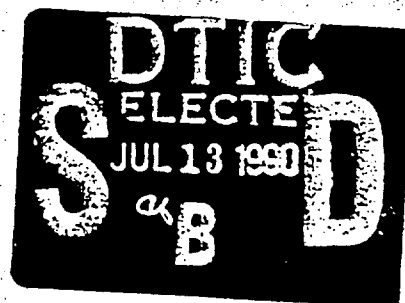


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**Recombinant MCF247 Virus, Leukemogenesis,
and Immunosuppression in AKR Mice**

by

David A. Reichman



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18. (cont.) retrovirus; genetic recombination; leukemogenic; viral supernatant (JG)

19. (cont.) that comparable amounts of viral particles are present per unit volume of viral SN from recombinant strains.

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BOSTON UNIVERSITY

GRADUATE SCHOOL

Thesis

RECOMBINANT MCF247 VIRUS, LEUKEMOGENESIS, AND
IMMUNOSUPPRESSION IN AKR MICE

by

David Alan Reichman

B.S., John Brown University, 1970

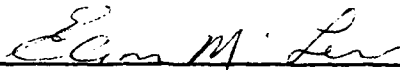
Submitted in partial fulfillment of the
requirements for the degree of

Master of Arts

1990

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RECOMBINANT MCF247 VIRUS, LEUKEMOGENESIS, AND
IMMUNOSUPPRESSION IN AKR MICE

DAVID ALAN REICHMAN

Boston University, Graduate School, 1990

Major Professor: Elinor M. Levy
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ABSTRACT

This paper traces the acquisition of the knowledge of thymic leukemia and lymphoma in the AKR mouse system. Early leukemia and lymphoma development in the AKR mouse strain is caused by the genetic recombination of an endogenous retrovirus and a xenotropic retrovirus in the thymic reticuloepithelial cells. This disease state is inevitable in all individuals in a high incidence strain (e.g. AKR), as the retrovirus is integrated into the germline and is transmitted by vertical transmission. The use of viral recombinants which only contain part of the parent genome cause decreasing amounts of disease with less of the parent genes. Restriction genes in some mouse strains restrict infection and growth of the retrovirus. Supernatant (SN) from viral recombinant strains of MCF247 virus have a suppressive effect both in vitro and in vivo depending on the amount of parental gene present. Reverse transcriptase assays of SN ensure that comparable amounts of viral particles are present per unit volume of viral SN from recombinant strains.

DISCLAIMER

The views presented in this paper are those of the author: no endorsement by the Department of the Navy has been given or should be inferred.

ANIMAL DISCLAIMER

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, DHEW, Pub. No. (NIH)78-23.

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INTRODUCTION

IMMUNOSUPPRESSION AND LEUKEMOGENESIS

Retroviruses which can cause leukemias have been identified in man, mice, birds, cats, primates, chickens, and cattle (Bentvelzen, 1982; Bishop, 1981, 1983; Chatis et al., 1984; Chattopadhyay et al., 1980; Chattopadhyay et al., 1981; Chattopadhyay et al., 1982; Cianciolo et al., 1980; Cianciolo et al., 1985; Cloyd et al., 1980; Cullen et al., 1983; Derse et al., 1986; Dunlap et al., 1979; Engleman et al., 1985; Grant et al., 1984; Greaves, 1986; Pesando et al., 1980; Poiesz et al., 1980; Rosen et al., 1985; Steffen and Robinson, 1982; Tsujimoto et al., 1985). The mechanism by which they cause leukemogenesis, however, is not understood (Chattopadhyay et al., 1982; Cianciolo et al., 1985). Although infection and viral replication (viral expression) seem to be required, they are not enough by themselves to induce leukemia (Jaenisch, 1980). Some evidence seems to indicate that suppression of the immune system may play a part in the induction of leukemia. Kumar, et. al., at Boston University Medical Center showed a correlation between the susceptibility of mice to the leukemogenic effects of Friend-Murine Leukemia Virus (F-MuLV) and the

ability of the virus to induce suppressor cells. Only those strains in which virus could induce suppressor cells developed leukemia (Kumar et al., 1976). The association of immunosuppression and leukemogenesis is also seen with other retroviruses such as MuLV, FeLV, and HTLV (Cianciolo et al., 1985; Engleman et al., 1985; Gallo and Wong-Staal, 1982; Hebebrand et al., 1979). In FeLV infections, the animals often die from opportunistic infections before the leukemia develops (Engleman et al., 1985). Although the mechanism of suppression sometimes involves suppressor cells, changes in either the number or the functional capability of other lymphocyte subsets are also seen. The linkage between suppression in the preleukemic state in the animal models and the eventual development of leukemia is suggestive of the importance of suppression but is not definitive.

It has been shown that when mice are injected with Mo-MuLV (Jaenisch, 1980; Ronchese et al., 1984) in a protocol that allows them to mount an immune response to virus - no leukemia develops and they retain a persistent infection. Older mice develop an immune response but do not develop leukemia. In contrast, if the infection protocol did not produce an immune response, the mice developed leukemia. This is seen in young mice, which when tolerized to Mo-MuLV virus do not generate an immune response, and do develop leukemia (Green et al., 1980; Green, 1980a, 1982,

1983, 1983a, 1983b, 1984, 1984a; Fallers and Hopkins, 1977). Evidence reported by Zanovello et al. suggests that continuous stimulation of virus specific T cells by M-MuLV infected cells might effectively prevent leukemogenesis vice cause lymphoma induction (Zanovello et al., 1984). This evidence correlates well with development of leukemia in an immunosuppressed situation (Jaenisch, 1980).

The results of a variety of experiments both in vitro (Klein, 1987; Langweiler et al., 1983; Mathes et al., 1979; Rudczynski and Mortensen, 1978; Tilkin et al., 1984; Wellman et al., 1984;) and in vivo (Klein, 1987; Mathes et al., 1979; Strayer and Dombrowski, 1988; Tilkin et al., 1985) clearly demonstrate that viral SN have a general suppressive effect on the normal activity of lymphocytes of several species of mice as well as a suppressive effect on the body's in vivo immune response. Several studies have also shown that suppression caused by the virus can be reversed by several means, including a T-cell proliferative response against the major viral envelope glycoprotein gp70. Associated with this response is the production of a lymphokine Interleukin - 3 (IL-3)(Ihle et al., 1981). Reversal also includes cytotoxic T-cell and macrophage induced reversal of immunosuppression by leukemia viruses (Blank and Lilly, 1977; Engers et al., 1984; Flyer et al., 1983; Ishikawa and Dutton, 1980; Merino et al., 1984; Specter et al., 1978;

Yasimizu et al., 1988), allogeneic bone marrow therapy, and active and passive immunization with appropriate oncornavirus (Schaller et al., 1977), immunization using a killed tumor vaccine (Olsen et al., 1976; Olsen et al., 1978) and inhibition of lymphocyte transformation using disrupted murine oncornavirus (Fowler et al., 1977). Purified interferon was found to be inhibitory on the replication of exogenous virus, activation of endogenous virus by IdUrd, and production of virus by chronically infected cells. Interferon did not abort exogenous infection or virus induction by IdUrd, but only delayed appearance of infectious virus. Virus production by chronically infected cells also was suppressed in the presence of interferon; however, after removal of interferon, rapid recovery of virus production occurred. These results indicate that unlike its effect on the majority of viruses, interferon does not inhibit MLV by a general inhibition of viral protein synthesis; rather it appears to inhibit one or more of the later steps in MLV replication which occur after the expression of viral gp antigen. The interferon block of acute exogenous or IdUrd-induced endogenous infection appeared to occur prior to virus assembly, resulting in a marked decrease in the number of free and cell-associated particles. In the chronic infection, however, the interferon treatment only partially prevented assembly and release of virus parti-

cles, but these had markedly reduced infectivity.

Zanovello et al. (Zanovello et al., 1984) found that intrathymic inoculation of Moloney leukemia virus (M-MuLV) would evoke a virus-specific T-cell response which would prevent lymphoma development in mice. Here C57BL6 (B6) adult mice when injected with M-MuLV developed a persistent viral infection of the thymus and T-cells, despite a strong cellular immune response, and then did not develop lymphoma.

Another situation in which outside forces affect the immune system is in the administration of a sublethal dose of radiation (Hooghe and Boniver, 1985). This leads to immunosuppression, and these mice develop radiation leukemia (Yefenof et al., 1984) in contrast to those mice which are not irradiated and do not develop leukemia. Therefore, as shown above, animals with an intact immune response do not appear to develop leukemia. In addition to leukemias caused by suppression of the immune response, there are also radiation leukemia viruses (RadLV). This virus was originally isolated from an X-ray induced lymphoma from a C57BL/6 (B6) mouse (Kaplan, 1950; Lieberman and Kaplan, 1959). Haran-Ghera isolated a retrovirus from the bone marrow of a B6 mouse exposed to fractionated X-ray irradiation that could induce low-incidence leukemias when inoculated intrathymically (i.t.) into B6 animals (Haran-Ghera, 1966). (It was designated D-RadLV. D stands for

dependency on irradiation for induction of high-incidence leukemias.) However, if the mice were exposed to sublethal irradiation (400 rads) shortly before or after virus inoculation, leukemia incidence was increased to 80 - 100 % (Haran-Ghera et al., 1977). Other variants of RadLV have since been isolated (A-RadLV)(Haran-Ghera and Rubio, 1977a; Otten et al., 1976) and evaluated for immunogenic, suppressive and genetic properties in relation to leukemogenic activity (Yefenof et al., 1984).

TROPISM OF VIRUS

The resistance of mouse cells to murine viruses is primarily controlled by the Fv-1 gene (Cloyd et al., 1981; Fallar and Hopkins, 1977; Hartley and Rowe, 1975; Kozak, 1985, 1985a; Mayer et al., 1978; Rassart et al., 1981; Yang et al., 1980). It exists in n or b alleles (named from the prototype tissue sections). Virus strains that grow in Fv-1(nn) cells (e.g., Swiss NIH mice) are said to be N-tropic. Those virus strains that grow in Fv-1(bb) cells (e.g., Balb/c mice) are said to be B-tropic (Gautsch et al., 1980; Ihle and Lazar, 1977). Heterozygous strains of mice (Fv-1(nb)) are resistant to both viruses (here resistance is dominant)(Odaka, 1975). Unlike interference, resistance affects a stage of infection between penetration and integration. Interference is the resistance to further

infection of cells already harboring an identical or related virus whose envelope glycoproteins block the cell receptors for the superinfecting virus. The block in interference is at the adsorption-penetration step, whereas resistance blocks at the next step, between penetration and integration. Retrovirus multiplication requires only three classes of virus-specified polypeptides: one for the outer envelope (env) proteins, a second for the inner capsid (gag) proteins, and a third for the reverse transcriptase (pol) protein. In many retroviruses, part of the gag, pol, or env sequences of the virus are replaced by a large functional oncogenic segment. These retroviruses are defective and unable to multiply by themselves, thus requiring the simultaneous presence of a nononcogenic "helper virus" to provide the missing gene product. This tropism of the virus is determined by a gag (inner capsid proteins) protein present in the virions. This restriction can be overcome by viral mutations which generate NB tropic viruses or by treating the cells with glucocorticoids (Lee et al., 1981; Letarte et al., 1980).

These viruses are also classified by their interaction with cells of different species as defined below:

Ecotropic strains - Viruses multiply only in species being studied, e.g. murine cells - AKR, Balb/c, etc.

Xenotropic strains - Viruses multiply only in cells of other species, e.g. mink cells.

Amphotropic/Dualtropic strains - Viruses multiply in cells of both ecotropic (species being studied, murine - AKR) and xenotropic (cells of other than the species being studied, e.g. mink) strains.

Viruses are also classified according to their life cycle as follows:

Provirus - Viral DNA transcript which has become integrated into cellular DNA.

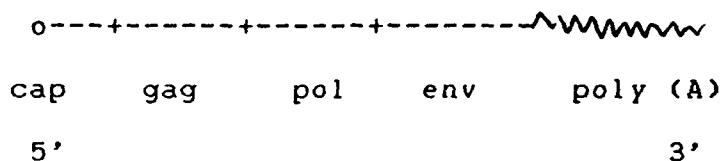
Endogenous Provirus - Retroviruses carried as proviruses in host genome (all cells), transmitted vertically through the germ line. Persist in the animal strain and are inherited like Mendelian genes.

Exogenous Provirus - Proviruses acquired by cells via infection. Exist only in cell clones derived from an originally infected somatic cell. Horizontally transmitted.

CLASSIFICATION AND REPLICATION OF RETROVIRUSES

The viruses of concern here are in the group of RNA-containing tumor viruses (oncornaviruses) - Retroviridae family (Dulbecco, 1980). Various oncoviruses induce sarcomas, leukemias, lymphomas, and mammary carcin-

omas. This family also contains nononcogenic members and is characterized by the presence of reverse transcriptase in approximately 30 molecules per virion. These enveloped viruses are ether sensitive and 100 nm in diameter. The capsid enclosing the single stranded RNA genome is probably icosahedral. Retroviruses are classified as types A, B, C, or D based on their morphological appearance in electron micrographs of thin sections. Mature virions of A particles are found only within cells and have a double shell with an electronlucent center. Mature virions of B particles have an eccentric core, mature C particles have a central core, and D particles have a morphology intermediate between B and C particles. Retroviruses are also classified/described on the basis of their antigenic and enzymatic properties. The outer membrane or envelope of a retrovirus consists of structural glycoproteins (involved in attachment) and lipid components. The inner core (nucleoid) contains viral RNA, reverse transcriptase, and structural proteins. Reverse transcriptase is an enzyme that synthesizes DNA from an RNA template. Reverse transcriptase is also known as RNA dependent DNA polymerase (Ross et al., 1971; Temin and Mizutani, 1970). The nucleic acid is a single-stranded linear molecule with a terminal repeat. Like mRNA, it has poly (A) at its 3' end and a cap at its 5' end. Each virion contains two identical RNA molecules attached by a linkage at one end. Total



The gag gene codes for internal structural proteins, the pol gene codes for reverse transcriptase , and the env gene codes for envelope glycoproteins (Bolognesi et al., 1978). From an immunologic standpoint, type specific and subgroup specific antigens are associated with viral envelope proteins. Group specific antigens are associated with the core protein.

