The Development of Recombinant Vaccines against Jembrana Disease

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Abstract.

Jembrana disease virus (JDV) is a lentivirus causing an acute infection with a 17% case fatality rate in Bali cattle in Indonesia. Control of the disease is currently achieved by identification of infected areas and restriction of cattle movement. A detergent-inactivated whole virus tissue-derived vaccine is sometimes employed in affected areas.

This thesis reports initial attempts to produce genetically engineered vaccines to replace the inactivated tissue-derived vaccine, which as it is made from homogenised spleen of infected animals, is expensive to produce and could contain adventitious agents present in the donor animals.

4 potential DNA vaccine constructs were created containing the JDV genes coding for the Tat, capsid (CA), transmembrane (TM) and surface unit (SU) proteins in a commercially available vaccine plasmid. These were assessed for functionality in a range of *in vitro* and *in vivo* assays. All proteins were expressed *in vitro* and administration of 2 of the constructs by a commercial 'gene gun' into the epidermis of mice resulted in antibody production to the appropriate protein. Due to the difficulties of licensing such a DNA vaccine in Indonesia, these vaccines were not progressed further.

A mathematical model was developed to describe the progression of the acute phase of Jembrana disease following experimental infection with JDV. The model divided the disease into 6 phases based on the rates of viral replication and clearance calculated from data on sequential plasma viral RNA load detected by quantitative reverse-transcription polymerase chain reaction. This allowed statistical comparison of each phase of the disease and comparison of the severity of the disease process in groups of animals. The use of the model overcame the difficulty of comparing the disease in different animals as a consequence of the animal-to-animal variation in the disease process.

The mathematical model was used to identify differences in the pathogenicity of 2 strains of JDV. One strain, JDV_{TAB} caused a more rapid onset of disease in non-

vaccinated controls, a significantly higher virus load at the onset of the febrile period and a higher peak viraemia than in animals infected with JDV_{PUL}. This provided the first evidence of variation in pathogenicity of JDV strains.

The measurement of virus load also demonstrated that some JDV infected animals developed a clinical disease that was not typical of that which had been reported previously. When infected with less than 1,000 infectious virus particles, up to 20% of infected animals failed to develop a febrile response. Infection of these animals was confirmed, however, by the detection of a high titre of circulating virus particles in plasma. These atypical infections had not been reported previously.

Application of the mathematical model describing the progression of the disease in individual animals was used to examine the effect of vaccination with the inactivated tissue-derived vaccine on the progression of the disease. Several effects were noted in vaccinated animals that were subsequently infected with JDV: a reduction in the duration of the febrile response, a reduction in the severity of the febrile response in the early phases of the acute disease, and a reduction in virus load in the early and later phases of the disease process.

The effect of vaccination with recombinant Tat, matrix (MA) and CA protein vaccines expressed in a bacterial expression system on subsequent JDV infection was also examined. A vaccine incorporating recombinant Tat and CA vaccine emulsified with Freund's incomplete adjuvant decreased the febrile response particularly in the later stages of the acute disease process, decreased the severity of the leucopenia in the later phases of the acute disease, and decreased the virus load in some but not all phases of the acute disease process. Vaccines administered with Freund's incomplete adjuvant were more efficacious than vaccines administered with QuilA, the latter actually exacerbating the disease process in vaccinated animals.

Declaration.

I declare that this is my own account of my research and contains work that has not previously been submitted for a degree at any tertiary educational institution.

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William Graham Fox Ditcham.

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For my mum.

Contents.

Abbreviations.

aa	Amino acid
Ab	Antibody
ADCC	Antibody dependant cellular cytotoxicity
AIDS	Acquired immune deficiency syndrome
ANGIS	Australian National Genomic Information Service
APD	Average pore diameter
APC	Antigen presenting cell
ARC	Animal Resources Centre
BIV	Bovine immunodeficiency virus
bp	Base pair
CA	Capsid protein
CAEV	Caprine arthritis-encephalitis virus
CCR5	C-C (beta) chemokine receptor 5
CDK9	Cyclin Dependent Kinase 9
cDNA	Complementary DNA
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C (alpha) chemokine receptor 4
DC	Dendritic cell
dH_2O	Distilled water
DLR	DNA loading ratio
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP)
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
EIAV	Equine infectious anaemia virus
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FITC	Fluorescein isothiocynate
FIV	Feline immunodeficiency virus
Gag	Group-specific antigen
GCG	Genetics Computer Group
gp	Glycoprotein

GST	Glutathione-S-transferase
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IAP	Intracisternal A particle
ID_{50}	50% Infectious dose
IFA	Immunofluorescence antibody assay
IL	Interleukin
IN	Integrase
IPTG	Isopropyl-β-thiogalactopyranoside
ISH	*In situ* hybridisation assay
JDV	Jembrana disease virus
LB	Luria-Bertani medium
LTR	Long terminal repeat
MA	Matrix protein
mAb	Monoclonal antibody
MDBK	Madin-Darby bovine kidney cells
MHC	Major histocompatibility complex
MHR	Major homology region
MLQ	Microcarrier loading quantity
mRNA	Messenger RNA
M-tropic	Macrophage tropic
MVV	Maedi visna virus
NA	Nucleic acid
NC	Nucleocapsid
Nef	Negative factor
ORF	Open reading frame
OD	Optical density
PI	Post-inoculation/infection
PID	Principal immunodominant domain
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Pre-integration complex
PLV	Puma lentivirus
PMSF	Phenyl Methyl Sulfonyl Fluoride
PR	Protease
Rev	Regulator of expression of virion proteins

RNA	Ribonucleic acid
RRE	Rev-responsive element
RSV	Rous sarcoma virus
RT	Reverse transcriptase
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SIV	Simian immunodeficiency virus
SIVagm	Simian immunodeficiency virus African green monkey
SIVcpz	Simian immunodeficiency virus chimpanzee
SIVmnd	Simian immunodeficiency virus mandrill
SIVsmm	Simian immunodeficiency virus sooty mangabey monkey
SIVsyk	Simian immunodeficiency virus Syke's monkey
SRLV	Small-ruminant lentiviruses
ssRNA	Single-stranded DNA
ssRNA	Single-stranded RNA
SU	Surface unit glycoprotein
TAR	*Trans*-activating response element
Tat	*Trans*-activator of transcription protein
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetra methyl ethylene diamine
T_m	Melting temperature of dsDNA
TM	Transmembrane glycoprotein
Tris	Tris(hydroxymethyl)aminomethane
WB	Western blotting
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Units.

°C	degrees Celsius
µg	micrograms
µL	microlitre
µm	micrometre
µM	micromolar
ρmol	picomoles
bp	base pairs
d	days
g	grams
g	times gravity
h	hours
ID_{50}	50% infectious dose
kb	kilobases
kDa	kiloDalton
kV	kilovolts
M	molar
mA	milliAmperes
mg	milligrams
min	minutes
mL	millilitre
mm	millimetre
mM	millimolar
mo.	months
ng	nanograms
nm	nanometre
OD	optical density
wk	week
rpm	revolutions per minute
s	seconds
U	Units of enzyme activity
V	volts
v/v	volume per volume
w/v	weight per volume

Chapter 1

Introduction.

The initial outbreak of Jembrana disease (JD) in 1964 on the island of Bali had a devastating effect on the Bali cattle (*Bos javanicus*) population, with estimates of mortalities varying from 25,000 to 70,000 out of a total cattle population of about 300,000 (Pranoto, 1967). The source of the virus was never determined. The disease is now endemic in Bali and has since been reported in the other islands of Sumatra, Java and Kalimantan in the Indonesian archipelago (Wilcox, 1997). As the disease appears specific for Bali cattle (Soeharsono *et al*., 1995a), and as significant numbers of these cattle occur only within Indonesia where they are used as draft animals for rice production and for beef production, the disease has not been reported in any other country.

The aetiological agent of JD was identified as a lentivirus (Kertayadnya *et al.*, 1993), closely related to bovine immunodeficiency virus (BIV), and designated Jembrana disease virus. Control strategies are based on containment of animals in affected areas and restriction of the movement of cattle from these areas to disease-free areas. The disease remains not only a major problem for cattle farmers but the restriction of the movement of cattle has had a significant effect on the economics of the Indonesian cattle industry.

Improved control procedures for the disease are needed. An inactivated whole virus vaccine prepared from homogenised spleen tissue from infected animals, inactivated with detergent and emulsified in a mineral oil adjuvant, has been reported for JD (Hartaningsih *et al.*, 2001). This vaccine had a sufficient level of efficacy, and it is currently used to vaccinate animals in areas surrounding an outbreak to decrease the likelihood of spread of disease. However, the possible presence of adventitious agents in the tissue-derived vaccine, the inability to distinguish serologically between vaccinated and infected carrier animals, the high cost of production and the difficulty of producing large quantities of the vaccine has led to the search for an alternative. This thesis reports an investigation of the suitability of selected

genetically engineered vaccines to replace the inactivated vaccine currently used in Indonesia.

As background to the thesis, a review of the literature relating to JD and possible vaccination strategies for lentivirus infections was undertaken and is reported in Chapter 2. The review includes the history of JD and how JDV compares in morphology, genome arrangement and pathogenic effects with the other members of the lentivirus family. The remainder of the review covers the history of both DNA and recombinant protein vaccination and how these technologies have been utilised to produce vaccines against the lentiviruses.

Chapter 3 describes preliminary attempts to develop a nucleic acid or 'DNA' vaccine against JD. There were many potential advantages to such a DNA vaccine which seemed applicable to the control of JD in Indonesia: the vaccines are stable, the response attained can be long lived from a single injection, the production of large amounts of vaccine is simple and low cost, and nucleotide sequences can be incorporated into the design to tailor the immune response attained (Whalen, 1996a). Several simple constructs were tested *in vitro* and *in vivo* in laboratory animals.

Assessment of the efficacy of vaccination against JD in cattle has previously relied on clinical indicators such as the effect on the duration and severity of the febrile period and case fatality rates (Hartaningsih *et al.*, 2001), but these indicators did not reveal the underlying dynamics of viral growth and clearance during infection. Real-time quantitative PCR methods were employed to assay the virus load during infection and to use this additional parameter to assess the response of cattle to infection by 2 strains of JDV. From the real-time PCR and other clinical data obtained from these experiments, a mathematical model of the acute infection was developed and this is reported in Chapter 4. The mathematical model was used to examine the pathogenesis infection using 2 different virus strains and the results are also reported in Chapter 4. The model was also used to re-examine the effect of vaccination with the inactivated tissue-derived whole virus vaccine on infection, and these results are reported in Chapter 5. The same model was then used to examine

the effect of vaccination with recombinant JDV Tat and CA protein vaccines on subsequent infection with JDV and these results are reported in Chapter 6.

A general discussion of the research reported in this thesis in the context of the need to produce a JD vaccine in Indonesia is presented in Chapter 7.

Chapter 2

Review of the literature.

In this chapter, a general review of the literature relevant to this research project is presented. The chapter is divided into 4 sections. The first of these includes a review of the history of JD and the subsequent identification of the causative agent as a lentivirus, JDV. The second section describes the features of the lentiviruses that differentiate them from other viruses and the relationship of JDV to other lentiviruses. The third section reviews the literature relating to different vaccination strategies. The fourth section examines the literature relating to vaccination of animal, non-human primate and human hosts against the diseases caused by the lentiviruses and the relevance of the current situation to the possibility of vaccinating Bali cattle against JDV infection.

By convention, when the name of a gene or open reading frame (ORF) is mentioned it is in italics and lower case, e.g. *tat.* The product of a gene or ORF is not italicised and the first letter is in upper case, e.g. Tat, or the name is abbreviated to 2 uppercase letters, e.g. capsid, CA, or nucleocapsid, NC.

History of Jembrana disease.

The first cases of an emerging infectious disease of banteng or Bali cattle (*Bos javanicus*) were reported in December 1964, on the island of Bali in the Indonesian archipelago. Within 12 months, the disease had spread across the whole island and had killed an estimated 25,000 to 70,000 of the total population of about 300,000 Bali cattle (Pranoto, 1967). Infected cattle suffered a febrile period of 2-10 days, with symptoms of diarrhoea, anorexia, nasal discharge, erosions on the oral mucosa and enlarged superficial lymph nodes. At necropsy, symptoms included splenomegaly, and haemorrhages in the myocardium, serous and mucous membranes (Dharma *et al*., 1991).

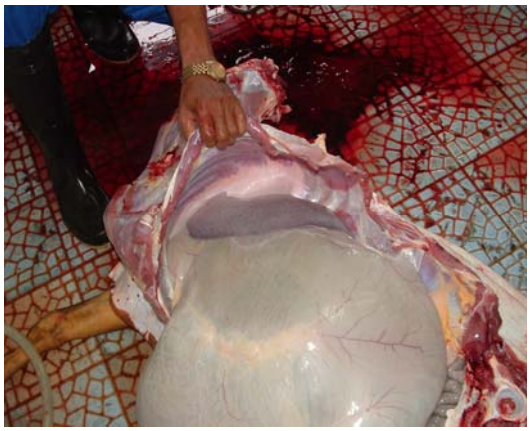


Figure 2.1 Necropsy of Bos javanicus infected with JDV, showing enlarged spleen. (Photo W. Ditcham)

The disease was named Jembrana disease after the Jembrana region of West Bali where it was first reported. Further outbreaks in Bali occurred in the Tabanan region in 1971 and in Karangasem in 1981. The disease was also detected in other provinces in other islands: Lampung (Sumatra) in 1976, East Java province in 1978, West Sumatra in 1992, South Kalimantan in 1992 and Bengkulu (Sumatra) in 1995.

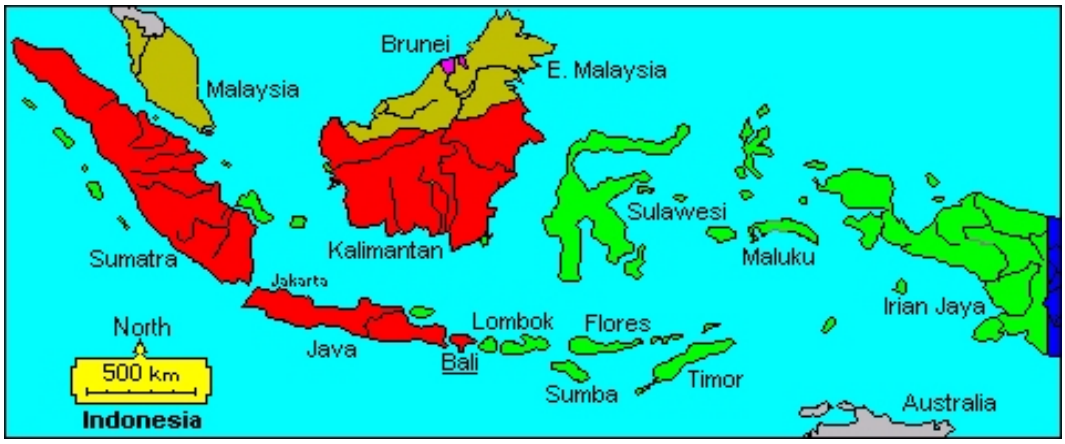


Figure 2.2 A map of Indonesia, with areas where Jembrana disease is endemic within the cattle population shown in red.

The initial outbreak in 1964 was mistakenly diagnosed as rinderpest and a mass vaccination against this disease was undertaken (Adiwinata, 1968). A ban on export of cattle from Bali was also introduced, except for cattle taken to Jakarta for slaughter, and coincidentally there were no outbreaks for several years. In the 1976

outbreak, investigations led to the hypothesis that the causative agent was rickettsial on the basis of the apparent detection within monocytes from infected cattle of intracytoplasmic particles similar in size to rickettsia (Budiarso and Hardjosworo, 1976). The clinical signs, pathology and haematology of JD were also similar to those seen in bovine ehrlichiosis and particularly Ondiri disease seen in East Africa (Ressang, 1985). However, only occasional monocytes from animals experimentally infected with the JD agent showed the rickettsial-like particles and anti-rickettsial drugs had no effect on the progress of the disease (Ramachandran, 1996).

Transmission studies revealed that the agent was present at a high titre in the plasma of animals during fever (Soeharsono *et al*., 1990). An infectious dose that infected 50% of inoculated animals (ID_{50}) was determined by titration of the infectious agent in naïve animals, where cattle were inoculated with serial dilutions of blood from an infected animal (Kertayadnya *et al*., 1993). It was determined that there were up to 10^8 ID_{50}/ml in the plasma of animals during the second day of the febrile response. There was a strong inverse correlation between inoculum concentration and an incubation period that ranged from 5-12 days, but no correlation between viral dose and severity of subsequent fever (Soeharsono *et al*., 1990). Other transmission studies proved that the agent of JD caused severe disease in only Bali cattle, but it could also at least transiently infect Ongole *(Bos indicus), Bos taurus* (Friesian breed) and buffalo *(Bubalis bubalus)* as well as Ongole x *Bos javanicus* cattle (Rambon and Madura breeds) and sheep. In those species other than Bali cattle, a transient subclinical disease was seen, marginally more severe in Friesian and Ongole cattle than in the other species (Soeharsono *et al*., 1990). The agent persisted in the blood and spleen of these animals for up to 9 months. Bali cattle that had survived the disease were resistant to further challenge for at least 2 years after the initial disease, and retained the agent in plasma for this period at least, and possibly for life (Soeharsono *et al*., 1990).

Haematological studies revealed that experimentally infected animals developed leucopenia, principally due to a lymphopenia but with a moderate neutropenia at the onset of fever, which persisted throughout the febrile period. During this period, cattle also showed elevated blood urea levels, decreased total protein levels and

thrombocytopaenia (Soesanto *et al*., 1990). Histologically, 3 phases of the disease were apparent in tissues. The initial phase in the first week of infection consisted of a general lymphoreticular reaction in the lymphoid organs, followed by an intense non-follicular proliferation of reticular and lymphoblastic cells, with infiltration of lymphoblastoid cells into the visceral organs including lungs, liver, kidneys and heart but not the brain. This phase lasted from 8-21 days post-infection (PI) and was followed by a third phase, commencing 5 weeks after infection when plasma cell formation occurred in the lymph nodes and spleen. These observations indicated that the acute phase of the illness was predominantly characterised by a T-cell response with a transient humoral immunosuppression (Dharma *et al*., 1991).

The discovery that the agent could be filtered through a 100 nm average pore diameter (APD) filter but was retained by a 50 nm APD filter indicated that it was a virus (Wilcox *et al*., 1992). It was subsequently identified as a retrovirus on the basis of electron microscopic studies demonstrating the presence of a spherical enveloped virus of about 100 nm diameter with an eccentric core, by detection of reverse transcriptase activity in purified virus preparations, and because antiserum reactive with the 26 kDa CA protein of BIV cross-reacted in Western blots (WB) with the 26 kDa protein of JDV, and vice-versa (Kertayadnya *et al*., 1993). Examination of the nucleotide sequence of a conserved region of the *pol* gene of JDV allowed classification of the virus as a lentivirus, most closely related to BIV (Chadwick *et al*., 1995a).

