Studies of the pathogenesis of Jembrana disease virus infection in *Bos javanicus*

I Wayan Masa Tenaya
D.V.M, M.Phil.

This thesis is presented in fulfilment of the requirements for the degree of Doctor of Philosophy

2010

Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for publication or degree at any other tertiary educational institution. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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I Wayan Masa Tenaya

2010

Abstract

Jembrana disease was reported initially in Bali cattle (*Bos javanicus*) on Bali island in 1964 and the causative agent was subsequently identified as a bovine lentivirus and designated *Jembrana disease virus* (JDV). This atypical lentivirus causes an acutely pathogenic disease that is associated with clinical signs and pathological lesions attributable to a disease primarily affecting the lymphoid system. Based on the intense proliferation of cells in the parafollicular (T-cell) areas of lymphoid tissue it has been assumed that the cellular tropism of the virus was for T-cells.

An initial investigation of the pathological changes following JDV infection provided morphological evidence that JDV infection occurred not in T-cells but probably in centroblast-like cells containing IgG and presumably of B-cell lineage. The identity of the infected cells was confirmed by double immunofluorescence labelling techniques as being IgG-containing CD79α^+ cells, indicating that the virus replicated in mature B-cells. Unlike other lentiviruses, no evidence of infection in T-cells or macrophages was obtained. These observations provide an explanation for suppression of the JDV-specific antibody response associated with JDV infection and the unique nature of the pathological response of Bali cattle to JDV infection.

Flow cytometric analysis of peripheral blood leucocyte populations was used to further the understanding of the pathogenesis of JDV infection. Changes in lymphocyte subsets during the course of Jembrana disease were investigated and analysis of the results showed that lymphopenia, a characteristic of the acute febrile phase of Jembrana disease, was at least partly due to a significant decrease in $CD4^+$ and $CD8^+$ T-cells and $CD21^+$ B-cells. In the immediate post-febrile recovery phase, virus-infected cells were not detected in lymphoid tissue but both $CD8^+$ T-cells and $CD21^+$ B-cells increased significantly and $CD4^+$ T-cells remained below normal levels resulting in a significantly reduced $CD4^+$:$CD8^+$ ratio.

Changes in expression of $CD8^+$ T-cell regulated cytokine genes was examined during the course of the acute disease process by quantifying cytokine mRNA expression using real-time reverse-transcription polymerase chain reaction (RT-PCR). The results showed that both IL-2 and IFN-γ cytokine mRNA were strongly expressed during the febrile and early post-febrile recovery phases, which coincided with the significant increase of $CD8^+$ T-cells and

reduction of viraemia during this phase. The results suggested the $CD8^+$ T-cell–associated cytokines IL-2 and IFN-γ probably play a significant role in the recovery process.

Acknowledgements

I would like to sincerely thank my principal supervisor, Prof. G. E. Wilcox, for his interest, support and invaluable guidance and assistance throughout the course of my PhD, especially his assistance in the preparation of this thesis. I am very grateful. I am deeply indebted to my co-supervisors, Dr. Moira Desport, Dr. Alexander McLachlan and Dr. Phil Stumbles for their advice and assistance during the initial stages of this work. I would also like to thank Assistant Prof. Kathy Heel and staff of the Centre for Microscopy, Characterisation and Analysis, the University of Western Australia, for allowing me to visit their laboratory and in guiding me during the experimental work described in Chapter 5 of this thesis.

This study was made possible by the provision of an Australian Centre for International Agricultural Research (ACIAR) John Allwright Fellowship for which I am most gratefully indebted to the Australian Government. I also wish to thank the Indonesian Government for giving me the opportunity to undertake postgraduate study in Australia. Many thanks are also addressed to the former and the current director of "Balai Besar Veteriner VI Denpasar" for allowing my absence during my time in Australia. I would like to express my appreciation to all staff members and technicians at the Laboratory Biotechnology "Balai Besar Veteriner VI Denpasar" for their help and humour when I collected my samples.

I would like to take this opportunity to thank all of my colleagues especially the post graduate students in the Veterinary Virology group: Joshua Lewis (for making good photo images), Tegan McNab (for helping me with RT-PCR techniques and providing homemade cakes), Linda Davies (for explaining English grammar) and Judhi Rachmat (for his Indonesian jokes). Many thanks also to all students, researchers and staff at the School of Veterinary and Biomedical Sciences and the WA State Agricultural Biotechnology Centre for their assistance and friendship.

Finally, I wish to express my sincere gratitude to my parents, brother, sisters and especially to my wife Nyoman Wiratningsih, my children Wayan Intan Martinez, Made Oni Martinez and Komang Melina for their support, encouragement and understanding while I was away from home for such a long time.

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List of publication arising from this thesis

Published paper

Moira Desport, **I.W. Masa Tenaya**, Alexander McLachlan, Tegan J. McNab, Judhi Rachmat, Nining Hartaningsih, Graham E. Wilcox (2009) *In vivo* infection of IgG-containing cells by Jembrana disease virus during acute infection. Virology 223: 221-227.

Paper submitted for publication

I Wayan Masa Tenaya, Phil Stumbles, Kathy Heel, Alexander McLachlan, Tegan Josephine McNab, Moira Desport , Nining Hartaningsih and Graham E. Wilcox. Flow cytometric analysis of changes in lymphocyte subsets in Bali cattle experimentally infected with Jembrana disease virus.

I Wayan Masa Tenaya, Alexander McLachlan, Tegan J. McNab, Linda. J. Davies, M. Slaven, G. Spoelstra, N. Hartaningsih, Graham E. Wilcox and Moira Desport . Histopathological and Immunohistochemical Characterisation of Experimentally Induced Jembrana Disease in Bali Cattle.

Oral presentations

I Wayan Masa Tenaya, Alexander McLachlan, Moira Desport and Graham E Cellular tropism of Jembrana disease virus in Bali cattle. Proceedings of the 19th Annual Combined Biological Science Meeting, Perth, UWA (2009).

Poster presentations

I Wayan Masa Tenaya, Alexander McLachlan, Moira Desport and Graham E. Wilcox. Cellular response to Jembrana disease virus in infected cattle. Murdoch University Poster Day (2007). **Prize for best 1st year poster**.

I Wayan Masa Tenaya, Alexander McLachlan, Moira Desport and Graham E. Wilcox. Target cell of Jembrana disease virus in Jembrana disease virus-infected cattle. Murdoch University Poster Day (2008). **Dean's prize for best overall poster**.

I Wayan Masa Tenaya, Alexander McLachlan, Phil Stumbles, Moira Desport and Graham E. Wilcox. Target cell of Jembrana disease virus in infected cattle. Murdoch University Research Poster (2009). **Prize for best immunological poster**.

I Wayan Masa Tenaya, Alexander McLachlan, Phil Stumbles, Moira Desport and Graham E. Wilcox. Target cell of Jembrana disease virus in infected cattle. Proceedings of the 19th Annual Combined Biological Science Meeting, UWA (2009).

Best animal research poster

I Wayan Masa Tenaya, Alexander McLachlan, Phil Stumbles, Moira Desport and Graham E. Wilcox. Target cell of Jembrana disease virus in infected cattle. Proceedings of the Australian Virology Group Meeting, Lorne (2009).

Abbreviations used in this thesis

aa	:	Amino acid
APC	:	Antigen presenting cell
BCIP	:	5-Bromo-4-chloro-3-indolyl phosphate
BIV	:	Bovine immunodeficiency virus
BLV	:	Bovine leukaemia virus
bp	:	Base pair
BSA	:	Bovine serum albumin
CA	:	Capsid protein
CAEV	:	Caprine arthritis-encephalitis virus
CCR5	:	C-C (beta) chemokine receptor 5
CD	:	Cluster of differentiation
cDNA	:	Complementary DNA
CTL	:	Cytotoxic T lymphocyte
CXCR4	:	C-X-C (alpha) chemokine receptor 4
DAB	:	3-3'diaminobenzidine
DAPI	:	4',6-diamino-2-phenylindole
DIG	:	Digoxigenin
DMEM	:	Dulbecco's modified Eagle's medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP)
dsDNA	:	Double-stranded DNA
EBP	:	Enhancer binding proteins (C/EBP)
EIAV	:	Equine infectious anaemia virus
FACS	:	Fluorescence-activated cell sorting
FITC	:	Fluorescein isothiocyanate
FIV	:	Feline immunodeficiency virus
Gag	:	Group- specific antigen
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
gp	:	Glycoprotein
HNPP	:	2-hydroxy-3-naphtoic acid-2-phenylanillide phosphate
HRP	:	Horseradish peroxidase
HIV		Human immunodeficiency virus
ID_{50}	:	50% Infectious dose
IL	:	Interleukin
IN	:	Integrase protein
IPTG	:	Isopropyl-β-thiogalactopyranoside
JDV	:	Jembrana disease virus
LSAB	:	Labelled streptavidin-biotin
LTR	:	Long terminal repeat
MA	:	Matrix protein
MAb	:	Monoclonal antibody
MHC	:	Major histocompatibility complex
MHR	:	Major homology region
mRNA	:	Messenger RNA
MVV	:	Maedi visna virus
NC	:	Nucleocapsid protein

Nef	:	Negative factor
OD	:	Optical density
ORF	:	Open reading frame
PIC	:	Pre-integration complex
PR	:	Protease
Rev	:	Regulatory of expression of virion protein
RNA		Ribonucleic acid
RSV	:	Rous sarcoma virus
RT	:	Reverse transcriptase
SD	:	Standard deviation
SIV	:	Simian immunodeficiency virus
SIVagm	:	Simian immunodeficiency virus African green monkey
SIVcpz	:	Simian immunodeficiency virus chimpanzee
SIVsm	:	Simian immunodeficiency virus sooty mangabey monkey
SPSS	:	Statistical package for the social sciences
SRLV	:	Small-ruminant lentiviruses
SSC	:	Sodium chloride and sodium citrate buffer
ssDNA	:	Single-stranded DNA
ssRNA	:	Single-stranded RNA
SU	:	Surface unit glycoprotein
TAR	:	*Trans*-activating response element
TAT	:	*Trans*-activator of transcription protein
TE	:	Tris-EDTA buffer
Th1	:	T helper 1
Th2		T-helper 2
Tm	:	Melting temperature of dsDNA
TM	:	Transmembrane glycoprotein
TNF-α	:	Tumour necrosis factor -alpha
X-gal	:	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

List of units

^{o}C	:	degrees Celsius
µg	:	microgram
µl	:	microlitre
µm	:	micrometre
µM	:	micromolar
ρmol	:	picomoles
bp	:	base pairs
g	:	grams
g	:	times gravity
h	:	Hour
ID_{50}	:	50% infectious dose
kb	:	kilobases
kDa	:	kiloDalton
ng	:	nanograms
nm	:	nanometre
rpm	:	revolutions per minute
U	:	Unit
V	:	Volts
v/v	:	volume per volume
w/v	:	weight per volume

Chapter 1

General introduction

The research results reported in this thesis involved a study of the cellular response to *Jembrana disease virus* (JDV), an acutely pathogenic bovine lentivirus causing Jembrana disease in Bali cattle (*Bos javanicus*) in Indonesia, with the specific aim of identifying the principal target cell of JDV. As a background to the investigations that were undertaken, a review of the literature on lentiviruses was undertaken and is provided in Chapter 2. The review included information on lentiviruses and included the bovine lentiviruses, JDV and the closely related *Bovine immunodeficiency virus* (BIV). The review concentrated on aspects of the pathogenesis of the various lentivirus diseases, the cellular tropism and the immune response to virus infection. Potential methodologies for investigating cellular responses to lentiviral infections were also reviewed.

An initial investigation of the histopathological lesions associated with JDV infection, in the febrile and immediate post-febrile phases, was undertaken and is reported in Chapter 3. The animal infections associated with this investigation were conducted in Indonesia and the study required the development of methods that could be used for the detection of JDV-infected cells and the identity of the various leucocyte subsets in formaldehyde-fixed tissues that could be imported into Australia from Indonesia. The characterisation and distribution of T-cells, B-cells and monocytes/macrophages was determined, as was the distribution of JDV-infected cells, in various lymphoid and visceral tissues of the infected animals.

The investigations described in Chapter 3 determined that JDV antigen-positive cells were probably antibody-producing cells, based on morphological observations and the distribution patterns of various leucocyte subsets. To confirm these observations, double immunofluorescence labelling techniques were developed for use on fixed tissues and then used to identify possible JDV infection in lymphocyte subsets and macrophages. These results are reported in Chapter 4.

To provide insights into the possible mechanism of recovery from the acute disease process associated with JDV infection, further animal infections were conducted and fixed peripheral blood leucocyte preparations were imported and used for analysis of

the changes in $CD4^+$ T-cells, $CD8^+$ T-cells and $CD21^+$ B-cells. These results are reported in Chapter 5.

Cytokines have an important role in both the inflammatory disease process and recovery from infection, and a preliminary investigation of cytokine expression during the acute Jembrana disease process was undertaken. These results are reported in Chapter 6. A marked up-regulation of the pro-inflammatory cytokines IFN-γ and IL-2 was found to correlate with the significant $CD8^+$ T-cell proliferation that had been detected during the recovery phase of the disease.

A general discussion of the major conclusions and recommendations for further investigations as a consequence of the research reported in Chapters 3-6 is presented in Chapter 7.

Chapter 2

Review of the literature

This Chapter presents a review of literature relevant to the research project, and contains 5 major sections. The first section contains general information on the classification and properties of viruses in the family *Retroviridae*. The second section focuses on the features of the genome and replication of viruses in the genus *Lentivirus*. The third section describes aspects of Jembrana disease including the historical aspects, subsequent identification of the causative agent as a lentivirus, mode of transmission, clinico-pathology and diagnostic methods that are useful to confirm the disease. The fourth section reviews the literature relating to lentivirus infections in other host species. The final section reviews aspects of the immune response to lentivirus infections with particular emphasis on changes in lymphocyte subpopulations and cytokine expression.

General features of *Retroviridae*

The *Retroviridae* (retroviruses) comprise a large and diverse group of viruses found in all vertebrate cells and include many important human and animal pathogens. Viruses in this family are characterised by their unique life cycle and replication mechanisms that utilise an essential reverse transcriptase to convert the viral single-stranded RNA (ssRNA) into a linear double-stranded DNA (dsDNA), entry and integration of this dsDNA into the genome of the host cells to form proviral-DNA. Transcription of RNA from the integrated proviral DNA with subsequent translation to form virus-coded proteins then results in the formation and release of new progeny virus (Baltimore, 1970; Luciw and Leung, 1992; Shibagaki and Chow, 1997)

Morphologically, retroviruses share a roughly similar structure but there are unique features of different genera. They are all typically 80-130 nm in diameter, enveloped, and have an inner capsid enclosing 2 copies of a positive-sense ssRNA genome (Flint et al., 2004a; Flint et al., 2004b). The envelope is derived from the host cell plasma membrane during the budding process and has inserted into it 2 viral-coded glycoproteins, the surface unit (SU) and trans-membrane (TM) glycoproteins. The envelope directly surrounds the matrix (MA) protein and the

internal capsid formed by the capsid (CA) protein that contains 2 identical copies of the viral RNA genome bound by nucleoproteins, and several viral encoded enzymes including reverse transcriptase (RT), integrase (IN), and protease (PR) (Coffin, 1992; Goff, 2007) (Figure 2.1).

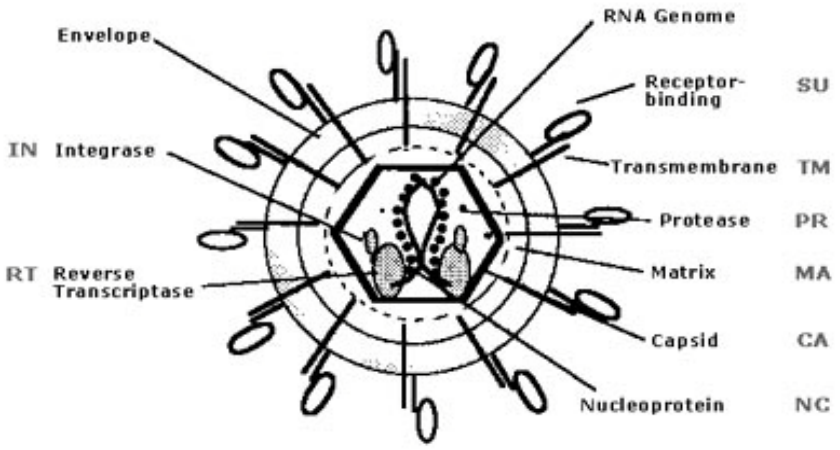


Figure 2.1. Diagrammatic representation of the structure of a typical mature virion of retrovirus. Reproduced from Coffin (1992).

Retroviruses can be divided into 3 subgroups based on their pathogenicity in host cells: spumaviruses, oncornaviruses, and lentiviruses (Burmeister, 2001; Wagner and Hewlett, 2004). They have also been grouped into 4 morphological types A, B, C and D, depending on their morphology during budding and maturation that can be observed by electron microscopy (Coffin, 1992; Luciw and Leung, 1992). Current taxonomy based on further genomic analysis distinguishes the retroviruses into 7 genera: *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, Spumavirus* and *Lentivirus* (Burmeister, 2001; Goff, 2007).

The genera *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus* are the oncogenic retroviruses and they may trigger a variety of leukaemias and sarcomas in several animal species including man (Burmeister, 2001). Members of the genus *Alpharetrovirus* cause sporadic lymphoid leukosis in avian species and these viruses are endemic in chicken flocks around the world. The genus *Betaretrovirus* contains 3 members that produce tumours in mammalian

species: *Mouse mammary tumour virus*, *Mason-Pfizer monkey virus* and *Jaagsiekte virus* (Bauerova-Zabranska et al., 2005; Cousens et al., 2004; Maeda et al., 2001). Viruses in the genus *Gammaretrovirus* are also responsible for leukaemias in mammalian species including cats, sheep and gibbons. The genus *Deltaretrovirus* contains the human and simian T-cell lymphotropic viruses causing T-lymphomas and neurological disorders in man and non-human primates, respectively, and *Bovine leukaemia virus* (Burmeister, 2001). The genus *Epsilonretrovirus* includes viruses of fish and reptiles, many of which are associated with tumour induction. Viruses in the genus *Spumaretrovirus* are also termed "foamy" viruses because of the nature of their cytopathic effects *in vitro*, characterised by marked syncytium formation, cytoplasmic vacuolation and cell death, but they have not been reported to cause disease *in vivo* (Coffin, 1992; Flint et al., 2004b; Jones-Engel et al., 2005; Meiering and Linial, 2001). Viruses in the genus *Lentivirus* induce a variety of clinical syndromes including immunodeficiencies in man, non-human primates and feline species, and chronic pneumonia, arthritis and encephalitis in sheep and goats (Chadwick et al., 1995a; Goff, 2007).

The genera *Alpharetrovirus*, *Betaretrovirus* and *Gammaretrovirus* are considered "simple retroviruses" as they encode only the 3 principal open reading frames (ORFs) *gag*, *pol* and *env* and require actively dividing cells for replication. The other genera are considered "complex retroviruses" as while they also encode the 3 principal ORFs they also encode a number of accessory proteins that are important for replication in non-dividing cells (Chen and Temin, 1982; Goff, 2007; Pfeifer et al., 2002).

Characteristics of lentiviruses

This genus contains species that have been divided into 5 groups based on their host specificities: primate lentiviruses (*Human immunodeficiency virus* [HIV-1 and-2] and *Simian immunodeficiency virus* [SIV]), bovine lentiviruses (*Bovine immunodeficiency virus* [BIV] and *Jembrana disease virus* [JDV]), equine lentiviruses (*Equine infectious anaemia virus* [EIAV], feline lentiviruses (*Feline immunodeficiency virus* [FIV] and ovine/caprine lentiviruses or the small lentivirus group (*Maedi-visna virus* [MVV] and *Caprine arthritis-encephalitis virus* [CAEV]).

A phylogenetic tree of several of these lentiviruses based on their *pol* genes is shown in Figure 2.2.

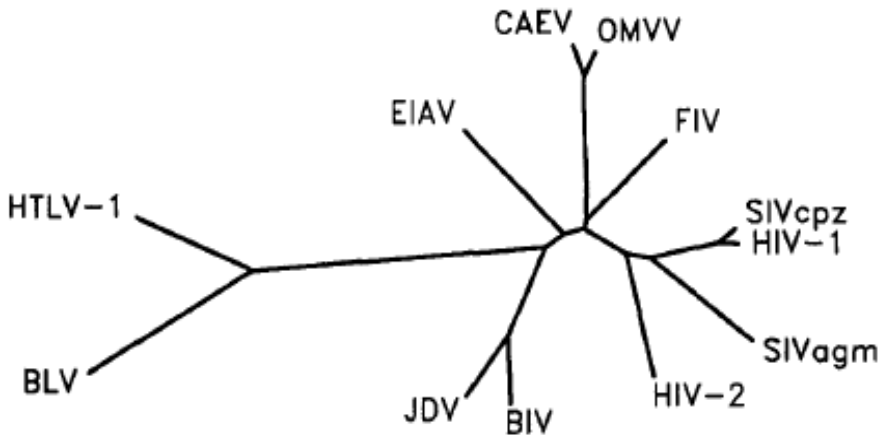


Figure 2. 2. Phylogenetic tree of lentivirus based on complete *pol* gene sequences. The relationship of 10 lentiviruses in 5 different host groups and 2 members of the leukaemia group of retroviruses (as outliers) are shown. JDV, *Jembrana disease virus*; BIV, *bovine immunodeficiency virus*; HIV-1, *Human immunodeficiency virus*-1; HIV-2, *Human immunodeficiency virus*-2; SIV_{agm}, *Simian immunodeficiency virus* (African green monkey); SIV_{cpz}, SIV (chimpanzee); FIV, *Feline immunodeficiency virus*; OMVV, *Maedi-visna virus*; CAEV, *Caprine arthritis encephalitis virus*; EIAV, *Equine infectious anaemia virus*; HTLV-1, *Human T- lymphotropic virus* type 1 and BLV, *Bovine leukaemia virus*. Figure sourced from Chadwick et al. (1995a).

Lentiviruses induce a number of clinical diseases, in a diverse array of mammalian hosts. They are typically slowly progressive diseases affecting a variety of organs depending on the virus involved, usually with long incubation periods and suppressed immune responses, that invariably lead to death (Clements and Zink, 1996; Goff, 2007). There are, however, a number of exceptions to this generalisation. At least 3 viruses, JDV, $SIV_{smmPBj14}$, and EIAV induce acute clinical diseases (Chadwick et al., 1995b; Fultz, 1991; Issel and Coggins, 1979; Sellon et al., 1994). Infection does not always lead to clinical disease or a fatal outcome: in some SIV infections the virus does not produce disease in its natural host, and disease occurs only if the virus is introduced into a different primate species (Brown et al., 2007; Cranage et al., 1992; Veazey et al., 2000). Some lentiviruses do not cause an invariably fatal disease: JDV infections do not always result in the death of animal

and the case fatality rate of the disease in experimentally infected animals is about 17% (Soeharsono et al., 1990). Recovered animals appear to be persistently immune and do not develop any further lentivirus-associated diseases. EIAV infections result in relapsing infections but animals that survive appear to eventually develop immunity and while still persistently infected appear able to control viral replication (Montelaro et al., 1993; Soesanto et al., 1990).

Lentiviruses differ from other retroviruses in that they lack the ability to induce neoplastic disease, they characteristically replicate in non-dividing/terminally differentiated cells and they are relatively species-specific (Clements and Zink, 1996; Lewis and Emerman, 1994). Their genome and replication cycle also differ and are more complex than the prototypic simple retroviruses (Vogt, 1997).

Genomic organisation of lentiviruses

As complex retroviruses, lentiviruses possess a number of regulatory and accessory genes that encode non-structural proteins in addition to those encoded by the 3 major ORFs *gag*, *pol* and *env* that are common to all retroviruses. They have similar genomic organisation to other genera, although there are species differences. The distinguishing feature of lentiviruses is the presence of additional and unique ORFs that encode accessory proteins involved in their replication (Vogt, 1997). The major distinctive regulatory and accessory genes of lentiviruses, found generally in the central region between the end of *pol* and the beginning of *env*, are shown in Table 2.1.

Table 2.1. Presence of accessory genes identified in lentiviruses.

Lentivirus	Regulatory and accessory genes									
	vif	*vpr*	*vpu*	*vpx*	*vpy*	*vpw*	*OrfA*	*nef*	*tat*	*rev*
HIV-1	+	+	+	ND	ND	ND	ND	+	+	+
HIV-2	+	+	ND	+	ND	ND	ND	+	+	+
SIV	+	+	+	+	ND	ND	ND	+	+	+
BIV	+	ND	ND	ND	+	+	ND	ND	+	+
JDV	+	ND	ND	ND	ND	ND	ND	ND	+	+
FIV	+	ND	ND	ND	ND	ND	+	ND	ND	+
SRLV	+	ND	ND	ND	ND	ND	ND	ND	+	+
EIAV	ND	ND	ND	ND	ND	ND	ND	ND	+	+

Data sourced from: HIV-1 and HIV-2(Flint et al., 2004b); SIV (Gibbs and Desrosiers, 1993); BIV (Gonda, 1994); JDV (Chadwick et al., 1995b); FIV (Zou et al., 1997); the SRLV (CAEV and MMV) (Harmache et al., 1995; Narayan et al., 1993); EIAV (Miller et al., 2000).

ND denotes not detected.

The most complex lentiviruses are the primate lentiviruses, typified by HIV-1 that has at least 6 additional genes including 2 regulatory genes (*tat* and *rev*) and 4 accessory/auxiliary genes (*vif*, *vpr*, *vpu*, and *nef*). The organisation of the 3 major ORFS and the additional genes of HIV-1 with the respective encoded proteins in the virion structure are presented in Figure 2.3. The *gag* ORF encodes the structural matrix (MA), capsid (CA) and nucleocapsid (NC) proteins. MA facilitates localisation of the protein in the cytoplasmic membrane which is associated with assembly of infectious virus (Coffin, 1992; Kiernan et al., 1998). CA serves as a major structural component that forms the core shell of the virion; it is the most immunodominant viral protein (Coffin, 1979; Coffin, 1992). CA has a highly conserved major homology region (MHR) responsible for antigenic cross-reactivity of many lentiviruses (Grund et al., 1994; Melamed et al., 2004). NC has an affinity

for the viral genome and is an essential protein for viral DNA synthesis is required for packaging RNA into the virion (Coffin, 1979; Coffin, 1992).

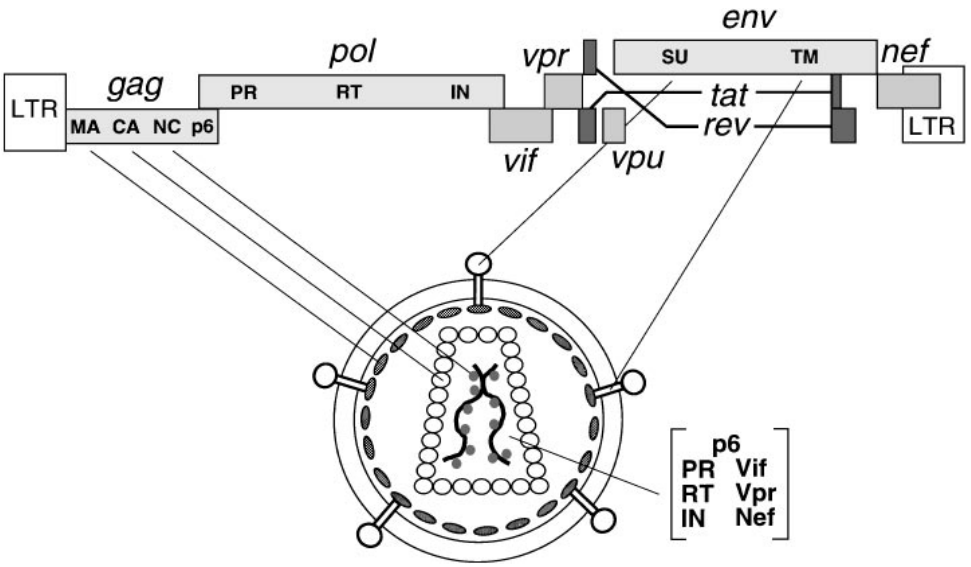


Figure 2.3. Structure and genomic organisation of HIV-1. Sourced from Frankel and Young (1998).

The *pol* ORF encodes 3 virion-associated enzymatic proteins, the reverse transcriptase (RT), integrase (IN), and protease (PR) that are incorporated into the capsid during assembly and are required for viral replication. RT has both RNAase and RNA-dependent polymerase activities that are essential for the transcription of the viral ssRNA genome to a dsDNA proviral form after entry of the virus into the host cell (Katz and Skalka, 1994; Temin, 1993). The gene encoding RT is relatively conserved among different genera of retroviruses, and therefore is useful for phylogenetic and evolutionary studies (Tobin et al., 1996). Integrase facilitates the stable integration of the provirus-DNA within the host cell DNA, and PR is essential for the proteolytic cleavage of the viral precursor polyproteins into individual subunit proteins during assembly and maturation of the virion (Coffin, 1979; Coffin, 1992). The PR therefore has a special role in the maturation of new virus, making it a major target for contemporary anti-viral therapy (Frankel and Young, 1998).

The *env* ORF encodes a polyprotein (Env) that is cleaved by proteases and modified in the endoplasmic reticulum to produce the 2 glycosylated envelope proteins, SU and TM (Coffin, 1992). The glycoproteins are translocated to and are incorporated into the host cell membrane, where the budding process takes place, and become exposed to the external environment (Luciw and Leung, 1992). The SU is required for the binding of the virus to target cell receptors. The TM projects from the envelope and anchors the SU component to the envelope and facilitates membrane fusion of the envelope with the plasma membrane of the host cell. Both TM and SU glycoproteins are determinants of tropism and virulence and are the major targets of the neutralising antibody response (Coffin, 1992).

Of the 6 additional (accessory) genes in lentiviruses, *tat* and *rev* control viral transcription and RNA transport and translation, and are expressed early in viral replication from multiply spliced transcripts (Chen et al., 1998; Feed and Martin, 2001; Frankel and Young, 1998; Miller et al., 2000). JDV Tat has a strong *trans*-activator ability and not only transactivates its own long terminal repeat (LTR) but is also able to strongly activate the heterologous BIV and HIV *in vitro*, to a greater extent than the homologous Tat proteins (Chen et al., 1999). The *vif* gene is found in the genome of all lentiviruses, with the exception of EIAV, and encodes the viral infectivity factor (Vif), a virion-associated protein that is functional during *in vivo* viral replication and antagonises the anti-viral activity of cellular components (Gonda, 1992; Li et al., 1998; Miller et al., 2000; Sheehy et al., 2002). The accessory genes *vpr, vpu*, *nef* and *vpx* are detected only in the genome of primate lentiviruses and have specific biological functions. The *vpr* and *vpx* genes encode nuclear proteins with a role in transporting the pre-integrated dsDNA from the cytoplasm into the nucleus of infected cells (Cheng et al., 2008; Hamaia et al., 1997). In HIV-2/SIV_{sm}/SIV_{mac} infections, Vpx is a virulence factor for monocyte-derived cells and dendritic cells, and is associated with the establishment of virus reservoir in macrophages (Fletcher et al., 1996; Goujon et al., 2007; Matsuda et al., 2009). Vpu is associated with release of the virion from the cell membranes (Binette et al., 2007; Ewart et al., 1996; Strebel et al., 1989). Nef is multifunctional, but mainly responsible for viral infectivity (Brugger et al., 2007; Marsh, 1999; Qi and Aiken, 2008; Sol-Foulon et al., 2004). The *nef* gene is not present in the bovine lentiviruses (Table 2.1) but they have a *tmx* gene in a similar location to *nef*, and 2 unique genes

vpw and *vpy* that seem to be analogous to the *vpr* and *vpu/vpx* genes of primate lentiviruses (Garvey et al., 1990).

Replication cycle of lentiviruses

The replication mechanism of lentiviruses involves conversion of the ssRNA genome to a dsDNA provirus and then integration of this provirus into the host cell DNA, typical of all retroviruses. The major steps are shown schematically in Figure 2.4. Briefly, the early phase commences with attachment of the envelope glycoproteins of the virus to a cellular receptor, CD4 in the case of HIV, in conjunction with a co-receptor, followed by fusion of the virus envelope with the plasma membrane of the host cell. Uncoated viruses then release their ssRNA genome which is converted into dsDNA using the virus RT and this dsDNA is transported into the nucleus. Subsequently the viral integrase mediates the integration of the viral dsDNA (proviral DNA) into the host genome. In the late phase, the proviral-DNA is transcribed using cellular enzymes to produce viral mRNAs, which then migrate to the cytoplasm for translation. The Env proteins undergo glycosylation and are inserted into the plasma membrane of the cell. Finally, the viral RNA and structural proteins are assembled into the immature virion core on the plasma membrane, and via a budding process is released from the cell to form free virus that then undergoes further maturation steps to form fully mature infectious virus.

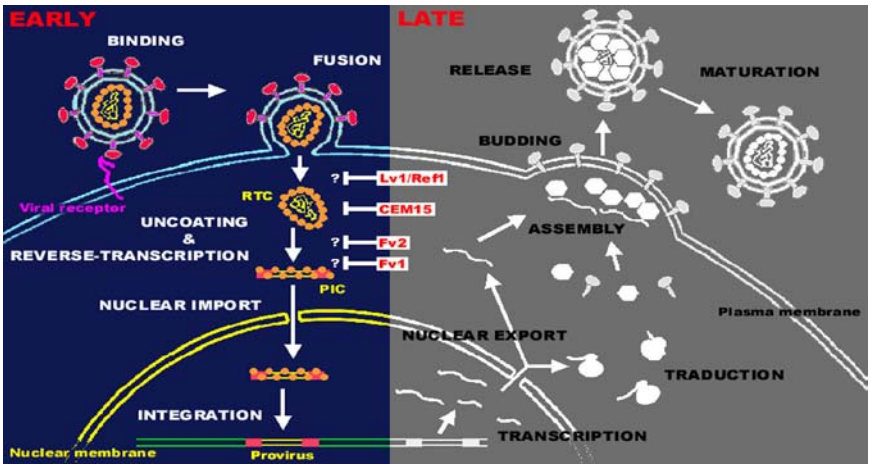


Figure 2.4. Major steps in the replication of a typical retrovirus. Reproduced from Nisole and Saib (2004).

Virion attachment and fusion to host cells

The first step in productive infection is the attachment of the virus to a host cell receptor, mediated by functional interactions between viral envelope glycoproteins and specific surface receptors on the target cell. This interaction is specific and selective between certain cell and virus types (Clapham and McKnight, 2002; Nisole and Saib, 2004; Overbaugh et al., 2001). This process has been extensively studied for HIV-1 and the related non-human primate lentiviruses as it offers a possible target for vaccines and antiretroviral therapy.

In HIV-1 infections, the attachment is mediated by the viral SU protein, gp120, to the host cell CD4 molecule. CD4 molecules are the major receptor on the surface of T-helper lymphocytes ($CD4^+$ cells) that are the primary targets of the virus, but are also present on other antigen-presenting cells such as macrophages, monocytes and dendritic cells. This interaction triggers a conformational change of the SU that enables the SU to contact host cell chemokine receptors. A variety of chemokine receptors are used, including CCR5 on macrophages, CXCR4 on $CD4^+$ cells, CCR3 on microglial cells of the brain, and a number of other co-receptors (Clapham and McKnight, 2001; Deng et al., 1996; He et al., 1997). These events in turn activate the TM to mediate fusion of the viral envelope with the host cell membrane (Clements and Zink, 1996; Sherman and Greene, 2002). Fusion leads to microinjection of the uncoated viral capsid component, containing the viral genome and enzymes, into the cytoplasm. Although HIV-1 is commonly viewed as a prototypical example of a virus that enters cells by fusion of the plasma membrane (Marsh and Helenius, 2006), more recently Miyauchi at al. (2009) reported that HIV-1 enters cells via endocytosis and complete viral fusion occurred subsequently in endosomes.