Replication of retrovirus requires entry of the virus into the cell by either fusion with the cell membrane or by phagocytosis. Once inside the cell, there are several steps involved in the macromolecular synthesis. First, cytoplasmic viral RNA is copied into DNA by viral reverse transcriptase. DNA is then transported to the nucleus and becomes cyclic. The DNA transcript next is integrated into cellular DNA. This integrated form of the virus is called the provirus. The integrated viral genome will then be replicated along with cellular DNA. In the next step,

cellular mRNA and progeny viral RNA are synthesized from the integrated DNA template. Finally, translation of viral polyproteins is accomplished, with subsequent cleavage into final protein products. Cleavage of these viral proteins and viral assembly occurs at the cell membrane. The virus is then released by budding, avoiding the death of the host cell by the viral replication process.

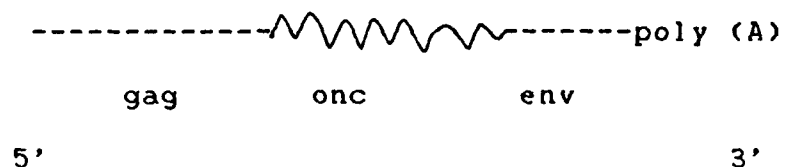
ONCOGENIC LEUKEMIC VIRUSES

We are interested in a subgroup of viruses that are type C oncornaviruses, because this group of oncoviruses contain the viruses which cause the lymphomas and leukemias we are studying - Mo-MULV, MCF-247, etc. There are two main categories of the type C oncornaviruses:

- 1) Nondefective leukosis viruses. These viruses have low oncogenic potential and are often recovered from animals with lymphomas or leukemias (Kozak, 1983, 1984; Kozak et al., 1983; Kozak et al. 1984, 1984a). They are able to multiply in many kinds of cells in vivo or in vitro without causing the cell to become transformed (Cloyd, 1983). They do not require helper viruses because they contain all the genes required for replication (they are frequently carried as proviruses in cellular genomes, and are transmitted vertically, i.e., in the germline). When they do transform cells they generally do so in only a

small proportion of specific target cells (generally in the hematopoietic-lymphoid system) that are in specific stages of differentiation. This oncogenic activity appears to be the result (in some cases) of recombination with another endogenous retrovirus and may entail some changes in the env gene (Kozak, 1983). It is also possible that there may be other mechanisms at work, to include, downstream promotion, or mutation of cellular genes by integration. These non-defective leukosis viruses are present in many animal species as silent proviruses. They can be activated to infectious viruses which are not usually oncogenic.

2) Leukosis or sarcoma viruses. These viruses are usually defective (Friend, Rauscher, Abelson, avian erythroblastosis virus (AEV), myelocytomatosis virus (MC29), and avian myeloblastosis virus) and require a helper virus for replication. The progeny virus usually has the host range of the helper virus. A defective transforming virus may sometimes be derived from its helper virus which has recombined with the cellular genome, acquiring an oncogene (see graphic below).



The viruses we are concerned with can be considered as derivatives of the first category (non-defective leukosis viruses) containing in their genome a fragment of cellular genetic material that confers a high oncogenic activity (Corcoran et al., 1984; Cuypers, 1984). Incorporation of this cellular fragment is often accomplished by a loss of viral genes essential for reproduction. These viruses thus need a non-defective virus as a helper for replication. Viruses in this class which cause leukemias do not transform all the various cells in which they multiply, but only transform specific target cells (generally hematopoietic-lymphoid cells) (Decleve et al., 1974; Graf and Berg, 1978; Jaenisch, 1976, 1977; Lilly and Pincus, 1973; Ostertag and Pragnell, 1978; Rosenberg et al., 1975).

In the 1930's viruses were recognized as causing leukemia in mice in several inbred strains (Bittner, 1936; MacDowell and Richter, 1935). The incidence of leukemia was shown to be selective and age related in several strains. AKR and C58 mice are high incidence strains which develop leukemias at a young age. Balb/c, C57BL, C3H/He mice are low incidence strains which get leukemia at an older age. NIH mice are essentially leukemia free (Chattopadhyay et al., 1974).

We are interested in one of the high incidence viruses in the first group. These viruses are often recovered from

animals with lymphoma or leukemia (Jaenisch, 1980; Haran-Ghera, 1980; Rowe and Pincus, 1972). They multiply without causing cell transformation, require no helper viruses (as they contain all the genes required for multiplication), and transform only a small portion of specific target cells (primarily the cells of the hemopoietic lymphoid system) in certain stages of differentiation (Herr and Gilbert, 1983; Waksal et al., 1976). There are three subgroups of this group in the murine system: 1) G - represented by the AKR strain; 2) FMR - represented by Friend, Moloney, and Rauscher viral strains; and 3) NZB - represented by the Xenotropic strains.

Non-defective, oncogenic murine type C viruses with highly related genomes can differ substantially in their disease-inducing phenotypes. Differences between viral isolates can include the latent period of disease induction or the type of leukemia or lymphoma induced (Cloyd et al., 1981; Datta et al., 1980). Knepper et al. found that the expression of endogenous mouse mammary tumor virus sequences varied over the course of development of the mammary gland during primary pregnancy and lactation in virus-free BALB/c mice. Both the level and temporal regulation of expression were different for long terminal repeat-, env-, and gag-pol-specific RNAs (Knepper et al., 1986). Zielinski et al. found that surface phenotypes in T-cell leukemia are determined by oncogenic retroviruses

(Zielinski et al., 1980). They also found that studies on bone marrow and thymic chimeras showed that the thymic epithelium is programmed to induce preleukemic changes in retrovirus expression and thymocyte differentiation in leukemia susceptible mice (See discussion for additional details). These phenotypes may also vary for a particular virus depending on the genetic constitution of the host (Cloyd et al., 1981; Famulari and Cieplensky, 1984). Two murine leukemia viruses that have been studied quite extensively are Moloney (Mo-MuLV) and the nondefective component of the Friend virus complex (sometimes called Friend helper virus) (DesGroseillers et al., 1983, 1983a; Engleman et al., 1985; Enjuanes et al., 1979). When injected into newborn NFS mice, Mo-MuLV induces T cell lymphomas while Fr-MuLV induces erythroleukemia. (Erythroleukemia is a leukemia of erythroblast-rbc stem cells). Rauscher "Mink Cell Focus-Inducing" (MCF) Virus has also been shown to cause erythroleukemia in mice (Van Griensven and Vogt, 1980).

AKR MICE AND LEUKEMIA

Some of the earliest work in the AKR mouse strain was done by Gross who found that one could take filtered extracts of AKR leukemia cells and transmit leukemia to newborn mice of low-leukemia potential (C3H/He) (Gross,

1951). Because of their immunological immaturity these mice could not reject the virus or tumor cells. The causative agent was identified as virus and became known as AKR virus or Gross virus, often called Gross passage A virus. The differential susceptibility of various mouse strains to spontaneous leukemia correlated with the frequency of spontaneous virus that was produced in the spleen or thymus. Tempelis showed that thymic epithelium controls the expression of preleukemic phenotype and leukemogenic retroviruses (Tempelis, 1987). The target cells are in the thymus and the earliest lesions seen are in the thymus (Cloyd, 1983; DesGroseillers 1983a). If the thymus is removed at birth the development of T-cell leukemia is prevented (Furth, 1946). Peled and Haran-Ghera also reported (Peled and Haran-Ghera, 1985) a high incidence of B cell lymphomas from thymectomized AKR mice which expressed TL.4 antigen. This effect noted by Furth of no leukemia development in thymectomized mice can be reversed if isologous (identical genotype) thymus tissue is subsequently grafted and virus is present. Gross also showed that animals of a high-leukemia strain did not need a post-natal infection to develop leukemia. In these animals, the virus is transmitted congenitally (existing at or usually before birth - vertical transmission) in the germ line. This was shown by putting fertilized ova from a high-leukemia strain into the uterus of a low-leukemia

strain mouse. These implanted ova developed into mice with a high incidence of leukemia. The cause was first thought to be a single virus. Later it was shown to be caused by ecotropic plus non-ecotropic viruses, both transmitted in the germ line (DesGroseillers et al., 1983). In contrast, Feline Leukemia Virus (FeLV) is transmitted horizontally among cats via saliva which contains high titers of infectious FeLV when secreted by persistently viremic carrier animals. In suburban environments, at least 50 % of all pet cats have received FeLV exposure by three years of age (Grant et al., 1984).

AKR mice have an endogenous non-leukemogenic virus - Akv (Pedersen et al., 1981). This virus recombines with a non-ecotropic (from another species) virus in the thymus to produce a virus termed Mink Cell Focus Forming virus (MCF) which is leukemogenic (Hartley et al., 1977; Rowe and Hartley, 1983; Staal et al., 1977). This recombination event is apparently a crucial step in the pathogenesis of the disease in AKR mice resulting in the generation in the pre-leukemic thymus of a novel class of MuLV (Datta et al., 1983). These MCF viruses are so named because of their ability to induce cytopathic foci in monolayers of a tissue culture line of mink cells, and they are recombinants between the endogenous ecotropic virus and endogenous genetic information related to xenotropic viruses (Datta et al., 1983). The viruses are negative in the XC assay, that

is they do not cause plaques (Nowinski et al., 1977; Rowe et al., 1970).

In the normal course of events, high levels of ecotropic MuLV are uniformly found among weanling mice, and the virus can be easily (Jaenisch, 1981) activated from early embryo cells in high ecotropic strains. The onset of disease varies some within individual animals but is generally from 6-12 months (Furth, 1946; Gross, 1951; O'Donnell et al., 1981; O'Donnell et al., 1984; O'Donnell et al., 1985; Pedersen et al., 1980, 1981; Peled et al., 1985). O'Donnell et al. described several stages of leukemogenesis that follow intrathymic injection of an MCF virus (MCF69L1) into young adult AKR/J mice. Stage I represents uniform virus infection of thymocytes without apparent changes in light-scatter properties of the cells or in the expression of several differentiation alloantigens on the major thymocyte subpopulations. Stage II was observed as early as 35 days postinjection and was distinguished by the presence of a subpopulation of cells with restricted transplantation properties which could be resolved from normal thymocytes by flow cytometry. Stage III was observed when considerable enlargement of the thymus had occurred and represented the outgrowth of fully transformed cells that replaced the normal thymocyte subpopulations. Mice at this stage did not yet display signs of frank leukemia, i.e., ruffled fur, hunched

appearance with chest enlargement, labored breathing, or lymph node enlargement. Few individuals survive longer than 12 months. Several studies have been done in which MCF virus has been injected either intraperitoneally (i.p.) or intrathymically (i.t.) into newborn mice (<2 days old). The method of inoculation did not appear to affect the initiation or course of the disease. It appeared to be more important for development of the disease that inoculation of newborn mice was accomplished as close to birth as possible. The disease was considered to have an accelerated onset if it occurred before 6 months of age in AKR mice (Datta, et al., 1980; Gross, 1951; Jaenisch, 1980).

The AKv virus and MCF virus differ almost entirely at three genes at the 3' end: 1) those coding for the long terminal repeat (LTR); 2) the gene for the viral envelope protein gp70; and 3) the gene coding for part of the p15E protein (Datta et al., 1983; Ikeda et al., 1976; Schmidt and Snyderman, 1988). The murine leukemia virus envelope protein is synthesized as a precursor molecule, Pr85env, which is proteolytically cleaved at an arginine residue to produce two mature envelope proteins, gp70 and p15(E).

The LTR gene is important for viral tropism and for regulating transcription of viral genes (DesGroseillers et al., 1983; Temin, 1982). The gp70 protein is a viral surface protein which may be involved in tropism, and

stimulates proliferation of T cells. The p15E protein anchors gp70 to the virus surface and can be immunosuppressive (Cianciolo et al., 1983, Cianciolo et al., 1984; Copelan et al., 1983; Snyderman and Cianciolo, 1984).

Dr. Christie Holland (now at UMASS Worcester) while at MIT in Professor Nancy Hopkins laboratory constructed a set of cloned recombinants between Akv and MCF 247 (Holland et al., 1985). MCF 247 is the prototype of leukemogenic MCF viruses isolated from high incidence leukemia mice. The goal of the construction of these cloned recombinants was to determine which of the genetic elements that distinguish MCF 247 from its ecotropic progenitor, Akv, control the ability of MCF 247 to induce T-cell lymphomas upon injection into AKR mice (Chatis et al., 1983; Holland et al., 1985a, 1987; Li et al., 1984). These molecular clones have been tested for leukemogenicity (Holland, et.al. 1985; Holland et al., 1985a; Holland, et.al. 1987). The recombinant which contains the LTR, gp70, and p15E region from the MCF 247, causes leukemias in approximately 90 percent of the mice within 3 months, just as the parental MCF 247 does. Clones containing any two of the three genes become somewhat less efficient, inducing leukemias in 36-82 percent of the mice in approximately four months. Clones with only one MCF 247 gene are still less efficient at inducing leukemia.

This study utilized supernatants (SN) harvested from cell lines transfected with several different recombinant murine retroviruses derived from MCF 247 obtained from Dr. Christie Holland. These viral SN were used in a variety of tests to try to determine the qualitative and semi-quantitative (relative effect) suppressive or stimulatory effects both in vitro and in vivo.

MATERIALS AND METHODS

Delayed Hypersensitivity Assay in Preleukemic AKR mice

Animals. - Mice of the AKR/J and DBA/2 strains were purchased from The Jackson Memorial Laboratory, Bar Harbor, ME, and were used at ages as indicated. CD-1 mice were a generous gift from Dr. Weldon Lloyd, Goldman School of Graduate Dentistry, Nutrition Department, Boston University, Boston, MA. Mice were housed in cages with constant access to standard mice chow and city tap water. Mice were segregated by age and sex with a maximum of six mice per cage. Newborn pups were kept with the mother in a separate cage until four weeks of age.

Harvesting of Spleen Cells. - Animals were sacrificed with 100 % carbon dioxide as described above, and the spleens removed aseptically and placed in approximately 10 ml of DMEM in a sterile petri dish. Spleen cells were harvested by the glass slide technique. In this technique, a small cut was made at several points around the edge of the spleen. Sterile glass slides were held so the frosted ends were opposing each other. The spleen was picked up between the two ends and gently macerated. Cells were then washed 2 X in Dulbeco's medium (Grand Island Biological Company,

Grand Island N.Y.) containing 10 % FCS (Flow Labs, Inc., McLean, VA), and counted using trypan blue (GIBCO, Madison, WI). The cells were resuspended at the appropriate dilutions as required by the test protocol.