Knowledge of the nucleotide sequence of JDV allowed *in situ* hybridisation studies, with riboprobes to detect viral RNA in tissues at different time points after inoculation with virus (Chadwick *et al*., 1998). These studies showed that 2 days PI, before disease was apparent, the majority of infected cells were seen in the parafollicular areas of the spleen, with very few in the lymph nodes, bone marrow, lungs, kidneys or myocardium. 2 days after the onset of the febrile period, virus started to appear in the lymph nodes and in the cellular infiltrate in other organs. After 4 days of fever, JDV-infected cells were disseminated widely in all tissues, with the follicular architecture of the spleen destroyed by proliferating cells. The specific cell type infected was not identified but the prevalence of infected cells in

the lymphoproliferative infiltrate in lymphoid tissues suggested that cells of a lymphoid origin or of the monocyte/macrophage lineage were infected (Chadwick *et al*., 1998).

Bali cattle are of considerable importance to the economy of Indonesia and in 1995 the species accounted for 26.8% of all cattle in Indonesia (Wiryosuhanto, 1996). They are utilised as pioneer animals in new settlements, as draft animals and for beef production. Although they grow slowly when fed a poor quality diet, with low fat deposition in the muscle mass, they have the capacity to fatten rapidly when concentrate is included in the diet. The cattle reach maturity at around 4-5 years of age, and there is a marked size difference and coat colour difference between the sexes. The animals display better heat tolerance than other cattle types, and better ability to survive drought conditions, which is offset by comparatively high calf mortality. The cattle are resistant to internal and external parasites, except liver flukes, but are very susceptible to malignant catarrhal fever and JD. The cattle are timid, and can revert to the wild state at any stage of life, an indication of their relatively recent domestication (Wiryosuhanto, 1996).

JD has spread in the 40 years since the first outbreak in Bali to several other islands of the Indonesian archipelago, probably through the illegal movement of cattle from Bali. In 2006, it is present in Bali, Java, Sumatra and Kalimantan; it is not known to occur in islands east of the Wallace Line, including Sulawesi and the Moluku group of islands where there are large numbers of Bali cattle (Wilcox, 1997). The movement of cattle from endemic areas has been banned in an attempt to prevent the spread of JD to currently JD-free areas. Locally, when an outbreak is suspected, control measures include preventing the free mingling of animals by stopping free grazing, spraying of barns with insecticide to kill insect vectors, and the rapid disposal of the carcasses of dead animals (Soeharsono, 1996). The use of insecticides is a residual belief by some in Indonesia that the disease has a rickettsial aetiology. In 1993, vaccination was started utilising a locally produced vaccine made from the spleen of infected animals emulsified in a mineral oil adjuvant. This vaccine decreases the duration and severity of disease in experimentally infected

animals, but did not induce a sterilising immunity, at least when animals were challenged with 100 ID_{50} of JDV (Hartaningsih *et al*., 2001).

Anecdotal reports suggest that the severity of the disease has been decreasing in the areas where it is endemic (Hartaningsih, personal communication), suggesting evolution of the host/pathogen relationship towards a more stable state, probably by selection for resistant animals in the population of the endemic areas (Coffin, 2004). New outbreaks in naïve populations still cause high case fatality rates among infected animals.

The *Retroviridae.*

The *Retroviridae* is a large and diverse family of viruses and contains many important human and mammalian pathogens, causing mainly but not exclusively, immunosuppressive and transformational diseases. They are, typically, enveloped positive-sense single-stranded RNA (ssRNA) viruses ranging from 60-120 nm in diameter, with each virion containing 2 identical copies of the genome, which are capped and polyadenylated in the manner of messenger RNA (mRNA). The viral genome is not processed directly as mRNA, however, but a virus encoded reverse transcriptase (RT) produces a double-stranded DNA (dsDNA) intermediate in the cytoplasm of an infected cell that is transported to the nucleus and incorporated into the host cell genome (Pringle, 1999). Incorporation into many sites of the genome is possible with a wide range of preferences, mediated by the central core of the virally encoded integrase (IN) (Shibagaki and Chow, 1997). The unique feature of the retroviruses is the subsequent expression of viral proteins from this integrated provirus (Flavell, 1995). Most retroviruses only replicate in actively dividing cells, with the exception of the lentiviruses which require terminally differentiated cells for replication.

Before the advent of nucleic acid sequencing, the classification of this group was based on electron microscopic studies revealing the morphology, subcellular location and the nature of the budding process as the virus is released from the cell (Pringle, 1999). Type-A viruses are non-infectious, replication-deficient and passed on to the daughter cells as provirus during mitosis. They are only found

intracisternally, surrounded by a membrane, forming what is termed an intracisternal A particle (IAP). Type-B virions are assembled by budding of pre-assembled immature particles, resembling IAP, through the plasma membrane to produce virus particles possessing an eccentric core, whilst Type-C particles are not seen in the cytoplasm, but are assembled at the plasma membrane during budding. Type-D viruses form in a similar manner to Type-B, but possess a characteristic cylindrical or cone-shaped core (Pringle, 1999). Differences in genome nucleotide sequences revealed by molecular techniques have superseded this original taxonomy, although it is still used to identify the mode of budding and viral assembly. Contemporary taxonomic techniques currently divide the *Retroviridae* into 7 genera (Pringle, 1999): *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, Lentivirus* and *Spumavirus*. In addition, there are endogenous retroviruses that have not yet been classified into genera.

The genus *Alpharetrovirus,* which contains the former avian type C retroviruses, includes the avian leucosis and avian reticuloendotheliosis viruses associated with transformation of cells and neoplasia (Pringle, 1999). The earliest evidence for the ability of a transmissible agent to cause neoplasia was obtained in a series of experiments in chickens early in the 20th century, and the alpharetrovirus responsible was ultimately named Rous sarcoma virus (Rous, 1979; Rous, 1983).

The genus *Betaretrovirus* contains the former mammalian type B and type D retroviruses. The type D retroviruses are morphologically similar to the type B but differ genetically and most members, including the prototype Mason-Pfizer monkey virus, cause an acquired immunodeficiency in primates (Pringle, 1999). The only non-primate virus in this group, Jaagsietke retrovirus, infects sheep and causes a pulmonary adenomatosis. This virus is closely related to an endogenous retrovirus in sheep and represents one of the few instances where both an exogenous and endogenous form of a retrovirus exists (Bannert and Kurth, 2004).

The genus *Gammaretrovirus,* consisting of the former mammalian type C retroviruses, includes the murine leukaemia virus-related viruses and the feline leukaemia virus group (Pringle, 1999).

The genus *Deltaretrovirus* contains the human and simian T-lymphotropic viruses together with bovine leukaemia virus, based on their genomic organisation. These viruses transform lymphocytes readily in an integration-site independent manner, causing T and B cell leukaemias (Pringle, 1999).

The genus *Epsilonretrovirus* comprises a group of fish retroviruses (Pringle, 1999).

The genus *Spumaretrovirus,* the foamy virus group (Delelis *et al*., 2004), originally consisted of a small number of isolates present in cell cultures derived from mammalian tissues, but recently they have been isolated directly from a variety of mammalian sources including non-human primates and these may have been transmitted to humans (Jones-Engel *et al*., 2005). They are not known to be associated with any disease syndrome.

Endogenous retroviruses, or transmissible retrovirus-like agents, are not yet subclassified within the family *Retroviridae.* These retroelements comprise about 5% of the human genome and the genome of many other animal species. Most are replication deficient, having acquired many deletions since their integration into the germ-line genomes of their hosts. "Younger" endogenous retroviruses have fewer deletions and one human endogenous retrovirus (HERV-K113), estimated to have integrated less than 200,000 years ago, contain ORFs for all retroviral genes and are theoretically capable of producing viable virus (Bannert and Kurth, 2004). The endogenous retroviruses are currently the subject of much research effort, as there are suspicions they may be implicated in the pathogenesis of many human and animal diseases. Oncogenesis, schizophrenia and multiple sclerosis are possible deleterious results of ancient endogenisation of retroviruses; conversely, the presence of an endogenous retrovirus can protect against infection with its exogenous counterpart (Bannert and Kurth, 2004).

The lentiviruses.

This group of retroviruses are a morphologically and genetically distinct genus infecting a wide range of mammalian host species. The name 'lentivirus' was coined because the disease caused by the first member of the group to be identified was a very slowly progressive chronic viral pneumonia in sheep, first seen in Montana in

1923 (Dawson, 1980). The genus includes equine infectious anaemia virus (EIAV), ovine maedi-visna virus (MVV) and caprine arthritis-encephalitis virus (CAEV), feline immunodeficiency virus (FIV), 2 bovine lentiviruses (BIV and JDV) and the primate lentiviruses. The primate viruses include several simian immunodeficiency viruses (SIV) and the 2 human immunodeficiency viruses (HIV-1 and HIV-2) (Joag, 1996).

Organisation of the lentivirus genome.

All of the known lentiviruses possess a characteristic genome organisation, increasing in complexity from the most simple, EIAV, to the most complex, HIV-1. In each virion, there are 2 identical strands of ssRNA joined by a 5' hydrogen bond (Coffin, 1992a). These strands each consist of the 3 obligatory *gag, pol* and *env* ORF characteristic of all retroviruses, flanked at each end by long terminal repeats (LTR) containing enhancer and promoter regions as well as 3' RNA processing sequences. The ORF *gag, pol* and *env* each encode polyproteins that are post-translationally cleaved to give the structural and functional proteins of the virus. The ORF *gag* encodes a precursor polyprotein that is cleaved to give the 3 major non-glycosylated structural proteins MA, CA and nucleocapsid (NC), as well as p6 in HIV-1. The ORF *pol* encodes the viral protease (VP), polymerase (POL), IN and reverse transcriptase (RT). The ORF *env* encodes the surface glycoproteins SU and TM. In addition to these 3 main ORFs encoding the major proteins, which are expressed from singly spliced mRNA's, multiple splicing of transcripts can give rise to a range of accessory genes, not all present in all virus species, including *tat, rev, vif, vpr, vpu* and *tmx* (Figure 2.3), each coding for proteins of the same names, with various functions described in the section outlining the viral replication cycle. The regions of *env* coding for SU and TM are proposed to also encode the Rev response element (RRE). The ends of the RNA strand consist of U3, R and U5 regions together making up the LTR which exists functionally only in the proviral genome. This contains the TATA box, essential for eukaryotic promoter recognition, the primer binding site used for initiation of negative-strand synthesis and transcription termination motifs, other enhancer sequences, the NF-k binding site and the *trans*-

activation response site (TAR), the binding site for the *trans*-activating protein Tat (Chadwick *et al*., 1995b).

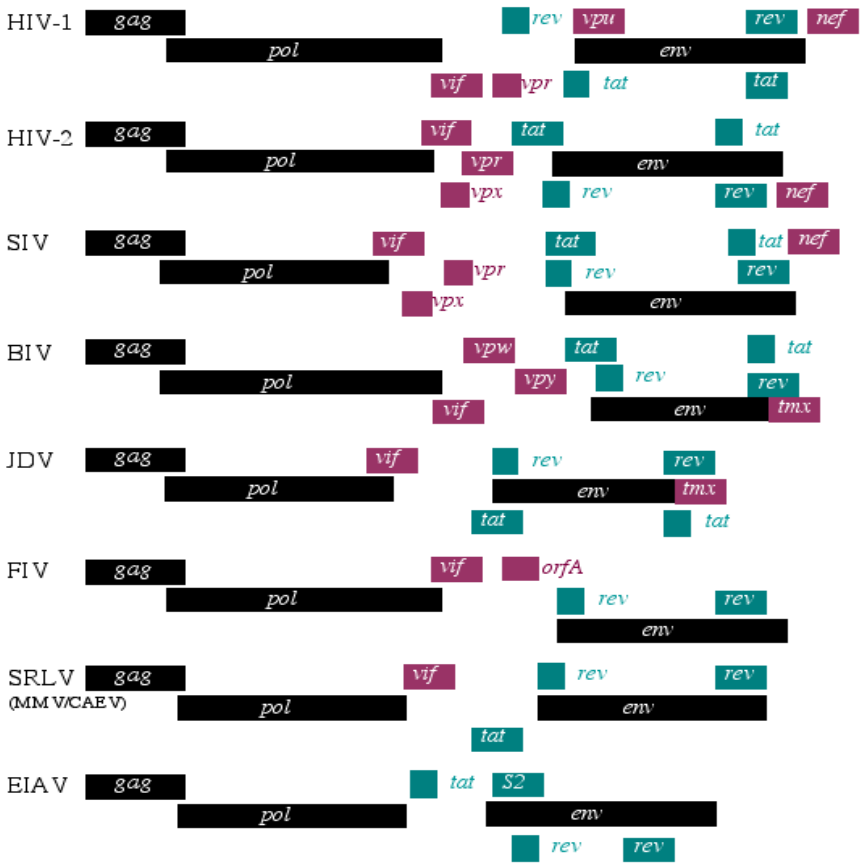


Figure 2.3 Schematic representation of the proviral genomic organisation of the lentiviruses: HIV-1, HIV-2 (Coffin, 1992b), SIV (Hayden *et al*., 1998), BIV (Gonda *et al*., 1994), JDV (Chadwick *et al*., 1995b), FIV(Zou *et al*., 1997), the small ruminant lentiviruses (CAEV and MMV) (Pyper *et al*., 1986; Harmache *et al*., 1995) and EIAV (Noiman *et al*., 1991). The ORF are depicted in 3 rows that are not indicative of the reading frame arrangement.

Morphology of the lentiviruses.

Figure 2.4 shows the typical morphology of a spherical encapsulated lentivirus particle, approximately 100 nm diameter. The envelope is derived from the lipid bilayer membrane of the host cell as the virus buds during the final maturation process. The surface projections consist of the *env*-encoded glycoproteins SU and

TM, formed into ‘spikes’ visible by electron microscopy. The viral membrane contains an eccentrically located bar-shaped nucleocapsid, distinct from the central spherical capsid seen in C-type retroviruses (Desrosier, 2001), surrounding the viral RNA and virion enzymes.

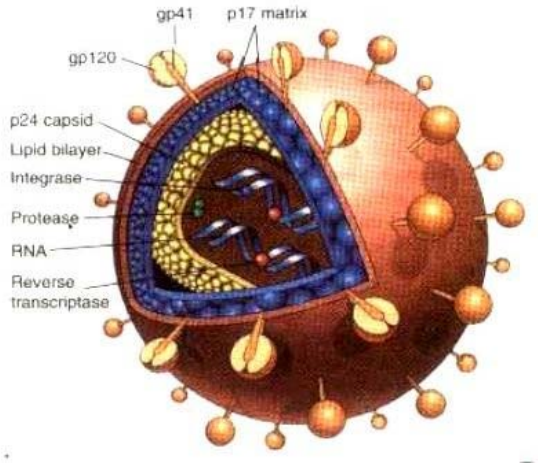


Figure 2.4 Schematic diagram of the structure of HIV, typical of other lentiviruses. The viruses are approximately 100 nm diameter and contains the inner core or capsid (CA) which may be bar or cone-shaped, within which is the 2 segments of the virus RNA genome and various virus-coded enzymes required for replication (IN, PR and RT). The capsid is surrounded by the matrix (MA) and an envelope derived from the membrane of the host cell as the virus matures by budding. Projecting from the envelope are 2 virus-encoded glycoproteins, SU and TM. Diagram reproduced from www.fsm.ac.fj (accessed 31/08/05).

Pathogenesis of lentivirus infections.

While there is an obvious similarity in the genome organisation of the 8 main genera, the pathology of the diseases caused by them varies markedly. The diseases they induce range from transient acute infections, chronic lymphoproliferative diseases associated with immunosuppression, chronic lymphoproliferative disease without immunosuppression but leading to organ failure, and an acute episodic disease with immunologically-mediated anaemia (Joag, 1996). The complex replication cycle of lentiviruses in target cells is in part responsible for the differing pathogenesis seen.

The primate lentiviruses HIV and SIV.

The type species of the genus *Lentivirus*, human immunodeficiency virus type 1 (HIV-1), infects $CD4^+$ lymphocytes leading to an immunosuppression and subsequent secondary infections. This virus has been the subject of an intense research effort since its discovery and identification as the causative agent of human acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Gallo and Wong-Staal, 1985). HIV-1 originated from a cross-species transmission from chimpanzees *Pan troglodytes troglodytes* of simian immunodeficiency virus (SIV_{cpz}) to humans on at least 3 or 4 separate occasions (Gao *et al*., 1999); AIDS is therefore, "a human infectious disease of zoonotic origin" (Apetrei *et al.*, 2004; Marx *et al.*, 2004; Keele *et al.*, 2006). Contaminated oral polio vaccines prepared in primate primary cell cultures were proposed as a possible route of infection of the virus to humans but this has since been refuted (Worobey *et al*., 2004) and contact with blood during slaughter of chimpanzees for food is thought to be the most likely source of the original transmission (Marx *et al*., 2004).

Another distinct retrovirus causing AIDS in humans, HIV-2, arose as a consequence of cross-species transmission from sooty mangabeys (*Cercocybus atys)* to humans of SIV_{sm} (Marx *et al*., 1991). These cross-species transmissions seem to occur relatively frequently between simian species as well as from simians to humans (Sharp *et al*., 2000) and analysis of the mutation rates of HIV-1 places the transmission of SIV_{cpz} to humans at approximately the middle of the 20^{th} century (Sharp *et al*., 2000). The human and simian retroviruses form the largest related group of retroviruses, and an indication of their variety and relatedness to each other and the non-primate lentiviruses is shown in Figure 2.5.

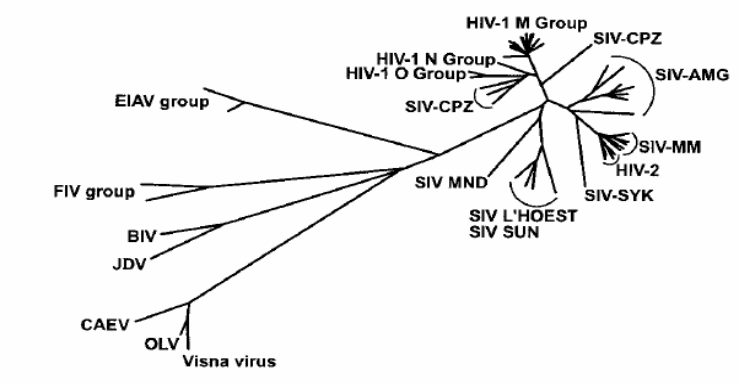


Figure 2.5 Phylogenetic relationship among representative primate and non-primate lentiviruses based on the sequence of gag genes. For HIV-1, 3 genetic groups (M, N and O) are shown. Strains of SIV have a subscript denoting the species from which they were isolated: chimpanzee (cpz), macaque (mm), African green monkey (agm), l'Hoest monkeys (lhoest), suntailed monkeys (sun), sykes monkeys (syk),and mandrills (mnd). Adapted from Lesnik (2002).

