The Koala and its Retroviruses:
Implications for Sustainability and Survival

edited by

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Leukemogenesis by Murine Leukemia Viruses: Lessons for Koala Retrovirus (KoRV)

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Abstract. Murine leukemia viruses (MuLVs) are the prototypical gammaretroviruses, and they have been extensively studied with regard to how they cause disease. Leukemogenesis by two MuLVs is reviewed here: the endogenous Akv MuLV of AKR mice, and exogenous Moloney MuLV. Important features of MuLV leukemogenesis include the in vivo generation of envelope recombinants (MCFs) through recombination with endogenous MuLVs, and induction of preleukemic changes typified by splenic hyperplasia secondary to bone marrow defects. Studies of MuLV leukemogenesis help to frame virological questions about how koala retrovirus (KoRV) may induce neoplastic or other diseases in koalas.

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The discovery of koala retrovirus (KoRV) in free-ranging and captive koalas *(Phascolarctos cinereus)* has been viewed with concern and interest. The primary concern is that KoRV-associated disease such as neoplasms, while yet to be conclusively proven to be KoRV caused, could increase the threats to survival of these animals. In the scientific community there is interest for several reasons: KoRV may be associated with lymphoma in koalas, it appears to be recently introduced into this species, and endogenization is an ongoing process. KoRV infection in koalas may provide an opportunity to study introduction and spread of a gammaretrovirus into a new host species and its accompanying effects. This process has happened in other species, notably mice, but in the more distant past, so some of the processes can only be deduced. At the same time, information learned from the relationship of murine gammaretroviruses and their hosts may provide lessons for understanding the potential relationships of KoRV and disease in koalas. The recent discovery of a second KoRV (KoRV-B) that may be associated with leukemogenicity (Xu *et al*., 2013) has similarities to oncogenesis in murine leukemia viruses (MuLVs). Leukemogenesis by MuLVs will be summarized here and possible implications to KoRV pathogenesis will be pointed out.

Murine leukemia viruses

MuLVs were first discovered in inbred mouse strains that had high incidences of leukemia. These studies resulted in isolation of several MuLV strains that cause leukemias of different hematopoietic lineages. For instance Moloney MuLV (M-MuLV) and Gross MuLV induce T-lymphoma, while Friend (F-MuLV) and Rauscher MuLV (R-MuLV) induce erythroleukemia and myeloid leukemia (Fan, 1997). These are the predominant MuLVs used in studies of MuLV leukemogenesis. They are prototypical retroviruses of the gammaretrovirus family.

MuLVs can be classified into types based on their envelope proteins and the kinds of cells that they infect, determined by the cell surface proteins that they bind. The leukemogenic MuLVs are mostly *ecotropic*; they infect cells of mice and rats, but they do not infect most

Table 1. Types of MuLVs according to host range.

virus class	susceptible cells	receptor name	function	examples
ecotropic	mouse, rat	CAT1	cationic amino acid transport	Akv-MuLV, Moloney MuLV
xenotropic	non-mouse	XPR1	phosphate export	xenotropic MuLVs
polytropic	mouse, non-mouse	XPR1	phosphate export	MCF MuLVs
amphotropic	mouse, non-mouse	PIT-2	phosphate import	amphotropic MuLV

other species. Their envelope proteins utilize the cationic amino acid transporter-1 (CAT1) molecule as the receptor (Table 1). Other MuLVs have been classified as *polytropic, xenotropic*, and *amphotropic*. Xenotropic MuLVs do not infect mouse cells, but they infect cells of other species. Polytropic MuLVs infect cells of both murine and non-murine origin; both xenotropic and polytropic MuLVs infect cells by interacting with the Xpr-1 molecule. Amphotropic MuLVs also infect mouse and non-mouse cells, but they infect by binding to the Pit-2 molecule.