Reverse transcription and integration of viral genome into the cellular genome

When virus enters into the cytoplasm, it is partially uncoated and the viral ssRNA is converted into dsDNA provirus via RT activity. This reaction is common to all retroviruses, and takes place within the viral nucleocapsid complex in the cytoplasm and is initiated by the RT and RNAse H activity of the viral RT that is packaged into the viral RNA (Zhang et al., 1993). The first step in the reverse transcription process is the binding of the cellular tRNA (tRNALys) to the primer binding site (PBS)

located in the 5'LTR of the viral genomic plus-stranded RNA (Freed and Martin, 2001; Gerdts et al., 1997). Subsequently, the minus-sense ssDNA that is complementary to the 5'U5 and R region is synthesised and the RNA portion of the newly formed RNA/DNA hybrid is digested by the RNAseH activity. The new minus-sense ssDNA is transferred to the 3'end of the RNA and the plus-stranded 3'R sequence hybridises with the R sequence. The minus sense DNA is elongated and most of the plus-sense RNA is then digested; only the polypurine tracts (PPT) are stable and these serve as a primer to synthesise the 3' part of the complementary strand of DNA. RNAse H then removes the PPT RNA sequences and a second strand transfer of the minus-sense DNA strand permits the hybridisation of PBS sequences of both DNA strands. At the end of this process, 3'ends for the completion of the synthesis of both minus and plus strand of DNA are provided to make the linear dsDNA that is contained in the pre-integration complex (PIC) ready for integration (Miller et al., 1997; Nisole and Saib, 2004).

Integration is not always successful and several blocks may occur before integration into the DNA of resting $CD4^+$ lymphocytes (Wong et al., 1997). Such blocks may delay reverse transcription due to a limited pool of dNTPs or to the inability to import the PIC into the nucleus. Because of these blocks, full-length viral DNA molecules can remain in the cytoplasm, and this is known as pre-integration latency (Bukrinsky et al., 1992; Korin and Zack, 1999; Lassen et al., 2004; Zack et al., 1990). Although, replication is delayed in this circumstance, mitotic stimuli are able to trigger further replication (Bukrinsky et al., 1991; Finzi et al., 1997; Zack et al., 1992).

Details of the integration of provirus with the PIC component and its migration into the nucleus remains unclear, as *in vitro* studies do not fully reproduce *in vivo* integration events (Devroe et al., 2003; Fletcher et al., 1997). In the simple retroviruses the viral PIC requires dividing cells for nuclear importation (Lewis and Emerman, 1994; Roe et al., 1993) but with lentiviruses this process can occur in non-dividing cells (Connolly, 2002; Lewis et al., 1992b; Naldini, 1998; Vodicka, 2001). The proviral DNA is assumed to cross the nuclear pore complex from the cytoplasm to enter the nucleus where it serves as precursor for the formation of the integrated virus dsDNA (Brown, 1997; Jenkins et al., 1998). The process of integration involves a specific interaction between the IN and 2 inverted repeats located at the

end of each LTR (Esposito and Craigie, 1998). As a product of integration, this process forms a gapped intermediate where the non-joined 5'-viral DNA ends are flanked by short single-stranded gaps in the host DNA, and typically HIV-1 integration sites are in introns of the relevant genes (Engelman, 2003; Esposito and Craigie, 1998; Lassen et al., 2004). It was reported that SIV integration preference is similar to that of HIV-1, suggesting that both lentiviruses may share a similar mechanism for target site selection (Crise et al., 2005).

Integration of the proviral DNA into the host genome allows survival of the virus as in this form it can persist in a latent form or it can directly proceed to productive replication, depending on the virus strain and the type of infected cells. In HIV infections, the virus can only replicate in activated cells (actively dividing $CD4^+$ T-cells and not naïve $CD4^+$ T-cells) that generally comprise 93-95% of productively infected cells. A majority of proviral DNA integrates into the host chromosome, and once integrated the *Orf-B* gene is responsible for latency via an interaction with cellular factor to slow down viral replication. However, most activated $CD4^+$ lymphocytes will die quickly as a result of infection, and only a small proportion become dormant, the so-called resting T-cells or memory T-cells that carry stable integrated provirus but are not permissive for viral replication unless they are further activated (Lassen et al., 2004; Marcello, 2006). $CD4^+$ lymphocytes reactivated by the same antigen and/or cytokines express both genes for immune responses and for HIV replication, leading to production of new virus particles. This unremitting replication is also reported for SIV, enabling them to replicate continuously in their natural host without $CD4^+$ lymphocyte depletion (Silvestri et al., 2003).

The integration of the provirus into the host genome is referred to as post-integration latency and is of immense practical importance because it provides a reservoir of virus that is protected from immune clearance and the effects of antiviral drugs (Chun et al., 1995; Chun et al., 1997; Finzi et al., 1997; Mok and Lever, 2007; Siliciano and Siliciano, 2004). There may be post-integration blocks to viral expression that can lead to viral latency in resting T-cells, which have an estimated half-life of at least 44 months (Finzi et al., 1999; Lassen et al., 2004; Williams and Greene, 2005). For these reasons, HIV-1 latency represents a known barrier to eradication of HIV infection (Lassen et al., 2004). Other types of infected cell such as monocytes, macrophages and dendritic cells are also considered as latently

infected resting cells and can provide reservoirs of HIV capable of escaping host immune surveillance and antiviral treatments (Blankson et al., 2002; Esposito and Craigie, 1998; Zhang et al., 1999). However, it was suggested (Marcello, 2006) that while dendritic cells and macrophages play an important role in viral spread and cell-cell transmission, their involvement in long-term latency has not been demonstrated unequivocally.

Transcription of mRNA

Transcription of lentivirus genes relies on their interaction with one of the 3 forms of RNA polymerase (Schmidt, 1993). Transcription produces an array of mRNA species that can be grouped into 3 classes based on the splicing process involved: an unspliced primary transcript (~9 kb), singly spliced RNAs (~4 kb) lacking the *gag-pol* coding region, and multiply spliced RNAs (~2 kb) lacking the *env* coding region (Purcell and Martin, 1993; Schwartz et al., 1990; Seguin et al., 1998). Approximately one half of the HIV-1 RNA transcripts that are unspliced are essential for *gag* and *pol* gene products. Singly spliced mRNAs encode the Env proteins and the viral regulatory proteins Vif, Vpr, Vpu, while the multiply spliced RNAs encode proteins Tat, Rev and Nef (Purcell and Martin, 1993).

The transcription of mRNA can be divided into early and late phases much like most virus infections. In the early phase, only multiply-spliced viral mRNAs are exported to the cytoplasm and are then translated into early non-structural regulatory proteins Rev and Tat, and in some viruses also Nef, and S2 (Purcell and Martin, 1993; Saltarelli et al., 1996). The late phase of replication is characterised by the production of unspliced or singly-spliced viral mRNAs that include the transcripts of *gag, pol* and *env* that will form the structural proteins and glycoproteins for new virions (Saltarelli et al., 1996). The accessory genes *vif, vpu, vpx, vpw, vpy,* and *Orf*A are also translated during this phase and are crucial in assembly, infectivity and viral pathogenicity (Saltarelli et al., 1996; Seguin et al., 1998).

Assembly and release from the cell

Virus assembly and release of the virus from the host cell takes place in areas adjacent to the plasma membrane and involves a number of sequential steps. The Env glycoproteins (SU and TM) that are translated from the singly-spliced *env* mRNA in the endoplasmic reticulum are transported to the plasma membrane via the

Golgi apparatus where they are glycosylated. At the same stage, the Gag and Gag-pol polyprotein precursor are translated and transported, by an unknown mechanism, and directed toward the plasma membrane. During and after transport, the Gag precursors, 2 copies of viral RNA genome, and other Gag-pol precursors form immature virus particles move close to the plasma membrane and assemble. Thereafter, the assembled Gag protein complex induces membrane curvature, leading to the formation of a bud into which the viral Env glycoproteins are incorporated. The bud develops and pinches off from the plasma membrane to produce a complete virion with an envelope acquired from the bilayered plasma membrane with incorporated Env glycoproteins (Freed, 1998; Grode, 2007).

After release from the host cell, there is further maturation of virus into the final mature forms. At this final stage, the Gag and Gag-pol polyprotein precursors are cleaved by the viral PR to the mature Gag (MA, CA, NC and p6) and Pol (PR, RT and IN) proteins, causing condensation of the core and formation of a mature infectious virion which is ready to initiate a new round of infection (Freed, 1998).

Bovine lentivirus diseases

Jembrana disease

Historical aspects

An outbreak of a highly infectious disease affecting Bali cattle (*Bos javanicus*) was first reported in the village of Sangkaragung, in the Jembrana district of Bali, Indonesia, in December 1964 (Adiwinata, 1967). The name Jembrana disease was derived from the district where the disease first occurred. During the initial stages of the outbreak, it was reported that an estimated 60% of Bali cattle were affected with a mortality rate of 98.9% (Ramachandran, 1996) but at the time there were no veterinary facilities on the island and this high case fatality rate has not been substantiated. Within 12 months, the disease spread to all 8 districts of Bali with a mortality rate of about 20% within a total Bali cattle population of approximately 300,000 cattle on the island (Pranoto and Pudjiastono, 1967; Wilcox et al., 1995). Some buffalo (*Bubalus bubalis*) were also reported to have died during the outbreak (Pranoto and Pudjiastono, 1967) although it was not confirmed that this was due to Jembrana disease and subsequent events suggest that buffalo are not clinically affected. The disease outbreak then waned and it was not reported further until 2

further smaller outbreaks were reported, one in 1972 and one in 1981 in the districts of Tabanan and Karangasem, respectively, where the clinical and pathological aspects of the disease detected were similar to those reported during the 1964 outbreak (Hardjosworo and Budiarso, 1973; Putra et al., 1983). However, although similar, the disease during these later outbreaks was considered milder with reduced morbidity and mortality rates, probably due to a level of immune protection among the local cattle population (Ramachandran, 1996). The disease is now endemic in Bali cattle on Bali island.

The first outbreak of a Jembrana-like disease outside Bali was in 1976 in Lampung province of the island of Sumatra. In this area, the disease was initially designated as "Rama Dewa disease," after the name of a village in which the cases were first reported and where it caused the death of 885 cattle (Prabowo, 1996). Interestingly, only Bali cattle were affected in this outbreak, although crossbred Bali cattle (*Bos javanicus x Bos indicus*), *Bos indicus* cattle, buffalo, goats and sheep were also present in the affected locations (Ramachandran, 1996). A further outbreak was reported in 1978 in East Java, which affected a total of 1,202 Bali cattle and caused 449 deaths (Ramachandran, 1996). A similar outbreak was reported in 1992 in West Sumatra where the morbidity rate was estimated as 70.8% and 133 out of 498 (26.7%) affected Bali cattle died (Tembok and Erinaldi, 1996). In 1993, serological evidence of Jembrana disease was detected in South Kalimantan, although mortalities attributed to the disease were relatively low, possibly associated with the low number of Bali cattle in that area (Hartaningsih et al., 1993). Jembrana disease is currently endemic in Bali, Java, Sumatra island and all 3 Kalimantan provinces of Borneo island (Hartaningsih et al., 1993).

The mechanism for the transmission of Jembrana disease to other islands where the disease is now endemic remains unknown. A recent genetic analysis of proviral-DNA samples obtained from cases of Jembrana disease in Bali and Sumatra showed that JDV strains from the 2 islands were very similar, with 97-100% nucleotide homology in *gag* sequences (Desport et al., 2007). These data would support the hypothesis that the most likely method for the spread of JDV to Sumatra was due to the illegal transportation of persistently infected cattle from Bali (Hartaningsih et al., 1993; Soeharsono et al., 1995a), although the origin of JDV in Kalimantan remains unclear.

The susceptibility of Bali cattle to Jembrana disease is important to the economy of Indonesia. These cattle have been widely distributed throughout Indonesia, they form about 27% of the total cattle population of Indonesia, and they make the highest contribution to beef production in Indonesia (Wiryosuhanto, 1996)

Aetiology

Rinderpest virus was initially considered the cause of the cases of Jembrana disease based on clinical signs and pathological lesions (Adiwinata, 1967; Pranoto and Pudjiastono, 1967). No serological test or virus isolation was conducted to support the diagnosis of rinderpest, and histopathological changes of Jembrana disease in infected Bali cattle were subsequently determined to be distinctly different to rinderpest (Ramachandran, 1996). A rickettsia was subsequently hypothesised as a causative agent on the basis of putative intracytoplasmic rickettsia-like particles observed within monocytes of infected cattle (Budiarso and Hardjosworo, 1976). The clinical signs, pathological and haematological changes of Jembrana disease were also similar to those found in bovine ehrlichiosis (Ondiri disease) in East Africa (Ressang et al., 1985). However, the presence of rickettsia was never confirmed and it was noted also that anti-rickettsial drugs had no effect on the recovery of cattle from experimentally induced disease (Ramachandran, 1996). Subsequently, a virus was suspected based on the ability of the infectious agent to pass through a 220 nm membrane filter, its resistance to antibiotics and the nature of histopathological changes (Ramachandran, 1981; Teuscher et al., 1981).

Soeharsono *et al* (1990) reported that during the febrile period of the disease, the infectious agent occurred in the blood and plasma to a high titre of about 10^8 cattle infectious doses per ml. The infectious agent persisted at a low titre of less than 10^2 infectious particles/ml of plasma in recovered animals for at least 25 months after recovery from the acute disease (Soeharsono et al., 1990). The infectious agent in the plasma was subsequently identified as a retrovirus on the basis of size, determined by filtration studies as less than 100 nm, by electron microscopic observations in tissues as a spherical enveloped virus of about 100 nm diameter with an eccentric core and C-type budding from the plasma membrane, and by the detection of RT activity in virus detected in plasma (Kertayadnya et al., 1993; Wilcox et al., 1992). Further genetic and antigenic analysis identified the virus as a lentivirus, closely related to BIV (Chadwick et al., 1995b; Kertayadnya et al., 1993). Further genetic

analysis of multiple isolates showed that it was a genetically stable lentivirus with only minimal genetic changes in isolates obtained from cattle in Bali over a 20 year period (Desport et al., 2007).

Clinical signs and post-mortem lesions

Infection of Bali cattle by intravenous inoculation of virus into susceptible cattle induces an acute disease syndrome after a short incubation period of 4-12 days. The short incubation period and acute nature of JDV infection with high morbidity rate and 17% case fatality rate is unusual for most lentivirus infections as lentiviruses generally result in a chronic and inevitably progressive disease terminating in death after a long incubation period. The major clinical signs of Jembrana disease during the acute disease are a fever persisting most commonly for 5-7 days, lethargy, anorexia, enlargement of superficial lymph nodes, a mild ocular and nasal discharge, diarrhoea with blood in the faeces, and pallor of the mucous membranes (Soeharsono et al., 1996; Soesanto et al., 1990). In natural cases, “blood sweating” (haemhidrosis) was reported on the back, flank, abdominal region and scrotum (Putra et al., 1983; Teuscher et al., 1981) but this is absent in experimentally infected cattle kept in insect proof stables, suggesting it was bleeding associated with biting arthropods (Soesanto et al., 1990).

The most easily observed and striking clinical feature of Jembrana disease is enlargement of the superficial lymph nodes, especially the prescapular and prefemoral lymph nodes, that can be a useful indicator of Jembrana disease under field conditions (Figure 2.5). Lymph node enlargement is not generally found in other common cattle diseases in Indonesia, including haemorrhagic septicaemia, bovine viral diarrhoea and bovine ephemeral fever, although it is observed in malignant catarrhal fever to which Bali cattle are particularly susceptible (Dharma, 1992).

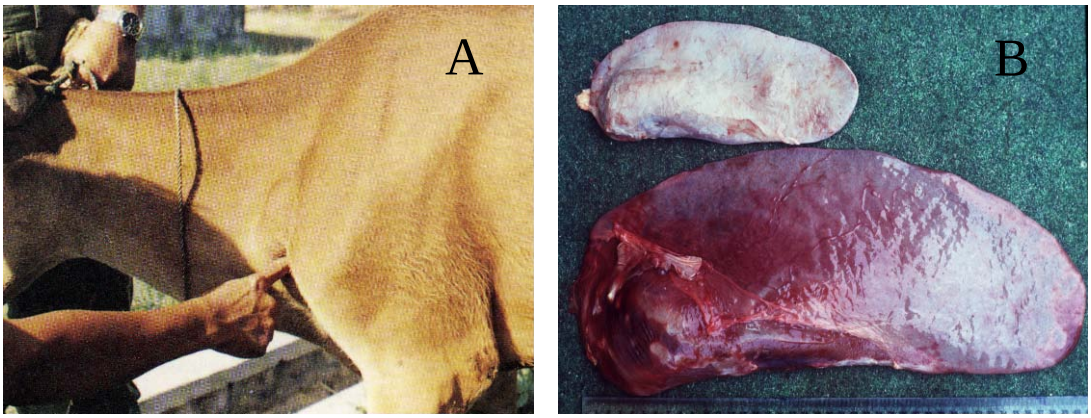


Figure 2.5. A. Marked enlargement of the prescapular lymph node and spleen are consistent feature of Jembrana disease. B. The spleen from affected cattle (bottom) is about 5 times larger than that in a normal animal (top). Photographs courtesy of the staff of BPPH, Indonesia.

The acute febrile phase of Jembrana disease experimentally induced by intravenous inoculation of virus is characterised by marked haematological changes that include leucopenia due to lymphopenia, eosinopenia, and slight neutropenia, thrombocytopenia, a normocytic normochromic anaemia, uraemia and hypoproteinaemia (Soesanto et al., 1990). The thrombocytopenia may be a factor contributing to the presence of the “blood sweating” reported in the field cases and likely associated with a poor clotting mechanism in association with biting arthropods such as tabanids.

The striking post-mortem lesions in Jembrana disease experimentally induced by intravenous inoculation of virus are lymphadenopathy and splenomegaly (Figure 2.5) and haemorrhages, and these are all invariably detected in cattle euthanised during the acute phase of the disease. Other lesions, including kidney lesions and lung consolidation, may also be observed (Dharma, 1992). Histopathological changes are found in all major organs except the central nervous system and reflect a rapid, intense lymphoproliferative disorder (Budiarso and Rikihisa, 1992; Dharma et al., 1991). The typical progression of pathological changes after JDV infection can be divided into 3 distinguishable phases (Dharma et al., 1991). Phase 1 is characterised by a generalised lymphoreticular reaction during the first week after infection, prior

to the development of clinical signs. In phase 2, from 1-5 weeks after infection, the spleen and lymph nodes become markedly enlarged, petechial haemorrhages may occur on serosal surfaces, and ulceration of the oral and intestinal mucosa can be detected. Phase 2 is associated with an infiltration of lymphoid cells into the parenchyma of most organs except the central nervous system (Dharma et al., 1991). In lymphoid organs particularly spleen and lymph nodes, there is a marked non-follicular lymphoproliferative reaction characterised by an intense proliferation of pleomorphic lymphoblastoid cells in the parafollicular (T-cell) regions and there is depletion of follicles (B-cell germinal centres) that results in total destruction of the normal follicular architecture. In addition, the prevalence of IgG-containing cells in tissues is decreased and the $CD4^+$:$CD8^+$ T-cell ratio in blood is also decreased significantly, which is associated with suppression of humoral responses and extensive T-cell proliferation (Dharma et al., 1994; Hartaningsih et al., 1994). A similar infiltrative and proliferative reaction was also detected in other organs including liver, kidneys, adrenal medulla and lungs. In the recovery phase or Phase 3 (4-5 weeks after infection) when there is remission of the clinical signs and a reduction in the viraemia, a marked lymphoid follicular reaction with plasma cell formation, and significant increase in $CD4^+$ and $CD8^+$ T-cell populations in lymphoid tissues is detected (Dharma et al., 1994; Hartaningsih et al., 1994).

The acute febrile phase is associated with a high titre of circulating infectious virus in the plasma of up to 10^8 infectious doses/ml (Soeharsono et al., 1990; Soeharsono et al., 1995a). This was determined by titration of the virus in susceptible cattle. Subsequent JDV-specific quantitative real-time reverse transcription PCR assay (RT-PCR) detected up to 10^{12} copies of the JDV RNA/ml plasma during the febrile phase (Stewart et al., 2005). The significance of the difference in titre determined by the infectious and the RNA genome assays has not been defined. Strain differences in the titre detected by qRT-PCR have been detected and on the second day of the febrile phase, the mean plasma viral titre in cattle infected with $JDV_{Tab/87}$ was significantly higher than in cattle infected with $JDV_{Pul/01}$ (Desport et al., 2007). The high titre of virus might be associated with JDV Tat that is a potent transactivator and thought to be at partly responsible for the high level of viral gene expression *in vitro* (Chen et al., 1999). A typical clinical response to JDV infection and viral load is shown in Figure 2.6.

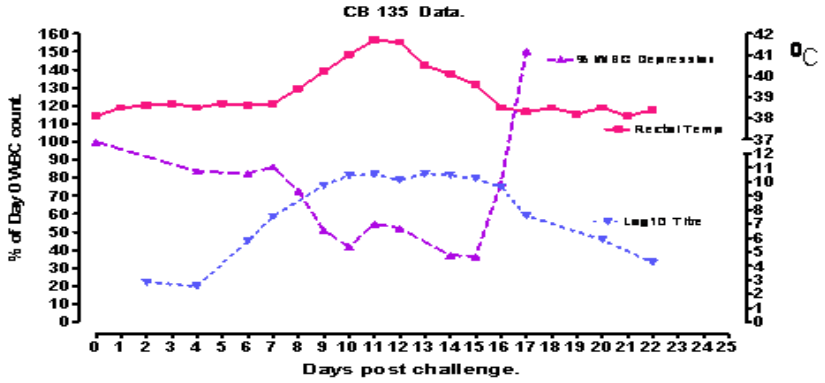


Figure 2.6. A typical clinical response of JDV-infected cattle. During the acute stage, an increased rectal body temperature coincides with an increased plasma viral load and a decreased leucocyte count. Reproduced from Desport et al (2007).

Animals that recover from the acute disease experimentally induced by intravenous inoculation of virus remain viraemic although at a low titre only, for at least 24 months and possibly for life, suggesting they may be a potential source of infection (Soeharsono et al., 1995a; Wilcox et al., 1992). JDV proviral DNA can be detected in the peripheral blood mononuclear cells (PBMC) of recovered animals for at least 18 months after the initial infection (Tenaya and Hartaningsih, 2004). There is no recurrence of disease following recovery from the acute clinical disease and animals resist challenge with homologous and heterologous strains of virus for at least 2 years after primary infection (Soeharsono et al., 1990). The lack of recurrence of disease in the recovered animals and the resistance to reinfection after recovery from the acute disease indicates the development of a protective immune response.

Experimental inoculation of JDV into other cattle types such as Friesian (*Bos taurus*), crossbred Bali cattle (*Bos javanicus* x *Bos indicus*) and Ongole cattle (Bos *indicus*), and buffalo (*Bubalus bubalis*) induces either an inapparent infection or a mild clinical disease that would be difficult to be detect under field conditions. This is consistent with the lack of reports of disease in these other cattle types in Indonesia. Experimental infection of sheep and goats was reported to induce a transient viraemia and no clinical signs (Soeharsono et al., 1990). The unique susceptibility of Bali cattle, descendent from wild banteng of South East Asia (Soeharsono et al., 1995b), is intriguing but the reason for this susceptibility, although apparently genetically based, is not understood.

The cellular tropism of JDV has not yet been defined and this is critical to understanding the disease process involved. The possibilities are that JDV could have a broad cell tropism like BIV in infecting B-cells (as shown by follicular atrophy early in the disease process), T-cells (as shown by the parafollicular hyperplasia and detection of virus in these cells), and macrophages (Chadwick et al., 1998; Dharma et al., 1991). There is unpublished evidence that JDV can be cultured in myeloma (NS1) cells fused with lymphocytes derived from non-JDV infected Bali cattle. The hybridoma cells could be infected with JDV and maintained up to one year during which time they continue to demonstrate a strong reaction with anti-JDV CA monoclonal antibody in Western immunoblots, suggesting that JDV may infect B-cells (Astawa, personal communication) but this report needs to be confirmed. However, as JDV strains in Bali are genetically stable with minor variation only in *env* (Desport et al., 2007). Therefore, it could also be possible that JDV has a narrow host cell range, potentially targeting long-lived cells with a slow turnover rate.

Mode of transmission of JDV

As consequence of the high titre of infectious virus in the blood of about 10^8/ml during the acute phase of Jembrana disease, transmission of JDV in 2 different ways has been hypothesised. First, mechanical transmission by vehicles such as multi-use needles during vaccination programs, and by blood sucking arthropods, has been considered to have a high probability of transferring infection from cattle during the acute disease phase (Soeharsono et al., 1995a). However, mechanical transmission of virus by arthropods is unlikely to be responsible for the extensive spread of Jembrana disease from island to island as the disease has not spread from Bali island to the closely adjacent islands of Nusa Penida and Lombok since the disease was initially reported in Bali in 1964 (Hartaningsih et al., 1993; Soeharsono et al., 1995a). There has also been limited spread of the disease from infected areas to neighbouring areas (Hartaningsih et al., 1993). During the acute phase when there is a high titre of virus in blood, close contact between animals might enable transmission of virus present in saliva of infected animals and infection of susceptible cattle by conjunctival, nasal or respiratory routes (Soeharsono et al., 1995a). Close contact between animals does result in transmission of MVV and CAEV in sheep and goats (Gufler et al., 2007; Shah et al., 2004a).

Transmission of JDV from the persistently infected animals wherein there is only a low titre viraemia, to susceptible animals has also been hypothesised (Soeharsono et al., 1995a) but how this occurs is unknown. However, transportation of persistently infected recovered cattle from JDV-infected areas to JDV-free regions is the most logical reason for the occurrence of Jembrana disease in other regions outside Bali (Soeharsono et al., 1995a).

Diagnosis of Jembrana disease

Diagnosis of Jembrana disease is based on clinical signs and pathological observations, in conjunction with the detection of JDV infection by immunological procedures and molecular techniques.

Serological assays

Two serological assays, an enzyme-linked immunosorbent assay (ELISA) and Western immunoblot, have been developed for the detection of an antibody response to JDV in infected animals (Hartaningsih et al., 1994; Kertayadnya et al., 1993). The initial ELISA utilised an antigen prepared by sucrose gradient centrifugation to purify and concentrate virus from plasma of acutely infected animals. With this assay, antibodies were not detected in a majority of cattle until 11 weeks after infection, with the maximum antibody response occurring between 23 and 33 weeks after infection. A Western immunoblot using a similar whole virus antigen indicated that the initial positive ELISA sera reacted strongly with the p26 JDV CA protein (Hartaningsih et al., 1994; Kertayadnya et al., 1993), typical of other lentivirus infections (Battles et al., 1992; Grund et al., 1994). The absence of a detectable antibody response until 11 weeks after JDV infection may be associated with the absence of a significant follicular reaction and scarcity of plasma cells in lymphoid organs during the acute phase and early recovery phase (Hartaningsih et al., 1994). ELISA, supported by Western immunoblotting, has been used as a routine surveillance technique for detection of JDV infection in the Indonesian cattle population (Hartaningsih et al., 1993; Soeharsono, 1996). The predominant antibody detected by ELISA and by Western immunoblotting was reactive with the p26 JDV CA, a response that was similar to that detected in other lentiviruses. There could therefore be a problem with the specificity of the assay as this protein is known to be cross-reactive in many lentivirus infections and particularly between JDV and BIV

(Kertayadnya et al., 1993). Potential problems with cross-reactivity could occur because of cross-reactions with a putative BIV-like virus detected in Bali cattle in Sulawesi, a Jembrana disease-free island, where positive ELISA results with this assay have been detected but where there is no evidence of Jembrana disease (Hartaningsih, personal communication). More recently, a recombinant JDV CA antigen was used in ELISA and Western immunoblotting procedures for the detection of BIV infection in dairy cattle in Australia, where it detected antibody presumably due to BIV infection in 3.8% of 690 serum samples (Burkala et al., 1999). Evidence of similar cross-reactivity of CA was reported in the small ruminant lentiviruses MVV and CAEV (Grego et al., 2002).

The use of recombinant CA antigens (Burkala et al., 1999) offers advantages compared to the whole virus antigen prepared from the plasma of infected cattle, although it has not reduced the potential problem of antigenic cross-reactivity between BIV and JDV. Attempts to identify possible type-specific epitopes in the MA, CA, and NC of JDV have been made by preparation of a series of truncated recombinant proteins, but none of these were able to differentiate sera from JDV-infected or BIV-infected cattle (Desport et al., 2005).

Immunohistochemistry

Immunohistochemical labelling techniques have been widely used for determining the pathogenesis of lentivirus-infected hosts, including SIV infection in monkeys (Zhang et al., 2007), HIV in humans (Bhoopat et al., 2001; Geijtenbeek et al., 2001; McCarthy et al., 2002; Wheeler et al., 2006), BIV in cattle (Heaton et al., 1998; Whetstone et al., 1997) and MVV in sheep (Brodie et al., 1995; Gendelman et al., 1985). An immunohistochemical test utilising a JDV p26 CA monoclonal antibody (Kertayadnya et al., 1993) was developed and used to detect JDV infection in tissues of infected cattle (Dharma et al., 1994).

In situ hybridisation (ISH)

Determination of the nucleotide sequence of the genome of JDV has allowed the development of an *in situ* hybridisation technique to detect viral RNA in tissues at different stage of the disease process (Chadwick et al., 1998). This ISH assay detected JDV RNA at the onset of the febrile period, mainly in the parafollicular areas of the spleen, and to a lesser extent in the lymph nodes, bone marrow, lungs

and kidneys. On the second day of the febrile period, viral RNA was detected at elevated levels in the lymph nodes and in the cellular infiltrate in other organs. On the fourth day of the febrile period, JDV-infected cells were widely distributed in all tissues. While the identity of the JDV-infected cells could not be determined, the prevalence and morphology of infected cells in lymphoid tissues suggested that the infected cells were of lymphoid origin and possibly of the monocyte/macrophage lineage (Chadwick et al., 1998).

Polymerase chain reaction (PCR)

PCR is the technique of choice for detection and analysis of minute amounts of DNA (Glick and Pasternak, 2003). It has been widely used for sensitive and specific detection of many lentiviruses, including HIV-1 (Nyambi et al., 1994) and BIV (Suarez et al., 1995).

For the detection of JDV proviral DNA, the first reported PCR assay involved a nested set of 4 oligonucleotide primers selected from sequences in the *gag* genes of JDV. These provided a sensitive assay that was specific for JDV, and did not recognise proviral DNA of the closely related BIV (Chadwick, 1995). Additional JDV-specific PCR assays have been developed to detect JDV proviral-DNA in PBMC isolated from JDV-infected cattle as early as 3 days after infection, before clinical signs developed, and until at least 18 months after infection (Tenaya and Hartaningsih, 2004; Tenaya et al., 2003). This test enabled the early detection of JDV after infection, whereas serological assays such as ELISA were generally unable to detect antibody until at least 11 weeks after infection (Hartaningsih et al., 1994). PCR assays have been used to monitor the effect of vaccination with a tissue-derived vaccine (Hartaningsih et al., 2001) on virus persistence and it was determined that the number of cattle in which proviral DNA could be detected in PBMC was reduced significantly after vaccination (Tenaya and Hartaningsih, 2005)

Bovine immunodeficiency virus

BIV was originally isolated in Louisiana from a dairy cow (R29) with persistent lymphocytosis, lymphadenopathy, progressive weakness and emaciation (Van der Maaten et al., 1972). The virus induced syncytium formation and was antigenically distinct from bovine spumavirus. Reinoculation of the R29 isolate into colostrum-deprived calves caused only a mild lymphocytosis and enlargement of subcutaneous

lymph nodes without any overt clinical signs, very different to the clinical signs observed in the animal from which it was isolated. Probably due to the absence of marked clinical signs in cattle experimentally infected with BIV, little further investigation of this virus did until the discovery that HIV-1 was also a lentivirus. It was not until later that Gonda *et al*. (1987) characterised the R29 strain of BIV as a lentivirus based on virion structure, budding characteristics and genetic homology with other lentiviruses. Antigenic cross-reactivity between the CA protein of BIV and the other known lentiviruses including HIV-1, EIAV, CAEV and MVV was demonstrated (Gonda et al., 1987; Jacobs et al., 1992).

Only rarely has BIV infection been associated with naturally occurring clinical disease and its distribution is worldwide, in contrast to JDV that seems to be confined to Indonesia (Brownlie et al., 1994; Chadwick et al., 1995b; Muluneh, 1994; Polack et al., 1996; Whetstone et al., 1990). Inoculation of cattle with R29 and other BIV-isolates has resulted in mild changes including a mild lymphocytosis predominantly due to B-cells, a transient increase of mononuclear cells and immune suppression (Whetstone et al., 1997; Zhang et al., 1997a). The difference in severity of the lesions between those observed in the cow from which the virus was isolated and those seen in the experimentally infected cattle has been attributed to loss of virulence of the R29 isolate since its original isolation, due to extensive attenuation following multiple passages *in vitro* (Suarez et al., 1993) but attenuation may not have occurred and other factors might well have been responsible for these differences. Experimental infections with other strains of BIV have also not produced lesions typical of those observed in the cow from which R29 was isolated. The apparent lack of pathogenicity of BIV, in marked contrast to the closely related JDV in Bali cattle, suggested that BIV may have a greater pathogenicity in Bali cattle. However, infection of 2 young Bali cattle with BIV did not induce clinical signs, although all animals became BIV positive by PCR and the virus was re-isolated from the infected cattle (Whetstone et al., 1996).

Recent studies with the R29 strain of BIV in Bali cattle confirmed previous observations (Whetstone et al., 1996) that this strain did not produce clinical signs of disease in this species and there was no greater susceptibility of Bali cattle to BIV as there is to JDV. Proviral BIV DNA was detected in PBMC from 4-60 days after infection when the experiment was terminated, with peak titres 20 days after

infection. There was a transient viraemia from 4-14 days after infection with a maximum 1 x 10^4 genome copies/ml of plasma. An antibody response to the TM protein occurred commencing 12 days after infection but an antibody response to the to the CA protein was detected in only 1 of 13 cattle before 60 days and only after 34 days (McNab et al., 2010). Apart from the lack of pathogenicity of BIV in Bali cattle there are interesting comparisons between BIV and JDV infection in Bali cattle: the transient viraemia soon after infection with BIV is similar to that observed after JDV infection but the level of viraemia after BIV infection is markedly less than that which occurs with JDV infection. The antibody response to BIV infection was also markedly earlier than that detected following JDV infection.

BIV is genetically closely related and shares common antigens with JDV (Barboni et al., 2001; Burkala et al., 1998; Desport et al., 2005; Kertayadnya et al., 1993). The similarity of the *gag* encoded proteins of BIV and JDV is approximately 62% at the amino acid level, the CA of both viruses share 75% identity whereas the identity of the TM of the 2 viruses is only 31% (Chadwick et al., 1995b; Kertayadnya et al., 1993). Using JDV CA in serological assays does not differentiate antibody to BIV and JDV (Barboni et al., 2001; Wilcox et al., 1995) even though unique epitopes have been described on the Gag protein of BIV and JDV (Lu et al., 2002).

BIV appears to have a broad host range and infection of sheep, goats and rabbits also induced a persistent infection and antibody response (Carpenter et al., 2000; Forman et al., 1992; Jacobs et al., 1996; Smith and Jacobs, 1993; Whetstone et al., 1991). BIV also appears to be pantropic and infect a wide variety of cell types including B-cells, T-cells and cells of the monocyte/macrophage lineage (Heaton et al., 1998; Rovid et al., 1995; Whetstone et al., 1997; Wu et al., 2003). The cellular receptor used by BIV has not been determined.

Lentivirus infection in other species

Human immunodeficiency virus type 1 (HIV-1)

HIV-1 was first identified in 1983 as the causative agent of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 2004; Clements and Zink, 1996). Although a second human immunodeficiency virus, HIV-2, was subsequently identified, HIV-1 is responsible for the majority of HIV infections globally, although

features of the 2 virus infections are similar (Azevedo-Pereira et al., 2005; Azevedo-Pereira et al., 2003; Reeves and Doms, 2002). The route of transmission of HIV-1 and HIV-2 are the same (Grant and De Cock, 2001), mainly as a cell-associated virus in semen transmitted by sexual contact, or in blood where it is transmitted as a consequence of needle-sharing amongst intravenous drug users or during blood transfusions, and maternally both *in utero* and from breast milk (Buonaguro et al., 2007; Friedland and Klein, 1987).