Transmission of HIV is caused mainly by transmission of cell-associated virus in semen during heterosexual sex, resulting in infection of dendritic cells in the mucous membrane, with alternative routes possible and including homosexual sex, blood borne transmission either through contaminated needles or contaminated blood products, and maternal transmission either during parturition or in breast milk (Pillay *et al*., 2000). The pathogenicity of the various human and simian retroviruses appears to be related to the length of time since the original infection of the host. In their natural hosts, SIV_{cpz} and SIV_{sm} are transmitted by both sexual means and via breast milk, and in these natural hosts they have adapted and produce asymptomatic infections (Emau *et al.*, 1991; Rey-Cuille *et al.*, 1998; Hirsch *et al.*, 1999). Cross-species transmission results in a range of outcomes, one of which is a conversion from an asymptomatic infection to a highly pathogenic infection that progresses to an AIDS-like state (Apetrei *et al*., 2004).

FIV.

The immunodeficiency resulting when humans are infected with HIV-1 or HIV-2, and when primates are infected with heterologous SIV isolates, is also seen when domestic cats are infected with heterologous members of the FIV group. 9 species of

Felidae including lions *(Panthera leo)* and puma *(Felis concolor)* and one member of the *Hyaenidae,* the spotted hyena, harbour FIV-like lentiviruses, mostly without any associated pathology, suggesting a commensalism analogous to that seen in primates infected with SIV. Cross-species transmission of these adapted viruses can result in disease that is similar to that seen after FIV infection of domestic cats (Troyer *et al*., 2005). The disease seen in domestic cats is very similar to that seen after human infection with HIV and therefore FIV infection of cats been used extensively as a non-primate animal model for the study of HIV infection in humans, especially for the study of vaccine strategies (Hosie *et al.*, 1995; Hosie *et al.*, 2000). FIV is spread horizontally by biting, and also vertically *in utero,* and to kittens while suckling, although sexual transmission has been demonstrated (Jordan *et al*., 1998).

The diseases induced by HIV-1 and HIV-2, the SIVs and FIV have many similarities. Initial infection and dissemination of the virus throughout the body is followed by an acute flu-like disease, sometimes with a range of other symptoms such as oral candidiasis, anorexia and neurological features, associated with high levels of virus replication (Vanhems *et al*., 1999). These high levels of virus replication then decrease to much lower levels (but are not eliminated entirely) initially by non-specific and later by specific host immune responses (D'Souza and Mathieson, 1996). The ability of the immune system to reduce virus load varies from individual to individual, and the level of the "set-point" (the steady state viral load attained after the initial clinical disease) is strongly correlated with the rate of progression to immune collapse and the onset of AIDS (Blattner *et al*., 2004). The incubation period prior to development of acute disease, as well as the duration of acute disease, is also prognostic of the rate of progression to disease, with longer incubation periods and a longer duration of the acute disease significantly correlated with delayed progression (Vanhems *et al*., 2000).

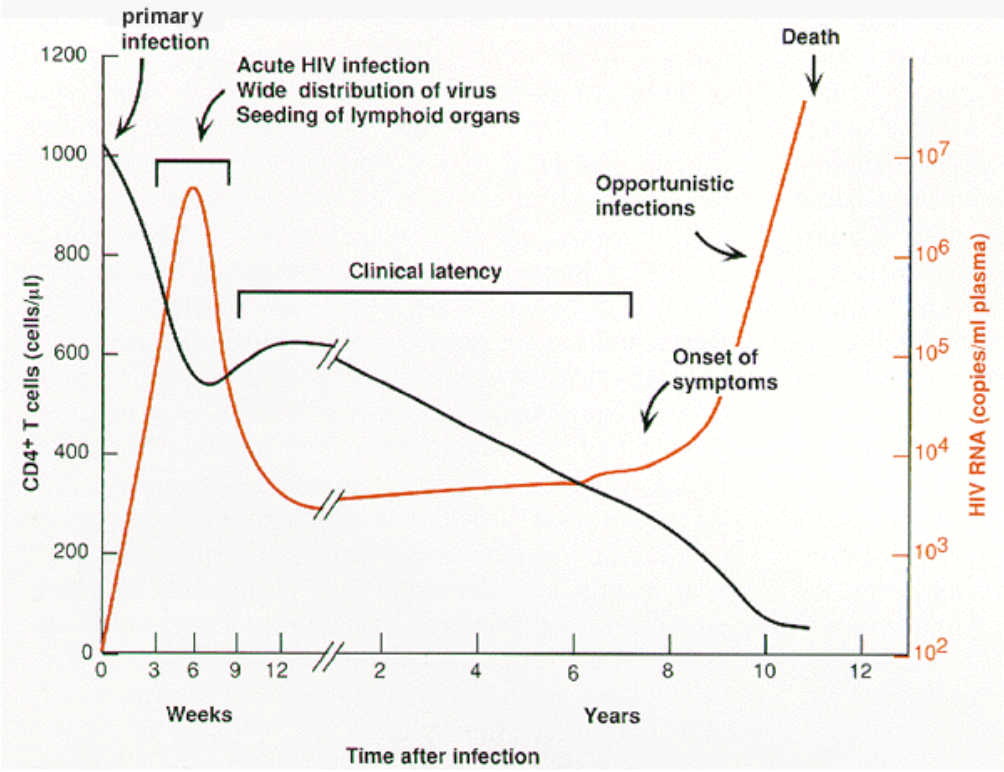


Figure 2.6 Infection time course of a typical HIV-1 infection, the best characterised lentivirus infection. Soon after primary infection an acute disease process occurs that is associated with flu-like symptoms, a transient high plasma virus load and a decrease in $CD4^+$ cells. This is followed by a long symptom-free period, with a progressive slow decrease in $CD4^+$ cells and eventual further increase in virus load leading to the onset of immunosuppression and opportunistic secondary infections (AIDS). Adapted from Weiss (1993).

The time course of a typical HIV-1 infection in humans is illustrated in Figure 2.6. After the initial acute disease process, a symptom-free period follows that can range in duration from a few weeks in SIV_{smm}PBJ14 infections of non-human primates to many years in HIV infection of humans. The lack of progression to AIDS in monkeys naturally infected with homologous SIV strains may be simply due to the clinically latent period exceeding the lifespan of the animal, as AIDS has recently been seen in a naturally infected sooty mangabey kept in captivity for 18 years (Ling *et al*., 2004). The initial acute phase of the disease is characterised by a transient depletion of circulating immune cells and very high plasma virus loads, with up to 10^9 virus particles/ml of plasma. Even in the asymptomatic period, the virus is not latent but is continually produced from newly infected cells. The virus load during

HIV-1 infections may slowly increase by about 0.1 $\log_{10}$/year until symptoms of AIDS appear (Sabin *et al*., 2000).

The increase in levels of virus replication during the later phase of infection is characterised by the development of AIDS, the onset of multiple opportunistic infections due to destruction of the $CD4^+$ T-cell population. Other effects include central nervous system effects such as dementia due to aberrant signalling from infected macrophages, and the wasting associated particularly with HIV-2 infections in Africa. Switching of receptor utilisation from CCR5 to CXCR4 late in the course of the infection hastens the onset of AIDS as these strains are more virulent (Weiss, 2000).

EIAV.

In contrast to the progressive immunodeficiencies caused by the lymphotropic retroviruses, the macrophage-tropic members of the family cause diseases with a different pathogenesis. EIAV, the only equine lentivirus, was the first non-plant virus to be discovered (Vallee, 1904) and was only characterised about 30 years ago (Charman *et al*., 1976). It causes a contagious disease in horses and mules and is capable of infecting donkeys without causing clinical disease. Initial infection is associated with an acute disease, characterised by anorexia and depression and a very high fever, which can be fatal (Dawson, 1988). In animals that survive this acute disease, this is followed by a persistent episodic anaemia, as erythrocytes become coated in viral components, leading to haemolysis and phagocytosis (Cheevers and McGuire, 1985; Sentsui and Kono, 1987). Central nervous system involvement is only occasionally seen (Oaks *et al*., 2004). The periodic clinical relapses, of a couple of days duration, are initially seen every 1-2 weeks but the intervals between relapses progressively lengthen until complete remission is attained. During the relapses, body temperature is elevated, there is anaemia and oedema of the abdomen, and an increase in the viral load in plasma, which has to reach a 'pathogenic threshold' to cause disease (Leroux *et al.*, 2004). Remission is a result of the establishment of immune control, involving both arms of the immune system, which reduces the rate of viral replication below the pathogenic threshold.

However, virus persists within macrophages in tissue sites (Harrold *et al*., 2000) and recrudescence of disease can be induced by administration of immunosuppressive drugs throughout the animals life (Leroux *et al.*, 2004). Transmission is by 3 methods: by biting flies that are able to mechanically transmit the infection; by vertical transmission to foals from infected dams; by prolonged contact with infected animals, either clinically affected or asymptomatic carriers, even in fly-free conditions (Dawson, 1988).

The small ruminant lentiviruses, MVV and CAEV.

MVV causes 2 conditions in sheep, a slowly progressive chronic pneumonia and a neurological disease. The respiratory form of the disease (maedi) is caused by the same virus associated with the paralytic form of the disease referred to as 'visna' (Cutlip *et al*., 1988). MVV is an exogenous retrovirus that can infect both sheep and goats (Shah *et al*., 2004) and is closely related to CAEV causing caprine arthritis and encephalitis in goats, and the 2 viruses are sometimes grouped together and referred to as small ruminant lentiviruses (SRLV) (Leroux *et al*., 1997). The 2 clinical forms of the disease, maedi and visna, occur in sheep over 3 years of age, and mastitis and arthritis are also seen occasionally (Dawson, 1987). Transmission is via nasal secretions when sheep are penned together for long periods, as is the case in Icelandic winter, and transmission from infected dam to lamb during suckling is also common (De Boer *et al.*, 1979; Preziuso *et al.*, 2004). In goats infected with CAEV, the arthritis occasionally seen in MVV-infected sheep is the dominant clinical manifestation in mature goats, with pneumonia and mastitis also manifest in many infected animals. The lesions are slowly progressive and can persist for several years. Transmission is through kids drinking milk from infected females, and spread from sheep to goats and *vice versa* of a subtype of the SRLV has also been demonstrated recently (Shah *et al*., 2004). The lesions are associated with viral infection of monocytes and macrophages.

The bovine lentiviruses, BIV and JDV.

BIV was originally isolated in the early 1970's in the USA from a cow with persistent lymphocytosis, emaciation and weakness (Van der Maaten *et al*., 1972).

Little research effort was expended on this virus until the emergence of HIV/AIDS in the human population, and the resultant upsurge in interest in all lentiviruses, when the nature of the virus was revealed by molecular genetics and antigenic studies (Gonda *et al*., 1987). Serological studies have indicated this virus or an antigenically related virus is widespread in the cattle population in many countries and while it has been reported in association with secondary disease due to immunosuppression, lesions of the legs and feet and CNS involvement presenting as 'dullness and stupor'(Snider *et al*., 1997), its association with these disease syndromes is unclear. The virus is strongly cell-associated, and has been identified in milk, suggesting a lactogenic pathway of transmission (Nash *et al*., 1995), as well as infection *in utero* (Moody *et al*., 2002)

JDV was classified as a lentivirus on the basis of its close genetic relationship to BIV even though the nature of the disease associated with JDV is very different to that detected in association with BIV (Chadwick *et al*., 1995a). Although the nature of the disease process, an acute disease process after a short incubation period with no recurrence of disease in those animals that survive the acute disease, is very different to that associated with most other lentivirus infections, there are similarities between the JDV-associated disease and the acute transient disease process observed soon after infection by some other lentiviruses.

Based on the finding of Bali cattle in Indonesia sero-positive to JDV in areas where JD is not present, an endemic infection of *Bos javanicus* with BIV or another antigenically-related non-pathogenic bovine lentivirus has been postulated (Hartaningsih, personal communication).

Replication of the lentiviruses.

A schematic illustration of the various steps involved in the replication of lentiviruses is shown in Figure 2.7. The cell surface receptors used by lentiviruses include molecules such as heparan sulphate (Nisole and Saib, 2004), and in the case of macrophage tropic (M-tropic) HIV-1, the macrophage mannose receptor via mannosylated resides on SU (Nguyen and Hildreth, 2003). In HIV-1 infections, the surface glycoprotein complex of SU (gp120) and TM (gp41) of which there are

about 100/virion, mediates entry of the virus into the cell via their specific interaction with cell receptors and co-receptors. SU initiates viral entry by specifically binding to cell surface receptors (CD4 in the case of HIV) and co-receptors CXCR4 (used by T-tropic HIV isolates) and CCR5 (used by M-tropic HIV isolates). Other strains of HIV have been found to utilise CCR3, CCR2b, Bonzo/STRL33 and BOB/GPR15 as co-receptors (Frankel and Young, 1998). SIV isolates utilise the same array of receptors and co-receptors as HIV (Muller-Trutwin *et al.*, 2000; Owen *et al.*, 2000) and it has been postulated that similarities in the amino acid profile of the C2-V3-C3 region of the SU protein to that of the chemokines whose receptors these viruses utilise indicates their ancestral acquisition of host chemokine genes (Shimizu and Gojobori, 2000). The identity of the receptor for the SRLV's, which are capable of infecting macrophages and dendritic cells *in vivo,* is not known. Icelandic and UK strains of MVV can infect a wide range of cells *in vitro*, while North American isolates are restricted to ruminant cell lines (Hotzel and Cheevers, 2002b) as is CAEV (Hotzel and Cheevers, 2001; Hotzel and Cheevers, 2002a).

EIAV has been shown recently to utilise a single molecule, related to the tumour necrosis factor receptor, to enter permissive host cells of the monocyte-macrophage lineage, without a requirement for co-receptors (Zhang *et al*., 2005).

FIV, despite its tropism for $CD4^+$ T-cells early in infection, does not use CD4 as a receptor, although it does use CXCR4 as a co-receptor. Instead, the receptor has been identified as CD134, which is up-regulated in activated $CD4^+$ T-cells cells (de Parseval *et al.*, 2004; Shimojima *et al.*, 2004).

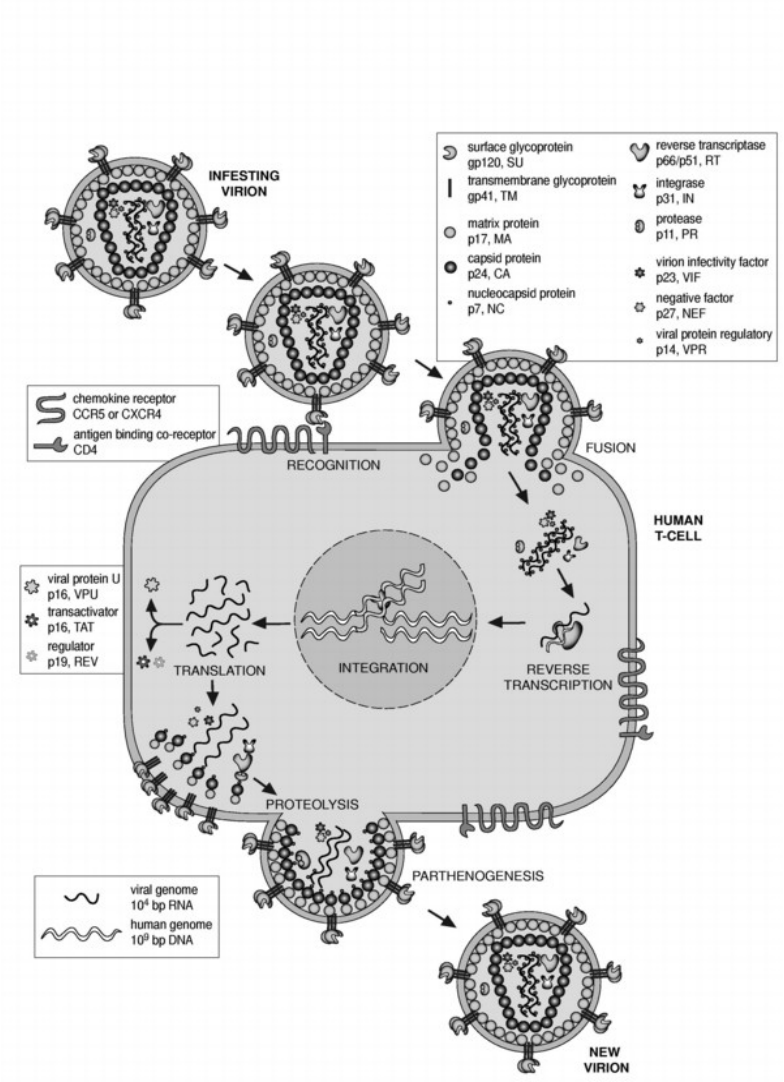


Figure 2.7 Steps in the replication of a typical lentivirus. Reproduced from www.molmo.be (accessed 28/07/06). Initial attachment via the viral envelope glycoprotein SU to cell surface receptors is followed by fusion of the viral envelope with the plasma membrane of the cell, involving TM, then uncoating of the virus particle, reverse transcription of the viral ssRNA genome to a dsDNA intermediate or provirus, integration of this provirus into one or more sites in the cellular genome, transcription and translational events involving the proviral DNA to produce virus proteins, followed by assembly involving budding from the plasma membrane and final maturation of the virus particle after release.

The receptor for BIV is not known but there are reports this virus is pantropic, infecting T-cells and B-cells as well as cells of monocyte-macrophage lineage (Whetstone *et al.*, 1997; Heaton *et al.*, 1998). This is in contrast to the tropism for monocyte/macrophage cell populations exhibited by the other ungulate lentiviruses.

The target cell and receptor for JDV are also not known, although a reported decrease in the ratio of bovine $CD4^+/CD8^+$ cells in the lymph nodes of Bali cattle infected with JDV (Dharma *et al*., 1994) suggests that the virus exhibits tropism for at least T-cells, again in contrast to the other ungulate lentiviruses. The close relationship of BIV and JDV may be reflected in a similar pantropic array of target cell types for JDV.

After the initial interaction of SU with its specific receptor and co-receptors, conformational changes bring TM into contact with the cell surface, initiating fusion of the viral envelope with that of the target cell. Fusion is thought to be initiated by an N-terminal hydrophobic peptide, with a transmembrane core region also important for fusion as well as anchoring the Env complex into the viral particle (Frankel & Young, 1998). The main feature of the TM molecule is a highly conserved region, bounded by 2 cysteine residues, that is highly immunogenic and termed the Principal Immunodominant Domain (PID). The TM monomer of JDV is slightly bigger than that of HIV-1, consisting of 359 amino acids with a molecular weight of 41.1 kDa (Chadwick *et al*., 1995b).

