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Studies on the MAIDS defective virus target cells

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## Preface

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

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**Abstract**

Murine acquired immune deficiency syndrome (MAIDS) shares several common characteristics with human AIDS, including immunodeficiency, hypergammaglobulinemia, susceptibility to infection, both B and T cell dysfunctions, lymphadenopathy, splenomegaly and aberrant cytokine production. The etiologic agent of MAIDS has been identified as a defective retrovirus which contains major deletions in its *pol* and *env* regions but has largely maintained its *gag* region. The only gene product of this virus is a 60 kDa *gag* fusion protein, $Pr60^{gag}$, which is necessary and sufficient to induce MAIDS in susceptible mouse strains. The target cells of the MAIDS defective virus have been identified as belonging to the B cell lineage. Infection of these target cells with the MAIDS defective virus leads to their proliferation, which is required for the full development of the disease.

We undertook several approaches in order to more clearly understand the role of the MAIDS defective virus target B cell population in the development of this disease. Studies using SCID, CD4 knockout, and nude mutant mice revealed that a relatively mature B cell population is the target of the MAIDS defective virus, and that these cells can be infected in the absence of $CD4^+$ T cells. Our knowledge of the nature of the MAIDS defective virus target B cells was furthered by the derivation of two independent MAIDS defective virus-infected B cells lines which have characteristics of both mature and immature B cells. These B cell lines were used to demonstrate an *in vivo* association between $Pr60^{gag}$ and c-Abl. Additional exploration of the $Pr60^{gag}$-c-Abl interaction revealed that

$Pr60^{gag}$ could induce a $CD8^-$ T cell-dependant rejection of v-Abl-transformed pre-B cell lines, and increase the tumor latency of mice inoculated with $Pr60^{gag}$-expressing B16F1 melanoma cells.

Taken together, these results enhance our knowledge of the identity and role of the MAIDS defective virus target cells in the pathogenesis of this disease and propose a possible mechanism by which $Pr60^{gag}$ activates the disease process. As well as inducing MAIDS, $Pr60^{gag}$ may also have potential positive use in cancer therapy.

## Résumé

Le syndrome d'immunodéficience induite de la souris (MAIDS), partage plusieurs charactéristiques communes au SIDA tel que l'immunodéficience, l'hypergammaglobulinémie, la susceptibilitée aux infections, la disfonction des cellules B et T, la lymphadénophathie, la splenomegalie, et une production anormale de cytokine. L'agent étiologique du MAIDS fût à l'origine identifié comme étant un rétrovirus défectif contenant des déletions d'importance dans les régions *pol* et *env*, mais ayant conservé sa région *gag*. Le seul gène de ce virus code pour une protéine de fusion *gag* de 60 kDa, $Pr60^{gag}$, nécessaire et suffisante à l'induction du MAIDS chez les souches de souris susceptibles. Les cellules cibles du virus défectif du MAIDS fûrent identifiées comme appartenant à la lignée de cellules B. L'infection de ces cellules cibles du virus défectif MAIDS mène à leurs prolifération, une étape nécessaire au dévelopement complet de la maladie.

Nous avons entrepris plusieurs approches afin de comprendre plus en profondeur le rôle des cellules cibles B dans le dévelopement du MAIDS. Des études utilisants les souris mutantes SCID, CD4 déficient et nue ont révélés qu'une population relativement mature de cellules B est la cible du virus défectif MAIDS et que ces cellules peuvent être infectées en l'absence des cellules $CD4^+$ T. Notre connaissance de la nature des cellules B cibles du virus défectif du MAIDS fût avancée par l'obtention de deux souches indépendantes de cellules B infectées par le virus défectif du MAIDS, qui ont des charactéristiques de cellules B matures et immatures. Ces souches de cellules fûrent utilisées pour démontrer l'association *in vivo* entre le $Pr60^{gag}$ et le c-Abl. L'exploration additionelle de cette interaction a révelée que $Pr60^{gag}$ peut induire un rejet des souches de cellules B transformées par le v-Abl uniquement en présence des cellules $CD8^+$ T. L'interaction entre le Pr60gag et le c-Abl peut augmenter la latence des tumeurs

chez les souris inoculées avec des cellules de mélanome B16F1 exprimant le $Pr60^{gag}$.

Dans l'ensemble, ces résultats accroient notre connaissance de l'identitée et du rôle des cellules cibles du virus défectif du MAIDS dans la pathogenèse de cette maladie et propose un méchanisme possible par lequel $Pr60^{gag}$ active le processus de la maladie. En plus d'induire le MAIDS, $Pr60^{gag}$ peut potentiellement être utilisé de façon positive en thérapie contre le cancer.

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## List of Abbreviations

| | |
|---|---|
| AIDS | acquired immune deficiency syndrome |
| ALV | avian leukosis virus |
| ALSV | avian leukosis-sarcoma virus |
| A-MuLV | Abelson murine leukemia virus |
| APC | antigen-presenting cell |
| BcR | B cell receptor |
| BLV | bovine leukemia virus |
| CA | capsid protein |
| CAEV | caprine arthritis-encephalitis virus |
| CMV | cytomegalovirus |
| CNS | central nervous system |
| ConA | concanavalin A |
| DNA | deoxyribonecleic acid |
| Du5H | molecular clone of the MAIDS defective virus |
| EBV | Epstein-Barr virus |
| EIAV | equine infectious anemia virus |
| FeLV | feline leukemia virus |
| FIV | feline immunodeficiency virus |
| GALV | Gibbon ape leukemia virus |
| HIV | human immunodeficiency virus |
| HTLV | human T cell leukemia virus |

| | |
|---|---|
| Ig | immunoglobulin |
| IgH | immunoglobulin heavy chain |
| IgL | immunoglobulin light chain |
| IL | interleukin |
| IN | integrase |
| INF | interferon |
| I.P. | intraperitoneally |
| IRES | internal ribosome entry sequence |
| kDa | kiloDalton |
| KS | Kaposi's sarcoma |
| KSHV | Kaposi's sarcoma-associated herpes virus |
| LDL | low-density lipoprotein |
| LP-BM5 | virus mixture containing uncloned ecotropic, MCF, and defective viruses used to induce MAIDS |
| LTR | long terminal repeat |
| MA | matrix protein |
| MAIDS | murine acquired immune deficiency syndrome |
| MCF | mink cell focus-forming virus |
| MHC | major histocompatibility complex |
| MMTV | mouse mammary tumor virus |
| Mo-MuLV | Moloney murine leukemia virus |
| MPMV | Mason-Pfizer monkey virus |
| NC | nucleocapsid protein |

| | |
|---|---|
| PBL | peripheral blood lymphocytes |
| PBS | primer binding site |
| PCP | *Pneumocystis carinii* pneumonia |
| PCR | polymerase chain reaction |
| PNS | peripheral nervous system |
| $Pr60^{gag}$ | the MAIDS defective virus 60 kDa fusion protein |
| $Pr65^{gag}$ | helper virus 65 kDa *gag* precursor protein |
| RadLV | Radiation leukemia virus |
| RAG | recombination-activating gene |
| RNA | ribonucleic acid |
| RS | Reed-Sternberg |
| RSV | Rous sarcoma virus |
| RT | reverse transcriptase |
| S.C. | subcutaneous |
| SCID | severe combined immune deficiency |
| SHIV | simian immune deficiency/human immune deficiency chimeric virus |
| SIV | simian immune deficiency virus |
| STLV | simian T cell leukemia virus |
| TcR | T cell receptor |
| TE | thymic epithelium |
| $T_H$ | T cell helper subset |
| WHO | World Health Organization |

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# Chapter 1

# Introduction and Literature Review

In 1904, Vallee and Carre (315) induced equine infectious anemia (swamp fever) by use of a filterable agent. This was the first disease shown to be caused by what is now known to be a retrovirus. A few years later, Peyton Rous made an even more important discovery when he demonstrated that cell-free extracts derived from a transplantable chicken sarcoma were able to induce tumors at the site of inoculation (275). This was the first isolation of a tumor virus, although the significance of this discovery was not appreciated for many years. Other researchers were also able to induce leukemia or sarcomas in chickens with cell-free extracts (83). These viruses are known as avian leukosis viruses (ALVs) and Rous sarcoma viruses (RSVs), respectively. Isolation of these retroviruses was possible because they induced an observable phenotype (neoplastic transformation) in infected cells. Many years would pass before the non-transforming retroviruses were isolated.

The first evidence for a transforming mammalian retrovirus was obtained in the 1930s, based on the study of mammary gland tumors in C3H mice. In 1942, Bittner (28) demonstrated that a filterable agent, now known as murine mammary tumor virus (MMTV) was responsible. The following decades led to the isolation of many murine leukemia and sarcoma viruses, such as Friend (94), Gross (117), Moloney (225) and Harvey (127). Concurrently, feline leukemia virus (FeLV), which is responsible for almost all cases of leukemia in cats, was discovered (154). The study of retroviruses has led to the isolation and identification of the viral transforming genes (*v*-onc) and their relationship to the their cellular counterparts (*c*-onc), as well as to an understanding of the additional mechanisms

by which these pathogens induce cellular transformation (300,306,307). An historical summary of retrovirus research is presented in Figure 1.

The success in isolating retroviruses from many vertebrate species during the 1950s and 1960s led to the reasonable expectation that human retroviruses would soon be isolated and be shown to cause cancer in humans (65). Despite the efforts of many laboratories, it was nearly 20 years before the first human retrovirus was isolated. In 1980, laboratories in the U.S. and Japan reported that a retrovirus, termed HTLV-I, was responsible for inducing a rare, unusually aggressive T cell leukemia (262,331). A closely related strain of virus, HTLV-II, was isolated from a patient with hairy cell leukemia (163). No other retroviruses have been isolated which induce cancer in humans, despite much effort.

The discovery in 1983 that the cause of the emerging acquired immunodeficiency syndrome disease (AIDS) in humans is a retrovirus (15,195,266) (termed HIV-1) (51) elicited renewed interest in the study of human retroviruses. Furthermore, the focus of interest shifted from the study of the mechanisms of cellular transformation to the examination of their ability to induce immunodeficiency in infected individuals. As AIDS has emerged as the major pandemic of this century especially in the developing nations, particularly in Africa, the resurgent interest in the study of retroviruses is quite justified.

The importance of AIDS as a major cause of morbidity and mortality, with its associated economic and social costs (both personal and societal), as well as its ability to be transmitted via the blood supply and by sexual contact, has made

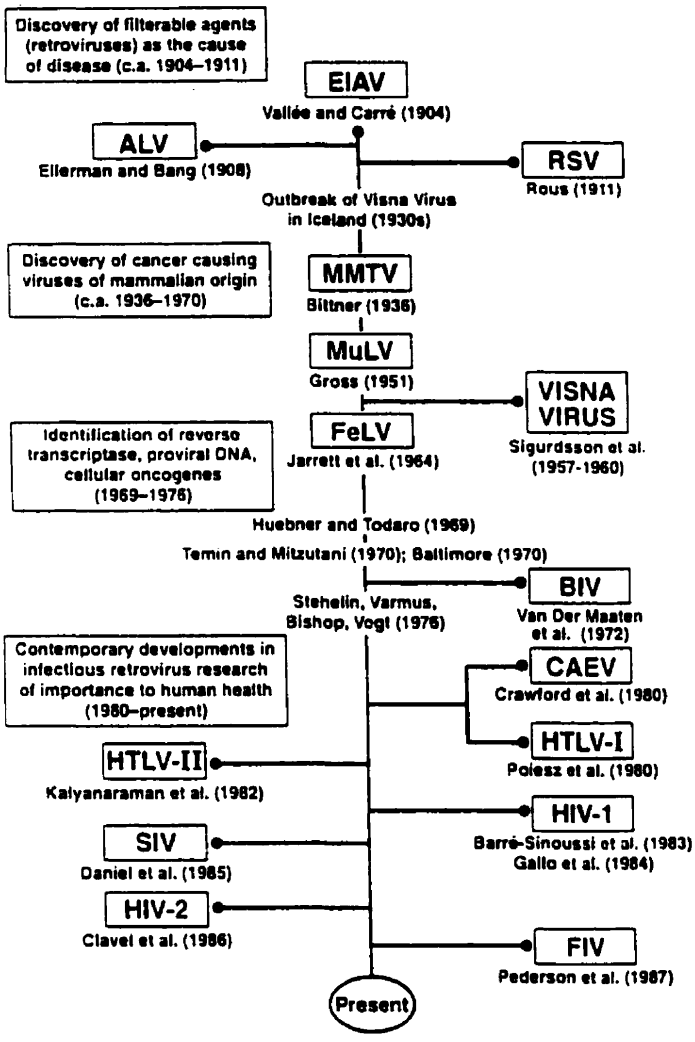


Figure 1: A historical perspective of retrovirus research. Taken from (111).

research in the field of AIDS/HIV a priority. For example, the funding allocated by the American National Institutes of Health to AIDS/HIV research stood at 10% of its total budget in 1994 (13) and today still consumes a large percentage of its budget. Central to the understanding of this disease has been the need to develop animal models which closely resemble the spectrum of phenotypes seen in humans in order to understand the mechanisms the virus uses to induce immunodeficiency, as well as for the testing of any potential vaccines.

The experimental work presented in this thesis describes several aspects of my research in the laboratory studying murine AIDS (MAIDS), a retrovirus-induced immunodeficiency syndrome of mice, which has some similarities to human AIDS (for reviews see (160,161,228)). Several experimental approaches have been utilised, both *in vivo* and *in vitro* to further extend our knowledge of the pathogenesis of MAIDS and, by extension, our knowledge of human AIDS. In order to provide a better understanding of to the reader, the first sections of the introduction will deal with some general background on retrovirology. These sections are not intended to be exhaustive, and the reader, if further interested, may refer to several detailed reviews on the subject (52,103,110,120,193,219,242,312,317,326). These sections will be followed by a review of the pathogenesis of AIDS, as well as reviews outlining the current animal models of AIDS, with an emphasis on the MAIDS model of the disease.

## 1- Definition and classification of retroviruses.

Retroviruses are characterised by two distinctive features: reverse transcription of viral RNA into DNA and a process of maturation outside of the host cell which transforms the initially non-infectious product of virus assembly into a mature, infectious virion (243). Retroviruses belong to the larger class of mobile genetic elements known as retrotransposons (196), which includes the Ty-1 element of yeast, and the Drosophila *gypsy* transposon.

Many criteria have been used to classify retroviruses. Bernhard (24,25) was the first to classify the viruses present in the tumors of mice, based on electron microscopy, as belonging to one of four groups (A to D). Other methods of classification include those based on host range; whether the virus is endogenous (i.e. integrated into the germline and transmitted in a Medelian fashion) or exogenous; whether or not the virus contains an oncogene; or other pathogenic properties. The most consistent classification, which is described below and shown in Table 1, divides the retroviruses into three subfamilies, the Oncovirinae, the Lentivirinae, and the Spumavirinae, based on their pathogenesis. The subfamilies are further divided into groups based on their nucleotide sequence and genome organisation.

### *1.1- The Oncovirinae:*

Most of these viruses were first described as tumor-inducing agents. There are five groups, which are not closely related, within this family. They are the following:

1) The avian leukosis-sarcoma (ALSV) group

2) The mammalian C-type virus group

3) The B-type virus group

4) The D-type virus group

5) The HTLV-STLV-BLV group

1) *The avian leukosis-sarcoma group* includes exogenous and closely related endogenous viruses of birds, such as RSV and AMV.

2) *The mammalian C-type virus group* comprises a large number of endogenous and exogenous viruses which have been isolated from mammals. Examples of this group include Moloney MuLV (Mo-MuLV), Abelson MuLV (A-MuLV) and FeLV. The human genome contains closely related defective endogenous proviruses, although no replicating endogenous viruses have yet been isolated.

The retroviruses of the above two groups can be further subdivided based on the presence or absence of an encoded oncogene. Those which do are replication-defective (described below) with the exception of some strains of RSV. The host range of the viruses from these two groups can also serve to further sub-divide them. For example, murine retroviruses can also be classified, based on their host range as being either ecotropic, xenotropic, or amphotropic. Ecotropic viruses replicate only on mouse cell; xenotropic viruses replicate in cells from many species, but not in murine cells; and amphotropic viruses replicate in both murine and non-murine cells.

As well, ecotropic viruses and recombinant MuLVs known as mink cell focus-forming viruses (MCF) can also be classified based on their relative efficiency of replication in cells from strains of mice bearing different alleles at

| Subfamily | Group | Example isolates | Comments |
|---|---|---|---|
| Oncovirinae | Avian leukosis-sarcoma | Rous sarcoma virus (RSV) | Exogenous; oncogene containing (*src*) |
| | | Avian myelobiastosis virus (AMV) | Exogenous; oncogene-containing (*myb*) |
| | | Avian erythroblastosis virus (AEV) | Exogenous; oncogene-containing (*erb*-A and *erb*-B) |
| | | Rous-associated virus (RAV)-1 to 50 | Exogenous; cause B-lymphoma, osteopetrosis, and other diseases |
| | | RAV-0 | Endogenous; benign |
| | Mammalian C-type | Moloney murine leukemia virus (Mo-MLV) | Exogenous; causes T-cell lymphoma |
| | | Harvey murine sarcoma virus (Ha-MSV) | Exogenous; oncogene-containing (H-*ras*) |
| | | Abelson murine leukemia virus (A-MuLV) | Exogenous; oncogene-containing (*abl*) |
| | | AKR-MuLV | Endogenous; benign |
| | | Feline leukemia virus (FeLV) | Exogenous; causes T-cell lymphoma, immunodeficiency, and many other diseases |
| | | Simian sarcoma virus | Exogenous; oncogene-containing (*sis*) (SSV) |
| | | Numerous endogenous and exogenous viruses, mostly in mammals | |
| | | Reticuloendotheliosis virus (REV); spleen necrosis virus (SNV) | Exogenous viruses of birds |
| | B-type viruses | Mouse mammary tumor virus (MMTV) | Endogenous and exogenous; mostly milk-borne; causes mostly mammary carcinoma, some T-lymphoma |
| | D-type viruses | Mason–Pfizer monkey virus (MPMV) | Exogenous; unknown pathogenicity |
| | | "SAIDS" viruses | Immunodeficiencies in monkeys |
| | HTLV–BLV group | Human T-cell leukemia (or lymphotropic) virus (HTLV) | Causes T-cell lymphoma; associated with neurological disorders |
| | | Bovine leukemia virus (BLV) | Causes B-cell lymphoma |
| Lentivirinae | Lentiviruses | Human immunodeficiency virus (HIV-1 and -2) | Cause of AIDS |
| | | Simian immunodeficiency virus (SIV) | Causes AIDS-like disease in certain monkeys |
| | | Feline immunodeficiency virus (FIV) | |
| | | Visna/maedi virus | Causes neurological and lung disease in sheep |
| | | Equine infectious anemia virus (EIAV) | |
| | | Caprine arthritis-encephalitis virus (CAEV) | |
| Spumavirinae | "Foamy" viruses | Many human and primate isolates [e.g., simian foamy virus (SFV)] | Exogenous, apparently benign |

Table 1: Retrovirus families. Taken from (52).

the *Fv-1* locus (for review see (159)). N-tropic viruses grow well in cells of *Fv-1*$^n$, but not *Fv-1*$^b$ mice, whereas B-tropic viruses grow well in cells of *Fv-1*$^b$, but not *Fv-1*$^n$ mice. The *Fv-1* alleles are co-dominant such that cells of hetrozygote *Fv-1*$^n$/*Fv-1*$^b$ mice restrict the replication of both N- and B-tropic viruses. The *Fv-1* gene has recently been cloned and appears to be derived from the *gag* region of an endogenous retrovirus unrelated to MuLV (26).

3) *The B-type virus group* contains only one infectious member-MMTV-although defective sequences are present in many species.

4) *The D-type virus group* includes the infectious viruses isolated from primates. Mason-Pfizer monkey virus (MPMV) is the prototype for this group.

5) *The HTLV-STLV-BLV group* includes HTLV-1, which induces T cell leukemia/lymphoma in humans, STLV, which causes malignancy in non-human primates, and BLV, which causes B-cell lymphoma in cattle.

None of the viruses of the last three groups encodes an oncogene, although they do contain novel sequences which contribute to their pathogenicity. MMTV, for example contains an open reading frame at its 3' end which has been shown to encode a superantigen (130,146,147,279). The members of the HTLV-STLV-BLV group contain novel regulatory sequences. MPMV has recently been shown to contain novel sequences in the 5' region which are important in the cytoplasmic export of the viral mRNA (39).

*1.2- The Lentivirinae:* These are exogenous viruses of both humans and animals which are responsible for a variety of neurological and immunological disorders (120,242), but have not been directly implicated in any malignancy. They are C-

type viruses. Their genome is characterised by a complex combination of genes in addition to the usual retroviral genes (discussed below). The best-studied members of this group are HIV-1 and HIV-2.

*1.3- The Spumavirinae:* The foamy viruses have been isolated from many species, including humans, but have not yet been associated with any disease (1). They acquire their name from the vacuolation of infected cells which is seen in tissue culture.

## 2- Structure of Retroviruses:

### *2.1- Chemical and physical properties of the virion:*

Retroviruses are formed when a core structure containing several polypeptides and the viral genome forms in juxtaposition to the plasma membrane of an infected cell. The core structure forms where the membrane has been modified by the insertion of virus-encoded glycoproteins, and in patches deficient in normal cell membrane proteins (65,271). The final steps in virus formation are the budding of the immature particles from the cell membrane followed by extracellular chemical and structural modifications. These processes have been observed by electron microscopy, and served as the first basis for retroviral classification (65).

The chemical and physical properties of a typical retrovirus reflects its mode of assembly. The virion contains internal structural proteins which, along with its RNA genome, forms the core. These internal proteins are known as the *gag* proteins (the word is derived from "group antigen"). Additionally, the core

contains reverse transcriptase (RT) and a tRNA primer needed to initiate provirus synthesis. The outer membrane of the virion contains the glycoprotein "spikes" (envelope proteins) which mediate the virus' binding to cell surfaces as the first step in infection.

Retroviruses have a density of 1.16-1.18g/cc in sucrose and 1.17-1.22g/cc in cesium chloride and are readily inactivated by heat, lipid solvents, and detergents, but are relatively resistant to inactivation by X or UV irradiation (215). Approximately 35% of the weight of the virion is lipid, 60% is protein, 3% is carbohydrate and 2% is RNA (215).

*2.2- Organisation of the retroviral genome:*

All retroviruses share a basic genomic organisation, which can be more easily explained by studying the proviral form of the virus, as seen in Figure 2. Retroviruses range in size from 7-10 Kb. At the virus' extremities are the long terminal repeats (LTRs) which are involved in regulating virus expression and viral integration into the host genome. The three coding regions common to all replication-competent viruses are found between these two LTRs. These genes are called *gag*, *pol-pro*, and *env*. Some retroviruses, mainly those of the family Oncovirinae, lack some or all of these genes, due to the presence of a v-onc, and are therefore unable to replicate by themselves. These viruses are replication-defective, and depend on other "helper" viruses to provide the missing functions required for their replication. In addition to these three genes, some viruses, particularly lentiviruses, contain additional genes which play a regulatory

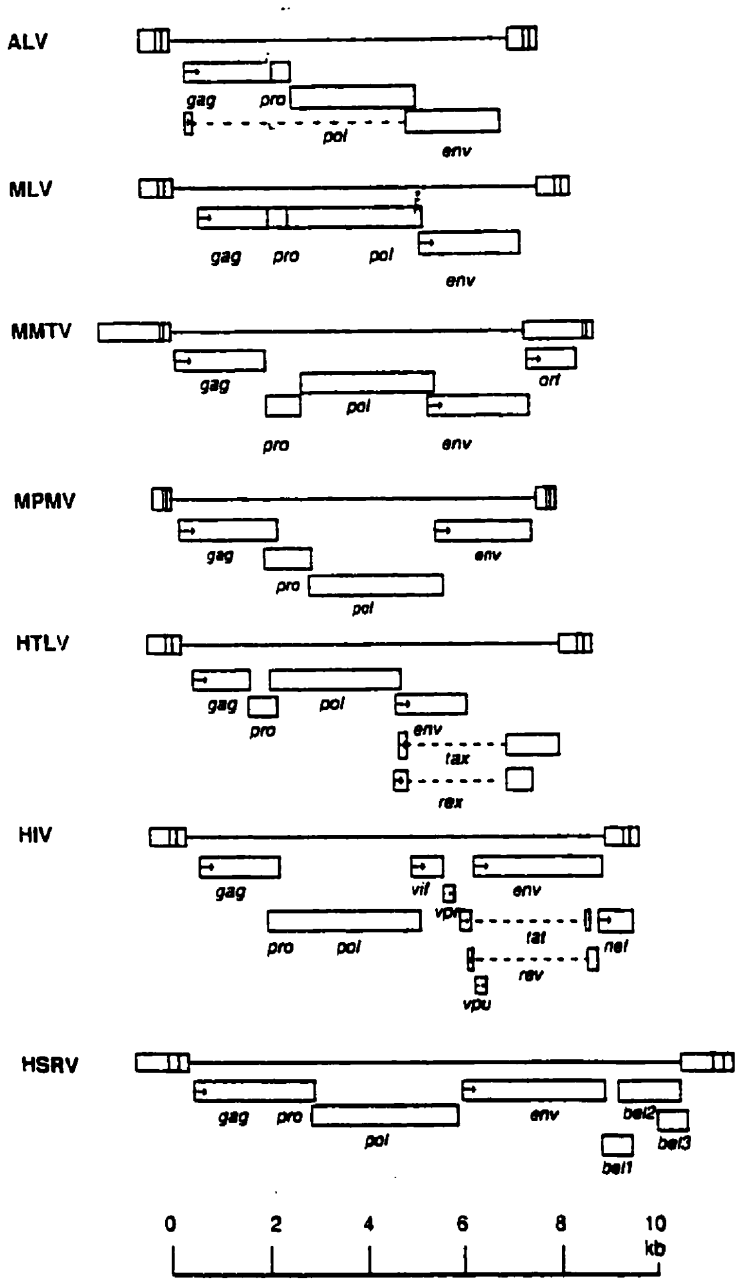


Figure 2: Coding regions of retrovirus genomes. Open reading frames in the genes of representative members of each retrovirus group are shown relative to the proviral structure. Small arrows show the sites of translation initiation. Taken from (52).

role during the viral life cycle. The presence of these additional genes has led to lentiviruses being termed "complex" viruses (59). The principal regions, both coding and non-coding, found in the retroviral genome, are described below.

**LTRs** are identical structures located at both extremities of the provirus which are divided into three regions- U3, R and U5 (203). With the exception of MMTV, the U3 region is non-coding and acts mainly as a promoter of viral transcription. A TATA box is located in U3, 20-30 bp upstream of the U3-R junction, which is where viral transcription initiates. Other promoter elements such as the CAAT box, are found about 100-150 bp distal to the transcription start site. Even further upstream in the U3 region lie enhancer elements which bind both ubiquitous and tissue-specific transcription factors (124,257,332). Additionally, the U3 region may also contain binding sites for virally encoded trans activating proteins, such as the Tax protein of HTLV-1 (297), or some coding sequences as found for the MMTV orf protein (53).

Adjacent to the U3 region lies the R region, so termed because its sequence is repeated and identical at both the 5' and 3' ends of the viral RNA (52). The main role of this region is during reverse transcription, where it permits the transfer of the minus DNA strand from one end of the viral RNA to the other (discussed below). Other sequences such as the 5' splice donor of HTLV-1 are occasionally found in this region as well (283,324). The U5 region is defined by its flanking sequences- R and the primer binding site (PBS). Mutational analysis shows a critical role for this region during reverse transcription and for the packaging of viral RNA (239).

**Primer binding site:** The 18 nt that form the PBS in the viral RNA are invariably complementary to the 3' terminal nt of the specific tRNA primer which initiates the process of reverse transcription.

**Leader:** This is the untranslated region between the PBS and the beginning of *gag*. Usually, the splice donor for all subgenomic messages is found in this region as is the signal for incorporation of the viral RNA into the virion (201).

***gag:***. The *gag* gene is translated from the full-length RNA to produce a precursor polyprotein that is subsequently cleaved to yield the structural proteins- matrix (MA), capsid (CA) and nucleocapsid (NC). Avian and mammalian C-type viruses contain an additional cleavage product, p12, that is located between MA and CA. N-terminal myristylation of MA allows the *gag* precursor to bind to the plasma membrane, which is critical for viral assembly (56). It has also been recently demonstrated that the MA of HIV-1 is needed for the import of the viral pre-integration complex into the nucleus of quiescent cells (100). Another group has shown an interaction of HIV-1 CA with cyclophilin A, which may act as a molecular chaperone or be required for viral assembly (61,93,309). The NC protein is found tightly associated with the viral RNA in the virion, and is required for viral assembly and for encapsidation of the viral RNA (78).

***pro:*** The viral protease PR, responsible for the cleavage of the *gag* and *pol* precursor polyproteins, is encoded by this gene. The location of *pro* between *gag* and *pol* varies depending on the virus.

***pol:*** This gene encodes the two essential activities required by the virus early in infection- the reverse transcriptase (RT) and the integrase (IN) needed to integrate

the viral genome into the cell DNA. Both *pol* and *pro* use mainly two mechanisms to be translated from the full-length viral RNA. These are the suppression of translation termination (readthrough) and frameshifting events (151).

**Internal Splicing Sites:** All replication-competent retroviruses contain splice acceptor sites located upstream of the *env* gene. This allows efficient translation of the *env* message. Additional splice acceptor sites are found in the lentiviruses and other complex viruses (254) to allow expression of their regulatory proteins. For example, the HIV-1 regulatory genes *tat, rev,* and *nef* are encoded in at least 12 different mRNA species (281).

***env:*** This gene encodes the two envelope glycoproteins which are also produced as a larger precursor protein. The glycosylated surface protein (SU) recognises the cell surface receptors and the smaller transmembrane protein (TM) anchors the *env* complex to the virion envelope (149).

**3' untranslated region**: This region is found in many oncoviruses and is located between the 3' end of *env* and U3. Its function is not entirely clear, however in MPMV this region has been shown to regulate the cytoplasmic export of the viral RNA (39).

**Polypurine tract:** This short sequence serves to initiate positive DNA strand synthesis during reverse transcription. It is located 5' to the beginning of the U3 region.

**Other genes:** Many viruses, such as the avian and mammalian C-type viruses, require only the *gag, pro, pol,* and *env* gene products for successful replication

(52). Other virus groups, especially the lentiviruses and other complex viruses encode additional proteins that play other roles during the viral life cycle. Many of theses proteins are classified as "*trans*-activators", analogous to those encoded by many DNA viruses. The best studied of these proteins belong to HIV-1 and will be presented below.

*tat:* This gene was named for its *trans*-activating potential and encodes a 14 kd protein essential to viral replication (60) that is translated from a multiply-spliced, subgenomic mRNA. This protein enhances viral expression by binding to an RNA loop structure (TAR) located in the 5' region of all HIV-1 transcripts. The interaction of *tat* with TAR stabilises the nascent transcript and facilitates its elongation. HTLV-1 encodes a protein, *tax*, with a similar function to that of *tat* (217).

*rev:* This small protein is a regulator of virus expression, and is responsible for the export of the viral mRNA from the nucleus to the cytoplasm (62). Like *tat*, it is also required for HIV-1 replication. It functions by binding to a specific RNA sequence, the Rev Response Element (RRE), which is located in the *env* region. Its mechanism of action is unclear, but is currently under study (30,91,95). HTLV-1 encodes a similar protein, *rex* (123,150).

The proteins described above, *tat* and *rev*, are the only additional proteins which are necessary for viral replication. The remainder of these proteins are dispensable for virus growth *in vitro*, and for this reason are referred to as "accessory" proteins (312).

***nef:*** All primate retroviruses, in addition to HIV-1, encode this protein of 27 kd (6,8). *nef* is bound to the cytoplasmic membrane via N-terminal myristylation. It was first believed to be a negative regulator, hence its name (from "negative factor"). More recent work in the SIV system using *nef*-deleted mutants has revealed its necessity in generating high levels of viral replication *in vivo* (170). The protein may also be involved in cellular activation (77).

***vif/vpr/vpu:*** These three small coding regions are derived from the single-spliced HIV-1 RNA (312). A role for shuttling the preintegration complex has been suggested for *vpr*, *vif* may play a role early in infection, and *vpu* has been implicated in viral release and in down-modulation of the viral receptor, although little is known about their precise functions. HIV-2 and most SIV strains do not encode *vpu*.

## 3- The Retroviral Life Cycle:

The retroviral life cycle can be divided into two phases. The first phase includes the entry of the virion core into the cytoplasm, reverse transcription of the viral RNA into double-stranded DNA, entry of the viral DNA into the host nucleus, and integration of the viral DNA into the host genome. All of these steps are mediated by proteins found within the virion, and do not require viral gene expression. The second phase of the retroviral life cycle includes the synthesis and processing of the viral genome, mRNAs, and proteins using host-cell factors, such as RNA polymerase II, and occasionally some specific viral products, such

as *tat*. Since the life cycle of retroviruses is circular, either one of the two above-mentioned stages could be viewed as the first phase.

*3.1- Cell attachment, penetration, and uncoating:*

Attachment of the virion to the cell is mediated by its *env* glycoprotein recognising a specific cell surface receptor. In the absence of the appropriate receptor, infectivity can be reduced by over seven orders of magnitude (52). Cell surface receptors have been identified for HIV-1, RSV, as well as for the ecotropic and MuLVs. They consist of CD4 (66,173), the LDL receptor (18), and the cationic amino acid transporter (3), respectively. GALV and the amphotrpic MuLV both use the same receptor, whose physiological role is sodium-dependant phosphate transport (330). Recently, members of the chemokine receptor family have been identified as co-receptors for HIV-1 (17,73,76,248). Following attachment, the virion enters the cell, most likely by receptor-mediated endocytosis. As the virion uncoats, the genomic RNA remains tightly associated with the RT, IN and *gag* proteins.

*3.2- Synthesis of viral DNA:*

Once the virion core has entered the cytoplasm, the process of reverse transcription of the RNA genome into double-stranded DNA occurs as depicted in Figure 3. This process is strictly dependent on the presence of the viral RT protein, which combines DNA polymerase and RNase H activities in one protein (12,308). RT lacks exonuclease (proofreading) activity, thus contributing to the genetic diversity seen in retroviruses. In particular, the RT of HIV-1 is quite

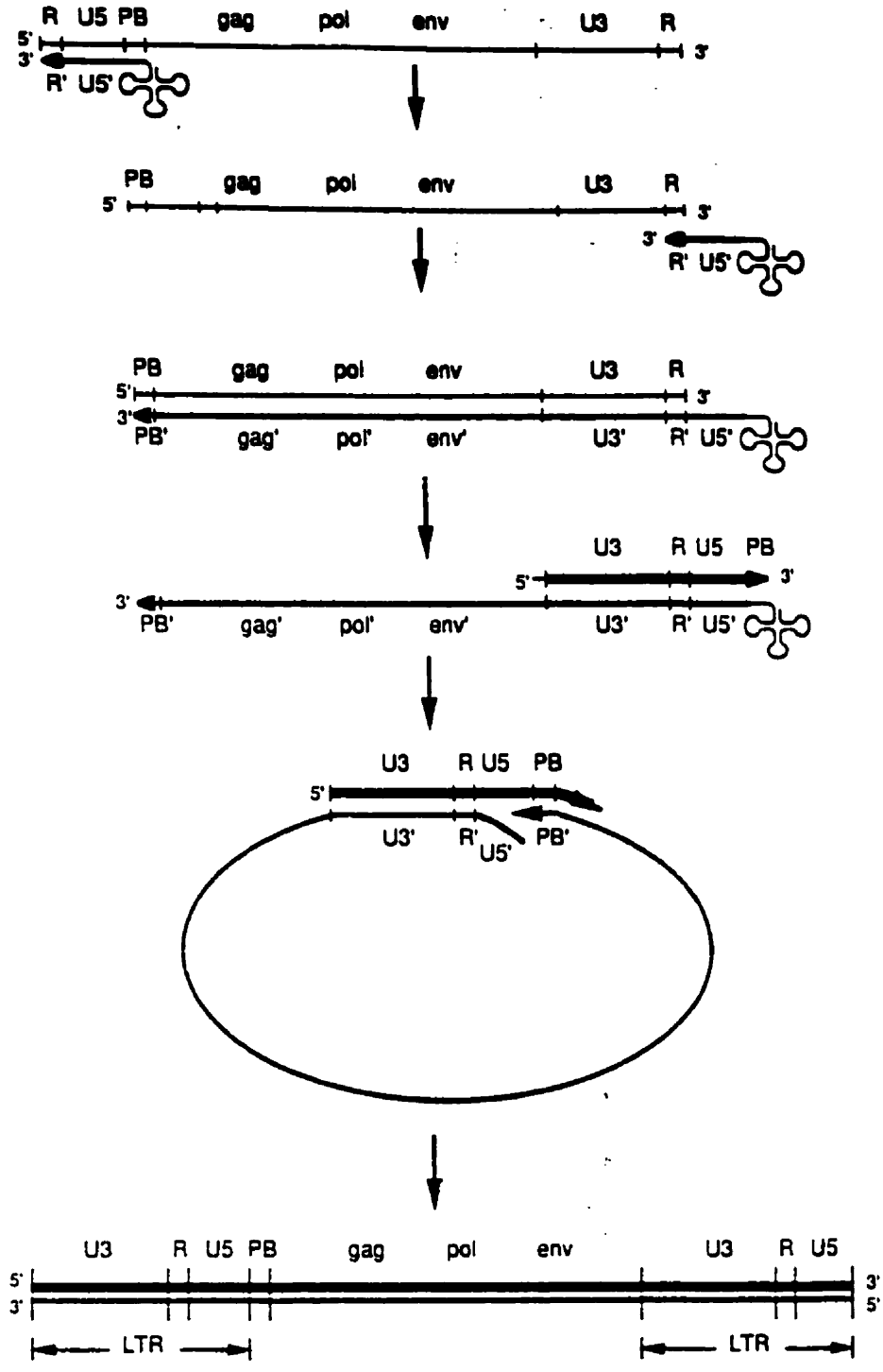


Figure 3: Mechanism of viral DNA synthesis. Thin lines represent RNA, medium lines represent negative-strand DNA and thick lines depict positive strand DNA. Negative-sense sequences are also denoted by a "prime". Taken from (52).

error-prone (31,54). Reverse transcription normally occurs in the cytoplasm of the infected cell, with synthesis of minus-strand DNA being primed by a specific tRNA that was incorporated into the virion. The resulting "minus-strand strong stop" DNA extends from the PBS into the U5 region, and ends in the R region. Base pairing of the R regions then allows this DNA to transfer or "jump" from the 5' end of the RNA to the 3' end of the RNA. DNA synthesis then proceeds from the 3' end of the viral RNA to the 5' end, resulting in a full minus-strand DNA molecule. With the exception of a polypurine tract in the 5' end of U3, the RNase H activity of RT is used to degrade the entire viral RNA. The remaining polypurine tract is then used as a primer to synthesise "plus-strand strong stop" DNA. At this point the tRNA primer is removed, permitting the "plus-strand strong stop" DNA to transfer to the 3' end of the minus DNA strand via base pairing of the PBS regions present in both DNA strands. DNA synthesis then proceeds for each strand thus generating the double-stranded DNA with LTRs that is the precursor of the integrated provirus.

*3.3- Integration:*

Integration is a process unique to retroviruses. A large majority of viral DNA molecules is integrated during every round of replication, and integration is probably necessary for proper expression of the provirus (52). These properties distinguish retroviral integration from the occasional aberrant "integrations" seen in some DNA virus infections. Following proviral DNA synthesis, the pre-integration complex composed of the proviral DNA, and the RT, IN and *gag* proteins is transported to the nucleus. For most retroviruses, breakdown of the

nuclear membrane during cell division is a requirement for integration. The notable exception to this rule is HIV, which can be actively transported across the nuclear membrane by aid of nucleophilic motifs present in MA (100). This retrovirus can therefore infect quiescent cells, unlike most retroviruses which can only infect dividing cells.

Sequence analysis of a large number of integrated proviruses has revealed several features which are common to all virus groups (29). First, the provirus is co-linear with the viral DNA and contains the genes in the order in which they are present in the genome, with the flanking LTRs. Second, the integration process leads to changes in both the viral and cellular DNA. The viral DNA is shortened by two bases (usually AA at each end), the exception being HIV, where only one nt is removed. The cell DNA flanking the integration site is not grossly rearranged. A short sequence (4-6 nt) at the site of integration immediately adjacent to the viral DNA is always duplicated, due to the introduction of a staggered break in the host DNA by the viral IN. The length of this duplication is characteristic of the virus group, and not the cell type. Third, the ends of the viral DNA are always 5' TG.....CA 3'. Finally, the ends of the LTRs are characterised by an inverted repeat spanning 2-10 nt. Mutations in this region greatly affect the integration process (55).

Integration of the provirus appears to be a random event, although it has been suggested that there is a tendency for integration into sites which are transcriptionally active (321,322). Once integrated, the provirus can be

considered to be perfectly stable. There is no known specific mechanism by which proviruses can be removed from the host genome.