HIV-1 induces a transient and acute clinical disease 3-4 weeks after infection, as do a number of other lentiviruses, which could be likened to a mild form of the acute clinical disease that is a consequence of JDV infection in Bali cattle. However, the acute disease following HIV infection is much milder than that which occurs as a consequence of JDV infection in cattle. HIV-infected individuals invariably recover and then go on to develop acquired immune deficiency disease (AIDS), whereas the case fatality rate associated with Jembrana disease is about 20% and animals that recover do not develop any further clinical disease. The major characteristics of the HIV disease process are depicted schematically in Figure 2.7. After infection, HIV-1 disseminates to and replicates in cells of regional lymphoid organs until a threshold of replication is reached 2-6 weeks post-infection (Alcami, 2004a; Brenchley et al., 2004; Weiss, 2000). There is then an occurrence of mild flu-like illness associated with a transient decrease in the number of circulating $CD4^+$ T-cells and a concurrent transient increase in the plasma viral load. This is followed by a rapid clearance of virus probably due to the ability of the immune system to generate an effective response to control replication (Blattner et al., 2004) and thought to be predominantly associated with cell-mediated responses but not with the development of neutralising antibodies (D'Souza and Mathieson, 1996). The ensuing virus infection is associated with a limited proliferation of the virus due to host defence mechanisms, but a minimal viraemia is nonetheless maintained (Tyler and Fields, 1996). In the presence of neutralising antibody, the viraemia is drastically reduced and becomes cell-associated to facilitate persistence of the virus and remission of clinical signs (Forthal et al., 2001; Tyler and Fields, 1996). Viral replication continues at a lower level during a long symptom-free period until the level of viraemia progressively increases again and this coincides with a progressive reduction in $CD4^+$ T-cells leading to onset of immunosuppression and opportunistic

secondary infection that manifest as AIDS (Figure 2.7). The period from initial infection until the occurrence of severe clinical symptoms leading to AIDS can range from months to years.

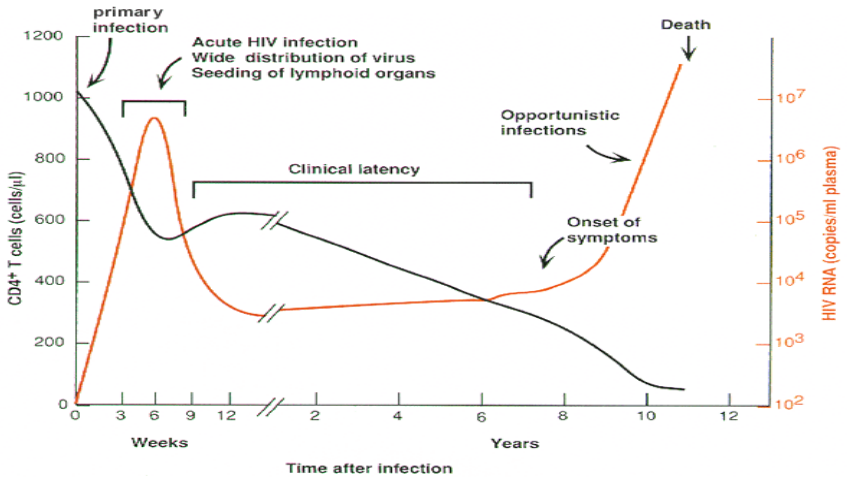


Figure 2.7. A typical time-course of HIV infection. After initial infection, $CD4^+$ cells decrease transiently in association with a transient high plasma virus load shortly after infection and this is associated with a flu-like illness. This is followed by a progressive slow decrease in $CD4^+$ cells during a long asymptomatic period but eventually there is a further increase in virus load and very low levels of $CD4^+$ cells leading to the onset of AIDS. Sourced from Weber (2001).

HIV-1 infects several primary cell types, predominantly the $CD4^+$ T-helper subset of lymphocytes, the macrophage lineage, and some populations of dendritic cells (Clapham and McKnight, 2002). The depletion of $CD4^+$ T-cells, the hallmark of HIV-1 infection, occurs predominantly in the gastrointestinal tract, similar to SIV infections (Brenchley et al., 2004; Canto-Nogues et al., 2001; O'Neil et al., 1999) and as a direct consequence of viral replication the infected cells are destroyed, leading to mass destruction of the immune system (Alcami, 2004a; Brenchley et al., 2004; Penn et al., 1999; Picker, 2006). The development of these changes is associated with a progressive change in virus replication from a slow replicating low-titre virus to a rapidly replicating high-titre virus, caused in part by a switch in the predominant receptor used by the virus that enables the virus to broaden its cell tropism (Fenyo et al., 1989; Weber, 2001).

Changes observed during AIDS include weight loss, diarrhoea and a range of secondary infections associated with opportunistic pathogens (Grant and De Cock, 2001; Mwachari et al., 2003; Stack et al., 1996; Ullrich et al., 1992). Immunosuppression leads to opportunistic infections (Mwachari et al., 2003; Zhang et al., 2007; Zhang et al., 2004) resulting in secondary infections, e.g. *Pneumocystis carinii* associated with pneumonia, *Mycobacterium* leading to tuberculosis, herpes zoster infections, toxoplasmosis, cytomegalovirus, oral candidiasis and other viral, bacterial, fungal and protozoal agents (Soriano et al., 2000; Sungkanuparph et al., 2003; Ullrich et al., 1992). Kaposi's sarcoma due to *Human herpesvirus type 8* is a common skin tumour seen in AIDS patients and growth of the tumour cells is thought to be stimulated by the extracellular Tat secreted from HIV-1 infected cells (Dupon et al., 1997; Engels et al., 2003; Rubartelli et al., 1998).

In individuals with AIDS, infected tissue macrophages have been detected in lung, colon, brain, liver and kidney (Cao et al., 1992; Donaldson et al., 1994). Other tissues that harbour persistently-infected macrophages include lymph node, spleen and bone marrow (Gorry et al., 2001). Infection of macrophages in certain organs results in some primary diseases including encephalitis, lymphoid interstitial pneumonia and bone marrow disorders leading to anaemia and thrombocytopenia (Hanna et al., 1998; Pise et al., 1992; Wheeler et al., 2006). About 30% of HIV-infected patients exhibit encephalitis in which HIV-1 is assumed to enter into the brain tissue via circulating infected monocytes that cross the blood brain barrier and mature into macrophages (Gonzalez-Scarano and Martin-Garcia, 2005; Wheeler et al., 2006).

The ability of HIV-1 to also infect and deplete $CD4^+$ lymphocyte populations is partly due to its ability to infect follicular dendritic cells in the germinal centres of lymphoid follicles. This causes gradual disruption of the follicular dendritic network and functional perturbations of B-cells in the tissue, ultimately leading to the onset of AIDS (Moir et al., 2003; Moir et al., 2001). B-cell dysfunction in HIV infection is largely associated with cell hyperactivation and can lead to hypergammaglobulinaemia (Shirai et al., 1992), increased spontaneous secretion of Ig (Conge et al., 1994; Fournier et al., 2002), and increased susceptibility to apoptosis (Muro-Cacho et al., 1995; Samuelsson et al., 1997). In HIV infections, B-cells respond poorly to mitogenic or antigenic stimuli *in vitro* and produce poor

antibody responses *in vivo* (Conge et al., 1998; Opravil et al., 1991). A similar phenomenon has been reported in SIV and FIV infections (Zhang et al., 2007; Zhang et al., 2004).

Dendritic cells are a main target for HIV-1 at the mucosal level and are among the first cell targets during early infection (Hu et al., 2000; Knight, 2001; Miller et al., 1994; Sewell and Price, 2001). These cells were the predominant cell type infected following vaginal exposure of macaques to SIV (Hu et al., 2000; Miller et al., 1994; Spira et al., 1996). They play a significant role as antigen-presenting cells (APC) that capture, transport and present the virus from mucosal membranes to $CD4^+$ and $CD8^+$ T-cells in lymph nodes (Cella et al., 1999; Geijtenbeek and van Kooyk, 2003; Rowland-Jones, 1999). Whether dendritic cells are directly infected by HIV-1 or are carriers of the virus has been controversial. The dendritic cell-specific protein (DC-SIGN) does act as a novel HIV-1 *trans*-receptor for binding and transmitting the virus to target cells but it does not function as a receptor for viral entry (Clapham and McKnight, 2001; Geijtenbeek and van Kooyk, 2003; Geijtenbeek et al., 2000b). Some have reported that HIV-1 infects dendritic cells that then secrete soluble HIV-1 gp120 which hampers $CD4^+$ T-cell proliferation and IL-2 production, and leads to immune dysfunction in AIDS patients (Fantuzzi et al., 2004; Kawamura et al., 2003). Others have reported that dendritic cells support viral replication dependent on their state of maturation (Bakri et al., 2001; Granelli-Piperno et al., 1998). Immature dendritic cells and Langerhans cells at the genital tract mucosal membranes were considered more permissive than mature cells to HIV-1 infection (Bakri et al., 2001; Bhoopat et al., 2001; Clapham and McKnight, 2001). These cells are different to other dendritic cells from other tissues in that they do not possess DC-SIGN associated with transmission events (Geijtenbeek et al., 2000a; Prakash et al., 2004), and they express C-type lectins such as mannose receptors that are thought to be involved in initial attachment of HIV-1 (Turville et al., 2003). Follicular dendritic cells that trap and retain HIV-1 in the follicles of secondary lymphoid tissues have also been considered as a significant reservoir of infectious virus (Brandon et al., 2008).

Equine infectious anaemia virus (EIAV)

Equine infectious anaemia was first described in 1904 but the causative virus was not identified as a lentivirus until more recently (Charman et al., 1976; Cook et al.,

2001; Montelaro et al., 1993). It is a lentivirus that occurs to a sufficient titre in blood that it can be transmitted mechanically by arthropods (Issel and Coggins, 1979; Montelaro et al., 1993; Sellon et al., 1994) but it can also be experimentally transmitted by inoculation of blood from naturally infected animals into susceptible animals (Spyrou et al., 2003).

EIAV induces a clinically variable disease with acute, chronic and inapparent carrier phases. The acute stage is atypical of most lentiviruses in that it is characterised by an early viraemic period after infection and this is associated with the rapid development of clinical signs including fever, depression, anorexia, weight loss, oedema and anaemia that sometimes results in death (Issel and Coggins, 1979; Valli, 1993). During this period, lymphocyte counts are reduced due to significant reduction of $CD4^+$ and $CD8^+$ T-cell populations (Murakami et al., 1999).

Animals that recover from the initial acute disease may develop intermittent or recurrent clinical episodes characterised by fever, thrombocytopenia, lethargy, inappetence, progressive anaemia and cachexia (Sellon et al., 1994; Valli, 1993). Periodic clinical relapses initially occur at 1-2 week intervals but the interval between relapses progressively increases until complete recovery is attained (Leroux et al., 2004) perhaps 12 months later. Most infected horses then develop a prolonged subclinical virus infection during which the cellular reservoir of virus is macrophages (Oaks et al., 1998; Sellon et al., 1994). The reduction of viral load is associated with the presence of neutralising antibodies (Sponseller et al., 2007).

Maedi-visna virus (MVV).

The MVV-related diseases, maedi and visna, were first reported in Icelandic sheep in Iceland (Narayan et al., 1993). Infected animals develop a chronic wasting disease characterised by interstitial pneumonia (maedi) or nervous signs (visna) (Benavides et al., 2006; Bolea et al., 2006; Cutlip et al., 1988) although mastitis is also common in infected lactating animals (Cutlip et al., 1988; Dawson, 1980; Dow et al., 1990; Lujan et al., 1991; Narayan et al., 1993). The diseases have been recognised in most sheep-rearing countries of the world, with the notable exception of Australia and New Zealand (Shuljak, 2006). The causative virus MVV is closely related to CAEV with which it shares approximately 75%, 78% and 60% identity to nucleotide sequences in *gag*, *pol* and *env* genes, respectively (Saltarelli et al., 1990).

Typical of lentivirus infections, infected sheep remain carriers for life (Dawson, 1987; Pepin et al., 1998) and transmission is possible in many ways but mainly by ingestion of infected colostrum or milk, or by aerosol transmission of virus from the respiratory tract of infected animals (Blacklaws et al., 2004; Leginagoikoa et al., 2006; Peterhans et al., 2004; Preziuso et al., 2004; Straub, 2004). There is a close genetic and antigenic relationship between MVV and CAEV (Gogolewski et al., 1985; Grego et al., 2002; Rosati et al., 1999) and natural cross-species transmission by close contact between sheep and goats is possible (Banks et al., 1983; Castro et al., 1999; Gjerset et al., 2007; Grego et al., 2007; Lacerenza et al., 2006; Pisoni et al., 2005; Rolland et al., 2002). The potential for cross-species transmission has been supported by recent phylogenetic analysis of sheep isolates that revealed the presence of strains that had greater genetic identity to CAEV than to MVV (Karr et al., 1996; Leroux et al., 1997; Shah et al., 2004b; Valas et al., 1997).

The virus predominantly infects fixed-tissue macrophages and it does not infect T-cells, in contrast to many other lentiviruses and including those of man, monkeys and cats (Brodie et al., 1995; Gendelman et al., 1985; Gendelman et al., 1986; Gorrell et al., 1992; Narayan et al., 1983). MVV has also been detected in dendritic cells that not only carry infectious MVV but are also host to virus replication (Ryan et al., 2000).

Caprine arthritis encephalitis virus

CAEV was first isolated in the early 1970s from synovial fluid of an arthritic goat and goat kids with encephalitis (Cheevers et al., 1988; Cork et al., 1974). The virus has a close genetic relationship to MVV and induces a similar disease in goats to those detected in sheep with MVV, and is widespread in many countries including Australia (Contreras et al., 1998; Cutlip et al., 1992; de la Concha-Bermejillo et al., 1998; Guiguen et al., 2000; Surman et al., 1987). In contrast to MVV infection, the main pathological forms described in infected goats are a chronic degenerative polyarthritis and a leukoencephalitis in mature goats and kids, respectively, and pneumonia less commonly (Cork et al., 1974; Rowe and East, 1997). As in sheep with MVV, horizontal transmission is mainly via ingestion by the newborn of infected colostrum and milk from the mother (East et al., 1993; Greenwood et al., 1995; Le Jan et al., 2005; Mselli-Lakhal et al., 1999; Peterhans et al., 2004). The virus in the ingested milk infects mononuclear cells of the monocyte/macrophage

lineage which are the primary target cells of CAEV, similar to MVV (Lechat et al., 2005; Narayan and Kennedy-Stoskopf, 1983).

Feline immunodeficiency virus (FIV)

FIV is a T-lymphotropic lentivirus of domestic cats first isolated from feline leukaemia virus (FeLV) serologically-negative cats showing an immunodeficiency-like syndrome (Pedersen et al., 1987). Although FIV and FeLV both may induce immunodeficiency, the infections can be specifically differentiated based on serological assays (Fontenot et al., 1992; Shelton et al., 1990).

Clinical manifestations of FIV infection occur in 3 phases: an acute, an asymptomatic, and a subsequent immunodeficiency condition, all of which appear analogous to that observed in HIV-infected patients (Ishida and Tomoda, 1990; Pedersen et al., 1987; Pedersen et al., 1989). The acute stage soon after infection is a transient disease with generalised lymphadenopathy, fever and leucopenia (Barlough et al., 1991; Obert and Hoover, 2002). This is then followed by a subclinical stage and finally by a terminal stage characterised by a number of chronic infections, wasting and a low level plasma viraemia (Pedersen et al., 1989). The terminal stage is similar to that seen in HIV and SIV infections (Pedersen et al., 1987; Yamamoto et al., 1988).

The virus targets activated $CD4^+$ T-cells by specifically binding to a CD134 receptor expressed on the surface of the cell and in conjunction with co-receptor, CXC chemokine receptor 4 (de Parseval et al., 2004; Shimojima et al., 2004). FIV infections cause a gradual depletion of $CD4^+$ T-cell subsets and impair immune function (Ackley et al., 1990; Olmsted et al., 1989; Talbott et al., 1989; Yamamoto et al., 1988). FIV, however, can also infect monocytes/macrophages and B-cells, and B-cells are considered to be a principal target in the later stages of infection (Brunner and Pedersen, 1989; Dean et al., 1999; Dean et al., 1996; English et al., 1993; Troth et al., 2008; Yang et al., 1996). Replication of the virus in cells of macrophage lineage has been considered to be responsible for disease manifestations of the central nervous system (Clements and Zink, 1996; Dow et al., 1990; Hartmann, 1998) and virus has been detected in the cerebrospinal fluid of seropositive cats experimentally infected with a Maryland strain of the virus (Prospero-Garcia et al., 1994).

Simian immunodeficiency viruses (SIV)

SIVs are a diverse group of nonhuman African primate lentiviruses that share many of the biological properties of HIV-1 and HIV-2 and tend to cause a disease remarkably similar to human AIDS, not in the primary host but in other heterologous primate species that are not the natural host (Brown et al., 2007; Desrosiers, 1990; Letvin et al., 1985; Veazey et al., 2000). SIV in their native hosts have adapted to survive in these hosts and the infected primates do not develop disease despite high levels of virus replication and a limited antiviral $CD8^+$ T-cell response (Broussard et al., 2001; Cumont et al., 2008; Rey-Cuille et al., 1998; Silvestri et al., 2003). These viruses are transmitted between individuals in association with sexual activity and male to male aggression (Whetter et al., 1999). An AIDS-like disease in non-human primates was first reported in rhesus macaques infected with SIV isolated from sooty mangabeys (Letvin et al., 1985). This finding subsequently provided a model for the study of human AIDS that is amenable to manipulation of a variety of experimental parameters. The major application of the model has been for study of pathogenesis (Hirsch and Johnson, 1994; Zink et al., 1998), transmission (Harouse et al., 2001; Tsai et al., 2004), genomic integration (Crise et al., 2005), treatment (Clements et al., 2005; North et al., 2005; Shen et al., 2003; Zink et al., 2005) and vaccine development (Egan et al., 2000; Haga et al., 1998; Hayami and Igarashi, 1997; Nath et al., 2000; Parker et al., 2001).

Clinical signs of simian AIDS generally include rapid weight loss, poor fluid intake, diarrhoea, loss of appetite and ataxia (Dykhuizen et al., 1998; Sopper et al., 1998). Primary components of the disease include giant cell interstitial pneumonia, meningitis, glomerulonephropathy, severe enteritis, persistent lymphadenopathy and bone marrow disorders (Brown et al., 2007; Dykhuizen et al., 1998; Kitagawa et al., 1991). Immunosuppression enables secondary opportunistic infections (Clements and Zink, 1996; Yanai et al., 1999). The development of lesions seen in the early stage of AIDS have been shown to be related to changes in the cellular tropism of the virus (Clements and Zink, 1996). Replication of some strains of SIV in cells of monocyte/macrophage lineage has been considered to cause primary neurological signs and pneumonia (Kim et al., 2003; Mankowski et al., 1998; Sharma et al., 1992) while strains that replicate in lymphocytes are predominantly responsible for decreasing absolute number of $CD4^+$ cells leading to opportunistic infections (Brown

et al., 2007; Dykhuizen et al., 1998; Mattapallil et al., 2005; Veazey et al., 2003; Veazey et al., 2000).

Infection with some strains of SIV has induced disease that is more rapidly progressive than is normally seen in lentivirus infections, and there are analogies of these infections to JDV infection in Bali cattle. Infection with SIV_{mac} or the hybrid simian/human immunodeficiency virus $SHIV_{89.6PD}$ caused rapid AIDS development in which some infected monkeys died within 6 months of virus infection (Dykhuizen et al., 1998; Smith et al., 1999; Steger et al., 1998; Zhang et al., 2007). In this rapidly progressive AIDS-like condition the plasma viral loads were found to be higher than is usual and there was frequently a lack of any virus-specific humoral immune response (Watson et al., 1997; Zhang et al., 2002; Zhang et al., 2004). In this condition, an early severe depletion of B-cells in germinal centres and disruption of the follicular dendritic cell network was evident, and were thought to be associated with the lack of antibody response that ultimately led to rapid disease progression (Dykhuizen et al., 1998; Zhang et al., 2007; Zhang et al., 2004).

Infection with $SIV_{smmPBj14}$ also produces a disease with some similarities to Jembrana disease but the infection is even more virulent than JDV infection. $SIV_{smmPBj14}$ is the most virulent primate lentivirus that has been described, causing a fatal disease in nearly all infected pig-tailed macaques (*Macaca nemestrina*) within days instead of months and years as in HIV and most SIV infections (Fultz et al., 1989). This highly atypical variant of SIV was originally isolated from lymphoid tissues of a macaque (PBj) that had previously been infected with SIV_{sm} for 14 months. The virus also infects and induces disease in other strains of monkeys including sooty mangabeys and rhesus macaques, but a more variable form of the acute disease is seen in rhesus monkeys (Fultz et al., 1989; Lewis et al., 1992a). Transmission of disease occurs only by means of experimental inoculation after which the virus induces a high titre viraemia, extensive cellular activation and proliferation and a high level of cytokine production, leading to acute severe clinical signs (Fultz and Zack, 1994; Lewis et al., 1992a). The acute clinical signs in pig-tailed macaques include depression, anorexia, fever, an erythematous skin rash, profuse diarrhoea and death, all within 6-10 days of infection (Fultz et al., 1989; Hodge et al., 1999; Lewis et al., 1992a; Mossman et al., 1996; O'Neil et al., 1999). Animals develop a severe lymphopenia involving all circulating lymphocytes, and a

moderate neutrophilia (Novembre et al., 1993; O'Neil et al., 1999; Schwiebert and Fultz, 1994). Pathological changes include a generalised lymphadenomegaly, splenomegaly and, unlike Jembrana disease, marked gastrointestinal lesions. The most prominent pathologic feature is a rapid and extensive lymphoid hyperplasia of the T-cell areas of lymph nodes, spleen and particularly the gut-associated lymphoid tissues (Fultz and Zack, 1994; O'Neil et al., 1999), and a similar T-cell proliferation is also seen in Jembrana disease. These expanded T-cell zones contained a high proportion of lymphoblasts, activated macrophages and syncytial cells, indicating high levels of viral replication and immune system hyperactivation. These histological changes are atypical of those observed with primary HIV-1 infections (Fultz, 1994; Fultz and Zack, 1994). $SIV_{smmPBj14}$ infects $CD4^+$ T-cells, macrophages, $CD8^+$ T-cells and B-cells *in vivo* (O'Neil et al., 1999). Additional *in vitro* studies have indicated that the virus also replicates efficiently in resting pig-tailed macaque PBMC (Fultz, 1991; Novembre et al., 1993), activates and induces proliferation of $CD4^+$ and $CD8^+$ lymphocyte subsets, and resting lymphocytes (Fultz, 1991; Fultz and Zack, 1994; Novembre et al., 1993; Schwiebert and Fultz, 1994). The mitogenic properties of Nef encoded by this virus have been assumed to play a major role in the activation of resting lymphocytes (Stephens et al., 1998). Animals that have survived the acute disease associated with $SIV_{smmPBj14}$ have shown a reduction of viraemia and production of antiviral-antibodies after about 2 weeks (Fultz, 1994). Attempts to develop prevention strategies have found that recombinant vaccines and a potent antiretroviral agent provided macaques with protection from lethal $SIV_{smmPBj14}$ challenge but not from natural infection (Hodge et al., 1999; Mossman et al., 1996).

Immune response to lentivirus infections

There are 2 main types of immune response to virus infections, involving the innate and adaptive immune systems (Cotran et al., 1999; Flint et al., 2000). The adaptive immune response can be humoral or cell-mediated, both of which seem important in lentivirus infections. The innate immune system can be activated within hours of infection and is the body’s first response to the virus. This system produces interferons, complement and inflammatory responses (Cotran et al., 1999). Interferons at this stage have many roles: they induce an antiviral state in neighbouring cells that inhibit virus replication and enhance clearance of infected cells by activating the complement cascade, natural killer cells (NK), dendritic cells,

macrophages, neutrophils and eosinophils, and releasing innate cytokines (Levy, 2001). In contrast to the innate immune system, the adaptive immune response is highly specific for a particular pathogen and takes longer to reach optimal activity (Roitt et al., 2001a). The longer time is required for activation of APC before the process of differentiation and maturation of the cell-mediated and humoral immune responses.

Perturbations of the immune system are a consequence of many lentivirus infections and can affect the virus-specific immune response. One example is the lack of a virus-specific humoral immune response seen in rapid progressors that can occur in HIV and SIV infections (Dykhuizen et al., 1998; Michael et al., 1997; Zhang et al., 2007). Another example is the late development of an antibody response seen in JDV infections in Bali cattle (Hartaningsih et al., 1994; Wareing et al., 1999) presumably due to the extensive early lesions in lymphoid tissues. A poor or minimal neutralising antibody response to HIV-1 can be a problem, permitting superinfection (Smith et al., 2006), or even when there is a robust neutralising antibody response this may fail to protect against infection with heterologous virus isolates (Blish et al., 2008; Yeh et al., 2009).

Cytokines produced by sensitised lymphocytes in response to lentivirus infections appear to have a significant role in the disease process associated with many lentiviruses. Lentivirus-infected cells and especially macrophages secrete pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumour necrosis factor-alpha (TNF-α) that tend to trigger clinical signs. They cause neural impairment at the late-stage of HIV and in SIV infections, by stimulating infiltration of macrophages into the central nervous system (CNS) and inducing production of neurotoxic substances (Orandle et al., 2001; Sopper et al., 1996; Xiong et al., 2000). In animals infected with virulent virus strains of EIAV, the activated macrophages predominantly produce pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (Lim et al., 2005). These cytokines are responsible for fever, anorexia and hypermetabolism leading to the wasting diseases that are commonly seen in HIV-induced AIDS (Chang et al., 1998). The mechanism by which the cytokines enhance *in vivo* virus replication include the recruitment of uninfected monocytes to the site of viral replication as found in HIV-1 and SIV (Schmidtmayerova et al., 1996; Zink et al., 2001), stimulation of an adhesion molecule that encourages monocyte migration into

tissues (Sampson et al., 2002), and the induction of viral activating molecules by non-monocytic cells as found in EIAV infection (Lim et al., 2005). Macrophages also secrete other cytokines and chemokines including IFN-type 1, RANTES, and macrophage inflammatory proteins-1α and 1β that regulate the replication of HIV in macrophages and CD^+4 T-cells (Fauci, 1996; Vicenzi et al., 1997).

Many lentivirus infections have a direct or an indirect affect on $CD4^+$ and $CD8^+$ T-cell populations, in the process affecting the production of cytokines. The kinetics of cytokine response in FIV, SIV and HIV infections and the level of their production is correlated with the development of clinical signs (Dean and Pedersen, 1998). $CD4^+$ T-cells, following contact with viral epitopes presented by MHC class II, secrete cytokines that promote the development of antigen-specific responses by both B and $CD8^+$ T- cells (Battegay et al., 1994; Clerici et al., 1994; DeKruyff et al., 1993). In FIV infections, activated $CD4^+$ T-cells release predominantly IL-2, IL-4, IL-10 and IFN-γ (Dean and Pedersen, 1998) but CD^+8 T-cells are also a source of IFN-γ (Dean and Pedersen, 1998; Maggi et al., 1997). In early HIV-1 infections, viral replication is also controlled by virus-specific $CD8^+$ T-cells (Pantaleo et al., 1997b).

A cytotoxic response by activated $CD8^+$ cells (cytotoxic T-cells; CTL) is critical in controlling viral replication. For example, the response to viral core proteins is associated with slower disease progression (Klein et al., 1995; Pantaleo et al., 1997b). $CD8^+$ T-cells inhibit virus replication by recognising and killing infected cells before completion of the virus replication cycle and the release of new virions (Mandl et al., 2007). Soluble factors secreted by $CD8^+$ T-cells include antiviral factors and cytokines that interfere with viral transcription and viral entry, respectively, and these are strongly associated with HIV non-progressive forms of HIV infection (Copeland et al., 1995; Zagury et al., 1998). Moreover, specific $CD8^+$ T-cell proliferation was associated with perforin expression that was maintained in nonprogressors (Migueles et al., 2002). However due to clonal exhaustion at later stage of disease the process, the HIV-specific CTL population decreases rapidly contributing to the inability of the immune system to control viral replication and spread of the virus (Pantaleo et al., 1997a). In order to investigate a candidate for anti-HIV-1 live-attenuated vaccines, gene-mutated HIV-1/SIV chimeric viruses were generated by recombination between HIV-1 and SIV_{mac} genomes (Haga et al., 1998; Hayami and Igarashi, 1997). The infection of the chimeric virus in naïve macaques

was associated with a depletion of $CD8^+$ T-cells, resulting in the inability of macaques to clear infection and led to a high plasma viral load, indicating that $CD8^+$ T-cells were actively involved in controlling the acute phase of primate lentivirus infections (Jin et al., 1999; Matano et al., 1998).

The humoral immune response is also important in controlling viral replication and this response is initiated by the presentation of antigens to immature B-lymphocytes that then develop into plasma cells secreting antibody. There are 5 classes of antibodies IgA, IgM, IgD, IgE and IgG, all of which have different antigen binding properties (Roitt et al., 2001a) and only 3 of the Ig classes appear important in viral infections: IgA involved in mucosal viral defence, IgM responsible for early viral agglutination and activation of the complement cascade, and IgG with a major role in viral clearance (Cavacini et al., 2003). The majority (about 75%) of all antibodies present in plasma are IgG subclasses that are released predominantly after consecutive exposure to an antigen. Although there are 4 IgG subclasses (IgG_1, IgG_2, IgG_3 and IgG_4), IgG_1 is the most abundant in human infections (Flint et al., 2000; Roitt et al., 2001a). Although the nomenclature describing human IgG subclasses is also used for bovine IgG subclasses, they may not necessarily be the same as the human equivalents. The presence of specific IgG subclasses has been correlated with the presence of certain types of cytokines, for example, *in vitro* studies have shown that the addition of IFN-γ (a Th1 cytokine) to stimulated bovine B-lymphocytes increased the turnover of IgG_2 mRNA and reduced the turnover of IgM and IgG_1 mRNA, suggesting that IFN-γ controlled IgG_2 production at the transcriptional level. In contrast, the addition of IL-4 (a Th2 cytokine) to stimulated bovine B lymphocytes enhanced the release of IgG_1 (Estes, 1996).

Neutralising antibody activity, while critical in controlling many virus infections, has a less obvious and uncertain role in lentivirus infections. In HIV-1 infections, IgG_1 and IgG_3 are involved in binding and neutralisation of virus, and essentially all neutralising antibodies are directed toward the envelope proteins, particularly gp120 (Cavacini et al., 2003; Parren et al., 1997; Rusche et al., 1988; Wyatt and Sodroski, 1998). HIV-specific antibodies are detectable in plasma of HIV-infected individuals within a few weeks of infection, but generally do not control viraemia (Aasa-Chapman et al., 2004; Binley et al., 1997) although several recent reports have indicated that neutralisation activity found within weeks of infection is associated

with early viral clearance (Frost et al., 2005; Richman et al., 2003; Wei et al., 2003). The neutralising antibody response improves with time and broader neutralisation activity against heterologous viral strains is found in chronic infections (Moog et al., 1997; Pilgrim et al., 1997). High levels of neutralising antibody have been detected in some long-term nonprogressors (Aasa-Chapman et al., 2004; Cao et al., 1995; Pilgrim et al., 1997; Zhang et al., 1997b). However, this non-progressor condition is not universal, and it is possible that the neutralising antibody response may contribute to inhibition of HIV replication in long term infections in some patients while not protecting against HIV superinfection in most patients (Bailey et al., 2006; Deeks et al., 2006). There was a strong correlation between the presence of a broadly cross-neutralising antibody response and non-progression to an AIDS-like syndrome in cynomolgus macaques infected with SIV_{sm}, indicating the important potential of neutralising antibodies to control viraemia in at least some lentivirus infections (Lauren et al., 2006).

Despite the comprehensively documented *in vivo* and *in vitro* activity of neutralising antibodies, some HIV isolates are highly resistant to neutralisation and this has been attributed to several factors. It has been attributed to the inaccessibility of antibody binding sites of the native Env complex (Moore et al., 1995; Wyatt and Sodroski, 1998; Zhu et al., 2006). It has been attributed to the variability of gp120, which allows new genotypes of HIV to escape neutralising antibodies produced in response to previous genotypes (Burton et al., 2004; Frost et al., 2005; Richman et al., 2003; Wei et al., 2003; Wyatt and Sodroski, 1998). Similarly in JDV infection, recovered animals resist challenge with homologous and heterologous strains suggesting the production of neutralising antibodies (Hartaningsih et al., 1994; Soeharsono et al., 1990) but it could be due to other immunological events. In Jembrana disease, the acute stage is associated with a significant reduction of peripheral blood lymphocytes and antibody production is delayed until about 11 weeks after infection (Dharma et al., 1994; Hartaningsih et al., 1994; Wareing et al., 1999). This suggests that T-cells may play an important role in controlling viral replication (Soeharsono et al., 1990). A significant reduction of the $CD4^+$:$CD8^+$ T-cell ratio in lymphoid tissues in the febrile and immediate post-febrile phase of Jembrana disease was reported (Dharma et al., 1994) suggesting that $CD8^+$ cells, increased during this phase and

may be involved but further studies of the lymphocyte response during Jembrana disease are required to determine this.

Chapter 3

Histological and immunohistochemical characterisation of experimentally induced Jembrana disease in Bali cattle

Summary

The principal lesions during Jembrana disease are detected mainly in the lymphoid system with a non-follicular proliferation of lymphocytes and these changes are associated with immunological effects including a delayed humoral response to the virus and transient immunosuppression. Histological and immunological studies were conducted to further characterise the cellular responses to the virus infection. The major histological changes during the febrile and early post-febrile phase were characterised by severe attenuation of follicles with depletion of the germinal centres and expansion of the parafollicular T-cell zone by proliferation of centroblast-like cells. JDV CA protein was detected primarily in these proliferating centroblast-like cells, and as they were morphologically similar to IgG-containing cells, the results suggested the main target of JDV was IgG-producing cells and not T-cells. Further studies utilising double-immunolabelling are required to confirm this.

Introduction

JDV produces a disease that is atypical of most lentivirus diseases. Infection of Bali cattle with JDV causes an acute and sometimes fatal disease after a short incubation period of 5-12 days, with clinical signs characterised by fever, depression, anorexia and generalised lymphadenopathy (Soeharsono et al., 1990). During the acute phase disease there is a high titre viraemia with about 10^8 infectious virus particles/ml of plasma (Soeharsono et al., 1990). Haematological changes include leukopenia, eosinopenia, a mild thrombocytopenia, a normocytic normochromic anaemia, hypoproteinaemia and elevated blood urea levels in association with kidney lesions (Soesanto et al., 1990).

Pathogenesis studies of JDV infection in Bali cattle have been undertaken (Dharma et al., 1991; Dharma et al., 1994) but many important biological questions remain unanswered. Initial studies describing the target organs and location of virus infected cells has been published (Chadwick et al., 1998) but comprehensive information regarding the location and identity of JDV-infected cells in infected organs is needed to facilitate a better understanding of the pathogenesis of JDV infection. Identification of target cells has played an important role in understanding the pathogenesis of many virus infections and of special value for this has been the use of immunoassays for detecting virus-specific antigens in tissue of infected animals, thereby enabling identification of the location of the target cells and their distribution in infected tissues. The availability of specific cell markers together with the development of fixation and antigen retrieval procedures with formalin-fixed, paraffin wax-embedded tissues, and improvement in immunolabelling kits, has increased the application and sensitivity of these tests (Gutierrez et al., 1999; Niku et al., 2006; Rathkolb et al., 1997).

In this Chapter, histological and immunohistological studies to characterise JDV-positive cells and other cells types in infected organs are described. Tissues obtained during the febrile and immediate post-febrile phases of the infection were tested, to provide comprehensive data related to the distribution and kinetics of the cellular response to JDV infection.

Materials and methods

Experimental animals

Bali cattle used in the experimental studies were female, approximately 12 months of age and weighed 80-100 kg. They were obtained from Nusa Penida, a small island adjacent to Bali, where Jembrana disease has never been reported and where antibodies to JDV have not been detected (Hartaningsih et al., 1994). Cattle purchased from the island have consistently developed clinical signs of Jembrana disease when infected with JDV (Soeharsono et al., 1990). Animals for these experiments were transported to Bali island to the Disease Investigation Centre Region VI Denpasar Bali. On arrival, they were sprayed with insecticide, kept in an insect-proof house and given water and elephant grass (*Penecetum purpureum*) *ad libitum*. Prior to use, all cattle were pre-treated with a broad spectrum antibiotic (oxytetracycline) at a dose rate of 5 mg/kg bodyweight for 3 consecutive days, a broad spectrum anthelmintic, and they were vaccinated against haemorrhagic septicaemia. Before inoculation with JDV, the absence of antibody to JDV was confirmed by ELISA test using a JDV recombinant CA antigen as described previously (Burkala et al., 1998).