Following fusion of the viral particle with the host cell membrane, and as depicted in Figure 2.8, the viral core enters the cytoplasm and is disassembled or "uncoated" to create sub-viral particles termed reverse transcription complexes (RTC) and pre-integration complexes (PIC). PICs contain the viral enzymes present in the viral particle, the accessory proteins Nef and Vif, and the host protein cyclophorin A (Nisole and Saib, 2004). Reverse transcription of the viral genome occurs during this phase, as the complex moves towards the nucleus. A $tRNA^{Lys}$ packaged into the virion acts as a primer for transcription initiation, binding to the site in the U3 region of the genome (Le Grice, 2003). Transcription proceeds to the 5' end of the genome, producing what is known as the strong-stop DNA (Huthoff and Berkhout, 2001). Continued synthesis of the negative-sense ssDNA utilises this strong-stop DNA as a primer for reverse transcription of the second strand of viral RNA from the 3'end. Degradation of the viral RNA occurs as the ssDNA is produced, except for the polypurine tract at the 5' end of the U3 region(Roda *et al*., 2003), which acts as a primer for synthesis of the positive-sense DNA strand also by the reverse

transcriptase. The error-prone nature of the RT (about 3.4 x 10^{-5} errors/bp/cycle) (Mansky and Temin, 1995) is responsible for the high mutation rate characteristic of the lentivirus family.

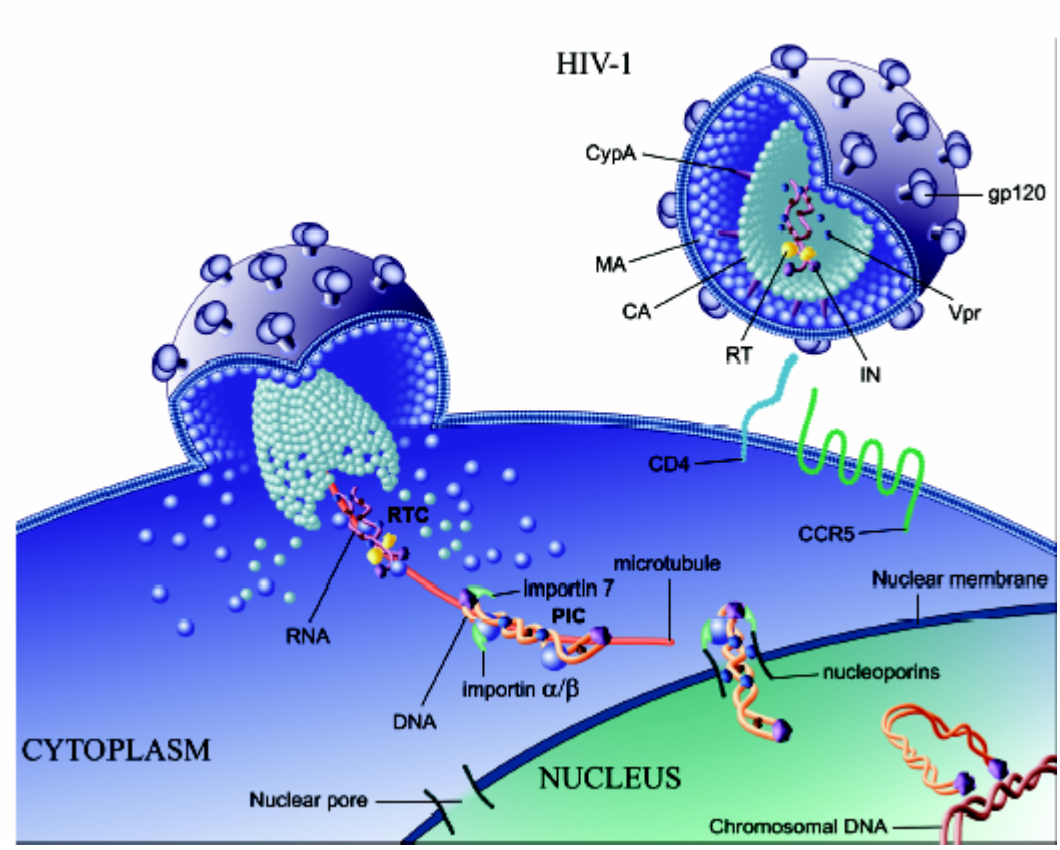


Figure 2.8 Steps involved in the replication of HIV until integration of the dsDNA intermediate provirus into the cellular genome. Further description of the events involved in provided in the text. Reproduced from Bukrinsky (2004).

The movement of the RTC through the viscous cytoplasm appears to be via interaction with the cytoskeleton of the cell, although the mechanics of the process are still unclear (Bukrinskaya *et al*., 1998). Phosphorylation of MA by virion-associated mitogen-activated protein kinase (MAPK) has been proposed as a possible targeting mechanism (Tang *et al*., 1999). The ability of the lentiviral PIC to enter the intact nucleus, rather than waiting for nuclear membrane breakdown as in the case of other retroviruses, confers the characteristic ability of lentiviruses to infect terminally differentiated non-dividing cells (Weinberg *et al*., 1991).

The entry of the PIC into the nucleus is probably reliant on the host cell nuclear import proteins. The viral proteins MA, IN and Vpr are proposed to interact with the host nuclear transport machinery. MA is found in close proximity to the PIC as well as in the extra-capsid layer, and in HIV-1 it carries 2 nuclear localisation signals (Dingwall and Laskey, 1991). The IN is karyophilic and interacts with importins presenting a possible route for the PIC to enter the nucleus via the nuclear pores (Bukrinsky, 2004). The role of Vpr in the entry of the PIC of HIV-1 into the nucleus is unclear; it is not strictly required, as viruses lacking Vpr can still replicate but with reduced efficiency in macrophages (Heinzinger *et al*., 1994).

Integration of viral dsDNA into the host chromosome ensures the survival of the virus, as it can then be transmitted from cell to cell as a provirus in the normal course of cell division. Integration requires the viral IN: initially, 2 3' nucleotides are removed from the linear viral DNA, leaving overhanging ends, which are then covalently joined to the 5' ends of the target DNA. The integration is completed as unpaired nucleotides are removed form the viral 5' ends, and these are joined to the target 3' ends (Frankel and Young, 1998). Integration can be at many sites within the genome (Shibagaki and Chow, 1997) but in HIV infection, mapping of over 500 integration sites has revealed a preference for genes actively transcribed by RNA Pol II (Schroder *et al*., 2002), thus ensuring transcription of viral genes. The process of integration is complex, requiring interaction of the viral integrase and DNA with numerous host proteins and culminating in the permanent integration of the viral genome into that of the host, where it resides ready for eukaryotic expression of viral genes (Nisole and Saib, 2004).

Within 30 min of infection of T-cells with HIV, changes in the expression levels of an array of host genes are seen, with about 8% being upregulated and 9% being downregulated. Integration into the upregulated genes is favoured (Schroder *et al*., 2002). The changes in expression levels occur before integration can have taken place, and several routes of activation, such as signalling post-CD4 binding events (Davis *et al*., 1997) or Nef-related activation of signalling pathways (Simmons *et al*., 2001) are postulated. The result is to engender an environment conducive to viral replication.

Initial transcription is controlled by constitutive host factors, including activation of the NF-κβ family of transcription factors involved in the response of the cell to infection, which interact with the LTR region of the proviral genome (Cullen, 1991). Early events in transcription produce multiply spliced mRNAs, encoding the regulatory proteins Tat and Rev, Nef and S2 (Saltarelli *et al*., 1996). Of these, Tat is responsible for greatly enhancing the synthesis of viral RNA through interaction with the transactivation response (TAR) element in the LTR region, increasing the processivity of the transcribing polymerases (Frankel and Young, 1998). Further regulation is provided by Rev. The default pathway is for the production of multiply spliced transcripts, and to overcome this, Rev binds to the RRE enabling the transport to the cytoplasm of unspliced mRNA transcripts coding for the viral structural proteins and glycoproteins, thus committing the virus to the final cytopathic stage of the replication cycle (Cullen, 1991). The RRE in HIV-1 is in *env* and of 2 putative RRE in JDV, one is postulated to consist of bases 6399-6590, lying across the junction between *su* and *tm* in *env,* while the other lies entirely within *tm,* spanning bases 7015-7423 (Lesnik *et al*., 2002). Nef is capable of downregulating CD4, and may thus aid release of the Env polyprotein from the endoplasmic reticulum, where newly synthesised molecules held by interaction with CD4 (Frankel and Young, 1998) allow incorporation of SU and TM into the cell membrane and aid in budding by removing CD4 from the cell surface (Weiss, 2000). This may also decrease subsequent entry of further viruses into a previously infected cell.

The translation of the lentiviral structural genes within the cytoplasm is accompanied by translation of a range of accessory genes that are more numerous in the complex primate lentiviruses and they aid in assembly and to confer infectivity and pathogenicity to the assembled particle. In the primate lentiviruses, these genes include *vif, vpr, vpu* in HIV-1, *vpx* in HIV-2 (Weiss, 2000) and in BIV they are *vpw* and *vpy*. JDV possesses *vif* only, which is likely to aid packaging of NC during assembly.

Assembly of the new virus is directed by the Gag protein and its interaction with a network of cellular proteins (von Schwedler *et al*., 2003). Gag is produced as a

polyprotein and cleaved by viral PR. The cleavage products are MA which is present between the viral membrane and the capsid in the assembled virion, CA that forms the core containing the RNA and NC which coats the RNA (Tritel and Resh, 2000). The p6 protein in HIV is responsible for controlling the size of the assembled particle (Garnier *et al.*, 1998; Garnier *et al.*, 1999) and for incorporation of Vpr during assembly of HIV-1 (Frankel and Young, 1998). Vpr is involved in cell-cycle arrest (Weiss, 2000). The assembly of the virus is an ordered process, with membrane-bound Gag, mediated by an N-terminal motif, progressively undergoing multimerisation under the plasma membrane, which contains SU and TM at this stage. Some host proteins are thought to be excluded, and viral proteins are requisitioned, as the new viral particle buds from the membrane (Tritel and Resh, 2000). As stated above, the retroviruses were originally classified according to the type of budding utilised, and JDV utilises type C budding (Kertayadnya *et al.*, 1993). In T-lymphocytes, the production of virus usually results in cell death, whereas in macrophages viral production can continue at a lower level for long periods (Weiss, 2000).

Natural and immune resistance to lentiviral infections.

The analysis of the protective immune response to infection provides an approach to rational vaccine design but the paucity of examples of natural immunity to HIV infection largely precludes this approach to identification of the biological correlates of protection. Though rare, examples of resistance to infection occur in some people (exposed-uninfected) who were exposed to the virus, often repeatedly, notably sex-workers, sexual partners of infected individuals, babies born to infected mothers, intravenous drug users, and haemophiliacs. Of interest in the search for immune correlates are people who are infected but in whom disease does not develop or its progression is slow, termed long-term non-progressors (Marmor *et al*., 2006). Natural resistance can be genetic, resulting from mutations in the viral receptor genes, differences in the MHC and HLA allotypes between infected and uninfected individuals (Trachtenberg *et al*., 2003) or differences in the regulation of cytokine production. Co-infection with the hepatitis C virus-related GB virus-C was linked to

long-term non-progression of HIV-1 infections in intravenous drug users [reviewed by (Marmor *et al*., 2006)]. Adaptive immunity-mediated resistance to infection in this group is rare although CTL responses may be important in the resistance to HIV infection of sex-workers in Kenya (Rowland-Jones *et al*., 1998) and in health care workers that do not develop infection after exposure to body fluids from infected individuals (Pinto *et al*., 1995). However, HIV-1 specific CTL were not deemed responsible for resistance to infection in one study of apparently resistant homosexual men (Koning *et al*., 2004).

High levels of mutation are responsible for lack of natural immunity.

The lack of examples of natural immunity highlights the problem of designing vaccines to engender immunity to lentiviruses, due in part to the extreme variability of the lentiviral genome. Lentiviruses lack an RT proofreading mechanism and so have a very high rate of spontaneous introduction of errors into their genome. Multiple insertions of viral genomes into a single cell also gives rise to heterozygous virus (Jung *et al*., 2002). Insertions, duplications, deletions and recombinations of genetic material all contribute to heterogeneity of the population. During HIV-1 infection, the high mutation rate ranging from 0.2-2 mutations per genome per cycle and the very high replication rate results in the generation of ~10^4-10^8 mutant virus particles/day in chronically infected individuals (Peyerl *et al*., 2004). This, combined with good tolerance of changes arising from the continual mutations, means that while any new infection probably arises from a fairly homogenous viral inoculum, the intrahost population quickly becomes a swarm of quasispecies (Saag *et al.*, 1988; Goodenow *et al.*, 1989). Under selection pressure from the immune system, the stochastic nature of the process of mutation gives rise, over periods of time calculated to be up to several years (Liu *et al*., 2006a), to new variants with mutations in epitopes previously recognised by both arms of the immune system, allowing escape from immune surveillance. These ‘escape mutants’ outgrow the original population under immune control. Escape from the CTL response is due to mutations in viral epitopes recognised by T-cell receptors in the context of MHC

class 1 presentation (Phillips *et al*., 1991), or in the initial infection by exhaustion of clonotypes specific for viral epitopes (Pantaleo *et al*., 1997b). The selection pressure exerted by the immune system on the intra-host viral population means that immunocompetent individuals tend to have a heterogeneous viral burden. In progressors, the constant attack on the immune defences leads eventually to immune collapse and as a result of the absence of continued selection pressure the viral population then tends to become more homogenous (Lukashov, 2001). The production of mutants by selection pressure can, however, be at a cost to the fitness of the virus and the mutations do not necessarily become fixed in the general viral population if the pressure is removed (Barouch and Letvin, 2004).

The early cell-mediated immune response to lentiviral infections.

The initial burst of viral replication in HIV-1 infection of humans is controlled by an oligoclonal expansion of virus-specific $CD8^+$ T-cells with a restricted pattern of variable β-chain T-cell receptor usage (Pantaleo *et al*., 1997a). The T-cell receptor repertoire of the HIV-specific CTL decreases rapidly due to clonal exhaustion, contributing to an inability of the immune system to control the replication and spread of the virus (Pantaleo *et al*., 1997b). The specificity of any T-cell response is closely correlated to the rates of disease progression and $CD8^+$ populations possessing restricted repertoires of TCR are more rapidly depleted in infected individuals than in those possessing wider repertoires, leading to more rapid progression of disease (Pantaleo *et al*., 1997b). The frequency of HIV-specific memory T-cells is also closely correlated to progression of disease (Musey *et al*., 1997). Virus control in horses infected with EIAV is similarly dependent on the induction of specific CTL following infection (McGuire *et al*., 2004) and depletion of $CD8^+$ lymphocytes was shown to interfere with the ability of macaques to clear primary infections with SHIV, a chimeric SIV-HIV (Matano *et al*., 1998), and lead to a rise in plasma viral load (Jin *et al*., 1999). In contrast, control of replication during primary MVV infection of sheep is not dependent on $CD8^+$ lymphocytes (Eriksson *et al*., 1999).

Control of viral replication is dependent on the quality of the CTL response, with the recognition that the response to viral core proteins is associated with slower disease progression in HIV-infected individuals (Klein *et al*., 1995). Constraints on the ability of different MHC class 1 alleles to bind viral epitopes, and mutation of viral CTL recognition epitopes giving rise to escape mutants, provides another method for virus to evade immune control (Phillips *et al.*, 1991; Borrow *et al.*, 1997). Soluble factors are secreted by $CD8^+$ T-cells, including an antiviral factor that acts at the level of viral transcription and associated with non-progression during HIV infection (Copeland *et al*., 1995); a range of cytokines are also secreted that are natural ligands for CCR5, and interfere with viral entry (Zagury *et al*., 1998). A role for $CD4^+$ cells in abortive HIV infections has been proposed, following recognition of viral epitopes presented by MHC class II alleles and subsequent secretion of cytokines and cell proliferation (Clerici *et al*., 1994).

Humoral responses to lentivirus infections.

Neutralising antibodies are also important in controlling virus replication. In HIV-1 infection the neutralising antibody response is largely directed to epitopes on the highly variable V3 loop of the gp120. This variability allows escape mutants to evade humoral immune control and replicate for a period until the humoral immune system responds by producing further neutralising antibody (Albert *et al*., 1990). Cynomolgus monkeys infected with SIV_{sm} showed a strong correlation between the presence of a broad repertoire of neutralising antibodies and non-progression to an AIDS-like condition. In HIV-1 infection this is not universal amongst non-progressors, and it is possible that neutralising antibody is merely one method of control of an established HIV infection, or that the broad array of neutralising antibodies is simply a result of long-term infection whilst remaining immunocompetent (Cao *et al.*, 1995; Pantaleo *et al.*, 1995; Montefiori *et al.*, 1996). A pre-existing humoral immunity could be effective at preventing the establishment of an infection after natural challenge with virus; passive immunisation studies in chimpanzees and macaques have shown solid protection in immunised animals when subsequently challenged with HIV and SHIV, respectively (Pincus *et al.*, 1996;

Mascola *et al.*, 1999). However, passive administration of antibodies prophylactically is only effective if administered within a 6 h period prior to exposure (Nishimura *et al*., 2003).

Subversion of the immune system by lentivirus proteins.

In the more complex lentiviruses, several viral proteins are able to manipulate the immune system of the host. Apart from the effects on viral transcription seen in infected cells, soluble Tat is secreted into the milieu surrounding infected cells and it has the capacity to enter uninfected cells directly through the cell membrane in a non-receptor-mediated manner (Frankel and Pabo, 1988). Upregulation of CXCR4 on uninfected T-lymphocytes and macrophages, and CCR5 receptors in macrophages is mediated in a dose-dependent manner by soluble Tat (Huang *et al*., 1998), postulated to enhance the susceptibility to infection with cell-free virus. However, Tat has also been shown to sensitise uninfected cells to apoptosis, whilst protecting infected cells from apoptosis (McCloskey *et al*., 1997). Extracellular Tat has profound effects on B-cell differentiation, depending on the receptor used, and this leads to the follicular hyperplasia seen in the asymptomatic period of the infection (Lefevre *et al*., 1999). Effects on the regulation of cytokines TNF-β, IL-2 and IL-4 have been reported in transgenic mice (Garza *et al*., 1996). Maturation of monocyte-derived dendritic cells is induced by biologically active HIV-1 Tat and the associated up-regulation of MHC and IL-12, TNF-α and β-chemokines drive Th-1 responses, presumably providing more target cells for the infecting virus (Fanales-Belasio *et al*., 2002). In HIV infections, Nef has been shown to down-regulate CD4 and MHC Class 1 receptors on the surface of infected cells (Stoddart *et al*., 2003) affecting cytokine activity in Th-1 cells (Collette *et al*., 1997) and predisposing T-cells to undergo apoptosis (Zauli *et al*., 1999). Vpu is also associated with CD4 degradation in the Golgi apparatus, allowing Env to be transported to the viral surface (Willey *et al*., 1992). In the bovine lentiviruses, the Vpw and Vpy proteins of BIV are postulated to have similar functions to Vpu and Vpr in HIV-1 (Gonda *et al*., 1990), and the Tmx protein of JDV and BIV may possibly be a Nef-equivalent based on its location in the genome (Gonda, 1992).