*3.4- Expression of the provirus:*

After integration, all further replication occurs via transcription of the provirus into RNA using cellular systems. This process can be quite efficient, and it has been estimated that up to 10% of the mRNA in an infected cell can be derived from one or a few integrated proviruses (318). Transcription of the provirus is accomplished by cellular RNA polymerase II (52), and begins at the U3-R junction and proceeds into the 3' LTR. The viral transcript is then cleaved at the end of the 3'R, and polyadenylated at this site. A 5' methylated cap is also added to the transcript. Full length transcripts may either be translated to give rise to *gag* and *pol* proteins, or may be used for virion assembly. As well, all retroviruses encode a single-spliced *env* message.

As discussed above, the "complex" retroviruses (58,59) express additional multiply-spliced messages which encode accessory or regulatory proteins. These viruses have evolved strategies to allow expression of these messages. Viral expression is controlled by two types of regulatory proteins. These are the viral *trans*-activators, such as *tat*, and the *rev*-like proteins, which control the levels of full length and single-spliced messages relative to the multiply-spliced messages. These two types of regulatory protein thus strongly influence whether the provirus is replicated or remains in a latent state (219).

*3.5- Translation and virion assembly:*

After transport to the cytoplasm, the different-sized transcripts are then translated to give the *gag*, *pol*, *pro*, and *env* proteins, as well as any accessory or regulatory proteins. As mentioned above, the translation of the *pol* and *pro* genes involves readthrough or frameshift events (151), which leads to levels of the *pol* and *pro* proteins which are about 5-10% the levels of *gag*. Readthrough events are also used to translate some multiply-spliced HIV-1 RNAs (281).

Assembly of virions is a poorly understood aspect of retroviral replication. By electron microscopy, two patterns can be discerned, which differ in the site of assembly. For most viruses groups, the process of budding and assembly are simultaneous. For B- and D-type viruses, capsid assembly occurs in the cytoplasm. This structure, an A-type particle, then associates with the membrane and buds out. In both cases, at the time of capsid formation, two copies of genomic RNA interact with the *gag* precursor protein and are encapsidated. As well, a structural rearrangement of the capsid to a more condensed form is seen. This maturation occurs at the same time that the precursor *gag* and *pol* proteins are cleaved by PR to give their individual proteins.

**4- Pathology induced by retroviruses:**

The Oncovirinae and Lentivirinae subfamilies of retroviruses are responsible for a wide spectrum of disease, as illustrated in Table 2. Retroviruses of the Spumavirinae subfamily have yet to be linked to any specific pathology

| Disease | Species |
|---|---|
| Leukemia/Lymphoma | Fish, chickens, cats, mice, rats, cows, sheep, primates, humans |
| Carcinoma | Chickens (renal), mice (mammary) |
| Sarcoma | Reptiles, chickens, mice, rats |
| Wasting and autoimmune diseases | Chickens, cats, primates, humans |
| Immune deficiency | Cats, mice, primates, humans |
| Anemia | Mice, horses |
| Arthritis | Sheep, goats |
| Neurological syndromes | Mice, sheep, goats, humans |
| Osteopetrosis | Chickens |

Table 2: Diseases caused by retroviruses. Taken from (185).

(307). The diseases most commonly associated with exogenous retroviruses can be divided into four groups: 1) malignant tumours; 2) proliferative diseases, both neoplastic and non-neoplastic; 3) anemias; and 4) the "slow", chronic, degenerative diseases (307). An examination of these retrovirus-induced diseases has been given elsewhere (52,185) and will be only be discussed here briefly.

The "acute" oncogenic retroviruses of the Oncovirinae subfamily (27), which are mainly defective, transform cells in tissue culture and induce tumors with a short latency period in the appropriate animal hosts (185). These viruses have incorporated into their genome copies of cellular genes (c-onc, or proto-oncogenes) which have been altered (v-onc) in such a way to transform the host cell. In most cases, expression of the viral oncogenes carried by a single virus is sufficient for tumor induction, although in other cases, additional genetic events are required (19,263).

The "slow", or non-transforming, retroviruses of the Oncovirinae subfamily, cause tumors with a long latency of 6 months or more (52,306,307). These viruses carry only genes involved in replication, and do not contain v-oncs. The majority of neoplasms induced by these retroviruses are either hematopoietic or epithelial in origin (307). Tumor induction by these agents is a complex multi-step process of continuous and dynamic evolution. Cellular transformation occurs mainly by viral disruption of cellular genes either by insertional activation of a proto-oncogene by one of many mechanisms, or more rarely, by gene inactivation (185). Virus-induced cellular transformation can also occur due to the presence of an "autocrine loop", where a viral gene product stimulates the growth of infected

cells (198). One example of this phenomena is the trans-activation of the interleukin-2 receptor gene in infected lymphoid cells by the *tax* protein of HTLV-1, thus favoring their proliferation (179). Additional, currently unknown events, are required in this example for full neoplastic transformation to occur (101).

Infection with retroviruses can also lead to the development of chronic degenerative diseases which are mainly observed in lentivirus infection (120,242). For example, Visna-maedi disease, a neurological disease of the central nervous system (CNS) of sheep, is caused by a lentivirus (242). Caprine arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) are two other lentiviruses which cause disease in goats and horses, respectively. Such diseases have been recognised for decades, and studies on these viruses have provided clues in the study of their human counterparts. The mechanisms by which these complex organisms cause disease are less well understood than those of the Oncovirinae, as can be seen by our current attempts to understand the pathogenesis of HIV-1 infection.

Lentiviruses share several common characteristics. These agents, especially HIV-1, have cytopathic effects which can observed *in vitro*. This cell death has been proposed to be due to direct toxicity of a viral gene product, extensive viral replication, and perhaps from cell fusion (reviewed in (193)). Furthermore, all lentiviruses demonstrate a T-lymphocyte and macrophage tropism (120,242). The ability of lentiviruses to elude the host's immune response against the infected cells is another factor which is related to the

persistent, chronic diseases they cause (255). This ability, especially in the case of HIV-1, is related to their ability to produce progeny of varying antigenic potential (31,54). Lentiviruses also have the capacity to produce a latent state by expressing certain of their regulatory genes (219). These modes of immune evasion are complemented by the fact that these viruses tend to infect cells of the immune system itself. This leads to a situation where the number of latently or productively infected cells remains a step ahead of the host's attempts to clear them, thus leading to a breakdown of the immune system (54,218,219).

Disease in lentivirus-infected hosts may be primary, caused directly by the lentivirus, or secondary, caused by the opportunistic pathogens that proliferate unchecked as a result of the loss of helper T lymphocyte function (see below). Since the advent of AIDS, enormous resources have been brought to bear on the mechanisms of pathogenesis of HIV. Several animal models have been developed in order to mimic one or more aspects of the human disease. These models, with an emphasis on the MAIDS model, will be discussed following a review of the pathogenesis of AIDS and the various animal models of this disease currently in use.

## 5- Pathogenesis of AIDS:

AIDS was first recognised in mid-1981 when unusual clusters of *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma (KS) were reported in young, previously healthy homosexual men in New York City, Los Angeles, and San Francisco (280). Soon afterwards, AIDS was identified in Europe, the

Caribbean, and Africa. Today, AIDS is a global pandemic, and the World Health Organisation (WHO) estimates that at least 10 million people are infected with the virus world-wide (211).

Typically, HIV-1 infection begins with an acute flu-like illness of variable severity, a prolonged period of clinical latency, followed by clinical disease marked by increased susceptibility to opportunistic infections and certain neoplasms such as lymphomas and Kaposi's sarcoma (251). There have been no reports of HIV-infected individuals who have cleared all virus after the acute illness phase of the disease (125). The period of clinical latency is marked, nevertheless, by a high production of HIV-1 virions in the face of a vigorous HIV-1-specific humoral and cell-mediated immune response (33,251,252). These early immune responses initially curtail HIV-1 replication, resulting in decreased viremia, but they do not eliminate all HIV-1 from the body (49,64,115). Results from antiviral therapy studies have also revealed that there is continual high-level HIV-1 replication, $CD4^+$ T cell infection, cell death and new $CD4^+$ T cell production during the clinical latent period (84,134,252,325)

The duration of the clinical latent period varies widely, and progression to AIDS occurs over a period of 8-10 years in 80% of HIV-1-infected individuals (42,89,199). Ten percent to 15% of HIV-1-infected patients are rapid progressors who develop AIDS within 2-3 years of infection; however there is also a group of 5-10% of infected individuals who remain healthy and do not develop AIDS and are termed long-term nonprogressors (42,80,194,285). One recently identified factor which confers some resistance to the development of AIDS in HIV-infected

individuals is the presence in these individuals of mutated CCR2 or CCR5 chemokine receptors, which also serve as co-receptors for HIV-1 (11,259,294). Detailed studies of these nonprogressing individuals may yield important insights into the control and prevention of HIV-1 infection and disease development.

*5.1- Depletion of CD4⁺ T cells:*

The hallmark AIDS is the extreme CD4⁺ T cell depletion seen in patients as the disease progresses. The CD4 molecule found on T cells and macrophages has been identified as the receptor for HIV, through which the viral *env* (gp120) binds and gains entry into the host cells, and very recently several groups have isolated other co-receptors for the virus which belong to the chemokine receptor family (17,63,73,76,248). Many groups have established a direct correlation between HIV burden and CD4⁺ T cell depletion (133,169,320). The mechanism(s) by which HIV causes this CD4⁺ T cell depletion is not well understood, although several hypotheses have been put forward to explain this phenomenon, as summarised in Table 3.

These hypotheses include a direct cytopathic effect of virus formation on infected cells due to viral budding (280), the binding of HIV *env* proteins to intracellular CD4 molecules (280) and the induction of apoptosis of HIV-1-infected lymphocytes (7,114,223). Indirect mechanisms of HIV cytopathicity include an autoimmune phenomenon, where anti-gp120 antibodies cross react with MHC class I and class II determinants (67) found on CD4⁺ T cells and a process by which uninfected CD4⁺ T cells bind circulating gp120 and become targets for lysis by antibody-dependent cellular cytotoxicity (280).

Potential Mechanisms of Depletion of $CD4^+$ T cells in HIV infection.

1. Direct cytopathic effect on HIV
2. Infection by HIV of a $CD4^+$ T-cell precursor or stem cell
3. Selective depletion of a subset of $CD4^+$ T cells or nonlymphoid $CD4^+$ cells that are trophic for $CD4^+$ T cells and thus critical for the propagation of the entire $CD4^+$ T-cell pool
4. Induction of secretion of soluble substances toxic to $CD4^+$ T cells by HIV-infected $CD4^+$ T cells and/or monocytes
5. Syncytia formation between uninfected and infected $CD4^+$ T cells
6. Autoimmune phenomena
7. Superantigen effects with nonrandom activation of $CD4^+$ T cells expressing particular Vβ T-cell receptors, enhancing their susceptibility to infection with HIV
8. Apoptosis or programmed cell death of stimulated $CD4^+$ T cells

Table 3: Taken from (280).

Another hypothesis to explain the $CD4^+$ T cell depletion seen with this disease has been put forward by Clerici and Shearer (50) who argue that T helper ($T_H$) type 1 and type 2 responses contribute to the immune dysregulation seen in HIV-1 infection. Their findings suggest that a $T_H1$ response which promotes cell mediated immune responses via IL-2 and INF-$\gamma$ production is protective whereas a $T_H2$ response leads to a less effective immune response to HIV-1 by augmenting B cell humoral responses via IL-4, IL-6, and IL-10 production. Induction of a $T_H1$ response by HIV-1 would therefore correlate with protection from $CD4^+$ T cell depletion and progression to AIDS while those individuals who respond to HIV-1 by mounting a $T_H2$ response would be susceptible to virus spread and the $CD4^+$ T cell depletion.

*5.2- Role of monocyte/macrophages:*

Macrophages and monocytes are also targets of HIV infection (274). HIV has been detected in monocytes/macrophages from the blood, brain, and lung of infected patients. HIV infection of these cells is persistent and poorly cytopathic, unlike the situation seen with $CD4^+$ T cells, as discussed above. The role of HIV-infected monocytes/macrophages in the pathogenesis of AIDS is unclear, although these cells may function as a reservoir of HIV within the host as seen for certain lentivirus infections in animals (242). Thus, infected monocytes/macrophages may spread HIV to uninfected $CD4^+$ T cells during normal antigen presentation. Since the monocyte/macrophage (microglial) cell is the one found to be most often infected in the brains of infected individuals, it is thought that these cells play a role in the neuropathogenesis of HIV infection (88,268), potentially

through the release of cytokines or other factors that are directly toxic to neurons or that lead to inflammation.

*5.3- Role of B cells:*

Along with a depletion of $CD4^+$ T cells, HIV-infected individuals also exhibit B cell dysfunctions, some of which are secondary to the T cell deficiency, while others are T cell-independent (274). B cells from AIDS patients show a spontaneously polyclonal proliferation and activation, increased immunoglobulin (Ig) secretion, and hypergammaglobulinemia, which all suggest a chronic B cell activation. A large number of these activated B cells are specific for HIV epitopes (280). In addition to the hyperactivity, the B cells also exhibit an intrinsic defect in antigen- and mitogen-induced responses at all stages of infection (280).

Interleukin-6 (IL-6) plays a critical role in the terminal differentiation of activated B cells. Exposure of peripheral blood lymphocytes (PBL) to HIV *in vitro* induces IL-6 production, mainly by monocytes (240). IL-6 also synergizes with other cytokines to induce HIV expression in infected monocytes (265). Oyaizu et al. (249) demonstrated that the gp120 and gp160 *env* glycoproteins can induce IL-6 secretion in PBL *in vitro*. Together these results suggest that monocytes and T cells contibute to the production of IL-6 which plays an important role in the pathogenesis of the B cell activation seen in HIV infection. There is also "cross-talk" between B cells and infected monocytes. Activated B cells secrete TNF-$\alpha$ and IL-6, and this is also seen in the spontaneously activated B cells in HIV-infected individuals. Production of these two cytokines has been shown to induce HIV expression in chronically infected monocyte lines (265).

B cell dysregulation in patients with AIDS is also manifested by their high incidence of B-cell lymphoma. It has been shown that the Epstein-Barr virus (EBV) plays a major role in the CNS lymphomas seen in AIDS (202), while its role in systemic lymphoma in AIDS patients is less clear. As well, CNS lymphoma in other immunodeficiency states was not associated with EBV (280). This strongly supports a pathogenic role for EBV in some AIDS-associated lymphomas.

## 6- Animal models for AIDS research:

A number of animal models are currently in use to study various aspects of HIV-1-induced pathology. These models include simian immunodeficiency virus (SIV) infection of macaques (104), infection of cats with feline immunodeficiency virus (FIV) (298), and HIV-1 infection of chimpanzees (99), rabbits (181) and SCID mice reconstituted with human tissues or cells (4,168,221,231,232). In addition to these models of lentivirus infection, transgenic mouse technology has also been employed to study the role of individual HIV-1 proteins in specific tissues (37,162,190,200,258,293,323). Finally, there is a C-type retrovirus infection of mice which reproduces several of the clinical features of AIDS and has been termed murine AIDS (MAIDS) (160,171,228). The experimental work in this thesis is based on this latter model of AIDS. The relevance of the above-mentioned models to AIDS will be discussed, followed by a comprehensive review of the MAIDS model.

In order to better understand the relevance of each of the animal models of AIDS, it is instructive to begin with an idealised perfect model, which is a scientific contradiction. The properties of an idealised model include the following:

- Induction by a lentivirus with the same regulatory genes as HIV-1;
- Infection of $CD4^+$ T cells and macrophages using the CD4 receptor and known co-receptors;
- Induction of a chronic disease, with a long period of low viral burden and a slow $CD4^+$ T cell depletion;
- Induction of a clinical spectrum of disease similar to that seen in AIDS, including neurologic dysfunction, lymphomas and opportunistic infections;
- Use of a small animal to allow large-scale experimentation at a reasonable cost, and whose genetics is well known.

Since no model is ideal, each has its own strengths and weaknesses which must be kept in mind when interpreting how the results apply to HIV-1 infection and to the development of AIDS.

*6.1- Infection of Chimpanzees with HIV-1:*

As the closest phylogenetic relative to humans, the chimpanzee (*Pan troglodytes*) has a critical role to play in advancing our understanding of HIV-1 infection and the development of AIDS. The chimpanzee can be an excellent model since its CD4 receptor is nearly identical to the human CD4 receptor (45), and its immune system has been well characterised (335). Chimpanzees do

support HIV-1 infection via a variety of routes (97,98) and with various isolates (241).

Over 100 chimpanzees have been infected with HIV-1 and until very recently, none had developed any AIDS-like ailments (247). The one animal that did finally develop AIDS had been infected for over 10 years (247). Most infected animals did not develop opportunistic infections, and only in rare cases was a decline in $CD4^+$ T cell numbers observed (172). It is also difficult to isolate virus from the peripheral blood of infected chimpanzees (172). The overall picture is one of persistent infection without progression to AIDS for the vast majority of infected animals.

Several explanations have been put forward to explain the lack of disease progression in HIV-1-infected chimpanzees (96), including the relative sterility of the animals' housing quarters which are free of other infectious agents which could potentially act as co-factors for disease progression. Another problem may be the use of tissue-culture adapted HIV-1 which may be attenuated to the point where they are inefficient in causing disease.

Despite the shortcomings of this model for HIV-1 pathogenesis, much value has been placed on this model for vaccine development. The fact that chimpanzees become chronically infected on challenge with HIV-1 makes this model an excellent tool to test the efficiency of vaccines to prevent or slow human HIV-1 infection (172). The long latency of disease is therefore analogous to the course of disease seen in humans, but limits this model's potential to gain wide

use, as does the fact that the chimpanzee is an endangered species and is very costly to obtain and maintain.

*6.2- Infection of Rabbits with HIV-1:*

It was previously known that the laboratory rabbit (*Oryctolagus cuniculus*) is readily infected by the human retrovirus HTLV-1 (224). It was later shown that rabbits support HIV-1 infection as well (90,181). The rabbits were infected by the intraperitoneal injection of cell-free virus following pre-treatment with agents that activate peritoneal macrophages, or by intravenous injection of HIV-1-infected human lymphoid cells.

HIV-1-infected rabbits produce antibody to HIV-1 proteins within 10 weeks of administration of the virus (172). HIV-1 can be detected in infected rabbits for a period of up to two years after infection by PCR, *in situ* hybridization, and virus isolation from rabbit cells and organs (313,319). Reports from several groups suggest that the brain may be a preferential target of HIV-1 infection in rabbits (172). There is no consistent evidence for clinical disease in infected rabbits, with the caveat that few infected animals have been observed for long periods of time. There is no obvious immunosuppression in infected animals, but animals infected with HIV-1 were reported to have diminished cellular responses to recall antigens (112).

Despite strong evidence showing persistent HIV-1 infection, there are several shortcomings to this model which render it less than ideal for testing antiviral agents or vaccines aimed at HIV-1. Both *in vitro* and *in vivo* studies demonstrated that large doses of virus are required for infection (90,181,182). As

well, the virus is difficult to isolate, and the infection, although persistent, does not lead to overt disease. Another problem with the rabbit model is lack of reagents available for study of their immune system, especially for understanding and analysing differences in T cell subpopulations. Until the means to exacerbate the course of infection are available or overt disease can be demonstrated, this animal model remains interesting but of limited experimental use.

*6.3- SIV infection of Macaques:*

Reports of outbreaks of opportunistic infections in captive macaque monkeys at several primate research centres in the early 1980s led to the identification of a simian immunodeficiency virus (SIVmac) that induces AIDS in macaques (68,191). SIVmac is one of a family of primate lentiviruses. In their natural hosts, primate lentiviruses typically produce a chronic asymptomatic infection. For example, sooty mangabeys infected with SIVsm, which is closely related to SIVmac, show no evidence of immunodeficiency. In contrast, cross-species transmission of primate lentiviruses out of their natural hosts may result in AIDS. SIVmac appears to have originated as a result of the accidental transmission of SIVsm from naturally-infected sooty mangabeys into macaques (212). It is hypothesised that a similar cross-species transmission of a primate lentivirus is likely to be responsible for the development of AIDS in HIV-1-infected individuals, although the nonhuman primate reservoir of HIV-1 has yet to be identified (158).

Infection of macaques with SIV resembles HIV-1 infection of humans in many respects. The one notable difference is in the latency periods of the two

viruses, with AIDS developing in SIV-infected macaques within 6 months, compared to the up to 10 years required to develop AIDS in HIV-1-infected individuals (172). In this respect, SIV infection closely resembles the typical course of paediatric AIDS. One must however bear in mind that the SIV studies normally use a virus inoculum selected for its ability to induce disease reproducibly, and in a time frame suitable for experimentation.

The clinical manifestations of AIDS in HIV-1-infected persons and in SIV-infected macaques are very similar. Inoculation of macaques with SIVsm or SIVmac leads to a persistent infection characterised by immunodeficiency, opportunistic infections, and eventual death. As in AIDS patients, the terminal stages of disease are marked by a reduction in $CD4^+$ T cells, loss of antibody reactivity to viral *gag* proteins and antigenemia. SIV-infected macaques develop a similar spectrum of opportunistic infections with such agents as cytomegalovirus (CMV), *Pneumocystis*, and *Candida*. Other frequent observations include a wasting syndrome and CNS disease that are virtually indistinguishable from those seen in human AIDS. Interestingly, Kaposi's sarcoma has never been observed in SIV-infected macaques (192). This last observation may be explained by the recent isolation of a novel Kaposi's sarcoma-associated herpesvirus (KSHV) (47,226) in humans which may not be present in macaques.

In most SIV-infected animals, lymphadenopathy, viremia, antigenemia and a decrease in $CD4^+$ T cells occurs within a few weeks of virus inoculation (192,217,270,336). Thereafter, three distinct clinical courses have been observed

(192,217,270,336). About one-third of infected animals develop persistent anaemia in the absence of a systemic SIV-specific antibody response, and go on to die in the first few months following infection. Another one-third to one-half of infected animals develop persistent viremia in the face of a strong SIV-specific antibody response. These animals generally survive 1 to 3 years, with clinical demise characterised by weight loss, a decrease in SIV-specific antibody titre, and low levels of circulating $CD4^+$ T cells. Thirdly, a small number of SIV-infected animals remain persistently infected for years. In these animals, SIV is not readily detected in PBL after the first few months of infection and a strong SIV-specific antibody response is generated.

At autopsy, end-stage disease is often marked by widespread distribution of SIV (16,132,192,217). Besides lymphoid tissues, primary lymphoid tissues associated with other organ systems (e.g. lung and intestines) are also common targets. Gut-associated lymphoid tissue is nearly uniformly infected and may account for the intractable diarrhea seen in most terminally ill macaques. SIV is also observed in non-lymphoid tissues such as kidneys (132).

A powerful feature of this animal model is the availability of molecular clones of proviral DNA that can give rise to infectious virions when transfected into susceptible cells in tissue culture. SIV derived by such a manner can then be used for experimental inoculation of macaques, and useful information can be obtained by studying the course of disease in these inoculated animals. Several molecular clones of SIVmac/sm have been characterised in such a fashion (reviewed in (157)). Manipulation of the proviral genome by exchanging or

deleting genetic information can pinpoint the determinants potentially involved in the development of AIDS. The strength of this technique has been shown by Kestler et al. (170), who studied the *nef* gene of SIVmac. It was found that mutants of SIVmac lacking *nef* did not induce AIDS in infected animals and that the same infected animals had a much lower viral burden. Furthermore, mutants of SIVmac containing a stop codon in *nef* reverted *in vivo* to a sense codon, suggesting a strong selective pressure for the *nef* protein. As for *nef*, other SIV genes can be evaluated for *in vivo* relevance to viral replication and AIDS development.

Another use of this model is in the testing of potential AIDS vaccine strategies using a variety of methods (69,139,269,301). For example, vaccine trials in macaques have demonstrated that a protective immune response can be elicited by whole inactivated virus vaccines, and is apparently mediated by humoral factors (157). Other trials using live attenuated SIV vaccines were found to protect macaques from disease but not from infection with a highly pathogenic strain of SIV (214). The use of simian/human immunodeficiency chimeric viruses (SHIV) has also been useful in studying immune responses to the envelope of HIV-1. SHIVs comprise the core of $SIV_{mac}$ surrounded by the envelope of HIV-1 and are infectious in macaques (197,277,287,288), but earlier SHIVs caused infection, but no disease. Nevertheless, these animals develop immune responses to the envelope of HIV-1 and therefore serve as a model for studying the evolution of the immune response to the envelope of HIV-1 and for testing vaccines utilizing the HIV-1 envelope glycoprotein. Recently, a SHIV

variant was isolated that leads to $CD4^+$ T cell depletion and AIDS in infected macaques (155). This model will be pertinent in evaluating the efficiency of vaccines and drugs directed at HIV-1.

In summary, SIV infection of macaques is currently the best available animal model for the study of the pathogenesis of AIDS as the disease spectrum in these animals closely resembles human AIDS. In combination with the use of SHIV infection of macaques, this model will be in the development of novel vaccine strategies and in the testing of antiviral drugs.

*6.4- Infection of SCID-hu mice with HIV:*

The C.B-17 SCID/SCID mouse carries a spontaneous recessive mutation that gives rise to a severe combined immunodeficiency (34). This strain has no functional B or T cells due to an enzymatic defect which causes incomplete recombination and rearrangement of the T cell receptor (TcR) and immunoglobulin (Ig) genes (for review, see (327)). Due to this defect, the SCID mouse may be engrafted with tissue from various sources with no possibility of rejection. This ability has made it possible to develop animal models for the study of HIV-1 infection of human lymphoid tissue engrafted onto the SCID mouse (220,229). One model is based on the transfer of human PBL into SCID mice, generating the hu-PBL-SCID mouse. The other approach taken involves transplantation of fetal human thymus and liver into the SCID mouse (SCID-hu thy/liv model). The fetal liver supplies human lymphoid precursor cells and the fetal thymus provides an environment for human T cell development. In addition to direct measurements of HIV infection in the human lymphoid grafts, certain

immune functions carried out by the grafted tissue can also be monitored for evidence of immunodeficiency caused by the virus (232).

*6.4.1-The hu-PBL-SCID model:*

Infection of these mice with HIV-1 results in changes in lymphocyte function consistent with those seen in infected individuals (232). In those experiments, the virus was inoculated intraperitoneally (I.P.), and a variety of techniques were used to detect the virus, including *in vitro* tissue culture of virus from PBL, *in situ* hybridization, and PCR (232). The human PBL graft is susceptible to every viral isolate tested, including those from paediatric patients (232,234). Infection with most HIV-1 isolates leads to $CD4^+$ T cell depletion within a few weeks, while $CD8^+$ T cell numbers are unaffected (230). HIV-1 infection of engrafted SCID mice leads to hypergammaglobulinemia , which is seen in primary infection of humans (183). As well, the EBV-associated lymphomas seen in AIDS patients have a counterpart in this mouse model of HIV-1 infection (235,260,276).

There are two important features of this system which mimic more closely the disease seen in humans as compared to the *in vitro* infection of primary human $CD4^+$ T cells. Firstly, the genotypic diversity of HIV-1 primary isolates is maintained in this model (35), and secondly, the role of the HIV-1 accessory genes is more readily ascertained (5,152). Experiments with *nef*-deletion mutants in this system confirmed results obtained by researchers using SIV-infected macaques as a model for HIV infection in humans (see above), showing that *nef* is a critical gene for pathogenesis (152,170).

Another important use of this model is in the development of potential anti-HIV-1 vaccines. PBL from human volunteers vaccinated with an anti-*env* vaccine were transferred to SCID mice. These mice were then challenged with the same strain of HIV-1 used to generate the vaccine. It was found that indeed these mice were resistant to HIV-1 infection (233). Other strategies are currently being employed with this model to further understand the humoral and cellular mechanisms that may contribute to resistance to HIV-1 infection (44,177,253,316).

*6.4.2- The SCID-hu thy/liv model:*

In this variation on the above theme, the observed effects of HIV-1 infection are generally similar to those for the hu-PBL-SCID model. It has been shown that 95% of SCID-hu thy/liv mice prepared with tissue from 18-23 week old fetuses may be infected by intravenous inoculation of HIV-1, with resulting viremia (168). Other groups have reported that the thy/liv implant can be infected with HIV-1 by direct injection of the virus into the implant (4,152). Depending on the strain of HIV-1 used and the size of the inoculum, depletion of $CD4^+$ cells is observed 3-9 weeks post-infection (4,32,152). This cell depletion is visualised histologically as hypocellularity, and thymic involution (4,32,299). The first cells to disappear are the $CD4^+CD8^+$ immature thymocytes, followed by a loss of mature $CD4^+$ T cells. Eventually, even the $CD8^+$ T cells are lost. Virus in infected mice can be detected by PCR, and in fact this method of detection was used to demonstrate the efficiency of AZT in infected mice (222,289).

While the majority of studies using the SCID-hu thy/liv mouse have focused on the impact of HIV-1 on the levels of $CD4^+$ T cells, HIV-1-induced pathology has also been observed in the thymic epithelial (TE) cells of the engrafted thymus (299). Degeneration of TE cells was observed, and these cells were associated with HIV-1 RNA, although it is not clear that the TE cells were productively infected. These results suggest that HIV-1 may disrupt the thymic microenvironment, thereby contributing to the depletion of $CD4^+$ T cells seen in HIV-1-infected individuals. This particular model may be especially useful as a tool for gaining insight into the impact of HIV-1 on the thymus and into the role of the pediatric thymus in the pathogenesis of HIV-1 infection in children.

Taken together, both variations of the SCID mouse model are important tools for studying the pathogenesis of HIV-1 infection, although they have some drawbacks. These models do not recapitulate all aspects of HIV-1 infection in humans, nor do they mimic the trafficking of virus and lymphoid cells between various tissues. As well, due to the lack of an immune system, the mice do not mount a response to HIV-1, although this can be viewed as an advantage in certain circumstances. These models will find use mainly for understanding the pathogenesis of HIV-1 as well as for testing anti-retroviral drugs and vaccines.

*6.5- Feline immunodeficiency virus infection of cats:*

Feline immunodeficiency virus (FIV) is a lymphotropic lentivirus first isolated in domestic cats suffering from various clinical syndromes suggestive of underlying immunodeficiency (256). FIV is a substantial health problem for its

natural host species, with a worldwide prevalence ranging between 1 and 30% among apparently healthy domestic cats and 10-20% among sick cats (23).

FIV-infected cats show a number of immunological abnormalities which closely resemble those seen in HIV-infected humans. Most notable are a progressive decline of $CD4^+$ T cells and a significant hypergammaglobulinemia (21). As in the human disease, early stages of the infection are characterised by a general peripheral lymphadenopathy with follicular hyperplasia, fever, and diarrhea (21). The acute infection is followed by a long, disease-free period, as in human AIDS. As in the SIV model of AIDS, the period of clinical latency varies greatly and it is hypothesised to be due to the result in differences in exposure to secondary pathogens, as experimental cats maintained in highly hygienic conditions remain asymptomatic for the longest periods (21).

Most cats infected with FIV eventually go on to develop AIDS, as characterised by multiple, often concomitant, superinfections with opportunistic agents (210), severe neurological disease, and neoplastic disorders such as lymphosarcomas and carcinomas (21). Renal pathology that closely resembles HIV-associated nephropathy has also been reported (264).

For most pathogenesis studies, cats have been inoculated with cell-associated or cell-free FIV by subcutaneous, intramuscular, intravenous, or intraperitoneal injection (22). The susceptibility of cats to infection and the severity of the primary-stage clinical signs decrease with age, and most experiments have been done with cats inoculated as kittens (107). In these

studies, virus can be re-isolated from blood after 1-3 weeks and antibody can be detected by ELISA after 3 weeks (22).

Cells susceptible to infection by FIV include $CD4^+$ and $CD8^+$ T cells, B lymphocytes, macrophages, and astrocytes (40,41,74). The CD4 antigen is not required for infectivity and several studies suggest that CD9 may be the FIV receptor (245,329). Acute infection is accompanied by a mild lymphadenopathy with pyrexia and leucopenia usually develops 4-6 weeks after infection and lasts several months (23,245). This period corresponds to a shift in virus distribution from predominantly T cells to macrophages and B cells (20,85). Acute infection is also marked by an increase in the $CD8^+$ T cell population (328), similar to that observed during the acute phase of HIV-1 infection in humans (250). Once recovered from the acute illness, the cats remain healthy for at least several years, although immune function, as measured by both *in vivo* and *in vitro* tests, progressively declines during this period (22).

Two areas in which FIV infection of cats has been especially studied as a model for HIV and AIDS are antiviral therapy and immunisation. Inhibition of FIV replication in cell culture has been reported for most nucleoside-analogue RT inhibitors tested, including AZT, ddI, ddT, and 3TC (57,82,246,295,303). Vaccination of cats with subunit vaccines has so far been unsuccessful (137,138,156), but some inactivated virus and cell-virus vaccines have provided protection against both homologous and moderately heterologous challenge viruses (136,156).

As a model for HIV-1 infection, this system has the advantages of being induced by a lentivirus in its natural host, with no risk of human infection. As well, the animals are easily obtainable and easily handled. There also exists a large reservoir of naturally infected animals. On the down side, the immunology of cats has not been well studied and it is difficult to produce, and wait for, end-stage disease. The pathogenesis of FIV infection of cats is probably best suited as a model of acute infection and for testing anti-viral therapeutics and vaccines.

*6.6- Transgenic animal models of HIV-1 infection:*

*6.6.1- Transgenic Mice:*

Transgenic mice are produced by direct micro-injection of cloned genes into the pro-nucleus of a fertilised egg (135). The tissue-specific and developmental patterns of the transgene expression are determined by the regulatory elements (endogenous or heterologous to the gene coding sequences) present in the injected DNA construct. Traditionally, transgenic mice have been used to study gene expression and function in a biological environment that is difficult to obtain and reproduce *in vitro*, especially in the fields of immunology, development and cancer research. In particular, transgenic animals have found wide use in the study of retroviral gene expression and pathogenesis due to several advantages of this system. By eliminating the steps involved in the complex process of retroviral infection, these animals allow us to bypass the restricted pattern of infectivity associated with retroviruses, and allow us, such as in the case of HIV-1, to study the pathogenesis of human retroviral infections in rodents. Secondly, transgenic animals allow the study of selected regions of the

retroviral genome, including regulatory elements, in an *in vivo* environment. Finally, by using tissue-specific promoters to direct the expression of selected retroviral gene products, transgenic animals allow the study of the effects of these gene products in a given cell population.

In the case of HIV-1, many groups have made use of this technology to study the effect of the whole virus, or of individual HIV-1 gene products, in a wide range of tissues (37,162,178,190,200,258,293,323). These transgenic mice have provided significant insight into the molecular pathogenesis of HIV-1 and its clinical manifestations. Leonard et al. (190) constructed transgenic mice by the direct micro-injection of the complete HIV-1 proviral genome into fertilised mouse eggs, yielding seven founders, one of which produced HIV-specific serum antibodies as measured in immunodetection assays. Progeny from this founder developed signs of disease and died within 25 days. Post-mortem analysis of these mice revealed epidermal hyperplasia, lymphadenopathy, splenomegaly and pulmonary lymphoid infiltration. As well, infectious HIV-1 particles could be recovered from this mouse line. Unfortunately, a continuous line of mice could not be maintained, and no further work was done on these animals. Furthermore, similar results could not be reproduced after many attempts.

Transgenic mice carrying mutations in the HIV-1 proviral genome have also been produced, with various phenotypes observed. Santoro et al. (278) constructed transgenic mice with a deletion in the *gag/pol* region of the provirus. Mice homozygous for this transgene had a similar phenotype to the mice of Leonard et al., suggesting that the AIDS-like cachexia seen in both transgenics

may be due to expression of the viral *env* or accessory genes. Interestingly, no significant depletion of $CD4^+$ T cells was observed in either of these two transgenic mice. In addition to the wasting syndrome seen in the above-described HIV-1 transgenic mice, other groups have reproduced aspects of HIV-associated nephropathy seen in AIDS patients using *gag/pol*-deleted HIV-1 proviral DNA (70,178).

AIDS patients often develop diseases of the peripheral nervous system (PNS) and the CNS. In order to study the effect of HIV-1 on the pathogenesis of these diseases, several researchers have expressed HIV-1 proviral DNA in the CNS of mice, using tissue-specific promoters. Thomas et al. (310) used the neurofilament promoter to target expression of HIV-1 to neurons, which led to peripheral neuron degeneration, while Goudreau et al. (113) targeted HIV-1 expression to oligodendrocytes, resulting in vacuolar myelopathy.

Attempts to produce high levels of HIV-1 proteins *in vivo* have also been successful. Jolicoeur et al. (162) expressed HIV-1 proviral DNA using the MMTV promoter. High levels of gp160, gp120 *env* proteins, and the p55 and p24 *gag* proteins were found in the mammary glands of four founders, with higher levels found in lactating females. Interestingly, these mice did not exhibit any signs of disease as seen with other proviral transgenics, most likely due to the specificity of the MMTV promoter.

Expression of single HIV-1 gene products in transgenic mice has also been accomplished, with much success. One of the most important transgenic models for understanding the pathogenesis of HIV-1 is the LTR-tat transgenic mouse

generated by Vogel et al. (323). These mice develop epidermal proliferation and dermal tumours. These tumours were suggested by the authors to have histological properties of Kaposi's sarcoma in humans. Although the exact mechanism by which tat induces this tumour remains enigmatic, it is known that tat is angiogenic *in vivo* (2,132) and is capable of stimulating the growth of cells derived from KS lesions of AIDS patients (86). Other transgenic mice expressing only the gp120 *env* protein of HIV-1 in the CNS exhibited a spectrum of neuronal and glial changes resembling abnormalities in brains of HIV-1-infected humans (311), pointing to a role for this protein in causing CNS damage.

Transgenic models have also supported an important role for the *nef* protein of HIV-1 in inducing $CD4^+$ T cell depletion. Skowronski et al. (293) generated transgenic mice expressing *nef* in T cells. These mice had a marked reduction in $CD4^+$ T cells with little or no change in $CD8^+$ T cell numbers. Further work on similar transgenics revealed a direct role for *nef* in down-regulating CD4 from the cell surface by direct intracellular sequestration of the CD4 molecules (38). Thus, SIV *nef*, as discussed above in the macaque model of AIDS, and HIV-1 *nef*, as shown in the transgenic mouse studies, appear to be critical for the reduction in circulating $CD4^+$ T cells. Other *nef* transgenic mice expressing the protein under the control of the TcR β-chain promoter/enhancer exhibited evidence of immune dysfunction in addition to alterations in $CD4^+$ T cell numbers (200).

To summarise, although mice do not support HIV-1 infection, the use of transgenic technology has largely circumvented this problem by allowing

researchers to express the whole HIV-1 genome, or parts thereof, in a specific tissue or tissues, in a small, inexpensive, and well-studied animal. The large number of transgenics tested to date has shed light on many aspects of the pathogenesis of HIV-1 infection and the development of AIDS such as the reduction in $CD4^+$ T cell numbers (200,293), the neurological damage seen in AIDS patients (113,310,311), HIV-associated nephropathy (178,272), and HIV-induced growth failure and cachexia (190,278). These models and constructs expressing high levels of HIV-1 proteins (162) may be useful in testing therapeutic drugs or strategies to either inhibit viral protein production and/or the resulting pathogenesis.

*6.6.2- Transgenic Rabbits:*

Another use of transgenic technology has been in the development of rabbits expressing the human CD4 molecule which is the receptor for HIV-1 (121,333). Although the laboratory rabbit can be infected with HIV-1, as described above, rabbit cell lines do not replicate virus as efficiently as human cells. Studies indicating that rabbit cells engineered to express human CD4 are more susceptible to HIV-1 infection (121,333) prompted the development of transgenic rabbits expressing human CD4 (296). There are few reports on studies with these human CD4 transgenic rabbits. One report, however, showed that transgenic rabbit lymphocytes support HIV-1 infection and are highly susceptible to HIV-1-mediated apoptosis (189). This is consistent with reports from other groups suggesting apoptosis as a mechanism for HIV-1-mediated $CD4^+$ T cell depletion seen in infected persons (7,114,223). Overall, the use of transgenic

rabbits remains limited and is surpassed by the better-studied and more versatile transgenic mouse models.

**7- Retrovirus-induced immunodeficiency syndrome of mice (MAIDS):**

MAIDS is caused by a unique mixture of murine leukemia viruses first isolated by Laterget and Duplan (184) from X-radiation-induced thymomas of C57BL/6 mice. They noticed that in mice inoculated with this virus mixture, now termed LP-BM5, the thymus was not involved, but lymphadenopathy and splenomegaly were observed. First thought to be a B-type reticulum cell sarcoma, the disease induced by this virus mixture has several common characteristics with human AIDS, including immunodeficiency, hypergammaglobulinemia (187,236), susceptibility to infection (43), and late onset B cell lymphomas (174). A comparison of the main features of AIDS and MAIDS is given in Table 4.