Mitogen Proliferation Assay. - Washed spleen cells at a concentration of 5×10^5 /well were plated in 96-well plates (Falcon Products, Becton Dickinson Labware, Oxnard, CA). 50 lambda (0.05 ml) of the respective viral SN diluted in media as appropriate was added to each well in triplicate groupings for each different condition. 50 lambda (0.05 ml) of the appropriate mitogen was added to each well. Control wells had media substituted for mitogen volume. Mitogen concentrations were as follows:

Concanavalin A (Con A) 1:20 dilution from stock solution;
Phytohemagglutinin (PHA) 1:32 dilution from stock solution.

Mitogens were diluted in sterile media in a 20X concentration and added to wells in volumes of 50 lambda (0.050 ml)/well (final concentration = 1-2 micrograms/ml PHA, and 1-4 micrograms/ml Con A). Total volume each test well 200 microliters (200 lambda). Plates were incubated at 37C, 5 % CO₂ for 48 hours. Plates were then pulsed with 50 microcuries of tritiated thymidine (³H-Thd)(New England Nuclear, Boston, MA) and returned to the incubator (Scientific Products, McGraw Park, IL) for 16 hours. Cells were harvested onto glass fiber filters (Gelman Sciences,

Inc. Ann Arbor, MI), washed, and individual wells counted in a beta scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Proliferation was expressed as counts per minute (cpm) and compared to control wells. Media control contained cells and media only. Mitogen control contained cells, media, and indicated concentrations of mitogen. The rest of the wells each contained cells, media, indicated concentrations of mitogen, and indicated concentrations of viral supernatants (SN).

In vitro Testing after In vivo Exposure and Treatment with Viral SN - The term "in vivo" mitogen studies as used here refers to fact that these mice were injected with appropriate SN at age of less than 2 weeks. Later these mice were used in the in vivo DTH Anergy testing on ears. Mice were sacrificed at the end of the DTH test. The ears were analyzed. Spleens were also harvested from these animals for "in vivo" mitogen proliferation studies, and the spleen cells looked at for their ability or lack of ability to respond to mitogen in tissue culture (no additional virus was added to the cells at this point). This testing arrangement looked at what effect the cells which had "seen" retrovirus particles in injected supernatant up to several months before had at this point in time. The type of cells looked at here is in contrast to the test conditions where naive cells see viral

supernatant in the well for the first time during the mitogen proliferation assay.

DTH Assay Protocol. - The quantitative isotopic ear assay for allogeneic challenge as originally described by Smith and Miller (Smith and Miller, 1979, 1979a) was used. Appropriate numbers of mice (2 - 3) were selected for each group for each mouse strain. Each animal received 2 mg cyclophosphamide by intraperitoneal injection (ip) (unless otherwise specified) on day -2. On day 0, mice were given a sensitizing dose of 5×10^7 lymphocytes from the donor strain or 10^9 SRBC by subcutaneous injection (lymphocytes given to test animals, sheep red blood cells given to control animals). On day 6, a suspension containing 4×10^6 lymphocytes or 4×10^7 SRBC in 0.010 ml of Dulbecco's Phosphate Buffered saline was injected intradermally into the pinna of the left ear. (The pinna is the projecting part of the ear lying outside the head). Mice were briefly anesthetized using chloral hydrate anesthesia during the injection procedure. Four hours later 0.1mM of flurodeoxyuridine (this was used to block endogenous synthesis) was injected ip. Twenty minutes later 2 microcuries I-125-iododeoxyuridine was injected ip. Sixteen hours later the mice were sacrificed by injecting 100 % Carbon Dioxide gas into a sealable glass container. The left and right ear pinnas were removed from each mouse.

The ears were counted individually in a gamma counter. The results are expressed as the average ratio of the counts in the test ear to the control ear for each mouse. Results for similar groups of mice were averaged and were expressed as percent of control ear.

Reverse Transcriptase Assay.

Reagents. - The procedure used was from Dr. C. A. Holland's Laboratory at the University of Massachusetts at Worcester, Massachusetts. Reagents utilized in this procedure included Nonidet P40 (NP 40)(Particle Data Laboratories, 115 Hahn Street, Elmhurst, IL 60126 (312)-832-5653), cold dTTP 2', 3', Dideoxythymidine 5'-Triphosphate(pfs)(Sigma Catalog Number D1013 or BMC Catalog Number 348732), Tritiated Thymidine 5'Triphosphate tetra sodium salt (methyl-3H)(3H-dTTP)(NEN), Polyadenylic Acid (5' poly rA)(Sigma Catalog Number P8652 or BMC Catalog Number), Oligo d(pT12-18)(pfs Sigma Catalog Number 02127 5 Units 33.10 Lithium Salt) or (Collaborative Research Catalog Number 12136), 0.1M Na pyrophosphate solution (Sigma).

RT reagent mixture consisted of the following: 1 M tris HCl pH 8.3, 0.5 ml; 1 M DTT, 0.2 ml; 0.02 M MnCl₂, 0.1 ml; 1 M NaCl, 0.6 ml; 1 % NP 40, 0.5 ml; 1 mM d TTP, 0.02 ml (20 lambda); 5 mg/ml poly rA, 0.02 ml; distilled water was added to bring volume of mixture to a total 5.0 ml.

Scintillation cocktail consisted of 4 L toluene, 15.2 g PPO, and 0.19 g POPOP. Bray's solution (available from NEN or National Diagnostics) was an acceptable alternative.

Assay Procedure. - Supernatant (SN) from plates of virally infected cell lines was harvested and pelleted in a tabletop IEC centrifuge at 1100 rpm for 10 minutes. One ml of this SN was pipetted into an Eppendorf microfuge tube with hinged lid. This was then pelleted in a Brinkman centrifuge for 30 minutes (approx. 20,000 x g - 15,000 rpm). The side of the tube on which the virus pellet will collect was marked (if the tube is always placed with the cap hinge on the outside or rim, the pellet will be located on that side of the tube). After centrifugation, the SN was removed with a pasteur pipette. PELLETS WAS NOT DISTURBED. The pellet was resuspended in 50 lambda of RT Mixture (diluted 1:1 with distilled water) and vortexed hard to resuspend pellet. The tube was incubated 60 minutes at 37 C. Twenty-five lambda of reaction mixture was spotted on to Whatman 540 filter paper circles (presoaked in 0.1M Na pyrophosphate and dried - will keep for months) and allowed to dry.

The filter paper circle (containing the dried reaction mixture and sample) was washed 4 times as follows: ALL SOLUTIONS WERE KEPT COLD AND ON ICE. Filter paper circles were washed 3 X with 5 % TCA (3 washes of 500 ml each on ice). The first wash was for 15 minutes, the next two

washes were for 10 minutes each. The filter paper disks were changed to a new beaker each time using forceps (the filter paper was ALWAYS handled with forceps - at all steps of preparation and testing). The fourth wash was with 95 % EtOH for 2 minutes. The filter papers were allowed to dry completely, then beta emissions of filter paper in 5 ml of the scintillation cocktail counted. The cocktail mixture contained approximately 500 microCuries of ³H dTTP.

Viral Supernatant Production. - Virally infected cell lines were plated in a sterile petri dish or tissue culture flask in media with protein (10 % FCS). The cell culture was allowed to grow to confluency. Media was removed, cell layer gently rinsed 2 X with 10 ml of media without protein and 10 ml of media without protein replaced. Plate or flask was then left in incubator overnight. SN was harvested after 24 - 48 hours. Supernatant was sterilized by filtration and tested on cells in lymphocyte proliferation assay. Aliquot was tested in reverse transcriptase assay to determine the number of viral particles per unit volume. Volume was adjusted (concentrate or dilute) as needed to obtain the desired concentration of viral particles. Sterile filtered viral SN was carefully labeled, aliquoted in cryopreservation vials and stored in liquid nitrogen vapors. Volume for storage was 0.5 - 1.0 ml/vial.

Tissue Culture Solutions. - The following tissue culture media solutions were used. Formulation is as described in the Tissue Culture Products handbook by Gibco Laboratories, 3175 Staley Road, Grand Island, N.Y. 14072. Media formulations included Penicillin-Streptomycin solution, RPMI 1640, HBSS without Ca^{++} , Mg^{++} , or Phenol Red, Fungizone, and Trypsin-EDTA solution.

RESULTS

The work of this paper looked at immunosuppression in vitro and in vivo, as part of the overall leukemogenesis picture. Most of the figures shown in this paper are from an individual experiment but are representative of the data obtained from multiple experiments with the various viral SN. In general we looked at the proliferation of lymphocytes in the presence of mitogen (Con A and PHA), in the presence of viral SN in young AKR mice, old AKR mice, and CD-1 mice, with a readout of radiolabeled thymidine incorporation by the cultured lymphocytes. Graphic comparisons were made between the three groups of mice for the same viral SN (on a separate Figure) and separate comparisons were made for each group of mice by themselves with all of the viral SN. Control values for each group are included on each figure. Data figures also included the DTH studies and compared those results to the in vitro mitogen studies. There are also summary figures which looked at in vitro mitogen studies, in vivo mitogen studies (DTH Anergy) of all mice as a whole and of mice by groups of mice (AKR vs CD-1, etc.), all looked at in the context of suppression vs no effect vs stimulation.

Previous work by Holland et al., (Holland et al., 1985) demonstrated that the degree of parental genes which were present in the genome of the viral recombinants had a

definite correlation with the occurrence and the severity of the disease. If at least three of the parts of the virion were present the viral recombinant SN was more suppressive and leukemogenic than those recombinants which had any two of the parts. Those which had only one part of the parental genome were not very leukemogenic. LTR sequences of murine leukemia viruses are not known to encode proteins, but do determine several viral functions (Lenz et al., 1984). Linney et al. showed that Moloney murine leukemia virus (M-MuLV) infection of embryonal carcinoma (EC) cells resulted in the integration of proviral DNA into the host cell genome, but not in virus production (Linney et al., 1984).

Virus derived from the three molecular clones of MCF247 all induced thymic lymphomas in AKR mice with a time course and incidence essentially like that of MCF247 virus itself. The recombinant Sst10/SC-1, in which the entire 3' half of the genome is derived from MCF was as leukemogenic as MCF247. The 3' half of MCF247 was divided into three fragments and used to construct recombinants that contain just a gp70 coding segment, just a segment Prp15E (P15E), or just the LTR of MCF247, or any two of these elements. Recombinant W5-1/SC-1 with just the LTR was weakly leukemogenic (less than one third of the animals becoming diseased after a long latent period. When the recombinant carried two of the three elements such as recombinant

W12-3/SC-1 with a gp70 and LTR from MCF247, it caused almost as high an incidence of disease as MCF 247, with only a longer latent period. Recombinant W16-1/SC-1 with the whole MCF247 genome except for the gp70 was the most surprising. Though this virus differed from another recombinant (1) only in having the 5' half of its genome derived from MCF247, it caused a significantly larger percentage of the animals injected with it to get leukemia (82 percent vs. 65 percent). This demonstrated that sequences in the 5' half of the genome can contribute to leukemogenicity in conjunction with appropriate 3'-end elements.

The first four figures (Figures 1 - 4) show what percentage of mice were suppressed, showed no effect, or were stimulated by the viral supernatants as indicated for each each group of mice. The features that each recombinant strain contains from the MCF247 parental strain are indicated at the bottom of each graph. The features of each parental strain are also included. Total mice tested and number of mice in each of the three categories are also indicated. Figure 1 looks at all mice for in vitro testing. In general the viral SN which had a complete retrovirus or 75% of it tended to be suppressive (MCF247, MCF30-2, W16-1/SC-1, and Sst10/SC-1). W12-3/SC-1 contained only two of the features of the parental strain and was less suppressive, whereas W5-1/SC-1 contained only one

Figure 1. In Vitro Mitogen Testing - All Mice. In vitro mitogen testing for purposes of this paper means that spleen cells "see" virus for the first time in the tissue culture plate during the 72 hour culture period. This is in contrast to the test conditions in Figures 19 and 20. Suppression was not defined in absolute terms but was based on the values shown in Figures 5-12 and Figures 13-18. In general, suppression was greater than 15 % less activity than control values, no effect was in the 30 % range ($\pm$ the control value) around the control, and stimulation was considered greater than 15 % more than the control value for a particular test category. This information was then looked at in terms of the percentage of mice that were tested that showed either suppression, no effect, or stimulation when in the presence of the various viral recombinant supernatants. These three categories were looked at in All Mice (Figure 1), AKR Mice (Figure 2), CD-1 Mice (Figure 3), and DBA Mice (Figure 4). Viral recombinant strain features are given on each graph as an aid to interpretation.

In Vitro Mitogen Testing

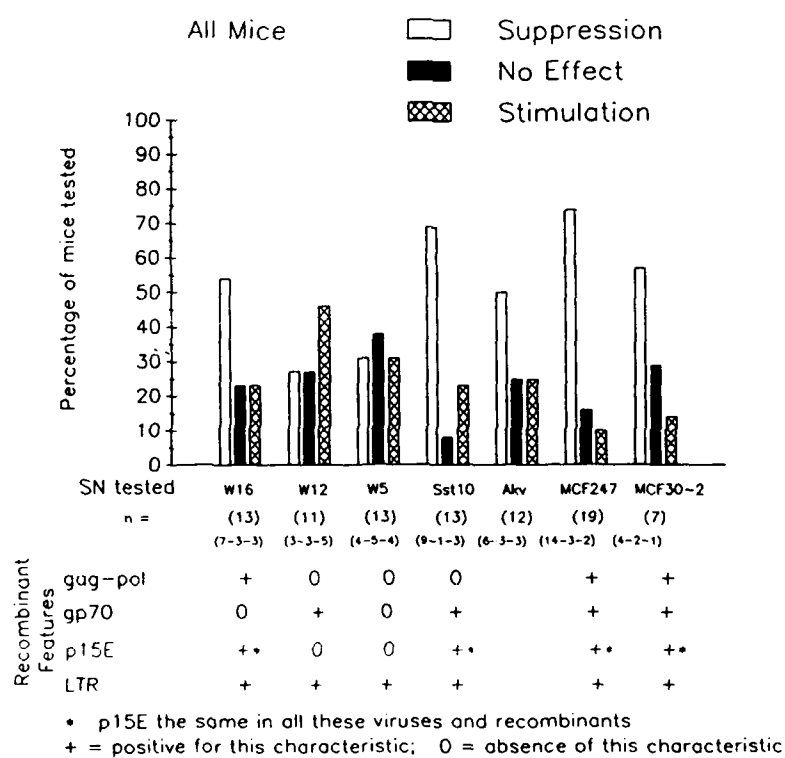


Figure 1

Figure 2. In Vitro Mitogen Testing - AKR Mice. This graph looks at in vitro mitogen testing for AKR mice as described in Figure 1. The experimental conditions and evaluation criteria are as described in Figure 1.