Vaccination strategies for the control of lentivirus infections.

The World Health Organisation (WHO) has identified vaccination, or more specifically immunisation, as the most cost effective method of increasing health in the global population. 9 major human diseases have now been controlled by global vaccination programmes. Many vaccines have been introduced in the 2 centuries since Jenner's experiments with variolation as a means of control of smallpox, including rabies (1885), plague (1897), diphtheria (1923), pertussis (1926), tuberculosis and tetanus (1927), yellow fever (1935), polio (1955, 1962), measles (1964), mumps (1967), rubella (1970) and hepatitis B (1981).

However; new diseases arise constantly and have included Ebola virus, Lassa fever, Nipah virus and many others. In 1983, HIV was identified as the causative agent of AIDS. The subsequent global pandemic of AIDS has led, as of 2006, to an estimated 65 million cases worldwide and 25 million deaths (Merson, 2006). 18,000 new infections are estimated to occur every day. A vaccine is urgently required.

The approach to vaccination has in the past been largely empirical, with the exact mode of action of effective vaccines and the reasons for the failure of others, often unclear. In the veterinary field, this approach has led to vaccines for a wide variety of companion and production animal diseases (Carter and Carmichael, 2003). However, the 'molecular revolution' in genomics, proteomics and bioinformatics and the advances in the knowledge of the precise functioning of the immune system, have enabled the concept of rational vaccine design, where the administered antigen can be tailored to induce the appropriate immune response to a pathogen by targeting the correct pathway for antigen display depending on the route of administration. There is, additionally, now a wide choice of adjuvants to induce a favourable cytokine milieu for the type of response desired (Singh and O'Hagan, 1999).

Many approaches have been tried in attempts to produce effective vaccines against lentivirus infections. The research effort invested in the past 25 years to develop a vaccine to halt the world-wide increase in HIV infection and the subsequent

pandemic of AIDS has been unprecedented, and yet no safe and effective vaccine has been produced.

Effective vaccines for HIV and probably for all lentivirus infections need to stimulate both the humoral and cell-mediated arms of the immune system (Letvin *et al.*, 2002; McMichael and Hanke, 2003; Moore and Burton, 2004). Broadly neutralising repertoires of antibody combined with robust CTL responses to viral epitopes are required for effective control of viral replication, if not sterilising immunity, and numerous strategies attempting to elicit these responses have been employed.

Attenuated virus vaccines.

While some very effective attenuated whole virus vaccines have been developed against a range of animal pathogens, problems exist with the use of these vaccines. Attenuation can be achieved by the repeated passage of a pathogen in culture but although attenuated vaccines may be effective, there is a danger of reversion to virulent forms or the failure of the attenuation process to completely remove virulence. There are also problems associated with the occurrence of antigenic variant organisms that are not controlled by a single vaccine. Additionally, the use of inactivated pathogens as vaccines potentially poses a risk of incomplete inactivation, as seen with polio vaccines in the 1950s (Brown, 1993; Furesz, 2006), and presents difficulties of finding methods of vaccine production and inactivation that do not reduce antigenicity and efficacy (Katz and Webster, 1989; Sawyer *et al.*, 1994).

Attenuation of lentiviruses for use as vaccines has been attempted with some success. Cats vaccinated with FIV attenuated by 381 passages in cell culture were completely resistant to systemic challenge with a heterologous pathogenic strain of FIV and showed greatly decreased clinical signs when challenged with the same strain intravaginally (Pistello *et al*., 2003). A targeted approach to attenuation, where individual genes are modified or removed from a vaccine strain of virus, has proved effective in several trials. Creation of deletions in the *nef* gene of a pathogenic strain of SIV attenuated the virus such that it could only replicate to a low level in rhesus monkeys, in which the virus normally replicated well. Monkeys infected with this

nef deletion mutant were resistant to challenge by a pathogenic strain of SIV but reversion of the attenuated vaccine strain to a pathogenic strain eventually occurred, with development of AIDS-like symptoms in the inoculated rhesus monkeys (Stahl-Hennig *et al*., 1996). The restoration of virulence of the originally attenuated strain was shown to be by recombination between the vaccine strain and the virulent challenge strain, which occurred after a long period (Gundlach *et al.*, 1998; Khatissian *et al.*, 2001). In horses, complete protection against both a rigorous, artificially high challenge dose and a low challenge dose repeated several times designed to mimic natural transmission, was attained in horses vaccinated with an EIAV strain modified by deletion of the S2 accessory gene; this mutant strain replicated to low levels but caused no disease and the continuous low level exposure to virus resulted in an immune response that enabled the horses to maintain an apparent sterile immunity post-challenge with a virulent strain (Li *et al.*, 2003). Efficacy of the vaccine was dependent, however, on the degree of attenuation of the virus, and the level of maturation of the immune response (Craigo *et al*., 2005), which is a drawn-out process (Hammond *et al*., 1997).

As with other lentivirus diseases, the most effective vaccine against CAEV has proved to be modified attenuated virus, a *tat* deletion mutant. Although challenge with pathogenic CAEV did not cause severe disease, sterilising immunity was not achieved (Harmache *et al*., 1998). Partial protection against MVV in sheep has also been achieved by intratracheal vaccination with an attenuated molecular clone (Petursson *et al*., 2005).

A different approach to attenuation is to utilise recombinant viruses, where entire genes of a pathogenic strain are replaced with that from a non-pathogenic strain. When this was tried with the pathogenic SIV_{mac239}, different levels of attenuation were attained by replacing the SU region of the genome with that from a non-pathogenic strain ($SIV_{mac1a11}$) or conversely by inserting the SIV_{mac239} SU gene into the non-pathogenic $SIV_{mac1a11}$. The levels of attenuation were determined by the initial period of viraemia following inoculation of the viruses into macaques, which ranged from 4-6 weeks for the non-pathogenic strain, to 6-8 weeks for the mostly $SIV_{mac1a11}$ strain to 27 weeks for the strain consisting of mostly SIV_{mac239}. The initial

length of viraemia correlated well with the protection achieved and only those monkeys vaccinated with the least attenuated strain resisted infection on challenge (Lohman *et al*., 1994).

The existence of closely related viruses infecting members of the same family, for instance the many species of cats infected with FIV, presents the possibility of utilising infectious but non-pathogenic virus from a closely related host species as a vaccine. Cats infected with puma or lion strains of FIV develop a persistent low level, cell-associated viraemia, and when challenged with FIV develop a lower viral load (VandeWoude *et al.*, 2002; VandeWoude *et al.*, 2003). A conceptually similar approach has been taken with vaccination of horses with EIAV attenuated by passage in donkey leucocytes, which induced specific CTL responses and good protection against heterologous and homologous challenge (Zhang *et al*., 2006). This suggests that perhaps BIV could induce a protective immunity against the closely related JDV.

An approach to the problem of attenuating virus to a safe level while still retaining its ability to engender an effective immune response has been addressed by co-expression of interleukins in a *nef*-deficient strain used to infect macaques before challenge with SIV_{mac239}. All vaccinated animals were protected against productive infection in this way, although pathogenic virus could be detected (Gundlach *et al*., 1998). Attenuation of a SIV strain that already had a stop codon mutation in *nef*, $SIV_{mac239nefstop}$, by replacing the promoter/enhancer region with the immediate early promoter region of human cytomegalovirus produced a virus that only replicated to a very low level when inoculated into macaques; on challenge with a pathogenic strain of SIV viraemia was decreased 1,000-fold (Blancou *et al*., 2004).

Inactivated viral vaccines.

Early studies with inactivated whole FIV showed good protection against low challenge doses (Yamamoto *et al*., 1991). However, initial experiments showed no correlation between anti-Env neutralising antibodies and protection in cats vaccinated with inactivated FIV, in fact an enhancement of infection occurred (Hosie *et al*., 1992). Further studies, though, involving vaccination of kittens with

paraformaldehyde-inactivated cultures of FIV with a deletion of the endocytosis motif from the *env* gene gave strong antibody responses and a decrease in the peak viral load post-challenge (Hosie *et al*., 2005).

Inactivation of an infectious molecular clone of SIV provided a protective immune response when used as a vaccine but interestingly, individual proteins cloned and expressed from the same source did not (Mills *et al*., 1992).

The commercially available FIV vaccine (Fel-O-Vax®; Fort Dodge) has shown up to 84% protection against intramuscular challenge with heterologous virus in vaccinated kittens (Uhl *et al*., 2002). The importance of a realistic challenge has been shown by demonstrating protection in vaccinated animals housed with infected cats, as opposed to the failure of protection in animals challenged by more "robust" parenteral routes (Matteucci *et al*., 2000). An apparent enhancement of infection in some cats vaccinated with Fel-O-Vax® has been noted where higher levels of virus in the acute phase have led to wider dissemination of virus (Dunham, 2006).

The use of formaldehyde-inactivated SIV_{mac} as a vaccine gave short-term protection in macaques against both homologous and heterologous challenge with a low dose of cell-free virus; the resistance induced was transient and approximately half of the animals succumbed when rechallenged 3 months after the final vaccination (Putkonen *et al*., 1992). The level of protection attained with this vaccine was reduced when the monkeys were challenged with PBMC from an infected macaque (Heeney *et al*., 1992). The protection attained with inactivated SIV seems dependent on the source of the challenge strain of the virus, as monkeys vaccinated with human T-cell culture-derived SIV_{mac251} were resistant to challenge with homologous virus grown in human T-cell culture, but not to virus grown in cells from rhesus monkeys (Le Grand *et al*., 1992).

Inactivated EIAV coupled to iron oxide beads was used as a particulate vaccine designed to activate the phagocytic pathway to obtain a strong CTL response; delayed progression to disease post-challenge was seen, although a reliable CTL response was not obtained (Hammond *et al*., 1999).

An inactivated viral vaccine has been tried for control of JD in Indonesia; the spleen from an infected animal was emulsified in a mineral oil adjuvant, the virus was inactivated by detergent treatment. This vaccine has shown efficacy in reducing clinical signs in experimentally infected animals (Hartaningsih *et al*., 2001) and anecdotally has been reported to contain the spread of disease in natural outbreaks (Hartaningsih, personal communication).

Subunit vaccines.

The first recombinant protein was expressed in a bacterial system in 1979 (Goeddel *et al.*, 1979), utilising the discovery that bacteria harbour self-replicating plasmids. The discovery of restriction enzymes and ligases allowed plasmids to be cut at specific sites and foreign genes to be inserted. These 'recombinant' plasmids could then be taken up into their host bacterium, normally *Escherichia coli*, in a process termed 'transformation', with subsequent expression of the protein encoded by the foreign gene. The advent of this technology promised inexpensive methods of producing pure proteins in bioreactors.

Recombinant protein vaccines have several advantages over the use of whole pathogens as vaccines. The principal benefit is the removal of the possibility of inadvertently infecting the vaccinate with adventitious organism that may be present in inactivated or attenuated vaccines, or of reversion to virulence of attenuated virus vaccines. It can overcome problems in culturing sufficient whole virus for use as vaccine.

HIV-1 has been described as a molecular entity consisting of '15 proteins and an RNA' (Frankel and Young, 1998). The majority of these proteins, or their functional analogues, are expressed by the non-primate lentiviruses, and have been the subject of research into their suitability as subunit vaccines for the control of animal lentivirus diseases, mirroring the effort of the research community in the hunt for an effective vaccine against HIV.

Env glycoprotein vaccines.

The structural envelope glycoproteins SU and TM together mediate viral entry into the target cell (described above) and so have attracted interest as potential vaccine targets, with the aim of blocking receptor recognition and so preventing the initial entry of the virus into the cell.

Vaccination trials with recombinant envelope proteins expressed in bacterial and baculovirus systems have given partial protection against homologous challenge with FIV in cats (Leutenegger *et al*., 1998) and EIAV in horses (Issel *et al*., 1992). However, heterologous challenge with EIAV of horses in the same trial showed a degree of potentiation of disease. Goats vaccinated with recombinant CAEV glycoproteins raised high titres of anti-glycoprotein antibodies, but were not protected against challenge with homologous virus (Cheevers *et al*., 1994). There is no information available regarding the immune response to the bovine lentivirus Env glycoproteins but there is considerable variation in the sequence of BIV *env* (Suarez and Whetstone, 1995) due to duplication and insertion of a 33-54 nucleotide fragment by template switching during the synthesis of the DNA minus strand (Li and Carpenter, 2001). However, this variation has not been observed in JDV, which displays little sequence variation over time (Desport, 2007).

Clearance of virus after the initial acute phase of lentivirus infection is thought to be associated with an effective CTL response. Difficulty in raising effective CTL responses to recombinant glycoproteins has led to research designed to dissect the specificity of CMI responses during natural infections. Elucidation of the viral epitopes required to induce an effective response has led to the technique of peptide-based vaccination of animals. Horses vaccinated with Env peptides shown to be CTL epitopes *in vitro* demonstrated a measure of protection against challenge (McGuire *et al*., 2004). Analysis of the T-helper responses to Env peptides in macaques infected with SIV is being used to develop peptide vaccines to induce anti-SIV $CD4^+$ T-helper (Th) cell activity (Sarkar *et al*., 2002).

Increasingly, the necessity for specific CTL-mediated immunity to lentiviruses has led to the use of chimeric viruses or virus-like particles where genes for the vaccine

antigen are incorporated into non-pathogenic or replication-deficient virus, cultured exogenously as distinct from DNA vaccination with viral genomes, to allow realistic presentation of correctly post-transitionally modified viral proteins to the immune system. This approach has shown varying levels of success: vaccination of macaques with recombinant Ankara vaccinia virus expressing genes from SIV primed the animals to produce a rapid neutralising antibody response following infection (Ourmanov *et al*., 2000). Addition of a baculovirus-expressed Env boost to this approach was also effective (Hu *et al*., 1992), on the other hand, vaccination of cats with Venezuelan equine encephalitis virus engineered to express FIV Env failed to confer any protection to subsequent challenge despite high levels of antibody to FIV (Burkhard *et al*., 2002).

Gag protein vaccines.

The Gag polyprotein is cleaved by viral protease to produce the structural proteins CA, MA and NC. These structural proteins show good conservation throughout the lentiviruses, and so are of interest as potential vaccines to induce broad immunity to a wide range of heterologous isolates. Of these proteins, CA is highly immunogenic, inducing an early and strong antibody response when administered as a vaccine and in animals infected with a lentivirus, including the bovine lentiviruses (Whetstone *et al.*, 1990; Burkala *et al.*, 1998). Both cell-mediated and antibody responses to CA may be important for control of natural lentivirus infection, as horses infected with EIAV possess Th cells responsive to an array of peptides containing both CTL and Th epitopes derived from EIAV CA, with multiple epitopes clustering in the major homology region (MHR) which is highly conserved throughout the animal lentiviruses (Chong *et al.*, 1991; Lonning *et al.*, 1999).

MA is important during viral assembly (Frankel and Young, 1998) and MA from HIV-1 has been shown to stimulate pro-inflammatory cytokine production, possibly creating a favourable milieu for viral replication. The stimulation is receptor-mediated and presents the opportunity of blocking the interaction with specific antibodies raised by vaccination with MA (De Francesco *et al*., 2002). Horses infected with EIAV also possess MA peptide-specific CTL responses (Lonning *et*

al., 1999), raising the possibility of inducing specific CTL against EIAV by vaccination with peptides derived from this protein.

The Gag proteins have been employed as candidate vaccine antigens for both HIV and the animal lentiviruses either as recombinant proteins expressed in bacterial or eukaryotic systems (Hanke *et al.*, 1994; Rafnar *et al.*, 1998; Leavell *et al.*, 2005) or as artificial peptides (De Francesco *et al.*, 2002; Fraser *et al.*, 2005) either singly, or in combination with others (McGuire *et al*., 2004). Many studies have attempted to induce specific CTL responses by incorporation of Gag proteins into chimeric virus-like particles, mimicking the particulate antigen properties of intact viral capsids (Wong and Siliciano, 2005) or utilising the self assembly properties of Gag to produce virus-like particles either designed to induce response to Gag itself or to other antigens included in the construct (Kang *et al*., 1999).

The sub-unit vaccine trials carried out with Gag-derived antigens against lentiviral infections have shown varying degrees of success. Preliminary trials with peptide antigens failed to induce protective immunity in horses (Fraser *et al*., 2005) despite attempts to circumvent the restriction of antigen presentation by Class 1 equine leucocyte alloantigen by administration of multiple peptides to induce an effective CTL response (Zhang *et al*., 1998). Cats vaccinated with HIV derived p24 were protected against FIV challenge by CTL-mediated immunity, indicating cross-reactivity of the response to CA in these animals (Coleman *et al*., 2005).

Tat protein vaccines.

There are several reasons to target Tat as a potential vaccine. Tat is multifunctional, it is produced early in the virus replication cycle and it is indispensable for virus replication (Arya *et al.*, 1985; Dayton *et al.*, 1986; Frankel and Young, 1998). The lentivirus regulatory genes tend to be more conserved than the structural proteins, and the resulting antigenic conservation of Tat reduces the lack of cross-reaction common with antisera to structural and envelope proteins (Demirhan *et al*., 1999). The protein is highly immunogenic and in HIV-infected humans, Tat antibodies have been shown to correlate with delayed progression to AIDS (Re *et al.*, 1995; Re *et al.*, 2001; Richardson *et al.*, 2003). Tat is secreted into the milieu surrounding

infected cells and can enter uninfected cells directly through the cell membranes (Ensoli *et al.*, 1993; Chang *et al.*, 1997) when through upregulation of the viral receptors it renders the cell more susceptible to viral infection (Huang *et al.*, 1998; Secchiero *et al.*, 1999). An immune response against this protein could therefore reasonably be expected to interfere with the viral replication cycle.

Candidate Tat antigens with potential for use as vaccines have been produced in several forms. Tat-derived peptides induced virus inhibitory antibodies in mice (Boykins *et al.*, 2000; Goldstein *et al.*, 2000) and have also been used in vaccines trials against SIV infection in macaques (Goldstein *et al*., 2000). Biologically active Tat protein has been used alone (Ensoli and Cafaro, 2000) and in combination with modified Env (Ensoli *et al*., 2005) as a vaccine in macaques. The potential toxic effects of biologically active Tat can be avoided by its conversion to an inactivated toxoid (Pauza *et al*., 2000).