Experimental design and infection with JDV

Two Bali cattle were infected with JDV and euthanised and sampled on the second day of the resulting febrile reaction. Five cattle were infected with JDV and euthanised and sampled 5-6 days after recovery from the febrile phase. Two cattle that had previously been infected with BIV-R29 as part of another experiment and housed separately were euthanised 42 days after BIV infection (Table 3.1) and used as controls. A total of 27 different tissues representing various organ systems were collected from all animals (Table 3.2).

Table 3.1. Animals and stage of infection when they were euthanised for tissue collection.

Animals	Virus infection	Time sampled
CB10, CB212	$JDV_{Tab/87}$	Febrile phase (2^{nd} day of febrile reaction)
CB203, CB205, CB206, CB208, CB.210	$JDV_{Tab/87}$	Post-febrile phase (5-6 days after febrile phase)
CB198, CB199	BIV-R29	42 days after infection

Table 3.2. Tissues collected from experimental animals.

Organ systems	Type of tissues
Central nervous	Cerebrum
Respiratory	Lung
Digestive	Pancreas, liver, rumen, reticulum, omasum, abomasum, duodenum. jejunum, ilium and colon
Lymphoreticular	Spleen, lymph nodes (prescapular, retropharyngeal, mediastinal, mesenteric), tonsil, thymus
Haematopoietic/ circulatory	Heart and bone marrow
Reproductive	Ovaries, uterus and mammary glands
Urinary	Urinary bladder, kidneys and adrenal cortex

Animals were infected with an estimated 10^3 ID_{50} JDV using methods very similar to those described previously (Stewart et al., 2005). Briefly, to obtain infectious virus a donor animal was inoculated intravenously with a suspension of frozen spleen from an animal infected with $JDV_{Tab/87.}$ On the second day of the resulting febrile

reaction, blood was removed and an approximate titre of infectious virus in the plasma was determined by an antigen-capture ELISA (Stewart et al., 2005). The plasma was then diluted to provide an estimated 10^3 ID_{50}/ml and 1 ml was inoculated intravenously into the experimental cattle.

Cattle were infected with the R29 strain of BIV as described previously (McNab et al., 2010).

Clinical signs in all cattle were recorded daily until the animals were killed for tissue collection.

Gross-pathological observation and sample collection

At necropsy examination, all fresh tissue samples listed in Table 3.2 were cut to an approximate 2 x 1 x 0.5 cm size and fixed in 10% neutral-buffered formalin pH 7.5 for 48-72 h. After further trimming, the tissues were then processed using an automatic tissue processor (Tissue Tek II) and embedded in paraffin wax. Sections of 4 µm thickness were cut from the formalin-fixed-paraffin wax-embedded tissues and these were mounted on silane coated glass slides (ProSciTech) and stored in incubator at 37^O C for not more than 3 days to ovoid tissue oxidation. Tissue sections were stained with haematoxylin and eosin (H&E) by standard procedures.

All sections were viewed using an Olympus BX51 photomicroscope and images were acquired using an Olympus DP 70 camera and associated Olympus DP controller software.

Immunohistochemical examinations

Source and characteristics of antibody reagents

A monoclonal antibody BD2 (mAb BD2) against JDV CA was produced by the Animal Virology Group, Murdoch University. Polyclonal rabbit anti-human CD3 (DakoCytomation) and monoclonal mouse anti-human CD79αcγ (DakoCytomation), monoclonal mouse anti-human MAC 387 that binds to a 34kDa protein, mb-1, a member of the B-cell antigen receptor complex (DakoCytomation), were reported to be reactive in formalin-fixed bovine tissues that were subjected to microwave pre-treatment (Gutierrez et al., 1999; Niku et al., 2006). Reactive antibodies were detected with a LSAB 2 system-HRP (DakoCytomation) that contained biotin-

labelled affinity isolated goat anti-rabbit and goat anti-mouse immunoglobulins coupled with streptavidin conjugated to peroxidase and used in conjunction with the chromogen 3-3' diaminobenzidine (DAB) for colour development. Polyclonal rabbit anti-bovine IgG was obtained from Jackson ImmmoResearch. The reactivity and properties of the antibodies used are summarised in Table 3.3. Normal mouse serum from adult Balb/C mice was used at a dilution of 1:50 as a negative control.

Table 3.3. Summary of antibodies used for immunolabelling.

Antibody	Antigen specificity	IgG isotype	Reactivity in cells	Antibody concentration
MAb BD2	JDV capsid (CA)	IgG1 kappa	Cytoplasmic	N/A
Anti-human CD3 (F7.2.38)	T-cell	N/A	Surface nor cytoplasmic	0.6 mg/ml
CD79αcγ (HM57)	B-cell	IgG1 kappa	Cytoplasmic	0.25 mg/ml
MAC387	Macrophage	IgG1 kappa	Cytoplasmic	0. 375 mg/ml
Rabbit anti-bovine IgG	Whole molecule bovine IgG			2.4 mg/ml

Immunoperoxidase labelling

Immunoperoxidase labelling was performed on tissue sections as previously described by Niku et al. (2006) with slight modification. Briefly, slides containing sequential tissue sections (one for a positive reaction and the other as a negative control) were heated 3 times for 3 min each time with 3 min between treatments with a hair dryer to ensure firm adhesion of the tissue to the glass slide. The tissues were then dewaxed by treatment with xylene and were then hydrated by immersion in an alcohol gradient (absolute, 95%, 70%) and finally water. The slides were then immersed in Tris-EDTA buffer (10 mM Tris, 0.1 mM EDTA, pH 9.2) in a plastic jar and then microwaved (2 times for 4 min at 805 W and 2 times for 4 min at 230 W with 1 min between each period of microwaving). The slides were then cooled by placing them in distilled water at room temperature. The sections were then treated

with 3% H_2O_2 in distilled water for 5 min to block endogenous peroxidise, then washed an additional 3 times with distilled water. The 2 tissue sections on each slide were circled using a wax pencil and 200 µl of primary antibody diluted in phosphate buffered saline (PBS) pH 7.2 containing 10% new born calf serum (NBCS) was added to the positive tissue sections, and PBS containing 10% NBCS to the control section. The dilution of antibody and time of incubation varied for each antibody and was determined by a series of preliminary experiments: MAb BD2 was diluted 1:200 and incubated for 30 min, the CD79αcγ (B-cell marker) was diluted 1:200 and incubated for 15 min, anti-human CD3 (T-cell marker) was diluted 1:50 and incubated for 30 min, and the anti-bovine IgG was diluted 1:1000 and incubated for 30 min. After incubation with the primary antibody, the slides were then washed 3 times with PBS. Two drops of biotinylated secondary antibody were then added to the sections and incubated for 15 min. The slides were again washed 3 times with PBS, and then 2 drops of peroxidase-labelled streptavidin was added to the slides and incubated for 10 min. After a further 3 washes with PBS the colour reaction was developed by adding 3-3'diaminobenzidine (DAB) for 3-5 min. The slides were then washed with distilled water, then the sections were lightly counterstained with Mayer's haematoxylin. The slides were then dehydrated in absolute ethanol, cleared with xylene and permanently mounted with DPX mounting medium before being examined by light microscopy. Negative controls were provided by omitting either the primary antibodies or secondary antibodies.

Results

Clinical signs and gross pathological findings

All JDV-infected cattle developed typical clinical signs of Jembrana disease: a transient increase in rectal temperature (commencing 9.86 ± 4.4 days after infection and persisting for 4.6 ± 2.3 days), anorexia, lethargy and a concurrent leucopenia (data not shown). Animals CB10 and CB212 that were euthanised on the second day of fever had very similar gross pathological changes including an enlarged spleen (Figure 3.1), moderate diffuse lymphadenomegaly, moderate lung consolidation in apical and cardiac lobes, and some haemorrhages in visceral organs. Animals CB203, CB205, CB206, CB208 and CB210 that were euthanised 5-6 days after the

resolution of fever had no macroscopic abnormalities. No gross pathological lesions were found in the control animals, CB198 and CB199, that had been infected with BIV 42 days previously.

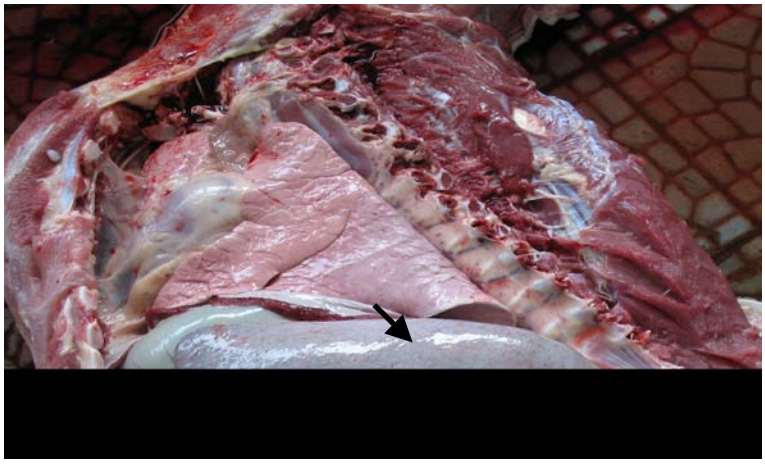


Figure 3.1. JDV-infected animal euthanised on the second day of the febrile reaction showing characteristic enlargement of the spleen to approximately 5 times normal size.

Histological examination

A range of tissues from 7 Bali cattle that were experimentally infected with $JDV_{Tab/87}$ were stained with H&E and examined for histological changes and immunolabelled to identify JDV-infected cells, B-cells, T-cells and macrophage/monocytes.

Histological changes during febrile phase

Tissues prepared from animals on the second day of the febrile reaction showed typical microscopic changes of Jembrana disease.

In the spleen the lymphoid follicles were severely attenuated with depletion and collapse of the germinal centre leaving focal aggregates of remnant mantle (dark zone) cells. The marginal zone (light zone) and periarteriolar lymphoid sheath were replaced by a population of medium sized pleomorphic round cells arranged in effacing sheets, leaving depleted remnants of the germinal centre and mantle zone (Figure 3.2A). These conditions were not observed in tissues prepared from control,

BIV-infected animals (Figure 3.2B). Many pleomorphic cells were observed infiltrating the parafollicular zone (red pulp), aggregating around penicilliary arteries and disseminated loosely throughout the red pulp. These pleomorphic cells were observed to have variable amounts of intensely basophilic cytoplasm, marked anisokaryosis with bizarre coarsely clumped chromatin, thus resembling centroblasts (Figure 3.3A) and they were not detected in tissue of BIV-infected control animals (Figure 3.3B). There were scattered apoptotic bodies and some mitotic cells. The periphery of the marginal zone (parafollicular zone) was demarcated by a circumferential ring of neutrophils.

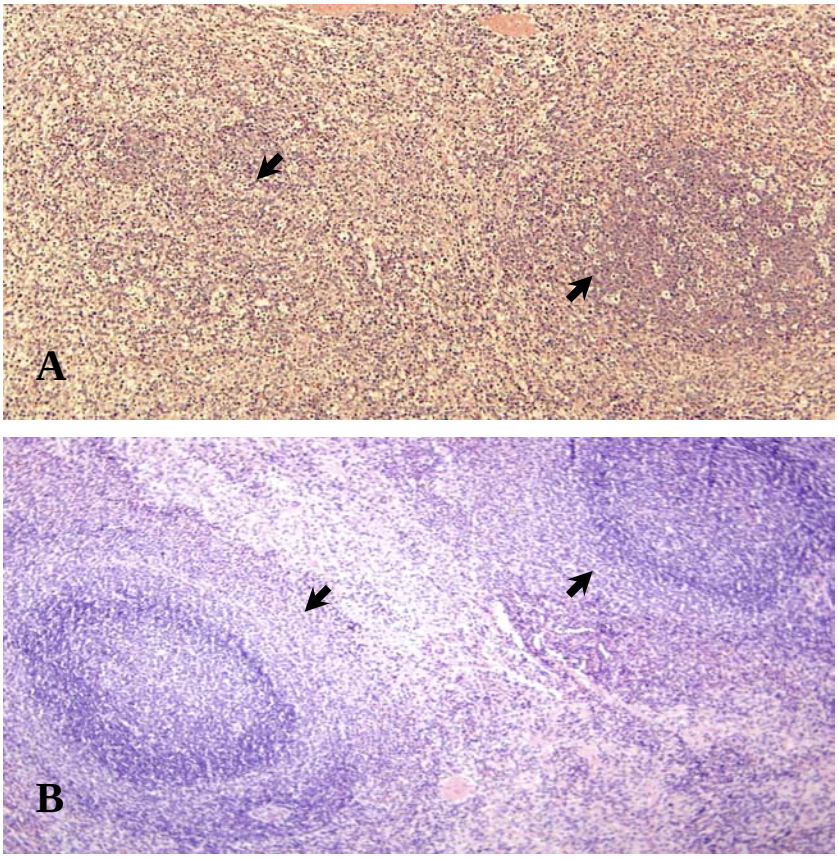


Figure 3.2. Spleen tissue from a JDV-infected animal on the second day of febrile reaction (A) and from control animal (B). There was a severe attenuation of follicles with depletion and collapse of the germinal centres. The depleted and normal follicular germinal centres are shown (arrow). H&E stain, 10X magnification).

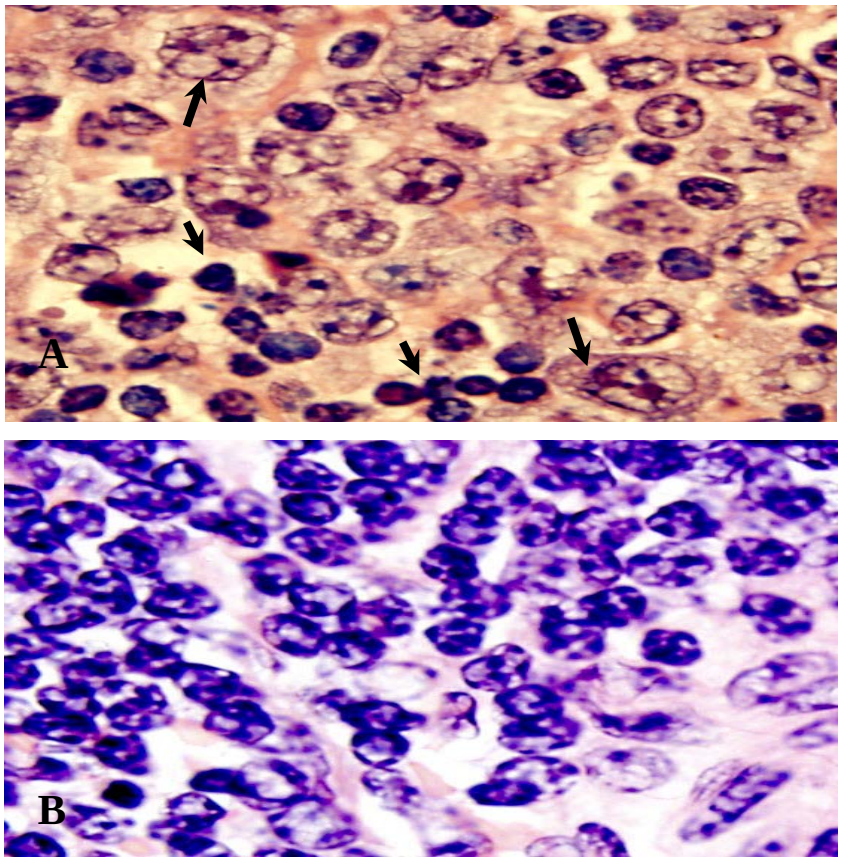


Figure 3.3. Paracortical area of spleen tissue from a JDV-infected animal on the second day of febrile reaction (A) and from a control animal (B). There was an abundant population of pleomorphic centroblast-like cells in JDV-infected tissue, mixed with a low number of small dark lymphocytes. Long arrows and short arrows point to representative pleomorphic centroblast-like cells and small dark lymphocytes, respectively. H&E stain, 100X magnification.

Lymph nodes displayed a consistent set of changes although the severity of the lesions varied within and between lymph nodes from different anatomical locations (Figure 3.4). The germinal centre of the follicles was depleted and the remnant mantle zone was surrounded by a wide zone of pleomorphic centroblast-like cells arranged in densely packed sheets that coalesced to efface the outer cortex. The pleomorphic centroblast-like cells were found surrounding large endothelial venules, filling the outer section of medullary cords, and infiltrating the surrounding cortex. There were a few pleomorphic centroblast-like cells in medullary sinuses. The lumen of large endothelial venules was often filled with neutrophils. Sometimes subcapsular sinuses and medullary sinuses were expanded by oedematous fluid.

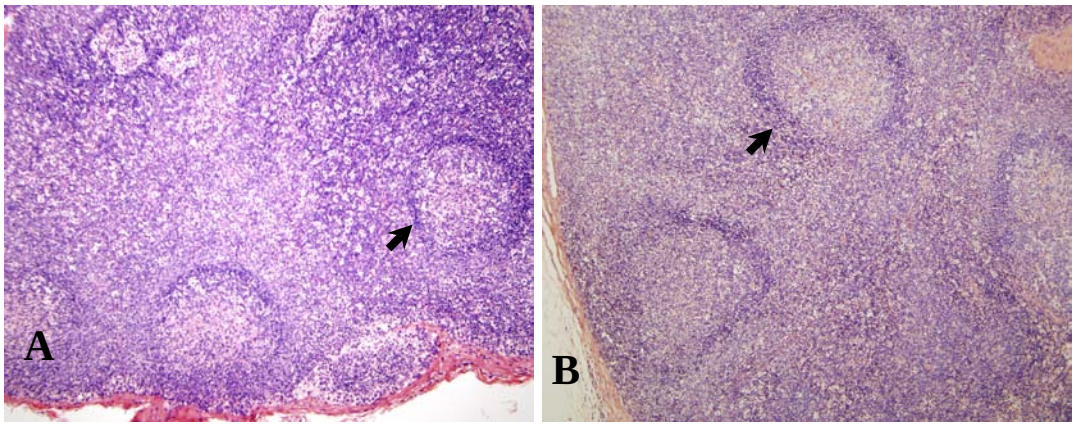


Figure 3.4. Mesenteric lymph node (A) and a prescapular lymph node (B) from a JDV-infected animal on the second day of the febrile reaction, showing depletion of follicular germinal centres. Arrows indicate representative depleted follicles. H&E stain, 10X magnification.

In the liver, scattered portal areas were expanded by infiltrates of mononuclear cells, including many pleomorphic centroblast-like cells.

In the kidney tissue of one (CB212) of the 2 animals, the interstitium surrounding some medium sized blood vessels was moderately expanded by a mononuclear cell infiltrate.

In the heart occasional vessels had a perivascular infiltrate of mononuclear cells.

In the bone marrow, there was a marked diffuse depletion of haematopoietic tissue, with a prominent lack of the myelocytic lineage storage pool band and segmented neutrophils. There were a few scattered pleomorphic centroblast-like cells in sinusoids.

In the thymus there was a severe diffuse depletion of the cortex with loss of cortical thymocytes, leaving a collapsed stroma.

In the lungs there was a mild diffuse expansion of the alveolar septa by fibrinous exudate, with some pleomorphic centroblast-like cells in the alveolar septa.

In the pancreas, aggregates of leucocytes were seen trapped in post-mortem blood clots within the lumen of medium to large diameter blood vessels in the pancreas. Some of the trapped circulating cells were pleomorphic centroblast-like cells.

No lesions were observed in the brain, adrenal, uterus, ovaries, mammary, urinary bladder, or the alimentary tract. Peyer's patches were small when present and could not be adequately examined in the tissues that were collected.

Histological changes in early post-febrile phase

In JDV-infected animals 5-6 days after the resolution of fever, there were no macroscopic abnormalities but there were a spectrum of microscopic lesions with a similar pattern of changes in all cattle.

In the spleen there were reduced numbers of follicles and the remaining follicles were variably attenuated, ranging from loss of the germinal centres and marginal zones leaving a remnant focal aggregation of mantle (dark zone) cells to depletion of the germinal centres and moderate expansion of the mantles. The marginal zone of severely affected follicles was populated by moderate numbers of centroblast-like cells and some tingible body macrophages. In the marginal zone of less severely affected follicles, there were a few centroblast-like cells but more apoptotic bodies and tingible body macrophages. The periarteriolar lymphoid sheaths were populated by sheets of small lymphocytes mixed with variable numbers of centroblast-like cells, apoptotic cells and tingible body macrophages. The parafollicular zone was loosely populated by neutrophils.

The lymph nodes of the recovered animals were similar to those of the febrile animals. The germinal centre of lymphoid follicles was depleted and the mantle zone was surrounded by a zone of pleomorphic centroblast-like cells. A striking difference, however, was that the corticomedullary junction was indistinct due to expansion of the paracortex by densely packed sheets of small dark lymphocytes. These were often mixed with many tingible body macrophages that were observed to have infiltrated and replaced the centroblast-like cells surrounding the follicles and populating the medullary cords. In some lymph nodes, the mantle zone of the follicles was expanded and some follicles had a sheet of centroblast-like cells occupying the germinal centres, characteristic of early reactive hyperplasia. In the thymus, the cortex was observed to be normal to mildly hyperplastic with loosely to densely packed sheets of small dark lymphocytes.

The haematopoietic tissue in the bone marrow was normocellular to hypercellular with a predominance of immature myeloid lineage cells with 10-20 % band neutrophils but no segmented neutrophils, indicative of a regenerative hyperplasia.

There were multifocal perivascular mononuclear infiltrates in the kidneys.

No lesions were apparent in other organs.

Distribution of JDV CA in tissues during febrile phase

JDV CA was detected in spleen tissues on the second day of the febrile phase and the distribution of immunolabelled cells was similar in the 2 animals (Figure 3.5A) and was not detected in tissues from BIV-infected control cattle (Figure 3.5B). In the spleen, numerous large pleomorphic cells with strong cytoplasmic labelling were disseminated throughout the red pulp, largely sparing lymphoid follicles and periarteriolar lymphoid sheaths. The JDV CA-positive cells were large pleomorphic cells with eccentric nuclei and resembled plasma cells and contained dark brown intracytoplasmic labelling (Figure 3.6A). The morphology of the cell type involved and the distribution of the immunoreactivity within the cells was similar to the morphology and distribution of IgG-containing cells (Figure 3.6B), although the JDV-CA labelling reaction was much stronger than the reaction with IgG.

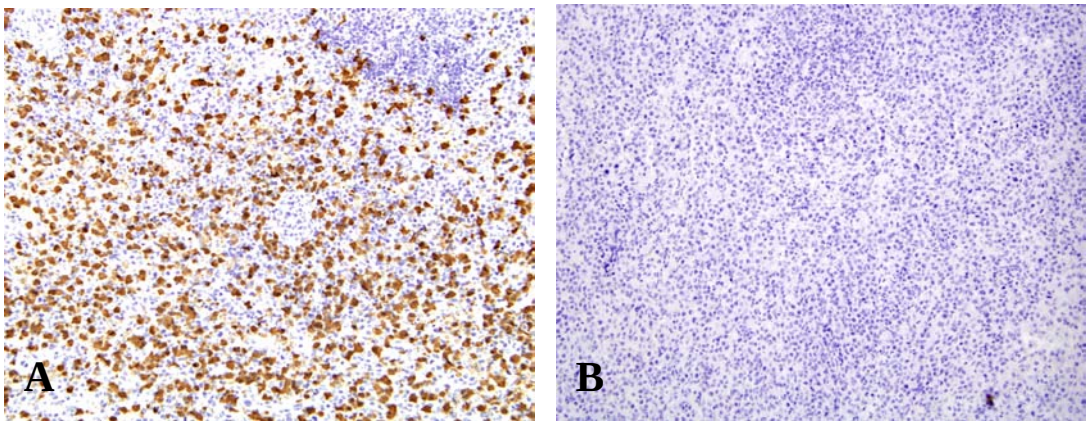


Figure 3.5. Spleen tissue from a JDV-infected animal on the second day of febrile phase (A) and from a control animal (B), both reacted with anti JDV CA MAb. JDV CA-containing cells (dark brown labelling) were prominent in the JDV-infected tissues (A) but not in the BIV-infected tissue (B). Immunoperoxidase labelling, 10X magnification. No labelling was detected when tissues were reacted with normal mouse serum or when reaction with the primary antibody was omitted.

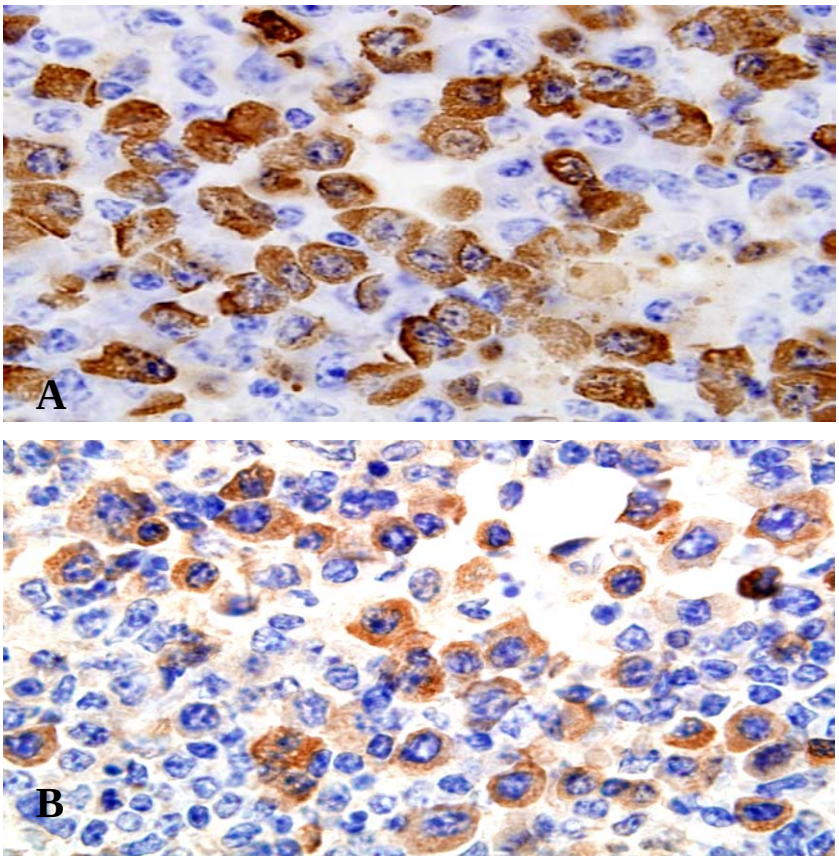


Figure 3.6. Spleen tissue from a JDV-infected animal on the second day of the febrile reaction, reacted with JDV CA MAb (A) and with polyclonal anti bovine IgG (B). The cells containing JDV CA were morphologically similar to those containing IgG: in both cases there was dark brown intracytoplasmic labelling of large cells with eccentric nuclei. Immunoperoxidase labelling, 100X magnification. No labelling was detected when tissues were reacted with normal mouse serum or when reaction with the primary antibody was omitted.

In the lymph nodes and tonsils there were variable numbers of large JDV CA-containing pleomorphic cells throughout the medullary cords, morphologically similar to those detected in spleen. There were fewer of these immunolabelled cells in the cortex and medullary sinuses and they were largely absent in the follicles. Occasionally, JDV CA was detected in cells in the lumen of large endothelial venules.

In the liver, JDV CA was detected in many cells in the mononuclear cell infiltrates in the portal areas of the liver. A few JDV CA-containing cells were also scattered randomly throughout the sinusoids, and were also detected in the lumen of blood vessels (Figure 3.7).

In the lungs, JDV CA was mainly detected in cells in the alveolar septa, and sometimes in the lumen of large blood vessels.

The kidney contained multifocal perivascular mononuclear cell infiltrates with a few JDV CA-positive cells and with low numbers of JDV CA-positive cells in the lumen of glomerular and interstitial capillaries.

In the heart, the multifocal perivascular mononuclear infiltrates also contained many cells with intracytoplasmic JDV CA.

In the bone marrow there were some cells containing JDV CA scattered diffusely throughout the sinusoids.

In all other tissues, including the thymus, there were rare JDV CA-containing cells within the lumen of vessels.

The characteristic reaction and the distribution of JDV-positive cells in a number of different tissues are presented in Figure 3.8 A-L, and the results are summarised in Table 3.4.

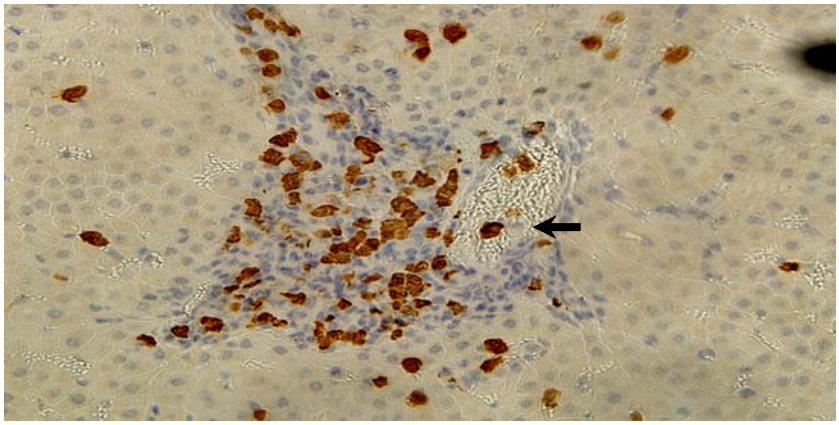


Figure 3.7. Liver tissue section from JDV-infected animal on the second day of the febrile reaction, reacted with anti MAb BD2 against JDV CA, showing reactive cells in the sinusoids and lumen of a blood vessel (arrow). Immunoperoxidase labelling, 40X magnification.

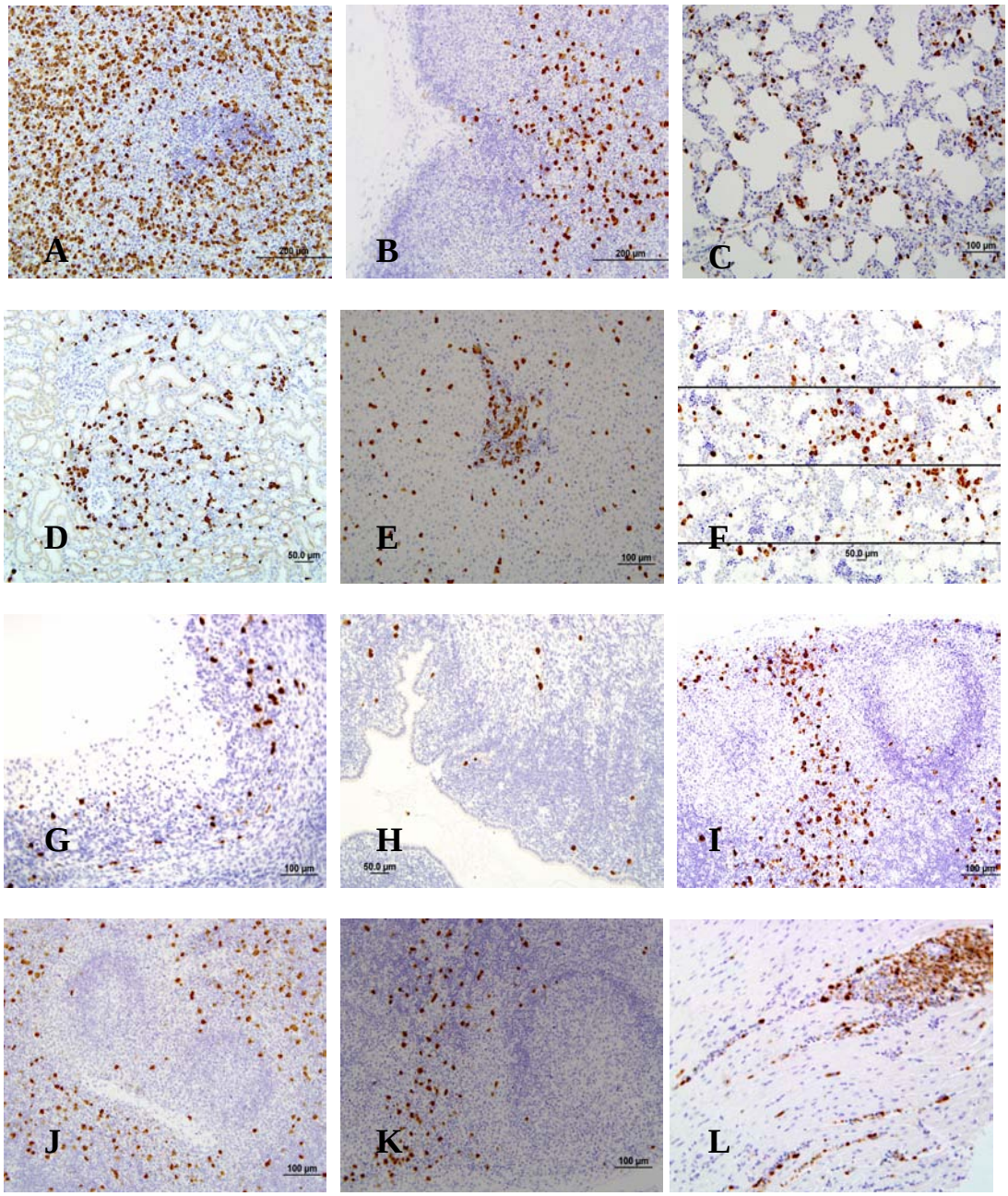


Figure 3.8. Representative tissue sections from JDV-infected animals on the second day of febrile reaction, reacted with MAb BD2 against JDV CA. JDV CA-positive cells were detected in a wide range of different tissues: A, spleen; B, prescapular lymph node; C, lungs; D, kidney; E, liver; F, bone marrow; G, ovary; H, uterus; I, retropharyngeal lymph node; J, mediastinal lymph node; K, mesenteric lymph node; L, heart. Immunoperoxidase labelling, 20X magnification.

Table 3.4. Summary of the distribution of JDV CA in tissues collected from 2 animals (CB10 and CB212) on the second day of the febrile reaction, determined using an immunoperoxidase procedure with MAb BD2 against JDV CA. Results were indistinguishable in the 2 animals.

Organ systems	Type of tissues	Results
Lymphoid	Spleen	+++
	Superficial lymph node	++
	Mesenteric lymph node	++
	Retropharyngeal lymph node	++
	Mediastinal lymph node	++
	Thymus	++
	Tonsil	+
Respiratory	Lungs	++
Digestive	Rumen	+
	Omasum	+
	Abomasum	+
	Intestine	+
	Liver	+
	Pancreas	+
Urinary	Adrenal cortex and kidneys	+
Haematopoietic/circulation	Bone marrow and heart	+
Reproductive	Ovaries and uterus	+
Nervous	Cerebrum	-

Positive (+) and negative (-) reactions were scored by qualitatively observing about 100 positive cells on 20X magnification: +++, very strong reaction (≥100 cells per microscope field); ++, strong reaction (50-100 cells per microscope field); +, weak reaction (≤50 cells per microscope field); - ,no positive cells.

Distribution of B-cells in tissue during febrile phase

In the spleen, B-cells were identified using the CD79αcγ MAb that produced dark brown intra-cytoplasmic labelling in reactive cells that were pleomorphic and centroblast-like and located in the marginal zone and periarteriolar lymphoid sheath of the spleen (Figure 3.9). In the lymph nodes and tonsils, similar pleomorphic

centroblast-like cells were observed within and surrounding the follicles, populating the cortico-medullary portion of medullary cords and in the paracortex. Similar cells were detected in the perivascular mononuclear infiltrate in the liver, kidney and heart, and megalokaryocytes and some round cells in the sinusoids of the bone marrow were also positive.

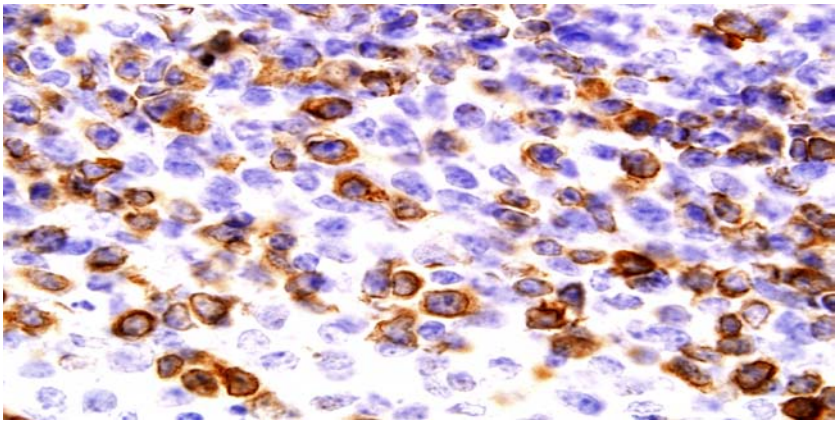


Figure 3.9. Spleen tissue section from JDV-infected animal on the second day of the febrile reaction, reacted with anti-human CD79αcγ (a B-cell marker). Note the CD79αcγ^{+} cells containing dark brown cytoplasmic labelling, around a depleted follicle. Immunoperoxidase labelling, 40X magnification.