In vitro Mitogen Testing

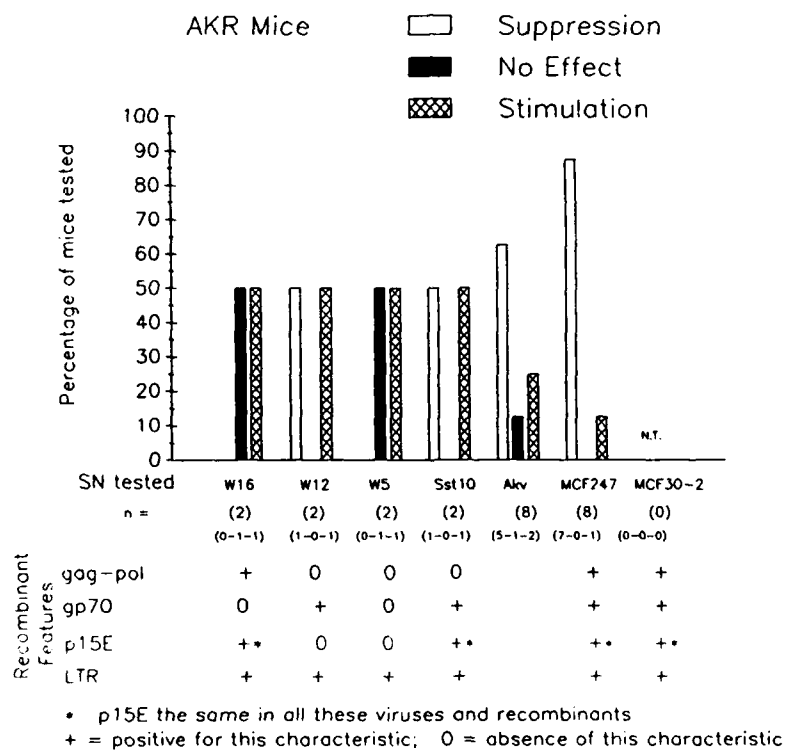


Figure 2

Figure 3. In Vitro Mitogen Testing - CD-1 Mice. This graph looks at in vitro mitogen testing for CD-1 mice as described in Figure 1. The experimental conditions and evaluation criteria are as described in Figure 1.

In vitro Mitogen Testing

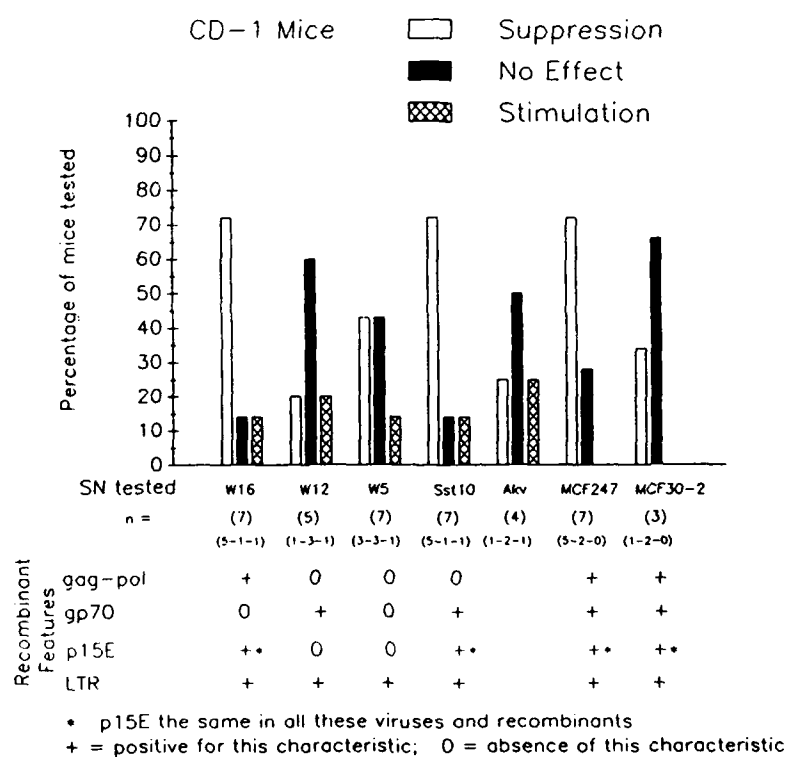


Figure 3

Figure 4. In Vitro Mitogen Testing - DBA/J Mice. This graph looks at in vitro mitogen testing for DBA/J mice as described in Figure 1. The experimental conditions and evaluation criteria are as described in Figure 1.

In vitro Mitogen Testing

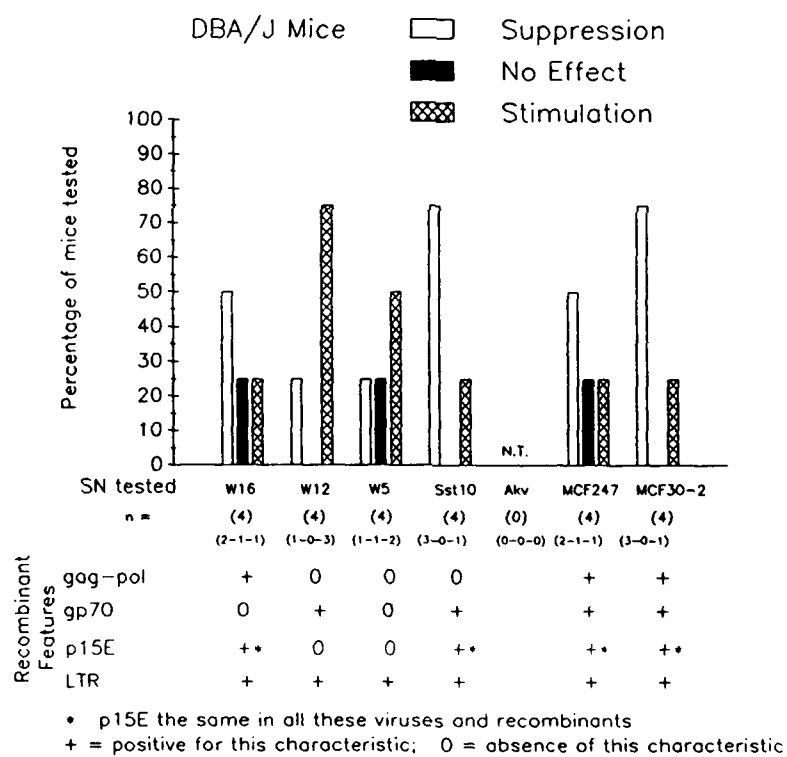


Figure 4

feature of the parental strain (LTR) and was essentially equivocal in it's ability to cause suppression, no effect, or to stimulate proliferation of lymphocytes in vitro.

Upon closer inspection, it is seen in Figure 2 that AKR mice in vitro mitogen testing with the recombinant viral SN was equally divided between the categories of no effect and stimulation. Both MCF247 and MCF30-2 were suppressive on in vitro mitogen testing. Only W16-1/SC-1, Sst10/SC-1, and MCF247 were suppressive on in vitro testing in CD-1 mice (Figure 3). DBA/J mice showed some suppression with W16-1/SC-1 and MCF247, and more suppression with Sst10/SC-1 and MCF30-2 (Figure 4).

Lymphocyte Proliferation Assay

Figures 5 - 8 show the effect of SN from four viral recombinants, W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 from parental MCF247 on in vitro proliferation with Con A mitogen on murine lymphocytes - from three sources - Young AKR mice, Old AKR mice and CD-1 mice. Figures 9 - 12 show the effect of SN from four viral recombinants, W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 from parental MCF247 on in vitro proliferation of murine lymphocytes with PHA from the same three sources. These graphs are the results of a single experiment performed in triplicate wells for each condition, but are representative of the data obtained in multiple experi-

Figure 5. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant W16-1/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (Con A). The results are from the spleen cells of one mouse, but are representative of multiple experiments. Three categories of mice were used: 1) Young AKR (<4 months old), 2) Old AKR (>6 months old), and 3) CD-1 mice (2-4 months old). Each test well contained 100 lambda of spleen cells at a concentration of 0.5×10^6 per ml or 5×10^4 per well, 50 lambda of Concanavalin A (Con A - 1:20 dilution from stock), and 50 lambda of SN from viral recombinant W16-1/SC-1 at indicated dilutions. Media control wells contained only media and cells. Mitogen control wells contained only media, cells, and mitogen (Con A 1:20 dilution from stock). All conditions were setup in triplicate wells. The abscissa represents the reciprocal of the dilution of the viral supernatant and the media and mitogen controls. The ordinate represents the the average counts (scintillations per minute) of ^3H -methyl-thymidine for each group of triplicate wells. Plates were cultured in 5% carbon dioxide, 37°C for 72 hours, trays then pulsed with 1 microcurie of radiolabeled thymidine diluted in media in a volume of 25 lambda/well. Plates were placed back in culture for 16 hours, then harvested onto glass fiber filter strips, dried at 37°C for one hour. Individual disks were then placed in scintillation vials, 3 ml of scintillation fluid added to each vial, vials shaken, and allowed to sit in a dark cool place for at least 45 - 60 minutes. Vials were then counted in a beta scintillation counter with appropriate settings to determine the counts/minute. These values are expressed here in average counts per minute.

Lymphocyte Proliferation Assay

SN from recombinant W16-1/SC-1

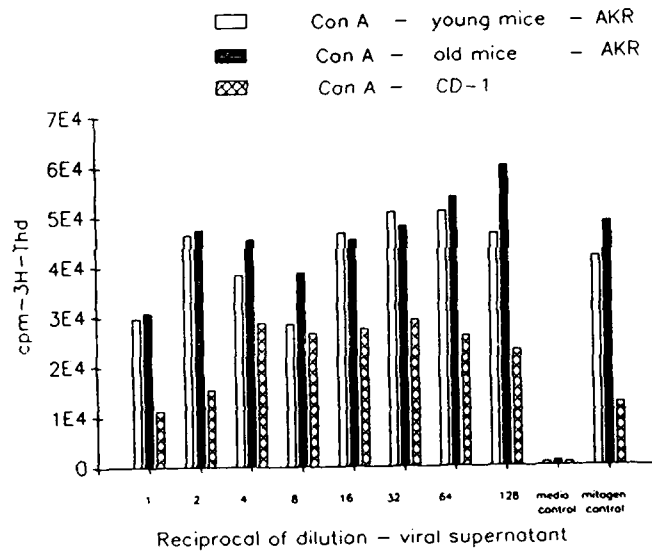


Figure 5

Figure 6. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant W12-3/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (Con A). These results are from the cells from one mouse spleen, but are representative of multiple experiments. The conditions for this experiment are as described in Figure 5.

Lymphocyte Proliferation Assay

SN from recombinant W12-3/SC-1

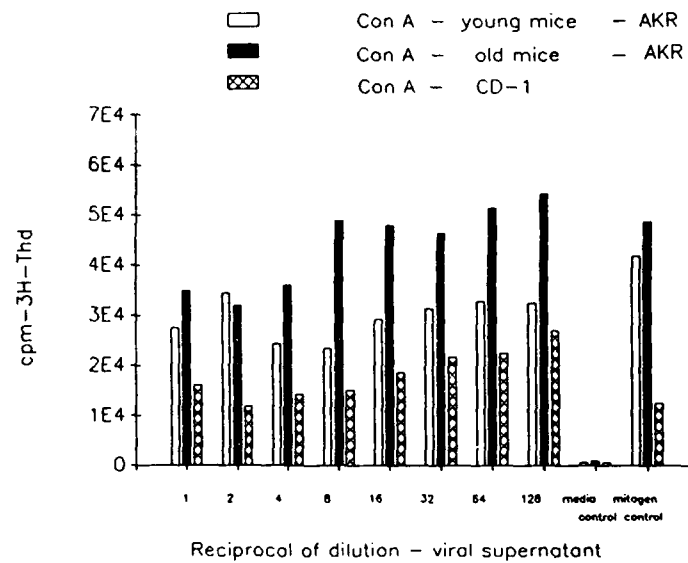


Figure 6

Figure 7. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant W5-13/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (Con A). These results are from the cells from one mouse spleen, but are representative of multiple experiments. The conditions for this experiment are as described in Figure 5.

Lymphocyte Proliferation Assay

SN from recombinant W5-1/SC-1

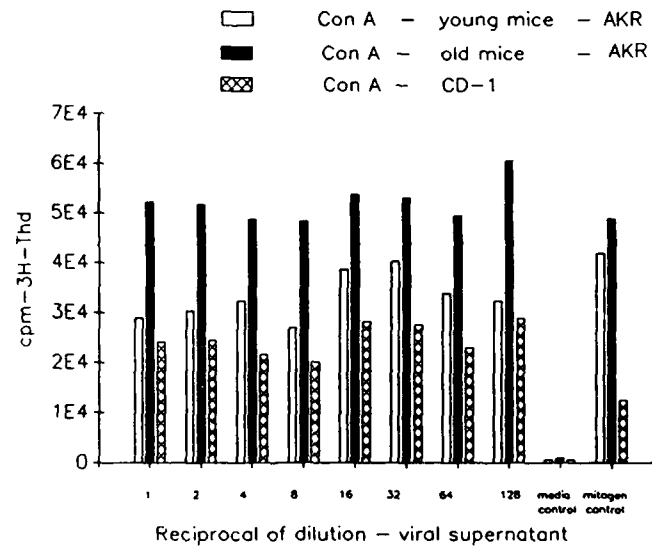


Figure 7

Figure 8. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant Sst10/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (Con A). These results are from the cells from one mouse spleen, but are representative of multiple experiments. The conditions for this experiment are as described in Figure 5.