The immunologic features of MAIDS virus infection of the susceptible prototype C57BL/6 mouse strain are well known, even if their causes are not. These features include:

- CD4 and CD8 T cell unresponsiveness (anergy)
- Activation of B cells with increased IgM, $IgG_1$, and IgE concentrations
- Lymphadenopathy and splenomegaly due to increased numbers of lymphoid and myeloid cells
- Aberrant cytokine production

The pathogenesis of this disease will be discussed in detail below. The interested reader is also directed to several reviews which have been produced on this topic (160,161,228).

*7.1- The etiologic agent of MAIDS is a defective virus:*

The virus mixture initially used to induce MAIDS in susceptible mice contains a complex assortment of replication-competent and -defective viruses which has not been fully characterised (9,118,119,186,188,209). For example, the LP-BM5 virus mixture, which is widely used to induce MAIDS, includes B-tropic ecotropic and MCF MuLV (119,236,334), which on their own do not induce MAIDS (48,186).

Two groups independently isolated the pathogenic viral genome responsible for causing MAIDS in susceptible mice, using molecular hybridization techniques (10,48). Interestingly, this 4.8 kbp viral genome was found to be defective, having suffered major deletions in the *pol* and *env* regions, with its *gag* region largely intact, but containing many point mutations and small insertions/deletions in the p12 domain of the *gag* sequences (10) (Figure 4). This virus, termed Du5H, appears to encode a single, 4.2 kb transcript which encodes the only gene product of this virus, a 60 kDa *gag* fusion protein, $Pr60^{gag}$ (141). Studies on $Pr60^{gag}$ indicate that it is myristylated, phosphorylated, and attached to the cell membrane, like other replication-competent MuLV *gag* precursors (141). It was also found in the same study that $Pr60^{gag}$ is not cleaved, and in the presence of helper $Pr65^{gag}$ appears to behave as a dominant negative mutant, interfering with the proper cleavage of the helper $Pr65^{gag}$.

## Comparisons Between AIDS and MAIDS

SIMILARITIES

T-cells

- *Increased proliferation
- *Impaired responses to mitogens
- *Impaired CD4+ cell function prior to changes in CD4+ cell frequency, including:
  - Help for CD8+ cytotoxic T-lymphocyte responses to modified self antigens
  - Responses to soluble antigen
  - Autologous mixed lymphocyte reaction
  - Help for normal B-cells

B-cells

- *Polyclonal activation
- *Differentiation to immunoglobulin secretion
- *Hypergammaglobulinemia
- *Autoantibody production
- *Circulating immune complexes
- *Impaired responses to mitogens
- *Impaired responses to help for normal T-cells
- *Decreased responses to helper T-cell-independent antigens
- *Development of lymphomas

Non-T, non-B-cells

- *Increased proliferation
- *Decreased natural killer cell function
- *Decreased natural killer responsiveness

Other

- *Lymphoproliferation
- *Enhanced susceptibility to infection
- *Effects of major histocompatibility complex polymorphisms on disease progression
- *Disease more rapidly progressive in neonates than adults

DIFFERENCES

- *Lentivirus in AIDS, C-type murine leukemia virus (MuLV) in MAIDS
- *CD4 as the prominent receptor for HIV, not for MuLV
- *Neurodegenerative disease in AIDS, not in MAIDS
- *Kaposi's sarcoma in AIDS, not in MAIDS
- *Opportunistic infections are a much greater problem in AIDS

CTL, cytotoxic T-lymphocyte.

Table 4. Taken from (228).

Mutational studies on the viral genome indicate a strong selection for an intact $Pr60^{gag}$ in causing MAIDS (140,142). Myristylation of $Pr60^{gag}$ is necessary for full induction of MAIDS (142). Other studies show a clear requirement for an intact $Pr60^{gag}$ in causing MAIDS. Huang et al.(140) constructed a series of $Pr60^{gag}$ truncation mutants, which revealed a need for intact $Pr60^{gag}$ and especially the p12 domain, confirming results obtained by others (267). Furthermore, deletions in the 3' end of the genome, which does not contain coding sequences, only slightly impair the ability of the viral protein to induce disease. Contrary to the results of others (267), Huang et al. (140) found that an intact $Pr60^{gag}$ is required for full induction of MAIDS, not only the MA(p15)-p12 domains.

*7.2- The MAIDS defective virus is sufficient to induce MAIDS in the absence of viral replication:*

Prior to the cloning of Du5H, it was postulated that viral replication was necessary for the development of MAIDS (43,126). To study the role of viral replication in the development of MAIDS, helper-free stocks of the MAIDS defective virus were constructed (143). These viral stocks were efficient at inducing MAIDS in the absence of viral replication. Further proof that viral replication is not needed for the induction of MAIDS comes from studies with anti-viral drugs. MAIDS was inhibited only in animals given 9-(2-phosphonylmetoxyethyl) adenine immediately after virus inoculation. If given later, this drug could not stop the development of MAIDS (102). The use of the

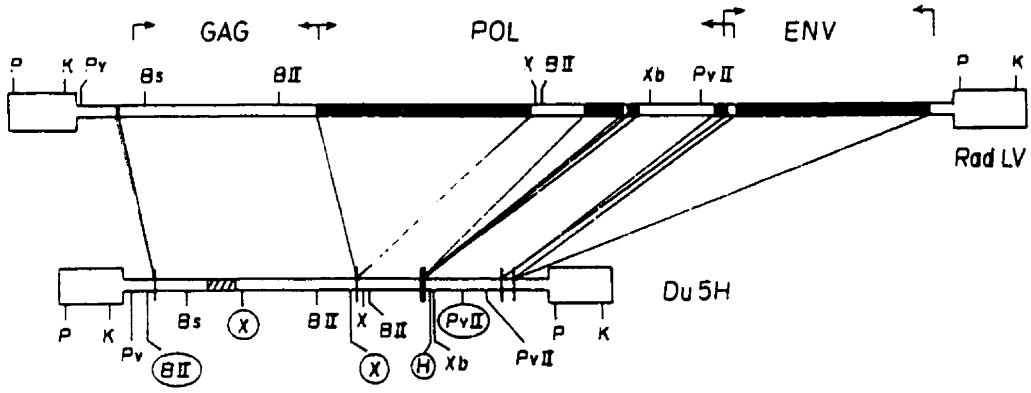


Figure 4: Structure of the MAIDS defective virus (Du5H) in comparison to the non-defective RadLV. Common restriction sites are shown. Note the large deletions in *pol* and *env*. The *gag* sequences are homologous in p15, p30, and p10. White box: homologous; black box: deleted; hatched box: unique sequences. BII, *Bgl*II; Bs, *Bst*E II; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; PvII, *Pvu*II; X, *Xho*I; Xb, *Xba*I. Circled restriction endonuclease sites are present in Du5H, but not in RadLV. Taken from (10).

helper-free system has greatly facilitated the study of MAIDS by eliminating many uncontrollable (or unknown) variables from the experimental design, such as the presence of replicating MuLV and MCF.

*7.3- The MAIDS defective virus infects cells of the B lineage:*

A critical role for B cells in the development was first suggested by experiments in which mice were depleted of B cells by treatment with anti-μ antibody (46). At this time it was already known that animals inoculated with the MAIDS virus mixture develop polyclonal B cell proliferation and hypergammaglobulinemia (175). Late stage disease in these mice is characterised by the outgrowth of clonal or oligoclonal B cell populations and the development of B lineage lymphomas (174). It was not clear, however, if these B cells were the targets of the MAIDS defective virus or if they were stimulated to proliferate by a secondary, indirect mechanism.

The use of helper-free MAIDS defective virus was instrumental in ascertaining that B cells are the primary target of this defective virus. By using a combination of Southern analysis and *in situ* hybridisation/immunohistochemistry, Huang et al. (145) were able to identify most infected cells as belonging to the B lineage. These authors argued that the infection and proliferation of these target B cells appear to be the critical events in the development of MAIDS. This is supported by both earlier and later studies on mice infected with either the helper-free MAIDS defective virus or the LP-BM5 virus mixture. Mice treated with the immunosuppressive drug cyclophosphamide were protected from the disease induced by helper-free MAIDS defective virus by

inhibiting the proliferation of the defective virus target cells (291). Later work using mutant mouse strains infected with either helper-free MAIDS defective virus (292) or the LP-BM5 mixture (171) confirmed the theory that relatively mature B cells are the target of the defective virus and that infection of this target cell population is critical for the development of MAIDS.

*7.4- The role of T cells in the development of MAIDS:*

*7.4.1- CD4$^+$ T cells:*

Functional T cells, and specifically CD4$^+$ T cells are required for the development of MAIDS as shown by the lack of disease in nude mice, or in mice depleted of CD4$^+$ T cells by antibody depletion (237,334). Although not infected by the MAIDS defective virus when helper-free stocks are used (M. Huang, C. Simard, and P. Jolicoeur, unpublished data), these cells are infected in mice inoculated with the LP-BM5 virus mixture (180,304). The CD4$^+$ T cells are required to trigger the proliferation of the infected B cells and the subsequent spectrum of disease (292,334).

Curiously, even though the CD4$^+$ T cells are required early in the disease to allow the target B cell expansion (292), these cells themselves become anergized as the disease progresses, even as their numbers increase. As early as two weeks post-infection an increase in CD4$^+$ T cell numbers and size is observed, and these cells exhibit a memory/activated phenotype (238). This change in CD4$^+$ T cell phenotype is paralleled by their inability to proliferate or to produce IL-2, IL-3, IL-4, and INF-$\gamma$ in response to stimulation with mitogens, superantigen, or anti-CD3 (238), although responsiveness could be restored with PMA and

ionomycin (92). This signalling defect has been shown to involve impaired calcium mobilization (206) as well as an uncoupling of the TcR from the phosphotylinositol hydrolysis pathway due to deficient activation of PLC-γ1 (92).

It has been suggested by several groups that the T cell anergy observed in MAIDS is a consequence of stimulation by the MAIDS defective virus $Pr60^{gag}$ (see below), and that this viral protein acts as a superantigen (148) with specificity for T cells expressing Vβ5-, 11-, and -12 chains (148,164).

Evidence for a superantigen effect has been provided by *in vitro* experiments using a single B cell lymphoma line, B6-1710, derived from mice inoculated with the LP-BM5 mixture (129,164,167). However, there is little or no evidence to support this superantigen theory. One group claims to have observed a preferential expansion and activation of Vβ5 $CD4^-$ T cells during the early course of MAIDS, although this expansion was very slight (284). The vast majority of evidence, both *in vitro* and *in vivo*, however, rules out any superantigen effect of the MAIDS defective virus (75).

Other groups have not observed any preferential expansion or deletion of $CD4^-$ T cells expressing particular Vβ genes during the course of MAIDS (176,238). As well, T cells from fully allogeneic $H\text{-}2^b \rightarrow H\text{-}2^d$ SCID chimeric mice fail to respond to the B6-1710 cell line, although these mice develop MAIDS (109). *In vitro* experiments in which the MAIDS defective virus was introduced into fibroblasts and B cells expressing MHC II did not elicit any stimulation of cells expressing Vβ5 (75). The same group also reported that mice lacking Vβ5 T cells did not show any decrease in susceptibility to MAIDS (75).

Interestingly, the same lab which initially reported the superantigen effect (148) has recently reported that mice which do not express any endogenous superantigens are susceptible to MAIDS (216), thus ruling out a requirement for these agents in the pathogenesis of MAIDS.

*7.4.2- $CD8^+$ T cells:*

Although most studies on MAIDS animals have probed the role of the $CD4^+$ T cells in the development of the disease, $CD8^+$ T cells have also been shown to play a role in controlling the development of MAIDS in certain strains of mice. Normal A/J do not develop MAIDS, but when these mice were depleted of $CD8^+$ T cells by chronic treatment with anti-CD8 monoclonal antibody they developed splenomegaly and the MAIDS defective virus was detected (204). Further studies on mice genetically deficient in $CD8^+$ T cells due to disruption of the β2-microglobulin gene also showed a role for $CD8^+$ T cells in eliminating MAIDS virus-infected target cells (305). The same study also suggested a role for perforin in the mechanism of viral resistance, although the resistance was not complete and other mechanisms involving $CD4^+$ effectors or cytokines may also be involved in mediating resistance to MAIDS in resistant mice (305). Precedence for multiple mechanisms of resistance to viral infections can be found in studies of Friend disease, a syndrome induced by a replication-defective virus distinct from Du5H and replication-competent Friend ecotropic helper viruses. Effective immune control against this virus complex requires both humoral and cell-mediated responses (128,273), and mice depleted of either $CD4^+$ or $CD8^+$ T cells fail to fully clear the infection (273).

Other groups have also analysed the CTL response generated by MAIDS-resistant and –sensitive mice (87,282). It was demonstrated that C57BL/6 mice, which are sensitive to MAIDS, can generate CTL to MAIDS-derived B-lineage lymphoma lines, although it is not clear to which epitope the CTL are induced, or even if it specific for $Pr60^{gag}$ (87). Another study examining the linkage between resistance to MAIDS and a vigorous anti-$Pr60^{gag}$ CTL suggests that the CTL response generated in resistant mice is not directed against the defective virus (282). The role of the $CD8^+$ T cells in the development of MAIDS is therefore far from clear.

*7.5- The Genetics of MAIDS:*

As alluded to above, inbred mice differ in their susceptibility to MAIDS, with the C57BL/6 strain used as the prototype susceptible strain. Several studies have been performed in order to understand the observed range of sensitivities to MAIDS seen in mice of different backgrounds. It has been reported that H-2-associated genes influence the development of MAIDS induced by the LP-BM5 virus mixture (122,205). The b, f, k, q, r, and s H-2 haplotypes were found to be associated with susceptibility to MAIDS (205), whereas the d and a H-2 haplotypes were associated with resistance to MAIDS (122). Other studies using helper-free N- or B-tropic pseudotypes of the MAIDS defective virus failed to show a clear correlation between a given H-2 haplotype and resistance or susceptibility to disease (144). The same study showed that the *Fv-1*$^b$ genotype does not confer susceptibility to MAIDS, as suggested by others (126) who used crude stocks of defective virus pseudotypes representing mixtures of B- and N-

tropic MuLV as well as MCF, all poorly characterised (160,161). It is as yet unclear as to whether the *Fv-1*$^n$ or *Fv-1*$^b$ alleles can block the development of MAIDS in mice inoculated with incompatible helper pseudotypes of the MAIDS defective virus. A study on the influence of H-2 Class II on the development of MAIDS reported that Eα molecules can also mediate some resistance to the disease, although the mechanism by which this occurs is unknown (207).

Despite the apparent complexity of the genetic factors controlling MAIDS, it was determined that there is a very strong correlation between the resistant and susceptible phenotype and the absence or presence of defective proviral DNA and RNA in the spleens of these animals (144). The presence of defective proviral DNA and RNA reflects the clonal or oligoclonal expansion of the infected target B cells, and these results suggest that this target B cell expansion is genetically controlled and is necessary and perhaps sufficient for the development of MAIDS.

Another interesting feature of this disease is the fact that susceptibility to MAIDS is dominant over resistance in $F_1$ crosses between susceptible and resistant strains (126,144,205). Resistance to MAIDS is therefore likely a consequence of the absence of a critical factor(s) essential for the expansion of the infected B cells and not due to the presence of a repressor which inhibits the infected B cell expansion.

*7.6- The role of cytokines in MAIDS:*

The T cell activation and dysregulation seen in MAIDS includes the aberrant expression of cytokines. In normal mice, analyses of T cell clones and *in*

*vivo* T cell responses to various antigens have shown that there exists several patterns of cytokine secretion by subsets of $CD4^+$ T helper ($T_H$) cells (for review, see (302)). The $T_H0$ subset is the precursor to the $T_H1$ and $T_H2$ subsets. $T_H0$ cells express IL-2, IL-4, IL-10 and INF-$\gamma$. $T_H1$ cells produce IL-2 and INF-$\gamma$, but not IL-4 or IL-10, while $T_H2$ cells produce IL-4 and IL-10, but not IL-2 or INF-$\gamma$.

Several studies have been carried out on the patterns of cytokine expression in LP-BM5 virus mixture-infected mice (36,105,131,261,314). It was found that early after infection with the LP-BM5 virus mixture a $T_H0$-like pattern of spontaneous cytokine expression was observed, with IL-2, -4, -6, and –10 and INF-$\gamma$ being produced. Later on, however, ConA stimulation of splenic cells led to increasing levels of Th2 cytokines, with a large decline in the levels of $T_H1$ cytokines (105). This suggested that a $T_H0$ to $T_H2$ switch may occur during the course of MAIDS and prompted further work to more closely examine this phenomenon.

Kanagawa et al. (165) studied (C57BL/6x129) mice bearing a deletion of the IL-4 gene ($IL\text{-}4^{-/-}$ mice). The time course of MAIDS in these mice was significantly prolonged, strongly suggesting an important role for this cytokine in the development of MAIDS. Parallel studies of $IL\text{-}4^{-/-}$ mice on a C57BL/6 background, however, showed no difference in susceptibility to MAIDS compared to wild-type mice (227). These contradictory results imply that in the case of the (C57BL/6x129) $IL\text{-}4^{-/-}$ mice, a gene(s) closely linked to the IL-4 gene on chromosome 11 mediates some aspect(s) of resistance to MAIDS. The results also exemplify the care that must be taken in interpreting results from knockout

experiments when different mouse strain backgrounds are involved, especially in a syndrome such as MAIDS which is known to be genetically complex (for example see (244)).

To further the hypothesis that a $T_H0$ to $T_H2$ switch is an important factor in the development of MAIDS, several experiments have been carried out in an attempt to induce a $T_H1$ response (or to prevent a $T_H2$ response) in MAIDS-susceptible mice (71,72,105,131). One approach used by several groups has been to inoculate other pathogens, apart from the MAIDS virus, into susceptible mice, that are known to induce a $T_H1$ response. For example, infection of C57BL/6 mice with Newcastle disease virus, which induces the expression of INF-$\alpha/\beta$, or the direct administration of INF-$\alpha/\beta$ significantly slowed the development of MAIDS (131). Similarly, co-infection of C57BL/6 mice with the LP-BM5 virus mixture and *Leishmania major*, which induces a strongly polarized $T_H1$ response in C57BL/6 mice, can modulate the development of lymphoproliferation and immunodeficiency caused by MAIDS (71,72). This was shown to be partially dependant on the induction of INF-$\gamma$ by *L. major*, while concurrent treatment with an anti IL-4 antibody synergized with INF-$\gamma$ to inhibit the development of MAIDS (71). These results show that INF-$\gamma$ and IL-4 play antagonistic roles in the pathology of MAIDS, which was implied by previous reports (14,81,106,286). Furthermore, it was also shown that *in vivo* treatment of C57BL/6 mice with IL-12 protected mice from MAIDS (105). The beneficial effects of this treatment were dependent on INF-$\gamma$ synthesis and were associated with inhibition of B cell proliferation and activation.

These studies suggest that contributing to the pathology seen in MAIDS may be the host's own response to the virus. The $T_H2$ pattern of cytokines observed in MAIDS could play a role in allowing expansion of the MAIDS defective virus-infected B cells rather than inhibiting an antiviral response. As mentioned above, a similar $T_H1$-$T_H2$ switch has been proposed as a possible mechanism in the pathogenesis of AIDS (50). While there are significant differences between HIV-1 and MAIDS virus infections, there are similarities between the two with respect to the possible contribution(s) of cytokines to the pathology of immunodeficiency seen in both syndromes. Understanding the role of cytokines in MAIDS may shed light on similar mechanism(s) operating in AIDS.

*7.7- How does the MAIDS defective virus cause MAIDS?:*

There are two, not necessarily mutually exclusive, main hypotheses regarding the central role of the MAIDS defective virus $Pr60^{gag}$ in inducing MAIDS. The first hypothesis, put forth by Hugin et al. (148), is founded on the premise that $Pr60^{gag}$ behaves as a superantigen, thereby anergizing the $CD4^+$ T cells that come into contact with this molecule when presented at the surface of the infected B cells, or other APC. Classical superantigens are defined by their ability, when complexed with MHC class II molecules, to engage a high proportion of T cells based predominantly on their expression of specific TcR $V_\beta$ genes (153,213,279). There are no superantigens known which anergize all $V_\beta$ subsets. These authors propose that the $CD4^+$ T cells are activated by the putative

Pr60$^{gag}$ "superantigen" and then proliferate and produce a variety of cytokines in response.

This hypothesis is based primarily on *in vitro* studies which used B cell lymphoma cell lines from LP-BM5 virus mixture-infected animals to stimulate T cell hybridomas bearing $V_\beta$ determinants 5.1, 5.2, 11 and 12 (129,148,164,167). *In vivo*, however, there is little or no evidence to support the theory that Pr60$^{gag}$ acts like a superantigen. Several groups have searched for a preferential expansion or deletion of $V_\beta$ subsets during the course of MAIDS, yet only one group reported a very modest expansion of $V_\beta 5$ CD4$^+$ T cells early in the course of disease (75,284), and other groups observed no difference in $V_\beta$ subsets during the course of MAIDS (238). As well, mice transgenic for TcR $V_\beta 3$, which should not recognise the MAIDS Pr60$^{gag}$ superantigen, developed MAIDS (176). Similarly, as discussed above, H-2$^b$→H-2$^d$ allochimeric SCID mice fail to recognise the putative superantigen, yet still develop MAIDS (109). Although this theory is appealing in many respects, it rests mainly on *in vitro* results obtained using two MAIDS defective virus-infected B cell lymphomas, and there is little or no evidence for an *in vivo* superantigen effect, although it has been reported that MHC class II expression is required for the development of MAIDS (108), implying a role for antigen, be it Pr60$^{gag}$ or another molecule, in the progression of MAIDS.

Our group has proposed a second hypothesis to account for the immunodeficiency induced by the MAIDS defective virus (143,160,161). In this model, immunodeficiency arises as a paraneoplastic syndrome, as a consequence

of infection of the target B cells with the MAIDS defective virus. Infection of the target B cells could lead to the production of a factor (or factors) harmful to the immune system, or these infected B cells could directly interact with other immune system cells, especially T cells, thereby leading to immunodeficiency. Supporting this hypothesis is the fact that infection of mice with helper-free MAIDS defective virus leads to the clonal outgrowth of B cells (143) with subsequent immunodeficiency. If the proliferation of the infected B cells is arrested by treatment with anti-neoplastic drugs, immunodeficiency is averted, again supporting a role for the proliferation and expansion of MAIDS defective virus-infected B cells in the development of MAIDS (291). We have also observed that infected B cells can influence T cells at a distance, where the infected B cells are not yet present (290).

Although $Pr60^{gag}$ is postulated to infect and reprogram its target B cells, leading them to proliferate and induce immunodeficiency, it does not appear to be directly oncogenic *in vitro* or *in vivo* (S. Klein, C. Simard and Paul Jolicoeur, unpublished results), and may therefore exert its effects by activating one or more signal transduction pathways in its target cells (79). We have also studied the potential of $Pr60^{gag}$ to act as a putative superantigen, and have found no evidence to support this hypothesis (75).

Despite the major differences in the two models put forward to explain the immunodeficiency induced by the MAIDS defective virus, they still share some common ground. In both models, B-T cell contact could play a key role in the induction of the polyclonal T cell anergy observed during MAIDS Support for

this concept is mounting from studies in which some aspects of B-T cell signalling have been disrupted *in vivo* by antibody treatment. For example, treatment of mice with antibody to the ligand for CD40 (gp39) inhibits MAIDS (116) as does antibody treatment to CD54 (ICAM-1) and CD11a (LFA-1) (208). It has also been reported that Fas/FasL interactions may play a role in MAIDS based on studies in *lpr* and *gld* mice (166). Further studies on the role of B-T cell interaction in this disease will yield more information on the central role of $Pr60^{src}$ in this complex disease.

As discussed above, B cells play a crucial role in the development of MAIDS, yet there is much to learn about their identity and the mechanisms by which their infection with the MAIDS defective virus triggers immunodeficiency. We therefore undertook both *in vivo* and *in vitro* studies in order to gain further insight into the identity of the target B cells and how the MAIDS defective virus-B cell interactions trigger this disease. These results are presented in the following four chapters.

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Chapter 2

# Studies of the Susceptibility of Nude, CD4 Knockout, and SCID Mutant Mice to the Disease Induced by the Murine AIDS Defective Virus

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## Forward

As discussed in the preceding chapter, both B and T cells play an important role in the development of MAIDS (2,4,6,7), even though only cells belonging to the B cell lineage appear to be infected by helper-free stocks of the MAIDS defective virus (1). It was also known that infection of these cells with the MAIDS defective virus leads to their proliferation and clonal expansion (3,5). It was not yet known, however, if a mature or an immature B cell population is the target of this virus, nor was it known if the presence of other cell populations is required for the proliferation of the infected B cells. In light of the importance of this B cell population for the development of MAIDS and to study the contributions, if any, of different lymphoid subsets to the development of MAIDS, we studied the susceptibility of SCID, $CD4^{-/-}$, and nude mice to the MAIDS defective virus.

Carole Simard was involved in the analysis of all the mutant mice examined in this manuscript. I was heavily involved in studying all aspects of the $CD4^{-/-}$ mice, including the breeding of these mice, as well as the FACS, Northern, and *in situ* hybridization/immunohistochemistry analysis of the mice. Additionally, I performed the Northern analysis of the (C57BL/6 X BALB/c)$F_1$ nude mice which were inoculated with the MAIDS defective virus. Tak Mak supplied the initial breeding group of $CD4^{-/-}$ mice. This manuscript was published in the Journal of Virology 71:3013-3022 (1997).

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# Studies of the Susceptibility of Nude, CD4 Knockout, and SCID Mutant Mice to the Disease Induced by the Murine AIDS Defective Virus

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**Murine AIDS (MAIDS) is induced by a defective retrovirus that infects lymphocyte cells of the B lineage. To determine whether functional T cells are required for the infection of B cells, T-cell-deficient mice (nude, CD4 knockout, and SCID) were infected with helper-free stocks of the MAIDS defective virus. Infection of B cells was monitored by Northern blot analysis and in situ hybridization. The C57BL/6 nude mice contained clusters of infected B cells, but less so than did the euthymic mice. In contrast, the (C57BL/6 × BALB/c)$F_1$ nude mice harbored more infected B cells than did their euthymic littermates when maintained in a pathogen-free environment. Clusters of infected B cells were also detected in the MAIDS virus-infected $CD4^{-/-}$ knockout mice despite the total absence of $CD4^+$ T cells in these mice. However, infected cells were not detected in SCID mice (deficient in mature T and B cells) inoculated with the same virus, indicating that precursor B cells are not a target of the virus in the absence of mature $CD4^+$ T cells. These data confirm that the primary event in the development of MAIDS is the infection of relatively mature peripheral B cells and that $CD4^+$ T cells are required to promote the expansion of these infected B cells.**

Murine AIDS (MAIDS) is characterized by splenomegaly, lymphadenopathy, hypergammaglobulinemia, T- and B-cell immune dysfunctions, increased susceptibility to opportunistic infections, and late appearance of lymphomas (for reviews, see references 22, 28, and 29). The disease is induced by a defective retrovirus (1, 7) which appears to encode a single gene product, the $Pr60^{gag}$ protein (7, 17). By using helper-free stocks of this defective virus, which allow initial cell infection but no cell reinfection and no virus replication (18), it has been possible to determine that most of the cells infected by the defective virus belong to the B-cell lineage (20, 38). These cells were found to proliferate markedly and to expand clonally after being infected (18, 20, 38). These studies did not determine whether mature B cells are initially the target of the virus or whether more immature B-cell precursors are initially infected and differentiate after being infected.

The mechanisms by which MAIDS develops are poorly understood. Although $CD4^+$ T cells are all profoundly anergized in MAIDS (1, 5, 12, 30, 32), they are rarely infected when helper-free stocks are used (38a). However, the presence of both T- and B-lymphoid cell populations has been reported to be required for MAIDS development (4, 14, 24, 31, 40). Mice depleted of $CD4^+$ T cells by anti-CD4 antibody treatment became resistant to MAIDS (40). Nude mice were also protected from lymphadenopathy, splenomegaly, and hypergammaglobulinemia after inoculation with the replication-competent LP-BM5 MAIDS virus stocks (14, 31). Similarly, mice depleted of mature B cells with anti-IgM antibody treatment and $\mu^{-/-}$ knockout mice were also resistant to MAIDS (4, 24). Together, these results suggested that the contribution of several cell populations might be required to allow the initially infected B cells to proliferate and to induce the severe immune defects seen in MAIDS.

It has not yet been determined whether B cells were infected and expanded in the MAIDS virus-infected nude or $CD4^+$ T-cell- or B-cell-depleted mice. In view of the importance of this B-cell population in MAIDS, it appears critical to determine whether the resistance to the disease prevents this B-cell infection and proliferation or whether resistance is manifested in the presence of proliferating infected B cells and whether $CD4^+$ T cells are required for the initial infection of B cells.

To gain additional information on the importance and contribution of various lymphoid cell subsets in the development of MAIDS, we studied the susceptibility of mutant mice (nude, $CD4^{-/-}$ knockout, and SCID mice) to the MAIDS defective virus. The nude mice suffer from a congenital thymic aplasia leading to the virtual absence of functional lymphoid T cells, with no impairment of B-cell numbers (21, 35). The $CD4^{-/-}$ knockout mice harbor a null mutation of the CD4 gene and have no mature $CD4^+$ $CD8^-$ T cells but have normal populations of mature $CD8^+$ $CD4^-$ T cells and of B lymphocytes (36). The SCID mice are severely deficient in both numbers and functions of mature T and B cells due to the impairment of the VDJ recombinase system required for the T-cell receptor and immunoglobulin (Ig) gene rearrangements (3, 37). Development of both T and B cells is aborted at an early stage of differentiation. This deficit in lymphocyte maturation is reflected by an abnormal thymus that is sparsely populated with immature lymphocytes and by the virtual absence of T and B cells in the peripheral lymphoid tissues (10, 11). However, the

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TABLE 1. Susceptibility of control and nude C57BL/6 mice to MAIDS

| Mouse | Phenotype[a] | Virus inoculated[b] | Time of sacrifice (days) | % of CD3 T cells[c] | In situ positive cells[d] | |
|---|---|---|---|---|---|---|
| | | | | | SP | LN |
| C-21 | WT | WT | 45 | ND | + | ++ |
| C-22 | WT | WT | 45 | ND | ++ | ++ |
| C-24 | WT | WT | 45 | ND | +++ | ++ |
| C-25 | WT | WT | 45 | ND | +++ | ++ |
| C-5 | WT | U3 | 45 | ND | + | ++ |
| C-6 | WT | U3 | 45 | 25.9 | ++ | ++ |
| C-7 | WT | U3 | 45 | 27.5 | < + | < + |
| C-26 | Nude | WT | 45 | ND | − | − |
| C-27 | Nude | WT | 45 | ND | < + | < + |
| C-28 | Nude | WT | 45 | ND | < + | − |
| C-1 | Nude | U3 | 45 | ND | < + | + |
| C-2 | Nude | U3 | 45 | ND | ++ | ++ |
| C-3 | Nude | U3 | 45 | ND | < + | + |
| C-4 | Nude | U3 | 45 | ND | < + | + |
| C-8 | Nude | U3 | 60 | 1.5 | ++ | + |
| C-9 | Nude | U3 | 60 | 0.7 | + | < + |
| C-11 | Nude | U3 | 60 | 0.6 | < + | < + |
| C-12 | Nude | U3 | 60 | 0.7 | < + | < + |
| C-13 | Nude | U3 | 60 | 0.5 | + | + |

[a] WT, wild-type euthymic.

[b] WT, wild-type Du5H MAIDS defective virus; U3, chimeric Du5H/Mo-LTR MAIDS defective virus. Mice received 1.5 ml of helper-free stocks of viruses i.p.

[c] Percentage of $CD3^+$ T cells as determined by FACScan analysis with the FITC-145-2C11 anti-murine CD3. ND, not done.

[d] Number of in situ positive cells, using the D30 (Du5H) or the U3 LTR (Du5H/Mo-LTR) RNA probes. −, no positive cell; +++, >50% positive cells, as shown in Fig. 1. SP, spleen; LN, lymph nodes (mesenteric, brachial, inguinal, cervical, mediastinal, and lumbar).

early B-cell precursors until the pro-B stage are present at normal levels in the bone marrow (16) and are highly susceptible to transformation by the Abelson murine leukemia virus (MuLV) (13). In the SCID mice, the myeloid lineage is not affected and develops normally (2, 11). Therefore, infection of SCID mice may help in identifying the stage at which cells of the B lineage are infected.

We report here that SCID mice are poorly infectable by helper-free stocks of the MAIDS defective virus whereas nude and $CD4^{-/-}$ mice exhibit modest populations of MAIDS defective virus-infected B cells but no enlargment of lymphoid organs. These data suggest that the infection of the primary target B cells by the MAIDS defective virus and the subsequent lymphoproliferative disorder have different requirements for $CD4^+$ T-cell factors.

## MATERIALS AND METHODS

**Animals and viruses.** Inbred C57BL/6 mice and athymic nude mice were purchased from Taconic (Germantown, N.Y.). The athymic (C57BL/6 × BALB/c)$F_1$ mice, their euthymic littermates, and the C57BL/6 SCID mice were from Jackson Laboratory (Bar Harbor, Maine). The BALB/c SCID mice were kindly provided by B. Phillips (Hospital for Sick Children, Toronto, Ontario, Canada). The $CD4^{-/-}$ knockout mice have been described previously (36) and were obtained from the colony at the Ontario Cancer Institute, Toronto, Ontario, Canada. They were bred on a C57BL/6 background for five generations before being used for the present experiment. These mice were screened by examining tail DNA with a probe for the gene for resistance to neomycin (Neo probe) or a probe derived from the mouse CD4 gene (15, 22), which allowed us to type heterozygote from homozygote knockout mice. The mice were housed in our conventional animal room or maintained in a pathogen-free environment, consisting of an air-filtered isolator (Isotec). For mice in the isolator, food pellets, water, and bedding were sterilized by autoclaving. Young (30- to 40-day-old) mice were inoculated twice intraperitoneally (i.p.), as described previously (18, 19, 20), with helper-free stocks of the defective MAIDS virus, either the wild-type Du5H (18, 20) or the chimeric Du5H/Mo-LTR, which harbors long terminal repeat (LTR) sequences from Moloney MuLV (20, 38). The mice were sacrificed about 45, 60, or 125 days postinoculation (p.i.).

**Tissue samples.** At the time of sacrifice, selected organs were fixed in freshly made 4% paraformaldehyde–phosphate-buffered saline and embedded in paraffin, as described previously (20, 23, 33), or were frozen by immersion in liquid nitrogen and stored at −80°C.

**Probes and in situ hybridization.** Hybridization of paraffin-embedded tissues was performed as described previously, with $^{35}S$-labeled RNA probes (20). Southern and Northern blot analyses were performed with $^{32}P$-labeled DNA probes. The specific Neo, CD4, $C_\mu$, D30, D34, ecotropic MuLV (Eco), and Moloney MuLV U3 LTR RNA probes have been described elsewhere (6, 15, 20, 27, 33, 38). The D30 probe was derived from the unique region of the MAIDS defective viral Du5H DNA (1). The D34 probe comprises nucleotides 3902 to 4184 of Du5H (18). The specificity of these probes has been discussed previously (1, 18). To detect 18S rRNA, a $^{32}P$-labeled oligonucleotide (5′-ACGGTATCT GATCGTCTTCGAACC-3′) was used.

**Flow cytometric analysis.** Freshly dispersed cells from the mesenteric lymph nodes or spleens were labeled with antibodies, as described previously (15, 34). Cells were stained directly with fluorescein isothiocyanate (FITC)-conjugated GK1.5 (murine anti-CD4) or FITC-145-2C11 (murine anti-CD3) monoclonal antibodies. The flow cytometric analysis was performed with a FACScan (Becton Dickinson) as previously described (15, 34).

**Double-label immunocytochemistry and in situ hybridization.** Immunocytochemistry to detect the B-cell-specific B220 antigen has been described before (20). For the double-label immunocytochemistry, the RA3-6B2 (rat IgG2) antibody (a kind gift of R. Coffman, DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, Calif.) and the rabbit anti-rat horseradish peroxidase were used. Following immunocytochemistry, slides were processed immediately for in situ hybridization.

**ConA stimulation test.** Cells harvested from mesenteric lymph nodes were resuspended in RPMI 1640–10% fetal calf serum–2 mM L-glutamine–$10^{-4}$ M β-mercaptoethanol–antibiotics (penicillin and streptomycin). The cells were stimulated by the mitogen concanavalin A (ConA) at increasing concentrations (from 0 to 20 μg/ml) as reported previously (1, 19, 39). Triplicates were done at each dilution, for each sample. The background (without ConA) was substracted from the mean counts.

**RNA extraction and hybridization.** Total RNA was extracted (9) and hybridized with $^{32}P$-labeled D30, D34, or the Eco probes, as previously described (19, 38, 39).

**DNA extraction and hybridization.** Cellular DNAs were prepared, digested with restriction endonucleases, and hybridized by the Southern technique, as described previously (18, 19).

## RESULTS

**Modest susceptibility of C57BL/6 nude mice to the proliferation-inducing potential of the MAIDS defective virus.** C57BL/10 and C57BL/6 nude mice, lacking most of their functional T cells, were reported to be resistant to MAIDS (14, 29, 31). These mice were inoculated with replication-competent stocks of LP-BM5; they developed no splenomegaly or lymphadenopathy and survived for more than 1 year after virus inoculation, in contrast to wild-type mice inoculated with the same virus (14, 31). However, the fate of the defective virus and the identity of the infected cells in these resistant mice were not studied extensively.

To determine whether the MAIDS defective virus was nevertheless able to infect its target B cells (20), which develop normally in nude mice (21, 35), C57BL/6 nude and control euthymic mice were given intraperitoneal injections of helper-free stocks of the MAIDS defective virus: namely, the wild-type Du5H (1, 18, 20) or the chimeric Du5H/Mo-LTR (20, 38) stocks. At 45 or 60 days p.i., all seven euthymic C57BL/6 mice inoculated with stocks of either virus showed a significant splenomegaly and/or lymphadenopathy (data not shown), as previously reported (18, 20, 38). In contrast, the C57BL/6 nude mice ($n$ = 12) inoculated with the same virus stocks had no significant enlargment of their spleen and lymph nodes compared to noninoculated C57BL/6 nude mice, confirming previous observations (14, 31).

Because the development of MAIDS has been reported to be dependent on the presence of functional T cells (14, 31, 40) and because nude mice are known to have very few functional

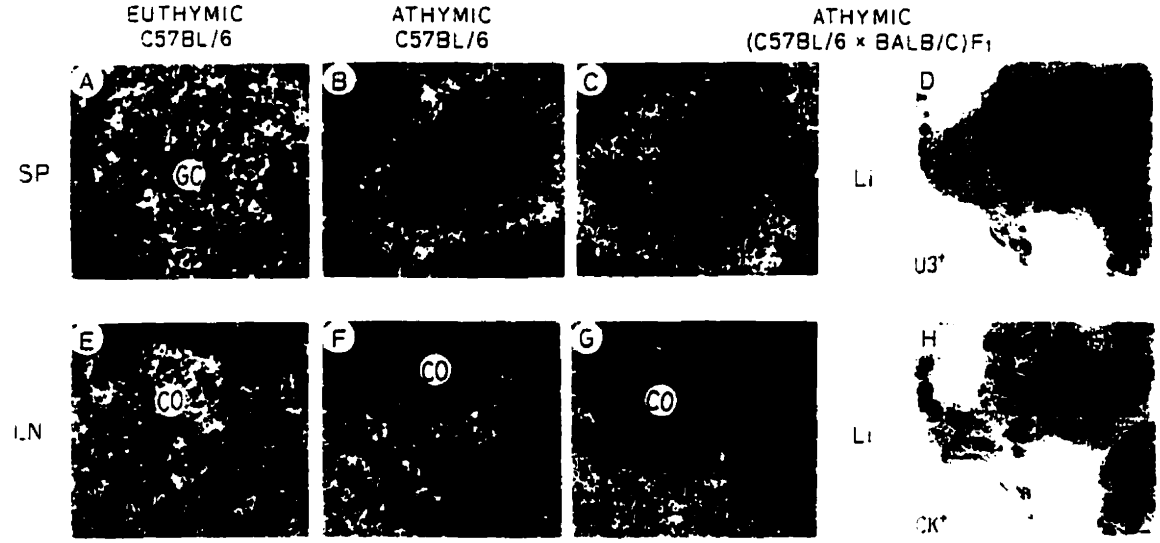


FIG. 1. In situ hybridization of lymphoid tissues of nude mice inoculated with the MAIDS defective virus. Euthymic C57BL/6 (A and E), nude C57BL/6 (B and F), and nude (C57BL/6 × BALB/c)F1 (C, D, G, and H) mice were given i.p. injections of helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus. Spleen (SP) (A to C), lymph nodes (LN) (E to G), and liver (Li) (D) sections were hybridized with $^{35}$S-labeled anti-sense LTR RNA probes (A to G). The liver in panel H is a section adjacent to the one shown in panel D and was hybridized with a $C_\alpha$ probe. Note the colocalization of the in situ signal (the U3 LTR RNA probes give a lower intensity signal than the $C_\alpha$ probe). GC, germinal center; CO, cortex. The counterstain is hematoxylin and eosin. Dark-field (A to C and E to G) and bright-field (D and H) sections are shown.

