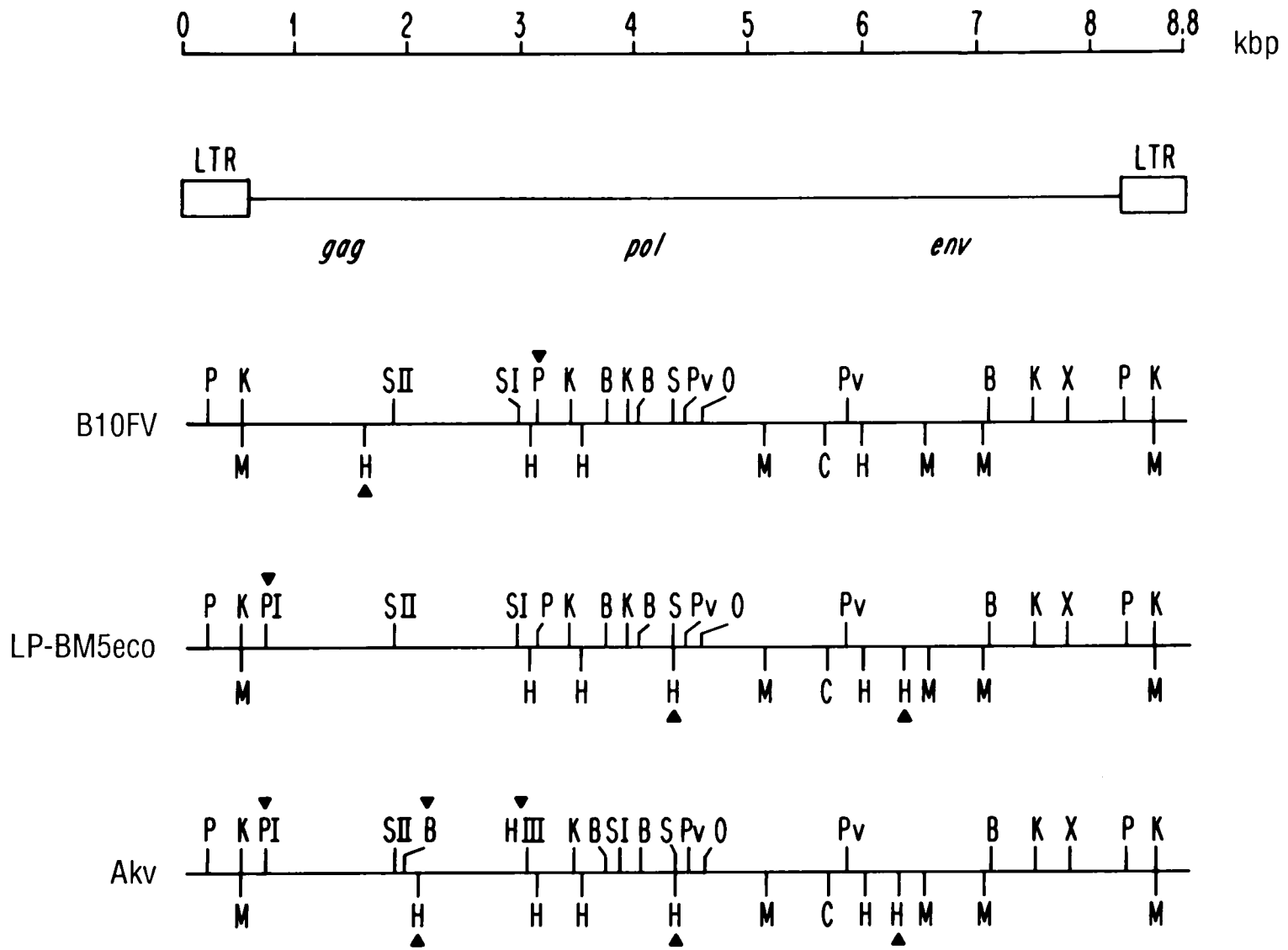

0 1 2 3 4 5 6 7 8 8.8 kbp
LTR
LTR
gag
pol
env
B10FV
P K SII SI P K B K B S Pv O Pv B K X P K
M H H H M C H M M M
LP-BM5eco
P K PI SII SI P K B K B S Pv O Pv B K X P K
M H H H M C H H M M M
Akv
P K PI SII B HIII K B SI B S Pv O Pv B K X P K
M H H H H M C H H M M M

TABLE 3.3

RESTRICTION FRAGMENT SIZES OF B10FV IN COMPARISON TO OTHER ECOTROPIC RETROVIRUSES

Restriction Enzyme	B10FV	B10.F(ash)	LP-BM5 eco	Akv	emv-2
PstI	3.0	3.0	3.0	8.2	(8.3)[a]
	5.2	5.2	5.2	0.5	
	0.5	0.5	0.5		
BamHI	3.4	3.4	3.4	1.4	(2.9)
	0.4	0.4	0.4	1.9	
	3.0	3.0	3.0	0.4	
	1.8	1.8	1.8	3.0	
				1.8	
PvuII	4.2	4.2	4.2	4.2	(3.0)[a]
	1.5	1.5	1.5	1.5	
	3.0	3.0	3.0	3.0	
SmaI	0.5	0.5	0.5	0.5	ND[b]
	4.7	4.7	4.7	4.7	
	1.4	1.4	1.4	1.4	
	0.4	0.4	0.4	0.4	
	1.7	1.7	1.7	1.7	
XbaI	7.7	7.7	7.7	7.7	(7.7)
	1.0	1.0	1.0	1.0	
SstI	2.9	2.9	2.9	3.7	ND
	5.8	5.8	5.8	5.0	

Restriction Enzyme	B10FV	B10.F(ash)	LP-BM5 eco	Akv	emv-2
HpaI	6.4	ND	6.4	3.1	ND
	2.3		2.3	3.3	
				2.3	
BstEII	1.2	ND	ND	0.7	ND
	3.6			3.5	
	2.4			2.9	
	1.5			1.2	
PvuI	*[c]	0.7	0.7	0.7	ND
		8.0	8.0	8.0	
HindIII	-[d]	-	-	5.8	(5.8)
				2.9	
KpnI	0.5	0.5	0.5	0.5	ND
	2.8	2.8	2.8	2.8	
	0.6	4.0	4.0	4.0	
	3.4	1.4	1.4	1.4	
	1.4				
SalI	4.2	-	4.2	4.2	(4.5)
	4.5		4.5	4.5	
SstII	1.7	ND	1.7	1.7	ND
	7.1		7.1	7.1	

Restriction Enzyme	B10FV	B10.F(ash)	LP-BM5 eco	Akv	emv-2
HincII	1.6	3.4	3.0	2.0	ND
	1.3	2.5	0.4	1.0	
	0.5	2.8	0.8	0.4	
	2.5		1.6	0.8	
	2.8		0.4	1.6	
			2.5	0.4	
				2.5	
BglII	2.3	0.7	ND	0.6	ND
	2.3	3.8		3.7	
	4.2	0.2		0.3	
		0.5		0.3	
		2.0		1.3	
		1.4		0.6	
				0.8	
				1.0	
ClaI	5.5	ND	5.5	5.5	ND
	3.2		3.2	3.2	

[a] (Size) - size detected with the env probe. Other fragments may exist but are not detectable with the env probe.

[b] ND - restriction sites not determined for this endonuclease.

[c] PvuI did not cut B10FV; see "Discussion" section.

[d] Restriction enzyme site not present in this provirus.

Sources: Chattopadhyay et al., 1981. Virology 113: 465-483.
Chattopadhyay et al., 1989. Proceedings of the National Academy of Sciences USA 86: 3862-3866.
Kozak and Rowe, 1980. In Animal Virus Genetics, Academic Press, New York, pp. 171-175.
Langdon et al., 1984. Virology 133: 183-190.
Sozer, 1989. Dissertation, University of Tennessee, Knoxville.

6.0 kb differ from those in LP-BM5eco and Akv at 5.7 kb. This restriction endonuclease produces small restriction fragments in B10FV (0.5 to 2.8 kb), and slight errors in measurement may produce the slight difference in placement of this HincII site in B10FV. Also, HincII and SalI cut at the same sequences in the DNA. The failure to identify a HincII site at the 4.2 kb SalI site is probably because a fragment was too small to detect easily on the Southern blots.

The 5' portion of B10FV contains some major restriction site differences when compared to Akv or emv-2. Both Akv and emv-2 have a HindIII site located at 3.0 kb; furthermore, Akv has additional BamHI and HincII restriction endonuclease sites near 2.0 kb that are not found in B10FV. Both Akv and emv-2 have been classed as N-tropic retroviruses (Liberman et al., 1978; Meruelo and Bach, 1983). The sequences between 2.0 and 3.0 kb contain the gag-pol junction region and have restriction endonuclease differences that are characteristic of N- and B-tropic retroviruses (Rassart et al., 1983). In addition to the N- and B-tropic differences, B10FV has an additional BamHI site at 3.7 kb that is also found in Akv and LP-BM5eco but not in emv-2. The restriction endonuclease mapping of emv-2 was done using genomic DNA from B10 mice digested with one or more restriction endonucleases and then hybridized with the env probe (Sozer, 1988). The env probe would not detect this additional BamHI site. The BamHI fragment consisting of the site located closer to the env region at 4.0 kb and the BamHI site 3' to the env region at 7.1 kb would be the only fragment detected in B10 genomic DNA by hybridization with env.

B10FV lacks the PvuI restriction endonuclease site at 0.7 kb that is found in B10.F(ash), LP-BM5eco and Akv; PvuI sites were not examined for emv-2 (Sozer, 1988). Several lots of PvuI were used in attempts to find this restriction site in B10FV,

and none of them produced altered linear B10FV fragments. In B10.F(ash), a BglII site was found at a site nearly indistinguishable from the PvuI site. No BglII site was found in this area in B10FV. These data suggest a change in the DNA of that area, which is located in the leader sequences of the gag region of B10FV, sufficient to alter these restriction sites in B10FV.

The two proviruses that B10FV resembles most, B10.F(ash) and LP-BM5eco, were both obtained from C57BL or C57BL-congenic mice. B10.F(ash) was molecularly cloned from the genomic DNA from the progeny of a single B10.F mouse found with a coat color mutation in a colony maintained by J. Stimpfling (Langdon et al., 1984). In addition to emv-2, F_2 progeny of the mutant animal possessed an additional germline provirus that was not linked to the coat color mutation. Restriction endonuclease mapping of the new provirus indicated that many restriction sites in B10.F(ash) were similar to sites in Akv.

B10FV resembles B10.F(ash), with some HincII and BglII restriction site polymorphisms; however, three major restriction site alterations occur in B10FV. The SalI site, not present in B10.F(ash) (Langdon et al., 1984), was found at 4.2 kb in B10FV, and additional BamHI and KpnI sites were found in B10FV at 2.4 and 3.9 kb, respectively. Xenotropic retroviruses that lack SalI and PvuI restriction sites have been described; the KpnI site at 3.9 kb has also been found in xenotropic MuLV (Chattopadhyay et al., 1981). Many xenotropic retroviruses from different mouse strains also contain PstI and PvuII sites at 3.0 and 4.4 kb, respectively.

LP-BM5eco was isolated from nonthymic lymphomas induced by ionizing radiation in C57BL/6 mice (Laterjet and Duplan, 1962; Haas and Meshorer, 1979; Haas and Reshef, 1980). Isolates from the nonthymic lymphomas contained xenotropic,

ecotropic and MCF MuLV. A mixture of the ecotropic retrovirus, LP-BM5eco, and a defective MCF-derived retrovirus (Chattopadhyay et al., 1989) causes an immunodeficiency syndrome characterized by lymphadenopathy, splenomegaly and eventual death of the infected animals from the massive enlargement of the lymphoid tissues (Pattengale et al., 1982). The restriction site map of B10FV is nearly identical to LP-BM5eco, with the exception of HincII site polymorphisms and the lack of a PvuI site in B10FV. B10FV and LP-BM5eco share the xenotropic retrovirus-associated restriction endonuclease sites that were discussed for B10.F(ash).

B10FV, B10.F(ash) and LP-BM5eco probably have a common origin based on the restriction site similarities among the three ecotropic retroviruses. The 5' portion of all three retroviruses share similarities with xenotropic and polytropic retroviruses, and all three were originated in C57BL (LP-BM5eco) or C57BL-congenic [B10.F(ash) and B10FV] mice. Xenotropic or polytropic proviral sequences contributed by F/St may have recombined with a defective endogenous ecotropic provirus from C57BL (emv-2) to produce a retrovirus with ecotropic-associated LTRs, the B-tropism determinant region from either a class of xenotropic retroviruses or a polytropic MuLV (Boone et al., 1988) which also contains the gag and pol coding regions, and an ecotropic env region from an endogenous ecotropic retrovirus.

Because B10 and B10.F mice are congenic, there is a limited region from which B10.F mice may carry additional xenotropic sequences with which ecotropic sequences can recombine. The H-2 region differs between B10 and B10.F mice, and retrovirus sequences have been found in the H-2 region. Several resistance factors associated with murine leukemia virus infections (Rmv1,2 and 3; Rfv-1,2 and Rrv-1) are found within the K, I and D regions, respectively, of the H-2 locus. The resistance to infections is

believed to be provided by H-2-linked retrovirus genomes that are expressed as cell-surface receptors, interfering with infection by viruses of similar type. A xenotropic regulation locus (Cxv-1) is also found near the H-2 locus. B10.F mice may have additional sequences in the congenic $H\text{-}2^a$ region that regulate retrovirus infection.

Conclusions

Infectious ecotropic retrovirus titers were measured from C57BL/10 (B10) and C57BL/10.F (B10.F) spleen cells. At two months of age, B10.F spleen cells produced high titers of ecotropic retroviruruses. The titer did not change through twelve months of age. B10 spleen cells did not produce measurable titers of ecotropic retroviruses.

Free viral DNA produced from B10.F spleen cell-infected SC_1 cells was used to analyze the types of retrovirus present in B10.F spleens. Using uncut and PstI-cut Hirt supernatant DNA and probes to the 5', 3' and env regions of ecotropic retroviruses, B10.F mice were found to have large amounts of MCF viruses in addition to a single ecotropic retrovirus, B10FV.

One infected SC_1 cell line, 513900, contained primarily B10FV with trace amounts of MCF virus. From this cell line, B10FV DNA was mapped for its restriction endonuclease sites. The restriction sites in the 3' region of B10FV were found to be similar to other ecotropic retroviruses. The 5' end, however, had restriction sites commonly found in some xenotropic retroviruses, especially in the leader and gag regions. Several restriction endonuclease site differences were found in B10FV when compared to a previously described B10.F provirus, B10.F(ash).

CHAPTER IV

THE PRODUCTION OF VIRUS-FREE BXF2NN H-2$^{n/n}$ MICE: CORRELATION OF H-2 GENOTYPE AND RETROVIRUS INFECTION WITH IMMUNE FUNCTION

Introduction

B10.F mice, congenic with B10 mice except at the H-2 region, express a milk-transmitted ecotropic retrovirus. B10.F mice differ histologically, hematologically and immunologically from B10 mice. All of these differences observed in B10.F mice may be caused by the ecotropic retrovirus infection present in all B10.F mice. However, B10.F mice also have a different H-2 region than B10 mice, so some of the differences found in B10.F mice may be caused by the different H-2 region.

To compare the effects of the retrovirus infection and H-2 region in B10.F mice, (B10 x B10.F)F_1 mice were raised and mated, and H-2$^{n/n}$ mice were selected from the F_2 progeny to establish BXF2NN mice, a retrovirus-free line of B10.F mice. These ecotropic retrovirus-free BXF2NN mice were injected as neonates with an known amount of B10FV obtained from infected SC_1 tissue culture cell supernatants. Using the uninjected, nonviremic BXF2NN mice as uninfected control mice, the effect of

ecotropic retrovirus infection in H-2$^{a/a}$ mice was examined.

This chapter describes: 1) the derivation of ecotropic retrovirus-free BXF2NN mice; 2) the ability of BXF2NN mice injected with B10FV to express ecotropic retroviruses; 3) the histology and 4) hematology of uninfected and infected BXF2NN mice; 5) the response of spleen cells from uninfected and infected BXF2NN mice to T- and B-cell mitogens; 6) the ability of spleen cells from uninfected and infected BXF2NN mice to form hemolytic plaques to SRBC in culture five days after primary immunization to SRBC in vivo; 7) the quantitation of splenic lymphocyte subsets in uninfected and infected BXF2NN mice using fluorescently labeled antibodies to lymphocyte cell-surface markers; and the restriction endonuclease analysis of 8) genomic DNA and 9) Hirt supernatant DNA originating from infected BXF2NN spleen cells.

Materials and Methods

Mice

BXF2NN mice were produced through (B10 x B10.F)F_1 hybrids mated to produce F_2 mice; the H-2$^{a/a}$ mice from the F_2 population were identified using hemagglutinating antibodies to MHC red blood cell antigens (described below).

Antibody production

Antibodies to H-2^a and H-2^b antigens were produced in (B10 x SWR)F_1 and (B10.F x SWR)F_1 mice, respectively. Spleen cells suspensions were prepared from

B10.F and B10 mice and injected i.p. into (B10 x SWR)F_1 and (B10.F x SWR)F_1, respectively, one time, again after thirty days, and then once every two weeks for three months. Sera from injected F_1 mice were tested for the ability to agglutinate RBC from B10 or B10.F mice using the hemagglutination assay described below. Mice whose sera agglutinated only B10 or B10.F RBC at significant titer were exsanguinated, the sera were collected from clotted blood, aliquoted into 4 ml tubes and stored at -20°C until use.

Hemagglutination assay

The H-2 serotypes of the (B10 x B10.F)F_2 animals were determined by the hemagglutination assay of Gorer and Mikulska (1954). Mice to be typed were bled (approximately 200 µl) from the orbital sinus into 2 ml of citrated saline (2% sodium citrate and 0.5% sodium chloride in water). The RBC were washed once by centrifugation at 600 g. The pelleted RBC were resuspended in physiological saline (0.85% sodium chloride) and washed two more times in saline at 600 g. After the third wash, the RBC pellets were resuspended in saline until the RBC mixture was the color of MEM medium; this color represented approximately a 2% RBC solution. The tube volumes were marked and the RBC were pelleted at 600 g. Human serum, previously absorbed on mouse RBC and heated to 56°C, was diluted 1:2 in saline and added to the RBC pellets to the premarked 2% RBC volume.