Lymphocyte Proliferation Assay

SN from viral recombinant Sst10

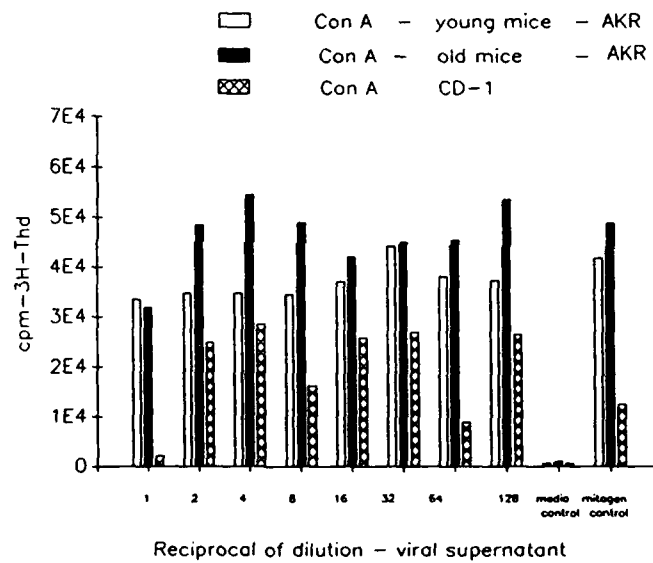


Figure 8

Figure 9. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant W16-1/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (PHA). Three categories of mice were used: 1) Young AKR (<4 months old), 2) Old AKR (>6 months old), and 3) CD-1 mice (2-4 months old). Each test well contained 100 lambda of spleen cells at a concentration of 0.5×10^6 per ml or 5×10^4 per well, 50 lambda of Phytohemagglutinin (PHA - 1:32 dilution from stock), and 50 lambda of SN from viral recombinant W16-1/SC-1 at indicated dilutions. Media control wells contained only media and cells. Mitogen control wells contained only media, cells, and mitogen (Con A 1:32 dilution from stock). All conditions were setup in triplicate wells. The abscissa represents the reciprocal of the dilution of the viral supernatant and the media and mitogen controls. The ordinate represents the the average counts (scintillations per minute) of ^3H -methyl-thymidine for each group of triplicate wells. Plates were cultured in 5% carbon dioxide, 37°C for 72 hours, trays then pulsed with 1 microcurie of radiolabeled thymidine diluted in media in a volume of 25 lambda/well. Plates were placed back in culture for 16 hours, then harvested onto glass fiber filter strips, dried at 37°C for one hour. Individual disks were then placed in scintillation vials, 3 ml of scintillation fluid added to each vial, vials shaken, and allowed to sit in a dark cool place for at least 45 - 60 minutes. Vials were then counted in a beta scintillation counter with appropriate settings to determine the counts/minute. These values are expressed here in average counts per minute.

Lymphocyte Proliferation Assay

Sn from viral recombinant W16-1/SC-1

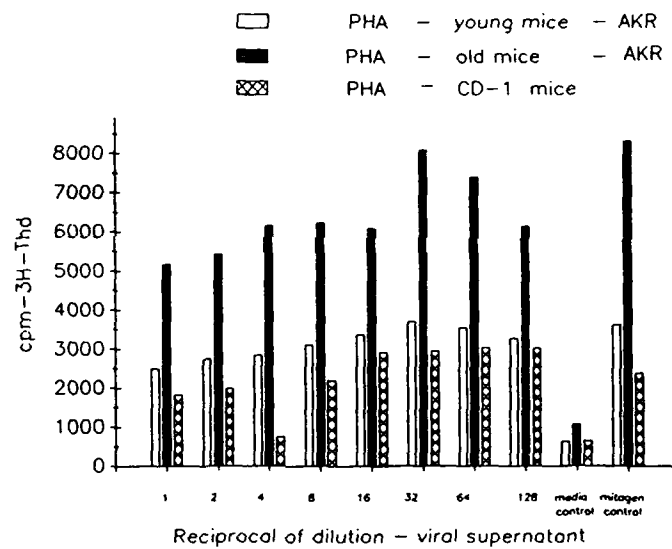


Figure 9

Figure 10. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant W12-3/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (PHA). These results are from the cells from one mouse spleen, but are representative of multiple experiments. The conditions for this experiment are as described in Figure 9.

Lymphocyte Proliferation Assay

SN from viral recombinant W12-3/SC-1

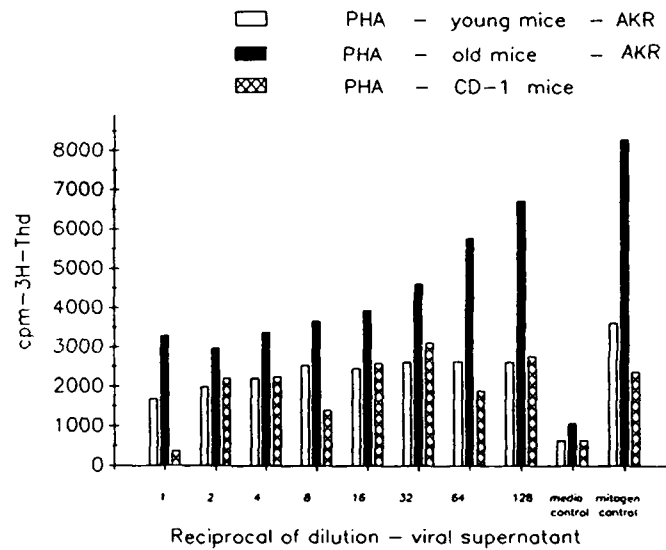


Figure 10

Figure 11. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant W5-1/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (PHA). These results are from the cells from one mouse spleen, but are representative of multiple experiments. The conditions for this experiment are as described in Figure 9.

Lymphocyte Proliferation Assay

SN from viral recombinant W5-1/SC-1

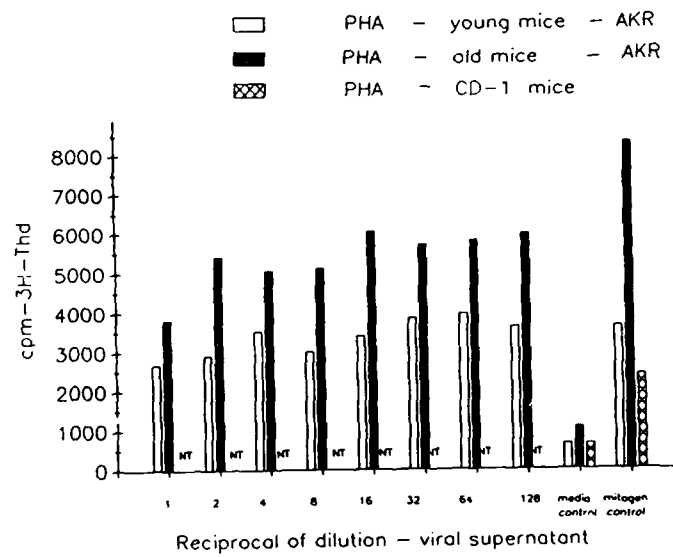


Figure 11

Figure 12. Murine spleen cells were tested in 96 well tissue culture plates in an in vitro assay to determine the effect of viral recombinant supernatant Sst10/SC-1 on lymphocyte proliferation in the presence of a standard tissue culture mitogen (PHA). These results are from the cells from one mouse spleen, but are representative of multiple experiments. The conditions for this experiment are as described in Figure 9.

Lymphocyte Proliferation Assay

SN from viral recombinant Sst10

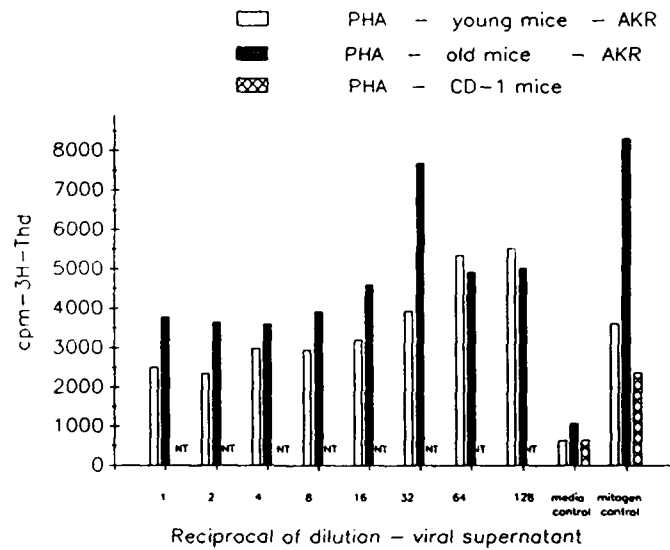


Figure 12

ments. Both control and experimental cultures were run in triplicate during each experiment. See Figures 1 - 4 and Figures 19 - 20 for the number of mice used in the various categories.

Figures 5 through 12 show the results obtained in cpm of radiolabeled 3H-methyl-thymidine incorporation from in vitro mitogen proliferation assays. Lymphocytes harvested from spleens of mice of various ages and species (See specific Figures) under sterile conditions were plated in microculture plates. The reciprocal of the dilutions of the viral SN tested were: 1, 2, 4, 8, 16, 32, 64, 128. The effect (suppressive, no effect, or stimulatory) of the viral SN in the presence of cellular mitogens Con A or PHA was evaluated in the lymphocyte proliferation assay. Several points can be made here after observation of the results. Young AKR mice were somewhat more suppressed relative to control than were the old AKR mice. Cells from the older mice tended to be more refractive to stimulation by the viral SN. This is borne out in further detail in the discussion of the DTH testing. There was also variation as to how dilute the supernatant could be and still have a suppressive effect. This is most likely a result of a variation in the concentration of the viral particles per unit volume in the various supernatants. This is discussed in more detail in the section on Reverse Transcriptase testing results. Variation was also seen in

the degree of suppression between the supernatant from parental virus strain (MCF247) and those of the recombinant viral supernatants (W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1).

Figures 13 - 18 examine the raw data for individual viral recombinant SN shown in Figures 5 - 12 from the perspective of percent of control by age group for each mitogen. Here it can be seen that Sst10/SC-1 SN which contains three of the four parts (gp70, p15E, LTR) of the parental virus MCF247 was generally more suppressive than W12-3/SC-1 and W5-1/SC-1. The remaining virus had high levels of suppressive activity.

There was also variability in the degree of suppression observed between the two mitogens used. PHA mitogen in the test system seemed to allow more suppression in the Old AKR mice than did Con A. All recombinant SN seemed to lose their suppression activity somewhat equally around a dilution of 1:8 or 1:16 in Con A in Old AKR mice (Figure 13) and somewhat less so in PHA in Old AKR mice (Figure 14). A similar result was seen in Young AKR mice (Figures 15 and 16) with the loss of suppression occurring around a dilution of 1:16 or 1:32. There appeared to be some stimulation by Sst10/SC-1 SN at very weak dilutions (1:64 or 1:128) in Young AKR mice in PHA (Figure 16) and minimal stimulation in Con A (Figure 15) in this group of mice.

Figure 13. Mitogen Proliferation in Old AKR Mice - Con A. This figure looks at the data generated in figures 5 through 8 from a perspective of age matched mitogen proliferation studies. The effect on mitogen proliferation of lymphocytes when cultured in the presence of supernatants (SN) from viral recombinants W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 at eight serial dilutions was examined. The results are presented as the percentage of lymphocyte proliferation activity relative to the control values for that mitogen stimulated age group of cells. The abscissa represents the reciprocal of the dilution of the viral supernatant from each of the recombinant viral strains. The ordinate represents the percentage of lymphocyte proliferation activity relative to the control values for each average of triplicate wells. This was determined by dividing the triplicate average by the control triplicate average for each data point.

Mitogen Proliferation in Old AKR Mice – Con A

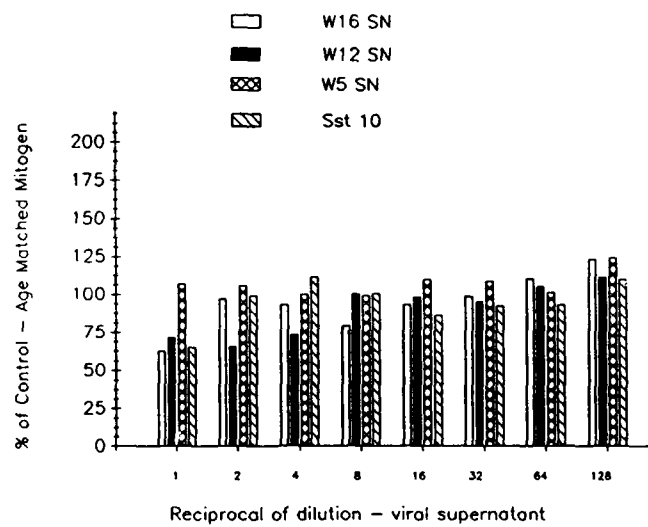


Figure 13

Figure 14. Mitogen Proliferation in Old AKR Mice - PHA. This figure looks at the data generated in figures 9 through 12 from a perspective of age matched mitogen proliferation studies. The effect on mitogen proliferation of lymphocytes when cultured in the presence of supernatants (SN) from viral recombinants W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 at eight serial dilutions was examined. The results are presented as the percentage of lymphocyte proliferation activity relative to the control values for that mitogen stimulated age group of cells. The abscissa represents the reciprocal of the dilution of the viral supernatant from each of the recombinant viral strains. The ordinate represents the percentage of lymphocyte proliferation activity relative to the control values for each average of triplicate wells. This was determined by dividing the triplicate average by the control triplicate average for each data point.