Endogenous MuLVs

Endogenous retroviruses result from infection of germ cells; progeny resulting from these germ cells will genetically transmit the integrated viral DNAs as endogenous viruses (Jern & Coffin, 2008). Endogenization of retroviruses has occurred throughout evolution (millions of years ago in some cases), but it is an ongoing process in some species. Mice genetically transmit multiple copies of endogenous MuLVs, most of which cannot produce infectious virus (Stocking & Kozak, 2008). Nevertheless some of these defective endogenous viruses can be expressed and have biological effects, as will be described below. The endogenous MuLVs have been genetically mapped and classified according to their envelope types. In laboratory mice, the predominant endogenous MuLVs are derived from xenotropic and polytropic MuLVs (Table 2). The genetic loci containing these endogenous MuLVs have been designated *Xmv*s and *Pmv*s/*Mpmv*s respectively. Endogenous ecotropic MuLVs (encoded by *Emv*s) are present in some but not all laboratory mice.

Insertional activation of proto-oncogenes

A common mechanism for tumorigenesis by non-acute retroviruses (retroviruses that do not themselves carry an oncogene) is insertional activation of proto-oncogenes (Fan, 1997; Hayward *et al*., 1981). During retroviral replication, the viral RNA genome is reverse transcribed into viral DNA, which is integrated more or less randomly into the host cell DNA. During reverse transcription, long terminal repeats (LTRs) at either end of the viral DNA are generated; in the inserted (proviral) DNA form, the LTRs carry the signals for initiation of viral RNA synthesis by cellular RNA polymerase II (enhancers and promoters). A hallmark of non-acute retroviral oncogenesis is that independent tumors show proviral integration in common insertion sites (CISs). The CISs contain proto-oncogenes (normal cell genes involved in positive stimulation of cell division) that are transcriptionally activated by integration of the inserted provirus nearby. This can result from read-through transcription from the retroviral LTR (promoter insertion) (Hayward *et al.*, 1981), or by activation of the proto-oncogene promoter by the nearby viral enhancer in the LTR (enhancer activation) (Fan, 1997). Identification of CISs in retrovirus-induced tumors has led to identification of new proto-oncogenes (Cuypers *et al.*, 1984; Nusse & Varmus, 1982), some of which are also involved in human cancers. Oncogene discovery through identification of CISs in retrovirus-induced tumors is continuing today (Kool *et al.*, 2010; Suzuki *et al.*, 2002).

One consequence of the LTR activation of proto-oncogenes in non-acute retroviral oncogenesis is that the LTRs (and in particular the enhancers) influence both efficiency and type of disease. For instance, enhancer sequences are frequently duplicated in MuLV LTRs, and these duplications or other alterations increase both the transcriptional activities of the LTRs and also the rate at which the viruses induce leukemia (Lenz *et al.*, 1984). In addition, when M-MuLV and F-MuLV were compared, the disease specificity (T-lymphoid vs. erythryoid leukemia respectively) could be switched by exchange of the enhancer sequences (Li *et al.*, 1987). This was correlated with the fact that the M-MuLV LTR is preferentially active in T-lymphoid cells while the F-MuLV is preferentially active in erythroid cells (Short *et al.,* 1987). Since the LTR enhancers are important in LTR activation of proto-oncogenes it is logical that an MuLV will induce tumors of the cell type where its LTR enhancers are most active.

While insertional activation of proto-oncogenes is a fundamental mechanism for oncogenesis by non-acute retroviruses, it has also become clear that other virus-induced events are also important. This will be discussed in the context of two well-studied MuLV systems: endogenous Akv MuLV of AKR mice, and M-MuLV.

Table 2. Endogenous viruses of laboratory mice. (Classification according to host range of the Env protein. Most, but not all, endogenous proviruses cannot encode infectious virus; some defective proviruses can participate in recombination with exogenously infecting MuLVs [e.g., Pmv's and Mpmv's]).

class	genetic loci	comments
xenotropic	Xmv's	Xmv1 is readily activated in some mouse strains.
polytropic	Pmv's	Envelopes from both classes bind Xpr1 receptor; multiple copies of both classes are in most mice.
modified polytropic	Mpmv's	
ecotropic	Emv's	Relatively few or no copies in most mouse strains.