Distribution of T-cells in tissue during febrile phase

In the spleen, T-cells that were identified by the presence of CD3 in the cytoplasm and cell surface, were unexpectedly scarce in the periarteriolar lymphoid sheaths due to apparent replacement by the pleomorphic centroblast-like B-cells (Figure 3.10). In the paracortex of lymph nodes and the tonsil there were multifocal but variably poorly demarcated islands of T-cells between the sheets of pleomorphic centroblast-like cells.

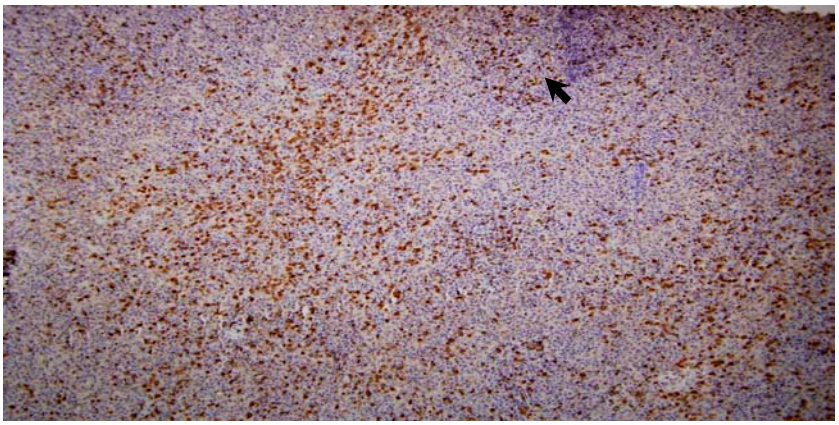


Figure 3.10. Spleen tissue section from a JDV-infected animal on the second day of the febrile reaction reacted with anti-human CD3, a T-cell marker. Note the scarce (relative to those detected in the post-febrile phase) population of $CD3^+$ cells (dark brown) in the periarteriolar lymphoid sheaths of a depleted follicle (arrow). Immunoperoxidase labelling, 10X magnification.

Distribution of cells of monocyte/macrophage lineage in tissue during febrile phase

Cells of the myelomonocytic lineage including granulocytes, circulating monocytes and a subset of (recent tissue emigrant) macrophages were identified using MAb MAC387. The majority of immunolabelled cells were identified as neutrophils on the basis of their segmented nuclei. Typically, a ring of neutrophils was observed surrounding the follicular mantle zone in the spleen (Figure 3.11) and the lumen of some large endothelial venules also contained neutrophils. Using serial sections, the distribution of these cells in other lymphoid compartment during the febrile phase of disease was less and differed to the distribution of cells that reacted with CD3, CD79αcγ and JDV CA MAb (Figure 3.12A-D).

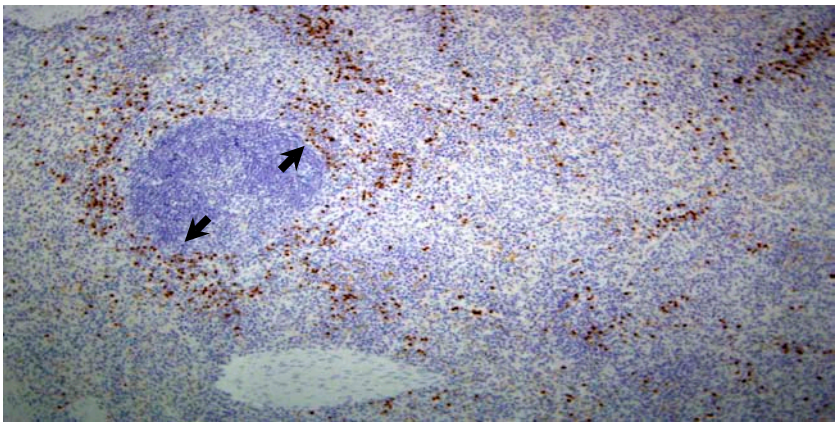


Figure 3.11. Spleen tissue section from a JDV-infected animal on the second day of febrile reaction reacted with anti-human monoclonal antibody, MAC387. MAC387^{+} cells are indicated by dark brown labelling and they were predominantly detected around the follicular mantle zone (arrow). Immunoperoxidase labelling, 10X magnification.

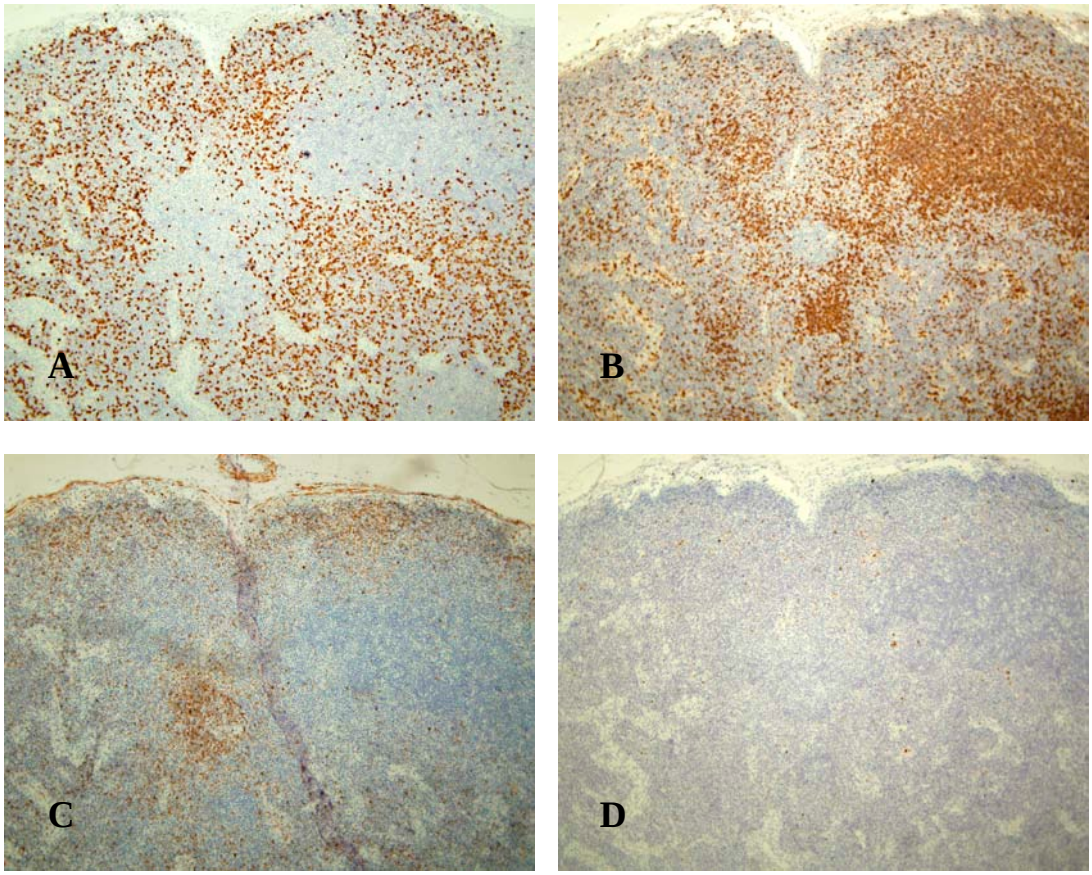


Figure 3.12. Serial sections of a mesenteric lymph node from a JDV-infected animal on the second day of febrile reaction reacted with anti JDV CA MAb (A), anti-human CD3, a T-cell marker (B), anti-human CD79αcγ, a B-cell marker (C) and anti-human MAC387 (D). The distribution patterns of the JDV CA-containing cells was generally different to the distribution of the CD3^{+} cells and the MAC387^{+} cells, but similar to the distribution of the CD79αcγ^{+} cells. Immunoperoxidase labelling, 10X magnification.

Distribution of JDV CA in tissues during early post-febrile phase

In contrast to the febrile phase, viral antigen was not detected in tissues prepared from cattle euthanised during the post-febrile phase.

Distribution of B-cells in tissues during early post-febrile phase

CD79αcγ^+ B-cells were identified in follicular mantles and were present in low numbers as centroblast-like cells surrounding follicles, and loosely disseminated throughout the periarteriolar lymphoid sheaths and red pulp of the spleen. In the lymph nodes the remnant mantle zone of follicles was CD79αcγ^+ and there were moderate numbers of centroblast-like cells with cytoplasmic labelling forming loose remnants of the coalescing perifollicular zones and sheets in the medullary cords.

Distribution of T-cells in tissues during early post-febrile phase

Numbers of $CD3^+$ T-cells in the periarteriolar lymphoid sheaths in the spleen and the paracortical areas of the lymph nodes were expanded by sheets of densely packed $CD3^+$ T-cells that infiltrated amongst and replaced the CD3-negative centroblast-like cells. In contrast to the acute phase, a sparse population of $CD3^+$ T-cells was detected in lymphoid tissues (Figure 3.13A), an abundant population of $CD3^+$ T-cells was found in these tissues during the early post-febrile phase (Figure 3.13B).

Distribution of cells of monocyte/macrophage lineage during early post-febrile phase

The distribution of $MAC387^+$ cells was essentially the same as in the febrile animals, but the large endothelial venules of the lymph nodes were not packed with neutrophils as was detected during the febrile phase (data not shown).

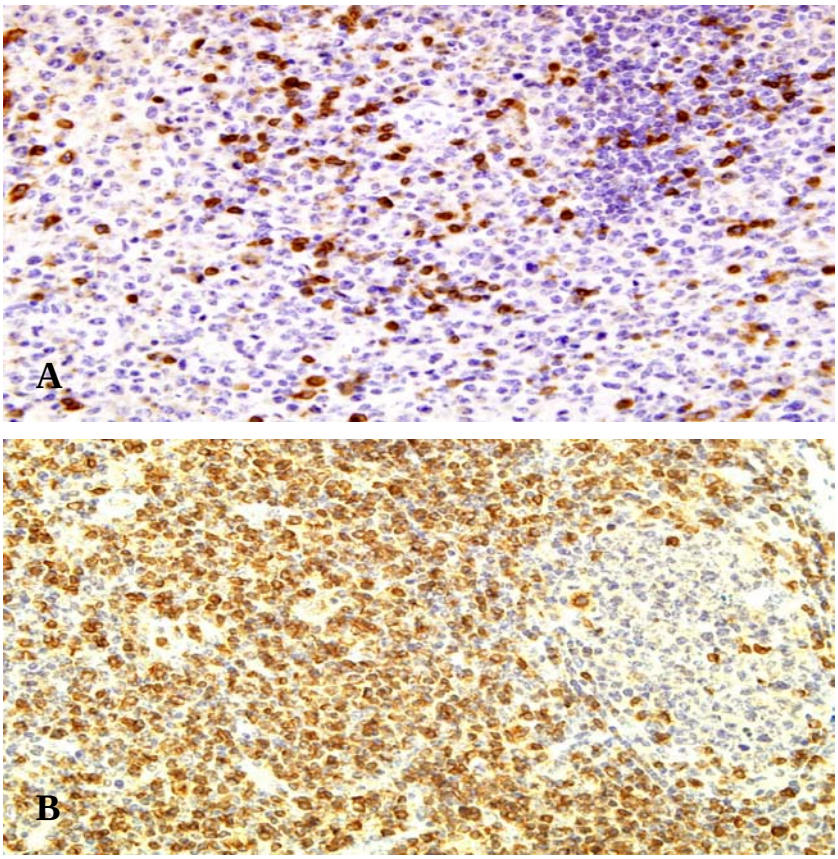


Figure 3.13. Mesenteric lymph node section from a JDV-infected animal on the second day of febrile reaction (A) and during the post-febrile febrile phase (B), demonstrating the distribution of $CD3^+$ T-cells. A sparse population of $CD3^+$ T-cells was detected around depleted follicles during the febrile phase, but an abundant population of $CD3^+$ cells was detected in the same region during the immediate post-febrile phase. The $CD3^+$ T-cells are indicated by dark brown cytoplasmic and/or cell membrane labelling. Immunoperoxidase labelling, 10X magnification.

Discussion

In the current study, typical clinical signs of Jembrana disease (Soeharsono et al., 1995a; Soesanto et al., 1990) were induced by infection with $JDV_{Tab/87}$. In the 2 control cattle that had been inoculated with BIV-R29 42 days previously there had been no clinical signs of infection as expected (McNab et al., 2010); these previously BIV-infected animals were used as controls in these current experiments to avoid the expense of purchasing additional non-infected cattle.

The major histological changes during the febrile phase of the acute disease induced by JDV occurred in lymphoid organs and were characterised by severe attenuation of

lymphoid follicles, with depletion of germinal centres, and a marked parafollicular reaction of the lymph nodes and the non-follicular compartment of the spleen. Significant histological lesions were not observed in several tissues, namely the adrenal, uterus, ovaries, mammary, urinary bladder and the alimentary tract. The microscopic changes in lymphoid organs are typical of those reported previously (Dharma et al., 1991) and are a hallmark of Jembrana disease.

The depletion of germinal centres during the febrile phase, a B-cell area, was thought to be associated with transient immunosuppression following JDV infection and the delayed development of antibody to the virus (Dharma et al., 1991; Dharma et al., 1994; Hartaningsih et al., 1994; Wareing et al., 1999), similar to that reported in rapidly progressive SIV infection (Zhang et al., 2007). It was also thought that the intense proliferative changes that occurred in the non-follicular area of lymphoid tissues during Jembrana disease were probably associated with proliferation of T-cells (Dharma et al., 1991). In the current studies, a marked proliferative reaction was also observed in the non-follicular regions but in the febrile phase the apparent proliferation was not due to an increase in $CD3^+$ T-cells but to an apparent infiltration of the areas with pleomorphic centroblast-like cells, which were not described previously (Chadwick et al., 1998; Dharma et al., 1991). The centroblast-like cells were predominantly observed in the parafollicular zone of red pulp of the spleen and in the medullary cords of lymph nodes. In the non-lymphoid tissues, they were mainly observed aggregating around small blood vessels, indicating they were probably derived from the circulating blood and may have originated from lymphoid tissues.

Five to 6 days after recovery from the febrile phase, a spectrum of microscopic lesions was still present in lymphoid tissues but they were of lesser severity and lesions were not detected in other organs. This change in the distribution of lesions and a decrease in the severity of the microscopic lesions in lymphoid tissues after the febrile period was also noted previously (Dharma et al., 1991). In the post-febrile phase, centroblast-like cells were still observed surrounding the follicles and around the medullary cords but their numbers were reduced and they were replaced by densely packed sheets of small dark lymphocytes.

What subpopulations of $CD3^+$ cells were involved in the tissue changes detected was not determined. The polyclonal rabbit anti-human CD3 marker that was used is

known to consist at least 4 different components (γ, δ, ε, ζ) with different distribution. In cattle, the distribution of γδ T-cells is localised to epithelial surfaces, particularly the skin and intestine and only 1-3% occur within lymph nodes, a percentage much less than the numbers of $CD4^+$ or $CD8^+$ T-cells that would be expected (Mackay and Hein, 1989). The dynamics of the T-cell response during the acute phase of Jembrana disease is interesting as, in the absence of an antibody response against the virus, it is probably related to a $CD8^+$ CTL-mediated immune response enabling recovery from the disease. In a previous study (Dharma et al., 1994) changes in the lymph node follicles were associated with a decrease in the $CD4^+$:$CD8^+$ T-cell ratio. A further study to examine the changes in $CD3^+$ T-cell subpopulations that occur from the onset of the febrile phase and recovery is required to determine details of the changes occur in this period.

There was no significant change in the number or distribution of $MAC387^+$ cells during the 2 phases of the acute disease process that were examined. The majority of $MAC387^+$ cells appeared to be neutrophils and these cells were mainly observed surrounding the marginal zone of the spleen and they occurred in only low numbers in other organs.

The presence of JDV CA in cells was used as an indication of JDV infection and the results obtained were similar to a previous study utilising the detection of virus RNA by ISH to demonstrate the distribution of virus in tissues (Chadwick et al., 1998). In both studies, the JDV-infected cells were detected predominantly in lymphoid organs, they were abundant in the non-follicular compartment of the spleen, and were also present in the paracortex of the lymph nodes and tonsils. They also infiltrated around perivascular area of the liver, kidney, heart and the bone marrow, but their distribution in these tissues suggested whey were derived from the circulation and they probably originated from the lymphoid organs. The JDV-infected cells were morphologically consistent with the morphology of the pleomorphic centroblast-like cells detected in the non-follicular areas of lymphoid tissues especially during the febrile phase of the acute disease. The CD79αcγ B-cell marker reacted with these cells and IgG was detected in morphologically similar cells. The available evidence strongly suggests that the virus-infected cells were antibody-producing cells but further studies utilising double-labelling techniques are required to confirm this, and these studies were undertaken and are reported in

Chapter 4. Both BIV and BLV have been shown to infect B-cells (Ban et al., 1993; Lavanya et al., 2008; Whetstone et al., 1997; Wu et al., 2003) and if confirmed in JDV infections it would explain the early loss of cells from the follicular compartments of lymphoid tissue and the delayed humoral immune response to JDV in infected cattle.

Chapter 4

Identification of the target cell of Jembrana disease virus in experimentally infected Bali cattle

Summary

A double immunofluorescent labelling method was developed to identify the subset of mononuclear cells in which the JDV CA protein could be detected. The protein was present in pleomorphic centroblast-like cells which were identified as B-lineage cells, possibly plasma cells, in lymphoid tissues. There was no evidence of infection of $CD3^+$ T-cells or $MAC387^+$ monocytes in tissues but large cells with a macrophage-like morphology in the lung were found to contain viral antigen, although they could not be conclusively shown to be productively infected. The tropism of JDV for mature B-cells may be relevant to the pathogenesis of Jembrana disease, particularly the delayed antibody responses and the genetic stability of this atypical lentivirus.

Introduction

Experimental infection with JDV initially causes a non-follicular lymphoproliferative response in lymphoid organs with a loss of IgG-containing cells and a decreased $CD4^+$:$CD8^+$ T-cell ratio in lymphoid tissues during the febrile phase of the disease (Dharma et al., 1991; Dharma et al., 1994). The distribution of infected cells in tissues at this stage of the disease was found to be predominantly in the parafollicular areas of the spleen and lymph node with little or no evidence of infected cells in the follicles (Chadwick et al., 1998). During the febrile phase, the follicular architecture was found to be obliterated by proliferating cells and marked follicular lymphoid reactions and plasma cell formation were only observed again from the fifth week after infection (Dharma et al., 1991). The phenotype of the proliferating cells in the parafollicular regions of the lymphoid tissue during the febrile phase of the disease was originally suggested to be T-cells. However, in Chapter 3, B-lineage cells ($CD79\alpha c\gamma^+$) were identified in the cortico-medullary region and were found to coalesce to efface the paracortex of lymph nodes and tonsils during the early stages of the febrile phase of the disease. A $CD3^+$ T-cell proliferative response was identified in lymphoid tissues in the immediate post-febrile phase (Chapter 3).

The cell-tropism of JDV was suggested to be lymphocytes and/or monocyte/macrophage lineage cells (Chadwick et al., 1998) but this has never been confirmed. The genetically related BIV exhibits a broad cell tropism *in vivo* which includes T-cells, B-cells and monocyte/macrophage cells although it is not clear whether all of these cell types are productively infected since only BIV proviral DNA was detected using PCR in these studies (Heaton et al., 1998; Whetstone et al., 1997). Other experimental infection studies in cattle have reported that BIV could only be isolated from monocytes but not from T-cells (Onuma et al., 1992) and when inoculated into rabbits, BIV antigen was detected in atypical blastic mononuclear cells in the red pulp of the spleen, cells which were presumed to be of the macrophage lineage (Pifat et al., 1992). JDV strains in cattle in Bali island are genetically stable with little variation even in *env* sequences in isolates from over a period of 20 years (Desport et al., 2007) which could indicate that JDV has a narrow host cell range, potentially targeting long-lived cells with a slow turnover rate.

Attempts to culture JDV in cell lines that support BIV infection have been unsuccessful and only *in vitro* cultivation of bovine mononuclear cells derived from peripheral blood could be shown to transiently support JDV replication when inoculated with plasma from infected cattle (Wilcox et al., 1992).

Perturbations of the immune system are a consequence of many lentiviral infections and a hallmark of Jembrana disease is the transient immunosuppression and delay in the development of virus specific antibodies until 5-15 weeks after infection (Desport et al., 2009; Hartaningsih et al., 1994; Wareing et al., 1999). A lack of a virus specific humoral immune responses has been reported in rapid progressor HIV and SIV infections (Dykhuizen et al., 1998; Michael et al., 1997) attributed to a progressive depletion of proliferating B-cells as early as 20 days after infection (Zhang et al., 2007).

To further define the tropism of JDV, double-immunofluorescent labelling was used to detect JDV CA in subsets of PBMC identified using specific antibodies in formalin-fixed tissues.

Materials and methods

Animals

The tissues examined were from 2 female Bali cattle (CB10 and CB212) 6-12 months of age purchased from Nusa Penida and confirmed as being free of JDV CA antibody using ELISA (Hartaningsih et al., 1994). The cattle were infected with 1 ml of a 10% homogenate of spleen in DMEM which had previously been prepared from an animal experimentally infected with $JDV_{Tab/87}$ (Soeharsono et al., 1995a). Rectal temperatures were monitored after infection and both animals were euthanised 2 days after developing rectal temperatures $\geq 39.5^{o}$C. Tissue samples were collected into 10% neutral buffered formalin from both animals, and included spleen, tonsil, lymph nodes, heart and bone marrow. Sections were prepared from each tissue at 4 μm thickness, mounted on silane coated glass slides (ProSciTech) and stored for ≤3 days before labelling to avoid tissue oxidation.

Generation and testing of JDV CA MAb hybridomas

Biotinylated recombinant JDV Gag protein constructs were expressed using Pinpoint Xa-1 vector (Promega) in *Escherichia coli* JM109 as described previously (Desport et al., 2005) and were kindly supplied by Dr Moira Desport.

A murine MAb produced against Jgag6, a construct encoding the entire JDV CA protein, was produced by Mr Judhi Rachmat in our laboratory. Briefly, the construct was expressed and purified according to the manufacturer's instructions and mixed with an equal volume of incomplete Freund's adjuvant. Eight-week-old female BALB/c mice were immunised 3 times at 2-week intervals. Three days after the final injection, mouse spleen cells were isolated and fused with the mouse myeloma cell line NS0 using 43% polyethylene glycol, (PEG, MW 1300-1600) (Sigma) as described previously (Chan and Mitchison, 1982). The screening assays were performed during cell growth in hypoxanthine aminopterin thymidine (HAT) selection medium. Positive clones were subcloned twice by limiting dilution. The specificity of the MAb was determined using the JDV Gag protein constructs in Western immunoblots as previously described (Desport et al., 2005). Isotyping of the selected MAb (LD1) was done on culture supernatants using a mouse isotyping kit (BioRad) and determined to have an isotype of IgG2b.

Antibodies and antigen retrieval

JDV CA in cells was visualised using the MAb LD1. Cells of the myelomonocytic lineage were identified using either MAC387 (as described in Chapter 3) or EBM11 (DAKO) and T-cells were identified using a human CD3 polyclonal antibody (DAKO) as described in Chapter 3. B-cells were identified using MAb anti-human CD79αcγ and a polyclonal rabbit anti-bovine IgG was used to identify IgG-containing plasma cells as described in Chapter 3. Alexa Fluor 488 (green) and 568 (red) conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen) were used to specifically recognise primary antibodies in double-immunofluorescence labelling. Immunoperoxidase labelling was performed after sections were deparaffinised in xylene and rehydrated through graded alcohols as described in Chapter 3. Antigen retrieval was required for all antibodies and consisted of microwave treatment in Tris-EDTA buffer pH 9.2 as described in Chapter 3. An alternative antigen retrieval method was required for MAb EBM11 which after

optimisation was found to be digestion in 0.1% proteinase-K (Invitrogen) for 5 min at room temperature.

Immunoperoxidase labelling

After antigen retrieval, all sections were treated with 3% H_2O_2 for 5 min before addition of primary antibody diluted in PBS containing 10% newborn calf serum for 15-30 min at room temperature. Streptavidin-biotin reagents (LSAB2, DAKO) with specificity for mouse IgG were used to directly detect primary mouse antibodies using DAB as the substrate chromogen, as described in Chapter 3. Specificity of antibodies was confirmed by omission of primary antibody and testing uninfected tissues from control animals.

Double immunofluorescence labelling

The slides were incubated with mixtures of 2 primary antibodies, using dilutions which had been established after preliminary titrations, for 15-30 min at room temperature, as described in Chapter 3. After washing 3 times with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.8), the appropriate secondary antibodies labelled with Alexa Fluor 488 or 568, either from different species or with differing Ig subclasses, were applied in TBS and incubated in the dark for 15-30 min. Additional sensitivity was obtained, where necessary, by using a rat anti-mouse IgG biotinylated secondary antibody that was subsequently detected using streptavidin Alexa Fluor 488 (Invitrogen). Finally, slides were washed with TBS and dried in the dark for 15 min, before being mounted in DAPI mounting medium (Vector Laboratories).

***In situ* hybridisation**

The protocol for ISH was as previously reported for detection of positive-sense JDV RNA using a digoxigenin-labelled riboprobe (Chadwick et al., 1998), with the following modifications. Prior to hybridisation, sections were pre-treated in a microwave in Tris-EDTA buffer (10 mM Tris, 0.1 mM EDTA, pH 9.2) as described for immunolabelling in Chapter 3. The digoxigenin-labelled probe was detected using a sheep anti-DIG-alkaline phosphatase conjugate (Roche) diluted 1:500 in the blocking solution and HNPP/Fast Red mixture (Roche). DAPI mounting medium was used to counterstain the nuclei (Vector Laboratories).

Results

Characterisation of JDV CA-specific MAb

To undertake further investigations into the cell tropism of JDV during acute infection, a virus specific MAb was produced which was selected so that it was of a different isotype (IgG2b) compared to the cell surface markers that were used (IgG1). MAb LD1 was successfully produced using a recombinant CA protein as the immunising antigen in mice and was screened against a range of JDV truncated Gag proteins to determine the specificity of the antibody binding. The JDV *gag* sequence encoded by each of the DNA constructs is shown in Figure 4.1 together with the Western immunoblot results showing that the MAb was reactive against Jgag2, Jgag 3, Jgag 5, Jgag 6 and Jgag 11. This indicated that MAb LD1 reacted with an epitope encoded by a region of the JDV genome between nucleotides 604-810 (Chadwick et al., 1995b).

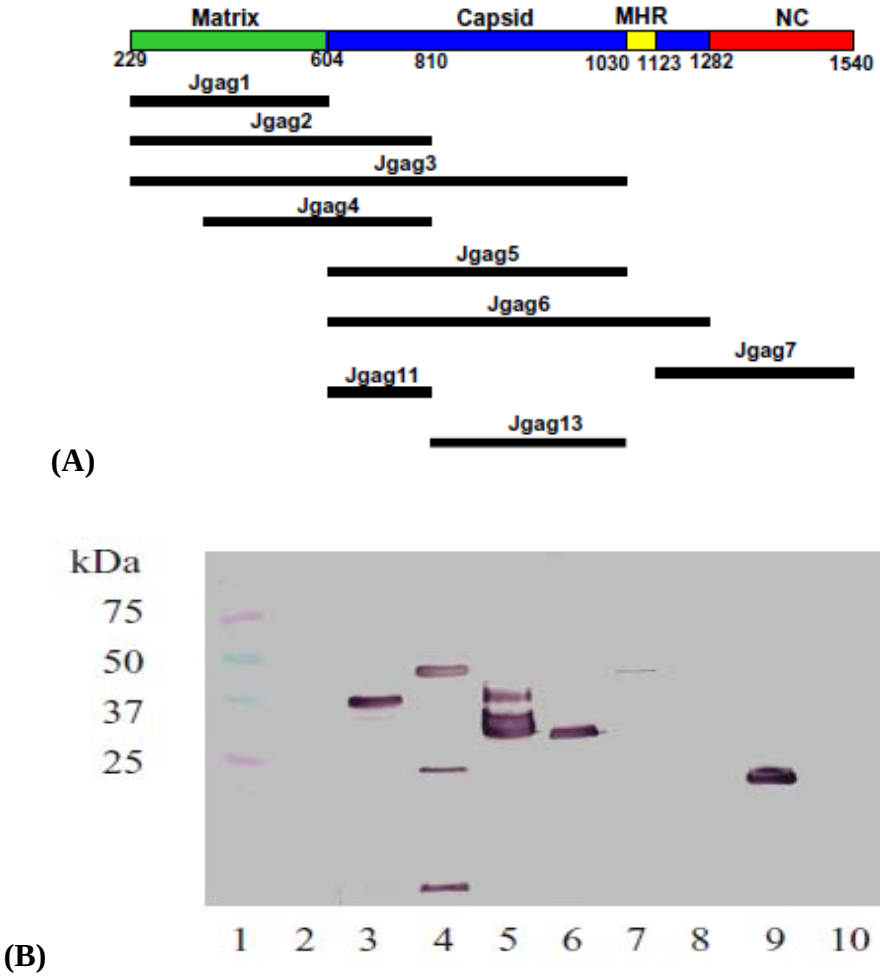


Figure 4.1 Mapping the reactivity of MAb LD1 against recombinant JDV CA. (A) Schematic representation of $JDV_{Tab/87}$ genome location (Chadwick et al., 1995b) of biotinylated JDV *gag* constructs expressed using Pinpoint X-A-1 system in *E. coli*. (B) Western immunoblotting with MAb LD1 revealed that its reactivity mapped to the amino terminus of CA in the region encompassed by Jgag11 (expressed by nucleotides 604–810). Lane 1, Marker; lane2, Jgag1; lane 3, Jgag2; lane 4, Jgag3; lane 5, Jgag4; lane 6, Jgag5; lane 7, Jgag6; lane 8, Jgag7; lane 9, Jgag11; lane 10, Jgag13.

Analysis of infected cell phenotype

Infection of bovine lymphocytes during acute infection with JDV was determined using immunofluorescence labelling techniques on formalin-fixed paraffin wax-embedded tissues. JDV- infected cells were identified using MAb LD1 and a secondary goat anti-mouse antibody labelled with Alexa Fluor 568, without any

further amplification of the fluorescent signal (Figures 4.2 A and B). $CD3^+$ T-cells were simultaneously labelled and were numerous in the lymphoid tissues examined (Figures 4.3 A and B). There was no co-localisation of signal when the fluorescent images for JDV CA and CD3 labelling were merged (Figures 4.4 A and B).

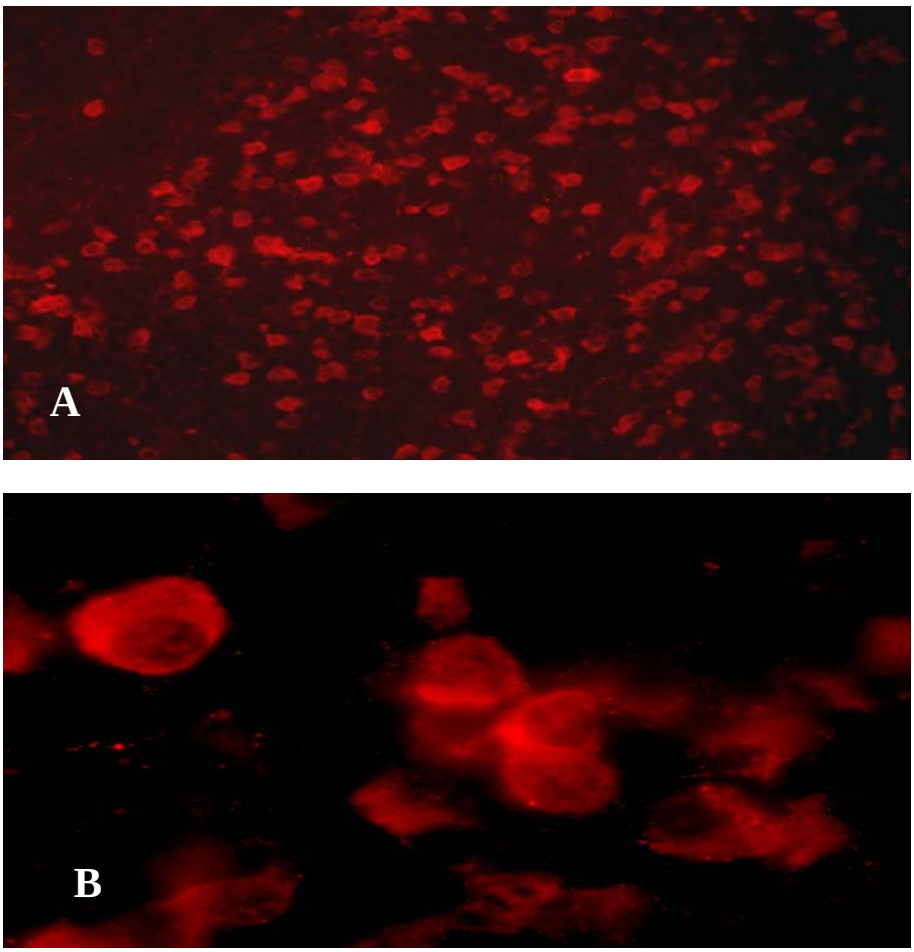


Figure 4.2 Mesenteric lymph node from a JDV-infected animal on the second day of the febrile reaction, reacted with MAb LD1 against JDV CA and a secondary goat anti-mouse antibody labelled with Alexa Fluor 568, showing characteristic strong cytoplasmic labelling. Magnification 40X (A) and 100X (B).

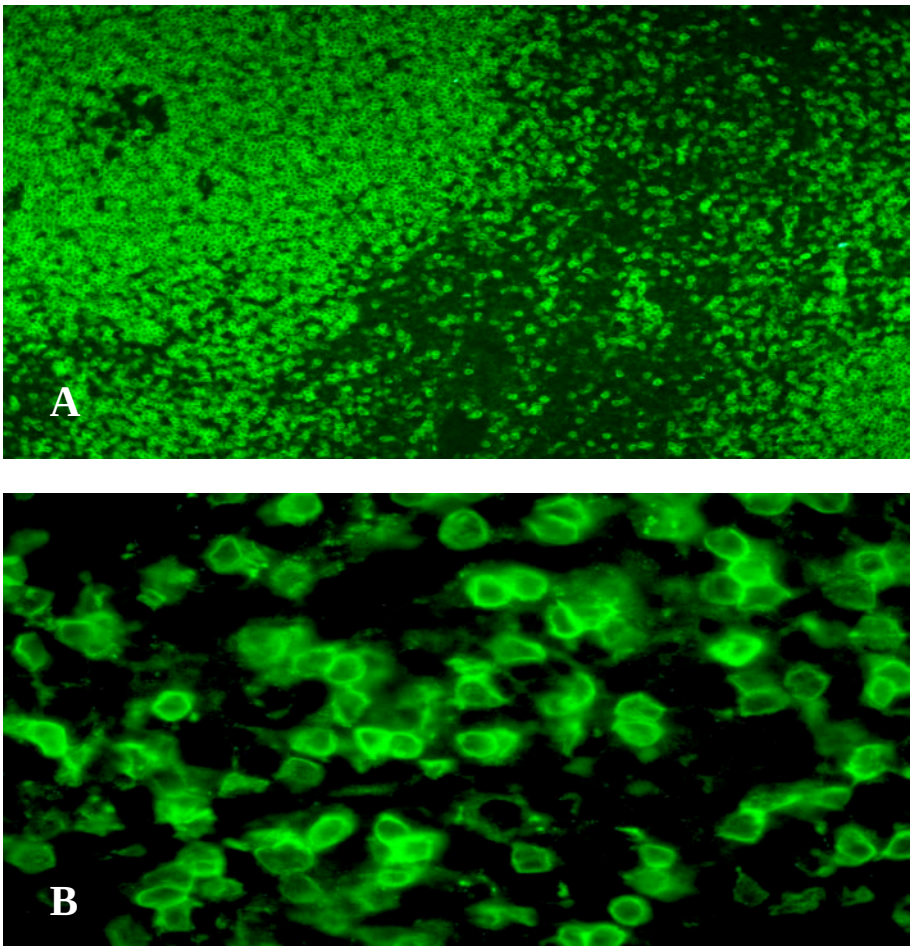


Figure 4.3 Mesenteric lymph node from a JDV-infected animal on the second day of febrile reaction, reacted with polyclonal rabbit anti-human CD3 (a T-cell marker) and a secondary goat anti-rabbit antibody labelled with Alexa Fluor 488, showing characteristic strong surface and or cytoplasmic fluorescence. Magnification 40X (A) and 100X (B).