T cells in the periphery (21, 35), we first quantified the percentage of T lymphocytes in the spleens of some of the infected wild-type and nude mice. Dispersed spleen cells were labelled with the FITC-conjugated anti-murine CD3 monoclonal antibody and analyzed with a FACScan. The percentage of CD3$^+$ T cells in the spleens of the infected nude mice was very low (0.80% ± 0.40%), as expected, compared to the percentage in wild-type-infected (26.7% ± 1.1%) and normal C57BL/6 (mean of 35%) mice (Table 1; data not shown).

The presence of the defective viruses in these mice was assessed in lymphoid tissues by Northern blot analysis with the D30 probe specific for the MAIDS defective viral genome. The levels of the expected 4.2-kb MAIDS defective viral RNA were lower in the spleens of the inoculated C57BL/6 nude mice tested than were those found in euthymic control mice (data not shown). With helper-free stocks of the virus, the levels of the MAIDS defective viral RNA or DNA reflect expansion of the infected cells but not cell reinfection (18). We confirmed the absence of the helper ecotropic MuLV RNA in tissues of these C57BL/6 nude mice by hybridization with the Eco probe (data not shown).

The presence in these organs of cells infected with the MAIDS defective virus was also assessed by in situ hybridization with highly specific riboprobes (20, 38). In euthymic mice, a significant but variable percentage of infected cells could be detected in lymphoid tissues (spleen, lymph nodes) by using the D30 (for the Du5H virus) or the U3 LTR (for the Du5H/Mo-LTR virus) probes (Fig. 1A and E; Table 1), as reported previously (20, 38). A significant percentage of in situ positive cells was also detected in most of the lymph nodes and spleens of most (11 of 12) of the nude mice (Fig. 1B and F; Table 1).

We have shown previously that most of the cells infected with the same helper-free stocks of the MAIDS defective virus are B cells and express high levels of $C_\alpha$ RNA in their cytoplasm, as detected by in situ hybridization with a $C_\alpha$-specific RNA probe (38). By using the same technique, we found colocalization of in situ signals on two adjacent sections of MAIDS virus-infected livers from C57BL/6 nude mice, when hybridized with the $C_\alpha$ or the viral probes (data not shown), indicating that the infected cells in the nude mice were also of B-cell origin.

Altogether, these data indicate that a target B-cell population of the C57BL/6 nude mice is infectable by the MAIDS defective virus, despite a very low percentage of functional T cells. However, clinical lymphoproliferation did not develop in these mice, and the percentage of the infected B cells was found to be lower in nude mice than in euthymic mice, suggesting a dependence of the infected B cells on the presence of a large number of functional T cells for their full expansion.

**The susceptibility of the (C57BL/6 × BALB/c)F$_1$ nude mice to the proliferation-inducing potential of the MAIDS defective virus resembles that of C57BL/6 mice.** To confirm our previous observations, we studied the effect of the nude mutation in a different mouse background, namely, in (C57BL/6 × BALB/c)F$_1$ mice. It has been reported previously that (C57BL/6 × BALB/c)F$_1$ euthymic mice are also susceptible to MAIDS but exhibit a slightly delayed clinical course compared to C57BL/6 mice (30). (C57BL/6 × BALB/c)F$_1$ nude mice and their euthymic littermates were inoculated with the helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus and were maintained in a pathogen-free isolator for about 125 days.

In contrast to the situation in euthymic C57BL/6 mice and to previously reported data (29), lymphadenopathy and splenomegaly were not evident in the euthymic (C57BL/6 × BALB/c)F$_1$ mice. At 125 days p.i., the lymphoid mass of these MAIDS-inoculated mice was 126.4 ± 17.9 mg ($n$ = 11) compared to 126.2 ± 7.1 mg ($n$ = 4) for the noninoculated mice (Table 2). In addition, in situ positive (infected) cells could not be detected with the U3 LTR probes in any ($n$ = 11) of these virus-infected mice (Table 2), confirming the absence of expansion of the infected cells in these euthymic mice.

Surprisingly, in 5 of 11 (C57BL/6 × BALB/c)F$_1$ nude mice kept in the same isolator and inoculated with the same virus stock, a significant increase in the size of the lymph organs (nodes and spleens) was clinically evident (Table 2). The mean

TABLE 2. Susceptibility of control and nude (C57BL/6 × BALB/c)F$_1$ mice to MAIDS

| Mouse | Phenotype[a] | Virus inoculated[b] | Weight of lymphoid organs (mg)[c] | In situ positive cells[d] | |
|---|---|---|---|---|---|
| | | | | SP | LN |
| B-30 | WT | - | 136 | - | - |
| B-40 | WT | - | 121 | - | - |
| B-41 | WT | - | 127 | - | - |
| B-42 | WT | - | 125 | - | - |
| B-43 | WT | - | 130 | - | |
| B-44 | WT | - | 141 | - | |
| B-45 | WT | - | 133 | - | |
| B-46 | WT | - | 132 | - | |
| B-47 | WT | - | 129 | - | |
| B-31 | Nude | - | 202 | - | |
| B-32 | Nude | - | 189 | - | |
| B-33 | Nude | - | 221 | - | |
| B-34 | Nude | + | 307 | + + + | + + + |
| B-35 | Nude | + | 196 | + + | + |
| B-36 | Nude | + | 513 | + + + | + + + |
| B-37 | Nude | + | 390 | + + + | + + |
| B-38 | Nude | + | 227 | + + | + + |
| B-39 | Nude | + | 278 | + | + |
| B-59 | WT | - | 165 | - | - |
| B-60 | WT | - | 125 | ND | |
| B-61 | WT | - | 109 | - | - |
| B-62 | WT | - | 98 | - | - |
| B-63 | WT | - | 113 | - | - |
| B-64 | WT | - | 121 | - | - |
| B-65 | Nude | + | 232 | + + + | + + + |
| B-66 | Nude | + | 212 | ND | ND |
| B-67 | Nude | + | 201 | + + + | + + + |
| B-68 | Nude | + | 188 | + + | + + |
| B-69 | Nude | + | 296 | + + | + + + |
| B-70 | Nude | + | 204 | | + |

[a] WT, wild-type euthymic.

[b] Mice were inoculated i.p. (+) or not (-) with 1.5 ml of helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus. The mice were killed at around 125 days p.i.

[c] Total weight of lymphoid tissues (spleen and various lymph nodes: mesenteric, cervical, mediastinal, brachial, inguinal, and lumbar).

[d] Number of in situ positive cells, using the 0.3 LTR RNA probes. -, no positive cell; + + +, >50% positive cells, as shown in Fig. 1. SP, spleen; LN, lymph nodes (mesenteric, brachial, inguinal, cervical, mediastinal, and lumbar).

[e] Clinical MAIDS was obvious macroscopically.

TABLE 3. ConA response of T lymphocytes from mesenteric lymph nodes of (C57BL/6 × BALB/c)F$_1$ nude mice inoculated with the MAIDS defective virus

| Mouse[a] | Virus[b] | % of CD4$^+$[c] | ConA[d] ($10^3$ cpm) |
|---|---|---|---|
| Nude | | | |
| B-31 | - | 2.1 | ND |
| B-32 | - | 1.2 | 47.8 |
| B-33 | - | 2.1 | 25.1 |
| B-34 | + | 2.6 | <1 |
| B-35 | + | 1.8 | 24.5 |
| B-36 | + | 2.6 | 2.4 |
| B-37 | + | 4.9 | <1 |
| B-38 | + | 1.7 | 1.6 |
| B-39 | + | 1.8 | 1.7 |
| WT | | | |
| B-30 | - | 37.5 | 419.3 |

[a] Mice were inoculated i.p. with 1.5 ml of the helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus. WT, wild-type euthymic.

[b] Mice received (+) or did not receive (-) the virus.

[c] Percentage of CD4$^+$ T cells in mesenteric LN as determined by FACScan analysis with the FITC-GK-1.5 monoclonal antibody.

[d] ConA tests were performed on mesenteric LN cells with 5 μg of ConA per ml, as described in Materials and Methods. ND, not done.

weight of the lymphoid organs of these infected mice was 267.3 ± 109.9 mg ($n$ = 11), compared to 204.0 ± 13.1 mg ($n$ = 4) for the noninoculated mice, a mean increase of 31% (Table 2). Such variation in the size of lymphoid organs of infected wild-type C57BL/6 mice has been observed previously (19). To ensure that these infected (C57BL/6 × BALB/c)F$_1$ nude mice were developing a typical MAIDS syndrome, an additional parameter of disease, namely, the response of their residual T cells to ConA, a T-cell-specific mitogen, was measured. The ConA stimulation response and the percentage of total CD4$^+$ T cells in the mesenteric lymph nodes of three uninfected and six infected nude mice were evaluated. The few residual CD4$^+$ T cells present in the mesenteric lymph nodes of the noninfected (C57BL/6 × BALB/c)F$_1$ nude mice (1.8% ± 0.5%) retained a good stimulatory response to ConA, although it was 10 times less than in the normal (C57BL/6 × BALB/c)F$_1$ mice (Table 3). In contrast, the few residual T cells (2.6% ± 1.2%) in five of the six tested (C57BL/6 × BALB/c)F$_1$ nude mice inoculated with the MAIDS defective virus were hyporesponsive to ConA (Table 3).

To determine the extent of infection in these mice, Northern blot analysis and/or in situ hybridization were performed with the probes specific for the MAIDS defective virus. The lymphoid tissues of most (C57BL/6 × BALB/c)F$_1$ nude mice ($n$ = 10) harbor defective viral RNA at very high levels, as determined by Northern blot analysis (Fig. 2A, lanes 3 to 5) and/or by in situ hybridization (Table 2). We could also detect in situ positive cells in mice inoculated at earlier times p.i. (38 and 72

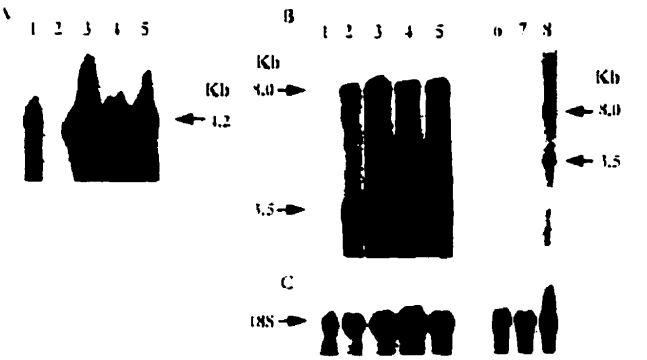


FIG. 2. Northern blot analysis of spleens of (C57BL/6 × BALB/c)F$_1$ nude mice inoculated with the MAIDS defective virus. Total RNAs (20 μg) were extracted and separated by electrophoresis as described in Materials and Methods. The samples were hybridized with $^{32}$P-labeled probes. Euthymic C57BL/6 (lane 1) or (C57BL/6 × BALB/c)F$_1$ (lane 2), and nude (C57BL/6 × BALB/c)F$_1$ (lanes 3 to 5) mice were given i.p. injections of helper-free stocks of the MAIDS chimeric Du5H/Mo-LTR virus. Lanes 2 and 6, spleen RNAs of noninoculated (C57BL/6 × BALB/c)F$_1$ nude and euthymic mice, respectively. Lane 8, RNA from MuLV-induced tumor as positive control. The mice were sacrificed at 120 days p.i. (A) The membrane was hybridized with the $^{32}$P-labeled D30 probe. The 4.2-kb RNA species (arrow) is specific to the MAIDS defective virus. (B) The same membrane shown in panel A was washed and rehybridized with a $^{32}$P-labeled Eco probe. In addition, another membrane (lanes 6 to 8) was hybridized with the same Eco probe. Note that the (C57BL/6 × BALB/c)F$_1$ nude mice express endogenous ecotropic MuLV RNA (8 and 3.5 kb) while the euthymic C57BL/6 and F$_1$ mice do not. (C) The same membranes were washed and rehybridized with a $^{32}$P-labeled 18S rDNA probe.

days p.i.) that were kept in the conventional room (data not shown). Also, MAIDS-infected cells that infiltrated the livers of (C57BL/6 × BALB/c)$F_1$ nude mice inoculated outside the isolator colocalized with $C_\alpha$ in situ-positive cells (Fig. 1 D and H). In addition, these (C57BL/6 × BALB/c)$F_1$ nude mice express high levels of full-length (8-kb) and *env*-spliced ecotropic MuLV-specific RNA (Fig. 2B, lanes 3 to 5), which was also present in noninoculated athymic $F_1$ mice (lane 2), indicating the presence of replicative endogenous helper ecotropic MuLVs in this strain of mouse. This contrasts with the absence of expression of ecotropic MuLV RNA in C57BL/6 mice (lane 1) as well as in euthymic (C57BL/6 × BALB/c)$F_1$ mice (lanes 6 and 7). Because of the presence of replicating ecotropic MuLVs in these nude mice, the number of infected cells and the levels of defective viral RNA may not result exclusively from the proliferation of infected cells but may also reflect a reinfection process. Although the characteristics of the virus-host interaction are no longer the same as those of a helper-free system, these results suggest that in a strain of a different background, the nude mutation is less inhibitory than in C57BL/6 mice inoculated with replicating (14) or nonreplicating (Table 1) MAIDS virus.

We characterized these infected cells further by studying their clonality. We have previously reported that in mice inoculated with helper-free stocks of the MAIDS virus, the proliferation of the infected B cells late in the disease is clonal or oligoclonal, as determined by $J_H$ or $C_\alpha$ gene rearrangements (18, 20). To determine whether the infected cells present in enlarged organs of these (C57BL/6 × BALB/c)$F_1$ nude mice inoculated with the MAIDS defective virus represent a clonal proliferation, we hybridized DNA from these organs with the $C_\alpha$ probe. No $C_\alpha$ gene rearrangement was detected in any ($n$ = 5) of the MAIDS-infected nude mice screened, in contrast to its detection in a MAIDS-infected control C57BL/6 mouse (data not shown), as reported previously (18, 20). This result suggested that the population of B cells infected with the MAIDS defective virus is still polyclonal, a phenotype consistent with reinfection occurring in these organs. Although the unexpectedly large number of infected cells in the (C57BL/6 × BALB/c)$F_1$ nude mice kept in the isolator is likely to reflect continuous reinfection via the presence of helper ecotropic MuLV in these mice, it nevertheless occurs despite a reduced number of peripheral T cells. Indeed, the percentage of $CD4^+$ T cells of the spleens or the lymph nodes of these mice was low compared to that of euthymic littermates, as expected (Table 3; data not shown).

Altogether, these results indicated that in a pathogen-free environment, the nude mutation in the (C57BL/6 × BALB/c)$F_1$ background did not prevent the infection of target cells by the MAIDS defective virus and the development of MAIDS, in contrast to its inhibitory effect in the C57BL/6 background.

**Modest susceptibility of $CD4^{-/-}$ knockout C57BL/6 mice to the proliferation-inducing potential of the MAIDS defective virus.** It has been reported that the presence of $CD4^+$ T cells is required for the development of MAIDS (14, 40). As seen above, T-cell-deficient nude mice bred from two distinct backgrounds are infectable by the MAIDS defective virus and exhibit a low to modest susceptibility to the disease. However, as seen by FACScan analysis (Tables 1 and 3; data not shown), these mice harbor a low percentage of $CD4^+$ T cells, which may contribute to the infectivity of the target cells.

To determine whether $CD4^+$ T cells are essential for the infection of target B cells, C57BL/6 $CD4^{-/-}$ knockout mice ($n$ = 10) and their control heterozygote or normal ($n$ = 19) littermates were injected with helper-free stocks of Du5H/Mo-LTR MAIDS defective virus. To ensure that the knockout mice were typed correctly, FACS analysis was performed for each mouse at the time of sacrifice (65 days p.i.). In contrast to their $CD4^+$ littermates, which harbored a high percentage of $CD4^+$ T cells (22.5% ± 6.0%) (Table 4), none of the knockout mice had $CD4^+$ T cells in their mesenteric lymph nodes (Table 4). As expected, all heterozygotes (+/−) and normal mice (+/+) developed MAIDS, as documented by the enlargement of their lymphoid organs (406.3 ± 171.3 mg), compared to uninoculated age-matched controls (155.7 ± 5.7 mg) (Table 4). However, the spleens and the lymph nodes of the $CD4^{-/-}$ knockout mice were not significantly enlarged (151.0 ± 24.2 mg), indicating that the presence of the $CD4^+$ T cells is required for the development of the lymphadenopathy and splenomegaly seen in MAIDS.

To determine whether cells were nevertheless infected in these mice, we performed in situ hybridization on tissue sections of their lymphoid organs. All the heterozygotes and the normal mice harbored a moderate to a high percentage of infected cells in their lymph nodes and spleens (Fig. 3A; Table 4), as previously reported (20, 38). Interestingly, the lymphoid organs of the $CD4^{-/-}$ mice also harbored infected cells, but in smaller numbers (Fig. 3B, C, and F; Table 4).

The presence of the defective viral genome in these organs was also assessed by Northern blot analysis with the D30 probe. The 4.2-kb MAIDS defective viral RNA was detected in the spleen of some $CD4^{-/-}$ knockout mice tested, although in most mice it was present at a lower level than in the heterozygote or normal control littermates (Fig. 4A, lanes 1, 2, and 7). We confirmed the absence of replication-competent ecotropic MuLV in these mice by hybridization of the same filter with an Eco probe (data not shown).

The identification of the infected cells was first assessed by in situ hybridization with a $C_\alpha$-specific RNA probe, as described above. We found colocalization of the in situ signals on two adjacent sections of the same lymph node tissue hybridized with the $C_\alpha$ or the viral probe (Fig. 3C and G), indicating that the infected cells in the $CD4^{-/-}$ knockout mice were probably of B-cell origin. To confirm the identity of the infected cells, we performed double-label immunocytochemistry and in situ hybridization with the B-cell lineage-specific B220 antibody. Most of the in situ-positive infected cells exhibited a weak immunostaining with B220 (Fig. 3D), as shown previously for normal C57BL/6 mice inoculated with the same helper-free MAIDS defective virus (20), confirming that the infected cells belong to the same B-cell lineage as in wild-type mice.

Altogether, these data suggest that the target B cells of the MAIDS defective virus can be infected in the total absence of $CD4^+$ T cells. Because these mice harbor a smaller number of infected cells than their control heterozygote or normal mice, these results suggest that $CD4^+$ T cells are required to trigger the full proliferation of these infected B cells and the subsequent development of the other manifestations of MAIDS.

**SCID mice are resistant to MAIDS.** It has been reported that mice depleted of mature B cells by treatment with anti-IgM antibodies (4) or as a result of a deletion of the $\mu$ gene (24) become resistant to MAIDS. We have also previously shown that most of the proliferating cells infected by helper-free stocks of the MAIDS defective virus belong to the B-cell lineage present in peripheral tissues (20, 38).

To determine whether the MAIDS defective virus was able to infect precursor B cells in the absence of mature T cells, as the Abelson MuLV does (13), we inoculated the mutant SCID mice. In these mice, mature T and B cells do not develop normally and only immature T and B lymphoid cells are present (3, 10, 11, 37). Ten C57BL/6 SCID mice (susceptible background), six BALB/c SCID mice (resistant background),

TABLE 4. Susceptibility of control and CD4$^{-/-}$ mice to MAIDS

| Mouse | Virus inoculated[b] | % of CD4$^+$ cells[c] | Lymphoid organ wt[d] (mg) | No. of foci of in situ positive cells[e] in: | | | |
|---|---|---|---|---|---|---|---|
| | | | | Spleen | | Lymph nodes | |
| | | | | S/M | L/TC | S/M | L/TC |
| CD4$^{+/+}$ | | | | | | | |
| 16 | + | 31.1 | 327 | 57 | 1 | 23 | 1 |
| 17 | + | 29.4 | 355 | 82 | 5 | 15 | 9 |
| 18 | + | 25.4 | 317 | 60 | 0 | 20 | 3 |
| 19 | + | 24.5 | 501 | 46 | 0 | 21 | 8 |
| 20 | + | 30.4 | 763 | 65 | 9 | 0 | 9 |
| 22 | + | 21.5 | 492 | 0 | 0 | 13 | 9 |
| 24 | + | 26.6 | 743 | 0 | 0 | 26 | 7 |
| 26 | + | 26.4 | 284 | 104 | 3 | 24 | 4 |
| 27 | + | 28.0 | 324 | 68 | 1 | 22 | 5 |
| 33 | + | 30.8 | 779 | 44 | 23 | 34 | 10 |
| 35 | + | 20.4 | 341 | 55 | 0 | 36 | 0 |
| 36 | + | 14.8 | 216 | 47 | 0 | 41 | 1 |
| 37 | + | 19.2 | 334 | 40 | 0 | 63 | 3 |
| 38 | + | 19.2 | 251 | 25 | 0 | 7 | 0 |
| 41 | + | 15.0 | 331 | 77 | 0 | 74 | 3 |
| 42 | + | 16.5 | 315 | 55 | 8 | 7 | 6 |
| 43 | + | 13.6 | 342 | 66 | 0 | 42 | 2 |
| 45 | + | 19.6 | 358 | 53 | 0 | 20 | 3 |
| 46 | + | 14.4 | 346 | 112 | 0 | 95 | 0 |
| Mean ± SD | | | 406 ± 171 | 56 ± 28 | 3 ± 6 | 31 ± 24 | 5 ± 3 |
| 29 | − | 28.0 | 150 | 0 | 0 | 0 | 0 |
| 30 | − | 29.2 | 159 | 0 | 0 | 0 | 0 |
| 47 | − | 27.5 | 152 | 0 | 0 | 0 | 0 |
| 48 | − | 27.5 | 162 | 0 | 0 | 0 | 0 |
| Mean ± SD | | 22.5 ± 6.0 | 156 ± 6 | 0 ± 0 | 0 ± 0 | 0 | 0 |
| CD4$^{-/-}$ | | | | | | | |
| 15 | + | 0.02 | 144 | 15 | 0 | 1 | 1 |
| 21 | + | 0.00 | 165 | 3 | 0 | 4 | 0 |
| 23 | + | 0.00 | 179 | 0 | 0 | 13 | 0 |
| 25 | + | 0.01 | 157 | 3 | 0 | 15 | 0 |
| 28 | + | 0.02 | 154 | 0 | 3 | 31 | 0 |
| 32 | + | 0.00 | 99 | 6 | 0 | 26 | 0 |
| 34 | + | 0.00 | 186 | 1 | 0 | 3 | 1 |
| 39 | + | 0.00 | 138 | 0 | 0 | 4 | 0 |
| 40 | + | 0.00 | 143 | 41 | 0 | 20 | 0 |
| 44 | + | 0.00 | 145 | 0 | 0 | 4 | 0 |
| Mean ± SD | | | 151 ± 24 | 7 ± 13 | 0 ± 1 | 13 ± 11 | 0 ± 0 |
| 31 | − | 0.00 | 166 | 0 | 0 | 0 | 0 |
| 50 | − | 0.02 | 144 | 0 | 0 | 0 | 0 |
| Mean ± SD | | 0.00 ± 0.00 | 155 ± 16 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |

[a] CD4$^{+/+}$ and CD4$^{+/-}$ mice.

[b] Mice were inoculated I.P. (+) or not (−) with 1.5 ml of the helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus. They were sacrificed at 65 days p.i.

[c] Percentage of CD4$^+$ splenic T cells as determined by flow cytometry with a monoclonal FITC-conjugated GK1.5 anti-murine CD4 antibody.

[d] Total weight of lymphoid organs: spleen and cervical, mediastinal, brachial, inguinal, and lumbar lymph nodes.

[e] Number of small (S), medium (M), large (L), or too many foci to count (TC) in situ positive (virus-infected) clusters in spleen and in nine randomly selected lymph nodes. Positive cells were labelled with a $^{35}$S-UTP riboprobe to the U3 LTR of Moloney MuLV.

and five euthymic C57BL/6 mice were inoculated with helper-free stocks of chimeric DU5H/Mo-LTR or the wild-type Du5H defective virus. The mice were killed at 3 months p.i., a time sufficient to allow MAIDS to develop (38). Respiratory failure was observed in the SCID mice due to late infections. At autopsy, multiple abscesses were found in the lungs and livers of the SCID mice, but their spleens and lymph nodes appeared relatively normal. In contrast, splenomegaly and lymphadenopathy were evident in the euthymic C57BL/6 mice (data not shown), as expected and as shown above.

In situ hybridization of the organs revealed no infected cells in the lymphoid tissues (Fig. 5B and C), in the lungs (Fig. 5D and E), or in the livers (data not shown) of these SCID mice of both strains ($n$ = 16). In contrast, and as expected, the lymphoid tissues of the inoculated wild-type C57BL/6 mice were all positive by in situ hybridization (Fig. 5A). These results indicated that SCID mice are unable to sustain the infection and/or proliferation of the target cells of the virus.

It has previously been reported that peritoneal macrophages were infected in mice given injections of the replication-com-

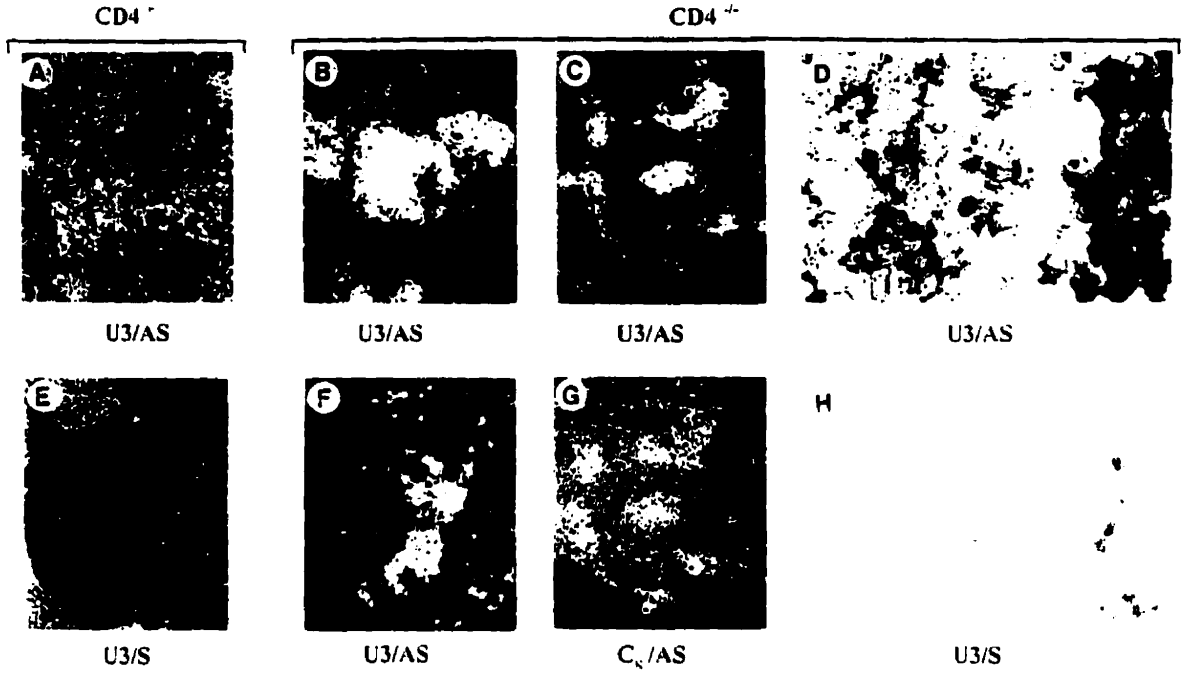


FIG. 3. In situ hybridization of lymphoid tissues of CD4$^{-/-}$ knockout C57BL/6 mice inoculated with the MAIDS defective virus. CD4$^{+/+}$ (A and E) and CD4$^{-/-}$ knockout (B to D and F to H) C57BL/6 mice were given i.p. injections of helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus. Lymph node sections were hybridized with $^{35}$S-labeled antisense (A to D and F) or sense (E and H) U3 LTR RNA probes or with the $C_\epsilon$ (G) probe. The section in panel G is adjacent to the one in panel C. Note the colocalization of the U3 LTR and $C_\epsilon$ in situ signals in panels C and G. An immunocytochemistry staining was done in panels D and H prior to the in situ hybridization, with antibody RA3-6B2 against the B-cell form of the B220 antigen (D) or with negative secondary antibody (H). The counterstain is hematoxylin and eosin. Dark-field (A to C and E to G) and bright-field (D and H) sections are shown. AS, antisense; S, sense.

petent LP-BM5 MAIDS defective virus stocks (5, 8, 40). However, using the same in situ hybridization technique as the one used for the present experiments, we were not able to detect infected peritoneal macrophages harvested from diseased C57BL/6 mice inoculated with helper-free stocks of the MAIDS defective virus that were induced by mineral oil inoculation i.p. 2 days prior harvesting the cells (38a).

To further confirm our results, we studied the state of infection of macrophages in SCID mice inoculated with helper-free stocks of the MAIDS defective virus. In SCID mice, macrophages are morphologically and functionally normal (2) and would be expected to be infectable, unless such infection requires the presence of T and B cells. Macrophages were not infected in our C57BL/6 and BALB/c SCID mice inoculated with helper-free stocks of the MAIDS defective virus, and numerous in situ negative macrophages in the lungs and liver of these mice were observed (data not shown). These results indicated that macrophages are not a target cell population of the MAIDS defective virus in SCID mice, confirming a result obtained in $\mu^{-/-}$ knockout mice (24).

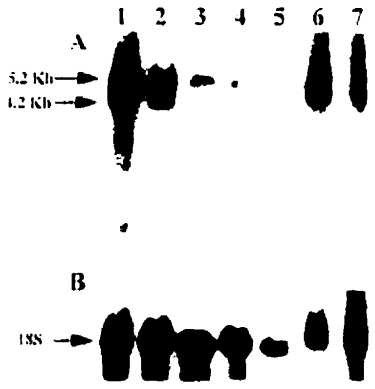


FIG. 4. Northern blot analysis of spleens of CD4$^{-/-}$ knockout C57BL/6 mice inoculated with the MAIDS defective virus. Total RNAs (20 μg) were extracted and separated by electrophoresis as described in Materials and Methods. CD4$^{-/-}$ knockout C57BL/6 (lanes 1 to 3, 5, and 7) and CD4$^{+/+}$ (lane 6) littermates were given i.p. injections of helper-free stocks of the MAIDS chimeric Du5H/Mo-LTR virus. Lane 4 contains spleen RNA of a noninoculated CD4$^{-/-}$ knockout mouse. The mice were killed at 6.5 days p.i. (A) The samples were hybridized with $^{32}$P-labeled D30 probes. The 4.2-kb RNA species (arrow) is specific to the MAIDS defective virus, while the 5.2-kb RNA is an endogenous species. (B) The same filter was washed and rehybridized with the $^{32}$P-labeled 18S rDNA probe.

## DISCUSSION

**T-cell-deficient nude mice exhibit some susceptibility to the MAIDS virus.** We used mutant nude mice lacking most functional T cells to study the role of T cells in the development of MAIDS. The C57BL/6 nude mice inoculated with the MAIDS defective virus showed less splenomegaly and lymphadenopathy than their euthymic counterparts, confirming previous reports (14, 31). Moreover, infected cells in the lymphoid organs of our inoculated C57BL/6 nude mice were, for most mice, less abundant than in wild-type mice but were nevertheless present. In contrast, Giese et al. (14) found that the levels of the MAIDS defective viral RNA in C57BL/6 nude mice were comparable to those detected in wild-type mice. The expansion of the infected cells in several strains of mice has previously been shown to be an important parameter of the MAIDS disease (19). The presence of a significant number of infected cells in C57BL/6 nude mice, at least those inoculated with helper-free viruses, suggests that their infection and initial proliferation is not totally dependent on CD4$^{+}$ T cells or depends on a very

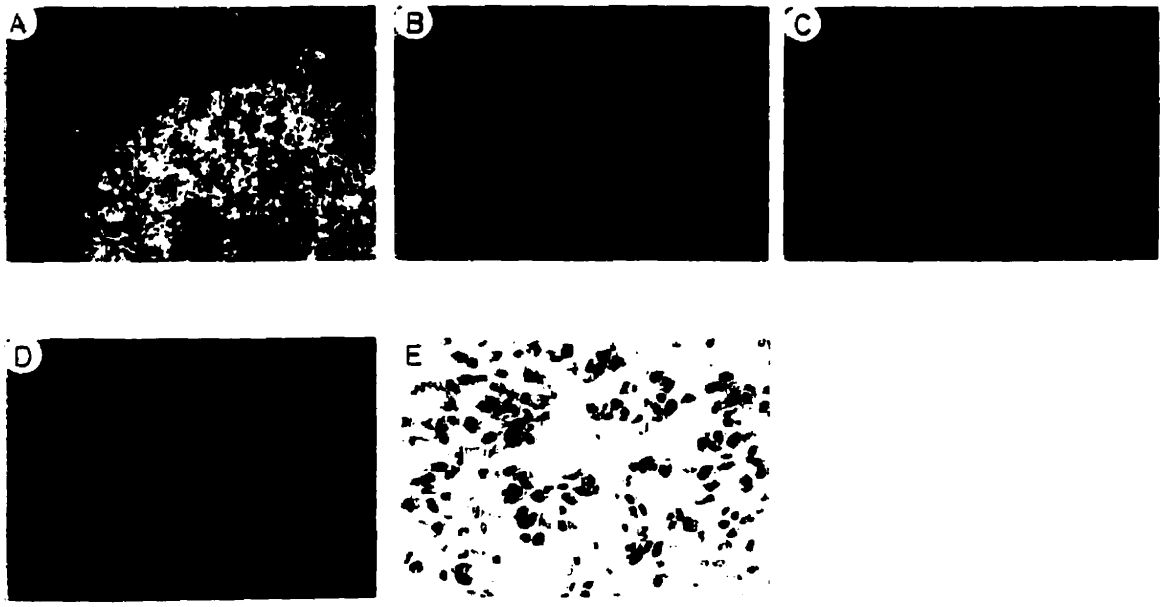


FIG. 5. In situ hybridization of lymphoid tissues of SCID mice inoculated with the MAIDS defective virus. Euthymic C57BL/6 (A) and SCID (B to E) mice were given i.p. injections of helper-free stocks of the MAIDS defective chimeric Du5H/Mo-LTR virus. Lymph node (A and B), spleen (C), and lung (D and E) sections were hybridized with the $^{35}$S-labeled antisense U3 LTR RNA probe. The counterstain is hematoxylin and eosin. Dark-field (A to D) and bright-field (E) sections are shown.

low percentage of residual CD4$^+$ T cells present in nude mice. The presence of CD4$^+$ T cells has indeed previously been reported to be essential for the development of MAIDS (14, 40).

Surprisingly, the effect of nude mutation on mice of a different background i.e., (C57BL/6 × BALB/c)$F_1$ inoculated with the MAIDS defective virus was distinct. These nude mice were much more susceptible to the virus than were their euthymic littermates maintained under pathogen-free conditions; they developed splenomegaly, lymphadenopathy, and anergy of their residual T cells. In addition, a large number of infected B cells was present in their lymphoid tissues whereas the euthymic mice were almost totally resistant to the disease. The resistance of the euthymic (C57BL/6 × BALB/c)$F_1$ mice to the development of MAIDS was unexpected, as these mice had previously been reported to be susceptible to the disease (29). One explanation for these apparently conflicting results may be the absence of endogenous ecotropic helper MuLV in these mice and the use of helper-free stocks of virus in our experiment and of helper-competent stocks by others (29). Alternatively, the different results may reflect the conditions under which these animals were kept, more specifically in a germ-free isolator in our experiment. In some instances, it appears that cofactors play an important role in the development and progression of MAIDS (24a). The resistance of these euthymic mice to MAIDS may also be immune system mediated and may reflect the clearance of the virally infected B cells by cytotoxic CD8$^+$ T cells. Indeed, cytotoxic CD8$^+$ T cells have previously been found to be responsible for the resistance of A/J mice to MAIDS: A/J mice depleted of their CD8$^+$ T cells became highly susceptible to MAIDS (26). The unexpected susceptibility of the (C57BL/6 × BALB/c)$F_1$ nude mice to MAIDS remains unexplained and may be related in part to the presence of helper MuLV and in part to their low levels of CD8$^+$ T cells.

**CD4$^+$ T cells are not required for the initial phase of MAIDS but play a role in the second-phase expansion of the lymphoid organs.** In experiments involving studies in nude mice, it has previously been shown that CD4$^+$ T cells are essential in the development of MAIDS (14, 40). Our results further extend this previous work by showing that this requirement for CD4$^+$ T cells occurs in a later phase of the disease. Indeed, our experiments with nude mice suggest that CD4$^+$ T cells are not required for the infection and initial polyclonal proliferation of B cells seen in MAIDS. However, these nude mice still harbor a low percentage of CD4$^+$ T cells, which may have contributed to the infection and the slow expansion of B cells that was observed in these mice. We used CD4$^{-/-}$ knockout mice and found that CD4$^+$ T cells were not required for the initial infection and proliferation of B cells. These knockout mice harbored a significant number of infected B cells in their lymphoid organs, and high levels of the 4.2-kb MAIDS-specific defective RNA could be detected in some of them. Interestingly, these CD4$^{-/-}$ mice did not develop the splenomegaly and lymphadenopathy characteristic of the MAIDS virus-infected CD4$^+$ littermates. Moreover, the percentage of the MAIDS virus-infected B cells was in general lower in these CD4$^{-/-}$ knockout mice than in their CD4$^+$ littermates. Altogether, these data suggest that CD4$^+$ T cells are required to trigger the full development of MAIDS. Although the CD8$^+$ T cells from CD4$^{-/-}$ mice have previously been shown to act as "helper" cells (25), the helper function of these cells does not appear to be significant for the full development of MAIDS, which was prevented in CD4$^{-/-}$ mice. We have previously observed that in mice inoculated with helper-free stocks of the MAIDS defective virus, the degree of enlargement of the lymphoid organs does not correlate with the number of infected B cells in these organs (18, 20) but, rather, depends on the expansion and/or recruitment of noninfected cells. The present results with the knockout mice would suggest that CD4$^+$ T cells play a critical role in this second-phase expansion of the lymphoid organs.

Therefore, studies with nude and knockout mice have helped to distinguish two phases in the development of MAIDS. The first phase appears to involve the infection and the initial polyclonal proliferation of the infected B cells. This

phase appears relatively CD4 T-cell independent. The second phase seems to be more dependent on $CD4^+$ T cells and involves the rapid enlargement of the lymphoid organs. This expansion most probably results from the proliferation and/or recruitment of noninfected cells, as well as a further proliferation of the infected B cells. As a low percentage of infected B cells is sufficient to trigger T cells into a hyporesponsive state (38), the proliferation of the infected B cells may represent a more stringent marker of disease progression than the lymphoid organ enlargement.

**Role of B cells in the development of MAIDS.** In our experiment with the C57BL/6 and BALB/c SCID mice, we found that these mice were resistant to MAIDS, because they did not harbor cells infected by the MAIDS defective virus and did not exhibit other signs of disease. Since these mice are deficient in mature T- and B-lymphoid cells (3, 10, 11, 37), two hypothesis may explain our findings. First, the absence of functional $CD4^+$ T cells may prevent the infection and/or expansion of cells of the B-cell lineage which are still present in these mice (pre-B, pro-B). This hypothesis seems unlikely, however, since infected B cells are detectable in nude mice which have a very small number of T cells, and in $CD4^{-/-}$ knockout mice which lack $CD4^+$ T cells. Second, the target B cells of the MAIDS virus may not be present in the SCID mice. This latter hypothesis is more consistent with previous data. We have indeed found that the majority of the target cells infected by the MAIDS defective virus are relatively mature B cells, having $C_\kappa$ and/or $J_H$ Ig gene rearrangement, expressing cytoplasmic $C_\kappa$ RNA and being located in the peripheral lymphoid organs and not in the bone marrow (20, 38). In addition, it has been shown that mice depleted of their mature B cells by treatment with antibodies against IgM or $\mu^{-/-}$ knockout mice, whose B-cell development is arrested at the pro-B stage, were resistant to MAIDS (4, 24). Altogether, these data suggest that the primary target cells of the MAIDS defective virus are relatively mature peripheral B cells.

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Chapter 3

# Establishment of MAIDS Defective Virus Target B Cell Lines and Their Characterization

Steven J. Klein, Carole Simard, and Paul Jolicoeur

## Forward

The central role of the B cell in the pathogenesis of MAIDS was reinforced by the results obtained through the study of the effect of the MAIDS defective virus on various mutant mice. The results in chapter 2 further strengthened the argument that a mature B cell population is the target of the MAIDS defective virus. We therefore felt that in order to more fully understand the nature of this population and its central role in MAIDS, it would be necessary to establish B cell lines derived from mice inoculated with the MAIDS defective virus. Success in this endeavor would give us excellent tools in order to further our understanding of the virus-cell and cell-cell interactions which lie at the heart of MAIDS.