Precharacterized anti-H-2^b and anti-H-2^a sera were diluted 1:8 in 2% dextran in saline. The anti-H-2 sera were aliquoted into 96-well culture plate wells in 25 µl volumes with additional 1:2 dilutions of the original 1:8 mixture of 2% dextran/saline to a final dilution of 1:64 for each antiserum. A 25 µl dextran/saline control well without

anti-H-2 sera was also made. Twenty-five µl of each 2% RBC solution were added to each set of anti-H-2 serum and dextran/saline dilution wells. The plates were covered and incubated at 37°C for 90 minutes.

The results of the hemagglutinations were read by pulling RBC from each sample well gently up into a pipette, streaking each sample onto a 50x75 mm glass slide, and gently rocking the slide two times to disperse the RBC in the control sample. The amount of hemagglutination in the anti-H-2 samples was observed through a dissecting microscope and graded on a scale from 0 to +++, based on the amount of hemagglutination observed at each dilution of anti-H-2 serum.

B10FV isolation for injection into BXF2NN mice

B10FV was obtained from supernatants of culture 513900, a chronically infected SC_1 cell culture produced as described in Chapter III. Southern blotting showed that the injected culture supernatants contained primarily B10FV with very small amounts of an uncharacterized MCF virus detectable only after long exposures of blots hybridized with pMR1 but not detectable on blots hybridized with the ecotropic-specific env probe.

Five- to ten-day-old BXF2NN mice were injected i.p. with 0.5 ml 513900 tissue culture supernatant (approximately 1.6×10^3 PFU ecotropic virus) filtered through 0.4 µm Acrodisc filters (Gelman). Uninfected and infected BXF2NN mice were analyzed at six months of age in the experiments described below.

Histology, hematology, mitogenesis, plaque-forming cells to sheep red blood cells (SRBC-PFC) analyses and fluorescence-activated cell sorter (FACS) analyses were conducted as described in Chapter II.

Extraction of genomic DNA

Spleen cells from BXF2NN mice sacrificed for mitogenesis or SRBC-PFC assays were pelleted in capped 18 x 75 polystyrene tubes and frozen until use.

Pelleted spleen cells were thawed and resuspended in TES (10 mM Tris pH 7.4, 40 mM EDTA pH 8.0, and 50 mM NaCl), approximately 2 ml per 100 mg spleen pellet; larger volumes were used for larger pellets, in proportionate ratios to spleen size and TES volume. To the resuspended pellet, 100 µg/ml Proteinase K and 100 µg/ml RNase A were added. The spleen cell suspension was vortexed briefly to mix and 5% SDS was added to make a 0.5% SDS solution. Each spleen cell mixture was vortexed again until the solution became viscous. The tubes were tightly capped and placed in a 1000 rpm rotary shaker heated to 55°C. The samples were digested for a minimum of six hours and preferably overnight.

After overnight incubation, the viscous fluid was extracted with an equal volume of PCA (25 parts Tris-saturated phenol: 24 parts chloroform: 1 part isoamyl alcohol) two times, or until no interphase was visible between the PCA and aqueous phases. After the final extraction, the aqueous layer was placed in a 15 ml Corex tube and 2.5 volumes of cold absolute ethanol was added, mixed by inversion and placed at -20°C overnight to precipitate the DNA.

The DNA was pelleted at 25,000 g at -20°C for 45 minutes. The ethanol was discarded and the DNA pellet was washed once in 5 ml cold 70% ethanol. The ethanol

was poured off and the pellet was air dried. After drying the pellet was resuspended in 3 ml TE (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0).

For genomic DNA analyses, 10 µg DNA were digested in PstI or PvuII according to the methods of the manufacturer (BRL).

XC plaque assay, Hirt supernatant DNA analyses and restriction endonuclease digestion, Southern transfer and autoradiography were performed as described in Chapter III.

Results

H-2 typing of (B10 x B10.F)F_2 mice

Red cells from 41 F_2 mice from seven (B10 x B10.F)F_1 X F_1 matings were tested for hemagglutination with antibodies against H-2^b and H-2^a MHC antigens. From these tests, 10 (24%) H-$2^{b/b}$, 19 (47%) H-$2^{b/a}$ and 12 (29%) H-$2^{a/a}$ mice were identified. These F_2 H-$2^{a/a}$ mice were mated to produce the BXF2NN offspring for these studies.

XC plaque assay for (B10 x B10.F)F_1 mice and uninjected and injected BXF2NN mice

Three (B10 x B10.F)F_1 mice were tested with the XC plaque assay for the production of infectious ecotropic retroviruses (Table 4.1). None of the three mice had a measurable titer of ecotropic retroviruses.

Progeny from the original twelve H-$2^{a/a}$ (B10 x B10.F)F_2 animals were used as

TABLE 4.1

ECOTROPIC RETROVIRUS TITERS IN SIX-MONTH-OLD UNINJECTED AND INJECTED BXF2NN MICE

Strain	Number of Animals	Average $\log_{10}$ PFU/ 10^7 Spleen Cells
(B10 x B10.F)F_1	3	0
Uninjected BXF2NN	3	2.12
	41	0
Injected BXF2NN	49	3.35

B10FV-injected and uninjected control animals. Injected and uninjected BXF2NN mice were tested for infectious ecotropic retrovirus production from spleen cells (Table 4.1). Forty-four uninjected BXF2NN mice were analyzed; three of these mice had a low but measurable (2.12 $\log_{10}$ PFU/10^7 spleen cells) ecotropic retrovirus titer. The remaining forty-one mice were XC plaque negative for ecotropic retroviruses. Forty-nine B10FV-injected BXF2NN mice were analyzed, and all had high ecotropic retrovirus titers with an average titer of 3.35 $\log_{10}$ plaque-forming units (PFU) per 10^7 spleen cells.

Histology

Histological sections from twenty-eight uninjected controls and thirty B10FV-injected BXF2NN mice were examined, and the results are presented in Table 4.2 and Figures 4.1 and 4.2. More than half of the injected BXF2NN mice had increased hematopoietic activity in the splenic red pulp as compared to uninjected BXF2NN mice (Figure 4.1 a and b), the proliferating cells comprised of megakaryocytic, erythrocytic and granulocytic precursor cells. Two uninjected BXF2NN mice out of a total of twenty-eight also had increased hematopoiesis in the spleen. Follicular hyperplasia was observed in the majority (25/30) of injected BXF2NN mice (Figure 4.1c); increased follicular size with hyperplasia of all cell types, increased germinal center size and increased mitotic activity characterize injected BXF2NN splenic follicles. One uninjected BXF2NN mouse had increased follicular size in the spleen. Two injected mice had amyloid deposits in their spleens; no amyloid deposits were observed in uninjected BXF2NN mice. Plasmacytosis was also observed in the spleens of small numbers of both uninjected (2/28) and injected (1/30) BXF2NN mice.

Lymph nodes in injected BXF2NN mice had follicular hyperplasia and

TABLE 4.2

SUMMARY OF HISTOLOGY FOR UNINJECTED AND INJECTED BXF2NN MICE

Organ and Description	Number of Animals[a]	
	BXF2NN	Injected BXF2NN
SPLEEN		
Increased EMH[b]	2	17
Follicular hyperplasia	1	25
Plasmacytoid cell infiltration[c]	2	1
Amyloid	0	2
LYMPH NODE		
Follicular hyperplasia	0	21
Plasmacytoid cell infiltration	7	19
Amyloid	2	6
LIVER		
Hydropic change[d]	4	9
Increased EMH	1	1
Periarteriolar lymphoid cell aggregates	0	2
OTHER		
Lung - lymphocyte accumulation	0	1
Kidney - periarteriolar lymphocyte aggregates	0	2
Kidney - Glomerulosclerosis	0	1
Lymphoma/leukemia with nonlymphoid organ involvement	0	4

[a] Total number of animals: 28 uninjected BXF2NN, 30 injected BXF2NN

[b] EMH: Extramedullary hematopoiesis

[c] Plasmacytosis composed of immunoblastic B cells, plasma cells and Russell's bodies

[d] Hydropic change as characterized by centralized nuclei with clear, non-vacuolated, stringy cytoplasm.

Figure 4.1 Histological sections of spleens from uninjected and injected BXF2NN mice (H&E, 288X).

Fig. 4.1a The normal distribution of the myeloid precursor cells is shown, with a small amount of hematopoiesis. The splenic follicles of this uninfected BXF2NN mouse have small germinal centers and normal organization.

Fig. 4.1b The red pulp of the infected BXF2NN mouse shows expansion of the myeloid cell lineages, with large numbers of erythrocyte, megakaryocyte and granulocyte precursor cells.

Fig. 4.1c The follicles in spleens of injected BXF2NN mice are larger in size than in uninjected BXF2NN mice, and germinal centers are greatly expanded. In this section, a large group of plasmacytoid cells surrounds the splenic follicle.

RP - Red pulp
F - Splenic follicle
GC - Germinal center
P - Plasma cells
T - Trabeculum
G - Granulocyte precursor cells
M - Megakaryocyte
E - Erythrocyte precursor cells

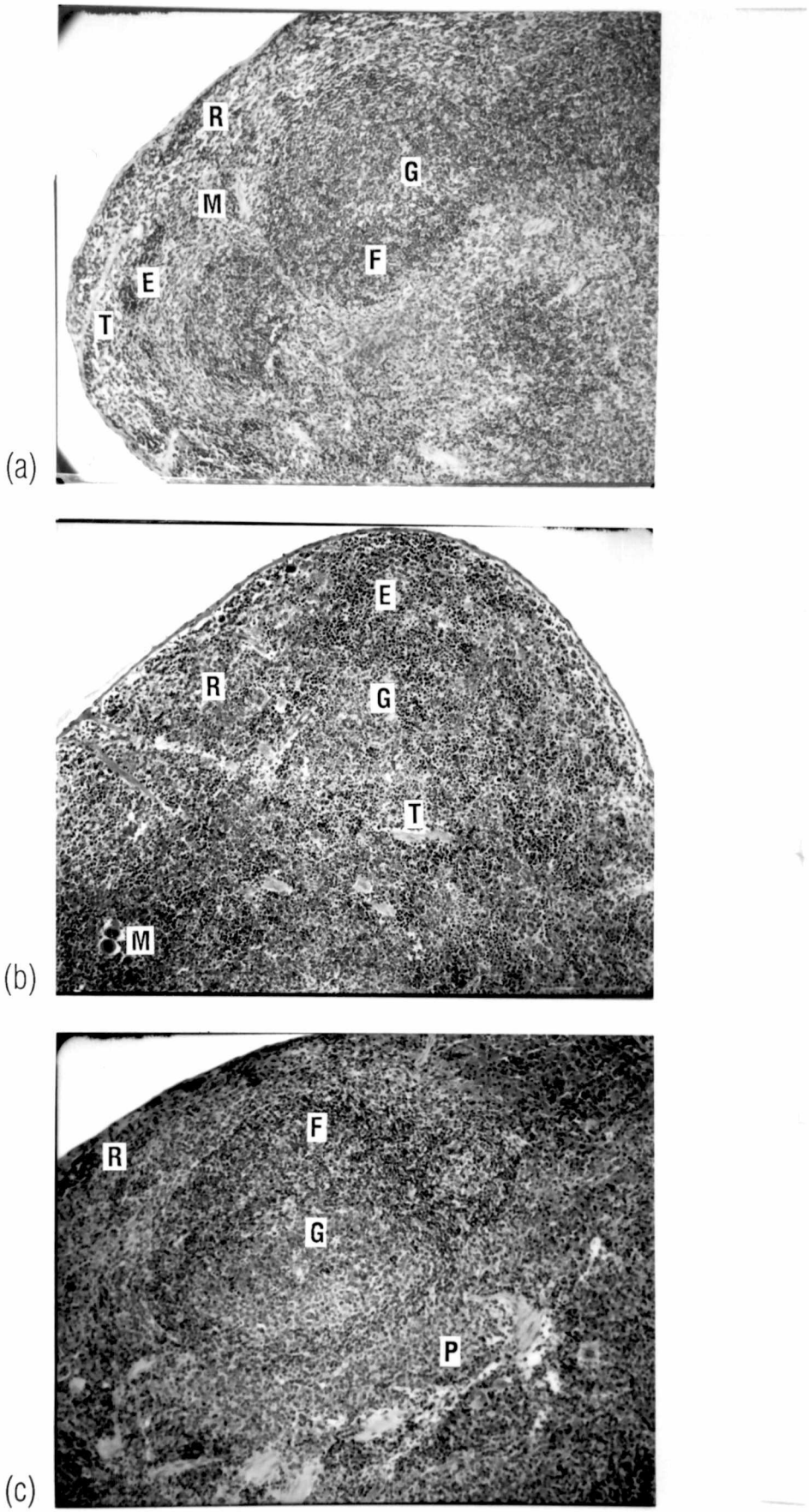
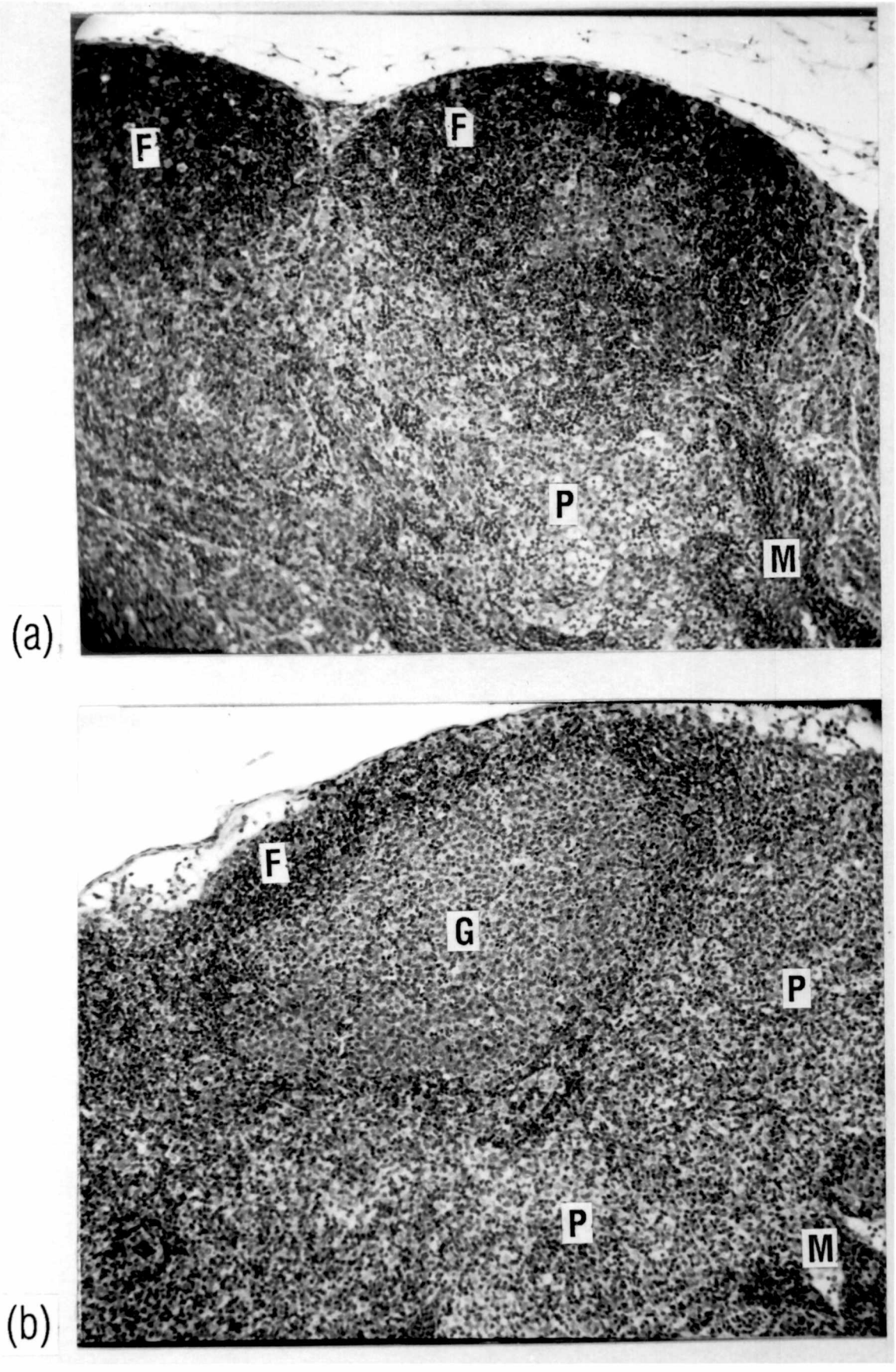

R
G
M
F
E
T
(a)
E
R
G
T
M
(b)
F
R
G
P
(c)

Figure 4.2 Histological sections of mesenteric lymph nodes from uninjected and injected BXF2NN mice (H&E, 288X)

Fig. 4.2a. Many small, dark lymphocytes surround the small germinal center in this section of mesenteric lymph node from an uninjected BXF2NN mouse. A large paracortical region surrounds the lymphoid follicle.

Fig. 4.2b. The germinal center is the primary feature of the mesenteric lymph node of an injected BXF2NN . The small, dark lymphocytes surrounding the germinal center in the lymphatic follicle are decreased in density. The paracortical region is larger than in mesenteric lymph nodes of uninjected BXF2NN mice. The medullary cords and sinuses are visible at the bottom right of the section.

F - Lymphoid follicle
GC - Germinal center
PC - Paracortical region
M - Medullary cords and sinuses


F
F
P
M
(a)
F
G
P
P
M
(b)

increased numbers of plasma cells in the paracortical regions of the lymphoid follicles (Figure 4.2b). The follicular hyperplasia in the lymph nodes was characterized by large numbers of B-immunoblast cells and large numbers of dividing cells. Some uninjected BXF2NN mice (7/28) also exhibited large numbers of plasma cells in the paracortical areas of the lymph node follicles. Small numbers of both uninjected (2/28) and injected (6/30) BXF2NN mice were observed with amyloidosis of the lymph nodes.