Several of the studies utilising Tat as a vaccine, both against HIV and animal lentivirus infections, have demonstrated this effectively ameliorated disease or prevented infection post-challenge, and established the importance of a Tat-specific immune response as one correlate of protection. Cynomolgus monkeys vaccinated with bacterially expressed Tat were able to control infection with highly pathogenic SHIV89.6P (Cafaro *et al.*, 1999; Maggiorella *et al.*, 2004). Vaccination with Tat toxoid attenuated disease in rhesus macaques, as demonstrated by a lower viral load, lower p24 levels and preservation of $CD4^+$ counts in the animals (Pauza *et al*., 2000). In humans, the strong correlation between high levels of antibody to Tat and slow progression to AIDS has led to vaccination experiments attempting to reproduce this state in immunocompromised individuals (Gringeri *et al*., 1998). The type of immunological responses needed for protection are still being elucidated but some studies are showing that both arms of the immune system are required for protection (Pauza *et al*., 2000). Despite the correlation of antibody levels with non-progression of disease in HIV-infected humans (Re *et al.*, 1995), studies in rhesus macaques vaccinated with Tat-derived peptides showed that even with the induction of a strong Tat antibody response no protection was achieved against challenge (Belliard *et al*., 2005).

Nucleotide vaccines.

In the early 1990's there were reports that the intramuscular administration of naked plasmid DNA encoding a foreign gene, under the control of a mammalian primer, could lead to the expression of the foreign gene product in cells within the muscle and that the immune response raised to this endogenously produced protein could be protective (Tang *et al.*, 1992; Fynan *et al.*, 1993; Robinson *et al.*, 1993; Ulmer *et al.*, 1993). Following these initial experiments the field of DNA or, more correctly, nucleic acid (NA) vaccination has grown rapidly. The potential advantages of the technology over more established methods of vaccine production are enormous. NA vaccination presents the possibility of achieving expression of a vaccine protein or peptide directly in the cells of a vaccinated animal or person, avoiding the potential problems of reversion to virulence when attenuated pathogen vaccines are used, or failure of inactivation with inactivated pathogen vaccines. Likewise, the problems of purification, correct conformation, solubility and stability associated with sub-unit vaccines produced with *in vitro* expression systems are avoided by the use of this technology (Whalen, 1996a). Furthermore, unlike conventional vaccines which tend to produce mainly humoral responses, NA vaccines can be tailored to produce a cell-mediated response, often essential in the control of viral diseases (Whalen *et al.*, 1995; Babiuk *et al.*, 1999a).

The simplest approach to NA vaccination is intramuscular injection of a naked plasmid DNA shuttle vector, encoding a gene derived from a pathogen. This approach has provided variable results; it proved effective in protecting dogs against canine distemper virus (Cherpillod *et al*., 2000) but was less effective in protecting cattle against bovine leukaemia virus (Brillowska *et al*., 1999) and bovine respiratory syncytial virus (Schrijver *et al*., 1997). However, improvements in the efficacy of these so-called 'first generation' NA vaccines have been achieved in a variety of ways. With intramuscular injection of plasmid, the DNA has to be taken up by myocytes within the muscle before any expression of the foreign antigen can occur. This antigen then has to be presented to the immune system for a response to be raised. The problems of poor response to vaccination of large animals with NA

vaccines developed in mouse models (Babiuk *et al.*, 1999b; Gerdts and Mettenleiter, 2001), due to unexpectedly high dose levels required, have led to the development of strategies designed to increase both the efficiency of antigen presentation and the strength of the response to that antigen. Targeting of antigen-presenting cells has proved effective at enhancing the efficacy of NA vaccination in sheep against *Corynebacterium pseudotuberculosis* (Chaplin *et al*., 1999) but not against *Taenia ovis* (Drew *et al*., 2001). Co-administration of cytokine genes has proved effective at enhancing protective responses to NA vaccination (Nobiron *et al.*, 2000; Wong *et al.*, 2002). Co-administration of genes for heat shock proteins (Chen *et al.*, 2000; Hauser and Chen, 2003), co-stimulatory molecules (Somasundaram *et al.*, 1999; Flo *et al.*, 2000) and chaperone genes (Lin *et al*., 2005) have all proved effective at enhancing the response to the antigens. The correct choice of adjuvants is as important in NA vaccination as in the more developed field of protein and inactivated pathogen vaccinology (Singh and O'Hagan, 2002; Sasaki *et al.*, 2003).

Microparticle bombardment of target cells, commonly termed 'gene gun vaccination', introduces foreign DNA, bound to a carrier, directly into target cells. The system can be used both *in vitro* or *in vivo,* and *in vivo* it has proved effective in raising a protective immune response to rabies virus in mice (Lodmell *et al*., 1998), and primates (Braun *et al.*, 1999; Lodmell *et al.*, 2001). These studies all utilised gold microparticles as carriers, chosen for their uniform size, inertness and high density, to carry DNA into the immune surveillance cells of the skin, such as dendritic cells. Other workers have targeted the skin-associated lymphoid cells by utilising liposomes, atelocollagen microparticles or simple topical application to the skin (Watabe *et al.*, 2001; Raghavachari and Fahl, 2002).

The route of administration of a NA vaccine effects the type, intensity and duration of the immune response obtained (Bohm *et al.*, 1998; Shkreta *et al.*, 2003). In addition to intramuscular or intracutaneous methods, routes used have included intranasal administration of DNA/liposome complexes to elicit mucosal IgA responses (Klavinskis *et al*., 1999) or as suppositories in cattle (Loehr *et al*., 2001). Administration of NA vaccines directly into lymph nodes has been shown to increase the efficacy of induction of immunity by up to 100-fold compared with

intradermal and intramuscular routes of administration (Maloy *et al*., 2001). Intracellular proteins will be presented to the immune system by MHC Class 1 receptors, and so activate the cell-mediated arm of the immune system (Mascola *et al*., 1999). This may be advantageous in raising a protective response to a viral pathogen, where antigens are produced intracellularly during the course of a natural infection. However, it may be necessary to produce an effective antibody response to clear a viral infection and as such a vaccine would need to also activate the humoral arm of the immune system. Tailoring of the induced immune response has been attempted by altering the destinations of the proteins expressed from the introduced plasmid (Mascola *et al*., 1999). Inclusion of oligonucleotides to code for tags to direct the foreign protein to be membrane bound, retained intracellularly, or to be secreted from the cell can change the response raised to that protein (Mascola *et al*., 1999). Changes in the cytokine microenvironment in the draining lymph node of a DNA vaccine administration site can be induced by the inclusion of CpG motifs, to switch the cytokine profile to a Th-1 biased response (Liu *et al*., 2005). Co-administration of genes encoding cytokines or other co-stimulatory molecules have also proved effective at altering the induced response (Leutenegger *et al.*, 2000; Nobiron *et al.*, 2003; Egan *et al.*, 2005).

NA vaccination has proved effective at inducing protective effects against animal lentivirus infections. Proviral infectious clones attenuated by removal of specific genes have been developed as vaccines against several lentiviral infections, including FIV (Flynn *et al.*, 2000; Lockridge *et al.*, 2000), CAEV (Cheevers *et al*., 2003) and SIV (Busch *et al.*, 2003; Liu *et al.*, 2006b). Constructs expressing viral proteins have been also employed in vaccine trials against MVV (Gonzalez *et al*., 2005) and with less success against EIAV (Cook *et al*., 2005). Administration of NA vaccines encoding viral proteins have proved more successful in protecting against SIV when coupled with a boost strategy, either with recombinant vaccinia virus expressing the same proteins (Someya *et al*., 2006) or with attenuated SIV (Amara *et al*., 2005).

Although the technology of NA vaccination shows promise, especially where cell-mediated immunity is required to control a pathogen, and although several vaccines

have proved efficacious, there are problems that still need to be overcome before the full potential of the technique is realised. Safety considerations present hurdles for the acceptance of NA vaccines for general use, especially the possibility of integration of the vaccines constructs into the germ line, or transfection of gut flora allowing dissemination of resistance and vaccine genes into the environment (Davis and Whalen, 1995; Ertl and Xiang, 1996; Donnelly *et al.*, 1997; Gregersen, 2001). Approaches utilising minimalistic immunologically-defined gene expression (or MIDGE) constructs, containing no bacterial resistance genes, and often consisting of a string of synthesised polynucleotides coding for multiple epitopes, or multiple copies of single epitopes rather than entire genes, have been examined as a solution (Leutenegger *et al.*, 2000; Moreno *et al.*, 2004).

Chapter 3

Preliminary studies on constructs for Jembrana disease virus DNA vaccines.

This chapter describes the production of several potential DNA vaccine constructs. The methods for PCR amplification of the vaccine genes, ligation into commercial vaccine plasmids, and the results of the subsequent *in vitro* and *in vivo* testing of the constructs for functionality and immunogenicity are described.

Introduction.

In 1992, it was discovered that injection of naked plasmid DNA encoding a gene under the control of an appropriate viral promoter could raise an immune response directed to the protein encoded by the transgene (Tang *et al.*, 1992). This raised the possibility of relatively cheap and simple production of nucleic acid vaccine constructs, to induce immune responses to vaccine proteins expressed endogenously has led to considerable research effort.

DNA vaccination with naked plasmids is often less effective in a large target animal than in the murine model systems used to develop the vaccine. The reasons for this are not clear, but such factors as low dosage relative to body mass in larger animals, or lower transfection efficiency, indicating a requirement for a more sophisticated vaccine delivery system, may play a part (Babiuk *et al.*, 2003). The potential for this novel vaccination strategy is so great, however, that despite initial discouraging results, much research has continued into engendering protective immune responses against several diseases of production animals. DNA-mediated vaccination of production animals has shown some promising results using cytokine or CpG adjuvancy (Pontarollo *et al.*, 2002; Nobiron *et al.*, 2003). Experimentation with different routes of administration, such as intradermal (Schrijver *et al.*, 1998), mucosal (Babiuk *et al.*, 2003), or even intramammary (Shkreta *et al.*, 2003), has given information on the best method to induce immune responses of different types and magnitude in large animals. Other promising trials in cattle have included DNA vaccination against bovine herpesvirus-1 (van Drunen Littel-van den Hurk *et al.*, 1998), and bovine leukaemia virus (Brillowska *et al.*, 1999).

Manipulation of the final destination of an expressed antigen, whether secreted, cytoplasmic or membrane bound, also affects the magnitude and type of immune response obtained (Lewis *et al.*, 1999; Morel *et al.*, 2004). It is apparent that careful design of the vaccine construct, and considered choice of adjuvant, route and site of administration can overcome the problem of obtaining a satisfactory response to vaccination in large animals. Several DNA vaccines for non-primate lentiviral infections have been trialled, with varying levels of success. Intradermal vaccination with a *tat*-deleted CAEV proviral genome proved able to establish a mild infection and confer protection against challenge with the homologous pathogenic virus in goats (Harmache *et al.*, 1998). Similarly, vaccination of cats with replication-deficient FIV proviral DNA clones conferred protection against the prototypic Petaluma strain of FIV, but not against challenge with a different strain (Hosie *et al.*, 2000). 2 out of 3 macaques in a small trial were protected against challenge with SIV_{mac251} by vaccination with plasmids containing proviral DNA with deletions in both LTR regions (Kent *et al.*, 2001), and deletion of 12 nucleotides from the nucleocapsid gene of $SIV_{(Mne)}$ produced a replication-deficient proviral clone that when inoculated into macaques protected against challenge with the pathogenic strain (Gorelick *et al.*, 2000).

In the light of the foregoing and, in addition, because of the potential of this technology to allow development of a low cost, easy to produce and stable vaccine, with no danger of inadvertantly spreading virus or reversion to virulence (as can occur with inactivated or modified viral vaccines) a project was initiated to design a DNA vaccine against JDV that would be applicable for use in Indonesia. Maintenance of an unbroken cold chain is often unreliable, so a stable vaccine not requiring refrigeration was desirable. As purification of large amounts of plasmid is a relatively simple operation compared with the often complex techniques required for protein purification, the simplicity of production of DNA vaccines was also seen as a great potential advantage. The possibility of attaining a long lasting protective response with only one vaccination, as was seen in macaques after a single DNA-mediated vaccination against rabies (Lodmell *et al.*, 2001) would also have been of

advantage in Indonesia as animals are often unmarked and hence difficult to find for a second vaccination.

To have the best chance of success in a DNA vaccine trial, it is necessary to ensure that constructs are functioning correctly before administration to the target animal species (Whalen, 1996b). This chapter describes the production and testing *in vitro* and *in vivo* of several vaccine constructs designed to raise an immune response to JDV proteins. Since little is known about the immunogenic or protective epitopes of JDV, each of the vaccine constructs included either whole genes, or a large proportion of a gene, from the JDV genome, in an initial attempt to raise and characterise an immune response. Intradermal delivery was chosen for the route of administration, using a "gene-gun" biolistic device to deliver DNA coated gold particles into the skin, for ease of use and reported ability to produce a stronger immune response to small amounts of DNA vaccine in large animals than other administration methods (Oliveira *et al.*, 2000). Use of the gene-gun to administer vaccines has given protection against the early stages of MVV infection in sheep (Gonzalez *et al.*, 2005), BHV-1 in cattle (Braun *et al.*, 1999), and partial protection of pigs against foot-and-mouth disease virus (Beard *et al.*, 1999).

Materials and methods.

Production of DNA vaccines.

2 commercially available plasmids designed for DNA vaccination were used in these trials. These were pCDNA3.1 His A (Invitrogen) and pVAX (Invitrogen) (Figure 3.1). The features of these plasmids include bacterial origins of replication and antibiotic resistance genes, to allow propagation and selection in *Escherichia coli*, a eukaryotic promoter to drive expression in mammalian cells, and the bovine growth hormone poly-A tail to stabilise the mRNA transcripts.

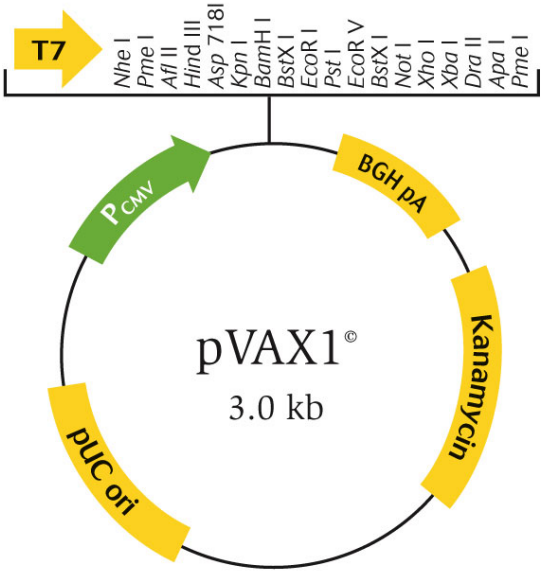


Figure 3.1 Map of pVAX1, a plasmid designed for eukaryotic DNA vaccination studies.

4 genes from the JDV genome were amplified by PCR for ligation into vaccine constructs. The entire gene encoding the CA protein was amplified from Clone #139, containing nucleotides 19 to 2881 of the JDV genome. The genes encoding exon 1 of the tat protein, a 5' truncated SU protein and the entire TM protein were amplified from Clone #55, containing DNA from nucleotide 3847 to 7732 of the genome of the type strain of JDV, Tabanan87 (JDV_{TAB}) (Chadwick *et al.*, 1995a). PCR primers were designed by eye for all PCR reactions required, and are shown in Table 3.1.

Amplification of vaccine genes.

Table 3.1 Primers used for amplification of SU and TM genes, and sequencing of all constructs. T_m denotes calculated melting temperature of the primer.

Primer	Sequence	T_m	Gene/protein
605JCaFOR	5'-CCACAACTTAGAAAGAACTTCC-3'	62˚C	*cap* / capsid protein (CA)
1282JCaREV	5'-GAATTCCATCTTCTGTTTACTTG-3'	62˚C	
5007JTatFOR	5'-GATATGCCTGGTCCCTGGG-3'	62˚C	*tat* / transactivating protein (Tat)
5301JTatEx1REV	5'-CGCGCAGTTAGGTGCCCT-3'	60˚C	
6463JTmKozFOR	5'-GCCACCATGGTCATATTCCTTCTCG-3'	76˚C	*tm* / transmembrane
7545JTmStopREV	5'-GTGTCACATCTCAACTGGG-3'	58˚C	protein (TM)
5194JSuFOR	5'-GACATGATGGAAGAAGGAAG-3'	58˚C	*su* / surface unit protein
6465JSuREV	5'-GGCTCTCTTTCCCCTAGG-3'	58˚C	(SU)
pVAX75FOR	5'-CGGGGTCATTAGTTCATAGC-3'	60˚C	Vector forward primer.
pVAX960REV	5'-GACACCTACTCAGACAATGC-3'	60˚C	Vector reverse primer.

PCR assays were conducted using a master mix consisting of the following final concentrations was prepared in Ultrapure water. 1:10 dilution of 10X Buffer, 1.5 mM $MgCl_2$, 0.15 mM of each dNTP, 1.2 mM of each primer, and 0.5 units of Taq polymerase. 24 µl of master mix was added to 1µl of target DNA, and the reaction mix was subjected to an initial denaturation step of 96˚C for 5 min followed by 25 cycles of denaturation at 96˚C for 1 min, annealing at 57˚C for 1 min (CA), 57˚C (Tat), 55˚C (TM), and extension at 72˚C for 1 min. A final extension step of 72˚C for 10 min was included to allow complete extension of all amplicons. Unincorporated nucleotides, contaminating enzymes and salts were removed with a QIASpin PCR cleanup kit (Qiagen) following the manufacturer's instructions.

Preparation of T-tailed vectors.

For the preparation of T-tailed vectors, pure plasmid was prepared from a culture (37˚C, overnight, 200 r.p.m) of the host strain Top10 (Invitrogen) harbouring the vector pVAX, in Luria-Bertani (LB) broth supplemented with 50 µg/ml kanamycin. The plasmid was purified from the stationary phase culture using an Aurum plasmid mini-preparation kit (BioRad) following the manufacturer's instructions. Purified plasmid was linearised with the blunt ending restriction enzyme *EcoRV,* (New England Biolabs) following the manufacturer's instructions. An overhanging T was added to the ends of the linearised plasmid by incubating (70˚C, 15 min) 0.5 µg of the

linearised pVAX in 10 µl of 1X PCR buffer containing final concentrations of 0.5 mM $MgCl_2$ and 0.2 mM dTTP, and 5 units of *Taq* polymerase. The unincorporated dTTP molecules, contaminating enzymes and salts, were removed with a QIASpin PCR cleanup kit (Qiagen) following the manufacturer's instructions.