Many previous attempts to derive such lines led uniquely to transplantable B and/or T cell lines (1-4). Such lines do not represent the true *in vivo* target of the MAIDS defective virus, which is not transplantable, and may reflect the use by these groups of the LP-BM5 mixture to induce MAIDS rather than helper-free stocks of the MAIDS defective virus used by our group. We had also been unsuccessful in transplanting primary tumors into normal or nude mice. We therefore attempted a novel approach to establish MAIDS defective virus-infected cell lines along with a traditional approach. Our efforts yielded two independent cell lines which appear to be of the B lineage and accurately represent the phenotype of the *in vivo* target cells of the MAIDS defective virus.

I was responsible for devising the transplantation protocol which was used to generate the SD1 cell line and which may be best suited for

generating other MAIDS B cell lines. Carole Simard isolated the CSTB5 cell line and partially characterized it. I fully characterized the SD1 cell line and completed the full characterization of the CSTB5 cell line. This manuscript is currently in press at Virology.

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# Establishment of MAIDS defective virus target B cell lines and their characterization

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## Abstract

Mice inoculated with the murine AIDS (MAIDS) defective virus develop severe B and T cell dysfunctions. The primary event in the development of this disease is the infection and polyclonal expansion of the target cells of this defective virus, which have been reported to belong to the B cell lineage. To further study the central role that these cells play in the development of MAIDS, we attempted to establish MAIDS defective virus-infected B cell lines *in vitro*. We succeeded in establishing two cell lines, SD1 and CSTB5, from the enlarged organs of C57BL/6 mice inoculated with helper-free stocks of the MAIDS defective virus. Both cell lines are not transplantable in syngeneic C57BL/6 mice or in nude or $CD8^{-/-}$ mice and are apparently not malignant. They both belong to the B lineage, as their immunoglobulin (Ig) genes, but not the T cell receptor (TcR) β locus, are rearranged, suggesting that they are relatively mature B cells. However, analysis of cell surface marker expression by FACS revealed a surface phenotype similar to that of pre-B cells (MHC $I^+$, MHC $II^+$, $B7.2^+$, $sIgM^-$, $sIgG^-$, $\kappa^-$, $B220^-$, $CD5^-$, $Thy1.2^-$, $TcR^-$, $CD3^-$, $CD4^-$, $CD8^-$, $Mac\text{-}1^-$, $33D1^-$). Additionally, the CSTB5 cells express CD40 and the SD1 cells express CD43. Both cell lines contain the MAIDS defective provirus and express the expected 4.2 kb viral RNA and the corresponding $Pr60^{gag}$ protein. The CSTB5 cells are non-producer, while the SD1 cell line produces what appears to be an endogenous MuLV. The phenotype of these cell lines is very similar to what is known about the target B cells of this virus *in vivo*. These new established cell lines are likely

to be useful in elucidating the mechanism(s) by which the MAIDS defective virus causes its target B cells to proliferate and induce T cell anergy in infected animals.

## Introduction

The murine acquired immunodeficiency syndrome (MAIDS) is induced by the Duplan strain of the murine leukemia virus (MuLV), and is characterized by lymphadenopathy, splenomegaly and severe dysfunctions of both B and T cells in susceptible mouse strains (for reviews, see (Morse III et al., 1992; Jolicoeur, 1991; Mosier, 1986)). This virus mixture contains non-pathogenic, replication-competent MuLVs (Chattopadhyay et al., 1991; Legrand et al., 1982; Haas and Reshef, 1980; Guillemain et al., 1980; Astier et al., 1982), as well as a pathogenic defective retrovirus (Chattopadhyay et al., 1989; Aziz et al., 1989). We have previously found that helper-free stocks of the MAIDS defective virus are pathogenic and induce a lymphoproliferative disease very similar to the disease observed after inoculation of the virus mixture (Huang et al., 1989). This observation has been recently confirmed with another helper-free stock (Pozsgay et al., 1993). Infection of mice with the crude virus mixture has been reported to lead to infection of many different cell populations, including B cells, T cells, and macrophages (Cheung et al., 1991; Bilello et al., 1992; Chattopadhyay et al., 1991; Klinken et al., 1988; Kubo et al., 1992; Hitoshi et al., 1993), as expected for replication-competent stocks. In contrast, the use of helper-free stocks of the defective retrovirus has allowed us to identify that cells of the B lineage are the primary targets of the pathogenic defective virus (Huang et al., 1991). This has later been confirmed by analysis of purified cell subsets obtained from mice inoculated with replication-competent stocks of the MAIDS virus (Kim et al.,

1994). It therefore appears that infection of B cells and their subsequent proliferation is the critical event in the initiation of the disease (Kim et al., 1994; Simard et al., 1997; Huang et al., 1989; Huang et al., 1991). We have found that the population of B cells initially infected *in vivo* is present in lymph nodes draining the site of inoculation of the virus (Simard et al., 1994). This suggests that these B cells are relatively mature, since there have been no reports of the presence of pre-B cells in this organ. The fact that mice deficient in mature B cells (Kim et al., 1994; Cerny et al., 1990) or with altered conventional B cell function due to the *xid* mutation (Tang et al., 1995) were resistant to the disease indeed indicated the strict requirement of this cell population for disease development and is consistent with our findings on the identity of the target cell population (Huang et al., 1991). However, $CD4^+$ T cells are also required for the development of MAIDS as shown by experiments which depleted $CD4^+$ T cells by antibody treatment *in vivo* (Yetter et al., 1988) or by use of mice genetically deficient for $CD4^+$ T cells (Giese et al., 1994; Simard et al., 1997; Mosier et al., 1987). It appears that $CD4^+$ T cells themselves do not have to be infected by the MAIDS defective virus for the disease to be induced (Huang et al., 1991, and C. Simard, M. Huang and P. Jolicoeur, unpublished data). It is also apparent that $CD4^+$ T cells are required at a post-infection stage to allow expansion of the infected B cells and the subsequent development of full-blown disease (Simard et al., 1997).

A major problem in studying the pathogenesis of MAIDS has been the lack of *in vitro* cell lines which mirror the *in vivo* phenotype of the target B cells.

Klinken et al. (1988) have established two B cell lymphomas from mice inoculated with the LP-BM5 crude virus mixture, but these cells are malignant, in contrast to the benign nature of the infected cells found in the enlarged lymphoid organs of MAIDS mice. The non-malignant nature of the MAIDS virus-infected B cells is inferred from their lack of transplantability in syngeneic or nude mice except in late stage disease (Mistry and Duplan, 1973; Klinken et al., 1988 and C. Simard, S. Klein and P. Jolicoeur unpublished data). The attempts by some groups, including ours, to establish cell lines *in vitro* led to the derivation of transplantable T cell tumors, or lines (Simard et al., 1995; Tang et al., 1992; Kubo et al., 1992). We found that expansion and transformation of T cells was a rare event in mice inoculated with helper-free stocks of the MAIDS virus, but occurred at a relatively high frequency when helper viruses were present in the virus inoculum (Simard et al., 1995). The infection and transformation of T cells in these mice likely represents a secondary event.

In the present study we report the establishment of two independent B cell lines which have a phenotype similar to that of *in vivo* virus target cells. These established cells do not express markers of the T, macrophage, or dendritic cell lineages, have rearrangements of their immunoglobulin (Ig) loci, but not their T cell receptor (TcR) β locus, and are not malignant.

## Materials and Methods

Animals and viruses: Inbred C57BL/6, *nu/nu* and SCID mice were purchased from Charles River Inc. (St-Constant, Quebec, Canada). C57BL/6 Beige mice were purchased from the Jackson Laboratory (Bar Harbor, ME). $CD8^{-/-}$ mice were initially provided by Tak Mak (The Amgen Institute, Toronto, Ontario, Canada), and were backcrossed for 6 generations onto the C57BL/6 background. Young (30-40 days) C57BL/6 mice were inoculated with helper-free stocks of the wild-type MAIDS defective virus, Du5H (Aziz et al., 1989), or with the Moloney long terminal repeat (LTR)-tagged (Du5H/Mo-LTR) defective virus (Huang et al., 1991). This chimeric Du5H/Mo-LTR virus was constructed by substituting the *PstI-KpnI* Du5H 3' LTR fragment with the homologous fragment from Moloney MuLV. These Moloney LTR sequences act as a molecular tag to aid in identifying and following the virus.

Transplantation of primary MAIDS lymphoid organs: For the SD1 cell line, enlarged spleen and lymph nodes were dissected from C57BL/6 mice which had been inoculated with the helper-free stocks of the Moloney LTR-tagged Du5H for 3-4 months. The organ mixture was homogenized in complete RPMI medium and immediately inoculated intraperitoneally, using a 23-guage needle, into SCID mice which were or were not treated with 50µl of anti-asialo GM1 antibody (Dako Inc., Japan) to deplete their NK cells. Anti-asialo GM1 treatment began on day -1 and continued on day 0 and every second day thereafter, for a period of three weeks. Mice were monitored for the development of ascites or other signs

of cell growth. Upon evidence of cell growth, the mice were sacrificed and the ascites were collected and seeded into RMPI-1640 medium/10% FCS (Hyclone)/50 x $10^{-5}$ M β-Mercaptoethanol and antibiotics, at approximately $10^6$ cells/ml. The cells were split weekly. The SD1 cell line which emerged from this protocol has been passed for many months and survive freezing in DMSO and the subsequent thawing.

For the establishment of the CSTB5 cell line, enlarged spleen and lymph nodes were aseptically removed from C57Bl/6 mice which had been inoculated with helper-free stocks of the wild-type MAIDS defective virus 3-5 months previously. The organs were homogenized in RPMI-1640 medium/10% FBS (Gibco)/50 x $10^{-5}$ M β-Mercaptoethanol and antibiotics and seeded at approximately $10^6$ cells/ml. The cultures were then monitored and split as necessary. From the culture of enlarged organs from over 30 MAIDS animals, only one cell line emerged.

Probes: The D30 (Aziz et al., 1989), Moloney U3M (Poirier and Jolicoeur, 1989), ecotropic MuLV *env* (Chattopadhyay et al., 1980), (Ig) $J_H$ (Alt et al., 1981; Poirier and Jolicoeur, 1989), (Ig) $C_K$ (Lewis et al., 1982; Poirier and Jolicoeur, 1989), (Ig) $D_H$ (Alt et al., 1984), and TcR β locus (Caccia et al., 1984; Poirier and Jolicoeur, 1989) probes have been described previously. For use in Southern or Northern hybridization, the probes were $^{32}$P-labeled by random-priming as before (Huang et al., 1989; Poirier and Jolicoeur, 1989).

DNA extraction and hybridization: Cellular DNAs were extracted, digested with the desired restriction enzyme, electophoresed through agarose, transferred to a membrane, and hybridized with the indicated probe, as described previously (Huang et al., 1989; Poirier and Jolicoeur, 1989).

RNA extraction and hybridization: Total cellular RNA was isolated by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987), and hybridized with $^{32}$P-labeled probes, as previously described (Huang et al., 1989).

Protein extraction and Western blotting: Proteins were extracted by lysis in RIPA buffer (150mM NaCl, 1% NP40, 0.5% Na desoxycholate, and 0.1% SDS) containing protease inhibitors, followed by SDS-PAGE and blotting with polyclonal goat-anti CA (p30) MuLV antibodies, as described previously (Dupraz et al., 1997).

Reverse transcriptase (RT) activity: The RT assay was performed on supernatants of cell lines as described previously (Gorska-Flipot et al., 1992; Huang et al., 1989).

Cell surface labeling and FACS: Cells from the established cell lines were labeled with the antibody of choice. The fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies 145-2C11 (murine anti CD3), GK1.5 (murine anti CD4), YTS 169.4 (murine anti CD8a), and H57-597 (murine anti- αβTcR) were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Biotinylated anti-CD43 (clone S7) was purchased from Pharmingen Canada (Mississauga, Ontario). FITC-conjugated anti murine CD5 (clone 53-7.313), MHC I (clone Y-3) and MHC II (clone D3-137.5.7), biotinylated Thy1.2 (clone

5A-8) and Mac-1 (clone M1/70.15.11) and the anti-CD40 hybridoma (clone FGK45.5) were kindly provided by Dr Patrice Hugo (Clinical Research Institute of Montreal, Montreal, Quebec). The RA3-6B2 (murine anti-B220) monoclonal antibody was a kind gift of Dr. R. Coffman (DNAX Research Institute, Palo Alto, CA), and was directly conjugated to FITC using the FITC QuickTag conjugation kit (Boehringer Mannheim, Laval, Quebec). Murine FITC-anti-μ antibody and FITC-anti-rat Ig (mouse adsorbed) was purchased from Kirkeengaard and Perry Laboratories (MD). Monoclonal anti-murine B7.2 antibody (clone GL-1), anti-murine $C_K$ (clone HB58) and anti-murine dendritic cell antibody (clone 33D1) were purchased from the American Type Culture Collection (MD). The FITC-anti-rat Ig was used as a second antibody. Cells were resuspended at a concentration of 2 X $10^7$/ml in PBS containing 1% FCS and 0.1% $NaN_3$ (wash solution) and $10^6$ cells were transferred to a conical-bottom microwell wells. Cells were then centrifuged and resuspended in a mixture of 5μg/ml of human immunoglobulins (Sigma Chemicals, Oakville, Ontario, Canada) and anti-Fc receptor antibody (clone 24G2)-containing cell supernatant at room temperature for 5-10 min. Cells were then washed, resuspended in wash solution containing the primary antibody and Incubated at 4° for 30 min. Cells were then washed 3 times and the secondary antibody was added, incubated at 4° C for 30 min and washed as above. Following staining, the cells were analyzed by flow cytometry using a Becton-Dickinson FACscan. Histograms were generated using CellQuest software (Becton-Dickinson).

Transplantation of established cell lines: The established cell lines (2-5 X $10^6$) were inoculated intraperitoneally into the desired mice in a volume of 250-500µl PBS. The mice were monitored for up to one month for evidence of tumor formation.

## Results

**Establishment *in vitro* of B cell lines from primary MAIDS enlarged lymphoid organs.** In order to further study the target B cell-MAIDS defective virus interactions which are critical in the development of MAIDS, we attempted to establish B cell lines *in vitro* from primary enlarged lymphoid organs from mice inoculated with helper-free stocks of the MAIDS defective virus. The choice of helper-free stocks was motivated by the hope of establishing non-producer cell lines. Two strategies were employed, the first being simply to dissect out the enlarged organs and placing the resulting cell suspensions in tissue culture. This strategy led to the establishment of one cell line, CSTB5, which has grown continuously in tissue culture for over one year. The CSTB5 cell line was the only line that emerged from the culture of several primary MAIDS organs from several (over 30) mice. The difficulty in establishing cell lines from mice inoculated with helper-free stocks of the MAIDS virus is in line with our inability to transplant primary MAIDS organs in syngeneic C57BL/6 mice or in nude mice.

In order to generate additional cell lines for study, we used an alternate approach involving transplantation of enlarged lymphoid organs from MAIDS animals into SCID mice depleted of NK cells by treatment with an anti-asialo GM1 antibody. This treatment is similar to the one used by Veronesi et al. (Veronesi et al., 1994) to establish Epstein-Barr virus (EBV)-infected human B cell lymphoma cell lines *in vitro*. Out of four SCID mice treated with anti-NK cell antibody (anti-asialo GM1) which received inoculations with primary MAIDS

lymphoid tissues, two developed ascitic growth within 3 weeks. When the ascites was seeded to tissue culture, one of the two cultures gave rise to a cell line, SD1, which has been cultivated *in vitro* for over one year. This cell line was derived from a mouse inoculated with a modified MAIDS defective virus (Du5H/Mo-LTR), containing the U3 LTR region of the Moloney MuLV (Huang et al., 1991). None of the five control $NK^+$ SCID mice injected with PBS alone and inoculated with the same tissues developed any tumor or ascites, indicating that NK cells had a significant negative effect on the growth of these cells *in vivo*.

**The SD1 and CSTB5 cell lines contain and express the MAIDS defective provirus.** Our first step towards studying the phenotype of these cells was to ascertain whether or not the cells contained the MAIDS defective virus. Southern blot analysis performed on DNA from both cell lines showed that the CSTB5 cells contain a unique, newly acquired provirus (in addition to endogenous proviruses) detected by the D30 probe (Figure 1B, lane 2). The same integration was observed with the 14810 cell line, a subline derived from CSTB5 (see below), as expected (data not shown). On the other hand, the SD1 cells harbor multiple proviruses of the MAIDS defective virus, as detected by the Moloney U3 LTR-specific probe (Figure 1A, lane 1). With the same U3 LTR probe, the CSTB5 cell line was negative, as expected, since it was derived from a mouse inoculated with the wild-type Du5H MAIDS defective virus (Figure 1A, lane 2).

Northern blot analysis was performed on these cells to determine whether they expressed the expected 4.2 kb MAIDS defective viral RNA. The cells of both lines expressed the 4.2 kb viral RNA species, the CSTB5 cells at lower

levels (Fig. 2A). Since the SD1 cell line was positive for reverse transcriptase (RT) activity, we also examined the expression of ecotropic viral RNA in the two cell lines. Hybridization with an ecotropic MuLV *env*-specific probe indicated that neither of the cell lines produced ecotropic viral RNA (Fig. 2B). This is consistent with the fact that the CSTB5 cell line had no RT activity (data not shown), and implies that non-ecotropic MuLV(s) is (are) produced by the SD1 cell line. Together, these results indicated that both cell lines were derived from primary cells initially infected *in vivo* by the MAIDS defective virus.

**The SD1 and CSTB5 cell lines express the MAIDS defective virus Pr60$^{gag}$ protein.** To determine whether the 4.2 kb MAIDS viral RNA found in these cells encodes the previously characterized MAIDS defective virus-specific 60 kDa *gag* protein (Pr60$^{gag}$), Western blotting was carried out on whole cell protein extracts using a goat polyclonal anti-CA (p30) MuLV antibody. This analysis revealed a 60 kDa protein in both cell lines (Fig. 3, lanes 1 and 2). In keeping with the RNA levels detected by Northern analysis, the CSTB5 cells produce less protein than the SD1 cells. Additionally, the SD1 cells produce a full-length 65 kDa *gag* protein (Fig. 3, lane 1), consistent with the fact that they are virus-producer. Curiously, the CSTB5 cell line, after transplantation into the NK-depleted SCID mice (cell line 14810), also became RT positive (data not shown) and now expressed both the MAIDS defective virus Pr60$^{gag}$ protein as well as a Pr65$^{gag}$ helper species (Fig. 3, lane 3). Passage of these cells into the SCID mice may have activated an endogenous non-ecotropic MuLV.

**The SD1 and CSTB5 cell lines have rearranged Ig loci.** To identify the lineage to which these MAIDS defective virus-infected cell lines belong, we first examined the status of their Ig and TcRβ loci by Southern blot analysis. Both cell lines had their TcR loci in germline configuration (Figure 4A, lanes 1 and 2), and both had their Ig κ locus rearranged (Fig. 4B, lanes 1 and 2). Furthermore, the SD1 cell line also had its Ig heavy chain locus rearranged (Fig. 4C, lane 1). These results strongly imply that both cell lines belong to the B cell lineage. However, it was somewhat surprising that the CSTB5 cell line did not have its Ig heavy locus rearranged, as one would expect for a B cell line. It is well documented indeed that the Ig heavy locus is the first to undergo rearrangement during B cell development, followed by the Ig κ or λ loci (for review, see (Alt et al., 1987)). To reconfirm that the heavy chain in the CSTB5 cells was unrearranged, we performed another Southern blot analysis on these cell line DNAs with a probe for the $D_H$ region of the Ig heavy chain locus. The CSTB5 cell DNA gave a positive signal with this probe (Fig. 4D, lane 2), while the SD1 cell DNA showed no detection of the D segments (Fig. 4D, lane 1), suggesting that only the SD1 line has undergone rearrangement of the Ig heavy chain locus.

**Surface phenotype of the SD1 and CSTB5 cell lines.** To further characterize the identity of these two cell lines, we studied the surface expression of a panel of markers specific for distinct cell lineages, using flow cytometry (Fig. 5). Both cell lines had an almost identical phenotype to each other. Neither cell line expressed any of the T-lineage markers tested (CD3, CD4, CD8, TcR, Thy1.2), Mac-1, which is specific for macrophages, 33D1, which is specific for dendritic

cells, or CD5, which is expressed on a specific sub-class of B (B-1a) cells found primarily in the peritoneum, and on T cells (Luo et al., 1992). Both cells expressed both MHC I and II as well as the CTLA-4 ligand, B7.2 (Ward, 1996), which is in keeping with their identity as being B cells. The CSTB5 cell line also expressed the ligand for gp39, CD40, another marker of B cells (Grewal and Flavell, 1996) while the SD1 cells expressed CD43 (Gulley et al., 1988), which is also found on B cells. Neither cell line expressed surface IgM, IgG, or κ, which are specific to mature B cells, suggesting that these cells are lacking some of the characteristics of fully mature B cells. Expression of B220, a B cell marker present in pro-B, pre-B, and mature B cells was very low in these cells, being detected at very low levels only occasionally. A summary of these data is given in Table 1. Taken together, the surface phenotype of these two cell lines indicates that they belong to the B cell lineage and are either arrested at a unique stage of development, or are fully mature but at the same time have sustained downregulation of some surface markers (IgM, IgG, κ, and B220) due to infection with the MAIDS defective virus.

**The SD1 and the CSTB5 cell lines are not transplantable.** To determine whether these cells also mirror their *in vivo*-infected counterparts in their inability to be transplanted, we inoculated both cell lines (2.5 and 5.0 X $10^6$ cells) intraperitoneally into a variety of hosts. We were unable to transplant these cells into syngeneic C57BL/6 mice nor into nude, SCID, syngeneic $CD8^{-/-}$ mice or syngeneic *beige* mice, which are deficient in NK cells (Roder and Duwe, 1979). As well, transplantation of these cell lines into C57BL/6 mice did not lead to

MAIDS in the receiving animals (observed for up to 3 months), again confirming that it is unlikely that these cells are producing replicating ecotropic MuLV. The resistance of these cells to transplantation is identical to that of the primary cells infected with the MAIDS virus stocks. A summary of the phenotypes of the SD1 and CSTB5 cell lines is given in Table 2.

## Discussion:

**The established MAIDS cell lines are B cells.**

In an attempt to study the target cells of the MAIDS defective virus, which has been reported to belong to the B cell lineage (Huang et al., 1991), we have established *in vitro* two B cell lines from lymphoid organs of MAIDS mice. The phenotype of these cells is that of relatively mature B cells which have rearranged their Ig genes, but not their T cell receptor (TcR) β gene and which express MHC class I and II, and low levels of B220, but do not express any surface Ig, Mac-1 or any T-cell markers. Moreover, both cell lines express the co-stimulatory molecule B7.2 which is found mainly on activated B cells (for review see Ward, 1996), and the CSTB5 and SD1 cell lines express, respectively, CD40 and CD43, two other B cell markers (for reviews see Grewal and Flavell, 1996 and Melchers et al., 1994). This phenotype is consistent with that of cells belonging to the B lineage, reflecting more closely the phenotype of a mature B cell population, although some characteristics of fully mature B cells (presence of surface IgM, IgG, κ, and B220) are lacking. This is consistent with our previous results showing that MAIDS virus-infected cells are initially localized in germinal centers (Simard et al., 1994), which lack pre-B cells. Curiously, the CSTB5 cell line has its Ig κ chain rearranged in the absence of Ig heavy chain rearrangement. We have previously reported and discussed such a phenotype in some MAIDS animals (Huang et al., 1991), suggesting that it is unlikely to be related to the establishment of these cells *in vitro*, although such a scenario is nevertheless

possible. Therefore, the preponderance of the evidence, as reiterated above, supports the notion that these two cell lines are of the B lineage.

Two other B cell lymphoma lines derived from mice inoculated with the LP-BM5 mixture of viruses (B6-1153 and B6-1710), which contain both ecotropic and MCF viruses in addition to the MAIDS defective virus, have previously been established (Klinken et al., 1988). They have a phenotype similar, but not identical, to the cells described here (Klinken et al., 1988). Indeed, neither SD1 or CSTB5 cells were transplantable into syngeneic C57BL/6 mice, nude mice, or $CD8^{-/-}$ mice, nor do they express CD5, in contrast to the above-mentioned B cell lymphoma lines.

The virological status of the SD1 and CSTB5 cells is also interesting. Both cell lines express the MAIDS $Pr60^{gag}$ protein at relatively high levels. This characteristic is worth pointing out as we have been unable to get high expression of $Pr60^{gag}$ in other B cell lines (CH33 and WEHI-231) that were infected *in vitro* with the MAIDS defective virus (S. Klein, P. Dupraz, and P. Jolicoeur, unpublished data), suggesting that this protein may not be tolerated well in B cells which have reached specific stages of differentiation. In addition, the CSTB5 cells were non-producer as documented by the absence of RT activity in the culture medium and of $Pr65^{gag}$ helper protein in Western blot analysis, strongly suggesting that the primary cell from which this line was derived was infected *in vivo* by the MAIDS defective virus. In contrast, the SD1 cell line, as well as the CSTB5 derivative 14810, expressed high levels of a 65 kDa protein species, which most likely represents a helper MuLV *gag* protein, even though these cell

lines were derived from mice inoculated with helper-free stocks. This helper MuLV, also detected as virions in the culture supernatant, is not ecotropic as determined by Northern blot hybridization with ecotropic *env* sequences. Moreover, this helper MuLV does not appear to replicate *in vivo* since inoculation of SD1 producer cells into syngeneic C57BL/6 mice did not induce MAIDS. This helper MuLV may represent an endogenous species and may have been acquired during passage into the SCID mice.

**Why is the establishment of infected target B cells from MAIDS mice so difficult?** The establishment of the CSTB5 MAIDS cell line *in vitro* was difficult and represented a rare success after many attempts. We had to rely upon a difficult protocol involving passage into NK-depleted SCID mice to derive another line, SD1. We and others have previously attempted unsuccessfully to derive *in vitro* target B cell lines infected with the MAIDS defective virus (Tang et al., 1994; Simard et al., 1995; Kubo et al., 1992). The transplantation protocols used gave rise to malignant T-cell lines or tumors which were transplantable into syngeneic C57BL/6 mice (Simard et al., 1995) or into nude mice (Kubo et al., 1992), respectively. The third protocol gave rise to clonal B and T cell outgrowths that were transplantable into SCID mice (Tang et al., 1994). We have rarely observed infected T cells during the course of MAIDS using our helper-free system (Huang et al., 1991), although up to 35% of mice inoculated with the LP-BM5 virus mixture experienced an expansion of T cells as determined by a clonal TcR β gene rearrangement (Klinken et al., 1988). This bias towards obtaining T cell tumors and lines may be due to several factors, such as the presence of

replicating MuLVs, especially in the LP-BM5-inoculated mice, or T cells from MAIDS mice may be more inherently transplantable than B cells. Regardless of the reason for increased transplantability of T cells from MAIDS mice, it is clear that in our helper-free system, the infected cell population is of the B lineage and less than one in 10,000 T cells are infected with the MAIDS defective virus (C. Simard, M. Huang and P. Jolicoeur, unpublished data). The relation of the reported transplantable T cells to the development of the disease is unclear (Simard et al., 1995; Kubo et al., 1992), although T cells, specifically $CD4^-$ T cells are required for the development of MAIDS (Simard et al., 1997; Mosier et al., 1987; Giese et al., 1994; Yetter et al., 1988).

The use of NK-depleted SCID mice was very important in establishing the SD1 cell line since our previous attempts is passing cells from MAIDS-infected tissues in syngeneic C57BL/6, nude, and SCID mice all failed. In addition, the CSTB5 cells were transplantable when passed into NK-depleted SCID mice, but not into unmanipulated SCID mice. NK cells play an important, although poorly understood, role in mediating resistance to intracellular pathogens and viruses as well as exhibiting anti-tumor activity (for review, see Whiteside and Herberman, 1995). It has been reported that NK activity is decreased during the course of MAIDS (Makino et al., 1993) and that mice treated with IL-12, which acts directly on NK cells, is protective in mice challenged with LP-BM5 (Gazzinelli et al., 1994). Our results suggest that NK cells may also play a role in mice inoculated with helper-free stocks of the MAIDS defective virus. The fact that these B cell lines are not transplantable into syngeneic C57BL/6 *beige* mice,

which have a defect in NK cell production (Roder and Duwe, 1979), nor into nude or $CD8^{-/-}$ mice implies that either both NK cells and $CD8^{+}$ T cells mediate resistance or that other host factors are involved in mediating the resistance of these cells to transplantation.

The NK-depletion protocol used to derive the SD1 MAIDS virus target cell line is similar to the one used by Veronesi et al (Veronesi et al., 1994) to derive B-cell lymphoma lines from human PBMC of EBV-infected patients. This protocol also appears to be useful in isolating target B cell lines from mice infected with the MAIDS defective virus. It may be worthwhile to further attempt to increase the efficiency of this transplantation protocol to generate additional B cell lines infected with the MAIDS defective virus since very little is currently known about how the virus reprograms these B cells and induces polyclonal T cell anergy.

**Do the established SD1 and CSTB5 cell lines represent the *in vivo* target cells of the MAIDS defective virus?** The identity of the *in vivo* target cells of the MAIDS defective virus has been initially studied with the LP-BM5 replication-competent virus mixture (Cheung et al., 1991; Bilello et al., 1992; Chattopadhyay et al., 1991; Hitoshi et al., 1993; Klinken et al., 1988; Kubo et al., 1992). As expected for a replicating virus stock, several cell populations were found to be infected, including B-cells, T-cells, and macrophages. Although the individual contribution of each of these infected populations to the disease process could not be ascertained, it was, however, clearly shown that $CD4^{+}$ T cells (Yetter et al., 1988; Mosier et al., 1987; Giese et al., 1994; Simard et al., 1997) and B cells

(Tang et al., 1995; Cerny et al., 1990; Kim et al., 1994) were essential to the development of MAIDS. The use of helper-free stocks of the MAIDS virus was instrumental in establishing that the primary target cell population of this defective virus belongs to the B-cell lineage (Huang et al., 1991). These target B cells were found to have rearranged Ig loci, and to express high levels of cytoplasmic κ mRNA and low levels of B220 (Huang et al., 1991) and to reside in peripheral lymph nodes (Simard et al., 1994). FACS analysis of cells in MAIDS enlarged lymph nodes has revealed that virtually all B cells (infected and non-infected) were $B220^{low}$ and $\kappa^{low}$ (Simard et al., 1994; Klinman and Morse, 1989; Hartley et al., 1989), strongly suggesting that the infected cells were indeed $B220^{low}$ and $\kappa^{low}$. However, it is impossible to get information on the MAIDS virus target B cells from other published FACS data because they were obtained on total cell populations present in the MAIDS lymphoid organs and did not distinguish between infected and non-infected cells (Simard et al., 1994; Klinman and Morse, 1989; Hartley et al., 1989).

Although little is known about the phenotype of the *in vivo* target B cells of the MAIDS defective virus, it appears that the phenotype of the established SD1 and CSTB5 cell lines described here is similar to what is known about the *in vivo* target B cells. Both primary and established cells have rearranged Ig loci, and express no sIgM, sIgG or κ and little or no B220. In addition, the difficulty in transplanting the two established cell lines reflects the phenotype of the early virus target cells *in vivo* which are not transplantable (Mistry and Duplan, 1973 and C. Simard, S. Klein, and P. Jolicoeur, unpublished data), but appear to

constitute a relatively benign population of proliferating cells crucial for the development of the disease. The MAIDS-derived B cell lymphomas established previously (Klinken et al., 1988) are transplantable and appear to represent a more aggressive phenotype than the MAIDS target B cells which initially proliferate upon infection.

The phenotype of the SD1 and CSTB5 cell lines described here is somewhat reminiscent of the target cells of the v-abl oncogene, which have been characterized as pre-B cells (Rosenberg, 1982). Not only do both cell lines have a surface phenotype similar to that of the v-abl target cells (Chen et al., 1994; Rosenberg, 1994), but the CSTB5 line has its κ locus rearranged in the absence of Ig heavy chain rearrangement, a molecular change which has also been observed in v-abl transformed pre-B cells (Schlissel and Baltimore, 1989). The potential role of c-abl in the development of MAIDS has recently been brought into focus by our work on the effectors of $Pr60^{gag}$. We recently demonstrated that a proline-rich domain in the p12 region of the MAIDS virus $Pr60^{gag}$ associates to the SH3 domain of c-abl (Dupraz et al., 1997). The MAIDS and the Abelson viruses may therefore infect and activate proliferation (in the case of MAIDS) or fully transform (in the case of v-abl) their respective target B cell populations, apparently via some common pathway(s).

Interestingly, CD43, which is normally found on pre-BI cells and on plasma cells (Melchers et al., 1994) is expressed on the SD1 cells. It has been observed that forced expression of this protein in a B cell lymphoma line led to enhanced survival of these cells upon serum deprivation (Misawa et al., 1996).

As well, transgenic mice expressing CD43 in peripheral B cells develop immunodeficiency (Ostberg et al., 1996). It will be of interest to study the expression of this marker during the course of MAIDS.

The presence of co-stimulatory molecules on the SD1 and CSTB5 cell lines may reflect the importance of B-T cell signaling in MAIDS which has been postulated to play a role in the development of T cell dysfunctions which are a hallmark of MAIDS (Gilmore et al., 1993; Makino et al., 1995; Kanagawa et al., 1995; Green et al., 1996). Green et al. (Green et al., 1996) have recently reported that antibody to gp39, the ligand for CD40, which is expressed on the CSTB5 cell line, can inhibit the development MAIDS in C57BL/6 mice. Both cell lines described here also express the co-stimulatory molecule B7.2, which is also important in signaling T cells via CTLA-4 and CD28 (Ward, 1996). Therefore, these B cell lines may be effective tools to further study the B-T cell interactions that occur during MAIDS and the induction of T cell anergy *in vitro*. Additionally, these cells are likely to be instrumental in understanding how the $Pr60^{gag}$ protein induces the proliferation of its target B cells.

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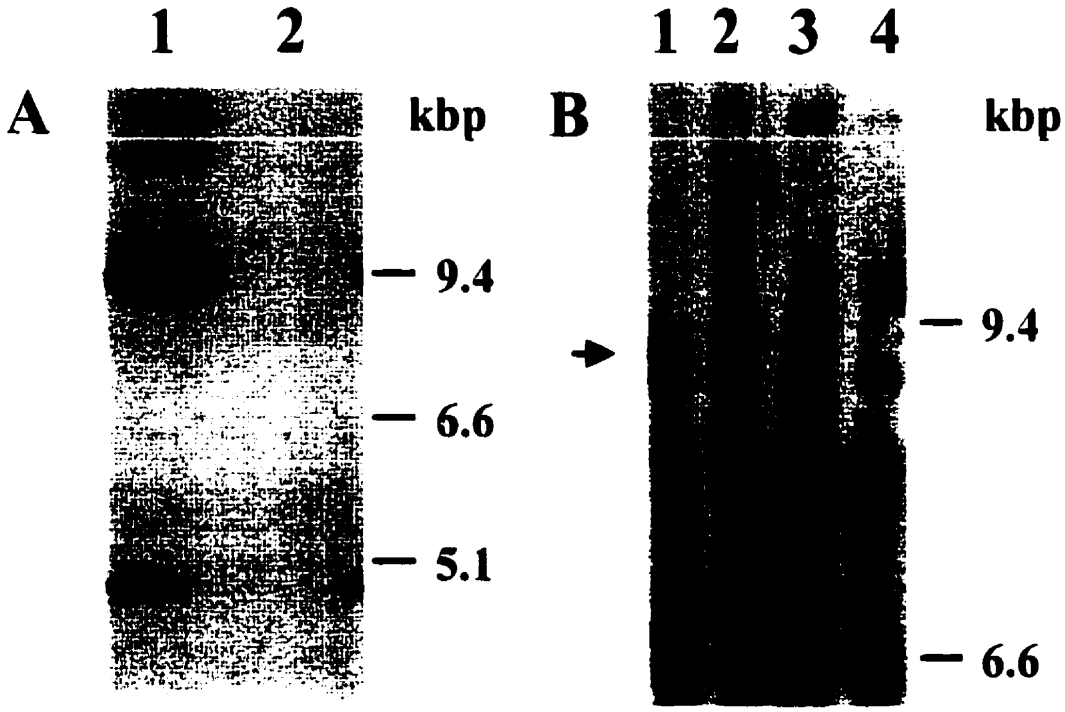


**Figure 1**

Legend to Figure 1: Southern blot analysis of MAIDS defective virus DNA in the SD1 and CSTB5 cell lines. DNA was extracted from cells or tissues, digested with either EcoRI (A) or SacI (B) and hybridized with a Moloney MuLV U3 LTR-specific probe (A) or with the p12-specific probe D30 (B). (A) DNA from SD1 (lane 1) and from CSTB5 cells (lane 2). (B) DNA from CSTB5 primary MAIDS tissue (lane 1); from CSTB5 cells (lane 2); from lymph node from another, unrelated MAIDS animal (lane 3); normal C57BL/6 spleen (lane 4). Arrow indicates the unique provirus integration observed in both the CSTB5 primary MAIDS tissue and in the established cell line.

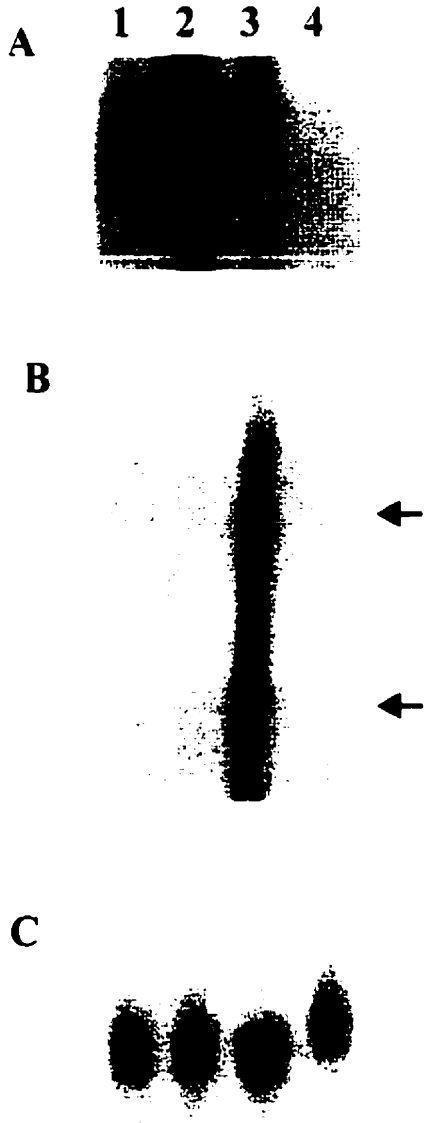


**Figure 2**

Legend to Figure 2: Northern blot analysis of MAIDS defective and ecotropic viral RNA in SD1 and CSTB5 cell lines. RNA was extracted from cells and hybridized with the p12-specific D30 probe (A) and then stripped and re-probed with the ecotropic MuLV *env*-specific probe (B). Lane 1, CSTB5 cells; lane 2, SD1 cells; lane 3, NIH/3T3 cells infected with helper-free MAIDS defective virus and ecotropic MuLV; lane 4, uninfected NIH cells. Arrows in (B) indicate the expected full-length 8.0 kb and spliced 3.5 kb ecotropic MuLV RNA species. (C) Hybridization of the same filter with an 18S rRNA oligo to show equal loading of RNA.

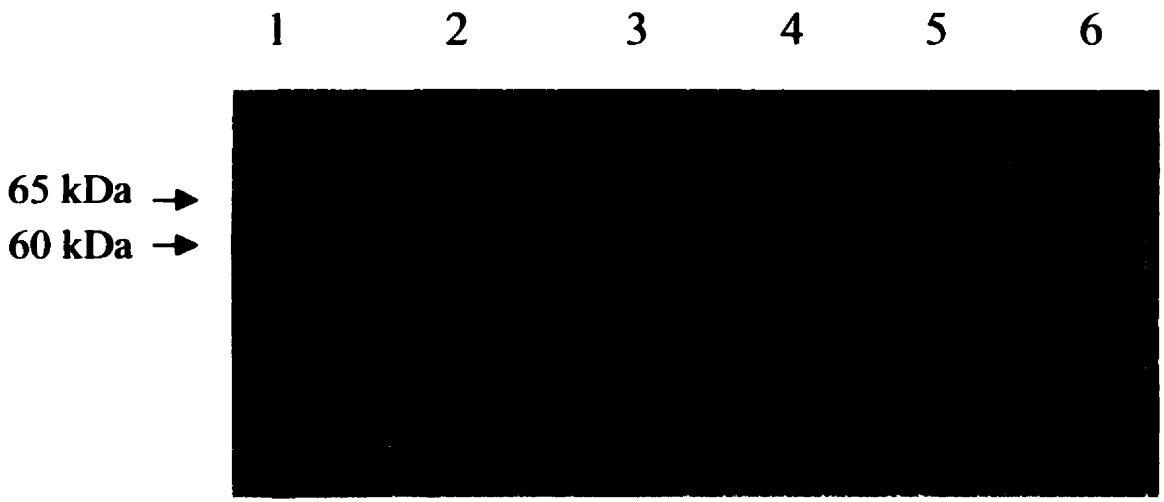


**Figure 3**

Legend to Figure 3: Western blot analysis of viral proteins produced in the SD1 and CSTB5 cell lines. Proteins were extracted from cells and separated by SDS-PAGE, and probed with a polyclonal anti-p30 antibody. Lane 1, SD1 cells; lane 2, CSTB5 cells; lane 3, 14810 cells (derived from CSTB5 cells); lane 4, Rat-1 cells; lane 5, v-abl transformed 203-33 cells; lane 6, v-abl transformed 203-33 cells infected with helper-free MAIDS defective virus.