Non-lymphoid organs in uninjected and injected BXF2NN mice showed uniformity in their histological characteristics. One uninfected and one infected BXF2NN mouse had foci of extramedullary hematopoiesis in the liver. Hydropic changes in hepatocytes were also observed in both injected and uninjected BXF2NN mice. Twice as many injected (9/30) as uninjected (4/28) BXF2NN mice were observed with hydropic changes around hepatic portal areas.

Other changes, observed less often and only in the injected BXF2NN mice were lung infiltration of lymphocytes, periarteriolar lymphocyte aggregates in liver and kidneys, one animal with glomerulosclerosis and four animals with T-cell (2) or B-cell (2) lymphomas.

Hematology

Hematologic parameters from six-month-old uninjected and injected BXF2NN mice are presented in Table 4.3. Blood from uninjected and injected BXF2NN mice had similar RBC counts; however, hemoglobin concentration (HGB), mean corpuscular volume (MCV) and hematocrit (HCT) values were significantly lower in blood from injected BXF2NN mice.

Total WBC counts were not significantly different between uninjected and

TABLE 4.3

HEMATOLOGY OF SIX-MONTH-OLD UNINJECTED AND INJECTED BXF2NN MICE

Strain	WBC ($x10^6$/ml)	RBC ($x10^9$/ml)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg/RBC)	MCHC (g/dl)	LYM (%)	MON (%)	GRAN (%)
Uninjected BXF2NN (15)[a]	5.70 (0.37)[b]	10.63 (0.12)	15.14 (0.15)	52.02 (0.56)	49.00 (0.31)	14.26 (0.13)	29.13 (0.31)	80.56 (1.25)	3.65 (0.44)	12.81 (0.93)
Injected BXF2NN (20)	6.91 (0.68)	10.39 (0.12)	14.54 (0.14)	48.24 (0.57)	46.42 (0.39)	14.03 (0.19)	30.23 (0.46)	68.98 (3.05)	7.19 (1.43)	21.12 (2.20)
p value	>0.05	>0.05	<0.025	<0.001	<0.001	>0.05	>0.05	<0.005	<0.05	<0.005

[a] Number of animals

[b] Standard error of the mean

injected BXF2NN mice. The distribution of cells that comprise the peripheral blood WBC count, however, in injected BXF2NN mice was different. The percentage of lymphocytes was significantly decreased while the percentages of monocytes and granulocytes were significantly increased in the peripheral blood from injected BXF2NN mice.

Mitogenesis

The mitogenic responses of spleen cells from six-month-old uninjected and injected BXF2NN mice to the T-cell mitogens Con A and PHA, and the B-cell mitogen LPS are presented in Table 4.4. Unstimulated spleen cells from both uninjected and injected BXF2NN mice statistically similar background levels of ^{3}H-thymidine incorporation, although the ^{3}H-thymidine incorporation was much higher in the injected BXF2NN spleen cell. The proliferative responses of spleen cells from B10FV-infected BXF2NN mice to all three mitogens were significantly lower than proliferative responses of spleen cells from uninjected BXF2NN mice. Spleen cells from infected BXF2NN mice responded one-third as well as spleen cells from uninfected BXF2NN mice to the mitogens Con A and PHA. The response of spleen cells from infected BXF2NN mice to LPS was one-half the response of spleen cells from uninfected BXF2NN mice.

SRBC-PFC

The data for SRBC-PFC are presented in Tables 4.5 and 4.6. The data are separated into male and female groups because there was a significant difference in the in vitro PFC response of spleen cells from male and female mice five days after

TABLE 4.4

MITOGEN RESPONSE OF SPLEEN CELLS FROM SIX-MONTH-OLD UNINJECTED AND INJECTED BXF2NN MICE

Strain	^{3}H-thymidine uptake[a]	Mitogen Dose		
		Con A 1 µg/ml	PHA 2 µg/ml	LPS 25 µg/ml
Uninjected BXF2NN (13)[b]	3197 (417)[c]	36.70[d] (4.05)	25.40 (2.45)	16.97 (3.66)
Injected BXF2NN (24)	10278 (2642)	11.54 (2.16)	7.65 (1.25)	7.85 (1.32)
p value	>0.05	<0.001	<0.001	<0.025

[a] Average counts per minute in unstimulated culture

[b] Number of animals used

[c] Standard error of the mean (sem)

[d] Stimulation index (SI) as calculated in "Materials and Methods", Chapter II

TABLE 4.5

FIVE DAY SRBC-PFC RESPONSES OF SPLEEN CELLS FROM UNINJECTED AND INJECTED BXF2NN MALE MICE

Strain	PFC/Spleen	PFC/10^6 Spleen Cells
Uninjected BXF2NN (14)[a]	84551 (6618)[b]	424 (39)
Injected BXF2NN (12)	51628 (8816)	227 (51)
p value	<0.025	<0.025

[a] Number of animals

[b] Standard error of the mean

TABLE 4.6

FIVE DAY SRBC-PFC RESPONSES OF SPLEEN CELLS FROM UNINJECTED AND INJECTED BXF2NN FEMALE MICE

Strain	PFC/Spleen	PFC/10^6 Spleen Cells
Uninjected BXF2NN	129852	626
(15)[a]	(13477)[b]	(75)
Injected BXF2NN	81260	270
(13)	(15819)	(29)
p value	<0.05	<0.001

[a] Number of animals

[b] Standard error of the mean

primary immunization to injected SRBC (Terres et al., 1968).

Spleen cells from both male and female groups infected with B10FV exhibited significantly decreased abilities to form hemolytic plaques to SRBC. The spleens of infected male and female BXF2NN mice had two-thirds as many SRBC-PFC as spleens of uninfected BXF2NN mice. The number of SRBC-PFC cells per 10^6 spleen cells for both infected male and female BXF2NN mice was approximately one-half the number of SRBC-PFC per 10^6 cells present in the spleens of uninfected BXF2NN mice.

FACS analyses of uninfected and infected BXF2NN spleen cells

Fluorescence-activated cell sorter (FACS) analysis of spleen cells from six-month-old uninfected and B10FV-infected BXF2NN mice indicated a decreased frequency of $Thy1^+$ cells and their subsets in the spleens of B10FV-infected BXF2NN mice (Table 4.7). The percentages of $Thy1^+$ (23.83±2.20 vs. 15.68±0.74) and $Lyt1^+$ spleen cells (23.49±1.38 vs. 15.99±1.09) were significantly higher in uninfected BXF2NN mice than in infected mice, respectively. The percentages of the $L3T4^+$ ($CD4^+$) and $Lyt2.2^+$ ($CD8^+$) T-cell subsets were also altered in infected BXF2NN mice. The frequency of $CD4^+$ T cells in the spleens of uninfected BXF2NN mice (17.42±0.97) was significantly higher than in infected BXF2NN mice (10.84±0.69). Infected BXF2NN mice had a significantly lower frequency of $CD8^+$ spleen cells (4.03±0.41) than uninfected mice (5.10±0.27). The CD4/CD8 ratio was significantly lower in infected BXF2NN mice because of the much lower percentage of $CD4^+$ cells in spleens of infected mice. $B220^+$ B cells were found at equivalent frequencies in spleens of uninfected and infected BXF2NN mice. Doubly positive Ly-1 B cells ($Lyt1^+$ and $B220^+$), while present at higher levels than the average for most mouse strains

TABLE 4.7

FACS ANALYSIS OF SPLEEN CELLS FROM SIX-MONTH-OLD UNINJECTED AND INJECTED BXF2NN MICE

Strain	Monoclonal Antibodies						
	Thy1	B220	Lyt1	Ly-1 B	L3T4 (CD4)	Lyt 2.2 (CD8)	CD4/ CD8
Uninjected BXF2NN (6)[a]	23.83 (2.20)[b]	56.90 (3.02)	23.49 (1.38)	9.35 (3.37)	17.42 (0.97)	5.10 (0.27)	3.42 (0.11)
Injected BXF2NN (6)	15.68 (0.74)	51.78 (4.12)	15.99 (1.09)	11.47 (2.02)	10.84 (0.69)	4.03 (0.41)	2.76 (0.23)
p value	<0.01	>0.05	<0.005	>0.05	<0.005	<0.01	<0.05

[a] Number of animals

[b] Standard error of the mean

(Hayakawa et al., 1983), were equivalent in both uninfected and infected mice.

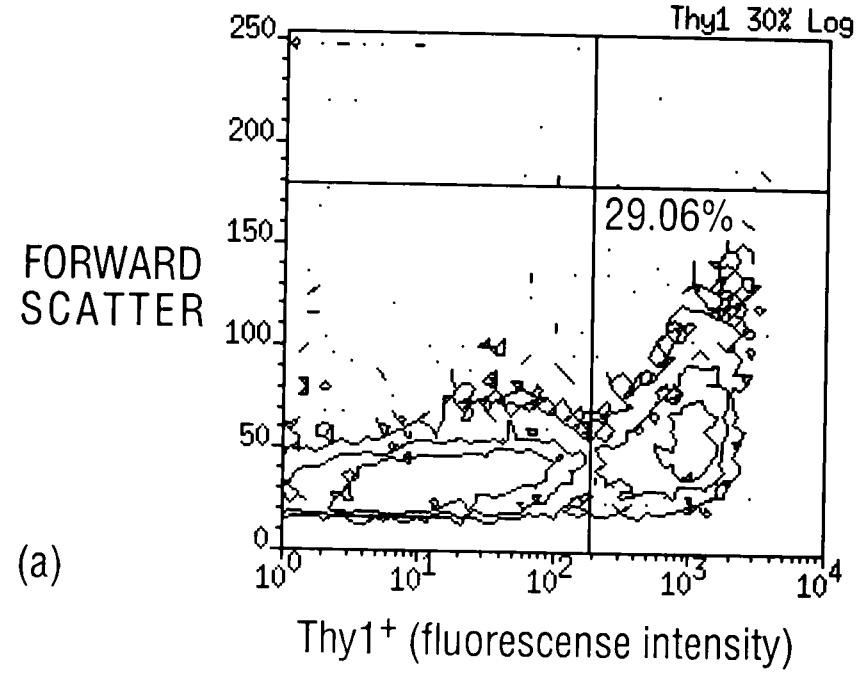
Figures 4.3 through 4.5 illustrate lymphocyte subset determination by the flow cytometer for representative animals using spleen cells from uninfected and infected BXF2NN mice labeled with antibodies to Thy1, Lyt1, B220, L3T4 (CD4) and Lyt2.2 (CD8). The figures are three-dimensional contour plots, with the *x* axis of each plot representing the increasing FITC green fluorescence intensity in logs scale, and the *y* axis representing either increasing cell size (Thy1) or increasing PE red fluorescence intensity (B220 and L3T4) in log scale. In Figure 4.3, $Thy1^+$ cells are shown. Spleen cells from an uninfected BXF2NN had 29.06% $Thy1^+$ cells, in comparison to only 10.61% $Thy1^+$ cells in the spleens of infected BXF2NN mice.

The frequency of $Thy1^+$ spleen cells for an uninjected and an injected BXF2NN and shown in Figure 4.3. The uninjected BXF2NN mouse in (a) had 29.06% $Thy1^+$ cells in its spleen, while the injected BXF2NN mouse had only 10.61% $Thy1^+$ spleen cells (Figure 4.3b)

Spleen cells that bind both anti-Lyt1 and anti-B220 are shown in the contour plot in Figure 4.4 a and b. $Lyt1^+$ spleen cell FITC fluorescence intensity follows the *x* axis, and $B220^+$ spleen cell PE fluorescence intensity is located on the *y* axis. Both the uninfected and the infected BXF2NN mice had similar percentages of $B220^+$ $Lyt1^-$cells (quadrant 1: 57.64% vs 61.43%, respectively). $Lyt1^+$ $B220^-$ spleen cells (Quadrant 4) were more numerous in uninfected BXF2NN spleens (25.85%) than in infected BXF2NN spleens (15.77%). Spleen cells that bind both the Lyt1 and B220 monoclonal antibodies, the Ly-1 B cells, are located in quadrant 2. In this example, the infected BXF2NN mouse had approximately one-third more Ly-1 B spleen cells (10.62%) than the uninfected BXF2NN mouse (7.45%).

Figure 4.3 Contour plots of fluorescence-activated cell sorter (FACS) analyses of spleen cells from uninjected and injected BXF2NN mice stained with FITC anti-Thy1 monoclonal antibody.

a. Spleen cells from an uninjected BXF2NN mouse
b. Spleen cells from an injected BXF2NN mouse


Thy1 30% Log
250
200
150
100
50
0
29.06%
FORWARD SCATTER
(a)
10^0
10^1
10^2
10^3
10^4
Thy1+ (fluorescense intensity)

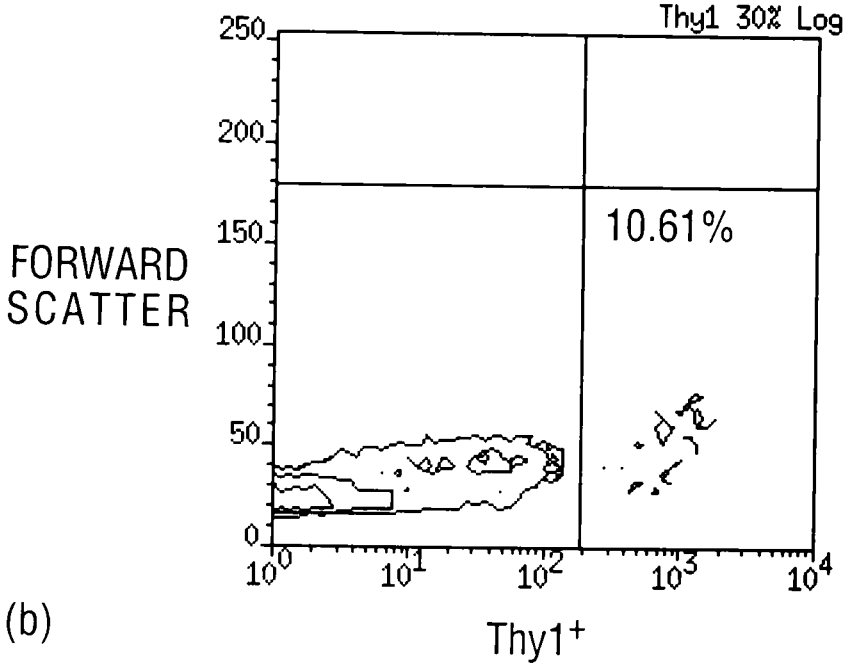
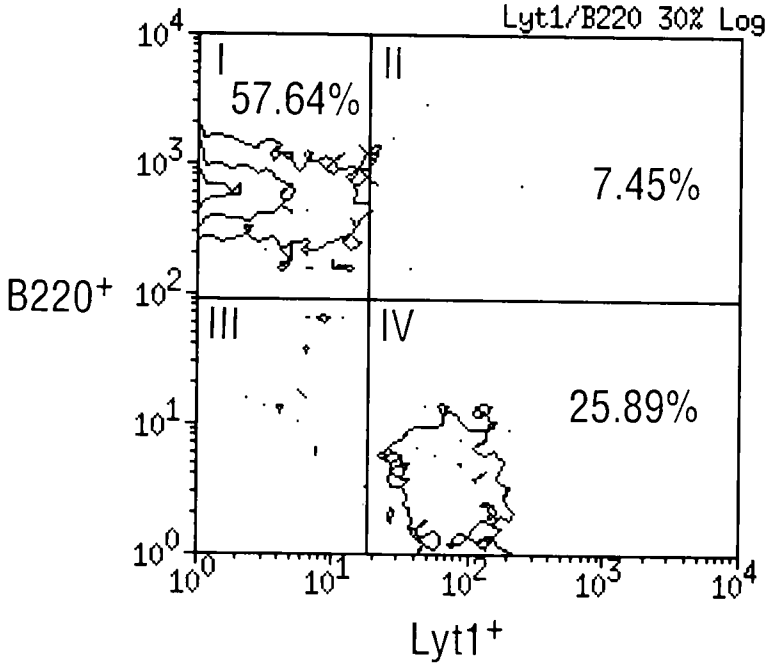

Thy1 30% Log
250
200
150
100
50
0
10.61%
FORWARD SCATTER
(b)
10^0
10^1
10^2
10^3
10^4
Thy1+

Figure 4.4 Contour plots of FACS analyses of spleen cells from uninjected and injected BXF2NN mice stained with FITC anti-Lyt1 and PE anti-B220 monoclonal antibodies.

a. Spleen cells from an uninjected BXF2NN mouse
b. Spleen cells from an injected BXF2NN mouse