B-cells identified using a monoclonal mouse anti-human CD79α were predominantly located in the germinal centres of the lymphoid follicles, as expected. No co-localisation of labelling with MAb LD1 and JDV CA was observed with $CD79\alpha^{+}$ cells in this location but approximately 10% of the $CD79\alpha^{+}$ cells outside the germinal centres were also reactive with MAb LD1 indicating that a proportion of B-lymphocytes were infected with JDV. The expression of CD79α on human B-cells ceases around the onset of plasma cell differentiation and plasma cells were identified in lymph node sections by detecting the presence of bovine IgG in the cytoplasm (Figure 4.5 A). A proportion of plasma cells were also found to contain JDV CA (Figure 4.5 B) and when the 2 images were merged, co-localisation between the IgG and JDV CA-positive cells was evident (Figure 4.5 C).

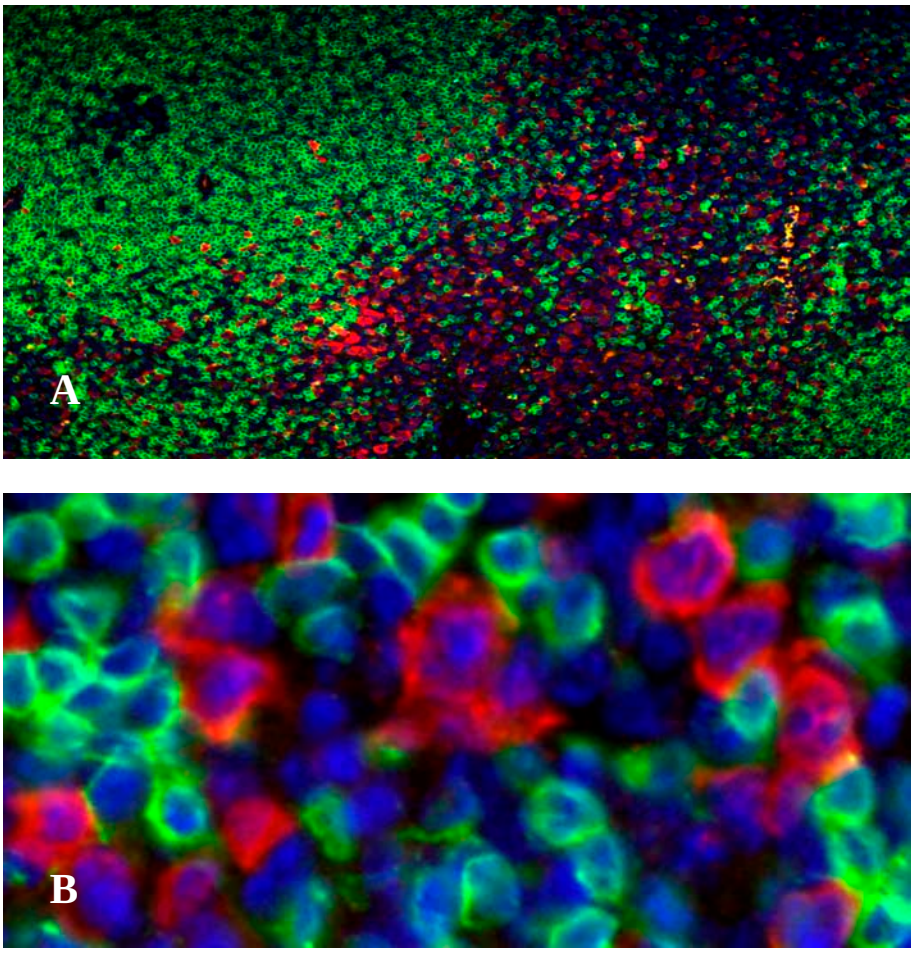


Figure 4.4 Mesenteric lymph node from a JDV-infected animal on the second day of febrile reaction, reacted with polyclonal rabbit anti-human CD3 (a T-cell marker) with a secondary goat anti-rabbit antibody labelled with Alexa Fluor 488 (green), and MAb LD1 with a secondary goat anti-mouse antibody labelled with Alexa Fluor 568 (red) to identify JDV CA-containing cells. No co-localisation was detected when the CD3 and MAb LD1 reactive cells were merged. Magnification 40X (A) and 100X magnification (B). Nuclei stain blue with DAPI.

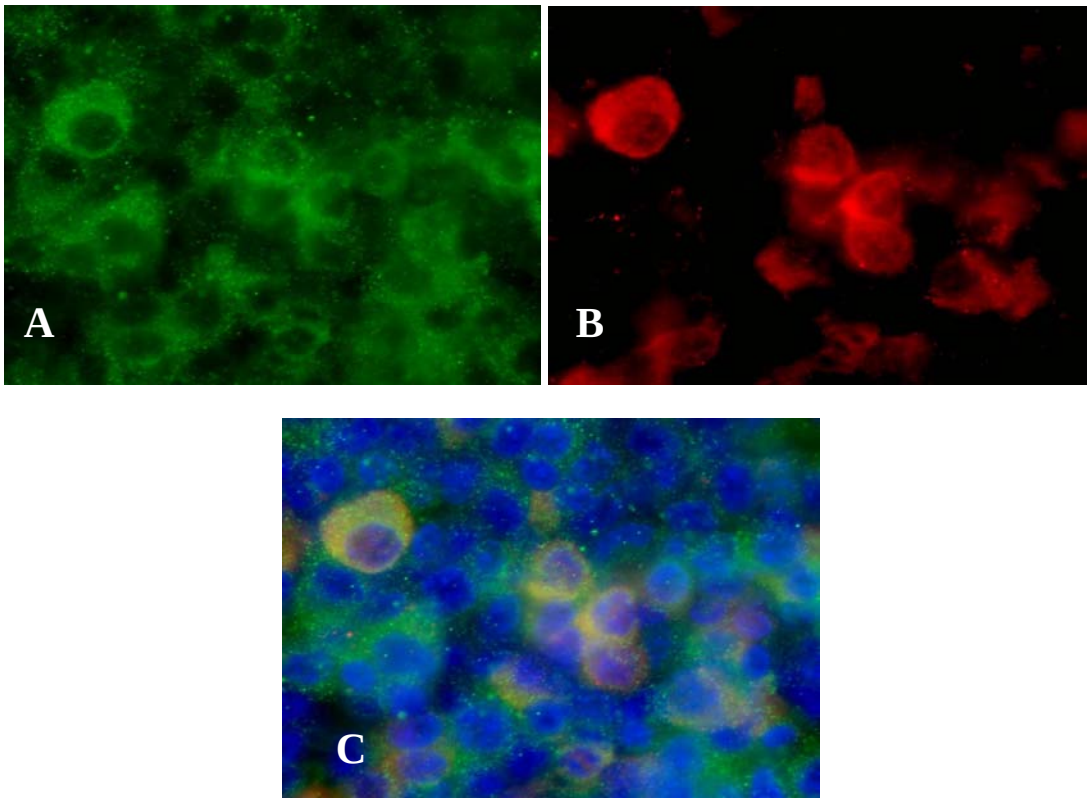


Figure 4.5 A mesenteric lymph node from a JDV-infected animal on the second day of the febrile reaction, reacted with rabbit anti-bovine IgG and a secondary goat anti-rabbit antibody labelled with Alexa Fluor 488 (A), and anti CA MAb LD1 with a secondary goat anti-mouse antibody labelled with Alexa Fluor 568 (B). Co-localisations were detected when the IgG and JDV CA-positive cells were merged (C). 100X magnification. Nuclei stain blue with DAPI.

Distribution of cells of monocyte/macrophage lineage

Blood-derived monocytes and neutrophils were identified in tissues using the MAb MAC387 (Figure 4.6). Despite similarities in the morphology and distribution of $MAC387^{+}$ cells and JDV CA-positive cells, there was no evidence of productive infection of monocytes or neutrophils. Antibodies directed against different regions of human CD68 and including MAb EBM11 have been used to successfully identify macrophages, and this has also been applied to identify macrophages in bovine tissues (Bielefeldt-Ohmann et al., 1988; Greywoode et al., 1990). Unfortunately, the antigen retrieval required for LD1 labelling was not compatible with that required for EBM11 and it was not possible to perform double immunolabelling with this combination of antibodies. However, when single immunolabelling was performed

on serial sections of lymph node, the distribution of JDV CA-positive cells in the medullary cords was different to the location of macrophages identified using EBM11, which were found predominantly in the medullary sinuses (Figure 4.7). The distribution of $MAC387^+$ cells and $EBM11^+$ cells was markedly different in the lung, confirming that these antibodies labelled different subsets of cells of the mononuclear phagocyte system.

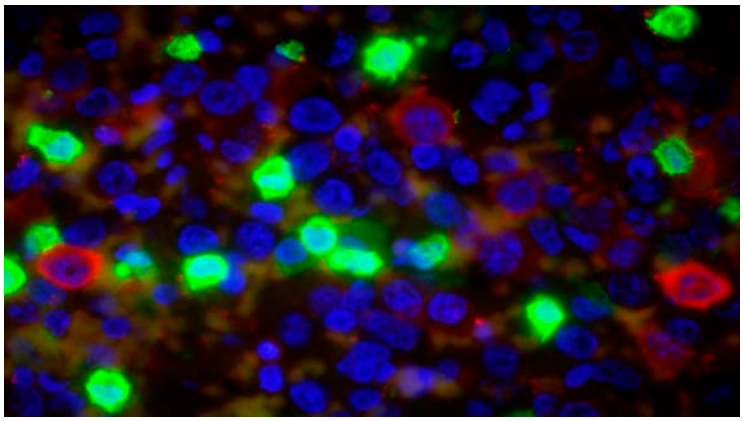


Figure 4.6. Spleen tissue from a JDV-infected animal on the second day of febrile reaction immunolabelled using MAb MAC387 and a secondary goat anti-mouse antibody labelled with Alexa Fluor 488 (green), and JDV anti-CA MAb LD1 JDV-CA with goat anti-mouse antibody labelled with Alexa Fluor 568 (red). The morphology and size of the reactive cells was different, and no co-localisations were detected when the $MAC387^+$ and JDV CA-positive cells were merged. Magnification 100X. Nuclei stain blue with DAPI.

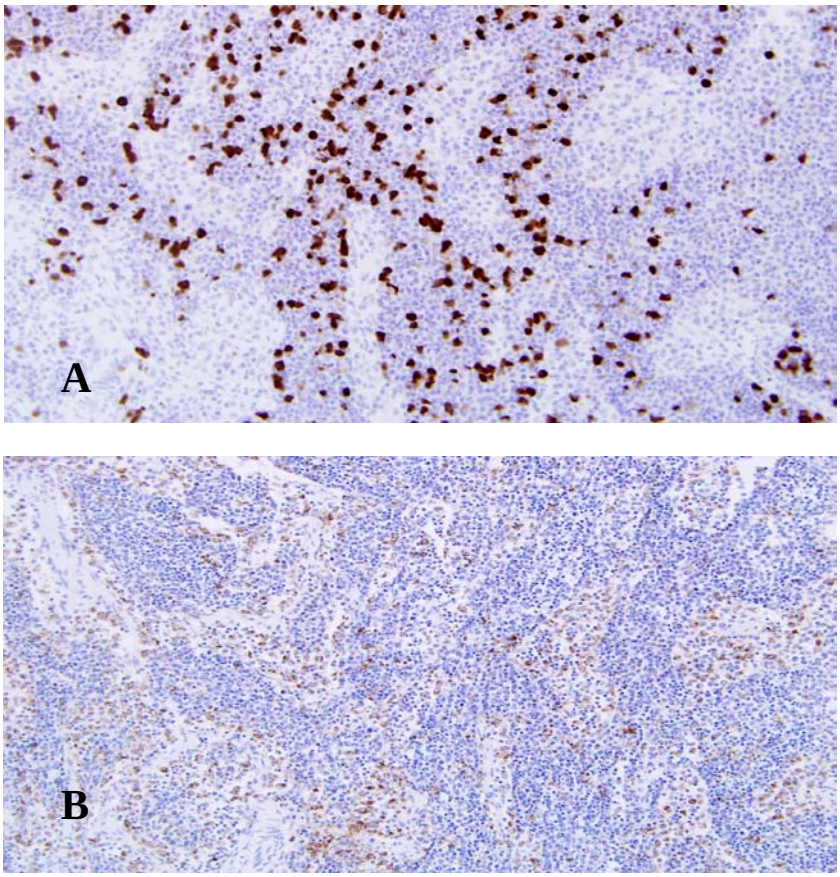


Figure 4.7. Consecutive sections of prescapular lymph node from a JDV-infected animal on the second day of the febrile reaction, reacted with JDV CA MAb (A) and EBM11 (B), showing the different locations of the reactive cells. JDV CA-positive cells showed a stronger labelling reaction and were mainly observed in the medullary cords but macrophages identified with EMB11 were generally detected in the medullary sinuses with weaker reactivity. Positive reactions indicated by dark brown labelling. Immunoperoxidase labelling, 40X magnification.

Morphology and distribution of infected cells

Many cells containing JDV CA were found in lymphoid tissues, particularly throughout the red pulp of the spleen (Figure 4.8 A), and these cells were large and pleomorphic with coarsely clumped chromatin and an abundant cytoplasm (Figure 4.8 B). Immunolabelled cells were often observed in close proximity to one another and were present mostly in non-lymphoid organs such as liver or kidney in mononuclear cell infiltrates. JDV CA was detected together with red blood cells in large vacuolated macrophage-like cells in the alveolar septae of the lung but it was not clear whether these cells were productively infected or merely phagocytosing

infected cells (Figure 4.9). IgG-containing cells had a similar morphology to the CA-containing cells with a large vacuolated cytoplasm and eccentric nucleus (Figure 4.10). The distribution and morphology of JDV RNA-positive cells was similar to that of JDV CA (Figure 4.11).

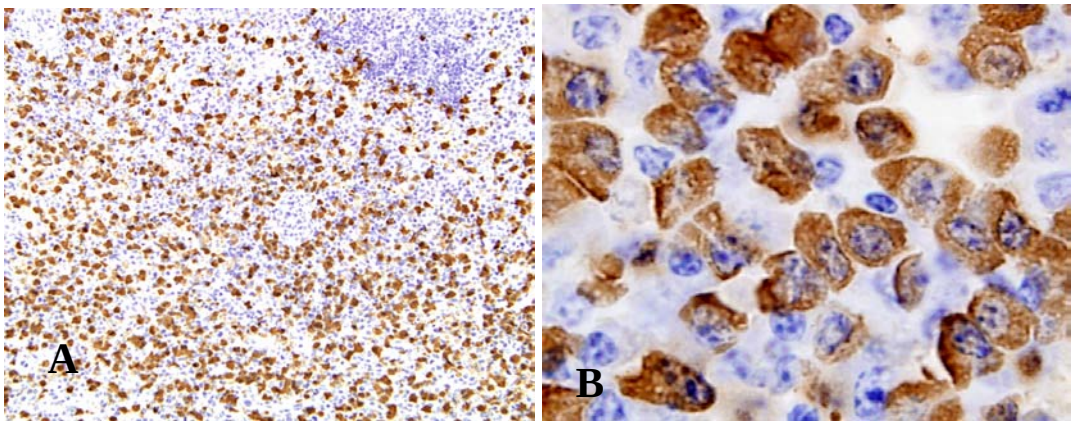


Figure 4.8. Spleen tissue from a JDV-infected animal on the second day of febrile reaction, reacted with JDV CA LD1 MAb, to show distribution of JDV CA-positive cells predominantly around the red pulp of the spleen, which were large and pleomorphic with coarsely clumped chromatin and abundant cytoplasm. Immunoperoxidase labelling, magnification 40X (A) and 100X (B).

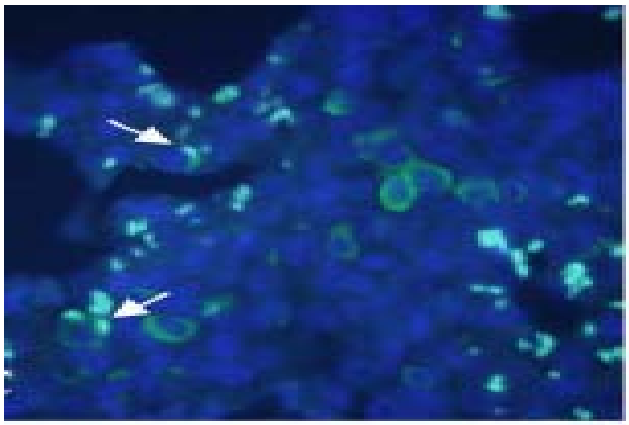


Figure 4.9. Lung tissue from a JDV-infected animal on the second day of the febrile reaction, immunolabelled using mAb LD1 and anti-IgG2b-labeled Alexa Fluor 488 (green), showing JDV CA in clusters of cells in this tissue and in occasional cells associated with red blood cell phagocytosis (arrows).

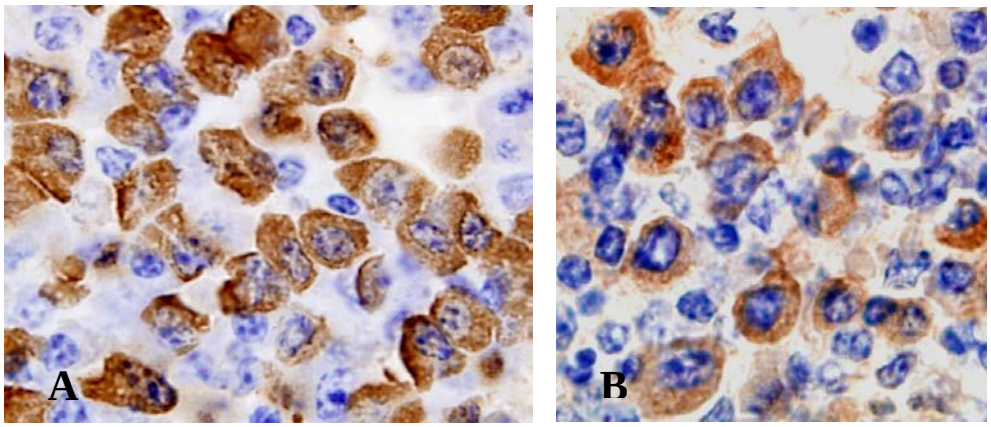


Figure 4.10. Spleen tissue from a JDV-infected animal on the second day of the febrile reaction, reacted with JDV CA monoclonal antibody (A) and rabbit polyclonal anti-bovine IgG (B), to show the similar morphology of the 2 reactive cell types, in both cases large cells with a vacuolated cytoplasm and an eccentric nucleus. The reactive cells contained dark brown cytoplasmic label. Immunoperoxidase labelling, magnification 100X.

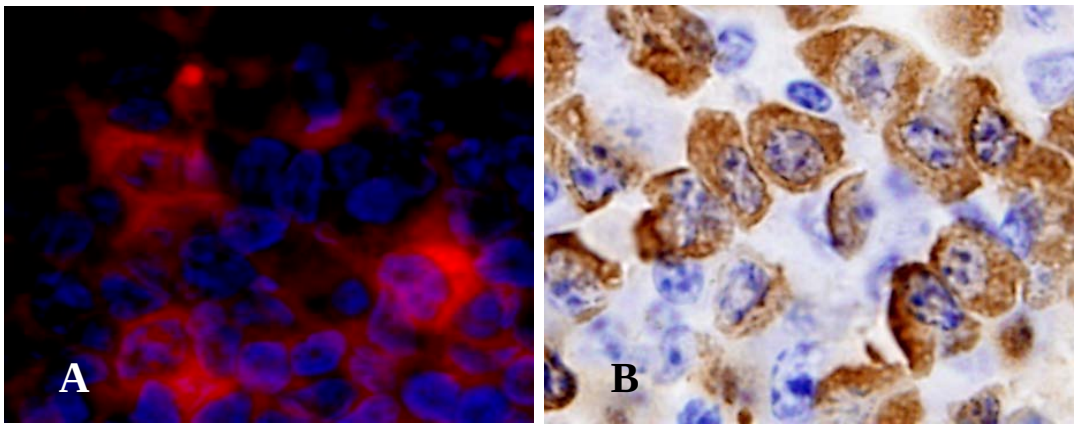


Figure 4.11. Spleen tissue from a JDV-infected animal on the second day of the febrile reaction, showing JDV RNA-positive cells detected using ISH with Fast Red (A) and JDV CA-positive cells detected using an immunoperoxidase labelling technique (B). In both cases, the reactive cells had a very similar morphology: large cells with vacuolated cytoplasm and an eccentric nucleus. Magnification 100X.

Discussion

The precise cell-tropism of JDV during the febrile phase of Jembrana disease in Bali cattle has not been identified previously but preliminary investigations reported in Chapter 3 suggested that JDV-infected cells were morphologically similar to cells that were immunolabelled with B-cell markers and expressed IgG, and therefore the virus probably replicates in mature B-cell lineage cells. The cell tropism of JDV was

further examined in the current study by the co-localisation and distribution of the major subsets of T-cells, B-cells, monocyte/macrophage lineage cells and cells containing JDV CA as an index that they were infected.

Observations reported in Chapter 3 indicated that the intense proliferative response detected in the T-cell areas of lymphoid tissues was not due to an expansion of $CD3^+$ cells but to an apparent infiltration of the areas with pleomorphic centroblast-like cells that appeared to be antibody-producing and probably of B-cell lineage, and that the distribution of T-cells and JDV-infected cells was different. The use of a pan T-cell CD3 marker and a JDV CA MAb for co-immunolabelling of T-cells and JDV-infected cells in the current study confirmed that $CD3^+$ T-cells were not infected with JDV. The apparent tropism of JDV for cells of a non-T-cell lineage is perhaps not surprising as there are large differences in the disease pathogenesis and genetic variability of JDV compared to most of the other lentiviruses, particularly those that are T-cell tropic (Desport et al., 2007; Soesanto et al., 1990).

The detection in the current study of JDV CA in a sub-population of B-lineage cells and including mature antibody producing B-cells, possibly plasma cells, confirmed that JDV can replicate in cells of this lineage. The location of the infected cells in the mantle zone, outside of the germinal centres, suggests that productive infection occurs in mature rather than proliferating immature B-cells. Infection of B-cells by lentiviruses is not unusual. They are infected at a low frequency by $SIV_{smmPBj14}$, an acutely lethal lentivirus infection in pigtail macaques (O'Neil et al., 1999) with similarities in pathogenesis to Jembrana disease. B-cells, particularly IgG-containing cells, are a major reservoir for FIV in chronically infected cats (English et al., 1993) and are both stimulated to proliferate and infected during the early stages of BIV infection (Heaton et al., 1998; Whetstone et al., 1997).

In the current study, pleomorphic centroblast-like cells were identified using CD79αcγ that effaced the normal T-cell population in the paracortex of lymphoid tissue during acute infection with JDV (Chapter 3). This suggests that, similar to what has been reported for BIV, there is a transient proliferation of B-cells during the acute stage of Jembrana disease (Whetstone et al., 1997). A putative polyclonal B-cell stimulatory epitope has been identified in the carboxyl-end of the envelope glycoprotein of HIV-1, specifically associated with Nef (Chirmule et al., 1994; Chirmule et al., 1990). Tmx, which is an accessory protein of unknown function

expressed from a similar region of the genome by the bovine lentiviruses, has been suggested to have a function analogous to Nef and it is possible that these viruses have evolved a mechanism to stimulate proliferation of their target cells *in vivo* (Chadwick et al., 1995b; Garvey et al., 1990).

Other non-primate lentiviruses exhibit a tropism for cells of the monocyte/macrophage lineage. Of these, FIV and BIV have been shown to have relatively broad cell tropisms (English et al., 1993; Heaton et al., 1998) whilst the small ruminant lentiviruses (CAEV and MVV) and EIAV are predominantly macrophage-tropic (Gendelman et al., 1985; Sellon et al., 1992; Zink et al., 1990). While there are similarities between the pathogenesis of EIAV infections in horses and Jembrana disease in Bali cattle, macrophages have not been identified as a target cell population for infection by JDV. The population of monocyte/macrophage cells identified using the MAC387 MAb was clearly not productively infected by the virus. MAC387 recognises leucocyte protein L1 (calprotectin) which is present in neutrophils and blood monocytes and is lost during their maturation into tissue macrophages (Brandtzaeg, 1988; Poston and Hussain, 1993). CD68 is an intracytoplasmic marker of mature tissue macrophages and is commonly used to identify macrophages infected by primate and other lentiviruses (Chakrabarti et al., 1991; Fischer-Smith et al., 2004). EBM11 has been successfully applied to identify cells bearing CD68 in fixed bovine tissues and was found to label a different population of cells when compared to MAC387 (Ackermann et al., 1994; Bielefeldt-Ohmann et al., 1988). The distribution of $EBM11^{+}$ cells in lymphoid tissues from JDV-infected cattle was not the same as the cells containing JDV CA although there were similarities in the size and morphology of the infected cells. Macrophage-tropic lentiviruses are often associated with neuropathology and infections in the brain (Andresdottir et al., 1998; Smit et al., 2001). The lack of convincing evidence for infection of macrophages by JDV is supported by the fact that the virus has never been detected in brain tissue (Chadwick et al., 1998) nor associated with any neurological signs (Soesanto et al., 1990). In addition, the genomes of the bovine lentiviruses differ from other non-primate lentiviruses by not encoding an identifiable dUTPase (Chadwick et al., 1995b; McGeoch, 1990). Retroviral dUTPases have a central role in productive viral replication in non-dividing cells, such as macrophages, where cellular dUTPases and the pool of available

deoxynucleotides are at low levels (Chen et al., 2002; Terai et al., 1991; Whetstone et al., 1997). The accumulation of G- to-A substitutions in CAEV has been shown to be prevented by the virally encoded dUTPase (Turelli et al., 1997) and replication in macrophages without a mechanism for preventing these substitutions leads to a drift of the genome towards poly(A).

The genomes of the bovine lentiviruses differ from the other lentiviruses by exhibiting dramatic differences in their genome compositions in their 5' compared to 3' halves. Whilst lentiviral genomes are characteristically A–rich, the BIV genome is only A-rich in the 5' half and both JDV and BIV are less A-rich in their *pol* sequences compared to the other lentiviruses (Foley et al., 2000). Both BIV and JDV appear to be genetically stable over time with much lower mutation rates than the other lentiviral genomes which may be related to their tropism for plasma cells with a long life span (Carpenter et al., 2000; Desport et al., 2007). The identification of cells containing JDV CA and RNA in the circulation as well as in tissues and the delayed humoral antibody response after the acute phase of infection are further indicators that the tropism of JDV is for B-cells rather than monocyte/macrophage-lineage cells. Circulating monocytes are only rarely infected in other lentiviral infections, for example in EIAV infections, and usually it is only after maturation to tissue macrophages that productive viral replication occurs (Sellon et al., 1992).

One of the hallmarks of JDV infection is the delay until at least 5 weeks and often much later after infection for seroconversion to viral antigens (Desport et al., 2009; Hartaningsih et al., 1994). This indicates that during the acute phase of the disease the normal process of antigen presentation and antibody production is disrupted. The presence of virus in plasma cells might be anticipated to affect the production of IgG and the decline in the number of these cells during the acute phase of the disease (Dharma et al., 1994) indicates that they do not survive being hijacked by JDV. This immunosuppressive effect is not restricted to JDV antigens as delayed responses have also been observed after vaccination with other antigens given at the end of the febrile response (Wareing et al., 1999).

JDV is often described as an atypical lentivirus because of the acute nature of the disease pathogenesis, the absence of viral variation, the delayed antibody response and the ensuing immunological events that develop after infection and prevent heterologous infections and relapses. The apparent tropism of JDV for B-cells and

lack of replication in T-cells and macrophages provides the basis for understanding these observations and indicates a fundamental difference from the other members of the lentivirus family. The role of macrophages in JDV infection of Bali cattle, however, is unclear. JDV proviral DNA is certainly present in the circulating PBMC population (Lewis et al., 2009) and yet monocytes identified using MAC387 are not infected and tissue macrophages identified using EBM11 are not in the same location as the CA-containing cells. All of the other members of this virus family, including BIV, are able to infect macrophages and JDV would indeed be an exceptional lentivirus if it were found to be solely B-cell tropic. The co-localisation of JDV CA and CD79αcγ^{+} labelling in approximately 10% of infected cells together with the close proximity of infected cells observed within the tissues indicate that other cells types could be involved and that infection of neighbouring cells without the necessary viral receptors may occur. Further studies with a larger panel of bovine cell surface markers are required to confirm the precise tropism of JDV.

Chapter 5

Flow cytometric analysis of changes in lymphocyte subsets in Bali cattle experimentally infected with Jembrana disease virus

Summary

Five Bali cattle were experimentally infected with JDV and all developed typical clinical signs of Jembrana disease characterised by a transient febrile response, enlargement of superficial lymph nodes and a significant leucopenia. Flow cytometric analysis of PBMC during the acute disease process showed that the reduced number of lymphocytes was due to significant decreases in $CD4^+$ and $CD8^+$ T-cells, and $CD21^+$ B-cells. At the end of the febrile phase, both $CD8^+$ T-cells and $CD21^+$ B-cells increased significantly but $CD4^+$ T-cells remained below normal values resulting in a significantly reduced $CD4^+$:$CD8^+$ ratio. These results suggest that in the absence of the production of specific antibodies to JDV for several weeks after recovery, a cell-mediated immune response involving $CD8^+$ cells may play a critical role in the recovery process.

Introduction

The majority of experimentally JDV-infected Bali cattle survive the acute clinical disease and do not develop any further clinical disease (Soeharsono et al., 1990; Soesanto et al., 1990) but the immune mechanism responsible for recovery from the acute disease and continued immunity has not been defined. There is no evidence that antibody plays a role in recovery as JDV-specific antibodies are not detectable until some weeks after recovery from the acute disease (Dharma et al., 1994; Hartaningsih et al., 1994; Wilcox et al., 1995). The cellular immune response, predominantly through interleukin activation of $CD8^+$ cytotoxic T lymphocytes, has been considered to play a critical role in EIAV infections (Murakami et al., 1999) and HIV infections (Migueles et al., 2002) and is also possible in JDV infections.

Hyperplasia of T-cell areas and depletion of B-cell areas of lymphoid tissues during acute Jembrana disease is a hallmark of the disease (Dharma et al., 1994). Depletion of the $CD4^+$ T-cell and $CD8^+$ T-cell populations was observed histologically in JDV-infected Bali cattle and significant differences were found during acute illness in follicular compartments of lymph nodes (Dharma et al., 1994). It was suggested (Dharma et al., 1994) that the gradual depletion of $CD4^+$ T-cells may have been due to the infection of T-cells. However, although T-cells are the predominant target cell of some lentiviruses, including HIV (Alcami, 2004b; Blankson et al., 2002; Brenchley et al., 2004; Clapham and McKnight, 2001; Penn et al., 1999; Samuelsson et al., 1997), SIV (Brown et al., 2007; Dykhuizen et al., 1998; Mattapallil et al., 2005; Picker, 2006) and FIV (Ackley et al., 1990), there is no evidence for infection of T-cells by JDV (Chapter 4) and the mechanism for the changes in T-cell populations in Jembrana disease remain unknown.

Although lymphopenia is a characteristic feature of Jembrana disease (Soesanto et al., 1990), changes in circulating lymphocyte subsets during the acute disease have remained uncharacterised. In this Chapter, flow cytometric analysis of the circulating $CD4^+$ and $CD8^+$ cell populations during the febrile and early post-febrile phases was undertaken to better understand the acute disease process associated with JDV infection, and the results are reported in this Chapter.

Materials and methods

Experimental animals and sample collection

Five Bali cattle were infected with JDV using procedures described in Chapter 3 and the febrile phase in the inoculated animals occurred from 5-11 days after infection. Blood samples were obtained daily from all animals for 14 days after infection and again at day 21 when the experiment was terminated. Sterile EDTA-containing vacutainer tubes (Greiner Bio-One) were used to collect blood samples for recovery of lymphocytes used for the studies described in this Chapter, and also for the cytokine expression studies that are reported in Chapter 6.

Lymphocyte preparation

Lymphocytes were isolated using Ficoll-Paque plus (Amersham Biosciences) following the manufacturer's instructions, then washed twice in FACS buffer (Dulbecco's phosphate-buffered saline [Thermo Scientific] supplemented with 5% heat inactivated foetal calf serum (FCS; Bovogen Biologicals) and 0.05% sodium azide [Sigma-Aldrich]). The washed lymphocytes were resuspended in FACS buffer and adjusted to a density of 1×10^7 cells/ml, and kept at 5^oC until they were immunolabelled on the same day. Some aliquots of lymphocytes were stored at -80^oC until RNA was extracted for the studies reported in Chapter 6.

Antibodies and cellular markers

Lymphocytes were labelled with either 2.5 μg/ml mouse anti-bovine CD4 MAb (Serotec), 5 μg/ml mouse anti-bovine CD8 MAb (Serotec) or 20 μg/ml mouse anti-bovine CD21 MAb (Santa Cruz), a B-cell marker. An Alexa Fluor (AF488) conjugated goat anti-mouse cross absorbed secondary antibody (Invitrogen) was used to detect all reactive MAb antibodies (Table 5.1).

Table 5.1. Primary and secondary antibodies used for flow cytometric analysis of lymphocytes from cattle infected with JDV.

Antibody	Source	Isotype / clone	Cat./Lot No
Primary antibody			
Mouse anti-bovine CD4	Serotec	IgG2a/CC8	MCA1653G
Mouse anti-bovine CD8	Serotec	IgG2a/CC63	MCA1653G
Mouse anti-bovine CD21	Santa Cruz	IgG2b/CC51	SC-101835
Secondary antibody			
Goat anti-mouse	Invitrogen	Alexa Fluor 488	A-11029

Cell surface labelling of lymphocytes

Lymphocytes were labelled for single-colour analysis using a previously published protocol (Foster et al., 2007; Rocchi et al., 2007) with slight modification. Following lymphocyte preparation, 1 ml of the lymphocyte suspension was incubated with 100 µl of primary antibody in FACS buffer for 30 min at 4^{o}C, followed by 3 washes with FACS buffer (by centrifugation for 1 min at 479 *g* at 4^{o}C). Secondary antibody (100 µl) diluted in FACS buffer was applied and incubated for 30 min at 4^{o}C in the dark. The cell suspensions were then gently washed 3 times in FACS buffer, then washed once with PBS and the cells then resuspended in 200 µl of fixation buffer (Dulbecco's phosphate-buffered saline supplemented with 4% paraformaldehyde) for 5 min at 37^{o}C. Finally, the cells were washed with 200 µl of ice-cold Dulbecco's phosphate-buffered saline supplemented with 1% bovine serum albumin (BSA; Sigma Aldrich) and resuspended in 1 ml of freezing medium (Dulbecco's phosphate-buffered saline supplemented with 1% BSA and 10% dimethyl sulfoxide [Sigma Aldrich]) before being transferred to freezing vials and then stored at -80^{o}C. Samples were stored for up to 2 months in Bali prior to transport to Australia for FACS analysis.

Flow cytometric analysis

Prior to flow cytometric analysis, cryopreserved lymphocytes samples were thawed rapidly at 37^{o}C in a water bath, then washed once with wash buffer (Dulbecco's phosphate-buffered saline supplemented with 0.1% BSA) and resuspended in 1 ml of labelling buffer (Dulbecco's phosphate-buffered saline supplemented with 10% heat-inactivated FBS and 0.1% sodium azide [Sigma Aldrich]). The immunolabelled samples were analysed using a BD FACSCalibur flow cytometer (BD Bioscience) with a 488 nm excitation laser. Lymphocytes were gated in a forward/side scatter plot (FCS vs SSC). AF488 fluorescence emission was collected with 530/30 nm band pass filter and acquired in the log scale. A bandpass-specific filter (FL1, 530 ± 15 nm) was used for Alexa Fluor 488. A minimum of 10,000 lymphocytes were examined per sample and an AF488 fluorescence histogram was used to compare the samples. Sample data were analysed using BD CellQuest Pro V5.2 (BD Biosciences) which is the standard operating software on the FACSCalibur. Experimental data were analysed and population statistics calculated using FlowJo V7.2.5 (Tree Star Inc., USA) flow cytometry analysis software.