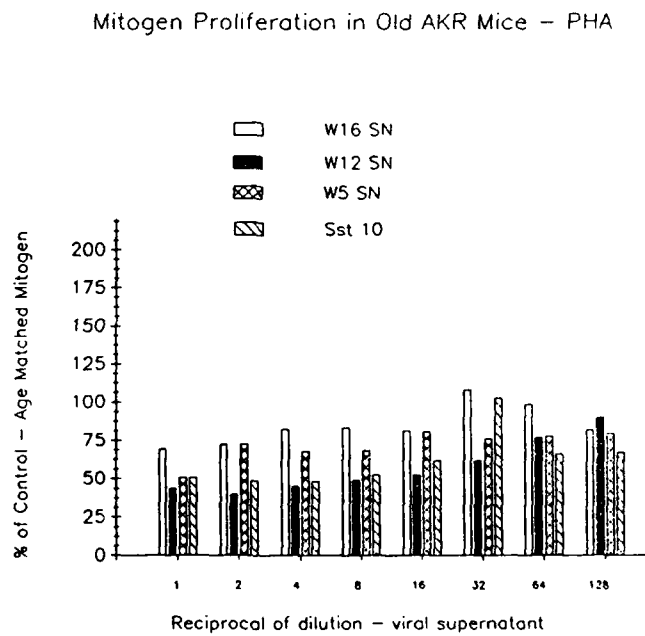


Figure 14

Figure 15. Mitogen Proliferation in Young AKR Mice - Con A. This figure looks at the data generated in figures 5 through 8 from a perspective of age matched mitogen proliferation studies. The effect on mitogen proliferation of lymphocytes when cultured in the presence of supernatants (SN) from viral recombinants W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 at eight serial dilutions was examined. The results are presented as the percentage of lymphocyte proliferation activity relative to the control values for that mitogen stimulated age group of cells. The abscissa represents the reciprocal of the dilution of the viral supernatant from each of the recombinant viral strains. The ordinate represents the percentage of lymphocyte proliferation activity relative to the control values for each average of triplicate wells. This was determined by dividing the triplicate average by the control triplicate average for each data point.

Mitogen Proliferation in Young AKR Mice – Con A

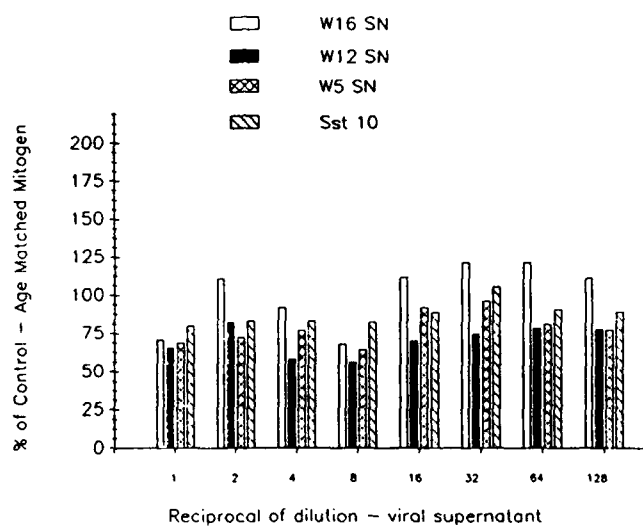


Figure 15

Figure 16. Mitogen Proliferation in Young AKR Mice - PHA. This figure looks at the data generated in figures 9 through 12 from a perspective of age matched mitogen proliferation studies. The effect on mitogen proliferation of lymphocytes when cultured in the presence of supernatants (SN) from viral recombinants W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 at eight serial dilutions was examined. The results are presented as the percentage of lymphocyte proliferation activity relative to the control values for that mitogen stimulated age group of cells. The abscissa represents the reciprocal of the dilution of the viral supernatant from each of the recombinant viral strains. The ordinate represents the percentage of lymphocyte proliferation activity relative to the control values for each average of triplicate wells. This was determined by dividing the triplicate average by the control triplicate average for each data point.

Mitogen Proliferation in Young AKR Mice - PHA

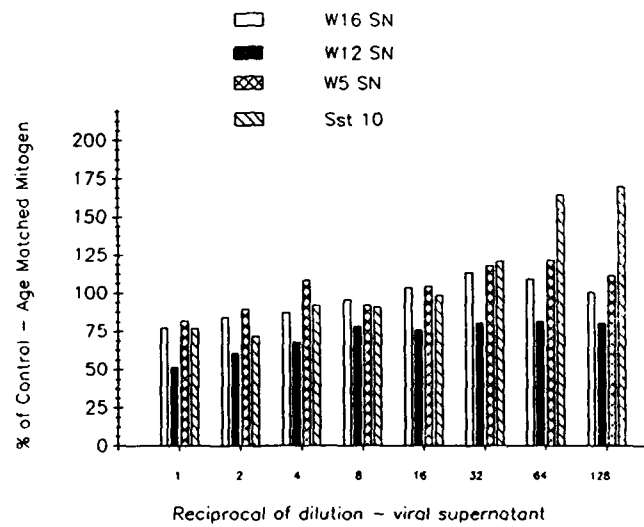


Figure 16

Figure 17. Mitogen Proliferation in CD-1 Mice - Con A. This figure looks at the data generated in figures 5 through 8 from a perspective of age matched mitogen proliferation studies. The effect on mitogen proliferation of lymphocytes when cultured in the presence of supernatants (SN) from viral recombinants W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 at eight serial dilutions was examined. The results are presented as the percentage of lymphocyte proliferation activity relative to the control values for that mitogen stimulated age group of cells. The abscissa represents the reciprocal of the dilution of the viral supernatant from each of the recombinant viral strains. The ordinate represents the percentage of lymphocyte proliferation activity relative to the control values for each average of triplicate wells. This was determined by dividing the triplicate average by the control triplicate average for each data point.

Mitogen Proliferation in CD - 1 Mice - Con A

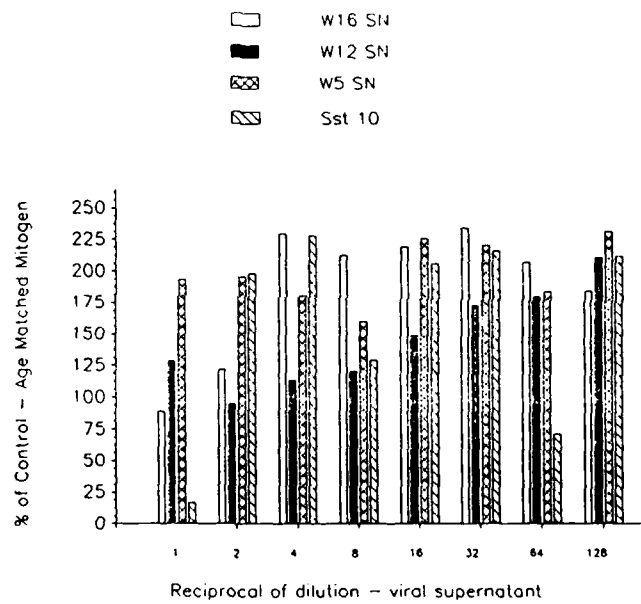


Figure 17

Figure 18. Mitogen Proliferation in CD-1 Mice - PHA. This figure looks at the data generated in figures 9 through 12 from a perspective of age matched mitogen proliferation studies. The effect on mitogen proliferation of lymphocytes when cultured in the presence of supernatants (SN) from viral recombinants W16-1/SC-1, W12-3/SC-1, W5-1/SC-1, and Sst10/SC-1 at eight serial dilutions was examined. The results are presented as the percentage of lymphocyte proliferation activity relative to the control values for that mitogen stimulated age group of cells. The abscissa represents the reciprocal of the dilution of the viral supernatant from each of the recombinant viral strains. The ordinate represents the percentage of lymphocyte proliferation activity relative to the control values for each average of triplicate wells. This was determined by dividing the triplicate average by the control triplicate average for each data point.

Mitogen Proliferation in CD -1 Mice - PHA

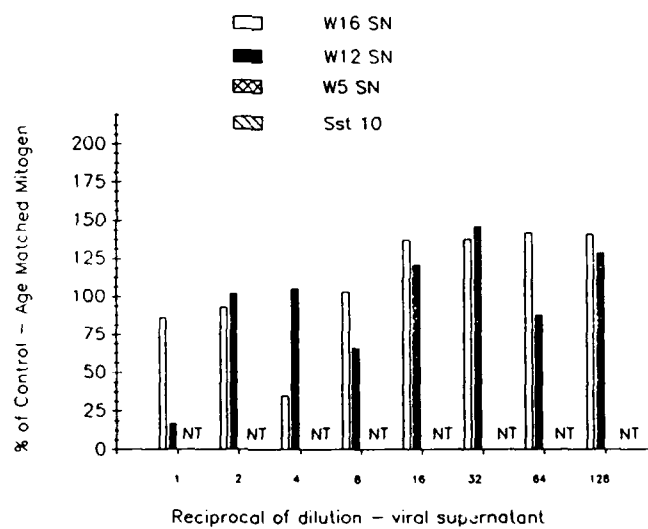


Figure 18

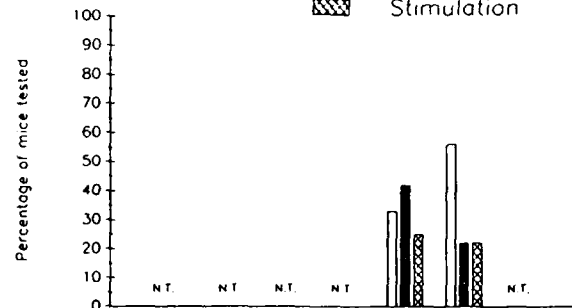
Mitogen proliferation studies in disease free "normal outbred mice" (low leukemogenic) showed interesting results as shown in Figures 17 and 18. W16-1/SC-1 and Sst10/SC-1, each of which contain three parts of the parent virus were slightly suppressive at a 1:1 test mixture but were slightly stimulatory at all other dilutions (1:2 - 1:128) in Con A (Figure 17). There was some variability in the effect in the PHA test group for mitogen proliferation on CD-1 mice (Figure 18).

Figures 19 and 20 examined in vivo mitogen testing. In vivo testing here refers to the testing (in vitro) of spleen cells from mice that were given ip injections of the various viral recombinant and parental viral SN as indicated as newborn (< 2 weeks old) mice. After a period of two to four months, mice were utilized in the DTH testing. Spleen cells were harvested from mice which were sacrificed after the DTH studies. Coordinated and correlated results are shown in Figure 19 for AKR mice, and Figure 20 for CD-1 mice. CD-1 mice served as the control mouse for the DTH studies. There was a good correlation between what was seen in the DTH studies and the "in vivo" mitogen studies in that those mice which showed suppression in the DTH assay were also suppressed in their mitogen induced lymphocyte proliferation response. The only suppression of lymphocyte proliferation in vivo for all mice was with viral recombinant SN Sst10/SC-1 and

Figure 19. In Vivo Mitogen Testing - AKR Mice. In vivo mitogen testing for purposes of this paper refers to the fact that these mice were injected with appropriate SN at age of less than 2 weeks. Later these mice were used in the in vivo DTH Anergy testing on ears. Mice were sacrificed at the end of the DTH test. Ears were analyzed, and spleen cells were looked at for their ability or lack of ability to respond to mitogen in tissue culture (no additional virus was added to the cells at this point (data not shown). This testing arrangement looked at what effect the cells which had "seen" retrovirus particles in injected supernatant up to several months before had at this point in time. The type of cells looked at here is in contrast to the test conditions in Figures 1 - 4. Again, suppression was not defined in absolute terms but was based on the values shown in Figures 5-12 and Figures 13-18. In general, suppression was greater than 15 % less activity than control values, no effect was in the 30 % range (+/- the control value) around the control, and stimulation was considered greater than 15 % more than the control value for a particular test category. This information was then looked at in terms of the percentage of mice that were tested that showed either suppression, no effect, or stimulation when in the presence of the various viral recombinant supernatants. These three categories were looked at in AKR Mice (Figure 19) and CD-1 Mice (Figure 20). Viral recombinant strain features are given on each graph as an aid to interpretation. AKR mice have been shown to have a single anergy for allogeneic stimulus which appears to develop in the preleukemic stage (see text for further discussion and reference). The readout for this anergy is a delayed-type hypersensitivity reaction in the pinna of the ear. This graph depicts those viral SN which were tested in all mice. CD-1 mice were used as the mouse species for the control mice (ears) and received received no injections of viral supernatant. Viral recombinant strain features are given on each graph as an aid to interpretation.

In vivo Mitogen Testing

AKR Mice  Suppression
  No Effect
  Stimulation

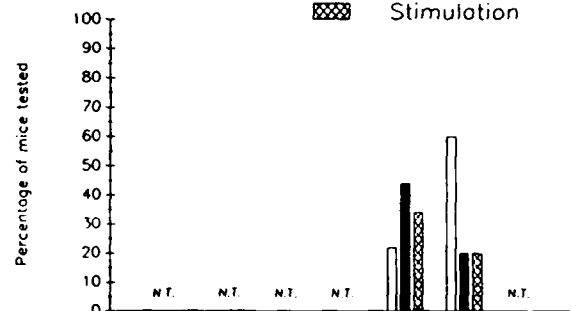


SN tested	W16	W12	W5	Sst10	Akv	MCF247	MCF30-2
n =	(0)	(0)	(0)	(0)	(9)	(12)	(0)
	(0)	(0)	(0)	(0)	(4-5-3)	(5-2-2)	(0)
gag-pol	+	0	0	0		+	+
gp70	0	+	0	+		+	+
p15E	++	0	0	++		++	++
LTR	+	+	+	+		+	+

+ p15E the same in all these viruses and recombinants
 ++ positive for this characteristic; 0 = absence of this characteristic

In vivo DTH Testing

AKR Mice  Suppression
  No Effect
  Stimulation



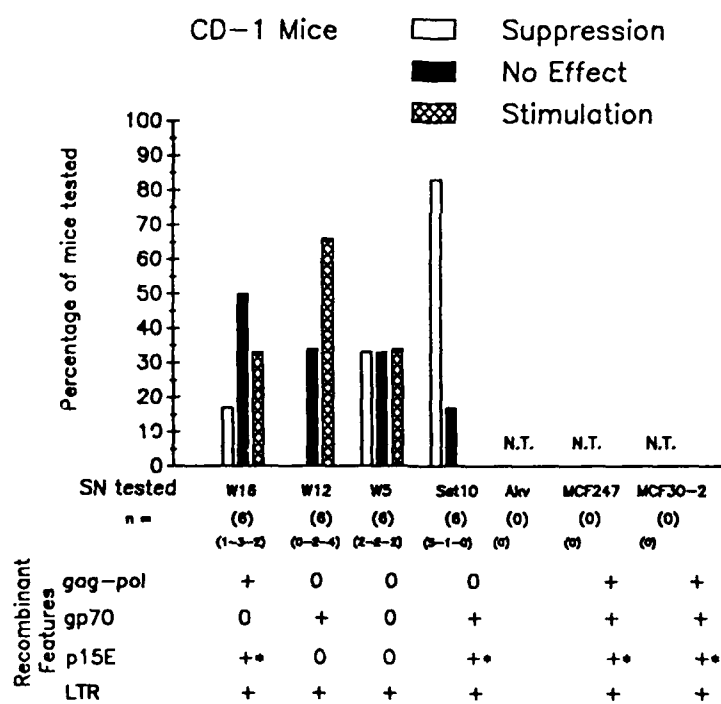
SN tested	W16	W12	W5	Sst10	Akv	MCF247	MCF30-2
n =	(0)	(0)	(0)	(0)	(9)	(10)	(0)
	(0)	(0)	(0)	(0)	(7-4-3)	(6-2-2)	(0)
gag-pol	+	0	0	0		+	+
gp70	0	+	0	+		+	+
p15E	++	0	0	++		++	++
LTR	+	+	+	+		+	+