Lyt1/B220 30% Log
I
57.64%
II
7.45%
III
IV
25.89%
B220+
Lyt1+

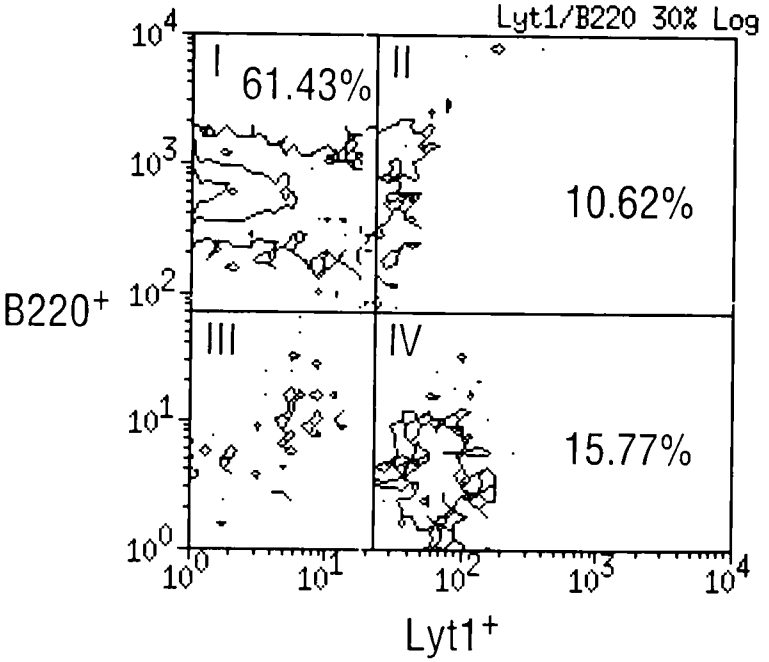
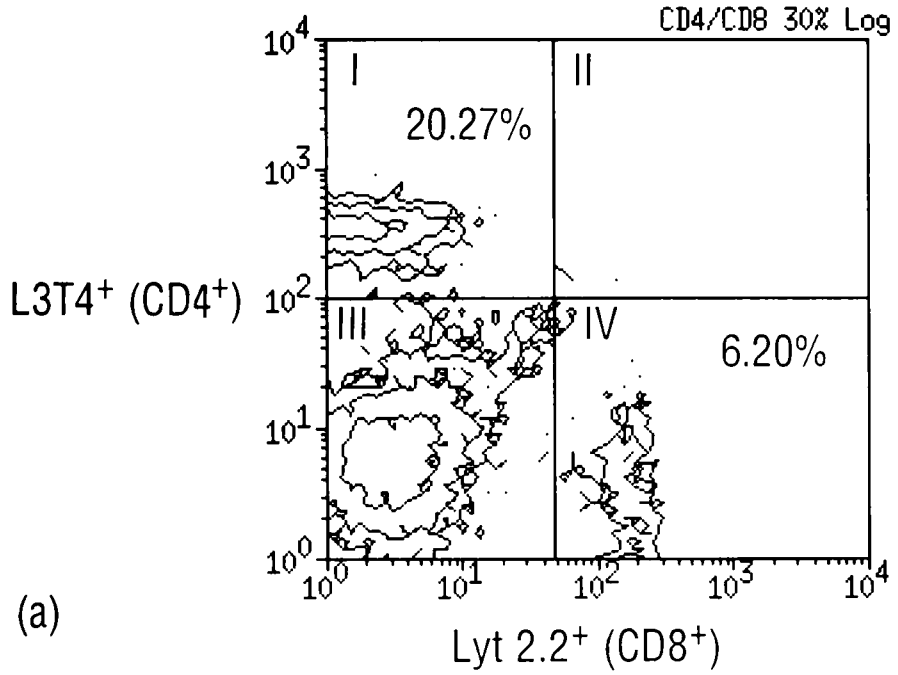
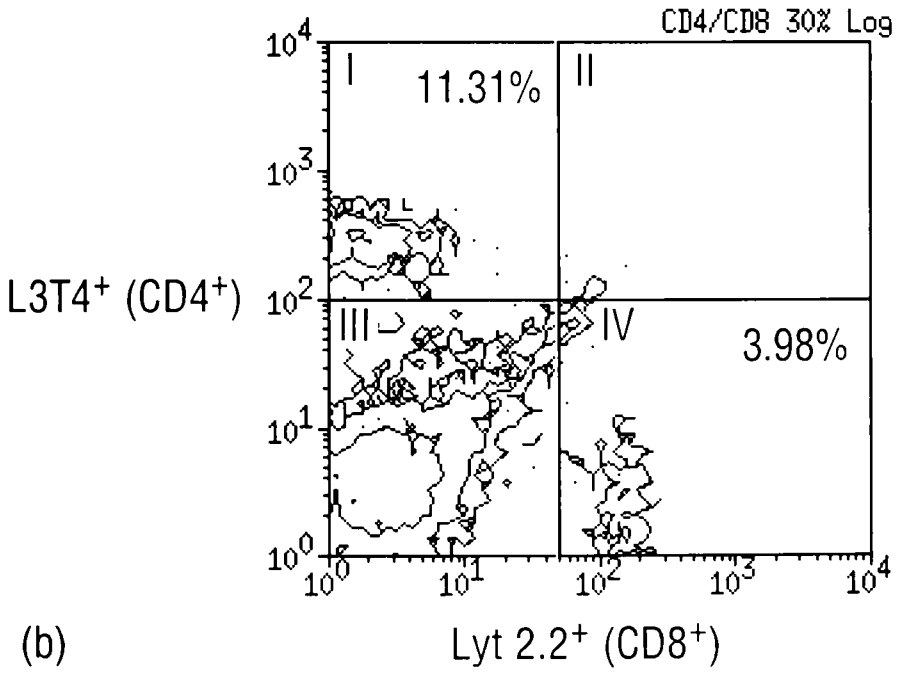

Lyt1/B220 30% Log
I
61.43%
II
10.62%
III
IV
15.77%
B220+
Lyt1+

Figure 4.5 Contour plots of FACS analyses of spleen cells from uninjected and injected BXF2NN mice stained with PE anti-L3T4 (CD4) and FITC anti-Lyt2 (CD8) monoclonal antibodies.

a. Spleen cells from an uninjected BXF2NN mouse
b. Spleen cells from an injected BXF2NN mouse


CD4/CD8 30% Log
I
II
20.27%
III
IV
6.20%
L3T4+ (CD4+)
Lyt 2.2+ (CD8+)

(a)


CD4/CD8 30% Log
I
11.31%
II
III
IV
3.98%
L3T4+ (CD4+)
Lyt 2.2+ (CD8+)

(b)

The percentages of spleen cells that bind antibodies to CD4 and CD8 antigens are shown in Figure 4.5 a and b. Quadrant 1 contains the $CD4^+$ $CD8^-$ spleen cells, while quadrant 4 contains the $CD8^+$ $CD4^-$ spleen cells. The uninfected BXF2NN mouse had nearly twice as many $CD4^+$ $CD8^-$ spleen cells (20.27%) as the infected BXF2NN mouse (11.31%). The infected BXF2NN mouse had two-thirds as many $CD8^+$ $CD4^-$ spleen cells (3.98%) as the uninfected BXF2NN mouse (6.20%).

Genomic DNA analyses of uninfected and infected BXF2NN mice

The genomic DNA from spleen cells of uninfected and infected BXF2NN mice was digested with the restriction endonucleases PvuII and PstI and Southern blots were hybridized with the ecotropic-specific env probe (Table 4.8). When emv-2, the endogenous ecotropic provirus common to C57BL strains, was digested with PvuII, the env probe recognized a 5.2 kilobase (kb) provirus-mouse DNA junction fragment formed by a PvuII site in the 3' portion of emv-2 extending into the mouse genomic DNA to a PvuII site 3 kb away. PstI cuts internally in emv-2 at the 5' and 3' LTRs, producing an 8.6 kb fragment that hybridized with the env probe.

When hybridized with the env probe, uninfected BXF2NN splenic DNA digested with either PvuII or PstI revealed only the emv-2-related ecotropic provirus fragments. B10FV-infected BXF2NN splenic DNA contained the expected emv-2-associated PstI and PvuII fragments, but an additional fragment was detected following digestion with PstI. When digested with PstI, a 5.2 kb fragment was detectable in infected BXF2NN splenic DNA. One BXF2NN animal that received an injection of B10FV did not show the B10FV-associated 5.2 kb PstI fragment, and it also had a very low ecotropic virus titer. Digestion of B10FV-infected BXF2NN splenic DNA with

TABLE 4.8

RESTRICTION ENZYME ANALYSIS OF SPLENIC DNA FROM UNINFECTED AND INFECTED BXF2NN MICE

Uninfected BXF2NN		Infected BXF2NN	
PstI	PvuII	PstI	PvuII
8.6[a] (10/10)[b]	5.2 (10/10)	8.6, 5.2 (21/22)	5.2 (18/22)
		unknown[c] (1/22)	5.2, 3.9 (1/22)
			5.2, 3.0 (1/22)
			12, 6.7, 5.2, 3.6, 3.4 (1/22)
			unknown[c] (1/22)

[a] Size, in kilobases, of ecotropic provirus fragment

[b] Number of animals with the indicated size of fragment/total number of animals

[c] No emv-2 ecotropic fragment was visible due to poor restriction endonuclease digestion

PvuII formed only the emv-2-related 5.2 kb fragment in most mice. Splenic DNA from two mice out of the twenty-two mice examined had one additional PvuII fragment, 3.9 or 3.0 kb in size, that was recognized by the env ecotropic probe, and splenic DNA from one mouse had four ecotropic-specific fragments of DNA in addition to the emv-2-related 5.2 kb PvuII fragment.

Hirt supernatant DNA analyses

The spleen cells from ten uninfected and twenty-seven infected BXF2NN mice were used to infect SC_1 cells for Hirt supernatant analyses (Table 4.9). All Southern blots of Hirt supernatant DNA preparations obtained from uninjected BXF2NN mice and uncut by restriction endonucleases contained an 8.8 kb virus species that hybridized with the 5' Moloney MuLV probe, pMR1. When the Hirt supernatant DNAs derived from the uninfected BXF2NN mice were digested by PstI, all contained three fragments, with sizes of 8.8, 4.9 and 3.0 kb. The same hybridization membranes containing these Hirt supernatant DNAs were rehybridized with the ecotropic-specific env probe; no ecotropic-specific fragments were visualized in any of the uninfected BXF2NN mouse Hirt supernatant DNA samples.

Hirt supernatant DNAs obtained from SC_1 cells infected with spleen cells from twenty-six infected BXF2NN mice were also analyzed on Southern blots following hybridization with the pMR1 and env probes. When uncut Hirt supernatant DNA derived from infected BXF2NN mice was hybridized with pMR1, an 8.8 kb virus species was present in most Hirt supernatants, and an additional 7.6 kb virus was present in four supernatants. Only the 8.8 kb species hybridized with the ecotropic-specific env probe. When the Hirt supernatant DNAs were digested by PstI, numerous

TABLE 4.9

ANALYSIS OF HIRT SUPERNATANT DNA FROM UNINJECTED AND INJECTED BXF2NN MICE

Spleen cell origin[a]	Treatment	Major Linear Species[b] (kb)	No.[c]	ENV-Specific Species[d] (kb)
Uninfected BXF2NN (10)	Uncut	8.8	9	-
		ND[e]	1	
	PstI	8.6, 4.9, 3.0	10	-
Infected BXF2NN (26)	Uncut	8.8	21	8.8
		8.8, 7.6	4	8.8
		ND[e]	1	-
	PstI	8.6, 5.2, 4.9, 3.0	10	5.2 (9) 8.6, 5.2 (1)
		8.6, 8.5, 5.2, 4.9, 3.0	3	5.2
		8.6, 5.2, 3.0	3	5.2
		5.2, 4.9, 3.0	3	5.2
		8.8, 8.6, 4.9, 3.0	2	5.2[f]
		8.6, 8.5, 5.2, 4.9, 4.2, 3.0	2	5.2
		8.8, 8.6, 4.9, 3.0	2	5.2[f]
		8.6, 6.5, 5.2, 4.9, 3.7, 3.0	1	8.6, 5.2
		8.8, 7.6	1	5.2[f]
		8.6, 5.2, 4.9, 4.5, 3.0	1	5.2
		8.6, 6.1, 5.2, 3.0	1	8.6, 5.2

[a] Spleen cells used to infect SC_1 cells for Hirt supernatant production

[b] Size of the major linear species, in kilobases, visualized following hybridization with the pMR1 probe

[c] Number of animals

[d] Size, in kilobases, of the linear species visualized following hybridization with the ecotropic env probe

[e] ND: Not determinable in that sample

[f] The 5.2 kb species was not visualized in the pMR1-probed sample but was visualized with the env probe

fragments were created but several fragments were common to most samples. Most PstI-digested samples contained the 5.2 kb (24/26), 8.6 kb (23/26), and/or the 4.9 kb (22/26) fragments that hybridized with pMR1. When the membranes containing these Hirt supernatant samples were rehybridized the with env probe, the only ecotropic fragment visualized in most samples was the 5.2 kb species, even in Hirt supernatant DNA samples where the 5.2 kb fragment was not initially detected by hybridization with pMR1. In four samples, in addition to the 5.2 kb ecotropic fragment, an 8.6 kb fragment was also detected by hybridization with the env probe.

Discussion

Production of ecotropic virus-free BXF2NN mice

The (B10 x B10.F)F_1 was chosen for the production of (B10 x B10.F)F_2 H-$2^{a/a}$ animals because the B10.F male does not appear to transmit retroviruses to its offspring unless the retrovirus is integrated into the germline. The B10 female does not possess large amounts of infectious ecotropic retroviruses, and, therefore, does not transmit large amounts of retroviruses through the milk (Popp, 1986). With the breeding scheme described, the H-$2^{a/a}$ F_2 offspring would not be exposed to maternal retroviruses and would remain ecotropic retrovirus-free until they receive injected viruses. Three (B10 x B10.F)F_1 animals were tested for the presence of infectious ecotropic retroviruses; none produced measurable titers of ecotropic retroviruses.

According to the Mendelian laws of segregation, intercrossing of (B10 x B10.F)F_1 mice to produce an F_2 population should yield approximately 25% H-$2^{b/b}$,

50% H-2$^{b/a}$ and 25% H-2$^{a/a}$ animals. The numbers of F_2 progeny found for each class (10:19:12) agree well with the expected numbers.

The spleens from (B10 x B10.F)F_2 H-2$^{a/a}$ (BXF2NN) mice were tested for infectious ecotropic retrovirus production. Out of 44 mice tested, three BXF2NN mice tested positive for infectious ecotropic retrovirus production. These positive animals were housed in a cage with three B10FV-injected BXF2NN mice. These mice may have become infected through some type of horizontal transmission of B10FV and were not included in any experimental results. The remaining forty-one uninfected BXF2NN mice had no detectable ecotropic retroviruses produced from their spleen cells.

Levels of ecotropic retroviruses in injected BXF2NN mice

Since the (B10 x B10.F)F_2 matings produced ecotropic virus-free mice, ecotropic virus-free H-2$^{a/a}$ mice were now available to study how B10FV affects the infected animals. B10FV obtained from infected SC_1 cell cultures as a cell-free culture supernatant was injected i.p. into five- to ten-day-old BXF2NN mice. The XC plaque assay was used to measure the amount of B10FV in the culture supernatant; approximately 1.6 x 10^3 PFU ecotropic virus was injected into each neonate. In addition to the ecotropic retrovirus, very small amounts of MCF virus may have been injected as part of the B10FV mixture. All BXF2NN mice that received an injection of B10FV expressed high levels of ecotropic retrovirus. When sacrificed at six months of age for experimental purposes, nearly all of the infected BXF2NN mice had greying fur, in contrast with the uninjected BXF2NN mice, whose fur remained black. This finding does not coincide with the findings of Jaenisch (1980), who found that C57BL embryos microinjected with Moloney MuLV at 8.5 to 9.5 days gestation, but not later,

developed grey fur. He concluded that Moloney MuLV infected the melanocyte precursor cells during this time of gestation. In the present study, nonviremic BXF2NN mice were infected with B10FV five to ten days after birth and were nursed on ecotropic virus-free mothers. Grey fur that developed in the injected BXF2NN mice resulted from infection of the melanocytes by B10FV after birth. Morse et al. (1985) and Popp (1986) also found that C57BL/10 mice foster nursed on viremic mothers later became grey with age. These studies suggest that either the melanoblast or the mature melanocyte could become infected with B10FV. Morse et al. (1985) used electron microscopy to show that C-type retrovirus particles were present in hair bulbs taken from areas containing grey fur.

Histology

Histological sections of tissues from uninfected and infected BXF2NN mice were compared to assess the effects of ecotropic retrovirus infection on the tissue organization of H-2$^{n/n}$ mice. The histological changes found in infected BXF2NN mice were located primarily in the lymphoid organs. In the spleen, increased numbers of myeloid precursor cells in the red pulp and follicular hyperplasia were observed in the majority of infected BXF2NN mice. The lymph nodes of infected BXF2NN mice had more follicular hyperplasia, often with plasmacytosis, than observed in uninfected BXF2NN mice. The histology of B10FV-infected BXF2NN mice suggests that the retrovirus infection results in increased hematopoiesis observed in the spleen and the follicular hyperplasia observed in the lymph nodes and spleens of these mice.

Moloney MuLV (MoMuLV) injected into neonatal mice induces a preleukemic state characterized by generalized hematopoietic hyperplasia in the spleen (Brightman

et al., 1990). The mechanism of the myeloid and erythroid progenitor cell hyperplasia induced by MoMuLV could include either the direct infection of hematopoietic stem cells and the resulting mitogenic effect of MoMuLV viral proteins, or the infection of a nonhematopoietic cell that indirectly leads to hyperplasia due to the increased secretion of hematopoietic cytokines. The infectivity of a variant of MoMuLV that is equally infective to animals but has reduced leukemogenicity (Davis et al., 1985) was compared to wild-type MoMuLV in spleen cell myeloid progenitor cell cultures from infected animals. Both the variant and the wild-type MoMuLV infected the splenic myeloid progenitor cells at high levels. Although the variant and the wild-type MoMuLV could infect hematopoietic progenitors in the spleen, only the wild-type MoMuLV could induce hematopoietic hyperplasia. This suggested that the variant virus was incapable of infecting the cell that generated the preleukemic hyperplasia.

Atypical histology observed in nonlymphoid tissue was observed in a small number of both uninfected and infected BXF2NN mice, e.g. the hydropic changes observed in the hepatocytes, occasional foci of extramedullary hematopoiesis in the liver and periarteriolar aggregates of lymphocytes in the liver, kidney and lung. These histological changes may be H-2$^{n/n}$-associated histologic features or effects of the C57BL background rather than changes caused by the effects of retrovirus infection.

Hematology

The hematology in infected BXF2NN mice showed altered RBC and WBC indices. The RBC counts were not different between uninfected and infected BXF2NN mice; however, infected BXF2NN mice had significantly lower HCT, MCV and HGB concentrations. The reduced HCT, HGB and MCV indicate that BXF2NN mice have a

chronic anemia characterized by a decreased RBC size and lower HGB concentrations per RBC. Blood films from infected BXF2NN mice show a higher incidence than normal of polychromatophilic RBC, indicating that there may be an increased production of RBC to compensate for a reduced RBC life span.

Anemias with decreased HCT, HGB and MCV values with elevated polychromasia are often observed in chronic inflammatory disorders, including many diseases such as AIDS, autoimmune disorders and malignant diseases. Chronic inflammation is associated with hypoferremia, probably as a result of decreased serum ferritin and increased iron retention in reticuloendothelial cells (Wintrobe et al., 1976). It is known that HGB concentrations are correlated with iron availability (Weinberg, 1984). Cytokines may also be involved with the development of inflammatory disease anemias (Torti et al., 1988). Tumor necrosis-α (TNF-α) may be involved in mediating the hypoferremia in anemias of chronic inflammation because TNF-α can function as an inducing agent for the synthesis of the ferritin heavy chain. Other cytokines, such as interferon-γ (INF-γ), while stimulating the immune response, can also inhibit bone marrow hematopoiesis (Broxmeyer et al., 1986).