Construction and identification of vaccine constructs.

The amplified genes were ligated into the T-tailed vector with T4 DNA ligase (Promega) following the manufacturer's instructions. Recircularised plasmids were transformed into chemically competent Top10 *E. coli* by the heat-shock method, and successfully transformed bacteria were identified by growth (37˚C, overnight) on selective LB Agar plates containing 50 µg/ml kanamycin.

Directional PCR was used to identify those transformants harbouring the insert in the correct orientation. 50 colonies were patched with sterile tips onto selective agar, and cultured (37˚C, overnight). Colonies were screened in pooled batches of 5. A small amount from each of 5 large colonies in the 10 batches was pooled into 50 µl of ultrapure water (Fisher Biotec) and boiled for 5 min. Following centrifugation (14,000 *g*, 1 min), 1 µl of the lysate was added to 24 µl of a PCR master mix, incorporating the relevant reverse primer of the gene of interest and the plasmid forward primer, pVAX75FOR. Cycling was carried out as above with an annealing temperature of 55˚C. Positive batches were then individually screened to identify the positive clone. This colony was then recultured and a single colony was inoculated into 50 ml of selective LB broth and incubated (37˚C, overnight, 200 r.p.m). This culture was used to prepare a glycerol stock (20% glycerol:80% log culture) of the bacteria harbouring the DNA vaccine plasmid.

Confirmation of inserts.

Plasmids were extracted from 2.5 ml of overnight culture using an Aurum MiniPrep kit (BioRad), following the manufacturer's instructions and the purified plasmids were sequenced using BigDye version 3.1 dye terminators (Applied Biosystems) in a ¼ volume reaction, following the manufacturer's instructions. Sequence data was viewed with Chromas version 2.3 (Technelysium), and sequence comparisons

performed with the GCG programme package, accessed via the Australian National Genomic Information Service (http://www.angis.org.au).

Extraction of plasmids for coating gold beads.

50 ml of LB broth containing 50 μg/ml kanamycin or ampicillin as appropriate were inoculated with a scrape taken with a sterile loop from the surface of the frozen glycerol stock of the host strain bearing the desired plasmid. The culture was incubated (37˚C, overnight, 200 r.p.m) and plasmid extracted from the stationary phase culture using an EndoFree plasmid extraction kit (Sigma) following the manufacturer's instructions. Final elution was in ultrapure water (Fisher Biotec). Yield was determined with a fluorometer (Pharmacia) and adjusted to 1μg/μl with ultrapure water for use in the gold bead coating procedure.

Coating of gold beads.

Gold beads (BioRad, 0.1 μm) were coated with the extracted plasmid according to the manufacturer's instructions, with a microcarrier loading quantity (MLQ) of 0.5 mg/shot, and DNA loading ratio (DLR) of 2 μg of each plasmid administered/0.5 mg of gold. Polyvinylpyrrolidone (PVP) was used at 0.1 mg/ml. Dryness of the ethanol used in the procedure was ensured by storing the ethanol over a molecular sieve to remove contaminating water molecules.

To verify the coating procedure, plasmid DNA was eluted from the gold coating 5 cartridges according to the manufacturer's instruction, by vortexing and sonicating the cartridges in TE8 (100mM Tris, pH 8, 100mM EDTA, pH 8). 1 μl of the resulting eluate was used as target nucleic acid in 25 μl PCR reactions, with vector forward primer and insert reverse primer, as described above in directional PCR, to confirm the presence and orientation of the gene of interest, and, by extension, the integrity of the construct.

Preparation of gene gun cartridges.

Cartridges were loaded following the manufacturer's instructions. While coating, the end of the tubing was clamped with artery forceps to prevent pressure fluctuations disturbing the suspension and destroying the smooth deposition of gold onto the tubing. Delaying the start of rotation of the tubing for up to 30 seconds after the initial 180˚ turn, rather than the 3-4 seconds suggested in the manual improved the coating markedly.

To ascertain the quality of the coating procedure, 3 cartridges from each coating run were discharged from the genegun into parafilm wrapped around a microscope slide, to check for even dispersal of the gold across the target area (Figure 3.2).

Cell-free *in vitro* transcription/translation of genes cloned into vaccine constructs.

A TnT rabbit reticulocyte coupled transcription/translation kit (Promega) with non-radioactive detection of expressed protein was used to confirm functionality of the constructs *in vitro,* following the manufacturer's instructions. Briefly, 0.2 µg of purified vaccine construct was incubated with 10 µl of cell free extract (1.5 reaction) containing all the requirements for gene transcription and translation, with the expressed protein labelled by incorporation of biotinylated methionine during translation. Expressed protein was subjected to PAGE followed by electrotransfer (30V, overnight) to nitrocellulose by conventional methods as described in Chapter 4). The resulting Western blot was washed in TBST (137mM NaCL, 10mM Tris, pH 7.4, 0.05% Tween) (3 times, 5 min/wash), blocked (TBST containing 1% w/v skim milk powder, room temperature for 1 h) and probed with streptavidin labelled with alkaline phosphatase (1:5,000 in TBST, 1 h, room temperature). After 3 further washes, labelled proteins were revealed by colour deposition where the specifically bound streptavidin/alkaline phosphatase interacted with the phosphatase substrate Western Blue (Promega).

Transfection of cultured cells.

Cos7 or MDBK cells were grown from stocks stored in liquid nitrogen. The stocks were rapidly thawed and initially cultured in 25 cm^2 cell culture flasks (Nunc) in growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with glutamine, antibiotics and 10% foetal bovine serum for 3 days. To expand the culture, the cell monolayer was washed with 1 ml of sterile PBS (NaCl 137 mM, KCl 27 mM Na_2HPO_4 10 mM, KH_2PO_4 2 mM, adjust to pH 7.4), dissociated by incubation with Multicel cell dissociation buffer (ThermoTrace), and split 1:3 in growth medium in 25 cm^2 tissue culture flasks. The cells were passaged every 3 days. For immunofluorescence assays, cells from a 25 cm^2 maintenance flask were seeded into 12-well or 48-well tissue culture plates (Nunc) and grown to near confluence. Transfection of the cells was achieved by incubation with 1 ml (12 well plates) or 500 µl (48 well plates) of DMEM containing either 1 µg of the purified DNA vaccine construct pVAX/*cap*, or 1 µg of purified pVAX/*tm*, complexed to 2, 4, 8 or 16 µl of lipofectamine (Invitrogen) following the manufacturer's instructions. 1 µg of pVAX vector (without any insert) complexed to 8 or 16 µl of lipofectamine was used as a control.

Immunofluorescent staining procedure.

Seventy two h after transfection the cells were fixed and permeabilised *in situ* with cold methanol, then reacted with either monoclonal anti-JDV CA BC10 (1:100 in PBS) or a polyclonal bovine anti-JDV serum with a high anti-TM titre (1:100 in PBS). The cells were then washed and specifically bound antibodies were detected with anti-species IgG conjugated to FITC (1:1000). The resulting fluorescence was detected with an Olympus BH-2 microscope, with UV excitation at 365-395 nm.

Transactivation of pTAR/lacz construct with pVAX/TAT.

The functionality of the pVAX/*tat* vaccine construct was tested by a pTAR/*lacz* assay. Near confluent *cos7* cells were transfected with pVAX/*tat* and a reporter construct, pTAR/*lacz* as above. After incubation for 3 h, the transfection solution was removed from the wells, replaced with growth medium, and incubated again for 18 h. Transactivation of the pTAR/*lacz* construct by expressed Tat protein from the vaccine construct was detected by the development of an intense blue colour in transfected

cells when incubated with the substrate solution (5 mM $K_4Fe(CN_6).3H_2O$, 5 mM $K_3Fe(CN_6)$, 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside [X-gal]).

Optimisation of levels of lipofectamine for transfection.

Cos7 cells were grown to near confluence in 24-well plates. Cells were transfected with the vaccine construct, pVAX/*tat*, the indicator plasmid, pTAR/*lacZ*, the positive control plasmid, pVAX/*lacZ,* and the negative control plasmid pVAX using different lipofectamine and plasmid concentrations, as detailed in Table 3.2.

500 µl volumes of these solutions were combined as detailed in Table 3.2, each was pipetted onto a washed monolayer of cells, and incubated for 30 min (37˚C, 5% CO_2-in-air atmosphere). Cell monolayers were washed, substrate solution applied and incubated (4 h, room temperature). Positive cells in 5 fields of view from each well were counted.

Table 3.2 Mixtures of lipofectamine and plasmid solutions used to transfect *Cos*7 cells cultured in 24 well plates.

Plate 1	1	2	3	4	5	6
A	5 + 1	5 + 2	5 + 3	5 + 4	6 + 1	6 + 2
B	5 +1	5 + 2	5 + 3	5 + 4	6 + 1	6 + 2
C	6 + 3	6 + 4	7 + 1	7 + 2	7 + 3	7 + 4
D	6 + 3	6 + 4	7 + 1	7 + 2	7 + 3	7 + 4

Plate 2	1	2	3	4	5	6
A	8 + 1	8 + 2	8 + 3	8 + 4		
B	8 + 1	8 + 2	8 + 3	8 + 4		
C	9 + 1	9 + 2	9 + 3	9 + 4		
D	9 + 1	9 + 2	9 + 3	9 + 4		

1. 250 µl DMEM (no antibiotics or lipofectamine)

2. 250 µl DMEM + 20 µl lipofectamine.

3. 250 µl DMEM + 40 µl lipofectamine.

4. 250 µl DMEM + 60 µl lipofectamine.

5. 250 µl DMEM + 10 µg pVAX.

6. 250 µl DMEM + 5.0 ug pVAX and 5.0 µg pVAX/*tat.*

7. 250 µl DMEM + 5.0 ug pVAX and 5.0 µg pVAX/*lacZ.*

8. 250 µl DMEM + 5.0 ug pVAX and 5.0 µg pTAR/*lacZ.*

9. 250 µl DMEM + 5.0 ug pVAX/*tat* and 5.0 µg pTAR/*lacZ.*

Comparison of fugene and lipofectamine for transfection.

Cos7 cells were grown to near confluence in 24-well tissue culture plates. Wells 1-6 of Row A were incubated with 100 µl of antibiotic-free, serum-free DMEM medium containing 0.6 µl of Fugene (Roche) and 1 µg of the positive control plasmid pVAX/*lacZ*. The 6 wells of Row B were incubated with 100 µl of medium as before, containing 3 µl of lipofectamine and 1 µg of pVAX/*lacZ*. The 2 remaining rows were incubated with medium containing fugene or lipofectamine, but without plasmid, to examine the effect of the transfection solution on the cells. The columns of the plate were incubated for 1 min, 10 min, 30 min, 1 h, 2 h and 3 h. The transfection medium was then removed and replaced with growth medium. The cells were re-incubated and levels of transactivation assessed as described above. Cell death was estimated and expressed as a percentage of the monolayer destroyed.

DNA vaccination of mice.

Groups of 5 ARC/Swiss mice were vaccinated and bled according to the protocol in Table 3.3. Each vaccine dose consisted of 1 µg of each plasmid coated onto 0.5 µg of gold with 0.1 mg/ml PVP. Initial doses were administered at 300 pounds per inch^2 (p.s.i.), subsequent doses at 250 p.s.i., into the shaved skin of the abdomen. All mice were bled before the initial vaccination, and following each subsequent vaccination, from the saphenous vein. The vein was occluded and pricked with a 30 G needle, then 2-3 drops of blood were sucked by capillary action into a haematocrit tube, and immediately expelled into a 500 µl Eppendorf microfuge tube to clot. Sera was collected after centrifugation of the clot. At the end of the experiment, the mice were killed and immediately bled out by cardiac puncture. Skin samples were cryoembedded in OCT 4583 (TissueTek) and stored at -80˚C. Spleens were removed and stored at -80˚C in RNAlater (Ambion).

Table 3.3 Protocol for DNA vaccination of mice.

Plasmid combination for each group of mice	1st vaccine	First bleed	2nd vaccine	2nd bleed	3rd vaccine	Final bleed
pVAX/pcDNA3.1*rev*	d0	d12	d21	d25	d55	d60
pVAX*tat*/pcDNA3.1*rev*	d0	d12	d21	d25	d55	d60
pVAX*tm*/pcDNA3.1*rev*	d0	d15	d20	d27	d56	d63
pVAX*cap*/pcDNA3.1*rev*	d0	d15	d20	d27	d56	d65
pcDNA3.1*su*/pcDNA3.1*rev*	d0	d12	d19	d21	d49	d56

d denotes day post initial vaccination

The sera from mice vaccinated with pVAX/*tat*:pcDNA3.1/*rev* and pcDNA3.1/*su*:pcDNA3.1*rev,* were screened for antibody by Western immunoblotting the appropriate antigen. His/Tat expressed in a eukaryotic system was a gift from Dr Ami Setiyaningsih, and SU/PIN was a gift from Dr Moira Desport. Immunoblots were initially stained with Ponceau red to identify the antigen band, which was marked with pencil. The blots were then blocked as above, and the section of the membrane bearing the antigen band carefully excised and cut into strips (to allow reassembly of the blot following probing with murine sera). Each section of the blot was probed with individual sera (1/10 in TBST) from the mice in both vaccine groups. Bound antibodies were detected with horseradish peroxidase conjugated anti-mouse IgG, with colour developed by incubation with H_2O_2 and 4-chloronapthol (1mg/ml in methanol, 1ml of this mixed with 5 ml of TBS containing 3 µl of 30% H_2O_2).

The sera from the pVAX/*tm*:pcDNA3.1/*rev* vaccinated group was screened by ELISA, using a synthetic peptide identical to the principal immunodominant domain of the JDV TM protein in an adaptation of a previously published method (Barboni *et al.*, 2001), utilising an anti-mouse IgG horseradish peroxidase conjugate to detect specifically bound antibody.

Sera from the pVAX/*ca*:pcDNA3.1/*rev* vaccinated group were also screened by ELISA, utilising a recombinant JDV CA antigen, Jgag6, expressed as a fusion protein in the Pinpoint system (Desport *et al.*, 2005). This was diluted in ELISA carbonate coating buffer (pH 9.0) at a concentration of 1.25 µg/ml and 50 µl pipetted into each

well. After overnight incubation at 4˚C, and subsequent washing with PBS/T (3 x 5 min/wash), the plates were blocked (PBST containing 1% w/v skim milk powder) for 1 h at room temperature. The plates were washed again, and 2-fold dilutions of test sera from an initial concentration of 1:10 in PBST were made in the rows, followed by incubation for 1 h at room temperature. The plates were washed, and specifically bound antibodies detected with anti-mouse IgG (1:2000 in PBST). Unbound antibodies were again removed by washing and the plate developed by incubation with a chromogenic substrate (HrP Substrate kit, BioRad, Cat no.172-1064). The colour development reaction was stopped with 2% w/v oxalic acid and the absorbance of the reaction read at 405nm in an ELISA plate reader (BioRad).

Results.

Plasmid constructs.

Approximately 20-30% of antibiotic resistant colonies obtained following transformation with vector constructs contained inserts in the correct orientation, detected by directional PCR on boiled colonies. Forward and reverse sequencing of the plasmids purified from selected positive clones confirmed that no errors had been incorporated into the vaccine genes during PCR amplification when compared to the JDV_{TAB} sequence.

Quality of gold coated plasmids loaded into cartridges.

The loaded cartridges were assessed for quality of coating by firing 3 cartridges from each batch into parafilm. Figure 3.2 shows the results of discharging 2 evenly coated cartridges and an unevenly coated one. The poor dispersal and delivery of the gold from the unevenly coated cartridge is clear, and evenly coated cartridges were selected for use in animal vaccination experiments.

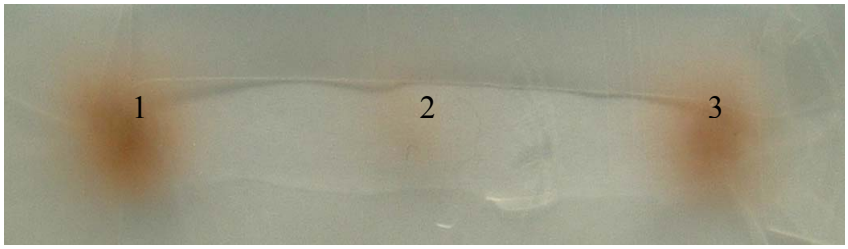


Figure 3.2 The appearance of gold microcarriers after discharge into parafilm to assess the quality of gold deposition during coating of the cartridge. Patterns 1 and 3 are from cartridges evenly coated with gold, the central shot, 2, is from an unevenly coated cartridge, and is unacceptable for use in animal experiments.

Expression of proteins *in vitro* by plasmid constructs.

Western blot analysis of proteins expressed *in vitro* (Figure 3.3) showed that the product of the *ca* gene was far better expressed that those coded for by the *tat* and *tm* genes, although all 3 proteins were successfully transcribed and expressed in this system.

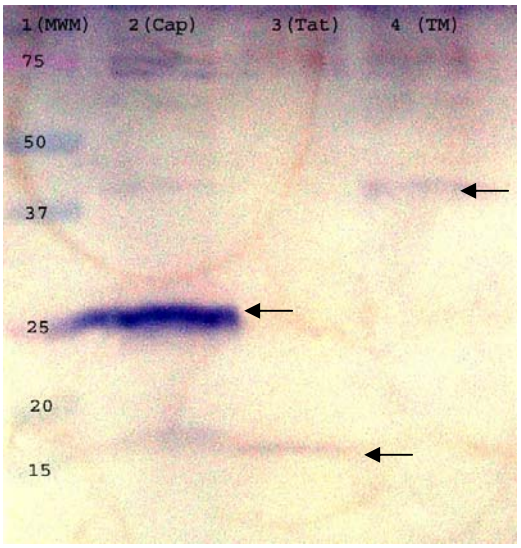


Figure 3.3 Developed Western blot of a gel following PAGE of the proteins expressed from vaccine constructs in the TnT rabbit reticulocyte cell-free expression system. The vaccine proteins JDV CA (lane 2), JDV Tat (lane 3) and JDV TM (lane 3) are arrowed. Molecular weight markers (MWM) are in kilodaltons (kDa).

Demonstration of expression of vaccine proteins by immunofluorescence.

Capsid expression in transfected cells, detected by monoclonal antibody, was demonstrated by strong cytoplasmic fluorescence in roughly 2% of cells in the *Cos7* monolayer. A similar proportion of cells in the monolayers transfected with pVAX/*tm* fluoresced strongly, with both nuclear and cytoplasmic fluorescence (Figure 3.4).