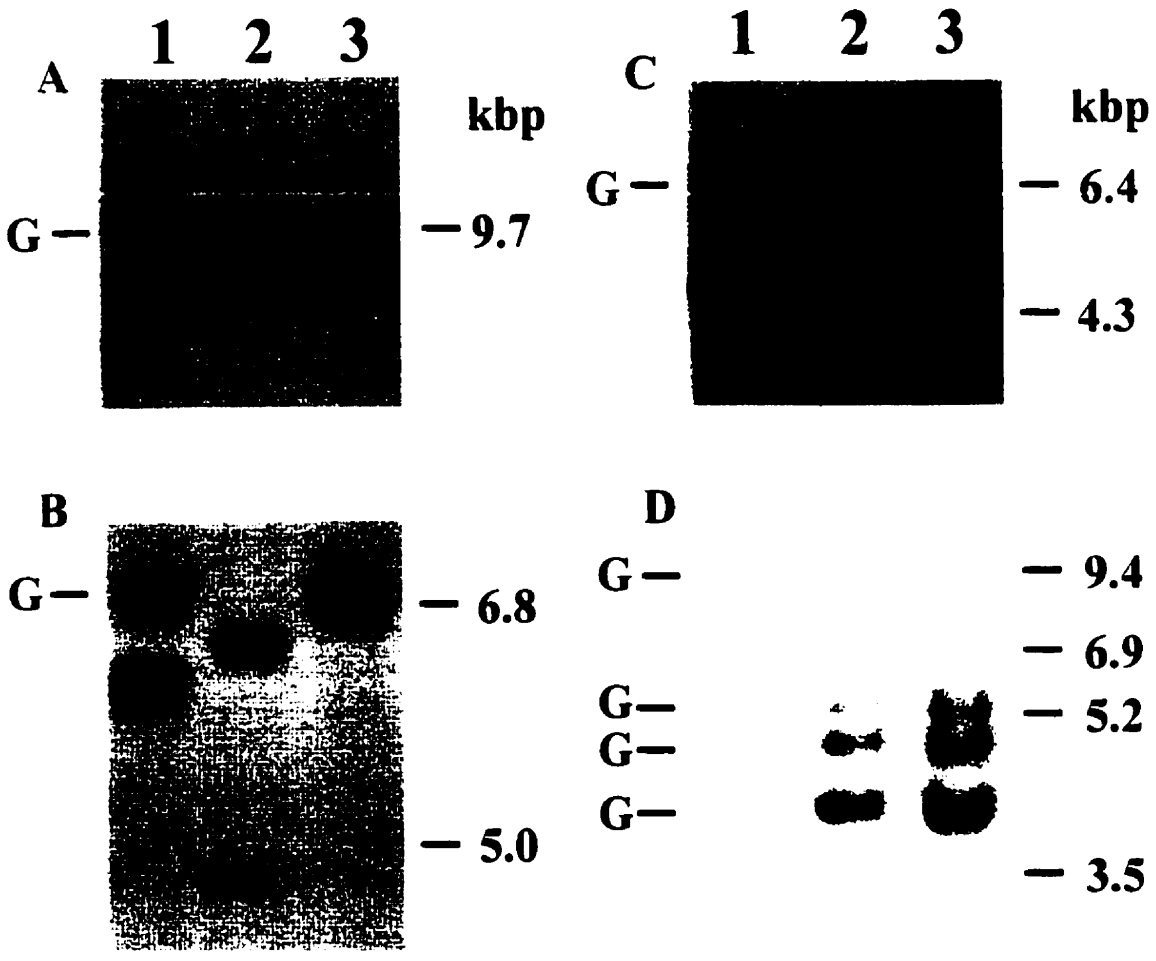


**Figure 4**

**Legend to Figure 4:** Southern blot analysis of rearranged immunoglobulin loci in the SD1 and CSTB5 cell lines. DNA was extracted from cells or tissue, digested with Hind III (A), EcoRI and BamHI (B) or EcoRI (C and D) and hybridized with either the TcRβ (A), $C_K$ (B), $J_H$ (C), or $D_H$ (D) probes. DNA from SD1 cells (lane 1); from CSTB5 cells (lane 2); from normal mouse tail DNA (lane 3). G, germline fragment.

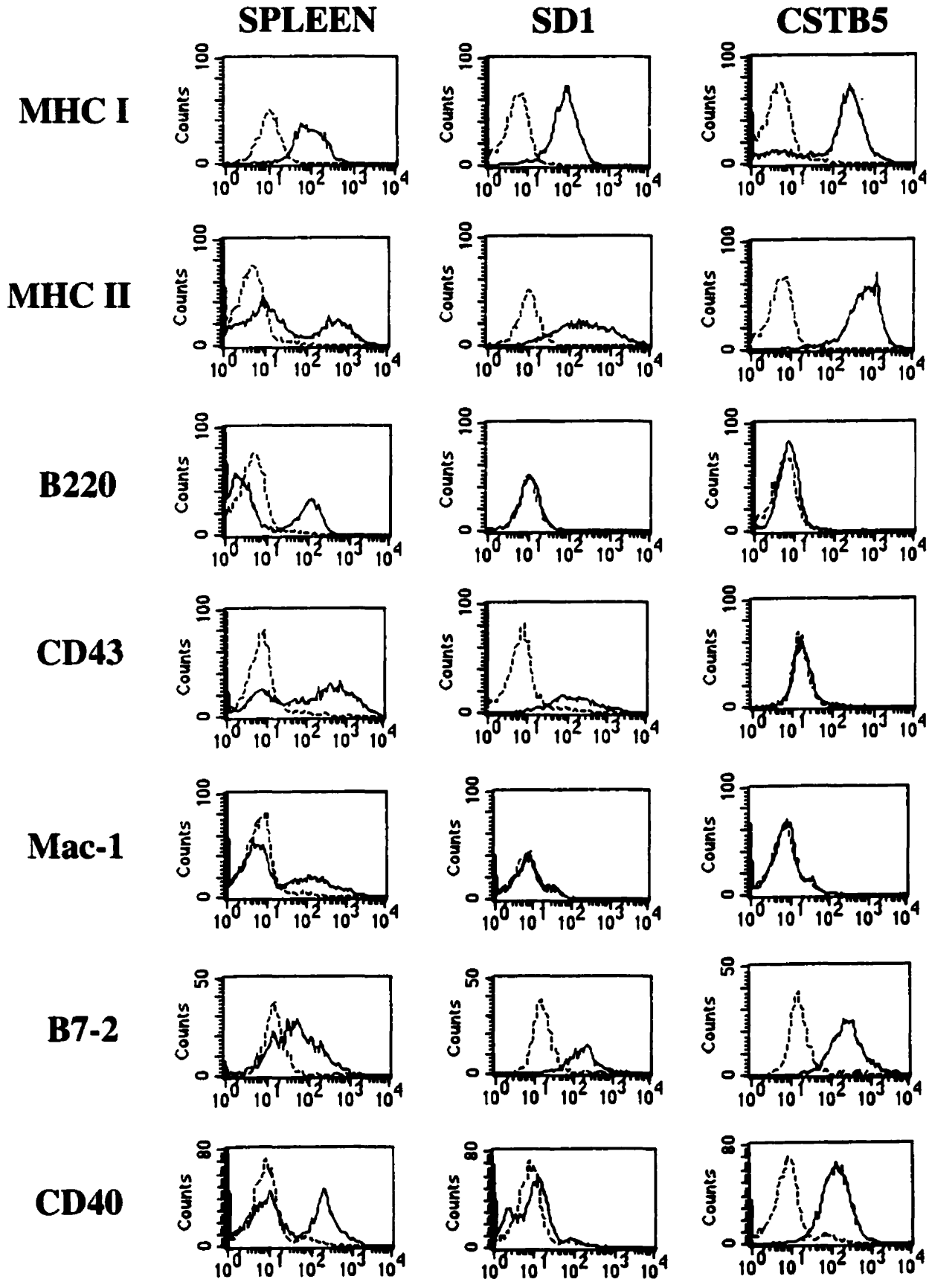


Figure 5

Legend to Figure 5: Flow cytometric analysis of the SD1 and CSTB5 cell lines. SD1, CSTB5, and C57BL/6 spleen cells were labeled with the indicated antibodies and analyzed by flow cytometry. Representative histograms are shown for several markers. The dashed line shows the level of staining of the negative control (unlabelled or secondary antibody only). The solid line indicates the level of staining observed on the cells with the indicated antibody (directly conjugated or primary and secondary together). Each histogram represents analysis of 5,000-10,000 gated cells.

**Table 1: Summary of Cell Surface Marker Expression on the SD1 and CSTB5 Cell Lines**

| Markers | SD1 | CSTB5 |
|---|---|---|
| MHC I | + | + |
| MHC II | + | + |
| B7.2 | + | + |
| CD40 | - | + |
| CD43 | + | - |
| TcR | - | - |
| CD4 | - | - |
| CD8 | - | - |
| CD3 | - | - |
| CD5 | - | - |
| Thy1.2 | - | - |
| Mac-1 | - | - |
| sIgM | - | - |
| sIgG | - | - |
| κ | - | - |
| B220 | - | -/+ |
| 33D1 | - | - |

**Legend to Table 1:** Summary of cell surface marker expression on the SD1 and CSTB5 cell lines. The two cell lines were analysed by flow cytometry using the antibodies indicated. Expression is indicated by either + (positive expression), - (negative expression), or +/- (weak expression) as compared to appropriate controls.

Table 2: Summary of the Characteristics of the SD1 and CSTB5 Cell Lines

| Cell Line | IgH Rearrangment | Cκ Rearrangment | TcRβ Rearrangment | Virus Integration | Pr60*gag* (*a*) | Pr65*gag* (*b*) |
|---|---|---|---|---|---|---|
| CSTB5 | - | + | - | + | + | - |
| SD1 | + | + | - | + | + | + |

| Cell Line | RT activity (*c*) | B cell markers | T/Macrophage Markers | Transplantablity (*d*) | Transplantation in NK$^-$ mice (e) |
|---|---|---|---|---|---|
| CSTB5 | - | + | - | - | + |
| SD1 | + | + | - | - | + |

(*a*): production of the MAIDS virus-specific 60 kDa protein
(*b*): production of a 65 kDa helper virus *gag* protein
(*c*): presence of reverse transcriptase activity
(*d*): transplantability into *nu/nu*, CD8$^{-/-}$, *beige*, or syngeneic C57BL/6 mice
(e): transplantability into NK cell-depleted SCID mice

Legend to Table 2: Summary of the characteristics of the SD1 and CSTB5 cell lines. The data presented in the manuscript are presented in tabular form in order to give a complete overview of the cell lines' qualities. Presence or absence of a tested property is indicated by either a + (positive) or a – (negative).

Chapter 4

# The Murine AIDS Virus *Gag* Precursor Protein Binds to the SH3 Domain of c-Abl

Philippe Dupraz, Najet Rebai, Steven J. Klein, Normand Beaulieu, and Paul Jolicoeur

## Forward

The MAIDS defective virus $Pr60^{gag}$ is both necessary and sufficient to induce disease, as shown by mutational analyses of this protein (2,4). Furthermore, it has been shown that a myristylation-negative mutant Du5H virus is non-pathogenic, strongly implying that a membrane association of $Pr60^{gag}$ is a requirement for the development of MAIDS (3). These results suggested that there are critical regions of $Pr60^{gag}$ which possibly interact with other cellular proteins at the cell surface. To further explore the mechanisms by which $Pr60^{gag}$ subverts its target B cell population and initiates the disease process, we searched for proteins which are capable of binding to $Pr60^{gag}$ by using the yeast two-hybrid system (1).

The majority of the work in this manuscript was performed by Philippe Dupraz, who isolated c-Abl as a binding partner of $Pr60^{gag}$ via the two-hybrid system, and demonstrated by mutational analysis as well as by Western blotting and immunoprecipitation that this interaction occurs via the SH3 domain of c-Abl and the p12 region of $Pr60^{gag}$. I provided the SD1 cell line which was originally used to confirm an *in vivo* association of c-Abl with $Pr60^{gag}$ and I participated in the Western blotting experiments. Najet Rebai produced the Du5H-overexpressing SB19 and SB21 clones. Normand Beaulieu was involved in the Western Blotting and co-immunoprecipitation studies. This manuscript was published in the Journal of Virology 71:2615-2620 (1997).

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# The Murine AIDS Virus Gag Precursor Protein Binds to the SH3 Domain of c-Abl

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**The $Pr60^{gag}$ protein of the murine AIDS (MAIDS) defective virus promotes the proliferation of the infected target B cells and is responsible for inducing a severe immunodeficiency disease. Using the yeast two-hybrid system, we identified the SH3 domain of c-Abl as interacting with the proline-rich p12 domain of $Pr60^{gag}$. The two proteins were shown to associate in vitro and in vivo in MAIDS virus-infected B cells. Overexpression of $Pr60^{gag}$ in these cells led to a detectable increase of the levels of c-Abl protein and to its translocation at the membrane. These results suggest that this viral protein serves as a docking site for signaling molecules and that c-Abl may be involved in the proliferation of infected B cells.**

Murine AIDS (MAIDS) is a severe immunodeficiency disease characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, T- and B-cell dysfunctions, and late appearance of B-cell lymphomas and opportunistic infections (19, 23). This severe immune disease is caused by a defective strain of murine leukemia virus (MuLV) (2, 4). The main target cells of this defective virus appear to be peripheral B lymphocytes which are induced to proliferate after infection (17). The defective viral genome encodes a single Gag precursor ($Pr60^{gag}$) protein (15) whose p12 region is highly divergent from those of the p12 proteins of other helper MuLVs. Mutational analyses have confirmed that the $Pr60^{gag}$ is necessary and sufficient for disease induction (14, 20, 25). In addition, myristylation-negative mutant MAIDS viruses were found to be nonpathogenic, indicating that myristylation and tight membrane association are required for $Pr60^{gag}$ to be pathogenic (16). These results suggested that intact $Pr60^{gag}$ may interact with some cellular effectors and possibly serve as a docking site at the membrane to initiate target cell expansion and pathogenesis. We searched for some proteins interacting with $Pr60^{gag}$ and found that the c-Abl protein is one of these interacting proteins.

## MATERIALS AND METHODS

**Construction of plasmids for yeast two-hybrid screening.** The construction of the clone encoding the complete $Pr60^{gag}$ fused to the LexA DNA-binding domain (DB) (pBTM/DuGAG) was made by PCR site-directed mutagenesis using oligonucleotides 5′-CCGGAATTCATGGGACAGACCGTAACCACTC-3′ (sense) and 5′-AGTACCATCTAGTGGCCACC-3′ (antisense). An *Eco*RI site was introduced at nucleotide (nt) 970 from Du5H (2). The amplified fragment was digested with *Eco*RI and *Aat*II (nt 1025) and subcloned into pBS-SK together with an *Aat*II (nt 1025)-*Hin*dIII (nt 3264) fragment from plasmid pDu5Hneo (2) to generate $Du5H^{R1-970}$, which was sequenced. Digestion with *Eco*RI and *Sal*I (of pBS-SK) from $Du5H^{R1-970}$ generated a fragment which was cloned into pBTM116 to generate pBTM/DuGAG. Plasmids pBTM/DuGAG/ΔCA, pBTM/DuGAG/Δ12, and pBTM/DuGAG/ΔMA were constructed by swapping the *Aat*II-*Hin*dIII fragment of plasmid $Du5H^{R1-970}$ with those of plasmids pDu5H-A, pDu5H-B, and pDu5H-C, encoding $Pr60^{gag}$ deletion mutants described previously (14), before subcloning into pBTM116. The G6T2 fusion was made by replacement of the *Bst*EII-*Hin*dIII fragment of G6T2 (26) in $Du5H^{R1-970}$ to generate pBTM/G6T2. The pGAL4-DB-DuGAG clone, which encodes the complete $Pr60^{gag}$ fused with the GAL4 DB, was constructed by subcloning the *Eco*RI-*Sal*I fragment of plasmid pBTM/DuGAG into plasmid pGBT9 (12). These GAL4 DB fusion constructs were tested in *Saccharomyces cerevisiae* YSF526 (*MATa his3 leu2 trp1 URA3::GAL1-lacZ*), and the LexA DB constructs were tested in strain L40.

**Yeast two-hybrid screening.** To identify proteins that interact with $Pr60^{gag}$, we used a partial 10.5-day-old mouse embryo (41) cDNA library fused to the VP16 acidic activation domain. A clone of *S. cerevisiae* L40 (*MATa his3 trp1 leu2 LYS2::lexA-HIS3 URA3::lexA-lacZ*) which contained pBTM/DuGAG was transformed with this cDNA library as described previously (35). An estimated $30 \times 10^6$ transformants were grown for 16 h in synthetic medium lacking leucine and tryptophan to maintain selection for the $Pr60^{gag}$ and library plasmids, respectively, and to allow expression of the *HIS3* reporter gene. Thereafter, the transformants were plated onto synthetic medium lacking histidine, leucine, tryptophan, uracil, and lysine. After 3 days at 30°C, $His^+$ colonies were picked, grown again for 3 days on plates lacking leucine, tryptophan, and uracil, and then assayed for β-galactosidase activity by a filter assay (3). To test for specificity, the 100 $His^+$ $LacZ^+$ colonies were tested by using a mating protocol with yeast strain AMR70 (*MATa his3 lys2 trp1 leu2 URA3::(lexAop)$_8$-lacZ*) as described previously (41) against the nonspecific bait lamin (pLexALamin). Plasmids from specific $His^+$ $LacZ^+$ colonies were extracted and retransformed into yeast strain L40 with either pLexALamin, pBTM116, pLexDA (encoding the *Drosophila* daughterless protein), or pBTM/DuGAG to further confirm their strict $Pr60^{gag}$ requirement for interaction. Three clones strongly transactivated the reporter in a $Pr60^{gag}$-dependent manner, and these plasmids were recovered.

**GST fusion constructs.** Plasmid pGST/c-Abl-SH3-SH2 was constructed by inserting the c-Abl *Not*I fragment of pVP16-Abl into plasmid pGEX-4T1. pGST/c-Abl-SH3 was constructed by subcloning the c-Abl *Bam*HI-*Hin*cII fragment from pVP16-Abl into plasmid pGEX-3X. Plasmid pGST/Abl SH2 was obtained by inserting the c-Abl *Hin*cII-*Eco*RI fragment from pVP16-Abl into *Sma*I-*Eco*RI-digested pGEX-3X. Glutathione *S*-transferase (GST)-p12 was generated by PCR amplification of a *Bgl*II subclone of the MAIDS *gag* region (nt 691 to 2228) with oligonucleotide 5′-CGGGGGATCCCCTTTTCCTTTATCGACACT T-3′ and the M13 universal primer. The PCR product was purified and digested with *Bam*HI (included in the oligonucleotide) and *Xho*I before ligation into pGEX-4T1. The GST-p12Δ*Not*I and GST-p12Δ*Sma*I deletion mutants were constructed like the wild-type GST-p12 plasmid except that the PCR product was cleaved with *Bam*HI-*Not*I and *Bam*HI-*Sma*I before subcloning in pGEX-4T1. To introduce a point mutation in the proline of the SH3-binding sites at residues 134, 158, and 181, the p12 region was amplified from GST-p12 with primers 5′-CCGGATCCCGGTCTGACCTTTACAC-3′ (sense), 5′-TATCGCCTCTAAA CCTCCTAA-3′ (P134A) (antisense), 5′-CCTCGCTAACCTTCCTCC-3′ (P158A) (antisense), and 5′-TTCCGCCCCTAATCCCATG-3′ (P181A) (antisense). These PCR fragments were then used as megnprimers. The mutant DNAs were cloned back into pGEX-4T1. GST fusions were purified as described previously (38).

**Biotinylation of proteins.** Purified GST fusion proteins were biotinylated in 100 mM sodium borate (pH 8.8) with biotinamidocaproate *N*-hydroxysuccinimide ester at a ratio of 50 μg of ester per mg of protein (22). The reaction was blocked with $NH_4Cl$, and excess biotin was removed by extensive dialysis against phosphate-buffered saline.

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TABLE 1. Specific interaction of $Pr60^{gag}$ with c-Abl in the yeast two-hybrid system[a]

| Plasmid transfected | Gene fused | Operator | β-Gal activity in yeast | |
|---|---|---|---|---|
| | | | pVP16/c-Abl SH3-SH2 | pVP16 |
| None | | LexA | − | − |
| pBTM116 | | LexA | − | − |
| pBTM/DuGAG | Du5H $Pr60^{gag}$ | LexA | +++ | − |
| pLexA/Lamin | Lamin[b] | LexA | − | − |
| pLexA/DA | Daughterless[b] | LexA | − | − |
| pBTM/DuGAG/ΔCA | Du5H $Pr60^{gag}$ (aa 1–372) | LexA | + | − |
| pBTM/DuGAG/Δp12 | Du5H $Pr60^{gag}$ (aa 1–180) | LexA | − | − |
| pBTM/DuGAG/ΔMA | Du5H $Pr60^{gag}$ (aa 1–53) | LexA | − | − |
| pBTM/G6T2 | RadLV $Pr65^{gag}$ | LexA | − | − |
| None | | $UAS_G$ | − | − |
| pGAL4-DB-DuGAG | Du5H $Pr60^{gag}$ | $UAS_G$ | +++ | − |
| pGAL4-DB-HG | HIV-1 $Pr55^{gag}$ [b] | $UAS_G$ | − | − |
| pGAL4-DB-MG | Mo-MuLV $Pr65^{gag}$ [b] | $UAS_G$ | − | − |

[a] Plasmids encoding a LexA DB or a GAL4 DB hunt fusion (21) and plasmids encoding the VP16 transactivating domain fused or not with the c-Abl SH3 and SH2 domains were cotransfected into yeast strain L40 (for testing the LexA DB fusions) or YSF-526 (for testing the GAL4 DB fusions). (In all cases, the *GAL1* promoter was used.) The transformants were tested for β-galactosidase activity (β-Gal) by using a standard filter assay. Entries indicate strong (+++), weak (+), or no (−) blue color after 7 h of reaction in X-Gal plates. RadLV, radiation leukemia virus; HIV-1, human immunodeficiency virus type 1; Mo-MuLV, Moloney MuLV; $UAG_G$, *GAL* upstream activation sequence.

[b] Previously described (21, 41).

**Far-Western blotting.** Equal amounts of proteins were separated on a 5 to 12% acrylamide gradient gel and transferred to nitrocellulose in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 11)–20% methanol (22). Filters were blocked in 10 mM Tris (pH 8)–0.9% NaCl (Tris-buffered saline [TBS])–0.05% Tween 20 (TBST buffer) plus 5% skim milk. Biotinylated probes were added in the same buffer at 0.5 μg/ml, incubated at room temperature for 2 h, and washed extensively in TBST plus 1% skim milk. Filters were incubated with avidin-conjugated horseradish peroxidase at 0.5 μg/ml in TBST plus 1% skim milk, washed extensively in TBST-milk, TBST, and TBS without Tween 20 before development by enhanced chemiluminescence (ECL) (Dupont Renaissance kit).

**Immunoprecipitation, Western blotting, and cell fractionation.** CST-B5 cells were transfected by electroporation with a plasmid containing the *Pst*I-*Hin*dIII $Pr60^{gag}$ fragment from the pDu5Hneo clone ligated into the pcDNA3 expression vector. Clones were screened for high protein expression by Western blotting. For immunoprecipitation, $2 \times 10^7$ cells were lysed in ice-cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, pepstatin, *Nα-p*-tosyl-L-lysine chloromethyl ketone [TLCK]). The clarified cell lysates were incubated with the appropriate antibody-coated protein G-agarose beads for 1 h at 4°C. The pellets were washed three times with 1 ml of lysis buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cell fractionation was performed as described previously (11). Western immunoblotting was performed as described previously (33) on polyvinylidene difluoride membranes and developed by using a Dupont Renaissance ECL kit.

**Antibodies.** The hybridoma producing the anti-Abl antibody 24-21 has been described elsewhere (36) and was kindly provided by Naomi Rosenberg. The hybridoma R187 against the viral Gag protein (anti-CA [capsid]) (5) was purchased from the American Type Culture Collection. The goat anti-MuLV polyclonal Gag antibodies were obtained from Program and Logistics, Viral Oncology, National Cancer Institute, Bethesda, Md., and have previously been characterized (15). Anti-CDC2 kinase and anti-Src were purchased from Santa Cruz Biotechnology, Inc., and Oncogene Science, respectively.

## RESULTS

**Interaction of $Pr60^{gag}$ with the c-*abl* SH3 domain.** We used the yeast two-hybrid system (12) to identify host proteins that bind to the MAIDS virus $Pr60^{gag}$ protein. With this assay, we identified one positive clone (DuGip111-3) (497 nt) which contained almost exclusively the SH3 and SH2 domains (amino acids [aa] 63 to 216) of the mouse proto-oncogene c-*abl*. This VP16-Abl clone was able to interact with a GAL4 or LexA DB/$Pr60^{gag}$ fusion construct, suggesting that the binding was not dependent on the specific promoter-DB fusion used to monitor the $Pr60^{gag}$-interacting potential (Table 1).

To demonstrate the specificity of the $Pr60^{gag}$–c-Abl interaction, the VP16-Abl construct was tested against different baits (Table 1). The VP16-Abl protein alone or in association with the control LexA DB protein (pBTM116) did not transactivate the β-galactosidase reporter gene. The VP16-Abl construct was also unable to interact with unrelated LexA DB or unrelated GAL4 DB fusion proteins and with closely related helper Moloney $Pr65^{gag}$ (pGAL4-DB-MG) (21) or ecotropic endogenous nonpathogenic radiation leukemia virus $Pr65^{gag}$ (pBTM/G6T2) DB fusion proteins. Thus, the SH3-SH2 Abl-$Pr60^{gag}$ interaction appeared to be specific.

To map the $Pr60^{gag}$ domain responsible for binding to c-Abl, we tested deletion constructs of $Pr60^{gag}$ in the two-hybrid system and compared their activities to that of the wild-type $Pr60^{gag}$ proteins (Table 1). Optimal binding was observed only with the full-length $Pr60^{gag}$ construct. A mutant that contains a stop codon in the CA region of $Pr60^{gag}$ (DuGAG/ΔCA) still retained some binding capability. Mutants deleted further into Gag (DuGAG/Δp12 and DuGAG/ΔMA) lost the ability to bind to c-Abl. Therefore, this analysis indicated that the C-terminal end of the matrix (MA) protein and the p12 region of $Pr60^{gag}$ were necessary to bind to c-Abl. This region has previously been shown to be required for pathogenesis (14, 20, 25).

To determine which of the c-Abl SH3 and SH2 domains of the VP16-Abl clone interacted with $Pr60^{gag}$, we carried out binding studies in vitro between recombinant proteins, using a far-Western assay (22). The complete VP16-Abl cDNA or truncated versions were expressed as GST fusion proteins, biotinylated, and used as probes on filters containing C-terminal MA and p12 domains of $Pr60^{gag}$ fused to GST. The GST–c-Abl SH3-SH2 probe was found to bind to the full-length GST-p12 protein in this assay (Fig. 1). The GST–c-Abl SH3 probe also bound, albeit with an apparent lower affinity than the SH3-SH2 fusion, to the GST-p12 protein. However, binding of the GST–c-abl SH2 and of the control GST probes was not detected. None of the probes bound to GST. We also tested whether other SH3 domains could bind to the same GST-p12 protein. The biotinylated GST–Nck 3x-SH3 (aa 1 to 761) probe was found to bind to the GST-p12 protein with an

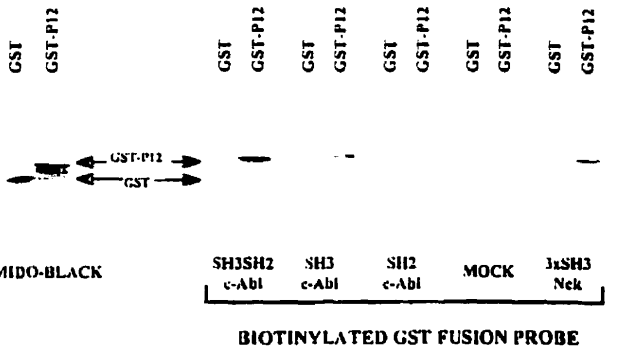


FIG. 1. The SH3 domain of c-Abl binds to $Pr60^{gag}$ in vitro. Purified GST or GST-p12 proteins were blotted onto nitrocellulose membranes, which were either stained with amido black or probed with various biotinylated GST–c-Abl or Nck proteins. Bound proteins were detected by ECL.

affinity slightly lower than that of c-Abl (Fig. 1). In contrast, the n-Src, c-Src, c-Fyn, c-Lck, Grb2, Gap, spectrin, Crk, and Fgr SH3 domains fused to GST and used as probes were unable to bind to the same GST-p12 protein (data not shown). These results indicated that the interaction of GST–c-Abl SH3-SH2 with GST-p12 was specific and was mediated by the SH3 domain.

The pattern of the c-Abl SH3 binding to the degradation product of the GST-p12 fusion protein as well as the inability of the p12 truncated LexA fusion (pBTM/DuGAG/Δp12) to interact in the two-hybrid system suggested that a critical SH3-binding site was located in the C-terminal region of p12. In this region there are three putative SH3-binding sites (termed MAIDS-binding site for SH3 [MB3-1] [LIPSKPPKSR], MB3-2 [ENLPNLPPLSK], and MB3-3 [RPPPPRYSPPNPMESR]) that contained the minimal consensus PxxP motif required for SH3 binding (Fig. 2A) (6, 27). To determine which motif was required for binding the c-Abl and Nck SH3 domains, we constructed two shorter GST fusion proteins (GST-p12Δ*Not*I and GST-p12Δ*Sma*I) and three single-amino-acid mutants disrupting each PxxP motif (Fig. 2A). Using the in vitro assay used for Fig. 1, we found that deletion of MB3-3 or MB3-3 plus MB3-2 of p12 abolished binding activity and that only the longer construct, which contains MB3-3, could bind to the c-Abl SH3 or Nck 3x-SH3 (Fig. 2B). Single-amino-acid substitutions at the MB3-1 (P133A) or MB3-2 (P158A) site did not inhibit c-Abl SH3 or Nck 3x-SH3 binding (Fig. 2B). However, disruption of proline 181 of the MB3-3 site (P181A) significantly reduced binding of c-Abl SH3 but not of Nck 3x-SH3 (Fig. 2B). It has recently been reported that the c-Abl SH3 domain preferentially recognizes long (10-aa) proline-rich peptides that have conserved three prolines: those at positions 7 and 10 in the PxxP sequence and another at position 2 (1). The MB3-3 site matches this consensus sequence (Fig. 2C).

**Detection of $Pr60^{gag}$–c-Abl complexes in mammalian cells.** We next studied whether c-Abl associates with $Pr60^{gag}$ in vivo, by coimmunoprecipitation. We used a nonproducer B-cell line (CST-B5) established from a MAIDS virus-infected mouse (10), in which $Pr60^{gag}$ could be overexpressed. We isolated two clones (SB19 and SB21) which overexpressed $Pr60^{gag}$ at levels 10- to 15-fold higher than those produced by the parental CST-B5 cells (Fig. 3A, upper left panel). Cell lysates were immunoprecipitated with either an anti-c-Abl or an anti-Gag antibody and immunoprecipitates were then subjected to Western blot analysis. In a control experiment, the 140-kDa

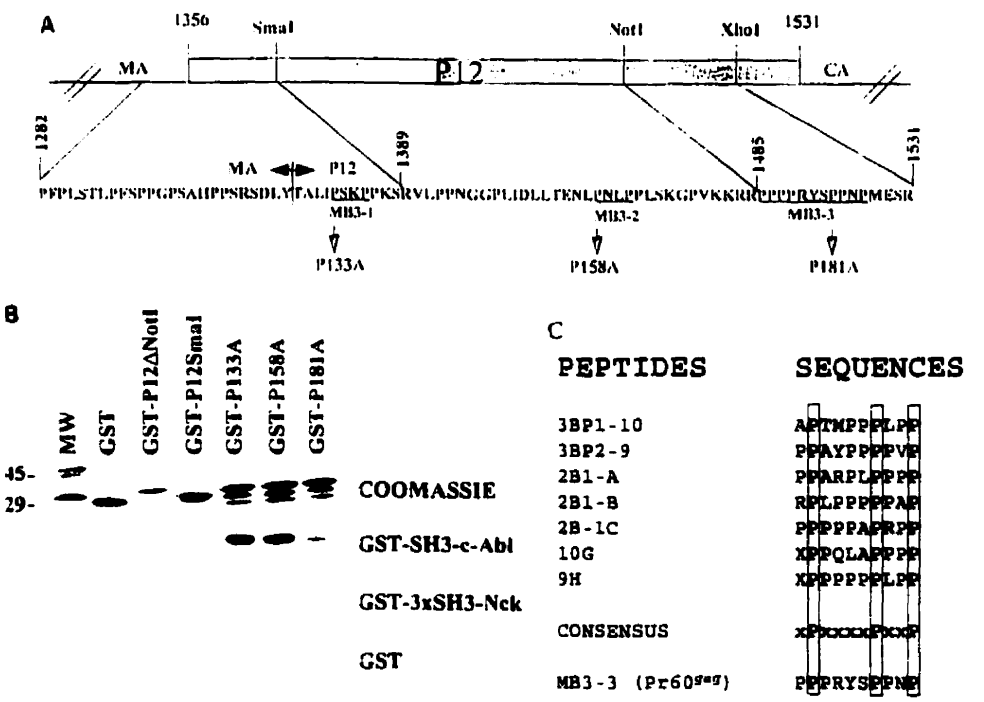


FIG. 2. Mutational analysis of the SH3-binding sites of $Pr60^{gag}$. (A) Schematic representation of the p12 domain of $Pr60^{gag}$. The amino acid residues are represented by single letters, and numbers indicate nucleotide positions in the MAIDS Du5H proviral DNA (2). Three putative SH3-binding sites are underlined. Single-amino-acid substitution introduced in GST-p12 are indicated. Restriction sites used to construct the GST fusion proteins are indicated. (B) Far-Western blot with GST-$Pr60^{gag}$ mutant proteins. Purified GST or GST-p12 mutant proteins were probed with the biotinylated GST-SH3 proteins of c-Abl or Nck as for Fig. 1. MW, molecular weight markers, positions of which are indicated in kilodaltons. (C) Alignment of the known c-Abl SH3-binding sites. Boxes, amino acids essential for binding (1). Nonconserved amino acids (x) are shown in the consensus sequence.

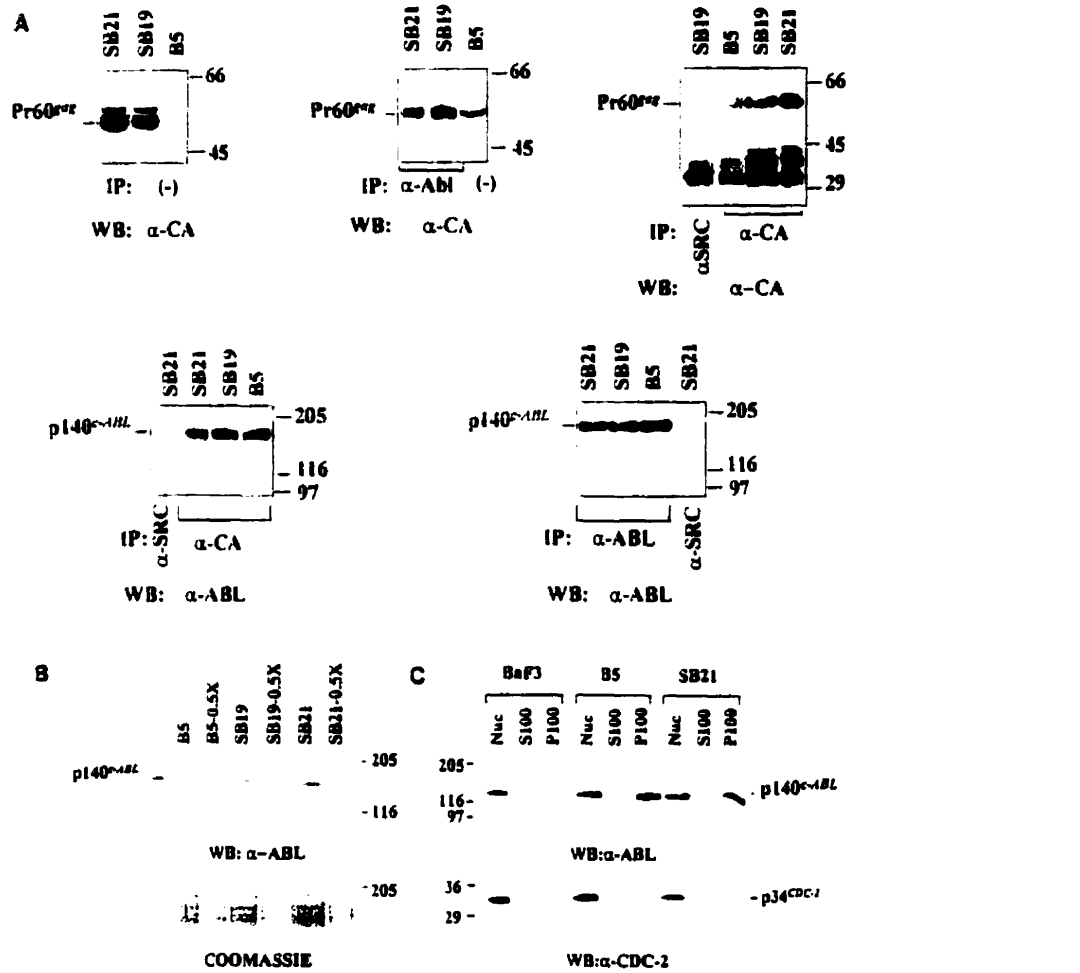


FIG. 3. Detection of c-Abl–Pr60$^{v\text{-}src}$ complexes in vivo. (A) Extracts of parental CST-B5 and Du5H-transfected CST-B5 (SB19 and SB21) cells, expressing low and high levels of Pr60$^{v\text{-}src}$, respectively, were subjected to immunoprecipitation (IP) followed by Western blotting (WB) with the indicated antibodies. IP (−) indicates that a total lysate (50 μg) was analyzed directly by WB without IP. The anti-CA (α-CA) antibody used for IP was MAb R187, while a goat anti-CA polyclonal antiserum was used in Western blotting. Numbers at the right represent molecular sizes in kilodaltons. In the upper right panel, limiting amounts of cell lysates were used during immunoprecipitation for semiquantitative analysis of Pr60$^{v\text{-}src}$. Immunoprecipitation with an unrelated MAb of the same isotype as anti-CA (anti-Src [αSRC]) was used as a control. (B) Levels of c-Abl in cells overexpressing Pr60$^{v\text{-}src}$. The c-Abl protein was detected by Western blotting of total cell lysates. A Coomassie blue-stained membrane is shown to document equal loading and transfer. (C) Localization of c-Abl in cells expressing Pr60$^{v\text{-}src}$. Equal amounts of the indicated cells were subjected to cell fractionation as described previously (11). Equal amounts of all fractions were analyzed by Western blotting with anti-Abl (α-Abl) or with control anti-CDC2 kinase (α-CDC-2) antibodies.

c-Abl was detected in total lysates or in immunoprecipitates with the anti-c-Abl antibody followed by Western blotting with the same antibody (Fig. 3A, lower right panel), as expected. Interestingly, in the anti-c-Abl immunoprecipitates analyzed by Western blotting with the anti-CA polyclonal antibody, a protein of 60 kDa comigrating with Pr60$^{v\text{-}src}$ was detected (Fig. 3A, upper middle panel). Conversely, in anti-CA immunoprecipitates analyzed by Western blotting with the anti-c-Abl antibody, the 140-kDa c-Abl protein could be detected (Fig. 3A, lower left panel). This band was not detected in immunoprecipitates obtained with a control monoclonal antibody (MAb) of the same isotype directed against a related cytoplasmic tyrosine kinase (anti-Src). These results indicated that c-Abl and Pr60$^{v\text{-}src}$ interact in vivo. This association of c-Abl with Pr60$^{v\text{-}src}$ was also confirmed in assays using another murine B-cell line (SD1) obtained from a MAIDS virus-infected mouse and expressing Pr60$^{v\text{-}src}$ at high levels (data not shown). The amount of c-Abl proteins coimmunoprecipitated with Pr60$^{v\text{-}src}$ in cells overexpressing Pr60$^{v\text{-}src}$ was not higher than in the parental B5 cells, suggesting that the levels of c-Abl, and not those of Pr60$^{v\text{-}src}$, were limiting in this system.