The anemia observed in infected BXF2NN mice may be caused by aberrant regulation of bone marrow hematopoiesis. When bone marrow cannot produce the sufficient numbers of cells for the blood, extramedullary hematopoiesis can occur in peripheral organs such as the spleen, liver and lymph nodes (Adler et al., 1977). In injected BXF2NN mice, the mild chronic anemia associated with the retrovirus infection could cause the observed splenic hematopoietic hyperplasia. Bone marrow defects have also been observed in mice infected by other retroviruses. Moloney MuLV (MoMuLV) infected mice, while exhibiting splenic hematopoietic hyperplasia, are

defective in bone marrow hematopoiesis in vitro (Li and Fan, 1990). It has been shown that Moloney MCF viruses (MoMCF), generated by the recombination of the injected MoMuLV with endogenous retroviral sequences, cause defective bone marrow hematopoiesis and splenic hyperplasia. Neither the MoMuLV nor the MoMCF viruses alone cause the defective hematopoiesis. The env genes of MoMuLV and MoMCF have been implicated in the effect on the bone marrow, and it was suggested that the receptors on the cell surface that interact with retrovirus env proteins in the process of infection may be cellular growth factor receptors. If MoMuLV and MoMCF simultaneously infect a hematopoietic cell, the blockage of the growth factor signals could cause the inability of that cell to grow and proliferate.

Infected BXF2NN mice also had perturbed WBC population frequencies, with a lower frequency of lymphocytes and higher frequencies of monocytes and granulocytes in the peripheral blood. Decreased lymphocyte frequencies and increased monocyte and granulocyte populations may be caused by an increase in colony stimulating factors (CSFs) secreted by macrophages during inflammatory processes (Metcalf, 1989). INF-γ enhances the secretion of CSFs, which induce the proliferation of monocyte and granulocyte precursors (Herrman et al., 1986). Lymphocytes, in turn, may be suppressed in infected BXF2NN and B10.F mice due to increased INF-γ and TNF-α levels (Balkwill, 1989).

Mitogens, SRBC-PFC and FACS analyses

The responses of spleen cells from uninfected and infected BXF2NN mice to T- and B-cell mitogens were compared to assess the impact of retrovirus infection on the ability of spleen cells to respond to mitogenic stimuli. When compared to uninfected

BXF2NN mice, spleen cells from B10FV-infected BXF2NN mice had lower responses to both T-cell mitogens, Con A and PHA, and the B-cell mitogen, LPS. Con A and PHA mitogen responses of spleen cells from infected BXF2NN mice responded only one-third as well as spleen cells from uninfected BXF2NN mice. LPS responses by spleen cells from infected BXF2NN mice were only half as great as the LPS mitogen responses obtained with spleen cells from uninfected BXF2NN mice. These data show that the lower response of spleen cells from infected BXF2NN H-$2^{n/n}$ and B10.F mice to T- and B-cell mitogens were caused by the effects of ecotropic retrovirus infections in these mice.

The presence of ecotropic retrovirus infections and the lower mitogen responses in infected BXF2NN mice make it attractive to postulate that the retrovirus infection influences all the mitogen responses similarly. To test this, Spearman ranking coefficients were calculated for the levels of ecotropic retrovirus and the stimulation indices to each mitogen in individual infected BXF2NN mice. A high correlation was not found between the titers of ecotropic retrovirus and either Con A (0.292) or PHA (0.261) stimulation indices; correlation values greater than 0.425 would have meant that the two groups of values were related. A slightly higher but not significant correlation between ecotropic virus titers and LPS responses (0.414) was observed in infected mice. The lack of correlation of retrovirus titer and mitogen stimulation indices may reflect a saturation of the infected animals with retrovirus. Any correlation of the mitogen responses with virus titer might be better observed in the early phases of the retrovirus infection.

High correlation was observed between the Con A and PHA stimulation indices (0.936) in infected BXF2NN mice. Whatever factor was influencing the suppression of

mitogenesis acted on both the Con A and PHA responses in a similar fashion. This suggests that a factor or cell type common to both Con A- and PHA-reactive spleen cells was affected by the retrovirus infection.

Many retroviruses or their protein products have been shown to inhibit mitogenesis. As discussed in Chapter II, the retroviral protein p15E from feline leukemia virus can inhibit Con A mitogen responses. In humans, both the whole virion and the large envelope glycoprotein (gp120) from HIV-1 can suppress the PHA mitogen response of human lymphocytes and lymphocytes from other species (Wainberg et al., 1985; Mann et al., 1987). The large glycoprotein also has been demonstrated to bind to CD4 molecules, and the binding of gp120 with CD4 on a cell's surface may render that cell less able to respond to the mitogen PHA.

Mouse retroviruses have also been shown to lower mitogenic responses of murine lymphocytes. Mosier et al. (1985) described that in LP-BM5-infected B6 mice, the spleen cells were unable to respond to the mitogens Con A, PHA and LPS as soon as four weeks after infection. Unlike in FeLV infection, no evidence was found to suggest that the viruses in the LP-BM5 mixture were directly immunosuppressive, so p15E-related immunosuppression was not likely to be involved.

The ability of primed spleen cells to form hemolytic plaques to SRBC was lower in infected BXF2NN mice than in uninfected BXF2NN mice. Both male and female infected BXF2NN mice were affected, and each group had two-thirds as many hemolytic antibody-producing cells per spleen as uninfected BXF2NN mice. When calculated per 10^6 spleen cells, infected BXF2NN mice of both sexes had half as many cells producing hemolytic antibodies to SRBC as uninfected BXF2NN mice. This discrepancy between the number of SRBC-PFC per spleen and per 10^6 spleen cells

indicated that the spleens of infected BXF2NN mice are more cellular than the spleens of uninfected BXF2NN mice, reducing the number of SRBC-PFC per 10^6 cells. Cell counts from uninfected and infected BXF2NN mice were made to show that indeed infected BXF2NN mice have a larger number of cells per spleen than uninfected BXF2NN mice. The larger cell counts in infected BXF2NN mice can be accounted for by the generalized splenic hyperplasia observed in B10FV-infected BXF2NN mice.

In addition to the reduced mitogen responses, primed spleen cells from mice infected with LP-BM5 were unresponsive to SRBC (Mosier et al., 1985). Those few cells that were able to respond to SRBC appeared to have arisen from the polyclonal B-cell activation found in these mice. Similar numbers of SRBC-PFC were found in the spleens of LP-BM5-infected mice whether or not the mice had been immunized to SRBC.

FACS analyses of spleen cells from uninfected and infected BXF2NN mice show that T-lymphocyte subsets were perturbed in infected BXF2NN mice. The percentages of $CD4^+$ and $CD8^+$ T-cell subsets were lower in B10FV-infected BXF2NN mice. Splenic B-cell percentages, however, remained unaffected in infected mice.

Mosier et al. (1985) reported some consistent changes in cell populations in B6 mice infected with LP-BM5 and, also, some variability in cell populations between individual infected mice. The $Thy1^+$ cell populations varied considerably in frequency among animals, and the majority of infected mice had slightly lower percentages of $Thy1^+$ cells in their spleens than control mice. The T-cell subsets Ly-4^+ ($CD4^+$) and Ly-2^+ ($CD8^+$) were decreased in LP-BM5 infected mice. The Ly-2^+ T-cell subset showed the greater decrease, with a 50% reduction in the frequency of $CD8^+$ cells (Morse et al., 1989). While the Ly-2^+ T-cell subset was lower in the spleens of the injected

BXF2NN mice, experiments by Morse et al. (1989) suggest that, although the Ly-2$^+$ (CD8$^+$) T cell subset was reduced in frequency in infected mice, there may be no functional defect in the remaining Ly-2$^+$ cells. Only the Ly-4$^+$ (CD4$^+$) lymphocyte subset population changed during the early disease process in the infected mice, having a loss of both antigenic phenotype and a loss of MHC self-restricted CD4$^+$ T-cell function.

B cells in LP-BM5-infected mice showed altered antigenic phenotypes and functions. Infected mice had 50% fewer B220$^+$ B cells in their spleens than uninfected control mice. B cells in infected mice had been shown to be critical for the development of the CD4$^+$ T-cell abnormalities (Cerny et al., 1990); however, CD4$^+$ cells were also needed for the induction of abnormalities in B cells, CD8$^+$ cells and macrophages, probably through the action or loss of action of some cytokine (Yetter et al., 1988). It has been suggested that B cells in animals infected with the LP-BM5 virus mixture present to CD4$^+$ T cells a self-like antigen that may be encoded by the Pr60^{gag} protein from the defective virus (Cerny et al., 1990; Rassart and Jolicouer, 1990). Such a protein was found associated with infected B cells, and it exerted a profound proliferative effect on CD4$^+$ T cells (Hügin et al., 1991). The protein was virally encoded because antibodies against the MuLV p30^{gag} antigen could inhibit the proliferative response. The virus-encoded superantigen may elicit lymphokines that create a generalized stimulation of T and B cells. This may deplete the number of cells available to respond to infecting viruses or tumors, leading to a progression of virus-induced disease.

Both uninfected and infected BXF2NN mice had higher frequencies of Ly-1 B cells in their spleens as compared to the frequencies observed in many other mouse

strains (Hayakawa et al., 1983). In the BXF2NN mice, higher frequencies of Ly-1 B cells did not appear to be associated with high ecotropic virus titers. The incidence of these cells may be influenced by some element contained within the H-2^{a} region or on the C57BL background.

Genomic and Hirt supernatant DNA analyses

Comparisons of genomic DNA analyses from the spleens of uninfected and infected BXF2NN mice show that the ecotropic B10FV retrovirus infected the H-2$^{a/a}$ mice and integrated into random sites in the somatic cell DNA. As found with B10.F mice (Sozer, 1989), all infected BXF2NN mice had the B10FV-associated PstI ecotropic fragment. PstI-digested splenic DNA from all infected BXF2NN mice carried the 8.2 kb emv-2-related fragment and also a 5.2 kb B10FV ecotropic fragment. The intensity of hybridization of this fragment on Southern blots may not accurately reflect the extent of B10FV proviral insertion into DNA from B10FV-infected mice because the genomic DNA extraction method used for these analyses also extracts free viral DNA from infected cells.

Digestion of infected BXF2NN splenic DNA with PvuII generated only the 5.2 kb emv-2-related ecotropic fragment in all uninfected and most infected BXF2NN mice. The appearance of an additional PvuII fragment would mean that the integrated provirus had become part of a clonally expanding cell line or had integrated into a stem cell and was carried by the progeny of that cell. Four of twenty-two infected animals were found with additional PvuII restriction fragments (Table 4.8).

The restriction fragment analyses of viral DNA from Hirt supernatants suggests that both uninfected and infected BXF2NN mice had several infectious retrovirus

species in common based on fragment size; similar fragment sizes were also found in B10 and B10.F Hirt DNA preparations. Most of these retroviruses in the Hirt supernatant DNAs from either uninfected and infected mice, however, did not hybridize with the ecotropic-specific env probe. A common ecotropic retrovirus identifiable by a characteristic 5.2 kb PstI restriction fragment was found in the Hirt supernatant DNA from B10FV-infected BXF2NN mice. In three of the twenty-six infected BXF2NN Hirt supernatant preparations, an 8.6 kb ecotropic virus species was also found in addition to the 5.2 kb fragment in PstI digests. The 8.6 kb species was either a mutated B10FV without the internal PstI site or B10FV partially cut with PstI; the enzyme may cut preferentially at the LTR PstI sites and less efficiently at the internal PstI site.

As described in both the histology and in the immunology discussions, B10FV-injected BXF2NN mice and other similarly retrovirus-infected mice can be described to have polyclonal immune cell activation. Krieg et al. (1988) have shown that polyclonal immune activators, such as injected LPS, can induce multiple MCF transcripts from spleen cells. Mice injected with LPS had MCF transcripts detectable from spleen cells six hours after injection, and the MCF transcripts remained elevated for eleven days. Four specific-sized, MCF-related transcripts were induced during the early period of LPS stimulation (Krieg et al., 1988).

C57BL/6 mice injected with the LP-BM5 retrovirus mixture develop massive B-cell proliferation in the early stages of the disease with large numbers of immunoglobulin-secreting cells (Mosier et al., 1985; Klinman and Morse, 1989). This immune cell activation is similar to the activation that occurs with LPS injection in normal mice. The hyperplasia observed in the spleens and lymph nodes of B10FV-

injected BXF2NN mice resembles the polyclonal B-cell activation described in LP-BM5-injected B6 mice. The presumed MCF viral species that were not detectable with the env probe in the Hirt supernatant DNAs prepared from infected BXF2NN mice could result from the immune cell activation caused by the retrovirus infection.

Conclusions

C57BL/10-congenic BXF2NN H-2$^{n/a}$ mice were identified by serotyping (B10 x B10.F)F_2 mice and mated to produce ecotropic retrovirus-free mice genetically identical to B10.F mice. After neonatal BXF2NN mice received an injection of tissue culture supernatant containing B10FV, the infected BXF2NN mice developed histologic, hematologic and immunologic perturbations similar to those observed in B10.F mice. Increased numbers of myeloid cells in the red pulp and follicular hyperplasia of the white pulp were found in spleens of infected BXF2NN mice. Lymph nodes of infected mice also showed hyperplasia of the follicles and plasmacytosis in the paracortical regions of the lymphoid follicles. Four infected BXF2NN mice had lymphomas; no uninfected BXF2NN mice were observed with lymphomas.

Uninfected and infected BXF2NN mice had similar numbers of peripheral blood WBC and RBC. In infected BXF2NN mice, however, the HGB, HCT and MCV values were lower, so the infected mice had a mild chronic anemia associated with the retrovirus infection. In the peripheral blood, the populations of the WBC were changed; blood from infected BXF2NN mice, like B10.F mice, had increased numbers of monocytes and granulocytes with lower numbers of lymphocytes.

In spleen cells from uninfected and infected BXF2NN mice, the immunologic responses and immune cell subset populations were altered. Cultured spleen cells from infected BXF2NN mice gave significantly lower responses to both T- and B-cell mitogens, and infected mice primed with SRBC had lower numbers of spleen cells able to produce hemolytic plaques to SRBC. There was a lower incidence of T cells in the spleens of infected BXF2NN mice; both $CD4^+$ and $CD8^+$ T-cell subsets were affected. Although $B220^+$ B-cell frequencies were unaltered by retrovirus infection, B-cell functions were altered. Either another B cell population which responds to LPS was reduced in frequency, or the B cells may not be responding to the cytokines that cause the B-cell proliferation in LPS responses. High ecotropic retroviruses titers did not correlate with mitogen responses by spleen cells of infected BXF2NN mice, possibly as a result of saturation of the infected animals' immune system with virus. Splenic Ly-1 B-cell frequencies were similar in uninfected and infected BXF2NN mice, but the frequencies are higher than found in most mouse strains.

DNA analyses showed that B10FV-infected BXF2NN mice, like B10.F mice, had B10FV integrated randomly in their spleen cell DNA. Numerous retroviruses were present in both uninfected and infected BXF2NN mice, but the dominant ecotropic retrovirus found in BXF2NN mice was the B10FV introduced via injection of infected SC_1 cell tissue culture supernatants into the neonatal BXF2NN mice.

CHAPTER V

CONCLUSIONS, SIGNIFICANCE AND RECOMMENDATIONS

C57BL/10 (B10) and C57BL10.F (B10.F) mice are congenic strains differing at the H-2 locus. B10.F mice have the $H\text{-}2^a$ haplotype originating from their maternal strain, F/St, while B10 mice carry the $H\text{-}2^b$ haplotype. B10 and B10.F mice not only differ at H-2, but B10.F mice also express high levels of ecotropic retroviruses from their spleen cells. Initially, it was observed that B10.F mice had histological changes in their lymphoid organs and had lower serum IgA levels than B10 mice. It was unknown whether the differences observed between B10 and B10.F mice were caused by the different H-2 or the retrovirus infection in B10.F mice. To control for the H-2 region differences, a group of (B10 x B10.F)F_1 mice were produced and intercrossed to produce F_2 animals. Mice homozygous for the $H\text{-}2^{a/a}$ haplotype were selected from the F_2 population and tested for the presence of ecotropic retroviruses. The $H\text{-}2^{a/a}$ mice, called BXF2NN mice, were found to be free of infectious ecotropic retroviruses. A small coloney of BXF2NN mice were raised and some of them were injected with tissue culture fluids containing B10FV, the ecotropic retrovirus found in B10.F mice. These injected BXF2NN mice were compared histologically, hematologically and immunologically to the uninjected BXF2NN mice to assess the impact of the retrovirus infection on $H\text{-}2^{a/a}$ mice.

Using unintegrated viral DNA obtained from SC_1 cell Hirt supernatant lysates, the types of retroviruses expressed in B10.F spleen cells were examined. The Hirt supernatant DNAs were digested with PstI, Southern blotted and detected by hybridization with pMR1, which is a probe to the 5' portion of Moloney MuLV (MoMuLV). Numerous retrovirus PstI fragments from B10.F spleen cell-infected SC_1 cells hybridized with pMR1. Most of these fragments did not hybridize with the ecotropic-specific env probe; therefore, the majority of these PstI fragments were derived from MCF viruses. Only a single ecotropic viral form was found in the B10.F-derived Hirt supernatant DNAs. The single 5.2 kb PstI fragment corresponded to the B10FV PstI fragment described by Sozer (1989).

One Hirt supernatant DNA, from SC_1 cell culture 513900, contained primarily B10FV with low levels of another non-ecotropic retrovirus. Viral DNA from this B10.F spleen cell-infected SC_1 cell culture was used to map restriction endonuclease sites for the B10FV virus. B10FV most resembled the B-tropic retroviruses B10.F(ash) and LP-BM5eco in 5' restriction endonuclease sites; the 3' regions of both N- and B-tropic ecotropic retroviruses showed great similarities in restriction endonuclease sites. While having many restriction sites in common with B10.F(ash), a provirus obtained from a coat-color mutant B10.F mouse (Langdon et al., 1984), B10FV had altered KpnI, HincII and BglII sites. B10FV had a SalI site at 4.2 kb not present in B10.F(ash), but B10FV lacked the PvuI site at 0.7 kb commonly found in other ecotropic retroviruses. This restriction enzyme site is sometimes missing from some xenotropic retroviruses (Chattopadhyay et al., 1984) and polytropic retroviruses (Boone et al., 1988), and this suggested that the 5' portion of ecotropic B10FV containing the B-tropism determinants and the gag and pol sequences may have had a nonecotropic origin.