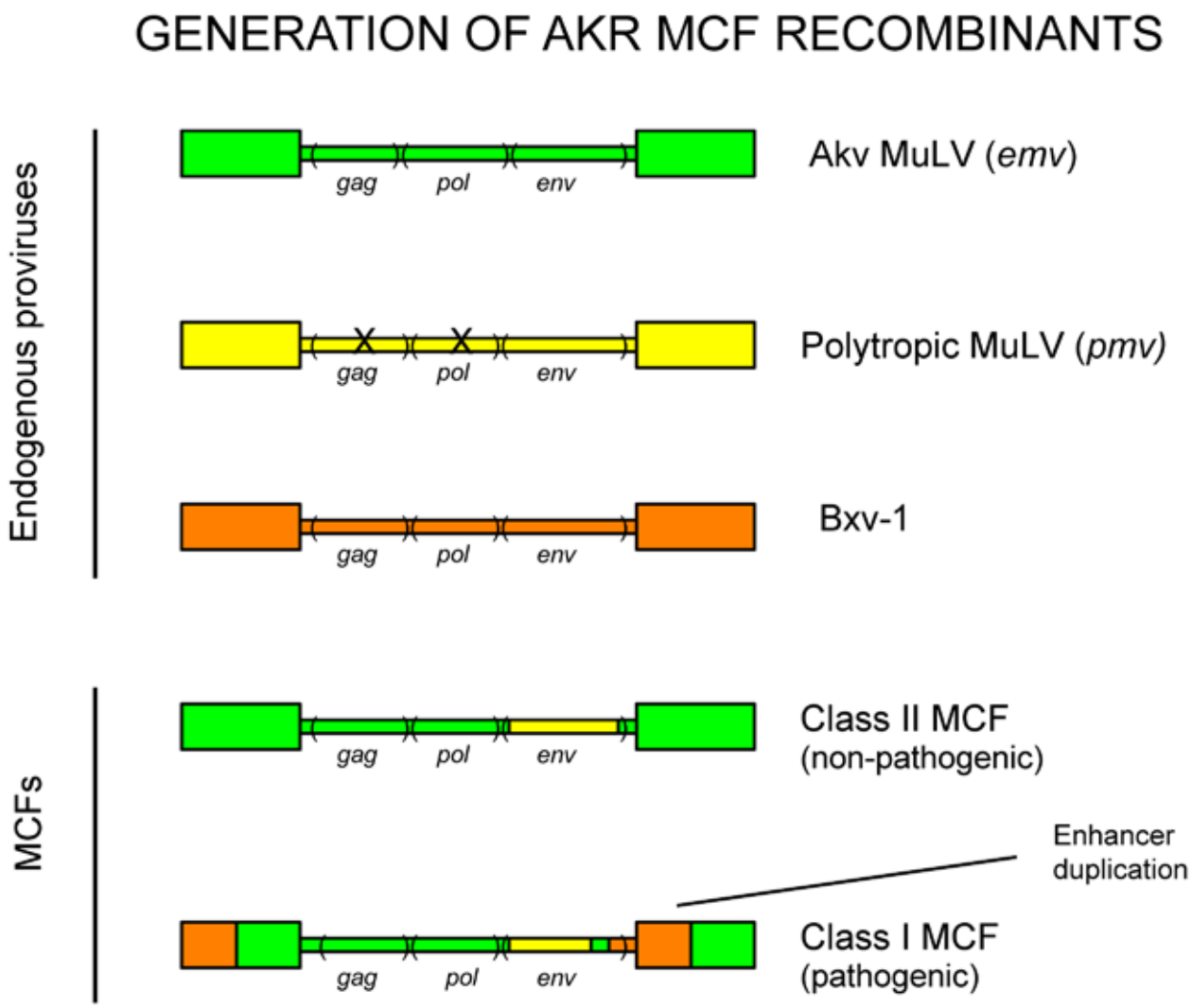

Figure 1. Generation of MCF recombinants in AKR mice. The organization of the endogenous proviruses that give rise to AKR MCFs is shown in the upper part of the figure. Akv-MuLV results from induction of one of two endogenous ecotropic proviruses (encoded by *Emv-10 or -12*). Recombination with a *Pmv or Mpmv* provirus gives MCF recombinants. The lower part of the figure shows class I and II MCFs; class II MCFs simply represent recombination between Akv-MuLV and an *Pmv* or *Mpmv* provirus, while class I MCFs result from additional recombination with *Bxv1* xenotropic endogenous virus.

Leukemogenesis in AKR mice

Inbred AKR mice develop T-cell lymphoma with a latency of 6–7 months. These mice genetically transmit two endogenous ecotropic MuLV proviruses (*Emv10* and *-12*) ; which both can encode replication-competent MuLV (termed Akv-MuLV). Akv-MuLV is activated in AKR mice after birth, and once activated it carries out additional rounds of infection in the animals. Activation of Akv-MuLV is required for leukemogenesis in AKR mice.

Hartley and Rowe made the seminal observation that AKR mice develop recombinant versions of Akv-MuLV close to the time when leukemia occurs (Hartley *et al.*, 1977). These recombinants result from recombination between Akv-MuLV and an endogenous polytropic virus, which results in the recombinant virus carrying polytropic envelope sequences in place of the Akv *env* sequences. (Fig 1) The resulting viruses were termed mink cell focus-inducing (MCF) recombinants because they cause cytopathic effect in vitro when