Interestingly, overexpression of Pr60$^{v\text{-}src}$ in CST-B5 cells also resulted in a modest but clear increase (two- to threefold) of the steady-state levels of c-Abl, as detected in total cell lysates from two clones (Fig. 3B).

To determine whether the formation of the Pr60$^{v\text{-}src}$–c-Abl complexes resulted in a subcellular redistribution of c-Abl, we performed a cell fractionation experiment. In control pre-B cells (BaF3) not expressing Pr60$^{v\text{-}src}$, the majority of c-Abl was nuclear (Fig. 3C), as reported previously (8, 40, 43). However, in cells expressing Pr60$^{v\text{-}src}$, the levels of c-Abl present in the membrane fraction were as high as in the nuclear fraction, suggesting that Pr60$^{v\text{-}src}$ helps in translocating c-Abl to the membrane in these cells.

Since c-Nck was found to bind to Pr60$^{v\text{-}src}$ in vitro (Fig. 1 and 2B), we used the same mammalian cells and the same technique as used with c-Abl to determine whether Pr60$^{v\text{-}src}$ interacts with c-Nck in vivo. We have been unable to detect such an

association of $Pr60^{gag}$ with c-Nck in vivo (data not shown), which suggests that these two proteins do not interact in vivo or that their interaction is too weak to withstand the coimmunoprecipitation procedure. Alternatively, the affinity of the antibodies used may not be strong enough to allow detection of this interaction.

## DISCUSSION

This work shows that the MAIDS virus $Pr60^{gag}$ acts as a docking molecule and binds to the c-Abl protein through its SH3 domain. This interaction appears to lead to the translocation of a fraction of c-Abl out of the nucleus, to the membrane, and to an increase of its steady-state levels. The vast majority of c-Abl in murine fibroblasts and murine hematopoietic cells is normally localized in the nucleus (34, 42). Overexpression of c-Abl in murine fibroblasts has been shown to block cell cycle progression (34). In contrast, the oncogenic forms of Abl, such as the chimeric bcr-Abl, v-Abl (Gag-Abl), or SH3-deleted mutant proteins, have been found to be mainly located outside the nucleus (13, 40, 42). Our results represent the first indication that a significant fraction of endogeneous nonchimeric murine c-Abl can be translocated outside the nucleus. It is tempting to suggest that this event is involved in the target B-cell proliferation observed in this disease. The v-Abl protein, which is similarly located at the plasma membrane and whose tyrosine kinase activity is activated, readily induces transformation and proliferation of its target pre-B cells (29, 30). Moreover, recent studies have shown that treatment of murine pre-B cells with type IV c-Abl- or type I c-Abl-specific antisense oligonucleotides induces apoptosis or differentiation, respectively (9). Similar effects have been reported for human $CD34^+$ hematopoietic cells (31). These results suggest that the MAIDS $Pr60^{gag}$ protein, through its binding to the c-Abl protein, may prevent apoptosis of its target B cells.

The mechanism by which the formation of $Pr60^{gag}$–c-Abl complexes outside the nucleus affects c-Abl function is not clear. Gross alterations of c-Abl kinase activity seem unlikely, as we have failed to detect any modulation of its tyrosine kinase activity upon binding of $Pr60^{gag}$ to its SH3 domain. However, undetectable but still functionally significant activation of c-Abl kinase activity in these complexes remains a possibility. Alternatively, recruitment or sequestration of c-Abl at the membrane by $Pr60^{gag}$ may allow access to novel substrates for its tyrosine kinase. Finally, binding of $Pr60^{gag}$ to the c-Abl SH3 domain may block access of this domain to a putative inhibitor of c-Abl thought to bind to the same SH3 domain (24). Mutations of the c-Abl SH3 sequences have indeed been found to activate c-Abl transforming activity, suggesting the presence of such an inhibitor (13, 18, 40). Recently, proteins interacting with both the SH3 domain of c-Abl and its C-terminal region have been identified, one of which has some of the properties of an inhibitor (7, 37).

The complex immune disease induced by $Pr60^{gag}$, the only gene product encoded by the MAIDS defective virus, is unique and involves lymphoproliferation as well as the loss of both T- and B-cell functions. Our data suggest that c-Abl is involved in this disease. However, more functional assays will be required to appreciate the contribution of c-Abl in the pathogenesis of MAIDS. Additionally, it is unlikely that this whole syndrome develops from the binding of only c-Abl to $Pr60^{gag}$. Rather, $Pr60^{gag}$ may serve as a docking site or scaffold for several signal transduction proteins involved in this disease. The Nck protein, which we have shown to bind to $Pr60^{gag}$ in a filter binding assay (Fig. 1 and 2A), may in fact represent such an additional signaling protein involved in MAIDS. Although we have been unable to detect this interaction in vivo, by coimmunoprecipitation studies, this does not definitely rule out its implication in this disease. It would be of interest to identify other cellular proteins binding to $Pr60^{gag}$ and to study their involvement in MAIDS and in other immunodeficiency diseases, including AIDS, since the human immunodeficiency virus type 1 Nef has been found to interact with the SH3 domain of Hck (32).

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Chapter 5

# Reduced Tumoriginicity of v-Abl-transformed pre-B Cells and B16F1 Melanoma Cells Expressing the MAIDS Defective Virus $Pr60^{gag}$ Protein

Steven J. Klein, Philippe Dupraz, and Paul Jolicoeur

## Forward

The data presented in the previous chapter revealed the *in vivo* association of $Pr60^{gag}$ with c-Abl, yet it was not clear how the association between these two proteins contributed to the development of MAIDS. One series of experiments initially designed to further explore this interaction was based on using pre-B cell lines expressing v-abl and which were infected with helper-free MAIDS defective virus in order to generate pre-B cell lines which expressed high levels of both v-abl and Du5H. Surprisingly, during the course of these studies we observed that the v-abl-transformed pre-B cell lines which were expressing Du5H were no longer transplantable into syngeneic C57BL/6 mice. We therefore decided to study this interesting finding in more detail and to examine the influence of expressing $Pr60^{gag}$ in another transformed cell line, the B16F1 melanoma.

Both Philippe Dupraz and I studied the effect of $Pr60^{gag}$ in the v-abl-transformed pre-B cell lines. Additionally, I bred the initial stock of $CD8^{-/-}$ mice onto the C57BL/6 background. Philippe Dupraz constructed the $Pr60^{gag}$-expressing vector and transfected the B16F1 melanoma cells with it. I isolated several of these B16F1 clones and analysed them by Northern and Western blot for expression of $Pr60^{gag}$. As well, I studied the latency of tumor development in mice inoculated with the $Pr60^{gag}$-expressing B16F1 clones.

# Reduced tumorigenicity of v-abl-transformed pre-B cells and B16F1 melanoma cells expressing the MAIDS defective virus $Pr60^{gag}$ protein

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## Abstract

The murine AIDS (MAIDS) defective virus induces in susceptible mice a profound immunodeficiency characterized by severe B and T cell dysfunctions when inoculated as a helper-free viral stock. The initiating event in the disease progression is the infection of a population of target B cells, followed by their polyclonal expansion and the later development of immune system failures. Consistent with this scenario, both B cells and $CD4^+$ T cells are required for the development of MAIDS. It is believed that in some cases, $CD8^+$ T cells are capable of mediating resistance to the disease by mounting a CTL response to the only gene product of the MAIDS defective virus, $Pr60^{gag}$. We have recently identified an *in vivo* interaction between $Pr60^{gag}$ and the c-abl non-receptor tyrosine kinase in B cells derived from mice infected with helper-free stocks of the MAIDS defective virus. While studying this $Pr60^{gag}$-c-abl interaction in further detail, it became apparent that expression of $Pr60^{gag}$ in several v-abl transformed pre-B cell lines led to an almost complete loss of tumor-inducing potential when these cells were inoculated into syngeneic C57BL/6 mice. Detailed experiments using MAIDS defective virus-infected v-abl-transformed 203-33 pre-B cells revealed a $Pr60^{gag}$-specific induction of a $CD8^+$ T cell-mediated rejection of these cells. Such an effect was not seen when the 203-33 cells were infected with Moloney MuLV or the neomycin-expressing N2 virus. A less pronounced, though statistically significant, reduction in tumor-inducing potential was also observed when $Pr60^{gag}$ was expressed in the B16F1 melanoma cell line.

These results indicate that $Pr60^{gag}$, or a protein induced by $Pr60^{gag}$ is indeed capable of inducing an immune response in normally susceptible C57BL/6 mice. Understanding the mechanism by which $Pr60^{gag}$ can induce diverse responses when expressed in different cell types can yield important clues into viral pathogenesis. Furthermore, modified forms of $Pr60^{gag}$ may be useful as a new tool for the gene therapy of cancer.

## Introduction

The murine acquired immunodeficiency syndrome (MAIDS) is characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, B- and T-cell dysfunctions, and the late appearance of B cell lymphomas (for reviews see (33,34,46)). The etiologic agent of this disease was found to be a defective murine leukemia virus (MuLV) (2,9) which appears to encode a single gene product, the 60 kDa *gag* fusion protein, $Pr60^{gag}$ (9,29), whose p12 region differs significantly from that of other MuLVs. The primary event in the development of the disease is the infection of a relatively mature B cell population by the MAIDS defective virus (32,36,58). Infection of these cells by the MAIDS defective virus leads to their polyclonal proliferation, which later in the disease becomes clonal or oligoclonal (30,31).

Although mature B cells are required for the development of MAIDS, T cells, specifically $CD4^+$ T cells, are required for the full induction of MAIDS, as supported by studies in nude, CD4 K.O., and C57BL/6 mice which had been depleted of $CD4^+$ T cells by *in vivo* administration of anti-CD4 antibody (49,58,64). We have shown previously that the $CD4^+$ T cells are not required for the infection of the target B cells, but are required at a post-infection stage to allow the full expansion of the infected B cells (58).

There exists a strong genetic component in MAIDS which confers resistance to the disease on some strains of mice, while other strains of mice are moderately resistant or completely susceptible to MAIDS (23,24,31,43). The prototype susceptible mouse strain is the C57BL/6 strain while BALB/c mice are

moderately resistant and SIM.R mice are completely resistant to the disease induced by the MAIDS defective virus (31).

It has been suggested that resistance or susceptibility to MAIDS is controlled by the H-2 haplotype, with $H\text{-}2^a$ and $H\text{-}2^d$ alleles conferring resistance to MAIDS while strains bearing the b, f, k, q, r, and s haplotyptes were found to be moderately to highly susceptible to MAIDS (23,43). A detailed examination of inbred strains of mice, however, revealed that their individual H-2 haplotypes were neither necessary nor sufficient for disease induction (31), suggesting that additional genes likely contribute to the development of MAIDS, or to its resistance. The same study concluded that susceptibility to MAIDS correlates with the target B cell expansion, while no target B cell expansion was seen in resistant mouse strains (31).

$CD8^-$ T cells also appear to play a role in controlling MAIDS in resistant mouse strains. In support of this theory, A strain mice depleted of $CD8^+$ T cells by chronic administration of anti-CD8 antibody are no longer resistant to MAIDS (42). Resistant mice which contained germline mutations in either the $\beta_2$-microglobulin or perforin genes developed a less severe form of MAIDS as compared to wild-type controls, demonstrating that the perforin-dependant functions of $CD8^+$ T cells play a role in resistance to MAIDS, but that other non-$CD8^-$ T cell-dependent mechanisms are equally important in controlling the disease (60).

Other groups have examined the CTL response against $Pr60^{gag}$ by both *in vivo* and *in vitro* techniques (16,57). It was reported that CTL can be raised

against tumors and infected cells from mice inoculated with the MAIDS virus mixture (16), which contains a complex mixture of MuLV in addition to the disease-inducing defective MuLV (39,47,48). However, these groups were unable to identify which viral epitope, either helper or defective, was responsible for generating the CTL response. Another study found that a CTL response to $Pr60^{gag}$ could be generated by mice susceptible to MAIDS (57), implying that in some cases resistance to MAIDS is not $CD8^+$ T cell-mediated.

We have recently reported an *in vivo* association of the c-abl non-receptor tyrosine kinase with $Pr60^{gag}$ (15) which may contribute to the B cell proliferation that is critical for the development of MAIDS. To further study this molecular interaction and its resulting signaling events, we attempted to express $Pr60^{gag}$ in B cell lineage cells which are transformed by the v-abl oncogene. Unexpectedly, we found that these MAIDS defective virus-infected v-abl-transformed cells were no longer transplantable into syngeneic C57BL/6 mice. We therefore studied this striking effect more closely and further studied this effect in the B16F1 melanoma cell model.

## Materials and Methods

Cell lines and viruses: The v-abl-transformed pre-B cell lines 203-33, 203-40 and 203-44 were kindly provided by Dr. Naomi Rosenberg (Tufts University, Boston, MA). These cells were maintained in RPMI medium supplemented with 10% Fetal Clone II serum (Hyclone) and 50 μM 2-mercaptoethanol. For selection of neomycin-resistant cells, 1 mg/ml G418 was added to the culture medium. The B16-F1 melanoma cell line (17) (catalogue # CRL-6323) was obtained from the ATCC (Rockville, MD). This line was maintained in DME supplemented with 10% Fetal Clone II serum (Hyclone) and 0.1 mM non-essential amino-acids. For selection of neomycin-resistant clones, 400 μg/ml of G418 was added to the culture medium. The modified helper-free MAIDS defective virus (Du5H/neo) has been previously described (32), as has the neo-expressing virus N2 (1).

DNA constructs, infections and transfections: The PvuI-XbaI fragment of the MAIDS defective virus, which encompasses the entire viral coding region ($Pr60^{gag}$), was cloned into the polylinker region of the pcDNA3 expression plasmid between its BamHI and XbaI sites. This construct (pcDNA3/Du5H), or the empty vector, was introduced into the B16-F1 cells by the calcium chloride technique, as described previously (30). 48 hr after the transfection, G418 was added to the medium and 4 independent clones (B16F1/M3, B16F1/M4, B16F1/M6, and B16F1/M17) expressing $Pr60^{gag}$, and two clones expressing the empty vector (B16F1/V1 and B16F1/V2) were selected and expanded for further study.

The 203-33 cell line was infected with the helper-free Du5H/neo (203-33/M cells) or N2 viruses (203-33/N2 cells), or with Moloney MuLV virus (203-33/Mol cells), by standard methods. Briefly, $10^6$ cells/ml were grown overnight in the presence of 4 μg/ml of polybrene. The following day, the cells were harvested and resuspended in 1ml of virus containing 4 μg/ml of polybrene. After being incubated at 37°C for 1 hr., the cells were diluted in 10 ml of medium. 48 hr. after the infection, the selective agent was added to the culture. Pools of cells resistant to G418 were used for further study. Du5H-infected 203-33 and 203-44 cells were generated as described above for the 203-33 cells. For cells infected with Moloney MuLV, infection was confirmed by assay for RT production, as described previously (19,30).

Mice: Young (30-40 days) C57BL/6 mice and C57BL/6 nu/nu mice were obtained from Charles River Inc. (St-Constant, Quebec). $CD8^{-/-}$ mice were initially provided by Dr. Tak Mak (Amgen Institute, Toronto, Ontario, Canada), and were bred for seven generation onto the C57BL/6 background. $CD8^{-/-}$, $CD8^{+/-}$ and $CD8^{+/+}$ mice were identified either by Southern hybridization of tail DNA and flow cytometry using antibodies to the CD8 molecule (Cedarlane, Ontario, Canada) or by PCR of tail DNA using the primer pair 5' GGACGCCGAACTTGGTCAG 3' and 5' CTCATGGCAGAAAACAGTTTCG 3' which spans the site of insertion of the neomycin cassette.

Transplantation of cells: Exponentially-growing cells were harvested by simple centrifugation for the non-adherent v-abl-transformed cells or by trypsinization for the B16-F1 cells, washed with PBS, and counted with a hemocytometer. The

desired amount of cells were resuspended in 0.2 ml of PBS and injected into the mice sub-cutaneously. The mice were then monitored for tumor development.

RNA extraction and hybridization: RNA was extracted from cells either by the method of Chomczynski and Sacchi (12) or with TRIzol reagent (Gibco), as per the manufacturer's protocol, and hybridized with $^{32}$P-labelled probes, as previousiy described (30,54).

Protein extraction and Western blotting: Proteins were extracted by lysis in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% Na desoxycholate, and 0.1% SDS) containing protease inhibitors, followed by SDS-PAGE and blotting with polyclonal goat-anti CA (p30) MuLV antibodies, as described previously (15).

Statistical analysis of animal survival times: The survival times (in days) of mice inoculated with the different B16-F1 clones were log-transformed and analyzed by one-way analysis of variance (ANOVA) to examine the significance of the differences in survival seen between the different groups of animals.

## Results

### The 203-33M cells express the MAIDS defective virus $Pr60^{gag}$

The 203-33M cells are derivatives of the v-abl-transformed 203-33 pre-B cell line. The pool of cells infected with the neo-expressing MAIDS defective virus construct was examined for its expression of the viral message by Northern blot analysis. As seen in Figure 1A, these cells expressed low but detectable levels of the MAIDS defective virus when the MAIDS defective virus-specific D30 probe was used. In contrast, RNA from 203-33 cells infected with a neo-expressing virus (203-33/N2) or with Moloney MuLV (203-33/Mo) did not hybridize with this probe (Figure 1A). Both the 203-33M and the 203-33/N2 cells expressed the neo message, as seen in Figure 1B, as expected. As a further control, cells infected with Moloney MuLV (203-33/Mol) were also generated. The rAdT-negative 203-33 cells became rAdT-positive upon infection with Moloney MuLV, indicating that they were successfully infected (data not shown).

### The 203-33M cells produce $Pr60^{gag}$

We next established that the 203-33M cells produced the expected 60 kDa protein, which represents the only product of this virus (9,29). Western blot analysis of these cells clearly shows a low, but easily detectable level of this protein (Figure 2). This contrasts with our previous attempts to express this protein in different lymphoid cell lines (S. Klein and P. Jolicoeur, unpublished data) which have been uniformly unsuccessful.

CD8$^-$ T cells are responsible for the lack of transplantability of the 203-33M cells

Upon attempting to transplant the 203-33M cells into syngeneic C57BL/6 mice, we found that a large percentage of the inoculated mice did not develop tumors and those that did develop tumors took a longer period to succumb to the tumor. This result was highly unexpected as the inoculation of the 203-33 parental cells into C57BL/6 mice led to 100% mortality (Table 1A). The 203-33/N2 and 203-33/Mo cells grew equally well when inoculated into C57BL/6 mice (Table 1A), suggesting that the expression of the MAIDS defective virus specifically inhibited the growth of these cells *in vivo*, and this inhibition was not due simply to the presence of any *gag* protein. A similar lack of growth in syngeneic C57BL/6 mice was observed for two other v-abl-transformed pre-B cell lines infected with the MAIDS defective virus (data not shown)

To examine further whether this phenomenon was due to a growth inhibitory effect of the MAIDS defective virus $Pr60^{gag}$ on these cells, or whether these cells were being actively rejected, we attempted further transplantation studies. Inoculation of the 203-33 and 203-33M cells into nude mice led to the growth of both cell populations (Table 1B), indicating that the 203-33M cells were not inhibited in *in vivo* growth by the presence of $Pr60^{gag}$, but rather that they were actively rejected by the T cells of the C57BL/6 mice. Further support for this contention comes from our transplantation of these cells into mice lacking CD8$^-$ T cells due to a targeted disruption at the CD8 locus. These mice have normal CD4$^+$ T cells and NK cells, but are unable to mount a cytolytic response. Both the 203-33 and 203-33M cells grew equally well in these mice (Table 1C),

strongly supporting the evidence that 203-33 cells infected with the MAIDS defective virus induced a CTL response when inoculated into C57BL/6 mice.

B16 cells express the MAIDS Pr60$^{gag}$ protein

The results described above were very intriguing as they supported the theory that the MAIDS Pr60$^{gag}$ protein has the potential to induce a CTL response that can lead to the rejection of what is normally a highly tumorigenic cell line. We were interested to see whether such a result could be obtained in another system. We chose to use the B16 melanoma model, since it has been fairly well studied and it allowed us to continue to use the C57BL/6 mouse.

The B16 cells were transfected with DNA encoding the MAIDS defective virus Pr60$^{gag}$. Several G418-resistant clones were isolated and examined for the expression of the expected message and protein. As seen in Figure 3A, Northern analysis of 4 independent clones using the MAIDS defective virus-specific D30 probe showed that they expressed high levels of the predicted RNA.

Western blot analysis of these clones using a polyclonal goat anti-CA (p30) antibody indicated that relatively high levels of Pr60$^{gag}$ were produced in all the clones (Figure 4). In contrast to the 203-33 cells, the B16 cells express a helper MuLV, as seen by the presence of a 65 kDa species reactive with the antibody. These cells also produced higher levels of Pr60$^{gag}$ than the 203-33M cells (compare Figure 2 and Figure 4), once again following a pattern we have often seen in which non-lymphoid adherent cell lines support moderate to high

expression of $Pr60^{gag}$, whereas lymphoid cell lines support little or no expression of this protein (S. Klein and P. Jolicoeur, unpublished data).

Pr60$^{gag}$-expressing B16F1 cells develop tumors with a longer latency than the parental B16F1 cells

C57BL/6 mice were inoculated with 4 independent B16F1 clones expressing the MAIDS defective virus $Pr60^{gag}$ (B16F1/M3, B16F1/M4, B16F1/M6, and B16F1/M17) as well as with two B16F1 clones transfected with the empty vector alone (B16F1/V1 and B16F1/V2) and the untransfected B16F1 parental cells. All the mice were inoculated subcutaneously on the same day with $2.5 \times 10^5$ cells. The mice were then observed for the development of tumors. We found that mice transplanted with the parental and the vector-transfected B16F1 cells died between 18 and 30 days post-transplantation with an average time to death of 26+/-5 days (n=6) for mice inoculated with the parental B16F1 cells and 20+/-3 days (n=5) and 23+/-4 days (n=5) for the mice inoculated with the B16F1/V1 and B16F1/V2 clones, respectively. In these animals, death occurred uniformly between 18 and 30 days post transplantation (Figure 5).

In contrast, mice inoculated with the B16F1 clones expressing the MAIDS defective virus $Pr60^{gag}$ exhibited an increased latency in the time to death. The average time to death of the inoculated mice was 35+/-2 days (n=5), 42+/-14 days (n=6), 34+/-5 days (n=5), and 37+/-7 days (n=6) for the M3, M4, M6, and M17 clones, respectively. None of the inoculated mice died before 25 days post-inoculation, and most mice survived well past the 30 day mark, by which time the

mice inoculated with the parental B16F1 cells or with the empty vector-transfected cells had all died (Figure 5). In fact most of the mice inoculated with the $Pr60^{gag}$-expressing B16 cells survived for close to 40 days. Statistically, there was no significant difference in animal survival times within the experimental and control groups ($P<0.0001$). Comparison of animal survival times between the four experimental groups (B16F1/M3, B16F1/M4, B16F1/M6 and B16F1/M17) (n=22) and the three control groups (B16F1, B16F1/V1, and B16F1/V2) (n=16) revealed a statistically significant difference in survival of animals inoculated with $Pr60^{gag}$-expressing B16F1 cells compared to the animals inoculated with the parental or control cells ($P<0.0001$). Thus, overexpression of $Pr60^{gag}$ in this highly aggressive melanoma cell line allowed the inoculated mice to survive nearly twice as long as those inoculated with the parental or control melanoma cell lines, suggesting an inhibitory effect of $Pr60^{gag}$ on tumor growth.

## Discussion:

In this manuscript we have presented evidence which indicates that the MAIDS defective virus $Pr60^{gag}$ protein can induce the rejection of a v-abl-transformed pre-B cell line by a $CD8^+$ T cell-mediated mechanism. This result was unexpected since it occurred in a C57BL/6 mouse-based experimental system, a system in which the MAIDS defective virus is capable of inducing disease, presumably because the host's immune system does not mount an adequate response to the viral $Pr60^{gag}$ protein. Our results were specific to the $Pr60^{gag}$, as a similar result was not seen when the 203-33 pre-B cells were infected with Moloney MuLV. Two other v-abl-transformed pre-B cell lines which were infected with the MAIDS defective virus also lost their ability to induce tumours in syngeneic C57BL/6 mice, but not in nude mice. This strongly suggests that these cells were rejected by a T cell-mediated event, most likely a $CD8^+$ T cell-mediated event, based on the results of the 203-33 cells infected with the MAIDS defective virus.

Many studies in MAIDS have focused on the role of $CD8^+$ T cells in mediating resistance or susceptibility to this disease (16,42,57,60). It has been known that *in vivo* depletion of $CD8^+$ T cells in resistant A strain mice renders these mice susceptible to MAIDS (42), implying a role for $CD8^+$ T cells in mediating resistance to MAIDS. More recent studies on mice lacking $CD8^+$ T cells due to a disruption of the $\beta_2$-microglobulin gene revealed a role for $CD8^+$ T cells in clearing virally-infected cells from the host via a perforin-dependant

mechanism (60). However, the same study concluded that other mechanisms involving $CD4^+$ T cells or cytokines may also contribute to resistance to MAIDS.

Studies employing $Pr60^{gag}$-expressing lymphomas derived from late-stage diseased animals demonstrated that C57BL/6 mice, which are sensitive to MAIDS, can in fact induce a CTL response (16). Unfortunately, it is not clear which antigen(s) is responsible for inducing the response, or even if the response is directed towards $Pr60^{gag}$ (16). In the studies presented here, although it is clear that $CD8^+$ T cells are critical for eliminating the $Pr60^{gag}$-expressing 203-33 cells, most likely through a cytotoxic mechanism, we cannot yet conclude with absolute certainty that it is definitely a CTL response. Such a conclusion can only be reached after a demonstration in challenged mice of CTL activity directed to the MAIDS defective virus-infected 203-33 cells. Further complicating matters is the fact that even if direct proof of a CTL is obtained, it would still not be clear to which antigen(s) the response is directed against. It is possible that infection of the 203-33 cells with $Pr60^{gag}$ leads to the upregulation of other, not normally expressed, cellular antigens which themselves provoke a CTL response.

One way to further exploit this system to help us understand how infection of 203-33 cells with the MAIDS defective virus leads to their rejection is to examine whether C57BL/6 mice which have been challenged with the $Pr60^{gag}$-expressing pre-B cells become resistant to challenge with the MAIDS defective virus. Since these mice do not appear to mount a CTL response when injected with helper-free MAIDS defective virus, it will be interesting to see if they remain susceptible to MAIDS or if challenge of these mice with $Pr60^{gag}$-expressing cells

leads to their resisting the disease. A positive outcome would imply that there is something particular about the MAIDS defective virus-infected 203-33 pre-B cells that allows them to initiate a rejection mechanism in the host. Alternately, the *in vivo* target cells of the MAIDS defective virus may be inherently inefficient at presenting the critical antigens to initiate the response, but once the animal is primed, infection of the target cells of the MAIDS defective virus may lead to a more easily activated memory response. It would still be necessary to determine if the CTL response is directed to $Pr60^{gag}$ or to another cellular protein(s) whose expression is activated by $Pr60^{gag}$.

Our results using the MAIDS defective virus-induced 203-33 cell line led us to explore the possibility that such a phenomenon could also occur in other systems. We therefore chose the B16F1 melanoma cell line to study since these cells readily give rise to tumours in syngeneic C57BL/6 mice (5,6,17,56). A further reason for using these cells is that it is one of few transformed cell lines available which are derived from C57BL/6 mice. We felt that this was an important consideration since the MAIDS defective virus has been best studied on this genetic background.

As seen from the data on the survival of mice inoculated with $Pr60^{gag}$-expressing B16F1 cells, there does seem to be some protection afforded to animals inoculated with these cells compared to animals inoculated with the parental or the neo-infected control cells. This protection is moderate compared to what was observed using the 203-33 cell line despite higher levels of MAIDS defective virus RNA and protein in the four B16F1 clones examined. There may

be several explanations for the discrepancy in animal survival observed between these two systems. Even though the $Pr60^{gag}$-expressing B16F1 clones express higher levels of this protein compared to the 203-33M cells, they are also much more aggressive than the 203-33M cells. It has been reported that as few as $5X10^3$ of these cells can lead to tumour formation and death of the animal (5). In our system, we inoculated $2.5X10^5$ of these cells. Such a large number of cells may overwhelm the host before an effective CTL response could be generated. It would therefore be of interest, in light of the present results, to attempt the same experiment but using fewer cells. This would necessitate titrating the number of cells in order to determine the minimum number needed to lead to tumour formation. Alternately, the 203-33 cells may be more efficient at priming the host immune system, or in processing and presenting $Pr60^{gag}$ in a form sufficient to induce an immune response as compared to the B16F1 melanoma cells, although little work has been done on the 203-33 cells.

The question of why some cells are capable of inducing a CTL response while others are not is an area of active research in the field of tumour immunology. Indeed, it has been shown that $CD8^+$ CTL play a major role in the rejection of immunogenic tumors (27,45), and CTL specific for autologous neoplasia can be derived from the blood of tumor-bearing patients (53). As well, several tumor Ag recognized by CTL have been identified so far (7,8,59) and several methods have been devised in order to isolate MHC class I restricted tumor antigens. The genetic approach involves the screening of either genomic or cDNA libraries derived from the tumor (14,41), while the biochemical approach

(55.61) involves isolating tumor-specific peptides directly from tumor MHC I molecules, followed by their purification. Of note, the latter approach was used to identify an immunodominant rejection antigen expressed by the murine colorectal line CT26 (28). The deduced antigenic sequence was found to derive from gp70, one of the products of the MuLV *env* gene. The *env* gene products are expressed in a variety of tumor cells, including lymphomas and leukemias of mice strains with high MuLV titres (25,26,52). One study has also identified a tumor antigen on a mouse spontaneous leukemia that derived from an intracisternal A-particle sequence (13). More recently, a *gag*-encoded CTL epitope which is shared by Friend, Moloney, and Rauscher MuLV-induced tumors was identified (10,38). CTL responses to *gag* proteins are also well documented in other systems, including HIV-1, SIV and FIV infections (11,37,40,63). Although the MAIDS defective virus does not encode *env* sequences (2,9,29) the precedent exists, as discussed above, for murine *gag* sequences acting as tumor rejection antigens. The possibility that $Pr60^{gag}$ is capable of acting in such a manner can not be discounted, as suggested by studies showing an *in vitro* CTL induction by lymphoma cell lines derived from MAIDS animals (16,20,57).

Much work has been done on the manipulation of the B16F1 cells in order to elicit a protective CTL *in vivo*. Several groups have shown that under certain circumstances, B16F1 cells can indeed induce a protective CTL response. These studies have generally relied upon manipulating the cells to express either cytokines, peptides or proteins in order to induce the CTL response (5,6,56). Apart from not inducing a CTL response directly, more recent evidence from a

variety of different systems, including the B16F1 melanoma model, indicates that tumor cells may utilise a variety of means to elude destruction. Recently, it has been shown that both melanoma cells and colon cancer cells express Fas(Apo-1/CD95) Ligand (FasL) (22,51), which is capable of interacting with its receptor, Fas, which is expressed by the responding T cells, thus triggering the death of these responding T cells by apoptosis(4,21,50). As well, the presence or lack of co-stimulatory molecules on many tumor cells also appears to influence whether or not the tumor cells will be rejected (3,18,44,62). A lack of expression of these co-stimulatory molecules, notably B7-1 and B7-2 is correlated with tumor growth, whereas tumor cells engineered to express these molecules show a dramatic loss of tumoriginicity (18,44,62), and can even lead to a host response against the B7$^-$ parental cells (44).

Although the results described in this paper are intriguing, we still need to understand their underlying mechanism in order to fully exploit this system. For example, it would be of much interest to study the expression of FasL as well as the B7 family members on both the uninfected and $Pr60^{gag}$-infected 203-33 and B16F1 cells to ascertain if our results follow the pattern seen in other systems. In fact, it has been reported that Fas-FasL interactions may play a role in the development of MAIDS (35). It will also be of interest to note if inoculation of syngeneic mice with the $Pr60^{gag}$-expressing 203-33 cells can protect those animals from challenge with either the unmanipulated 203-33 cells or with the MAIDS defective virus. Further studies are also warranted on the effects of $Pr60^{gag}$ on other tumor cells, including those not derived from C57BL/6 mice. Confirmation

and extension of these results in other systems may point to a possible use for $Pr60^{src}$ as a vaccine against established tumors as well as furthering our understanding about how some strains of mice resist developing MAIDS while other strains succumb to the virus.

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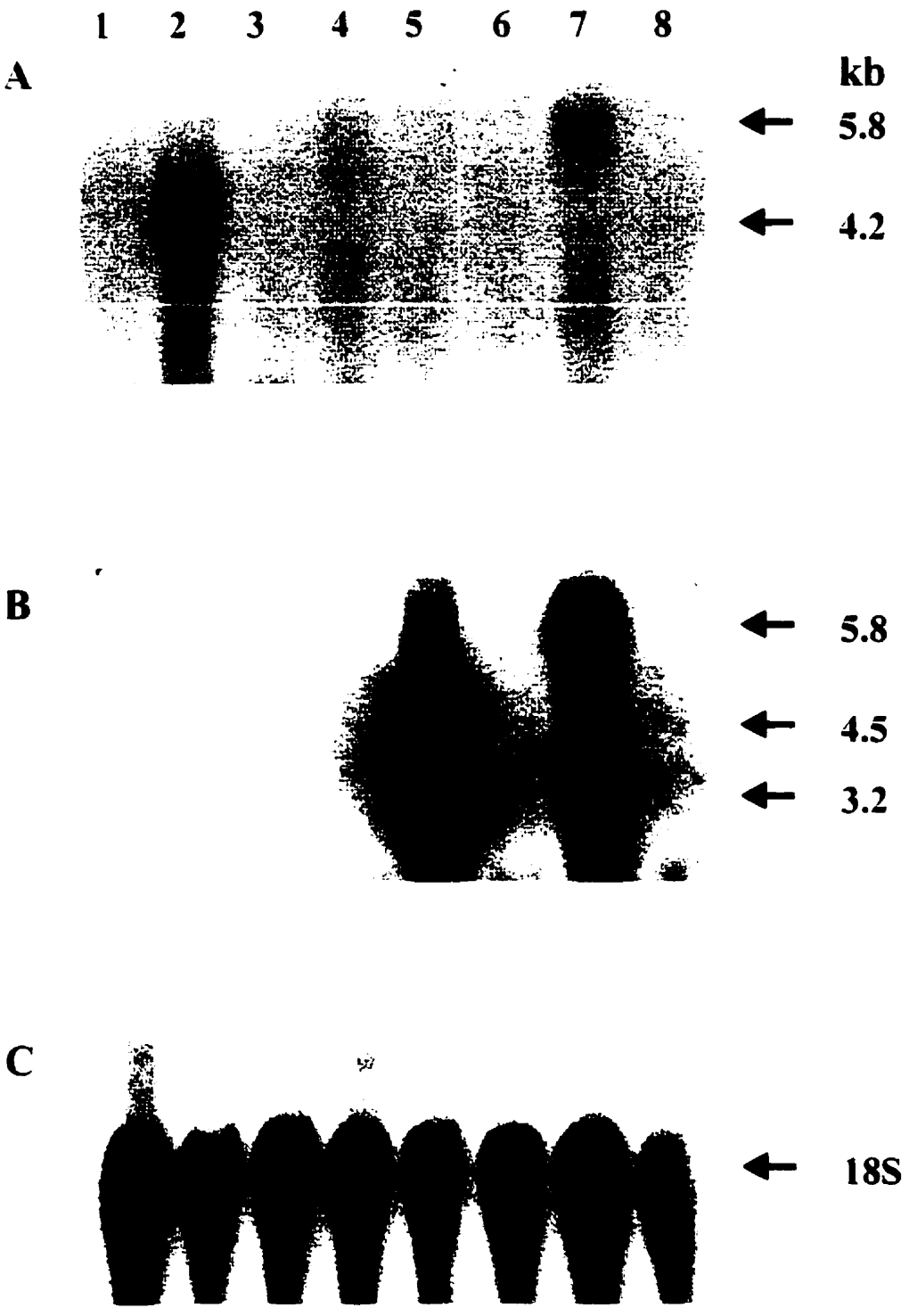


**Figure 1**

**Legend to Figure 1:** Northern blot analysis of 203-33 cells infected with the MAIDS defective virus. RNA was extracted from cells and resolved on an formalydehyde-agarose gel and transferred to a nylon membrane, followed by sequential hybridizations with $^{32}$P-labelled probes specific for the MAIDS defective virus p12 region (D30) (A), or for the neomycin resistance gene (B). Panel C shows the hybridization with a $^{32}$P-labelled oligo specific for the 18S rRNA to show equal RNA loading of the samples. Lane 1, normal spleen; lane 2, SD1 cell line; lane 3, B16F1 cell line; lane 4, normal thymus; lane 5, 203-33/N2 cells; lane 6, 203-33/Mol cell lines; lane 7, 203-33M cells; lane 8, 203-33 cells. Arrows in (A) indicate the expected 4.2 kb MAIDS defective virus RNA and the 5.8 kb transcript produced by the chimeric Du5H/neo virus. Arrows in (B) indicate the 5.8 and 2.5 kb transcripts produced by the chimeric Du5H/neo virus and the 4.5 kb transcript produced by the N2 virus. The neo probe detects 2 messages from the Du5H/neo construct (initiating from the viral LTR and from the internal SV40 promoter which drive the neo cassette), whereas the D30 probe detects a single message.

The arrow in (C) points to the 18S rRNA band.

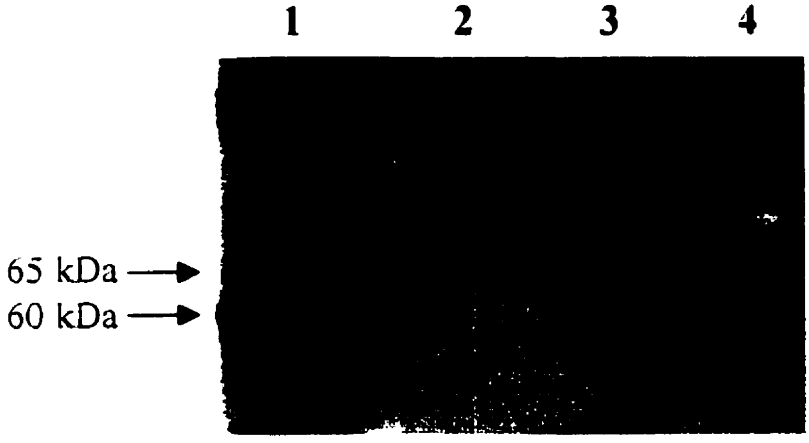


**Figure 2**

**Legend to Figure 2:** Western blot analysis of the 203-33M cells. Proteins were extracted from the cells, separated on an 8% SDS-PAGE gel, transferred to a PVDF membrane, and probed with an anti MuLV p30 antibody. Lane 1, SD1 cell line; lane 2, NIH cells; lane 3, 203-33 cells; lane 4, 203-33M cells. Arrows indicate the 60 kDa protein encoded by the MAIDS defective virus and an endogenous 65 kDa helper *gag* protein present in the SD1 cells, but absent in the 203-33 and 203-33M cells.

**Table 1: $Pr60^{gag}$-expressing 203-33 cells show a $CD8^+$ T-cell mediated growth inhibition**

**A**- 203-33 cells infected with the MAIDS defective virus show a decreased tumorigenicity

| Cells Inoculated | Animal Survival | Tumor Growth |
|---|---|---|
| 203-33 | 0/10 | 10/10 |
| 203-33/M | 12/15 | 3/15 |
| 203-33/Mol | 0/5 | 5/5 |
| 203-33/N2 | 0/5 | 5/5 |

**B**- Inhibition of growth of MAIDS defective virus-infected cells is T cell-mediated

Normal C57BL/6

| Cells Inoculated | Animal Survival | Tumor Growth |
|---|---|---|
| 203-33 | 0/2 | 2/2 |
| 203-33M | 5/6 | 1/6 |

Nude mice

| Cells Inoculated | Animal Survival | Tumor Growth |
|---|---|---|
| 203-33 | 0/5 | 5/5 |
| 203-33M | 0/5 | 5/5 |

**C**- Inhibition of growth of MAIDS defective virus-infected cells is $CD8^+$ T cell-mediated

Normal C57BL/6

| Cells Inoculated | Animal Survival | Tumor Growth |
|---|---|---|
| 203-33 | 0/3 | 3/3 |
| 203-33M | 3/3 | 0/3 |

$C57/CD8^{+/-}$

| Cells Inoculated | Animal Survival | Tumor Growth |
|---|---|---|
| 203-33 | 0/4 | 4/4 |
| 203-33M | 3/3 | 0/3 |

$C57/CD8^{-/-}$

| Cells Inoculated | Animal Survival | Tumor Growth |
|---|---|---|
| 203-33 | 0/5 | 5/5 |
| 203-33M | 0/8 | 8/8 |

Legend to Table 1: The indicated strains of mice were inoculated with 2 X $10^6$ of the indicated cells sub-cutaneously, in 0.2 ml of PBS. The animals were then monitored for evidence of tumor growth. Data are represented as both survival and tumor growth. In no case did an animal develop a tumor and survive. Data are shown in three separate panels representing three different experiments, as described in the table. All the animals in each group were inoculated on the same day and maintained under the same conditions.