Tissue culture supernatants from culture 513900 were used to produce B10FV for

injection into BXF2NN neonates. The pathobiology of injected BXF2NN mice was similar to that of B10.F mice. Six-month-old BXF2NN mice, when injected as neonates with 1600 PFU of ecotropic B10FV retrovirus, had grey fur and expressed high titers of ecotropic retrovirus from their spleen cells. Infectious B10FV viral DNA was found in every injected BXF2NN mouse, and no infectious ecotropic retrovirus was found in uninjected BXF2NN mice housed in pans separate from B10FV-injected BXF2NN mice.

Histologically, the lymphoid tissues of injected BXF2NN mice showed many of the same proliferative changes in the lymphoid follicles of the spleen and lymph node as observed in B10.F mice. Spleens and lymph nodes from B10FV-infected mice had hyperplasia of all cell types. Some other histological changes were found in nonlymphoid tissues in both uninfected and infected BXF2NN mice and in B10.F mice. In particular, hydropic change in the liver of the H-$2^{a/a}$ mice suggested that this may be an H-2-associated feature, or be influenced by some C57BL-related background genes.

In two separate groups of experiments, both B10 and uninjected BXF2NN mice were used as control animals. Prior to experiments for this dissertation, only B10 mice had been used to provide control materials in experiments using B10.F mice. While B10 and B10.F mice have different H-2 haplotypes they still possess the same genetic background (C57BL); however, B10.F have and B10 mice are free of infectious ecotropic retroviruses. Because B10.F mice not only expresses ecotropic retroviruses but also have a different H-2 haplotype, experimental results demonstrating significant differences between B10 and B10.F mice could not be attributable to the effect of only the retrovirus infection. Some H-2-related gene(s) might also cause some difference in B10 or B10.F parameters.

To assess the possible influence of MHC genes on the data, the results obtained from B10 and virus-free BXF2NN mice were compared (Table 5.1). Most of the

TABLE 5.1

STATISTICAL ANALYSES OF B10 AND UNINJECTED BXF2NN DATA

Parameter	B10	Uninjected BXF2NN	*p* value
HEMATOLOGY			
WBC ($x10^6$/ml)	7.14±0.31	5.70±0.37	<0.025
RBC ($x10^9$/ml)	10.17±0.13	10.63±0.12	>0.05
HGB (g/dl)	14.84±0.29	15.14±0.15	>0.05
HCT (%)	49.04±1.35	52.02±0.56	<0.05
MCV (fl)	48.17±0.99	49.00±0.31	>0.05
MCH (pg/RBC)	14.55±1.08	14.26±0.31	>0.05
MCHC (g/dl)	30.45±0.79	29.13±0.31	>0.05
LYM (%)	78.98±2.58	80.56±1.25	>0.05
MON (%)	3.69±0.72	3.65±0.44	>0.05
GRAN (%)	13.26±2.19	12.81±0.93	>0.05
MITOGENS			
Unstimulated (cpm)	1704±95	3197±417	<0.025
Con A (SI)[a]	37.80±4.99	36.70±4.05	>0.05
PHA (SI)	30.22±3.86	25.40±2.45	>0.05
LPS (SI)	12.55±1.21	16.97±3.66	>0.05
SRBC-PFC			
SRBC-PFC/spleen	129950±17072	84551±6618	<0.025
SRBC-PFC/10^6 cells	588±45	424±39	>0.05
FLOW CYTOMETRY			
Thy1 (%)	21.62±1.77	23.83±2.20	>0.05
B220 (%)	63.92±0.93	56.90±3.02	>0.05
Lyt1 (%)	18.80±1.02	23.49±1.38	<0.025
Ly-1 B (%)	4.88±0.41	9.35±3.37	>0.05
L3T4 (CD4) (%)	11.37±1.15	17.42±0.97	<0.005
Lyt2 (CD8) (%)	8.20±0.74	5.10±0.27	<0.005
CD4/CD8	1.39±0.06	3.42±0.11	<0.001

[a] SI: stimulation index, as described in Chapter II.

hematology results from B10 and BXF2NN mice were statistically equivalent. Only the total WBC counts and the HCT were significantly changed from B10 results. BXF2NN mice had significantly fewer WBC ($5.70x10^6$) per milliliter than B10 mice ($7.14x10^6$). The HCT in BXF2NN mice was significantly greater (52.02%) than in B10 mice (49.04%), indicating either a larger number of RBC per unit volume or larger RBC size in BXF2NN mice. B10 and BXF2NN mice had statistically similar numbers of RBC per milliliter, but BXF2NN mice had slightly, but not significantly, larger RBC than B10 mice.

Mitogen stimulation indices did not differ between B10 and BXF2NN mice. The uninduced, background ^{3}H-thymidine incorporation was significantly higher in BXF2NN spleen cells (1704 cpm vs. 3197 cpm). BXF2NN mice may have a higher rate of spontaneous proliferation of spleen cells than B10 mice because of the influence of an H-2 gene product.

Significantly fewer cells were available to respond to SRBC in BXF2NN spleens. The number of SRBC-PFC per spleen was significantly lower in BXF2NN mice (84551) than B10 mice (129950). When these numbers were compared as the number of SRBC-PFC per 10^6 spleen cells, however, the number of SRBC-PFC per 10^6 cells was essentially equivalent. This shows that although BXF2NN mice had fewer cells per spleen responding to SRBC, there were equivalent numbers of cells per 10^6 spleen cells that could produce hemolytic plaques in vitro.

Total T- and B-cell numbers did not differ in the spleens of B10 and uninjected BXF2NN mice. However, the T-cell subsets were significantly different in BXF2NN mice. L3T4$^+$ (CD4$^+$) T cells in BXF2NN spleens (17.42%) were significantly higher than in B10 mice (11.37%), while Lyt2.2$^+$ (CD8$^+$) T cells in BXF2NN spleens (5.10%) were significantly lower than in B10 mice (8.20%). The higher frequency of CD4$^+$ and lower

frequency of $CD8^+$ spleen cells in BXF2NN mice significantly raises the $CD4^+/CD8^+$ ratio (3.42) over the B10 value (1.39).

Because some values obtained from the H-$2^{a/a}$ BXF2NN mice differed from B10 values, the BXF2NN data were compared to B10.F data to find if the uninjected BXF2NN values could significantly change the results found comparing B10 and B10.F mice (Table 5.2). BXF2NN control values did not change the *p* values obtained for WBC or RBC numbers; these B10.F values did not differ significantly from BXF2NN. Lymphocyte (80.56% vs. 61.37%), monocyte (3.65% vs. 7.31%) and granulocyte ratios (12.81% vs. 27.93%) were still significantly different compared to BXF2NN mice. As observed with B10.F mice, lymphocyte, monocyte and granulocyte ratios were altered, with fewer lymphocytes and larger numbers of monocytes and granulocytes in B10FV-infected animals.

The HGB, HCT and MCV hematology values in B10.F mice were significantly lower than in BXF2NN mice. The lower MCV in B10.F mice indicated that the erythrocytes were smaller than normal; the reduction in HGB concentration and also in hematocrit (HCT) values were most likely due to the reduction in RBC size. The altered RBC indices suggested that the retrovirus infection in B10.F had chronically stimulated the hematopoietic system, causing a shortened RBC maturation time from the bone marrow producing smaller RBC with a decreased HGB content. This was consistent with observations from injected BXF2NN mice, which also had significantly lower HGB concentrations, HCT and MCV (Table 4.3).

Results obtained from mitogen and SRBC-PFC experiments with B10.F mice, remained significantly lower than results obtained from similar experiments with BXF2NN mice. Unlike the results obtained using B10 mice as the control mouse strain (Table 2.2),

TABLE 5.2

STATISTICAL ANALYSES OF UNINJECTED BXF2NN AND B10.F DATA

Parameter	Uninjected BXF2NN	B10.F	*p* value
HEMATOLOGY			
WBC ($x10^6$/ml)	5.70±0.37	9.80±2.42	>0.05
RBC ($x10^9$/ml)	10.63±0.12	9.73±0.24	>0.05
HGB (g/dl)	15.14±0.15	14.13±0.38	<0.05
HCT (%)	52.02±0.56	45.06±1.20	<0.005
MCV (fl)	49.00±0.31	46.40±0.64	<0.005
MCH (pg/RBC)	14.26±0.31	14.55±1.08	>0.05
MCHC (g/dl)	29.13±0.31	31.45±0.48	<0.001
LYM (%)	80.56±1.25	61.37±4.19	<0.001
MON (%)	3.65±0.44	7.31±0.77	<0.001
GRAN (%)	12.81±0.93	27.93±3.97	<0.001
MITOGENS			
Unstimulated (cpm)	3197±417	7391±1748	>0.05
Con A (SI)[a]	36.70±4.05	13.81±2.57	<0.025
PHA (SI)	25.40±2.45	9.10±1.45	<0.005
LPS (SI)	16.97±3.66	6.67±0.88	>0.05
SRBC-PFC			
SRBC-PFC/spleen	84551±6618	44828±10742	<0.005
SRBC-PFC/10^6 cells	424±39	151±89	<0.005
FLOW CYTOMETRY			
Thy1 (%)	23.83±2.20	19.35±0.28	>0.05
B220 (%)	56.90±3.02	47.87±3.67	>0.05
Lyt1 (%)	23.49±1.38	19.80±0.62	<0.05
Ly-1 B (%)	9.35±3.37	11.63±0.99	>0.05
L3T4 (CD4) (%)	17.42±0.97	13.98±0.82	<0.025
Lyt2 (CD8) (%)	5.10±0.27	4.91±0.39	>0.05
CD4/CD8	3.42±0.11	2.88±0.14	<0.025

[a] SI: stimulation index, as described in Chapter II.

one mitogen stimulation index did become nonsignificant at the 5% level of confidence. LPS mitogen stimulation indices did not differ significantly from BXF2NN values at the 5% level. This lack of statistical difference in LPS response between B10.F mice and BXF2NN mice may be caused by the large amount of variability in the BXF2NN data.

H-2$^{a/a}$ mice infected with B10FV had immune systems with altered cellular responses to mitogens. Both B10.F and injected BXF2NN mice had responses to the T-cell mitogens Con A and PHA that were one-third the normal, uninfected H-2$^{a/a}$ response. While the retrovirus infection did cause the lower responses of infected spleen cells to mitogens, there was no correlation of the titer of ecotropic retroviruses in injected BXF2NN mice with the response of the spleen cells to the mitogens. Con A and PHA responses did correlate with each other, suggesting that whatever influenced the Con A mitogen response also influenced the PHA response.

The number of B10.F spleen cells responding to injected SRBC remained significantly lower than BXF2NN SRBC-PFC numbers. Injected BXF2NN mice, while not as suppressed as B10.F mice, also had significantly lower SRBC-PFC per spleen and per 10^6 spleen cells. Thus, the retrovirus infection caused the lowered ability to respond to injected SRBC in B10.F and injected BXF2NN mice. The lower number of hemolytic antibody-producing B cells in infected mice was probably due to the lower number of CD4$^+$ T cells, which were also decreased to two-thirds that of normal, uninfected H-2$^{a/a}$ mice.

The percentages of cells in the spleens of B10.F mice expressing binding sites for fluorescently labeled monoclonal antibodies differed from B10 values when compared to BXF2NN spleen cell ratios. The frequency of T cells labeled with the Thy1 monoclonal antibody did not differ in B10.F or BXF2NN spleens; frequencies of Lyt1$^+$ T cells,

however, were significantly lower in B10.F spleens. The L3T4$^+$ (CD4$^+$) T cells were significantly lower in B10.F mice when compared with BXF2NN spleen cells; this value was not significantly different when compared to B10 values. Contrary to the B10 results, B10.F and BXF2NN mice did not have significantly different frequencies of Lyt2$^+$ (CD8$^+$) spleen cells. Infected BXF2NN mice not only had a lower frequency of Lyt1$^+$ cells in their spleens but also a lower frequency of Thy1$^+$ cells. The numbers of both L3T4$^+$ and Lyt2.2$^+$ T cells in the spleens of infected BXF2NN mice were significantly lower than in control mice. Despite the different CD4$^+$ and CD8$^+$ cell frequency in B10.F and infected BXF2NN mice, both B10.F and injected BXF2NN mice had similar CD4$^+$/CD8$^+$ ratios.

B cells, identified by the fluorescent antibody to the B220 antigen, were unchanged in frequency in the spleens of both B10.F and injected BXF2NN mice when compared to uninfected H-2$^{a/a}$ mice. When compared to B10 mice, B-cell frequencies appeared to be significantly lower in B10.F spleens. When B-cell frequencies in infected BXF2NN mice were compared to uninfected BXF2NN mice, however, no significant difference was found. B-cell frequencies were therefore higher in the spleen of H-2$^{b/b}$ mice as compared to H-2$^{a/a}$ mice.

One subset of B cells, the Ly-1 B cells, had a higher frequency in the spleens of B10.F, uninjected BXF2NN and injected BXF2NN mice than in B10 mice. This number was double to triple the number found in normal mouse strains (Hayakawa et al., 1983). This suggests that there is an H-2 region influence on the population of Ly-1 B cells in H-2$^{a/a}$ mice. No H-2 influence has been reported for Ly-1 B cell regulation.

The FACS data revealed some important differences in T- and B-cell populations in H-2$^{a/a}$ mice. When B10 and B10.F splenic lymphocytes were compared in Chapter II, T-cell totals were not statistically different, but there were differences in the T-cell subsets

within this population. Frequencies of $CD4^+$ T cells were essentially equal in B10 and B10.F spleens, while $CD8^+$ spleen cells were significantly lower in B10.F spleens. When B10 T cell subset frequencies were compared to those in uninfected BXF2NN mice, the $CD4^+$ T-cell subset was significantly higher in uninfected BXF2NN mice. B10 mice also had significantly higher numbers of $CD8^+$ T cells when compared to B10.F, uninfected or infected BXF2NN mice. The larger population of $CD4^+$ spleen cells and the smaller frequency of $CD8^+$ spleen cells altered the CD4/CD8 ratio in BXF2NN spleens as compared to B10 mice. Rather than raising the CD4/CD8 ratio, as observed when B10 and B10.F mice are compared, the retrovirus infection in BXF2NN mice significantly lowered the CD4/CD8 ratio.

As described in Chapter III, B10FV bears great similarity to the ecotropic helper retrovirus present in the LP-BM5 retrovirus mixture that causes a murine acquired immune deficiency syndrome known as MAIDS (Mosier et al., 1985). Recent evidence has suggested that MuLV p30 gag "superantigens" expressed by a defective retrovirus may be expressed on infected B cells in animals with MAIDS (Hügin et al., 1991), generating an expanded cell pool susceptible to MuLV infection and lysis. The action of this antigen is dependent upon MHC class II molecules on antigen presenting cells, and the antigen stimulates a particular set of T cells that have a rearranged $V_\beta 5$ T-cell receptor (TCR) β chain region. This variable region of the TCR is generally used along with MHC class II I-E molecules to induce deletion of cells with specific cell-surface markers in the thymus (Liao et al., 1990; Bill et al., 1990). The strains most susceptible to MAIDS have the I-E region deleted from their H-2 region (Hartley et al., 1989). In C57BL mice part of the I-E region is missing from their H-2 region, so $V_\beta 5$ T cells are not clonally deleted in their thymuses. The H-2 region of B10.F mice has not been molecularly characterized, but

evidence suggests that the I-E region of H-2^a mice may also be altered so as not to produce a product, because B10.F mice are susceptible to MAIDS (Hartley et al., 1989). While defective retroviruses have not been found in B10.F or infected BXF2NN Hirt supernatant DNAs, the few restriction endonuclease site differences in the gag region of B10FV may cause similar proteins to be expressed on B10FV-infected B cells.

The injection of B10FV into BXF2NN H-$2^{a/a}$ neonates has led to some conclusions for many unanswered questions about B10.F mice and their relationship with their congenic partner, B10. Many of the histologic, hematologic and immunologic differences observed in B10.F mice compared with B10 mice were associated with the retrovirus infection. An immunodeficiency manifesting itself as low spleen cell proliferative responses to T- and B-cell mitogens and the lower ability to form hemolytic antibodies to SRBC in B10.F mice were caused by the influence of the retroviral infection. $CD4^+$ T-cell subset frequencies in B10FV-injected BXF2NN mice appear to be unaltered compared to B10 mice but are actually reduced in frequency when compared to uninjected BXF2NN mice, and the lower frequency of $CD4^+$ cells was due to the actions of the retrovirus infection. B10.F mice had significantly lower B cell frequencies when compared to B10 mice, but the number of B cells in B10.F mice were similar to those in uninjected and injected BXF2NN mice.

Recommendations

The ecotropic retrovirus, B10FV, remains to be molecularly cloned and sequenced. Attempts to clone B10FV from large quantities of 513900 Hirt supernatant DNA resulted

in no positive clones. SC_1 or M.m. dunnii cells containing a higher ecotropic retrovirus titer must be prepared before B10FV can be cloned from Hirt supernatant DNA.

The early events in B10FV infection in BXF2NN mice need to be learned. Mitogen, SRBC-PFC and FACS data from two-month-old B10.F mice indicate that while mitogen responses are similar to control values, the number of T cells already is lower in frequency in the spleens of B10FV-infected animals. PFC-SRBC data also show an equal to slightly elevated ability to respond to SRBC. By injecting BXF2NN mice as late as 15 days after birth, a time course of events affected by B10FV infection could be constructed. Injected BXF2NN mice could be examined for viral clearance in the tissues by in situ hybridization. Cytokine levels for interferon-γ, tumor necrosis factor α and β, and interleukins 1, 2 and 3 could be measured as the retrovirus infection is established in the animals. Finally, determinations as to whether or not a hyperresponsive immune state exists during the early phases of B10FV infection could be made during the time-course investigations of B10FV-injected BXF2NN mice.