infecting mink lung fibroblasts. AKR MCF recombinants infect cells by binding to the Xpr1 receptor instead of the mCAT1 receptor. The fact that MCF recombinants were detected in AKR mice close to the time that leukemia developed led Hartley and Rowe to propose that MCF recombinants are the "proximal leukemogens" in these mice (Hartley *et al.,* 1977).

Additional studies of AKR MCF recombinants revealed another layer of complexity. The AKR MCF recombinants arising in these mice could be further subdivided into Class I and Class II MCFs. The class I MCFs were considered pathogenic because they could accelerate lymphomagenesis when infecting AKR mice; on the other hand the class II MCFs were not pathogenic by this acceleration assay (Holland *et al.*, 1985). Molecular analysis revealed that class II MCFs are recombinants containing an endogenous polytropic MuLV envelope, while class I MCFs actually result from two recombinations (Stoye *et al.*, 1991). In class I MCFs, the envelope sequences are polytropic, but additional recombination with another endogenous virus (the xenotropic *Bxv-1* provirus) results in the LTR and its enhancer sequences being derived from *Bxv-1* (Fig 1). The higher activity of the *Bxv-1* LTR compared to the Akv-MuLV LTR is thought to result in the pathogenicity of the class I MCFs.

There are several possible mechanisms by which class I MCFs contribute to leukemogenesis in AKR mice. First MCF recombinants would allow continued infection in animals where the majority of cells are already infected with Akv-MuLV. Cells infected by a retrovirus exhibit resistance