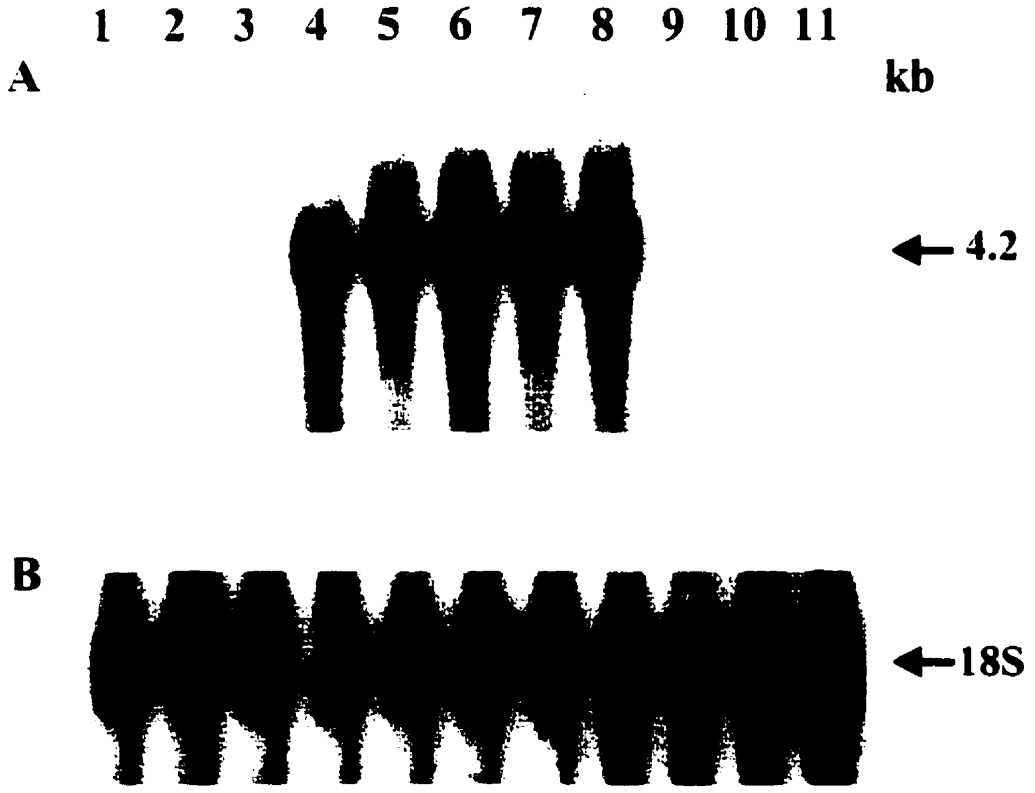


Figure 3

**Legend to Figure 3:** Northern blot analysis of B16F1 clones transfected or not with the $Pr60^{gag}$-expressing construct. RNA was extracted from cells and resolved on an formalydehyde-agarose gel and transferred to a nylon membrane, followed by hybridization with a $^{32}P$-labelled probe specific for the MAIDS defective virus p12 region (D30) (A). Panel B shows the hybridization with a $^{32}P$-labelled oligo specific for the 18S rRNA to show equal RNA loading of the samples. Lane 1, 203-33/N2 cells; lane 2, normal spleen; lane 3, normal thymus; lane 4, SD1 cell line; lane 5, B16F1/M17 cells; lane 6, B16F1/M6 cells; lane 7, B16F1/M4 cells; lane 8, B16F1/M3 cells; lane 9, B16F1/V2 cells; lane 10, B16F1/V1 cells; lane 11, B16F1 cells. The arrow in (A) indicates the expected 4.2 kb transcript produced by both the MAIDS *gag* construct and the MAIDS defective virus. The arrow in (B) indicates the 18S rRNA band.

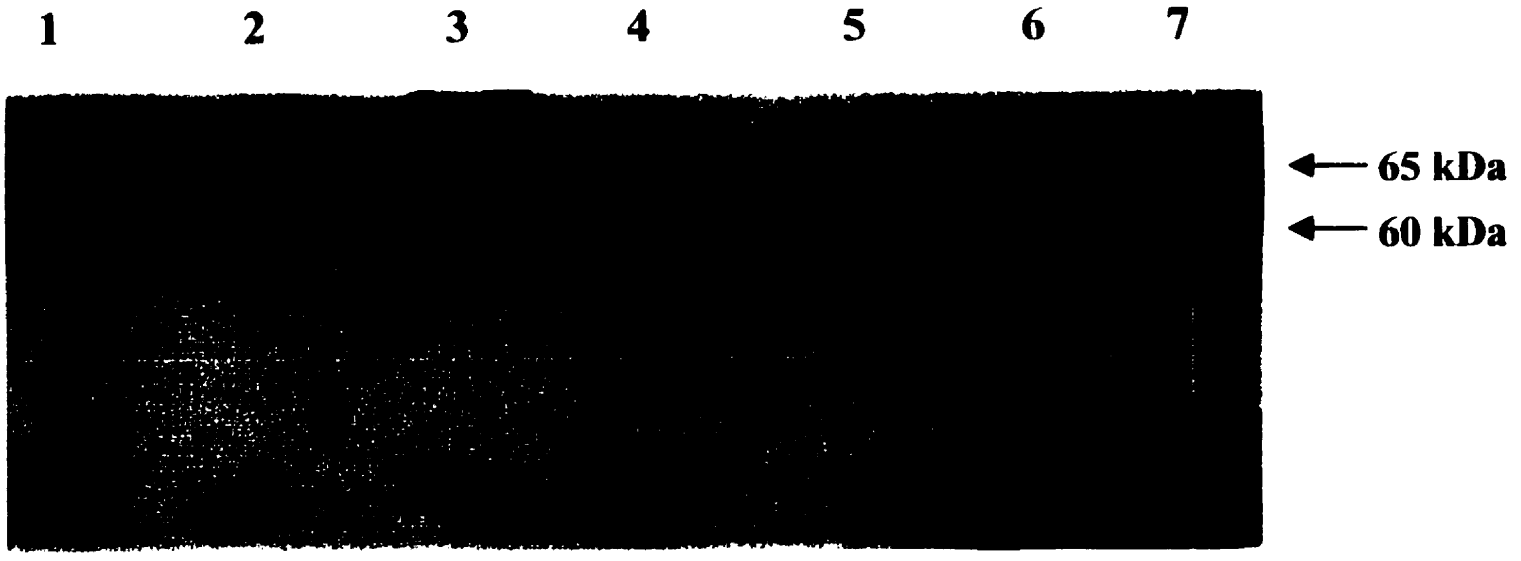


**Figure 4**

**Legend to Figure 4:** Western blot analysis of the MAIDS $Pr60^{gag}$-expressing B16F1 cells. Proteins were extracted from the cells, separated on an 8% SDS-PAGE gel, transferred to a PVDF membrane, and probed with a polyclonal anti MuLV p30 antibody. Lane 1, B16F1/M3 cells; lane 2, a MAIDS defective virus-expressing cell line; lane 3, B16F1 cells; lane 4, B16F1/V2 cells; lane 5, B16F1/M4 cells; lane 6, B16F1/M6 cells; lane 7, B16F1/M17 cells. Arrows indicate the endogenous 65 kDa *gag* protein and the 60 kDa protein product of the MAIDS defective virus. The lower band present in all lanes represents non-specific binding of the polyclonal antibody to the protein extract.

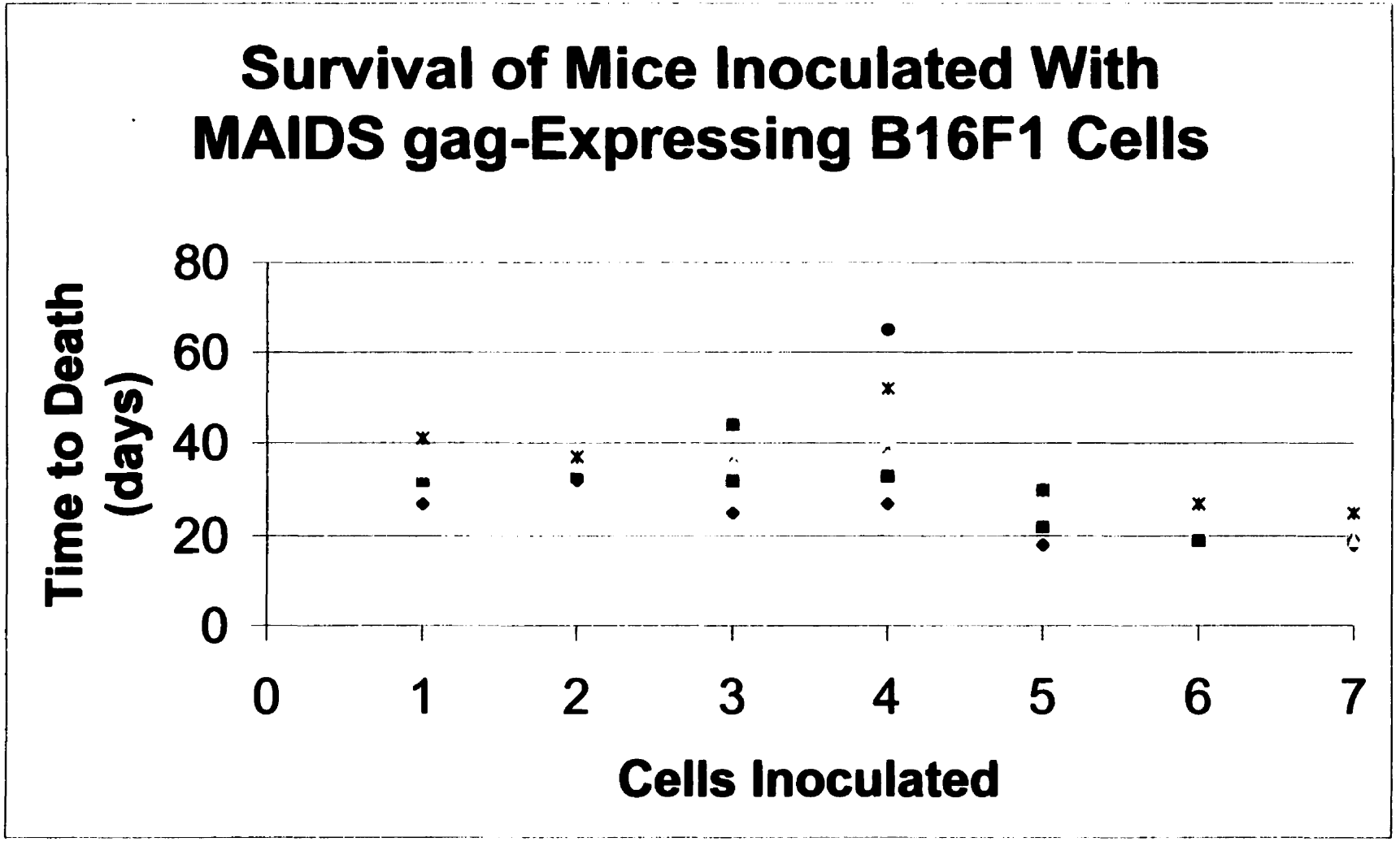
Survival of Mice Inoculated With MAIDS gag-Expressing B16F1 Cells
Time to Death (days)
80
60
40
20
0
0
1
2
3
4
5
6
7
Cells Inoculated

Figure 5

Legend to Figure 5: Survival of syngeneic C57BL/6 mice inoculated with the MAIDS $Pr60^{gag}$-expressing B16F1 cells. Mice were inoculated sub-cutaneously with 2.5 X $10^5$ of the indicated cells and monitored until the time of death. Each symbol represents the time of death of one animal in the indicated group. Group 1, B16F1/M6 (n=5); group 2, B16F1/M3 (n=5); group 3, B16F1/M17 (n=6); group 4, B16F1/M4 (n=6); group 5, B16F1 (n=6); group 6, B16F1/V2 (n=5); group 7, B16F1/V1 (n=5). All the mice in each group were inoculated on the same day and maintained under identical conditions.

Chapter 6

# General Discussion and Summary

The experimental work presented in this thesis has furthered our understanding of the pathogenesis of MAIDS in several respects: 1) our results studying the infection of various mutant strains of mice with the MAIDS defective virus revealed a central role for mature B cells in the early stages of this disease, with a post-infection role for $CD4^+$ T cells; 2) we successfully isolated two independent B cell lines which accurately reflect the *in vivo* phenotype of the MAIDS defective virus target cells; 3) these *in vitro* MAIDS defective virus target cells were instrumental in establishing that $Pr60^{gag}$ interacts with c-abl via its SH3 domain; and 4) studies using $Pr60^{gag}$-infected pre-B and melanoma cells revealed a potential role for this protein as an inducer of a host immune response.

## 1- The initiation of MAIDS requires mature B cells and is $CD4^+$ T cell-independent

We chose to study the contributions of various lymphocyte subsets to the development of MAIDS by examining the response of nude, $CD4^{-/-}$, and SCID mutant mice to the MAIDS defective virus. SCID mice, which lack mature B and T cells due to an enzymatic defect which causes incomplete recombination and rearrangement of the TcR and Ig genes (for review, see (71)) were found to be completely resistant to infection with the MAIDS defective virus, whereas nude mice, which lack mature T cells due to a defect in thymus development as a consequence of a mutation in a winged-helix transcription factor (51), were infectable with the MAIDS defective virus, albeit at a much lower level than seen in wild-type mice. The infected cells in the nude mice were characterised as

belonging to the B lineage. These results confirmed and extended previous results showing that the development of MAIDS is dependant upon the presence of both B and T cells (4,40,47,49,50,72). These results revealed that even in the absence of large numbers of mature T cells, infection of the target B cells can occur.

Previous studies also showed that the T cell dependence of the disease is due specifically to the $CD4^+$ subset (18,72), although the stage at which the disease was blocked was not examined, nor was there any information on which, if any, cells were infected in the absence of the $CD4^+$ T cells. Our work in this system has revealed that infection of the target B cells by the MAIDS defective virus does occur and that the infection of these cells is $CD4^+$ T cell-independent. More importantly, the experiments using the $CD4^{-/-}$ mice revealed that the development of MAIDS can be seen as proceeding in two distinct phases. In the first phase, infection of the target B cells by the MAIDS defective virus occurs in mice containing the target B cell population, regardless of whether or not $CD4^+$ T cells are present, as seen for the nude and $CD4^{-/-}$ mice. Infection of the target B cells, however, is necessary but not sufficient for MAIDS to develop. The second phase in the development of MAIDS is $CD4^+$ T cell-dependant. The $CD4^+$ T cells are required for the expansion of the infected B cell population, thus leading to the full development of MAIDS.

Interestingly, it appears that in the case of MAIDS, $CD4^-CD8^-$ T cells, which develop in mice lacking CD4 (45), cannot substitute for true $CD4^+$ T cells, contrary to what has been observed in other systems. For example, $CD4^{-/-}$ mice infected with *Leishmania* did not succumb to disease, as might have been

expected since resistance to this disease is dependent upon the presence of $CD4^+$ T cells (45). It was found that a population of MHC class II-restricted $CD4^-CD8^-$ T cells was responsible for this resistance. It therefore appears that these $CD4^-$ $CD8^-$ mature T cells can compensate for some helper functions in the absence of CD4, but not for others, as in the case of MAIDS, indicating that these $CD4^-CD8^-$ mature T cells may require the expression of CD4 for complete helper function.

One possibility for further exploring the apparent two-step development of MAIDS would be to try to rescue the development of MAIDS in $CD4^{-/-}$ mice by transferring into these mice $CD4^+$ T cells either at the time of inoculation or at various times post-inoculation. Alternately, conditioned medium from activated $CD4^+$ T cells or membrane preparations derived from $CD4^+$ T cells could be substituted for whole live $CD4^+$ T cells. Such experiments may be useful in determining 1) if the infected, non-expanding target B cells can be activated at a later time to give disease; 2) if B-T cell contact is required for the subsequent B cell expansion and/or; 3) if a soluble factor(s) can substitute for $CD4^+$ T cells in the development of MAIDS. The manipulation of the $CD4^{-/-}$ mouse system may therefore be quite useful in answering several important questions regarding the development of MAIDS.

**2- What is the nature of the MAIDS defective virus target cell?**

One of the most crucial unanswered questions regarding this disease is the nature of the target cell of the MAIDS defective virus. Our group and others have shown that a relatively mature B cell population is the target of the MAIDS

defective virus (30,40), although those groups using the crude MAIDS virus mixture observe infection of many different cell populations, including B cells, T cells, and macrophages (1,6,7,26,41,42). Our use of the helper-free system has alleviated the problem of studying infection of many different cell populations, some of which may have no impact on disease development. In our hands, inoculation of mice with helper-free MAIDS defective virus leads exclusively to infection of a B cell population, implying that is the infection of these cells that is crucial to the disease. Although the latency in MAIDS induced by the helper-free stocks of the MAIDS defective virus is longer than that seen with the crude virus stocks (29,54), the two diseases appear to be very similar, with infection of a mature B cell population being the critical event (30,40).

To fully understand the development of the disease it was therefore important to isolate and study these target B cells. Many attempts to generate cell lines that closely resemble the *in vivo* target of the MAIDS defective virus have been made, with limited success. In all cases, transplantable B lymphoma lines were derived (41,67,68) as were transplantable T cell lines (42,64,67,68). In our helper-free system, it has been impossible to transplant primary MAIDS defective virus-infected tissues into syngeneic animals (S. Klein, C. Simard, and P. Jolicoeur, unpublished data), nor do we observe the infection of T cells, which is a frequent occurrence in other systems. These transplantable B and T cell lines derived by others do not therefore appear to represent the true target population of this virus.

By using the two approaches described in chapter 3, we were successful in isolating two independent cell lines which accurately reflect the phenotype of the *in vivo* target cells of the MAIDS defective virus. Both of these cell lines contain integrated MAIDS defective virus and are clearly of the B cell lineage as assessed by the status of its Ig loci and by flow cytometric analysis of surface marker expression. Additionally, neither cell line was transplantable into a variety of mouse strains, consistent with what is observed *in vivo*. Curiously, these cell lines do not appear to represent a population of B cells which has been previously described, their phenotype falling between that of a pre-B and a mature B cell phenotype. As well, the CSTB5 cell line has rearranged its IgL locus in the absence of IgH rearrangement, a phenomenon seen occasionally in v-abl-infected cell lines. Further study of the unusual phenotype of these cells is warranted to expand on their identity. This, for example could include searching for the presence of B cell-specific transcription factors such as the STAT family members and BSAP (17,37,52) and signalling molecules such as Igα and Igβ (3,10).

Additionally, the established SD1 and CSTB5 cell lines may represent a population that has been rescued from apoptosis by the MAIDS defective virus. We have previously shown that MAIDS initiates in the germinal centre of the lymph node draining the site of inoculation of the virus (63), further suggesting that the MAIDS defective virus infects a relatively mature B cell population. Germinal centres are areas of the lymph nodes which contain large amounts of B cells which are undergoing apoptosis (39,55). It is possible that infection with the

MAIDS defective virus of these B cells destined to die may rescue them from apoptosis and reprogram them so that they become locked in a state of differentiation which may contribute to the development of MAIDS. Evidence in support of this hypothesis could be obtained by sequencing the rearranged Ig genes of the SD1 and CSTB5 cell lines, as well as those derived from primary clonal MAIDS tumors. Non-productive rearrangements of either the Ig heavy or light chain genes, which normally trigger the apoptotic pathway, would strongly suggest that these infected B cells are rescued from their normal fate. It has recently been shown that a subset of germinal centre B cells normally expected to die by apoptosis re-express the recombination-activating genes 1 and 2 (RAG1 and RAG2) and undergo secondary Ig gene rearrangement (24). An examination of the status of RAG1 and RAG2 in the SD1 and CSTB5 cell lines and MAIDS animals could further support the above hypothesis.

The phenotype of the SD1 and CSTB5 cell lines is reminiscent in several ways to that of the malignant Reed-Sternberg (RS) cells observed in Hodgkin's disease. Hodgkin's disease is characterized by the presence of a small number (usually less than 1% of the tumor mass) of the typical RS cells in a hyperplastic background of normal lymphocytes, plasma cells, neutrophils, eosinophils, and stromal cells (22). Indeed, it is not uncommon in animals infected with the MAIDS defective virus to observe a low (5-10%) percentage of infected cells in the enlarged lymphoid organs, suggesting a recruitment of normal, uninfected cells into the area by the infected cells. As with the MAIDS defective virus target cells, the RS cells have been difficult to characterize due to a lack of *in vitro* RS

cells which could be easily manipulated, and as such the cellular origin of these cells has been an issue of constant debate. Recent advances in single-cell manipulation of the RS cells has revealed that in many instances, these cells have Ig rearrangements, pointing to a B cell origin for these cells, while other groups have not detected this molecular event (32,43,44). Furthermore, the RS cells have been proposed to function as antigen-presenting cells (APC), since they express MHC class II and B7 molecules in addition to the adhesion molecules ICAM-1 and LFA-3 (11). As presented earlier in this thesis, the SD1 and CSTB5 cell lines also express MHC class II and B7-2, while the work of others has implicated a potential role for cell-cell contact in the development of MAIDS (19,36). Therefore there appears to be much in common between the target cells of the MAIDS defective virus and the cells which give rise to the RS cells seen in Hodgkin's disease.

Although most of the evidence points to a B cell population as the target of the MAIDS defective virus, it is still possible that it is another population that is infected with the MAIDS defective virus. This putative target cell would acquire a B cell phenotype as a consequence of being infected with the MAIDS defective virus. Such a scenario, although unlikely, cannot be entirely ruled out. In fact, it has been demonstrated that a conditional v-rel/estrogen receptor fusion protein not only causes an estrogen-dependent, v-rel-specific transformation of chicken bone marrow cells, but the same protein under different culture conditions yielded transformed B cells, antigen-presenting dendritic cells, or cells resembling polymorphonuclear neutrophils (2). This study suggests that other transforming

or growth-stimulating proteins (such as $Pr60^{gag}$) can alter the phenotype of a common progenitor cell.

In order to further explore the nature of the MAIDS defective virus target cell, it will be important to isolate and characterize these cells very early post-infection in order to compare their phenotype at this stage to what is seen later in the disease and to the SD1 and CSTB5 cell lines. It would be of great importance to be able to isolate the few infected cells from the overwhelming majority of non-infected cells. The use of vectors employing the poliovirus internal ribosome entry sequence (IRES) could be exploited for this purpose (59,66). These vectors allow for the expression of two separate proteins from the same mRNA, using the same promoter. To isolate the MAIDS defective virus-infected cells at their earliest stage, a construction containing $Pr60^{gag}$ coding sequences as well as a selectable marker would be required. The selectable marker should be expressed on the cell surface, allowing for rapid and easy isolation by cell sorting. For example, the human CD4 molecule (huCD4), which is obviously not expressed in mice, would be a good candidate as the marker. The huCD4 molecule is expressed at the cell surface, and antibodies to CD4 are commercially available. Isolation of cells infected with this chimeric MAIDS defective/huCD4 virus could then be accomplished at a very early stage of infection and shed even more light onto the nature of these cells.

The tools exist as well to examine whether or not infection of cells by the MAIDS defective virus leads to a switch from a dendritic or macrophage cell to a B cell lineage. Transgenic mice expressing huCD4 under the control of the

human CD4 promoter express huCD4 in $CD4^-CD8^-$ and $CD4^-CD8^-$ T cells, as well as in macrophages and dendritic cells, with no expression observed in B cells ((25) and Z. Hanna, N. Rebai and P. Jolicoeur, unpublished data). Infection of these mice with the MAIDS defective virus, followed by immunohistochemistry/*in situ* hybridization would allow us to ascertain whether cells infected with this virus express huCD4 at any point in the development of the disease, especially at the earliest times during which a lineage switch would most likely occur. Evidence of huCD4 expression in MAIDS defective virus-infected cells would indicate that a non-B lineage cell is the target of this virus.

**3- $Pr60^{gag}$ interacts with the c-Abl non-receptor protein tyrosine kinase**

Another major question in the field of MAIDS has been how infection of the target cells with the MAIDS defective virus initiates the disease by altering its target cells' function so as to cause their proliferation and the subsequent polyclonal T cell anergy. We have answered at least part of this crucial question by using the yeast two-hybrid system in order to identify proteins which are capable of interacting with $Pr60^{gag}$. We found that the c-Abl non-receptor protein tyrosine kinase binds to a proline rich region of $Pr60^{gag}$ via its SH3 domain (15). The use of the SD1 and CSTB5 cell lines derived from MAIDS infected animals was instrumental in revealing an *in vivo* association between the two molecules, and will certainly be useful as well for studying other *in vivo* associations between $Pr60^{gag}$ and other, as yet unidentified, proteins.

The interaction between $Pr60^{gag}$ and c-Abl is provocative for several reasons. It is well know that the v-Abl protein, which is membrane-bound as opposed to nuclear (for the most part), readily transforms pre-B cells (57,58). As discussed in Chapter 3, the phenotype of the SD1 and CSTB5 cells somewhat resembles the target cells of v-abl. It is conceivable that $Pr60^{gag}$ can activate cell proliferation via c-Abl, possibly via the same pathway used by v-abl to transform its target cells. In MAIDS, however, transformation of the target cells does not occur as in the case of infection with v-abl. This may be due to the relative kinase activities of c-Abl "activated" by $Pr60^{gag}$ as compared to that of v-Abl. Alternately, $Pr60^{gag}$ may bind several other proteins in addition c-Abl, thus affecting several intracellular signaling pathways, the sum of which leads to the observed phenotype (i.e. immunodeficiency as opposed to transformation). Such a scenario is likely, as several other putative binding partners of $Pr60^{gag}$ have been isolated but not yet identified (P. Dupraz and P. Jolicoeur, unpublished results).

The revelation that type IV c-Abl suppresses apoptosis while type I c-Abl induces differentiation (9) is also of interest. We have reported that the target cell population of the MAIDS defective virus resides in germinal centres, which are areas known to contain large numbers of cells undergoing apoptosis (39,55). As described above, it is possible that this target cell population is one that is destined to die by apoptosis, but is rescued from this fate by $Pr60^{gag}$, which blocks this pathway and instead causes the population to proliferate and induce immunodeficiency. In fact c-Abl-deficient progenitor B cell lines show an increased sensitivity to apoptotic stimuli (12). Preferential activation of type IV

c-Abl by its sequestration at the plasma membrane by $Pr60^{gag}$ may be one mechanism by which the MAIDS defective virus could accomplish this feat. Aberrant localization of type I c-Abl may affect the differentiation of the $Pr60^{gag}$-expressing cells, contributing to the phenotype observed in the SD1 and CSTB5 cell lines, as well as to what is seen *in vivo*. It is not yet known, however, if there is a preferential binding of type IV or type I c-Abl with $Pr60^{gag}$ or if the kinase activity of the c-Abl that associates with $Pr60^{gag}$ is higher than the non-associated c-Abl protein.

Despite the fact that other proteins are likely to interact with $Pr60^{gag}$ in a variety of ways, the association of $Pr60^{gag}$ with c-Abl warrants further study. While the data supporting an *in vivo* association of c-Abl with $Pr60^{gag}$ are strong, there is no evidence as yet supporting a direct role for c-Abl in the pathogenesis of MAIDS. Several courses of action can be taken in order to answer this important question. Firstly, there exists a compound which selectively inhibits the Abl tyrosine kinase activity and which was useful in inhibiting cellular proliferation and tumor formation by Bcr-Abl-expressing cells (14). It would be of great interest to examine the proliferation-inhibiting effect of this compound on the SD1 and CSTB5 cell lines. Concurrently, animals inoculated with the MAIDS defective virus could be treated with this compound in the same manner as was done for past studies involving treatment of MAIDS animals with anti-leukemic drugs (65). The results of such studies would be quite telling and would strengthen the argument that a $Pr60^{gag}$-c-Abl association is crucial for the development of MAIDS.

Further *in vivo* proof of the importance of the $Pr60^{gag}$-c-Abl interaction can also be obtained in another fashion. This would involve constructing novel MAIDS defective viruses which have mutations in the proline-rich p12 region which has been shown to be involved in binding the SH3 domain of c-Abl, or in other regions of p12 that are not implicated in this association. Inoculation of these viruses into susceptible mice would likely yield important differences in their respective pathogenicities and further demonstrate the importance of the MAIDS defective virus p12 region in binding to cellular effectors. The important caveat of this experiment is that if differences were to be observed between the wild-type and mutant viruses, it would not be known for certain that it was due to a disruption of the $Pr60^{gag}$-c-Abl association. It is possible that other, as yet unidentified, proteins would also be unable to bind the mutated $Pr60^{gag}$.

**4- Expression of $Pr60^{gag}$ in v-Abl-transformed pre-B cell lines leads to an inhibition of their growth via a $CD8^+$ T cell-dependant mechanism**

As discussed in chapter 5, expression of $Pr60^{gag}$ in three v-abl-transformed pre-B cell lines led to their lack of growth in normal syngeneic C57BL/6 mice. Inoculation of one of these $Pr60^{gag}$-expressing cell lines into C57BL/6 $CD8^{-/-}$ mice restored their ability to induce tumors, strongly suggesting that the lack of tumor formation by these cells in normal C57BL/6 mice resulted from a $CD8^+$ T cell-mediated response directed towards these cells. Extension of these studies using $Pr60^{gag}$-expressing B16F1 melanoma cells led to an increased latency of tumor formation of these cells compared to the control or parental cells. These results

were less dramatic compared to what was seen with the $Pr60^{gag}$-expressing pre-B cells, yet they were still statistically significant ($P<0.0001$). These results point to an unexpected potential role for $Pr60^{gag}$ as either a tumor rejection antigen, an inducer of a potential tumor antigen or as a regulator of other mechanisms which influence host-tumor cell interaction. We are unable at this point to exclude any of these possibilities, nor are they mutually exclusive.

Previous studies on the observed mouse strain differences in susceptibility to the MAIDS defective virus focused on the role of the $CD8^+$ T cells (16,20,46,60). It was initially believed that $CD8^+$ T cells were responsible for mediating resistance to this disease in resistant strains of mice, based on studies in which resistant mice were made susceptible by *in vivo* antibody depletion of their $CD8^+$ T cells (46). More recent studies, however, paint a more complex picture, with both $CD8^+$ T cell-dependant and –independent mechanisms postulated to play a role in mediating resistance. MAIDS-resistant mice deficient in $CD8^+$ T cells due to a targeted mutation of the $\beta_2$-microglobulin gene show an increased, but not complete susceptibility to MAIDS (69). Similar results were obtained with mice deficient in perforin (69), indicating that although $CD8^+$ T cells appear to be responsible for mediating resistance to MAIDS via a perforin-dependant mechanism, other factors contribute to resistance as well. It would be of interest to examine if the rejection of the MAIDS defective virus-infected 203-33 cells, which is known to be $CD8^+$ T cell-mediated, is effected via perforin. This could simply be done by inoculating these cells as well as the parental controls into mice which are genetically deficient in perforin production.

Another interesting point raised by these results is why these v-abl-transformed pre-B cells are capable of inducing a $CD8^+$ T cell-dependant immune response whereas the *in vivo* target cells of the MAIDS defective virus are seemingly incapable of doing so. Several reasons may explain this seeming contradiction. Firstly, the MAIDS defective virus $Pr60^{gag}$ may be more efficiently processed and presented to T cells in the v-abl-transformed cells than in its *in vivo* target cell population. This possibility could also contribute to the range of susceptibility to the disease-inducing potential of the MAIDS defective virus seen in different mouse strains. Some strains may be better able to process and present $Pr60^{gag}$ to T cells, and thereby avoid the disease by eliciting an immune response which eliminates the infected target B cells. Mice unable to eliminate the infected B cells due to an inability to present the viral peptides to T cells will therefore be at a great, although not absolute, risk for developing MAIDS. If such a mechanism is involved in mediating some aspects of resistance to MAIDS, it should be possible to shed light on this possibility by studying the fate of the infected target cells in resistant mice. These mice should show evidence of infection with the virus post-inoculation (e.g. by *in situ* hybridization), but there should be a steady decrease in the number of infected cells present at later times post-inoculation. $CD8^+$ T cells isolated from these mice could then be examined to ascertain whether they show any *in vitro* CTL activity. The caveat to this hypothesis is that even if anti-$Pr60^{gag}$ activity is shown in $CD8^+$ T cells from resistant, but not susceptible, mice it will still not be clear if susceptibility is due

to a defect in the $CD8^-$ T cells or in the infected target cells of the susceptible mice.

A second possibility for explaining the difference in immune system responsiveness to the v-abl-transformed pre-B cells compared to that of the *in vivo* target cells of the MAIDS defective virus may be that the latter population may express surface molecules which are incapable of providing sufficient "help" to elicit an immune response or which actively suppress the generation of an immune response. Although we do not have evidence to support this hypothesis, there is precedent for this mechanism in other systems (21,23,53,56) and therefore warrants further examination in the case of MAIDS.

**5- How do the MAIDS defective virus-infected target B cells trigger disease?**

It is clear from the work of others (4,40), as well as from the data presented in this thesis, that infection of the target B cells with the MAIDS defective virus is the initiating event in the disease process and that these infected B cells are central to the subsequent immunodeficiency which develops in tandem with their expansion. Despite this knowledge, little is known about the mechanism whereby these MAIDS defective virus-infected B cells cause the immunodeficiency seen in susceptible mice. In contrast to human AIDS, where the preponderance of evidence points to a depletion of $CD4^+$ T cells as a contributing cause of immunodeficiency (27,38,70), no such depletion of any T cells is seen in MAIDS. In fact, the absolute number of both B and T cells in MAIDS animals actually increases during the course of disease (5,28,48,61).

Despite this increase in B and T cell numbers, a corresponding polyclonal B and T cell anergy also develops (33,34,47), which is the underlying cause of MAIDS.

The work presented in this thesis has given us new tools to exploit in further studying MAIDS, and points to several potential mechanisms by which infection of the target B cells with the MAIDS defective virus could induce the polyclonal B and T cell anergy seen in MAIDS. Firstly, by isolating two independent cell lines which accurately reflect the phenotype of the *in vivo* target cells of this virus, we can now more easily study *in vitro*, rather than *in vivo*, the B-T cell interactions which appear to be at the heart of the immunodeficiency. One important avenue of research in this area could focus on using these SD1 and CSTB5 $Pr60^{gag}$-expressing cell lines to induce anergy or a decrease in mitogen responsiveness in primary T cells or in T cell lines. Further manipulations of the system could then be made to study the contributions of cell-cell contact and/or cytokines to this induction of anergy. *In vitro* success in this area could then be applied to preventing or reversing MAIDS in mice and may also suggest novel avenues of experimentation in the area HIV/AIDS research.

The phenotype of the SD1 and CSTB5 cell lines, as described above and in chapter 3 of this thesis, supports the hypothesis that infected target B-T cell contact is important for inducing immunodeficiency. It has in fact been shown by others that blocking the CD40-gp39 interaction in mice infected with the LP-BM5 MAIDS virus mixture abrogates the disease (19). The CSTB5 B cell line expresses strong levels of CD40, and both cell lines express the co-stimulatory molecule B7-2. As well, the SD1 cell line expresses the CD43 marker. These

three molecules have been implicated in B-T cell signalling and it is quite possible that they are involved in the B-T cell signalling which contributes to the development of MAIDS. The fact that the SD1 and CSTB5 cell lines do not appear to have a fully mature phenotype may also be of interest. Normally, B-T cell interactions involve mature populations, both of which are presumed to be capable of giving signals as well as receiving them. If the target cell population of the MAIDS defective virus is not a mature one, or if expression of $Pr60^{gag}$ in a mature B cell population leads to a "regression" in its signalling capacity, it is possible that the T cells which come into contact with these cells will receive an "incomplete" signal which will lead to their anergy rather than to their activation. The delivery of an "incomplete" signal to the T cells by $Pr60^{gag}$-expressing B cells may be a mechanism by which the MAIDS defective virus induces anergy in susceptible mice.

The use of the SD1 and CSTB5 cell lines was crucial in confirming an *in vivo* association between $Pr60^{gag}$ and c-abl. This is the first direct evidence that $Pr60^{gag}$ may act as a signalling molecule, either in a negative or positive manner. The fact that c-Abl is involved in both the regulation of both apoptosis and differentiation (9,12), combined with the unusual phenotype of the *in vitro* and *in vivo* target cells of the MAIDS defective virus, suggests that the interaction between $Pr60^{gag}$ and c-Abl may be responsible for some aspects of the novel characteristics of these infected cell populations. Further, the interaction of $Pr60^{gag}$ with c-Abl (or other proteins) in infected cells may "reprogram" the

$Pr60^{gcs}$-expressing cells to adopt a novel state of differentiation which leads to proliferation and subsequent immunodeficiency.

It is also highly likely that other molecules are recruited by $Pr60^{gag}$ to the plasma membrane where they may become activated and/or may contribute to aberrant intracellular signalling due to their association with $Pr60^{gag}$. At a minimum, it is probable that the $Pr60^{gag}$-c-Abl association disrupts the usual associations between c-Abl and its known binding partners (8,62) which may lead to altered c-abl activity. The interaction between the proline-rich p12 region of $Pr60^{gcs}$ and other SH2 domain-containing proteins may be another mechanism by which the MAIDS defective virus activates its target cell population and alters it in such a way so as to trigger both B and T cell dysfunction. This could be achieved by either causing an increase in the activity of these as yet unidentified molecules, or by blocking their inhibitory properties. The identification of other binding partners of $Pr60^{gag}$ will be of major importance in clarifying how this viral protein induces both the cell proliferation and immunodeficiency which result in MAIDS.

One of the earliest proposed mechanisms by which the MAIDS defective virus induces MAIDS revolved around the theory that $Pr60^{gag}$ acts like a superantigen (31,35,61). Although this theory has been largely discredited (13), it does not rule out the possibility that antigenic stimulation of T cells by $Pr60^{gag}$ is a factor in the development of MAIDS. In fact, it has been shown that the presence of MHC class II molecules is required for the development of MAIDS (18). However, it was not clear which population was required to express MHC class II

in order for MAIDS to develop. To answer the question of whether antigenic stimulation of the immune system is critical for the development of the disease, we could use gene knockout technology to produce chimeric mice which contain two populations of B cell which express or do not express MHC class II molecules. Inoculation of these mice with the MAIDS defective virus should still trigger MAIDS. If antigenic stimulation (i.e. infected B-normal T cell interaction) is required for the induction of MAIDS only those B cells which express MHC class II should proliferate. This proliferation could be assessed by a combination of immunohistochemistry/*in situ* hybridization to simultaneously detect the infected cells and the expression of MHC class II molecules.

Although MAIDS is induced by a relatively "simple" virus, the disease induced by this virus is very complex, involving both B cells, T cells, and perhaps other cell populations. As discussed above, there exists many possible mechanisms, many of them testable, by which the MAIDS defective virus $Pr60^{gag}$ could affect the immune system of its host. The results presented in this thesis have shed light on the earliest events of this disease which do not require $CD4^+$ T cells. The isolation and characterization of two cell lines which are representative of the *in vivo* target cell population of the MAIDS defective virus was also revealing in terms of their unusual phenotype. These lines substantiated the $Pr60^{gag}$-c-Abl association seen *in vitro* and also now give us the tools to further examine the B-T cell interactions which lie at the heart of MAIDS. The extension of the $Pr60^{gag}$-c-Abl studies was fruitful in revealing the potential application of $Pr60^{gag}$ as a tumor rejection antigen and as a possible cancer vaccine.

Understanding the many complexities of MAIDS can potentially benefit many areas of research, from signal transduction to the control of cancer.

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## Claims for Original Research

1) The studies involving the use of mutant mice, specifically the $CD4^{-/-}$ mice, provide us with new insights into the earliest phase of the disease, the infection of the target B cells by the MAIDS defective virus. MAIDS can now be seen as proceeding in two distinct steps- a $CD4^{+}$ T cell-independent infection of the target B cells by the MAIDS defective virus and a $CD4^{+}$ T cell-dependant expansion of the infected B cells which is required for the full development of MAIDS.

2) We have succeeded in isolating two independent cell lines which accurately reflect the phenotype of the *in vivo* target cells of the MAIDS defective virus. These lines will be invaluable tools to further study the $Pr60^{gag}$-B cell and the infected B cell-T cell interactions which lie at the heart of MAIDS.

3) The SD1 and CSTB5 cell lines described in this thesis were critical in proving an *in vivo* association between $Pr60^{gag}$ and c-Abl and will again be useful in isolating other binding partners of $Pr60^{gag}$.

4) The expression of $Pr60^{gag}$ in several v-Abl-transformed pre-B cell lines and in the B16F1 melanoma cell line led to a significant reduction in the tumoriginicity of these cell lines. This is the first evidence that $Pr60^{gag}$ can induce a protective immune response, either directly or indirectly, in addition to its ability to cause immunodeficiency in the same mouse strain.