LITERATURE CITED

Adler, S., F. Trobaugh and W. Knospe. 1977. Hemopoietic stem cell dynamics in ^{89}Sr marrow-ablated mice. Journal of Laboratory and Clinical Medicine 89: 592-602.

Aeby, P., A. Spicher, Y. de Chastonay, F. Muller and H. Tobler.1986. Structure and genomic organization of pro-retrovirus-like elements partially eliminated from the somatic genome of Ascaris lumbrioides. EMBO J. 5: 3353-3360.

Ahmann, G.B., D.H. Sachs and R.J. Hodes. 1978. Requirement for an Ia-bearing accessory cell in Con A-induced T cell proliferation. Journal of Immunology 121: 1981-1989.

Albritton, L.M., L. Tseng, D. Scadden and J.M. Cunningham. 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to retrovirus infection. Cell 57: 659-666.

Andersson, J., K. Grönvik, E.-L. Larsson and A. Coutinho. 1979. Studies on T lymphocyte activation. I. Requirements for the mitogen dependent production of T cell growth factors. European Journal of Immunology 9: 581-587.

Balkwill, F.R. 1989. Interferons. Lancet i: 1060-1063.

Baltimore, D. 1970. Viral RNA-dependent DNA polymerase. Nature 226: 1209-1211.

Bartholomew, C. and J.N. Ihle. 1991. Retroviral insertions 90 kilobases proximal to the evi-1 myeloid transforming gene activate transcription from the normal promotor. Molecular and Cellular Biology 11: 1820-1828.

Benade, L.E., J.N. Ihle and A. Decleve. 1978. Serological characterization of B-tropic viruses of C57BL mice: possible origin by recombination of endogenous N-tropic and xenotropic viruses. Proceedings of the National Academy of Sciences USA 75: 4553-4557.

Besmer, P. U. Olshevsky, D. Baltimore, D. Dolberg and H. Fan. 1979. Virus-like 30S RNA in mouse cells. Journal of Virology 29: 1168-1176.

Bill, J., O. Kanagawa, J. Linten, Y. Utsunomiya and E. Palmer. 1990. Class I and class II MHC gene products differentially affect the fate of $V^{\beta}5$ bearing thymocytes. Journal of Molecular and Cellular Immunology 4: 269-279.

Bittner, J.J. 1942. The milk-influence of breast tumors in mice. Science 95: 2470-2471.

Boone, L.R., P.L. Glover, C.L. Innes, L.A. Niver, M.C. Bondurant and W.K. Yang. 1988. Fv-1 N- and B- tropism-specific sequences in murine leukemia virus and related endogenous proviral genomes. Journal of Virology 62: 2644-2650.

Brightman, B.K., A. Rein, D.J. Trepp and H. Fan. 1991. An enhancer variant of Moloney murine leukemia virus defective in leukemogenesis does not generate detectable mink cell focus-inducing virus in vivo. Proceedings of the National Academy of Sciences USA 88: 2264-2268.

Britt, W.J. and B. Chesebro. 1983. H-2D control of recovery from Friend virus leukemia: H-2D region influences the kinetics of the T lymphocyte response to Friend virus. Journal of Experimental Medicine 157: 1736-1745.

Brown, P.O., B. Bowerman, H.E. Varmus and J.M. Bishop. 1987. Correct integration of retroviral DNA in vitro. Cell 47: 347-356.

Brown, P.O., B. Bowerman, H.E. Varmus and J.M. Bishop. 1989. Retroviral integration: structure of the initial covalent product and its precursors, and a role for the viral IN protein. Proceedings of the National Academy of Sciences USA 86: 2525-2529.

Broxmeyer, H.E., D.E. Williams, L. Lu,, S. Cooper, S.L. Anderson, G.S. Beyer, R. Hoffman and B.Y. Rubin. 1986. The suppressive influences of human tumor necrosis factor on bone marrow hematopoietic cells from normal donors and patients with leukemia: Synergism of tumor necrosis factor and interferon-gamma. Journal of Immunology 136: 4487-4495.

Cerny, A., A.W. Hugin, R.R. Hardy, Y. Hayakawa, R.M. Zinkernagel, M. Makino and H.C. Morse III. 1990. B cells are required for the induction of T cell abnormalities in a murine retovirus-induced immunodeficiency syndrome. Journal of Experimental Medicine 171: 315-320.

Chan, H.W., T. Bryan, J.L. Moors, S.P. Staal, W.P. Rowe and M.A. Martin. 1980. Identification of ecotropic proviral sequences in inbred mouse strains with a cloned subgenomic DNA fragment. Proceedings of the National Academy of Sciences USA 77: 5579- 5583.

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

Chattopadhyay, S.K., M.R. Lander, S. Gupta, E. Rands and D.R. Lowy. 1981. Origin of mink cell focus-forming (MCF) viruses: comparison with ecotropic and xenotropic murine leukemia virus genomes. Virology 113: 465-483.

Chattopadhyay, S.K., M.R. Lander, E. Rands and D.R. Lowy. 1980. Structure of endogenous murine leukemia virus DNA in mouse genomes. Proceedings of the National Academy of Sciences USA 77: 5774-5778.

Chattopadhyay, S.K., D.R. Lowy, T.M. Teich, A.S. Levine and W.P.Rowe. 1974. Qualitative and quantitative studies of AKR-type murine leukemia virus sequences in mouse DNA. Cold Spring Harbor Symposia on Quantitative Biology 39: 1085-1101.

Chattopadhyay, S.K., H.C. Morse III, M. Makino, S.K. Ruscetti and J.W. Hartley. 1989. Defective virus is associated with induction of murine retrovirus-induced immunodeficiency syndrome. Proceedings of the National Academy of Sciences USA 86: 3862-3866.

Chesebro, B., K. Wehrly and J. Stimpfling. 1974. Host genetic control of recovery from Friend leukemia virus-induced splenomegaly. Journal of Immunology 120: 1081-1088.

Cheung, S.C., S.K. Chattopadhyay, H.C. Morse III and P.M. Pitha. 1991. Expression of defective virus and cytokine genes in murine AIDS. Journal of Virology 65: 823-828.

Coffin, J.M. 1979. Structure, replication and recombination of retrovirus genomes: some unifying hypotheses. Journal of General Virology 42: 1-46.

Cohen J.C. and H.E. Varmus. 1979. Endogenous mammary tumor virus DNA varies among wild mice and segregates during inbreeding. Nature 278: 418-423.

Copelan, E.A., J.J. Rinehart, M. Lewis, L. Mathes, R. Olsen and A. Sagone. 1983. The mechanism of retrovirus supression of human T cell proliferation in vitro. Journal of Immunology 131: 2017-2020.

Cotter, S., W. Hardy and M. Essex. 1975. Association of feline leukemia virus with lymphosarcoma and other disorders in the cat. Journal of the American Veterinary Medical Association 166: 449-556.

Dalton, A.J., M. Potter and R.M. Merwin. 1961. Some ultrastructural characteristics of a series of primary and transplanted tumors of the mouse. Journal of the National Cancer Institute 26: 1221-1267.

Datta, S.K., P. Tsichlis, R.S. Schwartz, S.K. Chattopadhyay and C. Melief. 1978. Genetic differences unrelated to H-2 in H-2-congenic mice. Immunogenetics 7: 359-365.

Davidson, W.F., M. Chused and H.C. Morse III. 1981. Genetic control of B and T lymphocyte abnormalities in NZB mice in crosses with B10.D2 mice. Immunogenetics 13: 421-434.

Davidson, W.F., T.N. Fredrickson, E.K. Rudikoff, R.L. Coffman, J.W. Hartley and H.C. Morse III. 1984. A unique series of lymphomas related to the Ly-1$^+$ lineage of B lymphocyte differentiation. Journal of Immunology 133: 744-753.

Davis, B., E. Linney and H. Han. 1985. Suppression of leukemia virus pathogenicity by polyoma virus enhancers. Nature 314: 550-553.

DeLarco, J. and G.J. Todaro. 1976. Membrane receptors for murine leukemia viruses: characterization using purified viral envelope glycoprotein, gp71. Cell 8: 365-371.

Dickson, C., R. Eisenman, H. Fan, E. Hunter and N. Teich. 1984. Protein biosynthesis and assembly. In RNA Tumor Viuses, vol.1, R. Weiss, N. Teich, H. Varmus and J. Coffins, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 513-648.

Donehower, L.A., B. Fleurdelys and G.L. Hagar. 1983. Further evidence for the protein coding potential of the mouse mammary tumor virus long terminal repeat: Nucleotide sequence of an endogenous proviral long terminal repeat. Journal of Virology 45: 941-949.

Doolittle, R.F., D.-F. Feng, M.S. Johnson and M.A. McClure. 1989. Origins and evolutionary relationships of retroviruses. Quarterly Review of Biology 64: 1-30.

Dougherty, J.B. and H.M. Temin. 1988. Determination of the rate of base pair substitution and insertion mutations in retroviruses. Journal of Virology 62: 2817-2822.

Flaherty, L. 1981. Congenic strains. In The Mouse in Biomedical Research. H.L. Foster, J.D. Small and J.G. Fox, eds. Academic Press, New York, pp. 215-222.

Fuchs, D., A. Hausen, G. Reibnegger, E.R. Werner, G. Werner-Felmayer, P. Dierich and H. Wachter. 1991. Immune activation and anemia associated with chronic inflammatory disorders. European Journal of Haematology 46: 65-70.

Fujiwara, T. and R. Craigie. 1989. Integration of mini-retroviral DNA: a cell-free reaction for biochemical analysis of retroviral integration. Proceedings of the National Academy of Sciences USA 86: 3065-3069.

Fujiwara, T. and K. Mizuuchi. 1988. Retroviral DNA integration: Structure of an integration intermediate. Cell 54: 497-504.

Gallager, J.F., R. Griesemer, D.M. Popp and R.A. Popp. 1987. Correlation of retroviral integration and immunocompetence in the lymphatic tissues of B10.F mice. 15th Molecular and Biochemical Genetics Workshop, St. Croix, USVI (Abstract).

Gerard, G.F. and D.P. Grandgenett. 1980. Retrovirus reverse transcriptase. In: Molecular Biology of RNA Tumor Viruses, ed. by J.R. Stephenson, pp. Academic Press, New York.

Gorer, P.A. and Z.B. Mikulska. 1954. The antibody response to tumor inoculation. Improved methods of antibody detection. Cancer Research 14: 651-655.

Greaves, M. and G. Janossy. 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. Clinical and Experimental Immunology 9: 483-498.

Gross, L. 1951. "Spontaneous" leukemia developing in C3H mice following innoculation, in infancy, with AK-leukemic extracts, or AK-embryos. Proceedings of the Society for the Experimental Biological Medicine 76: 27-32.

Gross, L. 1958. Attempts to recover filterable agent from X-ray-induced leukemia. Acta Haematologica 19: 353-361.

Haas, M. and A. Meshorer, 979. Reticulum cell neoplasms induced in C57BL/6 mice by cultured virus grown in stromal hematopoietic cell lines. Journal of the National

Cancer Institute 63: 427-439.

Haas, M. and T. Reshef. 1980. Nonthymic malignant lymphomas induced in C57BL/6 mice by cloned dualotropic viruses. European Journal of Cancer 16: 909-917.

Harada, F., G.G. Peters and J.E. Dahlberg. 1979. The primer tRNA for Moloney murine leukemia virus DNA synthesis. Journal of Biological Chemistry 254: 10979-10985.

Hartley, J.W., T.N. Fredrickson, R.A. Yetter, M. Makino and H.C. Morse III. 1989. Retrovirus-induced murine acquired immunodeficiency syndrome: Natural history of infection and differing susceptibility of inbred mouse strains. Journal of Virology 63: 1223-1231.

Hartley, J.W. and W.P. Rowe. 1975. Clonal cell lines from a feral mouse embryo which lack host range restrictions for murine leukemia viruses. Virology 65: 124-128.

Hartley, J.W. and W.P. Rowe. 1976. Naturally occurring murine leukemia viruses in wild mice: characteriztion of a new "amphotropic" class. Journal of Virology 19: 19-25.

Hartley, J.W., W.P. Rowe, W.I. Capps and R.J. Huebner. 1969. Isolation of naturally occurring viruses of the murine leukemia virus group in tissue culture. Journal of Virology 3: 126-132.

Haseltine, W.A., D.G. Kleid, A. Panet, E. Rothenberg and D. Baltimore. 1976. Ordered transcription of RNA tumor virus genomes. Journal of Molecular Biology 106: 109-131.

Hayakawa, K., R.R. Hardy, D.R. Parks and L.A. Herzenberg. 1983. The "Ly-1 B" cell subpopulation in normal, immunodeficient and autoimmune mice. Journal of Experimental Medicine 157: 202-218.

Hayakawa, K., R.R. Hardy, M. Honda, L.A. Herzenberg, A.D. Steinberg and L.A. Herzenberg. 1984. Ly-1 B cells: Functionally distinct lymphocytes that secrete IgM autoantibodies. Proceedings of the National Academy of Sciences 81: 2494-2498.

Herrman, F. S.A. Cannistra and J.D. Griffin. 1986. T cell-monocyte interactions in the production of humoral factors regulating human granulopoiesis in vitro. Journal of Immunology 136: 2856-2861.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. Journal of Molecular Biology 26: 365-369.

Hoggan, M.D., C.E. Buckler, J. Sears, W.P. Rowe and M.A. Martin. 1983. Organization and stability of endogenous xenotropic murine leukemia virus proviral DNA in mouse genomes. Journal of Virology 45: 473-477.

Hsu, T.W., J.L. Sabran, G.E. Mark, R.V. Guntaka and J.M. Taylor. 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. Journal

of Virology 28: 810-818.

Huang, M. and P. Jolicoeur. 1990. Characterization of the gag/fusion protein encoded by the defective Duplan retrovirus inducing Murine AIDS. Journal of Virology 64: 5764-5772.

Hughes, S.H., P.R. Shank, D.H. Spector, H.J. Kung, J.M. Bishop, H.E. Varmus, P.K. Vogt and M.L. Breitman. 1978. Proviruses of avian sarcoma virus are terminally redundant, co-extensive with integrated linear DNA and integrated at many sites. Cell 15: 1397-1410.

Hügin, A.W., M.S. Vacchio and H.C. Morse III. 1991. A virus-encoded "superantigen" in a retrovirus-induced immunodeficiency syndrome in mice. Science 252: 424-427.

Ishimoto, A., J.W. Hartley and W.P. Rowe. 1977. Detection and quantification of phenotypically mixed viruses: mixing of ecotropic and xenotropic murine leukemia viruses. Virology 8: 263-269.

Jaenisch, R. 1980. Retroviruses and embryogenesis: Microinjection of Moloney leukemia virus into midgestation mouse embryos. Cell 19: 181-188.

Jenkins, N.A., N.G. Copeland, B.A. Taylor and B.K. Lee. 1981. Dilute (d) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. Nature 293: 370-374.

Jenkins, N.A., N.G. Copeland, B.A. Taylor and B.K. Lee. 1982. Organization, distribution and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of Mus musculus. Journal of Virology 43: 26-36.

Jenkins, N.A., N.G. Copeland, B.A. Taylor and B.K. Lee. 1987. Organization, distribution and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of Mus musculus. Journal of Virology 43: 26-36.

Jolicoeur, P. and E. Rassart. 1980. Effect of Fv-1 gene product on synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. Journal of Virology 33: 183-195.

Kawashima, K., H. Ikeda, J.W. Hartley, E. Stockert, W.P. Rowe and J.L. Old. 1976. Changes in the expression of murine leukemia virus antigens and production of xenotropic virus in the late preleukemic period in AKR mice. Proceedings of the National Academy of Sciences 73: 4680-4684.

Khan, N.S. 1984. Nucleotide sequence analysis establishes the role of endogenoous murine leukemia virus DNA segemnts in the formation of recombinant mink cell focus-forming murine leukemia viruses. Journal of Virology 50: 864-871.

Khan, A.S. and M.A. Martin. 1983. Endogenous murine leuemia proviral long terminal repeats contain a unique 190-base-pair insert. Proceedings of the National

Academy of Sciences 80: 2699-2703.

Khan, A.S., W.P. Rowe and M.A. Martin. 1982. Cloning of endogenous murine leukemia virus-related sequences from chromosomal DNA of BALB/c and AKR/J mice: identification of an env progenitor of AKR-247 mink cell focus-forming proviral DNA. Journal of Virology 44: 625-636.

King, S.R., B.J. Brown and R. Risser. 1987. Mechanism of interaction between endogenous ecotropic murine leukemia viruses in (BALB/c x C57BL/6) hybrid cells. Virology 162: 1-11.

Klein, J. 1986. Natural History of the Major Histocompatibility Complex.John Wiley and Sons, New York, New York.

Klinman, D.M. and H.C. Morse III. 1989. Characteristics of B cell proliferation and activation in Murine AIDS. The Journal of Immunology 142: 1144-1149.

Kozak, C.A. and W.P. Rowe. 1980. Chromosomal mapping of ecotropic and xenotropic leukemia-inducing loci in the mouse. In Animal Virus Genetics, Academic Press, New York, pp.171-175.

Krieg, A.M., A.S. Khan, and A.D. Steinberg. 1988. Multiple endogenous xenotropic and mink cell focus-forming murine leukemia virus-related transcripts are induced by polyclonal immune activators. Journal of Virology 62: 3545-3550.

Lai, M.C., C.S. Shimizu, S. Rasheed, B. Pal and M.B. Gardner. 1982. Characterization of genome structure of amphotropic and ecotropic wild mouse retroviruses. Journal of Virology 41: 605-614.

Langdon, W.Y., T.S. Theodore, C.E. Buckler, J.H. Stimpfling, M.A. Martin and H.C. Morse III. 1984. Relationship between a retroviral germ line mutation and a new mutation at the ashen locus in B10.F mice. Virology 133: 183-190.

Larsson, E.-L. and A. Coutinho. 1979. The role of mitogenic lectins in T-cell triggering. Nature 280: 239-241.

Laterjet, R. and J.F. Duplan. 1962. Experiments and discussion on leukemogenesis by cell-free extracts of radiation-induced leukemia in mice. International Journal of Radiation Biology 5: 339-344.