Statistical analysis

The absolute numbers of lymphocyte subsets was calculated by multiplying the percentage of each lymphocyte subset obtained from flow cytometry analysis with the total lymphocyte counts/ml, and were reported as a mean ± standard deviation (SD). A one-way ANOVA (SPSS® 17.0) was used to assess group differences in the lymphocyte populations, while differences between time points during infection were analysed using Bonferroni's multiple comparison. A value of $p<0.05$ was considered significant for all analyses.

Results

Evaluation of lymphocyte samples

In a preliminary experiment, sample preparation techniques were evaluated to optimise the quantity and quality of lymphocytes which were crucial for cell surface labelling. Flow cytometric analysis of normal lymphocyte samples isolated using Ficoll-Paque plus and immunolabelled following established protocols with a

representative cell-marker (CD4) showed that the samples had a high purity (66.4%), with few dead cells (less than 5%) or non-lymphocyte contaminants. The $CD4^+$ T-cell population (31.98% of the total population) (Figure 5.1) was in agreement with reported values for a normal bovine peripheral blood $CD4^+$ T-cells of 29 ± 4% (McBride et al., 1999).

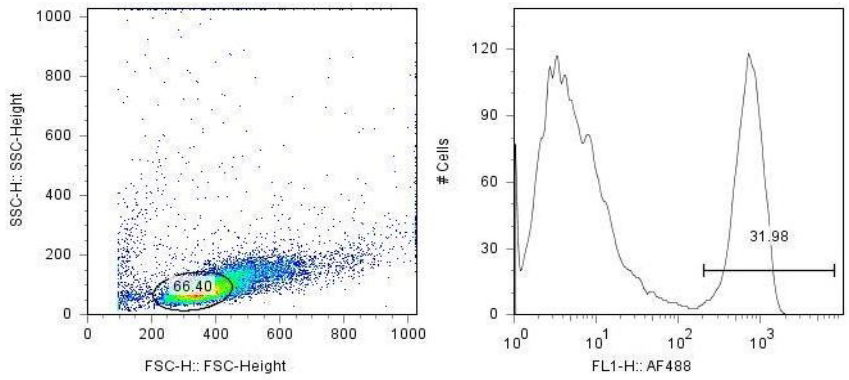


Figure 5.1. Flow-cytometry dot plots (left) and histogram (right) of normal lymphocytes prepared using Ficoll-Paque plus reacted with CD4 marker, showed a pure lymphocyte population and good surface labelling. Fluorescence intensity is depicted on the X-axis**.**

Flow cytometric analyses

There were significant differences in the mean (of 5 animals) absolute number of lymphocyte subsets at the 3 major time points: pre-infection (day 0 prior to JDV infection), during the febrile phase and during the immediate post-febrile phase (Table 5.2). During the febrile phase, the total number of $CD4^+$ T-cells decreased significantly ($p<0.001$) and remained below normal values until well after the febrile phase (Figure 5.2). Conversely, the total number of $CD8^+$ T-cells reduced slightly during this period but increased significantly ($p<0.001$) above normal values in the post-febrile phase (Figure 5.3). Due to the dramatic depletion of $CD4^+$ T-cell populations and significant increase in $CD8^+$ T-cells after JDV infection, the $CD4^+$:$CD8^+$ T-cell ratio also decreased significantly ($p<0.05$) from 0.5:1 at pre-infection to 0.25:1 and 0.01:1 during the febrile phase and post-febrile phase, respectively (Table 5.3). The population of $CD21^+$ B-cells reduced slightly during the febrile phase then increased significantly ($p<0.001$) as did $CD8^+$ T-cells during

the post-febrile phase (Figure 5.4). The changes in the lymphocyte subsets during the 3 time points after JDV infection are illustrated in Figure 5.5.

Table 5.2. Comparison of lymphocyte subsets during 3 major phases after JDV infection.

Lymphocyte population	Mean absolute number cells/ml ± SD		
	Pre-infection	Febrile phase	Post-febrile phase
T-helper cells, $CD4^+$	2418 ±277[a]	176± 171[b]	45 ±10 [b]
Cytotoxic T-cells,$CD8^+$	1210±206 [b]	768±489[b]	3187±601 [a]
B-cells, $CD21^+$	1799 ± 404 [b]	2065±823[b]	4225 ± 841 [a]

Means in a row with different superscripts are significantly different by Tukey's HSD (p<0.05).

Table 5.3. Changes in $CD4^+$:$CD8^+$ T-cell ratio during the course of JDV infection.

Days after infection	Mean $CD4^+$ (number/ml)	SD	Mean $CD8^+$ (number/ml)	SD	$CD4^+$:$CD8^+$ ratio
0	2418	713	1210	14	0.5:1
2	248	11	869	12	0.28 :1
4	217	8	740	12	0.29 :1
5	274	24	1236	31	0.22 :1
6	229	18	605	18	0.38 :1
7	138	11	367	11	0.37 :1
9	28	0.4	709	21	0.04 :1
19	36	0.2	3187	32	0.01:1

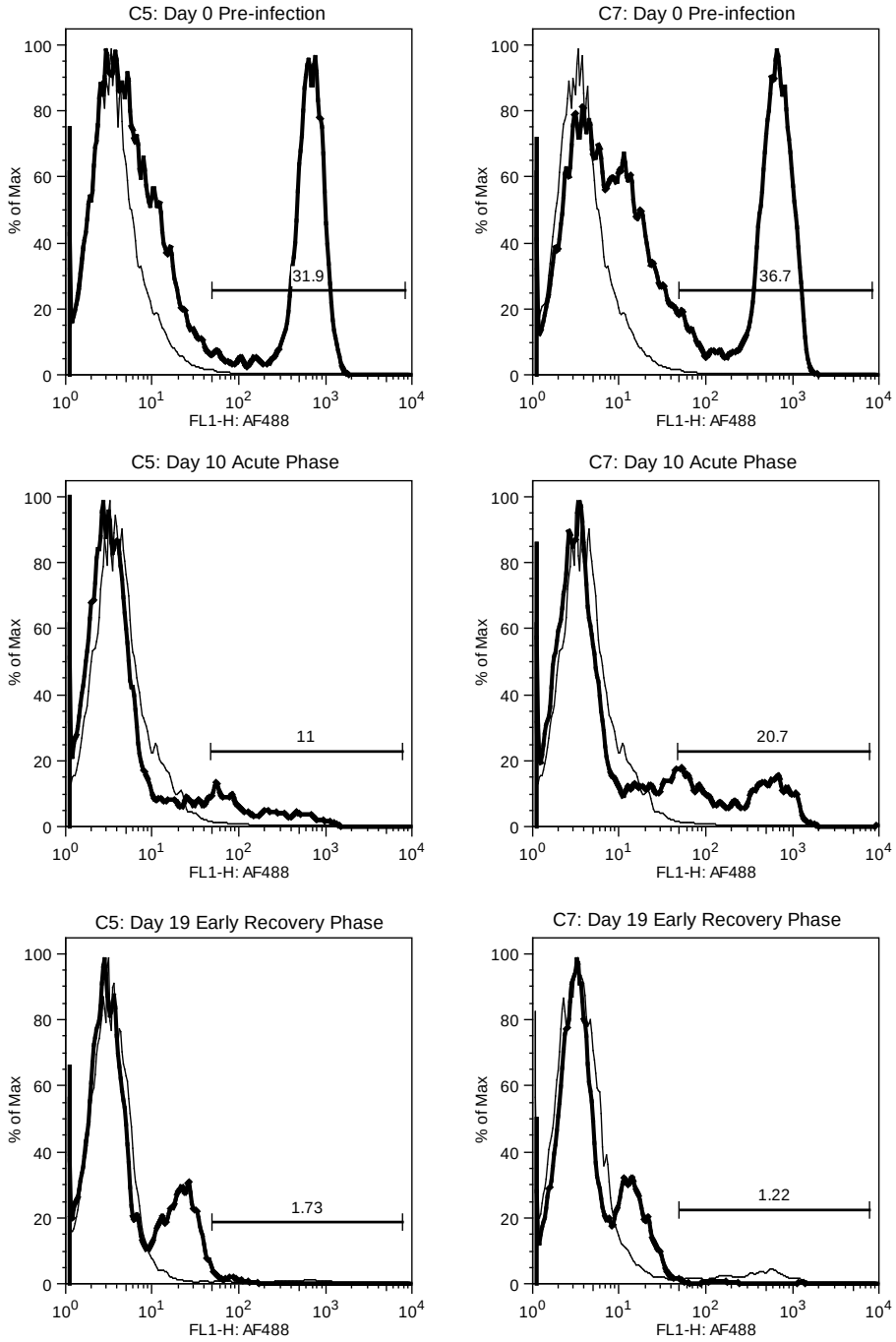


Figure 5.2. Flow cytometric analysis of lymphocyte $CD4^+$ T-cells before and after JDV infection. CD4-AF488 fluorescence histograms for representative cattle (left) CB5 and (right) CB7 showed a significant reduction of $CD4^+$ T-cells from pre-infection through the acute and early recovery phases. For each histogram, CD4-AF488 immunolabelled samples (thick black line) are compared to a non-labelled sample (thin black line).

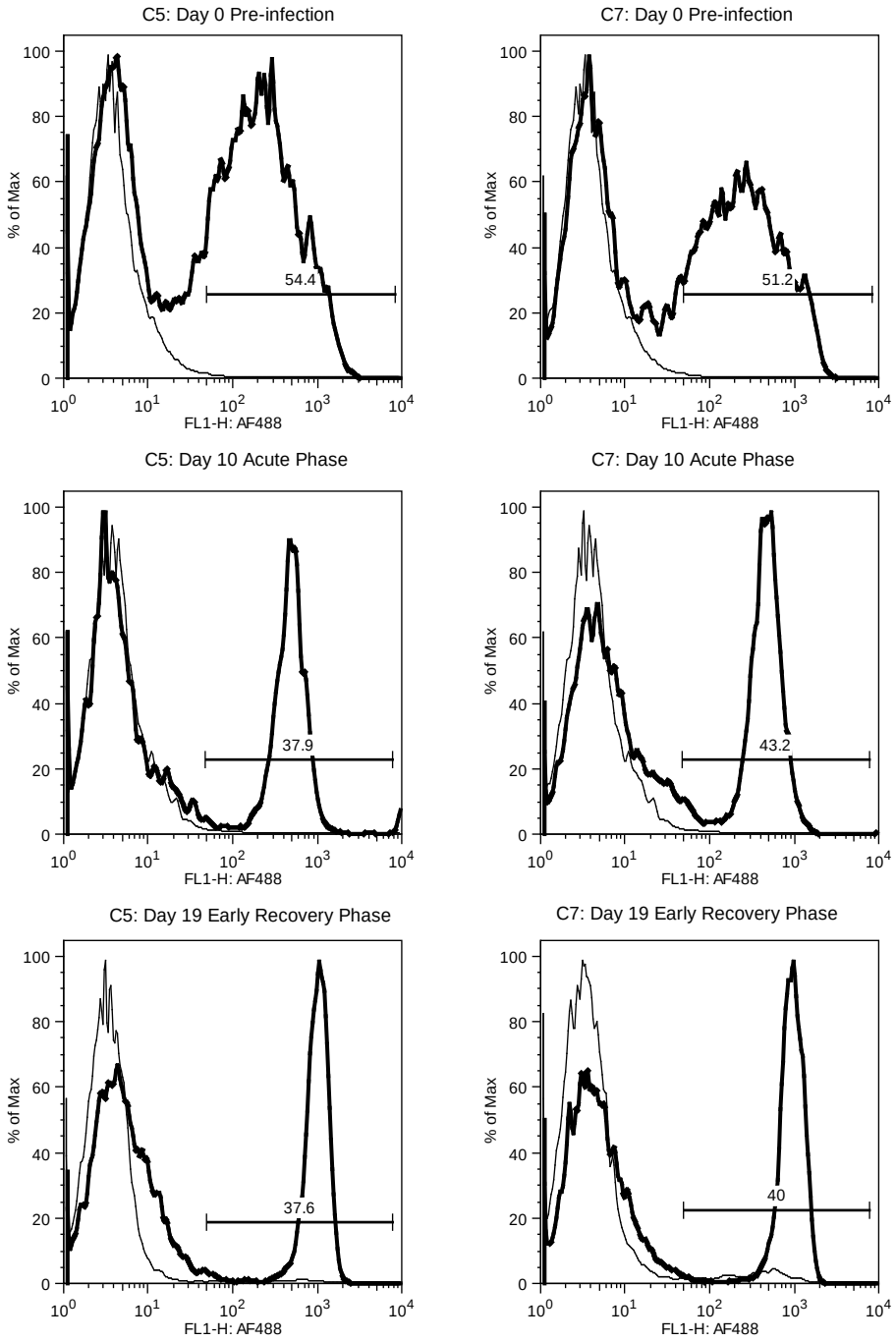


Figure 5.3. Flow cytometric analysis of lymphocyte $CD8^+$ T-cells before and after JDV infection. CD8-AF488 fluorescence histograms for representative cattle (Left) CB5 and (Right) CB7 showed a significant increase of $CD8^+$ T-cells from pre-infection through the acute and early recovery phases. For each histogram, CD8-AF488 immunolabelled samples (thick black line) are compared to a non-labelled sample (thin black line).

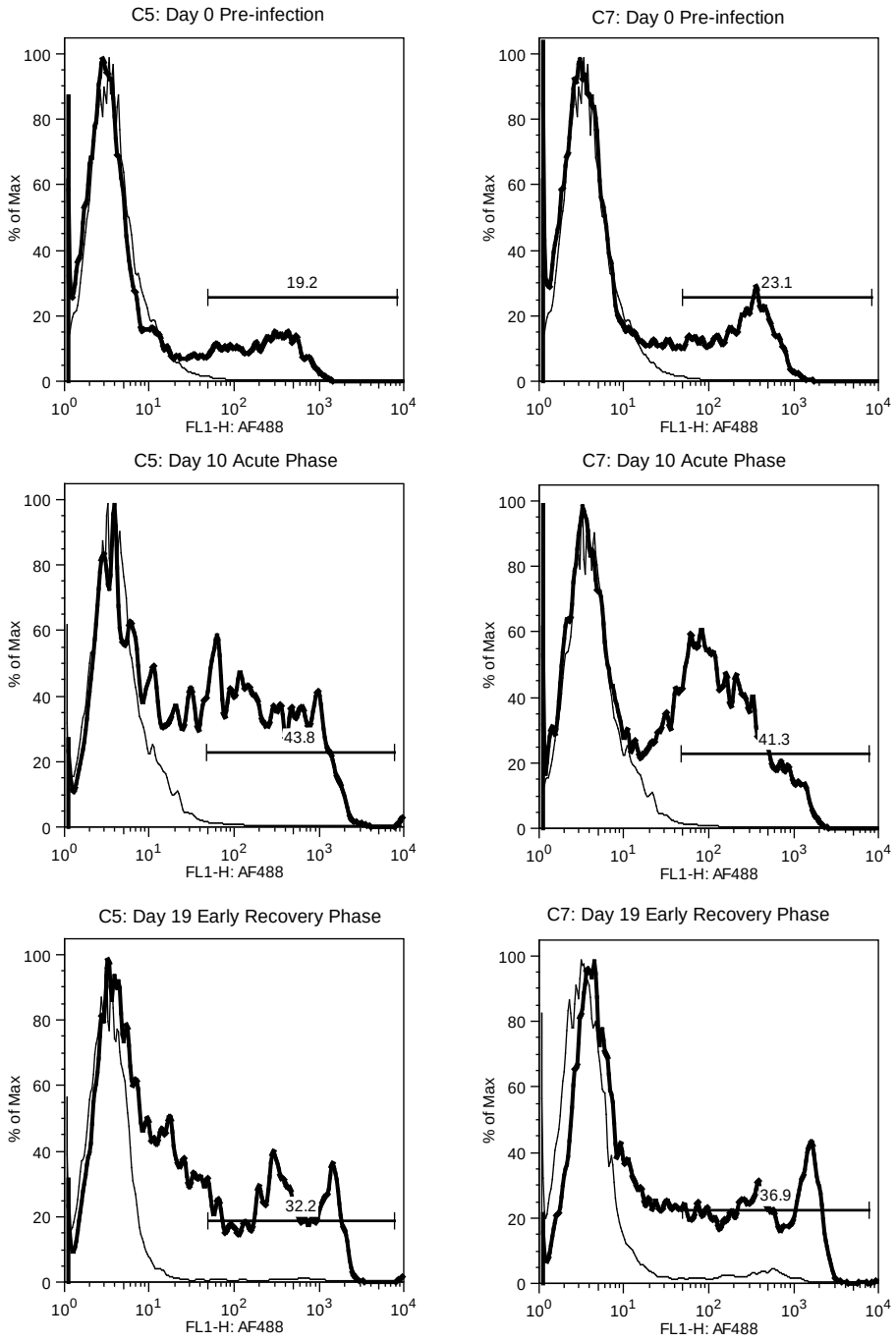


Figure 5.4. Flow cytometric analysis of lymphocyte CD21^{+} B-cells before and after JDV infection. CD21-AF488 fluorescence histograms for representative cattle (Left) CB5 and (Right) CB7 showed an increase of CD21^{+} T-cells from pre-infection through the acute and early recovery phases. For each histogram, CD21-AF488 immunolabelled samples (thick black line) are compared to a non-labelled sample (thin black line.

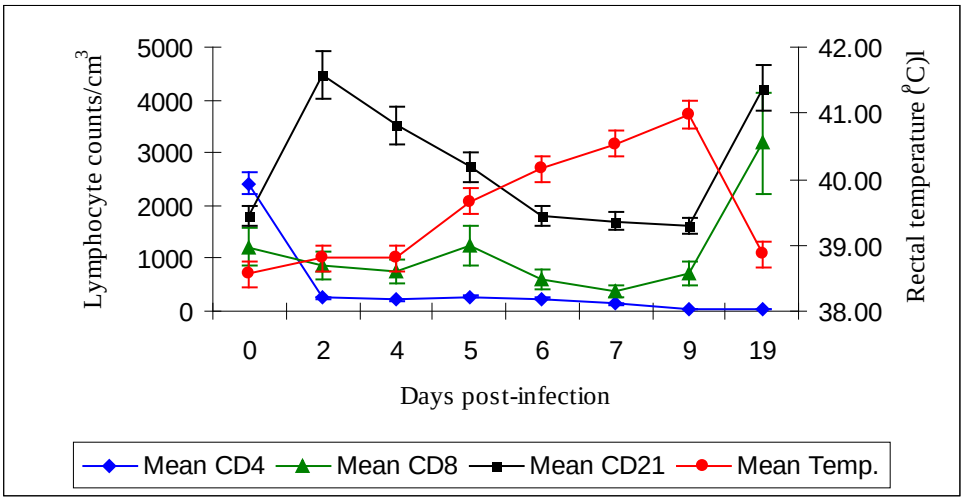


Figure 5.5 Lymphocyte subset changes following JDV infection. There was an elevated rectal temperature (red line) from 5-9 days after infection (febrile phase) and the population of $CD4^+$ T-cells decreased significantly ($p<0.001$) before the onset of the febrile phase and remained below normal values beyond the febrile phase. The total number of $CD8^+$ T-cells reduced slightly during the early febrile phase then increased significantly ($p<0.001$) above normal in the post-febrile phase. $CD21^+$ B-cells increased prior to the febrile phase, reduced gradually during the febrile phase then increased significantly ($p<0.001$) again at the end of the febrile phase. Data presented are means of values from 5 animals ± SD.

Discussion

The nature of the response of Bali cattle to JDV infection, an acute disease process with a short incubation period, a case fatality rate of about 17% and no recurrence of disease in those animals that recover, is unusual for a lentivirus. The lack of any recurrence of disease in animals that recover suggests the development of a strong protective immunity. The absence of JDV-specific antibody until at least 5 weeks and not in most cattle until 11 weeks after infection (Hartaningsih et al., 1994; Soeharsono et al., 1995b) implies that cell-mediated immune responses play a major role in the recovery of the infected animals and probably in their continuing immunity. The current study assessed the responses of peripheral blood lymphocyte subsets to JDV infection in experimentally infected animals to gain insights into the kinetics of the lymphocyte response following infection.

The use of flow cytometric analysis confirmed the previous report of the significant decrease in $CD4^+$:$CD8^+$ T-cell ratio of lymphocytes in lymphoid tissues during the acute phase of Jembrana disease but not during early post-febrile stages (Dharma et al., 1994). In this current study, both $CD4^+$ and $CD8^+$ T-cells in peripheral blood significantly decreased during the febrile phase compared to before infection, and this period corresponds to the duration of the lymphopenia reported during the febrile phase of Jembrana disease (Soesanto et al., 1990). The population of $CD8^+$ T-cells was greater than $CD4^+$ T-cells during the febrile phase but increased markedly during the post-febrile phase. Due to the significant increase of $CD8^+$ T-cell numbers at the end of the febrile phase and a continuous depletion of $CD4^+$ T-cells, this resulted in the dramatic increase in the $CD4^+$:$CD8^+$ T-cell ratio.

The significant increase in the $CD8^+$ T-cell population after the febrile phase strongly correlated with the expansion of $CD3^+$ T-cell numbers seen in lymphoid tissues during this stage and reported in Chapter 4. This positive correlation may indicate that the majority of the $CD3^+$ T-cells were $CD8^+$ T-cell subsets. Further, the increased population of $CD8^+$ T-cells during JDV infection, in the absence of JDV-specific antibody until several weeks after JDV-infection (Hartaningsih et al., 1994; Soesanto et al., 1990), provides additional support for the role of these cells in the recovery from the acute disease process. Virus-specific $CD8^+$ cytotoxic T-cells may play an important role in host defence against lentivirus infections (Levy, 1993; Salk et al., 1993). The antiviral role of $CD8^+$ cytotoxic T lymphocytes has been considered to be important in the inhibition of the progression of early EIAV infection before the production of virus neutralising antibody (Hammond et al., 1997; McGuire et al., 2004; McGuire et al., 1994). It is also thought to be important in non-progressor HIV-infected individuals (Cao et al., 1995; Migueles et al., 2002), and in controlling SIV replication and protection against SIV challenge (Genesca et al., 2009; Genesca et al., 2008; Jin et al., 1999). It is only in the transition to chronic infection that the impressive early potency of the antiviral $CD8^+$ cytotoxic T-cells may wane (Pantaleo et al., 1997a) possibly due to a reduction of perforin production linked with the inability the immune system to control viral replication and spread of the virus (Migueles et al., 2002; Pantaleo et al., 1997a; Zhang et al., 2003). As with other lentivirus infections, at least during acute infections, the result of this study tend to support to the current hypothesis that virus-specific $CD8^+$ CTL may play a

crucial role in host defence against lentivirus infections (Levy, 1993; Salk et al., 1993).

It is unclear why $CD8^+$ T-cells increased and $CD4^+$ T-cells were dramatically decreased despite the absence of infection of T-cells by the virus (Chapter 4). For the T-cell tropic lentiviruses, a gradual depletion of $CD4^+$ T-cell subsets is associated with infection of these cells, evident in HIV infections (Alcami, 2004b; Blankson et al., 2002; Brenchley et al., 2004; Clapham and McKnight, 2001, 2002; Penn et al., 1999; Samuelsson et al., 1997), SIV infections (Brown et al., 2007; Dykhuizen et al., 1998; Mattapallil et al., 2005; Picker, 2006; Shen et al., 2003; Steger et al., 1998; Veazey et al., 2003; Veazey et al., 2000) and FIV infections (Ackley et al., 1990). However, reduction of $CD4^+$ T-cell populations is not always related to their infection by viruses. In EIAV infections, for example, both circulating $CD4^+$ and $CD8^+$ T-cell subsets are reduced significantly during acute infection, although mature macrophages and not T-cells are the main target cells of the virus (Cook et al., 2001; Murakami et al., 1999; Oaks et al., 1998; Sellon et al., 1992). In EIAV infection, depletion of the T-cell subsets is possibly an indirect affect of the virus infection or virus components (Murakami et al., 1999).

The population of $CD21^+$ B-cells increased prior to the febrile phase, indicating a transient proliferation of B-cells or release of B-cells into the peripheral blood during this phase, similar to that reported during BIV infection (Whetstone et al., 1997). The reason for this is unknown but in HIV-1, a putative polyclonal B-cell stimulatory epitope has been found in the carboxyl end of the envelope glycoprotein of the virus, specifically associated with Nef (Chirmule et al., 1994; Chirmule et al., 1990). Tmx, an accessory protein of unknown function that is expressed from a similar region of the genome as is *nef* by bovine lentiviruses (Chadwick et al., 1995b; Garvey et al., 1990) might be involved similar to Nef in the proliferation B-cells *in vivo* but this hypothesis would need to be confirmed. During the febrile phase, there was a progressive reduction in the numbers of $CD21^+$ B-cells which may be associated with replication of virus in these cells. This is supported by evidence presented in Chapter 4, not only that B-cells were infected with virus but that at least some infected B-cells were mature IgG-containing cells or plasma cells.

In conclusion, the present study has clearly demonstrated dramatic changes in the population of T-cell subsets and B-cells during the course of Jembrana disease. B-

cells, possibly mature B-cells, that appear to be the host of JDV, increased in peripheral blood prior to the onset of the febrile phase and then declined in numbers and this decline corresponded to the decrease in numbers of these cells in tissues during the febrile phase of the disease. $CD8^+$ T-cell numbers increased during the acute disease and may well play a role in the recovery process before the production of neutralising antibody.

Chapter 6

A preliminary investigation of cytokine expression in Bali cattle experimentally infected with Jembrana disease virus

Summary

Real-time RT-PCR was used to investigate the expression of pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α in PBMC following JDV infection. There was an up-regulation of IFN-γ mRNA expression during the febrile phase, and there was significant up-regulation of both IL-2 ($p<0.05$) and IFN-γ ($p< 0.001$) cytokines during the post-febrile phases that coincided with the significant reduction of JDV RNA ($p<0.001$) to undetectable values in this phase, suggesting these cytokines may have a significant role in the recovery process. The up-regulation of IL-2 and IFN-γ genes correlated with a significant increase of $CD8^+$ T-cells during the febrile and immediate post-febrile phases of Jembrana disease that were reported in Chapter 5, and support a hypothesis that the cell-mediated immune response is a significant factor in recovery of animals.

Introduction

The pathogenesis of infection with the acutely pathogenic JDV in Bali cattle is poorly understood. The disease process is an unusual one for a lentivirus, with an acute disease process after a short incubation period, recovery of most (~80%) animals, and no subsequent recurrence of any disease attributable to the virus infection. There is a delayed antibody response to the virus until several weeks after the acute disease process (Dharma et al., 1994; Hartaningsih et al., 1994) perhaps attributable to replication of the virus in IgG-producing cells (Chapter 4). The absence of an antibody response until several weeks after the apparent recovery from the acute disease has suggested that a T-cell-mediated immunity must be responsible for recovery and the survival of cattle. A significant increase of $CD8^+$ T-cells in the post-febrile phase was demonstrated (Chapter 5) and provides evidence that they are involved.

Cytokines play a crucial role in regulating adaptive immune response, and T-cells are an important source of cytokines of both primary and memory immune responses. $CD4^+$ T-cells are a main source of T-cell cytokines, and based on their cytokine profiles these cells are divided into a Th1 type (producing IL-2, IFN-γ and TNF-α) and a Th2 type (producing IL-4, Il-5, IL-6 and IL-10) (Mosmann and Sad, 1996; Mosmann et al., 1986).

Much of the research on the antiviral $CD8^+$ T-cell response has focused on the cytolytic abilities of these cells (Binder and Kundig, 1991; Kagi et al., 1994; Lukacher et al., 1984). However, like $CD4^+$ T-cells, $CD8^+$ T-cells have also been considered as the major source of T-cell-derived cytokines including IFN-γ, TNF-α, IL-2, granulocyte macrophage-colony stimulating factor (GM-CSF), RANTES (regulated upon action T-cell expressed and secreted), macrophage inflammatory protein MIP-1α and MIP-1β during viral infection (Kristensen et al., 2004; Paliard et al., 1988). Three of these cytokines, IFN-γ, TNF-α and IL-2, have been widely investigated and associated with many aspects of infection. IFN-γ is also produced by natural killer (NK) cells and NK T-cells as part of the innate immune response, which is involved in many functions including inhibition of viral replication, tumour control, macrophage activation, and up-regulation of both major histocompatibility complex (MHC) class I and II (Boehm et al., 1997; Rosenzweig and Holland, 2005).

The pro-inflammatory cytokine TNF-α is produced mainly by macrophages and mast cells in addition to T-cells, with a primary role in the regulation of immune cells, induction of apoptotic cell death, activation of macrophages and cytotoxic cells, induction of inflammation and inhibition of tumorigenesis and viral replication (Corral et al., 1999; Locksley et al., 2001). Elevated levels of TNF-α have been associated with the fever, malaise, and weight loss that accompany chronic infections, and reduction in levels of TNF-α have been linked with the reduction of clinical signs in a number of disease states (Moreland et al., 1997; Tracey and Cerami, 1992). The cytokine IL-2 synthesised by T-cells is crucial for proliferation and activation of mainly cytotoxic $CD8^+$ T-cells but also $CD4^+$ T cells and B-cells; it maximises the killing efficacy of macrophages and regulates T-cell proliferation (Karasuyama et al., 1989; Roitt et al., 2001b).

In response to viral infection, T-cell subsets may function in different ways. In lymphocytic choriomeningitis virus infection, $CD4^+$ T-cells are not essential for virus-induced T-cell-mediated inflammation (Marker et al., 1995). In HIV-infection, the reduction of $CD4^+$ T-cells causes dysfunction of $CD8^+$ T-cells, NK cells and B-cells which results in high levels of virus production in the chronic (AIDS) stage of infection (Flint et al., 2004b). In some lentiviral infection, $CD8^+$ T-cells play a critical role in the early stages of infection (Jin et al., 1999; Matano et al., 1998; Pantaleo et al., 1997a) and also during late stages of non-progressor infections, suggesting these cells secrete antiviral substances (Copeland et al., 1995; Migueles et al., 2002; Zagury et al., 1998).

In JDV infections, although populations of $CD4^+$ T-cells were depleted, $CD8^+$ T-cells increased significantly during the febrile and post-febrile phases (Chapter 5) and as a majority of JDV-infected animals survive the acute disease without further recurrence of clinical signs, suggests that $CD8^+$ T-cells are important in recovery. These and other cells, such as NK cells and macrophages, might also produce pro-inflammatory cytokines and these cytokines could play a significant role not only in the inflammatory process but also in recovery from infection.

The studies reported in this Chapter were conducted to examine the expression of cytokines IL-2 , IFN-γ and TNF-α by PBMC following infection with JDV and to determine the correlation between these responses and the apparent hyperplasia of

$CD3^+$ T-cells in lymphoid tissues observed during the early post-febrile phase of Jembrana disease (Chapter 3) and the significant proliferation of $CD8^+$ T-cells in the circulation during the early post-febrile phase that was demonstrated by flow cytometric analyses (Chapter 5).

Materials and methods

Experimental animals, sample collection and PBMC preparation

The 5 Bali cattle infected with $JDV_{Tab/87}$ described previously in Chapter 5 were also used as a source of blood for the derivation of PBMC for detection of cytokine mRNA. Blood samples were obtained daily from the animals for 19 days following JDV infection using EDTA-containing vacutainer EDTA tubes (Greiner Bio-One). The blood samples were centrifuged to recover PBMC and plasma, as reported in Chapter 5, and the preparations were frozen at -80^{o}C until they were used for extraction of RNA.

Primers

Oligonucleotide primers for cytokine genes IL-1, IL-2, IL-6, TNF-α and IFN-γ, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control, were as described previously (Konnai et al., 2003; Leutenegger et al., 2000). All oligonucleotide primer sequences (Table 6.1) were checked using the BLASTIN program (Zhang and Madden, 1997). Oligonucleotide primers for JDV RNA quantification were those described previously (Stewart et al., 2005).

RNA extraction

For cytokine mRNA analysis, total RNA (tRNA) was extracted from PBMC with RNeasy Plus with genomic DNA (gDNA) eliminator columns (Qiagen), following the manufacturer's instructions. To remove any residual gDNA, the extracted tRNA was treated with 1 U of DNAase (Promega) per 1 ug tRNA at 37^{o}C for 30 min followed by inactivation of the enzyme with DNAase Stop solution at 65^{o}C for 10 min. Concentrations of the enzyme-treated tRNA were determined using a spectrometer (Nano-drop 1000), after which the samples were stored on ice until they were tested on the same day; they were not frozen to avoid RNA degradation. A normalisation procedure was used to correct for experimental error during the

extraction and processing of the RNA, as previously described (Bustin, 2000, 2002; Bustin and Nolan, 2004). Forty ng per reaction of enzyme-treated tRNA was used as template for amplification in each RT-PCR.

Table 6.1. Sequence of oligonucleotide primers for real-time RT-PCR.

Gene	Primer sequences (5'-3') (forward & reverse)	Length	
		Primer(bp)[a]	Product (bp)
IL-1α [b]	GATGCCTGAGACACCCAA	18	173
	GAAAGTCAGTGATCGAGGG	19	
IL-2[b]	TTTT TAC GTC CCC AAG GTT AA	20	217
	CGT TTA CTG TTG CATCATCA	20	
IL-6 [b]	TCCAGAACGAGTATGAGG	18	236
	CATCCGAATAGCTCTCAG	18	
TNF-α[c]	TCTTCTAAGCCTCAAGTAACAAGT	N/A	103
	CCATGAGGGCATTGGCATAC		
IFN-γ [c]	TGGATATCATCAAGCAAGACATGTT	N/A	151
	ACGTCATTCATCATCACTTTCATGAGTTC		
GAPDH [c]	GGCGTGAACCACGAGAAGTATAA	N/A	120
	CCCTCCACGATGCCAAAGT		
JDV *pol*[d]	GGGAGACCCCGTCAGATGTGGA	N/A	121
	TGGGAAGCATGGACAATCAG		

[a] bp: base pairs
[b] Konnai et al (2003).
[c] Leutenegger et al. (2000).
[d] Stewart et al. (2005).

Viral RNA was extracted from thawed plasma samples using a QIAamp Viral Mini Kit (Qiagen) according to the manufacturer's instructions. Prior to extraction, plasma samples were clarified by centrifugation (8,000 x *g* for 5 min). Viral RNA was then extracted from 140 µl of plasma, eluted in a final volume of 50 µl of elution buffer (AVE buffer; QIAGEN), and stored at -80^{o}C until tested.

cDNA synthesis from RNA

A 1 µg tRNA sample was transcribed to cDNA using SuperScript III RT (Invitrogen) following the manufacturer's instructions. The primer pairs listed in Table 6.1 were used to synthesise cDNA and the PCR products were sequenced to verify their specificity using a standard sequencing procedure (Applied Biosystems 3730 DNA sequencer, DNA Sequencing Facility, Murdoch University).

Cloning of cDNA

DNA samples remaining after sequence analysis were electrophoresed in a 2.5 % agarose gel for 2 h at 65 V. DNA bands were visualised by labelling with SYBR Green (Promega) and were excised from the gel and the DNA extracted using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. The purified PCR products were cloned into pCR 2.1 using a TA Cloning System (Invitrogen) as recommended by the manufacturer. Following transformation of *E. coli*, selected white (transformed) *E. coli* colonies were cultured in YT medium pH 7.2 containing 10 g bacto-yeast extract and 5 g NaCl in 1 l of double distilled H_2O. The cloned DNA plasmids were purified from bacterial cells using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Plasmids were screened for the appropriate inserts by *Eco* RI digestion (Promega), according to the manufacturer's instructions.

For further sequence analysis and preparation of standard curves, the purified cloned plasmid DNA was amplified by PCR. Briefly, 2 μl of plasmid DNA was mixed with 2.5 μl 10X buffer, 1.25 μl of 25 mM $MgCl_2$, 0.2 μl of 2.5 mM DNTPs, 1 μl of 20 pM forward and reverse primers, 0.125 μl *Taq* polymerase (5.5 unit/μl) and made up to a total 25 μl by addition of extra PCR grade water. The PCR assays were conducted using a 3 min denaturising step at 94^{o}C, 35 cycles of 94^{o}C for 30 seconds, 52^{o}C for 1 min, 72^{o}C for 1 min, 72^{o}C for 10 min, and then held at 14^{o}C. After visualisation of the products in agarose gels as described above, DNA bands were extracted from the gels and the concentration of the extracted DNA was determined spectrophotometrically at 260 nm, and the concentration was adjusted to 1 μg DNA/ml. The purified DNA was directly sequenced to confirm the identity of the PCR products.