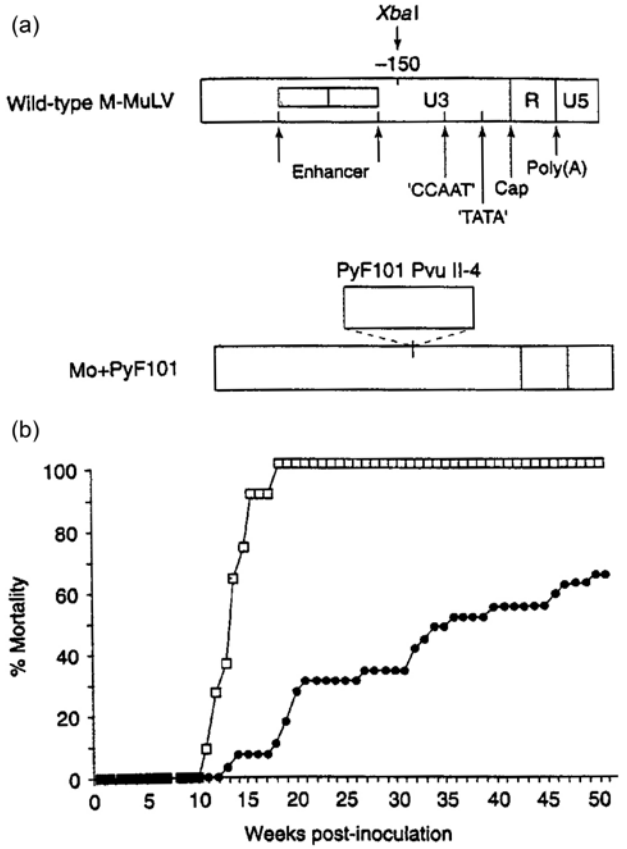

Figure 2. The Mo+PyF101 variant of M-MuLV. *(a)* The organization of the wt M-MuLV LTR is shown at the top; the location of the inserted PyF101 enhancers is shown below. *(b)* NIH Swiss mice were inoculated subcutaneously with wt or Mo+PyF101 M-MuLV. The per cent mortality from T-cell leukemia is shown as a function of time. Filled symbols represent animals infected with Mo+PyF101 M-MuLV and open symbols represent animals infected with wt virus.

to superinfection by viruses with envelopes that bind to the same cellular receptor, but they can be infected by viruses that utilize a different receptor. Thus, in an AKR mouse, cells infected by Akv-MuLV could be re-infected by an MCF. Second, since the range of cells in an AKR mouse that Akv-MuLV infects is determined by those cells expressing the ecotropic mCAT1 receptor, MCF recombinants could potentially infect additional cell types that express the Xpr1 receptor but not mCAT1. Third, MCFs could have physiological effects that contribute to tumorigenesis. It has been reported that MCF envelopes bind to cellular growth factor receptors such as the interleukin-2 receptor (IL-2R), leading to factor-independent growth of IL-2 expressing T-lymphocytes (Li & Baltimore, 1991). Other studies have indicated that MCFs lead to premature thymic atrophy resulting from lysis of infected thymocytes (Haran-Ghera *et al*., 1987). This could lead to repopulation of the thymus with cells with leukemic potential ("preleukemic cells").

A final point should be made about AKR leukemogenesis. While the appearance of class I MCFs is associated temporally with the development of leukemia, and infection of AKR mice with a class I MCF accelerates the rate of disease, class I MCFs do not cause leukemia when used to infect mice that are not infected with an ecotropic MuLV. Thus the rapid leukemia in AKR mice appears to result from infection by both ecotropic Akv-MuLV and a class I MCF.

Leukemogenesis by M-MuLV

M-MuLV induces T-cell lymphoma in 3–4 months when inoculated into newborn mice (Fan, 1997). M-MuLV-induced leukemias show proviral activations of cellular proto-oncogenes such as *c-myc* and *pim-1* and a variety of others. M-MuLV-infected mice also generate MCF recombinants, although M-MCFs retain the M-MuLV LTR likely because it is highly active in T-lymphoid cells. Like AKR MCFs, M-MCFs do not efficiently induce disease when inoculated into mice in the absence of M-MuLV. Thus many of the virological principles for leukemogenesis in mice infected with exogenous M-MuLV are shared with spontaneous leukemia in AKR mice.