Lazo, P.A., J.S. Lee and P.N. Tsichlis. 1990. Long-distance activation of the myc protooncogene by provirus insertion in Mlvi-1 or Mlvi-4 in natural T cell lymphoma. Proceedings of National Academy of Sciences 87: 170-173.

Lazo, P.A. and P.N. Tsichlis. 1990. Biology and pathogenesis of retroviruses. Seminars in Oncology 17: 269-294.

Leeson, T.S. and C.R. Leeson. 1981. Histology. W.B. Saunders Co., Philadelphia.

Li., Q. and H. Fan. 1990. Combined infection by Moloney murine leukemia virus and mink cell focus-forming virus recombinant induces cytopathic effects infibroblasts or in long-term bone marrow cultures from preleukemic mice. Journal of Virology 64: 3701-3711.

Liao, N.S., J. Maltzman and D.H. Raulet. 1990. Expression of $V^{\beta}5.1$ gene by murine peripheral T cells is controlled by MHC genes and skewed to the $CD8^{+}$ subset. Journal of Immunology 144: 844-848.

Liberman, M., H.S. Kaplan and A. Decleve. 1976. Anomalous viral expression in radiogenic lymphomas of C57BL/Ka mice. In Biology of Radiation Carcinogenesis, J.M. Yuhas, R.W. Tennant and J.D. Regan, eds., Raven Press, New York, pp. 237-244.

Lieberman, M. and H.S. Kaplan. 1959. Leukemogenic activity of filtrates from radiation-induced lymphoid tumors in mice. Science 130: 387-388.

Lilly, F. 1970. Identification and location of gene governing spleen focus response to Friend leukemia virus in mice. Journal of the National Cancer Institute 45: 163-169.

Link, C.D., J. Graf-Whitsel and W.B. Wood. 1987. Isolation and characterization of a nematode transposable element from Panagrellus redivivus. Proceedings of the National Academy of Sciences 74: 5325-5329.

Lowy, D.R. 1986. Transformation and oncogenesis: Retroviruses. In Fundamental Virology, B.N. Fields and D.M. Knipe, eds, Raven Press, New York, pp. 235-263.

Lung, M.L., J.W. Hartley, W.P. Rowe and N.H. Hopkins. 1983. Large RNase T_1-resistant oligonucleotides encodong p15E and the U3 region of the long terminal repeat distinguish two biological classes of mink cell focus-forming type C viruses of inbred mice. Journal of Virology 45: 275-290.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mann, D.L., F. Lasane, M. Popovic, L.O. Arthur, W.G. Robey, W.A. Blattner and M.J. Newman. 1987. HTLV-III large envelope protein (gp120) suppresses PHA-induced lymphocyte blastogenesis. Journal of Immunology 138: 2640-2644.

Martin, P.J., J.A. Hansen, A.W. Siadek and R.C. Nowinski. 1981. Monoclonal antibodies recognizing normal human T lymphocytes and malignant human B lymphocytes: a comparative study. Journal of Immunology 1920-1923.

McChesney, M.B. and M.B.A. Oldstone. 1987. Viruses perturb lymphocyte functions: Selected principles characterizing virus-induced immunosuppression. Annual Reviews of Immunology 5: 279-304.

McCubry, J. and R. Risser. 1982. Genetic interactions in the spontaneous production of endogenous murine leukemia virus in low leukemic strains. Journal of Experimental Medicine 156: 337-349.

McGeady, M.L., C. Jhappan, R. Ascione and G.F. Vande Woude. 1983. In vitro methylation of specific regions of the cloned Moloney sarcoma virus genome inhibits its transforming activity. Molecular and Cellular Biology 3: 305-314.

Melief, C.J.M., A. Vlug, R.E.Y. de Goede, C. de Bruyne, W. Barendsen and P. de Greeve. 1980. Naturally occurring leukemia viruses in H-2 congenic C57BL mice. I. High lymphoma incidence following milk-borne transmission of virus. Journal of the National Cancer Institute 64: 1179-1189.

Meruelo, D. and R. Bach. 1983. Genetics of resistance to virus-induced leukemias. Advances in Cancer Research 40: 107-188.

Metcalf, D. 1989. Haematopoietic growth factors 1. Lancet i: 825-827.

Mishell, R.I. and R.W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. Journal of Experimental Medicine 126: 423-454.

Moelling, K., D.P. Bolognesi, H. Baur, W. Busen, H.W. Passman and P. Hausen. 1971. Association of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature 234: 240-243.

Morse III, H.C. 1981. The laboratory mouse - a historical perspective. In The Mouse in Biomedical Research, H.L. Foster, J.D. Small and J.G. Fox, eds. Academic Press, New York, pp. 1-16.

Morse III, H.C., C.A. Kozak, R.A. Yetter and J.W. Hartley. 1982. Unique features of retrovirus expression in F/St mice. Journal of Virology 45: 1-7.

Morse III, H.C., R.A. Yetter, J.H. Stimpfling, O.M. Pitts, T.N. Fredrickson and J.W. Hartley. 1985. Greying with age in mice: Relation to expression of murine leukemia viruses. Cell 41: 439-448.

Morse III, H.C., R.A. Yetter, C.S. Via, R.R. Hardy, A. Cerny, K. Hayakawa, A.W. Hugen M.W. Miller, K.L. Holmes and G.M. Shearer. 1989. Functional and phenotypic alterations in T cell subsets during the course of MAIDS, a murine retrovirus-induced immundeficiency syndrome. Journal of Immunology 143: 844-850.

Mosier, D.E., R.A. Yetter and H.C. Morse III. 1985. Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice. Journal of Experimental Medicine 161: 766-784.

Mosier, D.E., R.A. Yetter and H.C. Morse III. 1987. Functional T lymphocytes are required for a murine retrovirus-induced immunodeficiency disease (MAIDS). Journal of Experimental Medicine 165: 1737-1742.

Mullins, J.I., C.S. Chan and E.A. Hoover. 1986. Disease specific and tissue-specific production of unintegrated feline leukemia virus variant DNA in feline AIDS. Nature 319: 333-336.

Nikbakht, K.N., C.-Y. Ou, L.R. Boone, P.L. Glover and W.K. Yang. 1985. Nucleotide sequence of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites of glutamine tRNA. Journal of Virology 54: 889-893.

Odaka, T. 1975. Genetic-transmission of endogenous N- and B-tropic murine leukemia viruses in low-leukemic strain C57BL/6. Journal of Virology 15: 332-337.

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

Orosz, C.G., N.E. Zimm, R.G. Olsen and L.E. Mathes. 1985. Retrovirus-mediated immunosuppression I. FeLV-UV and specific FeLV proteins alter T lymphocyte behavior by inducing hyperresponsiveness to lymphokines. Journal of Immunology 134: 3396-3403.

Ou, C.-Y., L.R. Boone and W.K. Yang. 1983. A novel sequence element and the nucleotide structural features in the long terminal repeat of a BALB/c mouse genomic leukemia virus-related DNA clone. Nucleic Acids Research 11: 5603-5620.

Pattengale, P.K., C.R. Taylor, T. Twomey, S. Hil, J. Jonasson, T. Beardsley and M. Haas. 1982. Immunopathology of B-cell lymphomas induced in C56BL/6 mice by dualotropic murine leukemia virus (MuLV). American Journal of Pathology 107: 362-377.

Peebles, P.T. 1975. An in vitro focus-induction assay for xenotropic murine leukemia virus, feline leukemia virus C and feline-primate viruses RD-114/CCl/M-7. Virology 67: 288-291.

Persson, U., P.H. Bick, L. Hammerström, E. Möller and C.I.E. Smith. 1978. Different requirements for T cells responding to various doses of concanavalin A. Scandinavian Journal of Immunology 8: 291-301.

Peters, R.L., G.J. Spahn, L.S. Rabstein, G.J. Kelloff and R.J. Huebner. 1973. Murine C-type RNA virus from spontaneous neoplasms: In vitro host range and oncogenic potential. Science 181: 665-667.

Pierce, J.H. and S.A. Aaronson. 1982. BALB- and Harvey-murine sarcoma virus transformation of a novel lymphoid progenitor cell. Journal of Experimental Medicine 150: 873-887.

Pincus, T., J.W. Hartley and W.P. Rowe. 1971a. A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture studies of naturally occurring viruses. Journal of Experimental Medicine 133: 1219-1233.

Pincus, T., W.P. Rowe and F. Lilly. 1971b. A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to Friend murine leukemia virus. Journal of Experimental Medicine 133: 1234-1241.

Pinter, A. and E. Fleissner. 1977. The presence of disulfide-linked gp70-p15(E) complexes in AKR murine leukemia virus. Virology 83: 417-422.

Popp, D.M. 1978. Use of congenic mice to study the basis of degenerative disease. In Genetic Effects on Aging, D. Bergsma and D.E. Harrison, eds. Alan R. Liss, New York : pp. 261-279.

Popp, D.M. 1979a. Basal serum immunoglobulin levels. I. Evidence for genetic control in B10 and B10.F mice. Immunogenetics 9: 125-135.

Popp, D.M. 1979b. Basal serum immunoglobulin levels. II. Interaction of genetic loci and maternal effect on regulation of IgA. Immunogenetics 9: 281-292.

Popp, D.M. 1982. An analysis of genetic factors regulating lifespan in congenic mice. Mechanisms of Aging and Development 18: 125-134.

Popp, D.M. 1990. Lymphocyte subsets and abnormalities in retrovirally infected mice. In Critical Reviews in Immunology, Vol. 10. CRC Press, Boca Raton : pp. 73-112.

Popp, D.M., Otten, J.A and R.A. Popp. 1986. Interaction of H-2 genotype and basal serum immunoglobulin A level influences longevity. Mechanisms of Aging and Development 36: 79-93.

Popp, R.A., P.A. Lalley, J.W. Whitney and W.F. Anderson. 1981. Mouse alpha globin and alpha globin-like pseudogenes are not syntenic. Proceedings of the National Academy of Sciences 78: 6362-6366.

Rasheed, S., M.B. Gardner and E. Chan. 1976. Amphotropic host range of naturally-occurring wild mouse leukemia viruses. Journal of Virology 19: 13-18.

Rassart, E. P. Sankar-Mistry, G. Lemay, L. Des Groseillers and P. Jolicouer. 1983. New class of leukemogenic ecotropic recombinant murine leukemia virus isolated from radiation-induced thymomas. Journal of Virology 45: 565-575.

Rein, A., M.R. McClure, N.R. Rice, R.B. Luftig and A.M. Shultz. 1986. Myristylation site in $Pr65^{gag}$ is essential for virus particle formation by Moloney murine leukemia virus. Proceedings of the National Academy of Sciences 83: 7246-7250.

Risser, R. and J.M. Horowitz. 1983. Endogenous mouse leukemia viruses. Annual Review of Genetics 17: 85-121.

Robaye, B., R. Mosselmans, W. Fiers, J.E. Dumant and P. Galand. 1991. Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in

vitro. American Journal of Pathology 138: 447-453.

Rous, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. Journal of Experimental Medicine 13: 397-411.

Rowe, W.P. 1973. Genetic factors in the natural history of murine leukemia virus infection: G.H.A. Clowes Memorial Lecture. Cancer Research 33: 3061-3068.

Rowe, W.P. 1978. Leukemia virus genomes in the chromosomal DNA of the mouse. Harvey Lectures 72: 173-192.

Rowe. W.P. and J.W. Hartley. 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv-1^b strains of mice. Journal of Experimental Medicine 136: 1286-1301.

Rowe, W.P., W.E. Pugh and J.W. Hartley. 1970. Plaque assay techniques for murine leukemia viruses. Virology 42: 1136-1139.

Scolnick, E.M., W.C. Vass, R.S. Howk and P.H. Duesberg. 1979. Defective retovirus-like 30S RNA species of rat and mouse cells sre infectious if packaged by type C helper virus. Journal of Virology 29: 964-872.

Selton, G., H.T. Cuypers and A. Berns. 1985. Involvement of c-myc in MuLV-induced T cell lymphomas im mice: Frequency and mechanisms of action. EMBO J. 4: 1793-1798.

Severinson, E. and E.-L. Larson. 1986. Lymphocyte responses to polyclonal B and T cell activation. In Handbook of Experimental Immunology, volume 2, D.M. Weir, L.A. Herzenberg, C. Blackwell and L.A. Herzenbeerg, eds. Blackwell Scientific Publications, Boston: 63.1-63.18.

Shelton, G.H., M.L. Linenberger, C.K. Grant and J.L. Abkowitz. 1990. Hematologic manifestations of feline immunodeficiency virus infection. Blood 76: 1104-1109.

Shultz, A.M. and A. Rein. 1989. Unmyristylated Moloney murine leukemia virus $Pr65^{gag}$ is excluded from virus assembly and maturation events. Journal of Virology 63: 2370-2373.

Snell, G.D. 1948. Methods for the study of histocompatibility genes. Journal of Genetics 49: 87-108.

Sozer, A.C. 1989. Ecotropic retroviruses of nonirradiated and irradiated C57BL/10.F mice. Dissertation for Degree of Doctor of Philosophy, University of Tennessee, Knoxville.

Steffen, D.L., R. Mural, D. Cowling, J. Mielcarz, J. Young and R. Roblin. 1982. Most of the murine leukemia virus sequences in the DNA of NIH/Swiss mice consist of two closely-related proviruses, each repeated several times. Journal of Virology 43: 127-135.

Stoye, J.P. and J.M. Coffin. 1987. The four classes of murine leukemia virus: structural relationships and potential for recombination. Journal of Virology 61: 2659-2669.

Strand, M. and J.T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: Multiple antigenic determinants of the major internal protein and envelope glycoprotein. Journal of Virology 13: 171-180.

Teich, N., J. Wyke, T. Mak, A. Bernstein and W. Wardy. 1982. Pathogenesis of retrovirus-induced disease. In RNA Tumor Viruses, R. Weiss, N. Teich, H. Varmus and J. Coffin, eds. Cold Spring Harbor Press, Cold Spring Harbor, New York., pp. 785-998.

Temin, H.M. 1964. Homology between RNA from Rous sarcoma virus and DNA from Rous sarcoma virus-infected cells. Proceedings of the National Academy of Sciences 52: 323-329.

Terres, G., S.L. Morrison and G.S. Habicht. 1968. A quantitative difference in the immune response between male and female mice. Proceedings of the Society for Experimental Biological Medicine 127: 664-667.

Torti, S.V., Kwak, E.L., S.C. Miller, L.L. Miller G.M. Ringold, K.B. Myambo, A.P. Young and F.M. Torti. 1988. The molecular cloning and characterization of murine ferritin heavy chain, a tumor necrosis factor-inducible gene. Journal of Biological Chemistry 263: 12638-12644.

Tucker, H.St.G, J. Weans, P. Tsichlis, R.S. Shwartz, R. Khiroya and J. Donnelly. 1977. Influence of H-2 complex on susceptibility to infection by murine leukemia virus. Journal of Immunology 118: 1239-1243.

Varmus, H.E. 1982. Recent evidence for oncogenesis by insertion mutagenesis and gene activation. Cancer Surveys 1: 309-317.

Varmus, H. 1988. Retroviruses. Science 240: 1427-1435.

Varmus, H., N. Quintrell and S. Ottiz. 1981. Retroviruses as mutagens: Insertion and excision of a non-transforming provirus alter expression of a resident transforming provirus. Cell 25: 23-36.

Varmus, H. and R. Swanstrom. 1982. Replication in retroviruses. In: Molecular Biology of Tumor Viruses, R.A. Weiss, N. Teich, H. Varmus and J. Coffins, eds. Cold Spring Harbor Press, New York: pp. 75-117.

Wainberg, M.A., B. Spira, M. Boushira and R.F. Margolese. 1895. Inhibition by human

T-lymphotropic virus (HTLV-I) of T-lymphocyte mitogenesis: failure of exogenous T-cell growth factor to restore responsiveness to lectin. Immunology 54: 1-7.

Weinberg, E.D. 1984. Iron withholding: A defense against infection and neoplasia. Physiology Reviews 64: 65-102.

Werner-Favre, C., T.L. Vischer, D. Wohlwend and R.H. Zubler. 1989. Cell surface antigen CD5 is a marker for activated human B cells. European Journal of Immunology 19: 1209-1213.

Wintrobe, M.M., G.R. Lee, D.R. Boggs, T.C. Bithell, J.W. Athens and J. Foerster. 1974. Clinical Hematology, 7th edition. Lea and Febiger, Philadelphia.

Wyke, J.A. 1986. Viruses and cancer. In Introduction to the Cellular and Molecular Biology of Cancer, L.M. Franks and N.M. Teich, eds. Oxford University Press, Oxford, pp 176-199.

Yetter, R.A., J.W. Hartley and H.C. Morse III. 1983. H-2-linked regulation of xenotropic murine leukemia virus expression. Proceedings of the National Academy of Sciences 80: 505-509.

Yetter, R.A., R.M.L. Buller, J.S. Lee, K.L. Elkins, D.E. Mosier, T.N. Fredrickson and H.C. Morse III. 1988. $CD4^+$ T cells are required for development of retrovirus-induced immunodeficiency syndrome (MAIDS). Journal of Experimental Medicine 168: 623-635.

Zijlstra, M., W.L.E. Vasmel, T. Radaskiewicz, E. Matthews and C.J.M. Melief. 1986. The H-2 complex regulates both susceptibility to mouse viral lymphomagenesis and the phenotype of the virus-induced lymphomas. Immunogenetics 13: 69-76.

Zon, L.I., C. Arkin and J.E. Groopman. 1987. Haematologic manifestations of the human immunodeficiency virus (HIV). British Journal of Haematology 66: 251-261.

VITAE

Jane Frances Gallager was born in Chester, Pennsylvania on April 8, 1963. She attended elementary schools in Concordville, Pennsylvania and Calhoun, Georgia, and graduated from Calhoun High School in June 1981. The following August she entered Oxford College of Emory University as an Oxford Scholar, and in June 1985 received her Bachelor of Science degree in Biology from Emory University in Atlanta, Georgia. In August 1985, she entered the University of Tennessee through the Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee, and in August 1991 received a Doctor of Philosophy degree in Biomedical Sciences.

Jane is married to Dr. Richard Mural. She is presently pursuing post-doctoral research in Environmental Sciences at the Oak Ridge National Laboratories, Oak Ridge, Tennessee.