+ p15E the same in all these viruses and recombinants
 ++ positive for this characteristic; 0 = absence of this characteristic

Figure 19

Figure 20. In Vivo Mitogen Testing - CD-1 Mice. In vivo mitogen testing for purposes of this paper refers to the fact that these mice were injected with media without serum as a set of control mice at an age of less than 2 weeks. Later these mice were used in the in vivo DTH Anergy testing on ears. Mice were sacrificed at the end of the DTH test. Ears from these mice were used as control ears and their values were subtracted from the values seen in the AKR mice. Their spleen cells were looked at for their ability or lack of ability to respond to mitogen in tissue culture (no additional virus was added to the cells at this point (data not shown). This testing arrangement looked at what effect the cells which had "seen" retrovirus particles in injected supernatant up to several months before had at this point in time. The type of cells looked at here is in contrast to the test conditions in Figures 1 - 4. Again, suppression was not defined in absolute terms but was based on the values shown in Figures 5 - 12 and Figures 13 - 18. In general, suppression was greater than 15 % less activity than control values, no effect was in the 30 % range (+/- the control value) around the control, and stimulation was considered greater than 15 % more than the control value for a particular test category. This information was then looked at in terms of the percentage of mice that were tested that showed either suppression, no effect, or stimulation when in the presence of the various viral recombinant supernatants. These three categories were looked at in All Mice (Figure 19), AKR Mice (Figure 20), and CD-1 Mice (Figure 21). aid to interpretation. Figure 20 does not contain in vivo ear graphs as the CD-1 mice were used as the "ear" controls for the other mice tested. Because AKR mice have been shown to carry the gene for leukemia in their germline, and because some studies indicate that there is a fine degree of early suppression or at least viral interaction with the lymphocytes in question it was necessary to choose a species which is considered a low leukemia risk.

In vivo Mitogen Testing



* p15E the same in all these viruses and recombinants
 + = positive for this characteristic; 0 = absence of this characteristic

Figure 20

MCF 247. These mice had significant suppression with MCF247 in the DTH testing. Only in vivo mitogen testing was done on CD-1 mice. Sst10/SC-1 SN was the only recombinant viral SN which was suppressive. The suppression of CD-1 mice "in vivo" mitogen proliferation was very significant in that over 80% of the mice were suppressed.

Delayed-Type Hypersensitivity Testing - Burdick and Williams (1985) discovered a singular anergy for delayed-type hypersensitivity (DTH) in preleukemic AKR mice. This total anergy for DTH against allogeneic cells which developed in AKR mice by 4 to 5 months of age was not related to the route of sensitization or other assay parameters. They did not directly address the relationship of the anergy to lymphoma but the discovered anergy strongly suggests a biological relationship to the leukemic state.

The following procedure was used to assay the effect of viral constructs on DTH activity. Four to six mice per group which had been injected with viral SN at age < 2 weeks were injected on day -2 with 2 mg of cyclophosphamide ip. On day 0, these same mice were injected subcutaneously (sc) with a sensitizing dose (5×10^7) of lymphocytes from donor strains (DBA/J mice) or 10^9 SRBC. On day 6 the mice were injected with a suspension of 4×10^6 lymphocytes or

4 x 10E7 SRBC in 0.01 ml (10 lambda) of Dulbecco's PBS intradermally in the pinna of the left ear. Chloral hydrate suspension was used as the anesthesia. Four hours later all mice were injected ip with 0.1 mM fluorodeoxyuridine to block the endogenous synthesis. Twenty minutes later 2 microCuries of radiolabeled I-125-iododeoxyuridine was injected ip. The mice were sacrificed 16 hours later with 100% carbon dioxide gas. The left and right ear pinnas were removed by transection at the hair line and counted individually in a gamma counter and expressed as average ratio of counts in the test ear to control ear.

Reverse Transcriptase Assay. -

Viral particle concentration assays of viral SN were performed. The tests showed (data not shown) a wide range for the number of viral particles present in various "batches" of viral SN. Premature conclusion of the experimental period did not allow adjusting each viral SN to contain an equal number of viral particles per unit volume and repeating all of the above testing. A repeat of the above (Figures 1 - 20) tests plus additional tests after standardization of the number of viral particles per unit volume for each SN would have been more ideal. The results may not have been any different, but the data would have a higher confidence factor and have been "cleaner" in that there would have been a higher "n" as well as the use

of reagents that were more standardized as to relative equivalency to each other. One would then be in a better position to propose with a high degree of certainty and definity that the effects seen were attributable to a specific part of the retrovirus genome at a particular concentration (dilution).

Additional Testing - If time had been available the following testing would have been completed. First, all viral SN would have been completely analyzed for viral particle concentration and individual SN pooled and/or adjusted by concentration or dilution to a common starting level for all viral SN. Several concentrations of the viral SN could then be tested utilizing serial dilutions of these SN in an effort to determine their effect on in vitro mitogen proliferation, in vivo mitogen proliferation testing, DTH testing, and cytotoxicity testing. An assessment of the state and degree of suppression could include an evaluation of the T-cell and B-cell types and their subtypes. These results would have been generated from a much larger number of total mice and mice in each category and thus reduce the effect individual animals might have on the overall outcome.

DISCUSSION

Leukemogenesis

The mechanism by which retroviruses cause leukemias in many animal species including man is not understood (Chattopadhyay et al., 1982; Cianciolo et al., 1985). It takes more than just viral infection and viral replication (viral expression) to induce leukemia (Jaenisch, 1980). The work by Kumar, et. al. (Kumar et al., 1976) indicating that suppression of the immune system is probably an integral part of the induction of leukemia in the murine system was later expanded upon by the work of others (Cianciolo et al., 1985; Engleman, et. al. 1985; Gallo and Wong-Staal, 1982; Hebebrand, et. al. 1979). When mice, for example, are injected with a dose of Mo-MuLV in a protocol which permits them to mount an immune response to virus, they will not develop leukemia, but will maintain a persistent infection (Huebner et al., 1976; Jaenisch, 1980; Ronchese et al., 1984). This condition of immune non-responsiveness is also seen in young mice which if injected on a protocol in which they do not generate an immune response, do develop leukemia. This is very similar to that seen when leukemia develops in an individual that is immunosuppressed or immunocompromised. Sekine et. al. found that CBA mice

when tested with a rapidly lethal transplanted lymphoma could survive challenge when they were pretreated with allogeneic lymphoma cells or other material expressing murine leukemia virus (MuLV). Protection only resulted when the injected material was positive when assayed for virus (Sekine, et. al. 1975). Those mice which did not produce an immune response developed leukemia.

The antigen phenotype for pertinent antigens is variable throughout the course of the disease. A survey of age-related expression of thymus-leukemia (TL) alloantigens (TL 1,2,4) among bone marrow, spleen, and thymus cells of grossly normal and leukemic AKR/J showed a transient expression of TL antigens on cells in the BM population in 1-20 day old AKR/J mice. The TL antigens then became undetectable until three months of age with reappearance at that time point. Thymocytes expressed TL at 4 months with a maximum level at 6-8 months. TL antigens were expressed on spleen cells from newborn mice with a maximum level of 5-6 months. After the maximum TL expression in all types of cells, levels soon dropped to undetectable levels (Peled 1984).

Virus and the Organ/Tissue Relationship

The viruses we have been concerned with are retroviruses of the group of RNA-containing tumor viruses

(oncornaviruses) - Retroviridae family (Dulbecco, 1980). Within this group we are interested in the related group of viruses that are type C oncornaviruses and contain the viruses which cause the lymphomas and leukemias of interest, namely Mo-MuLV, MCF247, etc. They are non-defective leukosis viruses, have low oncogenic potential, and are often recovered from animals with lymphomas or leukemias (Haran-Ghera, 1980; Jaenisch, 1980; Lilly, et. al. 1975; Rowe and Pincus, 1972; Yanagihara et al., 1982).

They are able to multiply in many kinds of cells in vivo or in vitro without causing the cell to become transformed (Cloyd, 1983), and do not require helper viruses because they contain all the genes required for replication. These viruses are frequently carried as proviruses in cellular genomes and are transmitted vertically, i.e. in the germline (Quint et al., 1982). Those cells which they transform are generally only a small, very specific group of target cells, e.g. cells in the hematopoietic-lymphoid system that are in specific stages of differentiation. When this oncogenic activity does occur, it is often a result of recombination event with another endogenous retrovirus. This recombination event with silent proviruses that are non-defective leukosis viruses may cause activation to a non-oncogenic but infectious virus.

It is interesting to note that there are several strains of mice which have a gene (designated Rmcfr) which may be either dominant or semidominant - mapping to chromosome 5, that specifically restricts infectivity of mink cell focus-forming (MCF) murine leukemia virus. This gene is closely linked to the morphologic marker gene Hm and may be of use in determining the role of MCF viruses in various forms of leukemogenesis (Hartley et al., 1983).

Viruses have been recognized as the causative agent of leukemia in several inbred mouse strains since the 1930's. Early work showed that the incidence of leukemia was selective and age related in several of these inbred strains. Two of these strains are AKR and C58 mice (Dulbecco, 1980; Chattopadhyay, et. al., 1975). The degree of disease manifestation which occurs with these viruses is variable, and differences between viral isolates can include the latent period of disease induction or the type of leukemia or lymphoma induced (Datta et al., 1980; Cloyd et al., 1981). Phenotypes may also vary for a particular virus depending on the genetic constitution of the host (Cloyd et al., 1981).

Gross found that one could take filtered extracts of AKR leukemia cells and transmit leukemia to newborn mice of low-leukemia potential (C3H/He) (Gross, 1951). Because these mice were still immunologically immature, they could not reject the virus or tumor cells. The causative agent,

identified as virus, became known as AKR virus, Gross virus, or Gross passage A virus. The target cells of this virus are in the thymus (Nowinski, et al., 1977), the earliest lesions are observed in the thymus (Cloyd, 1983; DesGroseillers et al., 1983a) and it was found that removal of the thymus at birth prevented the development of T-cell leukemia (Furth, 1946) which was reversible if isologous thymus tissue containing virus material was grafted into the subject animal.

The env gene has been implicated in the cause of the leukemia. In an effort to determine exactly how the inclusion of these sequences in the genome causes the leukemia, Laigret, et al. used a synthetic 16-base-pair MCF-env-specific oligomer probe to identify subgenomic MCF-related mRNAs present in the thymus tissues of AKR mice prior to the appearance of full-length (8.4-kilobase <kb>) recombinant MCF viral RNAs (Laigret et al., 1988). These potential MCF precursors consisted of 7.2-, 3.0-, and 1.8-kb RNA species. They then determined the structure of the MCF-related m-RNAs on the basis of Northern (RNA) blot hybridization analyses, determined the nucleotide sequence of a cloned cDNA segment representing the 3' portion of the 7.2-kb mRNA, and studied the expression of ecotropic and xenotropic MuLV sequences by using env-specific DNA probes. Their results indicated that ecotropic, xenotropic, and MCF-related transcripts were constitutively and concur-

rently expressed exclusively in thymus tissue of two month old AKR mice prior to detection of MCF viral RNAs and molecularly characterized the thymic MuLV RNAs which may participate in formation of recombinant MCF viruses. They then identified a novel recombinant ecotropic viral RNA which is a good candidate for a possible intermediate in the stepwise generation of leukemogenic MCF MuLVs. Martin et al. (Martin et al., 1981) reported in 1981 that through the use of molecular cloning they had identified murine leukemia virus (MuLV)-like sequences from human DNA. Repaske et al. (Repaske et al., 1983; Repaske et al., 1985) further characterized with determination of part of the nucleotide sequence of endogenous type C retrovirus segments in human chromosomal DNA.

It was soon discovered that the leukemia was not caused by a single virus agent, but was caused by the result of a recombination event. Leukemogenic mink cell focus-forming (MCF) viruses of AKR mice are believed to originate in thymic tissue via recombination between ecotropic, xenotropiclike, and endogenous MCF-related murine leukemia virus (MuLV) sequences.

In the AKR mice this recombination event involves the recombination of the endogenous non-leukemogenic virus Akv with a non-ecotropic (from another species) virus in the thymus to produce a virus termed Mink Cell Focus Forming virus (MCF) (Vogt et al., 1985). Elder, et al. (Elder et

al., 1977) suggested that not only were the MCFs the product of recombination between endogenous ecotropic and xenotropic viruses, but that the recombination has taken place within the envelope (env) gene which encodes the surface glycoprotein (gp70) of the virion. Tryptic peptide analysis allowed them to make the following conclusions: (1) the gp70s of the MCF viruses are not identical to one another and are different from the possible parental viruses tested; (2) the MCF virus gp70s have tryptic peptides in common with xenotropic virus gp70s as well as with ecotropic virus gp70s; and (3) the gag region protein, p30, of the MCFs tested is identical to the p30 of AKR virus (Akv-1 or Akv-2) and distinct from the p30 of xenotropic viruses, suggesting that the 5' end of the recombinant viruses is of Akv origin. This virus is leukemogenic (Holland et al., 1985a). This recombination event is evidently a crucial step in the pathogenesis of the disease in the AKR mouse, the result of which is the generation in the preleukemic thymus of a novel class of MuLV (Thomas and Coffin, 1982; Thomas, 1986). These viruses are termed Mink Cell Focus-Forming virus (MCF) because they have the ability to induce cytopathic foci in monolayers of a tissue culture line of mink cells. These new viruses are recombinants between the endogenous ecotropic virus and the genetic information related to xenotropic viruses. The viruses do not cause plaques in

the XC assay (Rowe et al., 1970).