We have studied M-MuLV leukemogenesis, using a virus with a modified LTR, Mo+PyF101 M-MuLV. This virus has enhancer sequences from the F101 variant of murine polyomavirus inserted into the M-MuLV LTR between its enhancer and promoter (Fig. 2) (Linney *et al.*, 1984). When inoculated subcutaneously into NIH Swiss mice, Mo+PyF101 M-MuLV shows reduced leukemogenicity (Davis *et al.*, 1985). Comparative studies revealed a virus-induced preleukemic state induced by wt but not Mo+PyF101 M-MuLV, typified by mild splenomegaly and hyperplasia of multiple hematopoietic lineages (Davis *et al.*, 1987). Thus this preleukemic hyperplasia was correlated with the efficient induction of leukemia by wt but not Mo+PyF101 M-MuLV. Further studies indicated that the splenic hyperplasia was secondary to a virus-induced defect in bone marrow hematopoiesis, and the reduced leukemogenicity of Mo+PyF101 M-MuLV was correlated with the absence of the bone marrow defect (Li & Fan, 1991). These effects are reminiscent of myelodysplastic syndrome in humans, where defects in bone marrow hematopoiesis lead to compensatory extramedullary hematopoesis (e.g., splenic hyperplasia) and increased incidence of leukemias.

An explanation for the bone marrow defect and splenic hyperplasia was provided by the observation that mice inoculated subcutaneously with Mo+PyF101 M-MuLV do not generate MCF recombinants (Brightman *et al.*, 1991). Moreover, infection of NIH-3T3 fibroblasts or primary mouse bone marrow cultures with a combination of M-MuLV and M-MCF was growth inhibitory, while infection with either virus alone did not inhibit cell growth (Li & Fan, 1990). These results indicate a role for M-MCF recombinants early in the disease process, i.e. induction of the preleukemic state, although they do not exclude involvement of MCFs in later stages of M-MuLV leukemogenesis. The roles of M-MuLV and M-MCFs in multiple steps of leukemogenesis are shown in Fig 3. In addition to the early events described, re-infection of M-MuLV induced T-lymphomas and activation of proto-oncogenes in tumor progression (tumor progression loci) has been documented (Bear *et al*., 1989; Gilks *et al.*, 1993).

In summary murine leukemia viruses not only activate proto-oncogenes during leukemogenesis, but they induce changes that promote development of tumors both during preleukemic phases, and also during tumor progression. Envelope recombinants (MCFs) are involved in some of these processes.