Preparation of standard curve for quantitative analysis

To quantitate cytokine mRNA expression, calibration curves were prepared using the measured fluorescence of serial 10-fold dilutions from 10^{-2} (0.01 ng/ml) to 10^{-6} of the synthesised cDNA that had been adjusted to 1 μg DNA/ml as described above, and the concentrations were derived by extrapolation from the standard curve. The mass of every plasmid (Table 6.2) was converted to moles and multiplied by Avogadro's number, to estimate the copy number of cytokines in each reaction

mixture. The number of cytokine copies detected in each reaction was automatically determined using a software package (SYBR-green, Corbett Research). Generation of standard curves and regression analysis were performed by using a Rotor-Gene program (Corbett Research).

Standard curves for quantifying JDV RNA were prepared as previously reported (Stewart et al., 2005).

Table 6.2. Plasmid mass used to estimate cytokine copy number.

Gene	Gene size (bp)	Vector size (bp)	Total size (bp)	RNA copy number (x 10^8/µl)
IL-1	173	3015	3188	2.91
IL-2	217	3015	3232	2.87
IL-6	236	3015	3251	2.85
IFN-γ	151	3015	3166	2.93
TNF-α	103	3015	3118	2.97
GAPDH	120	3015	3136	2.96

Analysis of bovine cytokine expression by real-time PCR

RT-PCR assays were performed using a Rotor-Gene 3000 (Corbett Research) according to the manufacturer's instructions. The SYBR Green RT-PCR reaction was performed with 2 µl RNA containing 40 ng of DNAase-treated RNA as the template. The 2 µl RNA sample was added to 8 µl reaction mix consisting of 5 µl of 2X SYBR Green RT-PCR reaction mix (Bio-Rad), 1 µl of 300 nM of each primer, 0.2 µl iScript RT for one-step RT-PCR (Bio-Rad) and 0.8 µl of nuclease-free water (Bio-Rad). The PCR mixtures were analysed in duplicate but the standards were analysed in triplicate.

Statistical analysis

A one-way ANOVA (SPSS® 17.0) was used to assess group differences in the expression of cytokine, and differences between time points during JDV infection were analysed using Bonferroni's multiple comparison. A value of $p \leq 0.05$ was considered significant for all analyses.

Results

RNA preparation

As shown in Figures 6.1 and 6.2, the addition of 40 ng DNAase per reaction completely eliminated gDNA from tRNA samples.

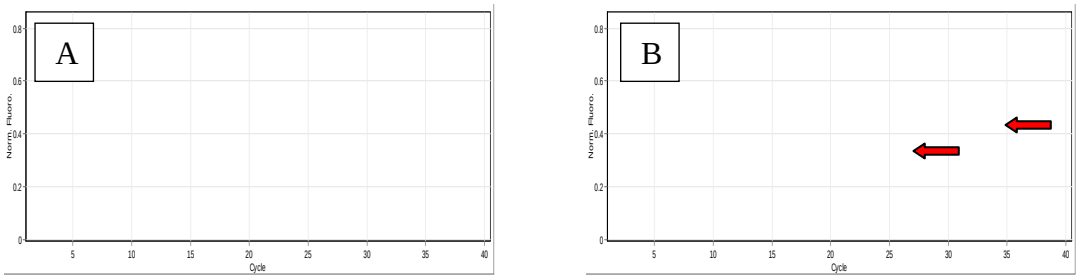
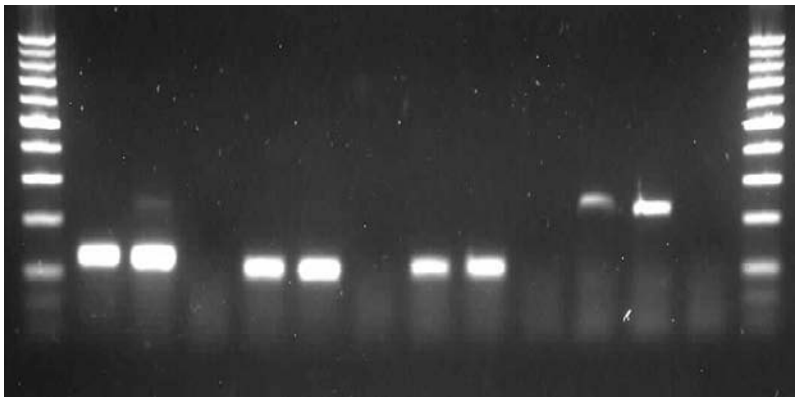


Figure 6.1 Real-time RT-PCR analysis of TNF-α showing a complete removal of gDNA from tRNA samples. A. Example of a standard curve for TNF-α, linear representation. B. Non-DNAase-treated tRNA (left arrow) and DNAase-treated tRNA (right arrow). No amplified products were detected when RT was omitted, indicating gDNA was completely removed. The reaction patterns from other genes were similar.

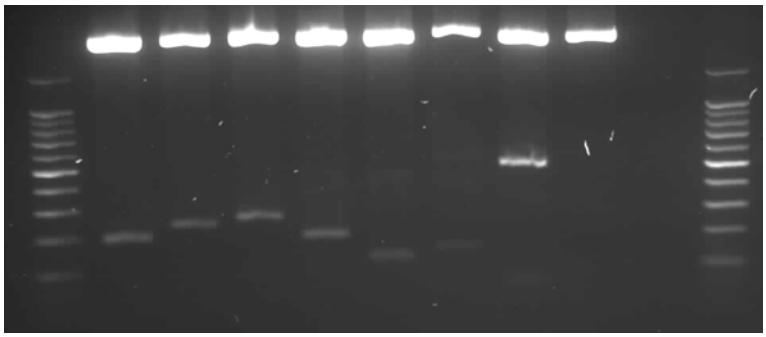


1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 6.2. Agarose gel electrophoresis of RT-PCR products showing a complete removal of gDNA from the extracted RNA and the reaction products for 4 different primer pairs. Lanes 1 and 14, 100 bp DNA marker (Promega); lanes 2, 5, 8, and 11, DNAase-treated RNA of GAPDH, TNF-α, IFN-γ and IL-2, respectively; lanes 3, 6, 9 and 12, non-DNAase treated RNA of GAPDH, TNF-α, IFN-γ and IL-2, respectively; lanes 4,7,10 and 13, RT^- control for GAPDH, TNF-α, IFN-γ and IL-2, respectively. The PCR reaction products of GAPDH were ~120bp, TNF-α ~103 bp, IFN-γ ~151 bp and IL-2 ~217 bp. Although IL-1 and IL-6 assays were developed, these cytokines were not analysed in JDV-infected cattle.

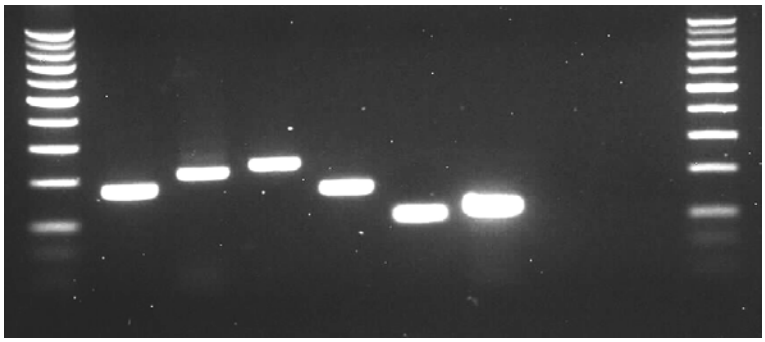
Confirmation of successful cloning and sequencing

The DNA products generated using the cytokine primers (Table 6.1) were successfully cloned into pCR 2.1 (Figure 6.3). The purified DNA inserts were amplified by PCR to provide DNA samples for sequence analysis and preparation of standard curves (Figure 6.4). Sequence analysis of the PCR products confirmed their identity as the respective bovine cytokine genes (Figure 6.5).



1 2 3 4 5 6 7 8 9 10 11

Figure 6.3. Agarose gel electrophoresis of the non-PCR product of plasmid DNA constructs, digested with EcoR1, to confirm the successful cloning. Lanes 1 and 11: 100 bp DNA ladder (Promega); lane 2, IL-1 (173 bp), lane 3, IL-2 (217 bp), lane 4, IL-6 (236 bp), lane 5, IFN-γ (151 bp); lane 6, TNF-α (103 bp); lane 7, GAPDH (120 bp); lane 8, control plasmid with DNA insert supplied by the manufacturer (from a white colony); lane 9, control plasmid (from a blue colony) with no insert; lane 10, water control. Although IL-1 and IL-6 assays were developed, these cytokines was not analysed in JDV-infected cattle.



1 2 3 4 5 6 7 8 9 10

Figure 6.4. Agarose gel electrophoresis of PCR-products shown in Figure 6.3. Lanes 1 and 10, 100 bp. DNA ladder (Promega); lane 2, IL-1 (173 bp), lane 3, IL-2 (217 bp), lane 4, IL-6 (236 bp), lane 5, IFN-γ (151 bp); lane 6, TNF-α (103 bp), lane 7, GAPDH (120 bp), lane 8, primer control (IL-1 DNA) and lane 9, water control. The DNA products correspond to the plasmid DNA constructs shown in Figure 6.2. Although IL-1 and IL-6 assays were developed, these cytokines were not analysed in JDV-infected cattle.

1. GENE ID: 280943 TNF|tumor necrosis factor(TNFsuperfamily, member 2) [Bos taurus] (Over 10 PubMed links) Score = 91.6 bits (49), Expect = 1e-15 Identities = 49/49 (100%), Gaps = 0/49 (0%) Strand=Plus/Plus

```
Query  29   TCTCCGGGGCAGCTCCGGTGGTGGGACTCGTATGCCAATGCCCTCATGG  77
            |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  330  TCTCCGGGGCAGCTCCGGTGGTGGGACTCGTATGCCAATGCCCTCATGG  378
```

2. GENE ID: 281237 IFNG | interferon, gamma [Bos taurus] (Over 10 PubMed links) Score = 104 bits (114), Expect = 3e-19 Identities = 58/59 (98%), Gaps = 0/59 (0%) Strand=Plus/Plus

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Query  16   TGNACTCATCAAAGTGATGAATGACCTGTCGCCAAAATCTAACCTCAGAAAGCGGAAGA  74
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  TGAACTCATCAAAGTGATGAATGACCTGTCGCCAAAATCTAACCTCAGAAAGCGGAAGA  46
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3. GENE ID: 280822 IL2 | **interleukin 2 [Bos taurus]** (10 or fewer PubMed links) Score = 255 bits (282), Expect = 5e-65 Identities = 167/180 (92%), Gaps = 3/180 (1%) Strand=Plus/Plus

```
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            || ||||| ||||||| |||||| |||| ||||||||||||||||||||| ||||||||
Sbjct  234  ATCTTAAGTGTTTACTAGAAGAACTCAAACTTCTAGAGGAAGTGCTAAATTTAGCTCCAA  293


Query  66   GCACAAAGG-GAAACCCAGAGAGATCAAGGATTCAATGGACAATATCAACCGAATCGTTT  124
            ||| |||   ||| |||||||||||||||||||||||||||||||||  |||||||||
Sbjct  294  GCAAAAACCTGAACCCCAGAGAGATCAAGGATTCAATGGACAATATCAAGAGAATCGTTT  353

Query  125  TGGAACTACAGGGATCTGAAACAAGATTCACATGTGAATATGATGATGCAACAGTAAACG  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  TGGAACTACAGGGATCTGAAACAAGATTCACATGTGAATATGATGATGCAACAGTAAACG  413
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4. gb|EU276071.1| **Bos taurus interleukin 6 (IL6)** mRNA,complete cds Length=641 GENE ID: 280826 IL6 | interleukin 6 (interferon, beta 2) [Bos taurus] (10 or fewer PubMed links) Score = 320 bits (354), Expect = 3e-84
Identities = 177/177 (100%), Gaps = 0/177 (0%) Strand=Plus/Plus

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Query  26   CAGAACACTGATCCAGATCCTGAAGCAAAAGATCGCAGATCTAATAACCACTCCAGCCAC  85
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  446  CAGAACACTGATCCAGATCCTGAAGCAAAAGATCGCAGATCTAATAACCACTCCAGCCAC  505

Query  86   AAACACTGACCTGCTGGAGAAGATGCAGTCTTCAAACGAGTGGGTAAAGAACGCAAAGAT  145
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  506  AAACACTGACCTGCTGGAGAAGATGCAGTCTTCAAACGAGTGGGTAAAGAACGCAAAGAT  565

Query  146  TATCCTCATCCTGAGAAACCTTGAGAATTTCCTGCAGTTCAGCCTGAGAGCTATTCG  202
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  566  TATCCTCATCCTGAGAAACCTTGAGAATTTCCTGCAGTTCAGCCTGAGAGCTATTCG  622
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Figure 6.5 Sequence of PCR-amplified bovine cytokine genes. The percentage identity (92% to 100%) with reference standards is shown.

Clinical signs, JDV RNA and cytokine response in JDV-infected cattle

RT-PCR assays were used to quantitate JDV RNA load in plasma and the expression of IL-2, TNF-α and IFN-γ mRNA in PBMC. A representative melting curve analysis of the RT-PCR assay for IFN-α with a coefficient of correlation of 0.998 is shown in Figure 6.6, and similar results were obtained with all other RT-PCR assays.

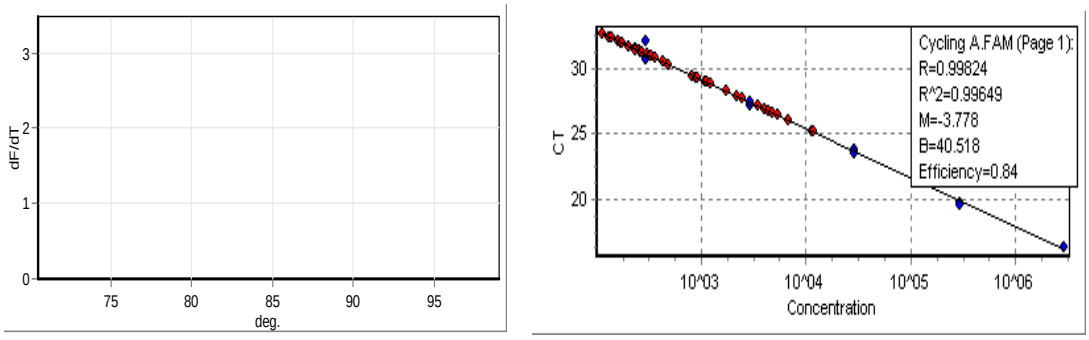
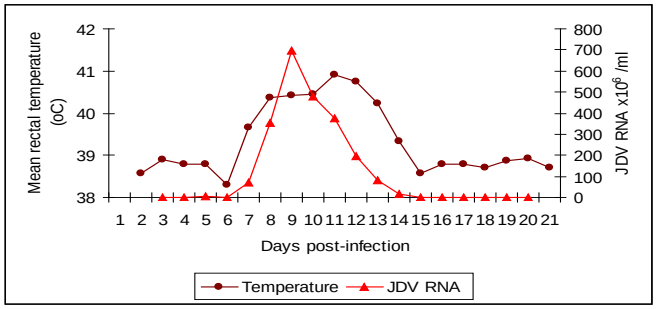


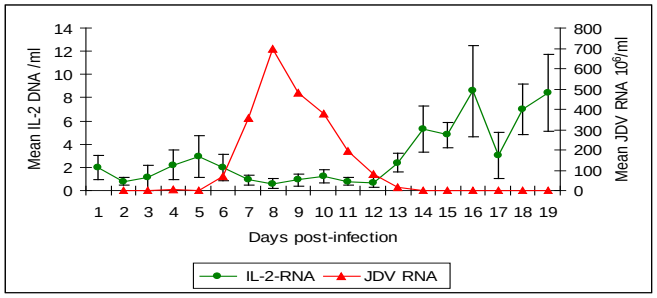
Figure 6.6. Melting curve analysis (left) and coefficient of correlation (right) for the RT-PCR for IFN-γ expression in one animal (CB5), showing a single peak for a specific PCR reaction, and the expected R value and gradient (M value).The melting curve analysis and coefficient of correlation patterns for the RT-PCR for other cytokines tested and from other animals used in this study were similar.

There was a significant increase in plasma JDV RNA concurrent with the development of the transient febrile period 4 to 7 days after infection (Figure 6.7A). Peak levels of JDV RNA in plasma occurred 9 days after infection. The levels then decreased and JDV RNA was undetectable in plasma in the post-febrile period (Figure 6.7A). IL-2 mRNA expression was low before and during the febrile period but significantly increased ($p<0.05$) in the post-febrile period and then remained above the pre-infection values until the termination of the experiment 21 days after infection (Figure 6.7B). TNF-α mRNA expression was not significantly increased ($p=0.284$) during the pre-febrile and early febrile phase of JDV infection but levels increased slightly during the later stages of the febrile phase and in the immediate post-febrile phase (Figure 6.7C). IFN-γ mRNA expression increased in a biphasic mode: it increased during the early stages of the febrile phase, it then decreased but remained above pre-infection levels during the remainder of the febrile phase, it then again increased significantly ($p <0.001$) again at the end of the febrile phase and remained high until about 16-17 days after infection (Figure 6.7D).

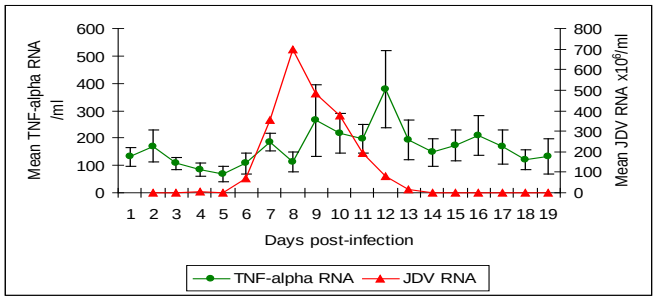
Representative patterns of gene expression as determined by RT-PCR in the 5 JDV-infected cattle are shown in Figure 6.8.



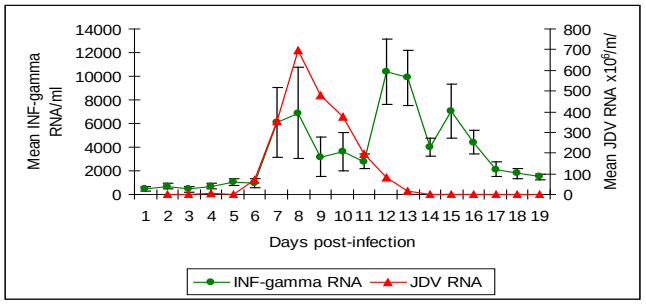
A



B



C



D

Figure 6.7. Summary of febrile response, virus load and cytokine mRNA expression (genome copies) of 5 cattle following infection with $JDV_{Tab/87}$. A. Febrile response and plasma JDV RNA levels. B, IL-2 mRNA expression. C. TNF-α mRNA expression. D. IFN-γ mRNA expression. Results represent the mean values in the 5 infected cattle and error bars showing SD are indicated.

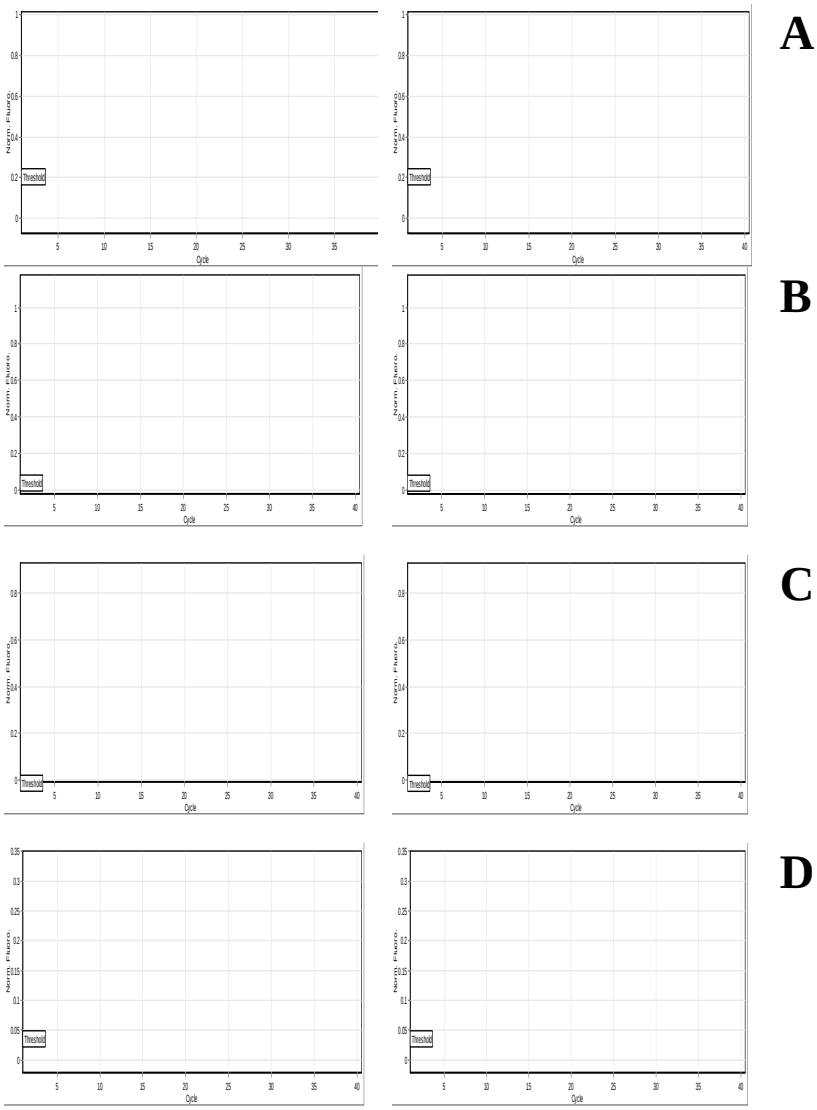


Figure 6.8. Representative pattern of the expression of cytokine mRNA and JDV RNA in a single animal (CB7) which was the strongest responder, although all data from other animals used in this study were similar. Standard curves are shown on the left and test samples on the right. (A) IL-2; (B) TNF-α; (C) IFN-γ; (D) plasma JDV RNA.

Discussion

The real-time RT-PCR methodology used in this investigation has been previously used for *in vivo* studies of cytokine gene expression in cattle (Konnai et al., 2003; Usui et al., 2007; Waldvogel et al., 2000; Zaros et al., 2007). The technique is sensitive and simple and has advantages over other techniques for the quantification of cytokine mRNA (Blaschke et al., 2000; Giulietti et al., 2001; Malinen et al., 2003;

Wang and Brown, 1999). The technique enabled samples to be collected in Indonesia and their subsequent importation to Australia where the assays were conducted. It was also determined that plasma IFN-γ protein levels detected using a commercial ELISA correlated with IFN-γ gene expression determined by RT-PCR (data not shown), confirming that the IFN-γ mRNA was translated into protein *in vivo*. GAPDH was selected as a control general housekeeping gene to verify the quality of RNA extracted and to standardise the assays; it is widely employed as an endogenous control gene in quantifying cytokine expression (Dheda et al., 2004; Lang and Heeg, 1998; Leutenegger et al., 2000; Pico de Coana et al., 2004).

The cytokines IL-2, IFN-γ and TNF-α were investigated to verify if changes in the level of their expression would correlate to the pathological changes occurring in Jembrana disease. Expansion of this study to include additional cytokines would have provided more comprehensive information and while methodology was developed for quantification of bovine IL-1 and IL-6, these assays were not conducted and reported in this thesis due to time limitations. However, the significant expression of IL-2 and IFN-γ mRNA correlated well with the significant increase of $CD8^+$ T-cells detected by flow cytometry (Chapter 5), suggesting that these cytokines were probably produced by the proliferating $CD8^+$ T-cells and, in the absence of JDV-specific antibody until well after recovery, these events have a crucial role in controlling JDV infection and enabling the majority of JDV-infected cattle to survive the acute disease.

A role of other cell types in expression of these cytokines is also possible but is unlikely to be due to the production of cytokines by Th1 $CD4^+$ T-cells as the number of $CD4^+$ T-cells was reduced significantly until well after recovery (Chapter 5). The reason for the reduction of $CD4^+$ T-cells during the febrile period is unknown, but in HIV-infection, expansion of these cells can be affected by circulating IL-2 (Ganusov et al., 2007; Stapleton et al., 2009). However, while IL-2 expression was significantly up-regulated during the post-febrile phase of Jembrana disease, the $CD4^+$ T-cell populations in plasma remained low (Chapter 5).

The cytokines IL-2 and IFN-γ have a well defined role in the pathogenesis of other viral infections. IFN-γ is produced predominantly by NK and NK T-cells as part of the innate immune response, and by $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T-

lymphocyte effector T-cells once antigen-specific immunity develops (Schoenborn and Wilson, 2007). It plays a critical role in the immune process, enhancing the microbicidal action of macrophages and stimulating the production of IgG (Boehm et al., 1997; Harrington et al., 2006; Schoeborn and Wilson, 2007; Weaver et al., 2006). The up-regulation of IFN-γ expression during the febrile phase of Jembrana disease, and its known role in the anti-viral effector function of CD8+ T-cells, correlated with the decrease in viral load at the end of the febrile phase.

IL-2 is an example of an autocrine growth factor, produced by all T-cells early in their activation and is very important in up-regulating T-cell proliferation and the activation of macrophages and cytotoxic $CD8^+$ T-cells, maximising the killing efficacy of these cells (Karasuyama et al., 1989; Roitt et al., 2001b). The low levels of IL-2 expression during the febrile phase of Jembrana disease, when viral loads in plasma are very high, paralleled the low levels of $CD4^+$ T-cells and $CD8^+$ T-cells in plasma during this period (Chapter 5). The up-regulation of IL-2 expression determined during the post-febrile phase of JDV infection coincided with expansion of $CD8^+$ T cells (Chapter 5) and suggests a role for IL-2 in the proliferation of these cells and therefore in the recovery process and survival of a majority of JDV-infected cattle.

The role of TNF-α during Jembrana disease is unclear. TNF-α is involved in the regulation of immune cells, inducing acute phase reactions, triggering apoptotic cell death, and inhibiting viral replication (Abbas et al., 1996). In the JDV-infected animals, TNF-α was only slightly up-regulated during the febrile and post-febrile phase of acute Jembrana disease and while it may contribute to the induction of the clinical signs of Jembrana disease and in reducing viral load at the end of the febrile phase, the evidence for this is minimal.

In conclusion, this is the first report quantifying cytokine expression during Jembrana disease. The results indicated up-regulation of IL-2 and IFN-γ expression, which was associated with the expansion of $CD8^+$ T-cells during this period, suggesting these events may be responsible for viral clearance and recovery from the disease. Further investigations are required to broaden the range of cytokines examined and to investigate the role of these cytokines in the acute disease process.

Chapter 7

General discussion

Bali cattle, descendent from wild Banteng, are important to beef production in Indonesia (Martojo, 2003; Wiryosuhanto, 1996). They are adapted to the tropical climate experienced in many regions of Indonesia, they can be used not only for beef production but also as draught animals for preparation of rice fields for planting, and they have therefore been invaluable in the development of new transmigration areas within Indonesia (Martojo, 2003). These cattle are now widely distributed through Indonesia from Aceh in the west to West Papua in the east. They are the predominant cattle breed in Indonesia, about 2.6 million of a total cattle population of about 5.3 million distributed in several areas but especially Bali, Sumatra, Kalimantan, Sulawesi and Maluku (Talib et al., 2002). Unfortunately, Bali cattle are extremely susceptible to Jembrana disease and this disease is therefore a major problem that continues to affect the cattle industry in Indonesia. There is not only a direct economic loss as a consequence of the effects of the disease but an indirect loss as a consequence of the restriction of the transportation of cattle from areas where the disease is endemic (Bali, Java, Sumatra and Kalimantan) to disease-free regions.

Although the cause of the disease was identified as a previously unknown bovine lentivirus (Chadwick et al., 1995a) and this enabled a series of studies that have resulted in an improved understanding of the response of Bali cattle to JDV infection, many aspects of the pathogenesis of the infection in Bali cattle have not been investigated. In particular, the precise cell-tropism of JDV and the immunopathological response to the virus have not been explored. Because of histological observations of an intense cellular proliferation in the non-follicular (T-cell) compartments of lymphoid tissues and haematological findings of lymphopenia, which are hallmark features of Jembrana disease (Soesanto et al., 1990), there has been an assumption that JDV probably infects T-cells (Dharma et al., 1991; Dharma et al., 1994), even though the closely related BIV is pantropic (Heaton et al., 1998).

The studies reported in Chapter 3 of this thesis demonstrated for the first time that the intense proliferation of cells in the non-follicular areas of lymphoid tissues

during the febrile phase of the disease was not due to proliferation of $CD3^+$ T-cells but instead was due to infiltration of these areas by centroblast-like cells that expressed IgG and therefore appeared to be antibody-producing cells of the B-cell lineage. The population of these centroblast-like cells decreased during the post-febrile phase when a significant proliferation of the $CD3^+$ T-cell population in parafollicular areas was detected. JDV infection, evident by the detection of JDV CA by immunoperoxidase techniques, appeared to be of cells with the same distribution and morphological appearance to the centroblast-like cells, and not of T-cells or macrophages. Confirmation of the identity of the infected cells was sought using double-immunolabelling techniques as used by others for investigating viral tropism (Espinoza and Kuznar, 2009; Mason et al., 2000; Valnes and Brandtzaeg, 1984) and concordance between cells containing JDV CA detected by immunofluorescence and some IgG-containing cells and $CD79\alpha^+$ cells supported the identification of the JDV-infected cells as antibody-producing B-cells.

Because of the marked differences in the disease process following JDV infection and that of other lentiviruses (Soesanto et al., 1990), especially the acute nature of the disease and the unusual genetic stability of the virus (Desport et al., 2007) ,the apparent predilection of the virus for B-cells and not T-cells or macrophages as with most lentiviruses is perhaps not surprising. The tropism of the virus for cells of B-cell lineage would explain the depopulation of B-cell (follicular) areas (Dharma et al., 1991) and the delayed antibody response to JDV and to other immunogens (Hartaningsih et al., 1994; Wareing et al., 1999) during the post-febrile phase. It is probable that the normal process of antibody production is disrupted by the infection and death of these cells. Both BIV and JDV appear to be genetically stable over time with much lower viral mutation rates than the other lentiviral genomes and this may be related to their tropism for plasma cells with a long life span (Carpenter et al., 2000; Desport et al., 2007).

The lack of replication of JDV in macrophages might explain the absence of neurological lesions in Jembrana disease. Neurologic lesions reported in HIV-infected individuals were associated infection of microglia cells (Gonzalez-Scarano and Martin-Garcia, 2005) and in SIV infections, the presence of CNS disease has also associated with the presence of SIV-infected monocytes/macrophages in the brain (Bissel et al., 2008).

The apparent predilection of JDV for cells of the B-cell lineage and apparent lack of replication in T-cells or macrophages is unusual and has not been observed with other lentiviruses. Although some lentiviruses do infect B-cells they infect other cell types as well. Of these, the genetically related BIV infects B-cells, T-cells and monocytes (Heaton et al., 1998), SIV infects $CD4^+$ and $CD8^+$ T-cells, macrophages and B-cells *in vivo* (O'Neil et al., 1999), and HIV-1 infects $CD4^+$ T-cells, macrophages and dendritic cells, although it is not clearly associated with direct infection of B-cells (Clapham and McKnight, 2002; Conge et al., 1998; Muro-Cacho et al., 1995; Shirai et al., 1992). Other lentiviruses producing acute disease syndromes are not specifically B-cell tropic: EIAV in horses targets tissue macrophages (Murakami et al., 1999; Oaks et al., 1998; Sellon et al., 1994) and $SIV_{smmPBj14}$ in pig-tailed macaques targets macrophages (Fultz et al., 1989). It will be interesting to determine the nature of the receptor utilised by JDV and its distribution in different cell types, and if the receptor involved has an unusual distribution in Bali cattle that might help to explain the specificity of the disease for Bali cattle and the mild or subclinical nature of the disease in other ruminants (Soeharsono et al., 1990; Soeharsono et al., 1995a; Soeharsono et al., 1995b).

To understand the cellular mechanism responsible for the recovery process in the majority of experimentally JDV-infected cattle, changes in lymphocyte subsets during JDV infections were investigated (Chapter 5). Flow cytometric analysis confirmed that the lymphopenia, a characteristic haematological change occurring during the febrile phase of Jembrana disease (Soesanto et al., 1990) was due, at least in part, to a significant reduction of both $CD4^+$ T-cells and $CD8^+$ T-cells. As the virus appeared to not infect $CD3^+$ T-cells, including $CD4^+$ and $CD8^+$ T-cells, it is probable that the mechanism for the reduction of the $CD4^+$ T-cells found in JDV infection is similar to that reported in EIAV infection, an indirect affect of products produced during viral replication (Murakami et al., 1999). The reduction in $CD4^+$ cells during Jembrana disease would likely contribute to the immunosuppression and the increased occurrence of secondary diseases, such as haemorrhagic septicaemia, reported in cattle affected with Jembrana disease (Dharma et al., 1994).

A significant expansion of $CD3^+$ T-cells in lymphoid tissue during the early post-febrile phase (Chapter 3) coincided with significantly increased $CD8^+$ T-cell population, demonstrated by flow cytometric analysis (Chapter 5). The trigger for

the marked proliferation of these cells after recovery from the febrile phase is not known. In the acutely pathogenic $SIV_{smmPBj14}$, Nef was assumed to have mitogenic properties and to activate resting lymphocytes (Stephens et al., 1998), and although *nef* is not present in JDV, a *tmx* gene is located in the same region of the genome as *nef* in the primate lentiviruses (Chadwick et al., 1995b) and might express a protein with a Nef-like function.

The increased $CD8^+$ T-cell population might be associated with the recovery of animals and the survival of a majority of JDV-infected cattle from the acute disease (Soesanto et al., 1990). It might also be associated with their resistance to further infection and the absence of any further clinical signs of disease attributable to JDV infection (Soeharsono et al., 1990). An increased $CD8^+$ T-cell population was reported in asymptomatic HIV-1 positive individuals (Copeland et al., 1995; Zagury et al., 1998), and in SIV infections (Mandl et al., 2007) even though it failed to eradicate viral infection at the later stages of infection (Migueles et al., 2002; Pantaleo et al., 1997a). While the results obtained and reported in Chapter 5 suggest qualitative factors within the $CD8^+$ T-cell response might be the principal determinants of control over JDV replication and disease progression during the acute disease, it will be essential to extend this finding to determine the kinetics of the response after the immediate post-febrile phase, beyond the duration of the current experiment.

The *in vivo* studies reported in Chapter 6 indicated that JDV infection induced changes in cytokine gene expression, determined by measurement of cytokine-specific mRNA activity. Increased IFN-γ mRNA expression detected using RT-PCR was in accordance with increased IFN-γ expression detected by an IFN-γ ELISA. Although similar ELISA kits were not used to check the concordance between mRNA activity and protein expression of the other cytokines it is likely that a correlation would occur. The increased mRNA expression occurred primarily with IL-2 and IFN-γ under Th1 cell regulation (Abbas et al., 1996). The changes in the IL-2 and IFN-γ cytokine mRNA correlated with the significant increase in peripheral blood $CD8^+$ T-cells, which provided additional evidence that up-regulation of these cells *in vivo* and the synergistic actions of these cytokines may have been associated with recovery from the acute disease process. A similar up-regulation of both IL-2 and IFN-γ cytokine mRNA was considered responsible for the absence of persistent

lymphocytosis in *Bovine leukaemia virus* infection (Kabeya et al., 1999). More extensive investigation of the kinetics and duration of the expression of these cytokines and other related cytokines should be undertaken not only in peripheral blood but also in lymphoid tissues to increase our understanding of the role of cytokines in the inflammatory response and recovery.

In conclusion, the research undertaken and presented in this thesis has greatly improved the understanding the cellular response of animals to JDV infection. Determination of a significant role of B-cells in the pathogenesis of JDV infection has paved the way for a better understanding of the disease process. It might also facilitate development of methods for the cultivation of JDV *in vitro*. The changes in T-lymphocyte sub-populations associated with recovery provide additional support for the importance of a cell-mediated immune response in the recovery of a majority of JDV-infected animals. The demonstration of increased expression of cytokine mRNA by $CD8^+$ T-cells, particularly IFN-γ and IL-2, suggests a role for these genes in the infectious process and recovery.

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