High levels of this ecotropic MuLV virus are found among young mice (Jaenisch, 1981) and can be activated from embryo cells as early as around 10-12 days into the gestation period. Though there is some variability in the disease presentation, the average time is in the 6-12 month range (Burdick and Williams, 1985; Elder et al., 1977; Wustrow and Good, 1985). It is very rare to have an AKR mouse that is older than 12 months. Several studies have found that the disease can have an accelerated onset (occurs in AKR mice before 6 months of age) if newborn AKR mice (<2 days old) are injected with a concentrated virus stock of various viral strains either intraperitoneally (i.p.) or intrathymically (i.t.) (Elder et al., 1977; Gross, 1951; Jaenisch, 1980).

Riesenfeld and Alm (Riesenfeld and Alm, 1977) found that embryonic thymus organ cultures could be used for studies on relations of MuLV, lymphoid cells, and microenvironment to lymphoma development. They found that thymuses of 14-day old CBA and AKR embryos could be maintained in organ cultures for at least nine weeks with sustained production of lymphocytes. Use of an indirect immunofluorescence technique demonstrated the spontaneous appearance of MuLV-antigen-containing cells in AKR, but not CFA thymuses. This spontaneous MuLV expression generally occurred at just over two weeks in culture.

Kahn, et. al. (Kahn et al., 1976) also noted that even the red blood cell (rbc) metabolism in AKR mice in the pre-lymphomatous phase is affected. AKR rbc had an increased glucose utilization and 2,3,diphosphoglycerate production (2,3-DPG), and a decreased adenosine triphosphate (ATP) levels when compared with controls. The data would imply that there was a direct effect of the virus infection either on the mature rbc or on its precursor.

Comparison of Akv and MCF virus.

As mentioned earlier, the Akv virus and MCF viruses are very different at three genes at the 3' end. These are (1) the gene which codes for the long terminal repeat (LTR), (2) the gene which codes for the viral envelope protein gp70, and (3) the gene which codes for part of the p15E protein. The important effect these genes have on the host environment is as follows. The LTR gene is involved in viral tropism and helps regulate the transcription of viral genes. The gp70 protein is a viral surface protein and may have a role in tropism, but also stimulates proliferation of T-cells. The p15E protein is responsible for anchoring the gp70 to the virus surface. The p15E protein can be suppressive (Copelan, et al., 1983).

Immunosuppression is commonly associated with retrovirus-induced animal tumors. Studies that have looked at both the murine and feline retrovirus systems would suggest that the 15,000 dalton protein (p15E) of the virion may contribute to the immunosuppression by interfering with normal lymphocyte function (Copelan, et. al. 1983).

Copelan, et. al. (Copelan, et. al. 1983), evaluated the mechanism of retrovirus suppression of human T-cell proliferation in vitro. In this study they looked at the effect of ultraviolet inactivated feline leukemia virus (UV-FeLV) and purified p15E virus on human T-cell proliferation stimulated/driven by the mitogen Concanavalin A (Con A). Purified p15E virus a 15,000 m.w. virus envelope protein (p15E), was fractionated from purified FeLV by freeze thawing, ultracentrifugation, ether extraction, and liquid chromatography). They first observed an increase in the inhibition of mononuclear cell (MNC) proliferation when the amount of virus was increased to 40 micrograms UV-FeLV/well of 100,000 human MNC cells. No significant additional decrease in the proliferative response (increase in inhibition) was seen at 80 micrograms/well of UV-FeLV. To attempt to determine the mechanism of UV-FeLV mediated inhibition of lymphocyte proliferation, they next showed that there was no effect on IL-1 production with either mouse thymocytes or human T-cells. Monocytes were recognized as accessory cells but their accessory cell

function was not hindered by the presence of inactivated virus (UV-FeLV) in the system. Even when IL-1 was substituted for monocytes, 40 micrograms of virus protein would still markedly inhibit the proliferation of T-cells. The presence of 40 micrograms of UV-FeLV almost completely inhibited IL-2 generation in vitro, but when monocytes were added back to the cultures, they reduced the inhibitory effect of purified p15E on T-cell proliferation. Similar results were seen when inactivated virus was substituted for purified p15E.

Copelan, et. al. (Copelan et al., 1983) suggested that the mechanism for the induced suppressor of lymphocyte proliferation may involve suppression of the T-cell function through the inhibition of response to IL-2 and inhibition of secretion of IL-2. These studies did not address the mechanism by which the monocytes provided partial protection of the T-cells from purified p15E and inactivated virus. It is not known if the monocytes bind, degrade, or phagocytize the virus and protein. Alternatively monocytes may secrete factors that stabilize or protect the T-cell membrane from virus and viral protein effects.

Additional evidence for the immunosuppressive nature of p15E came in 1985, when Cianciolo and others found a region of p15E conserved among murine and feline retroviruses (Cianciolo et al., 1985). A homologous region was

also found in the transmembrane envelope proteins of the human retroviruses HTLV-I and HTLV-II. A peptide (CKS-17) was synthesized to correspond to this region of homology and then examined for its effect on lymphocyte proliferation. They first showed the amino acid sequences for the conserved region of retrovirus transmembrane envelope proteins and for the synthetic peptide sequences. When the synthetic peptide CKS-17 (coupled to BSA) was added in an IL-2 assay it was shown to cause a dose-dependent inhibition of growth of the IL-2 dependent CTLL-2 cell line. They also showed that CKS-17 inhibited the proliferation of murine and human splenocytes in a two-way mixed lymphocyte reaction (MLR) culture. Significant inhibition of lymphocyte proliferation was not seen when uncoupled CKS-17 was tested. Biological activity might therefore be dependent on a particular conformation that is conferred to the peptide molecule when it is coupled to a protein carrier.

Cloyd showed that lymphomagenic MCF viruses of thymic origin (AKR-247 and C58L1) were found to infect and replicate selectively only in those immature lymphocytes present in the thymic cortex of AKR mice whereas non-lymphomagenic MCF viruses of splenic origin (C58v-1-C77 and C58v-2-C45) selectively infected and replicated in cells that appeared to be B-cells. Virus binding studies suggested that neither the T- nor B-cell tropisms of the MCF virus were

due to differential binding to the cells (Cloyd et al., 1980).

In addition to the synergistic effects of certain combinations of the viral proteins mentioned above, time or rather timing also seems to play an important role. Hunsmann, et.al.(Hunsmann et al., 1981) showed that vaccination of mice with gp85 (a virus envelope glycoprotein of Friend virus) in a large enough dose (100micrograms) completely protected mice challenged with (F-MuLV) if given in the early phase of the disease. Passive antibody therapy was not effective in later stages of the disease. Passive antibody therapy also did not appear to influence the anti-gp85 titer, but in those animals given lower immunization doses (those mice which died from leukemia), the antibody titers fall below detectable levels 2-4 weeks before death.

Anergy is diminished reactivity to specific antigen(s) and may take the form of diminished immediate hypersensitivity or diminished delayed type hypersensitivity (DTH), or both; a condition in which there is no response to the injection of an antigen. Strong DTH can generally be produced in mice by sensitization and challenge with allogeneic splenocytes. Burdick and Williams (1985) while performing experiments to define the allogeneic incompatibilities that are effective antigens in DTH, found that most mouse strains tested showed a strong reactivity

against major and multiple minor antigens. The one exception was preleukemic recipient mice of the AKR strain. These mice seemed intermittently incapable of producing DTH responses against any allogeneic cells. This anergy could have implications in the study of in vivo immune responses and could have relevance to the lymphoma that develops in older AKR mice. Additional experiments to determine whether there was a real difference between AKR mice and other strains showed that although younger AKR mice did intermittently show a detectable DTH response, by the age of 4 months, these mice were completely anergic for DTH against allogeneic cells. This immune impairment was seen well before the age of six months, the earliest point at which AKR mice begin to demonstrate lymphoma(69-1), and would strongly suggest that this is an example of anergy related to the preleukemic state.

Wustrow and Good recently looked at the expression of antigens coded in murine leukemia viruses on thymocytes of allogeneic donor origin in AKR mice following syngeneic or allogeneic bone marrow transplantation (Wustrow and Good, 1985). AKR mice were the irradiated recipients of bone marrow cells from donors either syngeneic (leukemia sensitive AKR cells) or allogeneic (leukemia resistant C57BL/6 cells). What they saw here was that allogeneic chimeras were "resistant" and did not develop any evidence of leukemia for up to thirty months after bone marrow

transplantation although the C57BL/6 thymus cells developing in the AKR mice did have viral antigens on their surface.

The nature of this resistance is very interesting and should be studied further. Allogeneic chimeras and syngeneically transplanted mice both showed evidence of accelerated viremia and of recombinant virus formation. The findings suggested that an event essential to leukemogenesis which occurs within the AKR lymphoid cells or their environment is lacking in the allogeneic chimeras. The nature of this influence of a resistance gene or genes introduced into AKR mice by allogeneic bone marrow transplantation is unknown. As indicated earlier, protection from or delayed presentation of leukemia symptoms in AKR mice has been accomplished by several techniques or approaches. Some of these include neonatal thymectomy, continuous treatment by immunosuppressive agents and interferon, and bone marrow transplantation in conjunction with a sublethal dose of gamma irradiation (Anklesaria et al., 1987). In a series of reports, Pollard and Truitt (Pollard and Truitt, 1973; Truitt et al., 1974) combined sublethal irradiation with allogeneic bone marrow transplantation to create a chimeric AKR mouse with transferred DBA to bone marrow. DBA/2 mice do not develop leukemia spontaneously. The explanation for the ability to alter the disease in chimeras was not clear. Three possibilities

were proposed: a) irradiation may have deleted a cell type from AKR mice which was responsible for the development of leukemia; b) it may have been replaced by a genetically resistant cell type from donor bone marrow; or c) the immunological reconstitution of the chimeric AKR mouse was incomplete. Chimeric germfree AKR mice could be maintained in apparent good health with no development of spontaneous leukemia up to 15 months. Conventional AKR mice with bone marrow cells from conventional DBA/2 mice died of GVH disease. Leukemia appeared spontaneously in conventional AKR mice transplanted with AKR bone marrow cells. Leukemia could thus be prevented in the germfree AKR mice with allogeneic chimerism.

Zielinski et al. showed that (AKR x NZB)F1 (ANF1) hybrid mice do not develop leukemia because they severely restrict the expression of ecotropic and xenotropic related retroviruses in their thymuses (Zielinski et al., 1982). The exact reason and relationships, however of just how the development of thymic leukemia in AKR mice depends on a series of genetically programmed events involving endogenous retroviruses and thymic lymphocytes, and the thymic reticuloepithelial environment remains somewhat of a mystery. Though these ANF1 mice inherit dominant loci for ecotropic and xenotropic virus expression from their AKR and NZB parents, they fail to develop leukemia. This protection can be correlated with a thymus-specific

restriction of the expression of retrovirus in these hybrids. However when ANF1 thymic lymphocytes differentiate in the thymic environment of irradiated young AKR mice, they manifest a specific high grade augmentation in the expression of murine leukemia virus (MuLV) antigens and retroviruses, including polytropic viruses, as well as leukemic transformation. By contrast, those phenotypic changes do not occur when the ANF1 thymocytes differentiate in leukemia-resistant irradiated hosts, such as the ANF1, and C57BR strains. Zielinski and others (Zielinski et al., 1982) feel that there may be some intrinsic, perhaps abnormal, property of AKR thymic epithelium which contributes to leukemogenesis. The contribution of thymic epithelium to the development of AKR leukemia was emphasized by the demonstrations that thymectomy greatly reduces the incidence of leukemia, and that leukemia susceptibility is restored by thymic grafts. The component of the graft that allows the expression of leukemogenic potential resides in its reticuloepithelium. Using thymectomized mice deliberately infected with a highly leukemogenic virus, it has been shown that that the development of leukemia was restored even by thymic grafts from normal, "low-leukemia" strains. When the development of spontaneous leukemia in thymectomized F1 hybrids was studied, however, thymic epithelial grafts from the AKR parents were found to be more efficient in restoring

leukemia susceptibility than those from the low leukemia parents. Furthermore, the AKR thymic epithelium is more effective in inducing T lymphocyte differentiation and remains functional much longer in life than that of low leukemia strains. This intrinsic, perhaps abnormal property of AKR thymic epithelium which appears to have a central role and significant contribution to leukemogenesis is distinct from radiation-induced thymic leukemias in which leukemic transformation occurs in the bone marrow and the thymic environment merely allows it to become manifest.

Summary.

In summary, we have traced the acquisition of the knowledge of thymic leukemia and lymphoma in the AKR mouse system. This has taken us from the realization that the early leukemia and lymphoma development in a highly inbred strain of mice (AKR) is caused by a virus and is related to the thymus (neonatal thymectomy will eliminate the development of the disease). We have learned that it is by no means a simple disease caused by a simple virus, but is a very complex disease brought about by the genetic recombination of an endogenous retrovirus and a xenotropic retrovirus in the thymic reticuloepithelial cells. Suppression is involved but is not a major part of the

disease. It has been shown by the use of viral recombinants which contain only parts of the virus genome that those viral recombinant strains which contain at least three parts of the features of the parental strain (gag-pol, gp70, p15E, LTR), cause leukemias in approximately the same frequency and time frame as the parental virus. Those which contain only two of the features cause leukemias in about 65 -75 % of the mice injected, and those which contained only one of the features of the parental virus cause only about one third of the leukemias with an extremely long latent period. Selected works have shown that the reticuloepithelial cells of the thymus in the AKR mouse are a critical area for the recombination event to take place and for these new cells to develop. There appears to be a restriction gene common to some species of mice (low-leukemia mice) which restricts the growth of the retrovirus. There are critical sequences in the env protein p15E that are suppressive in vitro. Synthesized sequences matching these "natural" sequences are suppressive in vitro only when coupled to a protein carrier. This may speak to the more natural conformation of a regular antibody in its suppressive effect. Bone marrow transplantation of young animals or newborns with or without immunosuppression has caused delay of leukemia development. The development of leukemia can apparently be avoided even though the bone marrow chimeric cells will

will display a surface phenotype with demonstrable thymic leukemia antigens/surface markers. We are getting much closer to not only understanding this disease, but also understanding leukemia in general.

We have demonstrated that the leukemogenic MCF virus is more suppressive than the endogenous Akv virus. We have also shown that the viral recombinant supernatant from Sst10/SC-1 which contains three of the four features of the parent retrovirus is almost as suppressive as the parental virus with its complete genome. We have demonstrated that these recombinant SN are suppressive both in vivo and in vitro.

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