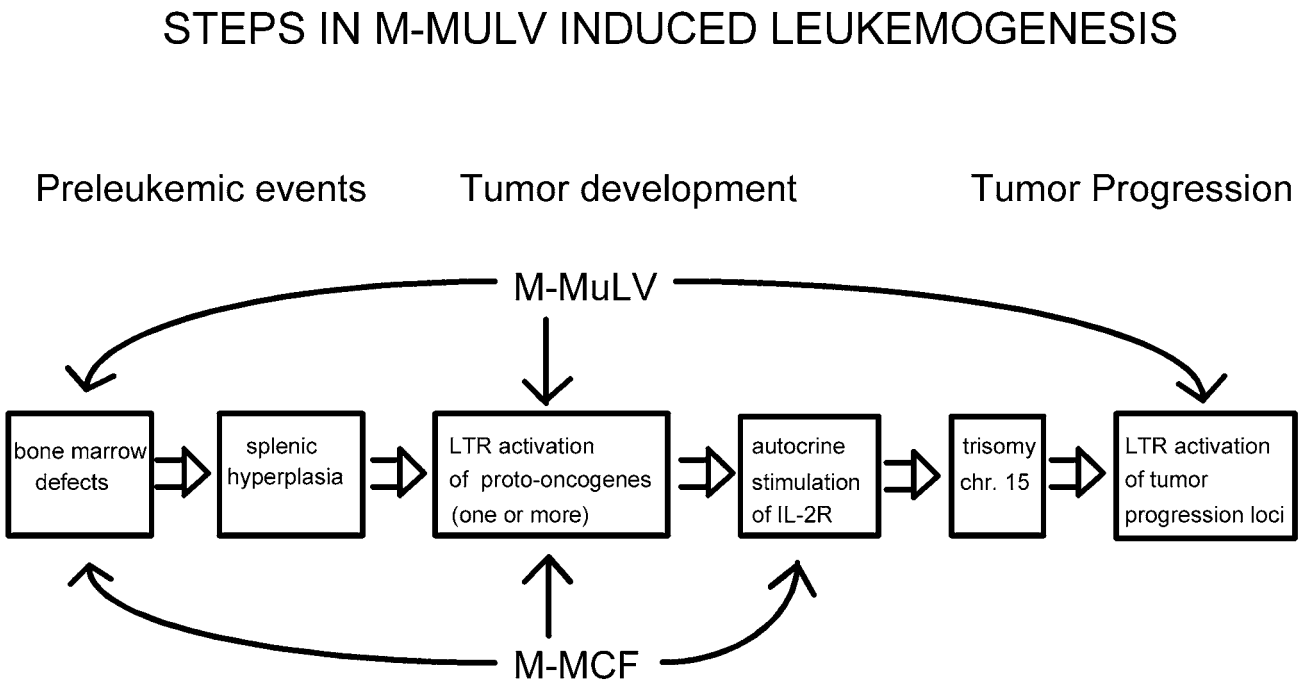

Figure 3. Steps in M-MuLV-induced leukemia. Different steps in development of leukemia after M-MuLV infection are shown, and the roles of M-MuLV or MCF recombinants are indicated.

Lessons and perspectives from MuLVs on potential KoRV leukemogenesis

As discussed elsewhere in this volume, the high incidence of lymphoma in koalas along with other neoplastic or pre-neoplastic conditions is highly suggestive of KoRV causing some of these diseases, particularly the lymphomas. This is supported by the close evolutionary relatedness of KoRV and MuLV, and the similarity of the koala diseases to MuLV-induced diseases. However, definitive proof that KoRV is inducing leukemia in koalas (e.g., integrated KoRV DNA at CISs in tumors) has not been reported. Hopefully ongoing investigations will provide such proof.

The recent description of a second KoRV strain, KoRV-B that may be associated with enhanced leukemogenicity is particularly noteworthy (Xu *et al.*, 2013). KoRV-B differs from the original KoRV (A) sequence by having an envelope protein that recognizes a different cellular receptor (the thiamine transporter vs. Pit-1), and it also has a triplication in the enhancer motif in the LTR. These differences are quite reminiscent of pathogenic Class I AKR-MCFs, which contain polytropic Env as well as altered LTR enhancers from the Bxv-1 endogenous virus. Other investigators have obtained evidence for other Env region variants of KoRV in infected animals (Shojima *et al., 2*013) (P. Young, unpublished), but it is not yet clear if these Env variants bind to different cellular receptors, and how frequently they are observed. The origins of the new Env sequences in KoRV-B and the other new variants is also interesting. Do these viruses represent recombination with endogenous KoRV-related proviruses, analogous to the contributions of endogenous MuLVs to generation of MCFs? Alternatively, do they represent recombination with other enveloped viruses? Ongoing sequencing of the koala genome should provide insight into these questions.

By analogy to MuLV leukemogenesis, if a causative role for KoRV in development of lymphoma or other neoplasms is confirmed, it may be useful to consider KoRV-A as analogous to ecotropic Akv-MuLV or M-MuLV, while KoRV-B might be analgous to an MCF recombinant. In this light the following questions can be asked:

1 Is KoRV-A by itself able to induce leukemias, and if so how efficiently?

2 Is formation of Env recombinants (KoRV-B and others) a common feature of KoRV-A infection or leukemogenesis in koalas?

3 Is KoRV-B capable of inducing leukemia by itself, or is co-infection with KoRV-A required?

4 Are some of the hematopathologies in KoRV-infected koalas analogous to preleukemic changes in M-MuLV-infected mice (bone marrow dysplasia, splenic hyperplasia)?

While it may be difficult or impossible to address these questions experimentally, in any event considering them conceptually will help to clarify virological aspects of KoRV leukemogenesis.

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