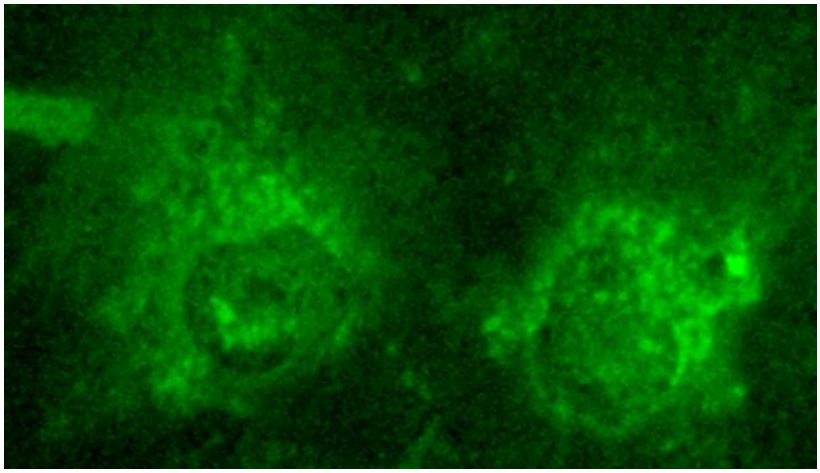


Figure 3.4 Photomicrograph showing the fluorescence resulting from intracytoplasmic immunostaining of expressed CA in *cos7* cells transfected with pVAX/*ca,* following permeabilisation and labelling of expressed protein with anti-CA MAb and fluorescein conjugated anti-mouse IgG.

Transactivation study.

Transfection of cells was more effectively achieved when lipofectamine was used as a transfectant, compared with fugene (Figure. 3.5). However, more cell death was seen after prolonged incubation (over 60 min) with almost all cells detached from the well when this transectant was used (Figure 3.6). To minimise cell death, a transfection time of 10 min was used to determine the optimum ratio of lipofectamine to plasmid DNA for transfection. Cells were optimally transfected with pVAX/*tat*:pTAR/*lacZ* at a lipofectamine:DNA ratio of 4 µl:1 µg (Figure. 3.7).

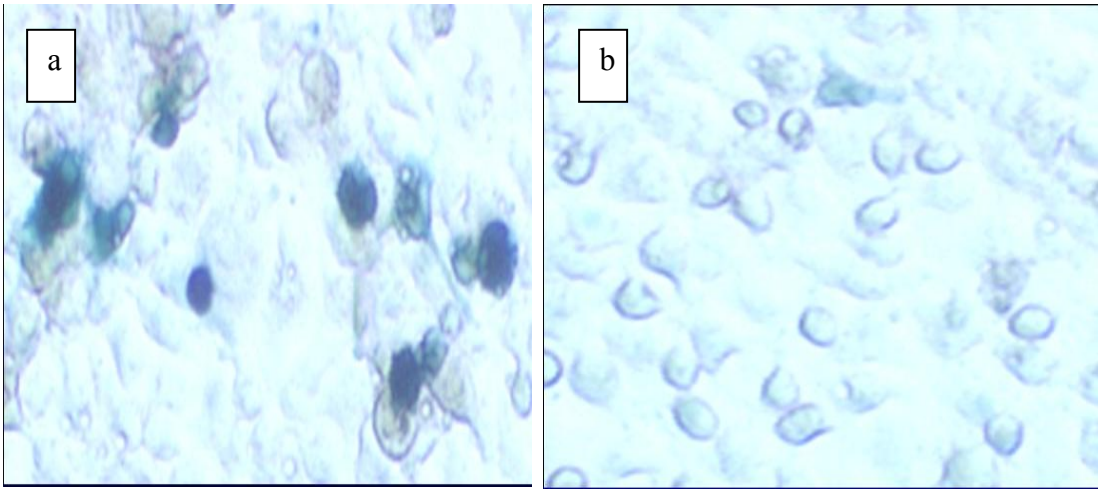


Figure 3.5 (a) Intense blue staining of *cos7* cells co-transfected with pVAX*tat* and pTAR*lacZ* resulting from *trans*-activation of the TAR by Tat expressed from the vaccine construct. (b) Negative control transfected with pTAR*lacZ* and empty pVAX vector

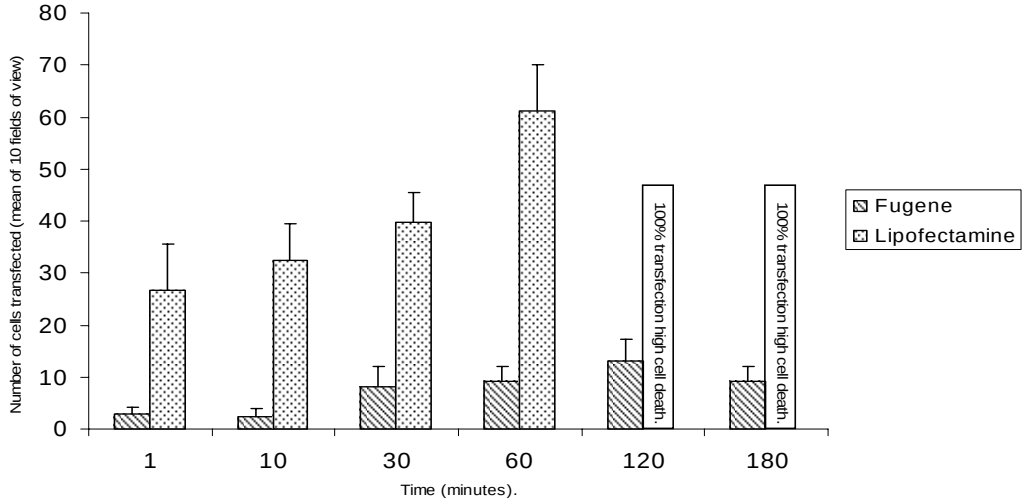


Figure 3.6 Comparison of the transfection rates of *cos7* cells when 2 transfectants were used to introduce pVAX/*tat* and the marker plasmid pTAR/*lacZ* into the cells. Lipofectamine was more effective but incubation for longer periods (over 60 min) resulted in a high proportion of cell death.

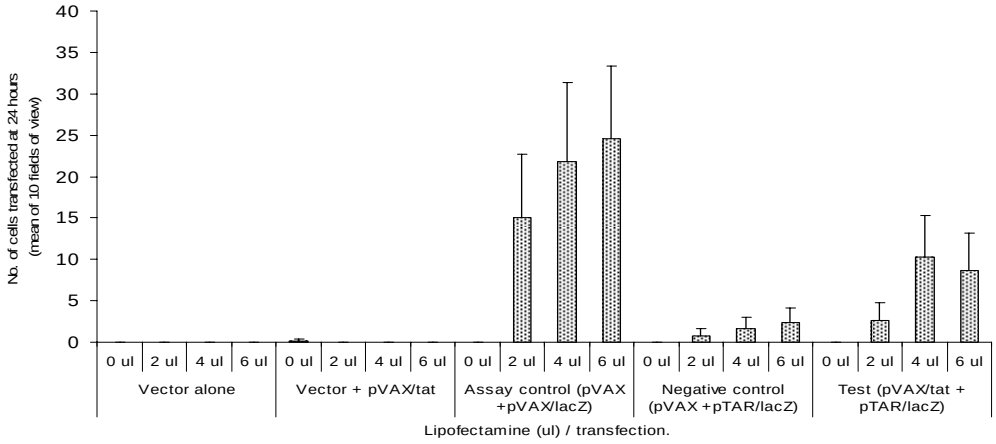


Figure 3.7 Determination of the optimal ratio of lipofectamine:plasmid DNA for transfection of *cos7* cells. DNA was complexed to lipofectamine in 500µl of medium at a ratio of 1µg total DNA to 0,2,4,6 µl of lipofectamine, and the resulting mix used to transfect near confluent cells in 24 well plates.

DNA vaccination of mice.

After injection of mice, all the cartridges used were checked visually after firing, and all gold had been removed at both firing pressures. The area of impact of the gold on the skin was clearly visible immediately after firing, and the gold from the previous vaccination was still visible at the next vaccination 12 to 15 days later. The skin subjected to gold bombardment at the first vaccination with a firing pressure of 300 p.s.i. showed signs of reddening and petechiae, and subsequent vaccinations were administered at 250 p.s.i, which prevented this effect. No mice showed any ill effects or change in demeanour after the administration of the DNA.

All of the mice vaccinated with the pVAX/*tat*:pcDNA3.1/*rev* construct on days 0 and 21 seroconverted to Tat produced in a eukaryotic cell culture system by 4 days after the second vaccination; the reaction was weak. This weak response was not detectable 5 days after the third vaccination.

One of 5 mice vaccinated with pcDNA3.1/*su*:pcDNA3.1*rev* seroconverted at the first test bleed 12 days after the first vaccination. Two of the 5 mice were antibody-positive by 3 days after the second vaccination, on day 19. All the Western

immunoblot reactions were weak and reactivity could not be detected in the third test bleed 7 days after the third vaccination on day 49.

Sera from the mice vaccinated with pVAX/*ca* and pVAX/*tm* coadministered with pcDNA3.1/*rev* were screened by ELISA. No evidence of specific antibody was detected in any of the sera of the mice after vaccination with these 2 constructs.

Discussion.

The experiments described in this chapter indicated that all of the constructs were capable of producing protein *in vitro,* and that 2, pVAX/*tat* and pVAX/*su,* administered intradermally resulted in an antibody response in mice.

Several methods were used to demonstrate *in vitro* expression of the proteins from the plasmid constructs. One of these was a coupled transcription/translation kit which allowed transcription of mRNA from the T7 promoter incorporated in the pVAX plasmid (Figure 3.1), with subsequent translation of proteins utilising the cell machinery in the reticulocyte lysate. An advantage of this procedure was that it allowed non-radioactive detection as the kit allowed incorporation of biotinylated methionine into the translated proteins, with subsequent detection on Western Blot with streptavidin-alkaline phosphatase. The system suggested there were higher levels of expression of CA than Tat and TM. This result is similar to that seen with CA protein in the bacterial expression systems reported later in this thesis (Chapter 6). This high level of CA expression in the bacterial systems was thought to be due to either greater solubility of the expressed protein, the codon utilisation in the gene, its secondary mRNA structure, or its low bacterial toxicity. In the TnT system, RNA structure may have played a part in the low expression of Tat and TM, but codon preference should not have been a problem for these genes as viral proteins are normally produced in eukaryotic cells. The apparent low levels of Tat expression by this system may be due to low levels of incorporation of methionine, as methionine is under-represented in this protein. However this cannot account for the apparent difference in the expression of CA and TM and the results do indicate that the different levels of expression are real. Immunofluorescent staining of transfected cells with monoclonal anti-CA antibody again demonstrated expression of CA protein, with about 2% of transfected cells exhibiting strong cytoplasmic staining. Detection of expressed TM was with a hyperimmune polyclonal bovine anti-JDV serum, and although fluorescent cells were present in the transfected monolayer, cross reaction with intranuclear Rev protein expressed from the coadministered pcDNA 3.1/*rev* construct could have been responsible for some of the staining seen, especially the

intranuclear staining, as BIV Rev is localised to the nucleus *in vivo* (Oberste *et al.*, 1993; Schoborg and Clements, 1994; Schoborg *et al.*, 1994). The construct encoding Rev was co-administered with the vaccine constructs to investigate any requirement for Rev in expression of the vaccine genes. The transport of singly spliced or unspliced viral mRNA transcripts from the nucleus of infected cells is dependant on the interaction of Rev with the Rev responsive element (RRE) (located in the *env* region of the viral genome) to overcome the default pathway where mRNA transcripts are spliced before nuclear export to produce the transcripts coding for the regulatory proteins (Frankel and Young, 1998). However the inclusion of this construct was erroneous, as the vaccine genes were amplified from cDNA where splicing had already occurred, removing any Rev-dependancy from the expression of the administered vaccine genes (Chadwick *et al.*, 1995b).

While the transcription/translation kit used seemed to demonstrate only low levels of Tat expression, the strong transactivation of the pTAR/lacZ construct suggests there was significant expression of Tat from pVAX/*tat.* This assay was developed in our laboratory (Setiyaningsih, 2006), to enable functionality assessment of vaccine constructs for quality control during production of plasmids for animal trials, and modifications described in this Chapter were developed to reduce problems of cell death in the transfected cultures by using alternative transfectants, decreasing the time of interaction of the plasmid/transfectant complex with the cells, and very gentle treatment of the cells post-transfection. The assay is dependent on a transactivation region or TAR motif, which in HIV-1 is a hairpin structure at the 5' end of the nascent viral RNA transcript. The multifunctional viral protein, Tat, binds to this region of the 5' LTR and is capable of increasing the transcription of viral RNA by several orders of magnitude (Frankel and Young, 1998). Replacement of the promoter region in the pVAX/*lacZ* reporter plasmid with the TAR region from JDV gave a construct that would not express unless transactivated by Tat, and so successful transfection of cells was indicated by upregulation of the *lacZ* gene in co-transfected plasmid and development of the intense blue colour when the substrate, X-gal, was incubated with the cells. The high levels of cell death in transfected cells may also

have been indicative of Tat expression, as extracellular Tat has toxic properties (Gallo, 1999).

Having shown functionality of the constructs *in vitro* with several assays, activity *in vivo* was assessed by the inoculation of mice using intradermal bombardment with gold particles to deliver the vaccine constructs. This technique has several advantages over the intramuscular injection of DNA. Most importantly, many workers have attained strong cellular and humoral responses to several antigens after administration of small (<1-2.5 μg) amounts of DNA to mice with a biolistic device (Chen *et al.*, 1997; McCluskie *et al.*, 1999). This contrasts with the much larger (up to 1 mg) amounts per injection required to raise an immune response in mice when the DNA is administered intramuscularly (Morel *et al.*, 2004). The difficulty in raising a strong response in larger animals (Beard *et al.*, 1999), is suspected to be in part dose-related, and it was hoped that biolistic transdermal vaccination would remove the need for large amounts of foreign DNA to be injected into the muscle, as found necessary in other attempts to DNA vaccinate cattle (Cox *et al.*, 1993; Babiuk *et al.*, 2003). The smaller amounts of DNA required would have ultimately made for a reduced cost of production of the vaccine in Indonesia, and as animals would ultimately be used for food at some time post-vaccination, an intradermal rather than intramuscular injection site would provide for greater safety.

To reduce costs, a mouse model rather than a bovine model was used to test the constructs for functionality *in vivo*. Surprisingly, mice did not appear to respond to CA vaccination whereas this protein was well expressed in both the TnT cell free system and in transfected cells. The CA antigen for screening of sera was produced as a soluble fusion protein in bacteria and has been shown to be recognised by sera from both naturally infected, and recombinant CA vaccinated cattle (Desport *et al.*, 2005), indicating that the mice had failed to seroconvert rather than the antigen being undetectable due to conformational changes. The mice also failed to respond to the TM DNA vaccine construct, for reasons that were not clear.

While mice did not seem to respond to the CA and TM vaccine construct, all mice vaccinated with pVAX/*tat* produced antibody to Tat. The bands obtained in Western

immunoblots were not strong, indicating that high levels of antibody had not been produced. A similar weak antibody response was obtained against SU protein on a blot probed with sera from 2/5 of the pcDNA3.1/*su* vaccinated mice. The Western immunoblot antigen in this case was a bacterially expressed GST fusion protein and may not have possessed the array of epitopes present on a correctly folded glycosylated protein, expressed in a eukaryotic system. This same antigen was not recognised strongly by sera from naturally JDV-infected recovered cattle, and the lack of appropriate glycosylation of the bacterial protein may well have prevented strong recognition by the murine sera. Induction of an antibody response to the intracellularly expressed proteins was probably secondary to the induction of a cell mediated response, as endogenously produced protein in APC would be presented to T-cells in the context of the MHC class 1 pathway. However, re-presentation of antigen, produced in transfected keratinocytes, released, and processed by APC to be presented to the immune system in the context of MHC class II pathway, could give rise to a humoral response and antibody production. Tat protein in natural HIV-1 infections is known to be released from infected cells into the milieu (Chang *et al.*, 1997), where it is capable of penetrating bystander cell membranes in a non-receptor mediated manner. It is possible that the seroconversion seen in the *tat* and *su* vaccinated mice was a result of release of Tat and SU by transfected keratinocytes and its uptake and processing by APC in these animals. The seroconversion to Tat in these mice was a significant finding, as anti-Tat antibody levels are inversely correlated with rate of progression to AIDS in humans, and antibodies to Tat can inhibit viral replication in HIV-1 infected cells in culture (Re *et al.*, 2001). Other functions of the Tat molecule, such as transactivation of transcription factors and protection against apoptosis, are also successfully stopped by addition of anti-Tat antibody to infected cell cultures (Re *et al.*, 2001). SU is not known to be released from transfected or naturally infected cells. Cell death may have released some expressed protein and the poor rates of seroconversion may reflect this route of antigen interaction with the immune system.

The non–response to vaccination with TM and Capsid constructs, and the weak responses to SU and Tat constructs, may have been overcome by the use of an

adjuvant. Many DNA vaccine experiments have highlighted the need for adjuvancy to enhance the reactogenicity of the administered construct (Sin *et al.*, 1998; Somasundaram *et al.*, 1999; Nobiron *et al.*, 2001). First generation vaccines, incorporating live attenuated, or killed organisms contain a wide range of antigenic determinants, capable of providing sufficient of the signals required for good immunogenicity (Schijns and Tangeras, 2005). The quest for ever more molecularly defined antigens has often led to an decrease in immunogenicity as the non-specific determinants are removed from the vaccine formulation (O'Hagan, 1998; Singh and O'Hagan, 1999). In the case of DNA vaccination, the requirement for these costimulatory signals has led to attempts to replace the traditional adjuvants with a defined molecular adjuvant, either from incorporation of CpG motifs into the construct, co-administration of cytokine or interleukin genes, or chemical adjuvants such as alum (Scheerlinck, 2001; Sasaki *et al.*, 2003). The efficacy of adjuvants is due to activation, by these co-stimulatory signals, of the APC which are then capable of activating naïve T-cells (Lima *et al.*, 2004). CpG motifs, present in bacterial DNA at a far higher level of occurrence than in eukaryotic DNA, are potent activators of APC, but the amount of DNA administered by gene gun inoculation may be too low for CpG motifs to act as an effective adjuvant. Future work will require investigation of the adjuvant requirements of JDV DNA vaccines.

It is also possible that expression but non-release of the CA and TM proteins led to a solely CMI response, that was not detected in cell proliferation assays attempted by another member of the research group (unpublished data). Nucleic vaccination involves endogenous expression of foreign genes, and induction of humoral responses is enhanced by tailoring the administered genes to include secretion signal peptides, allowing direction of the proteins produced down the secretion pathways of the cell (Lewis *et al.*, 1997; Boyle *et al.*, 1998; Bucht *et al.*, 2001). Further modification of the constructs coding for the vaccine genes to include secretion signals would be required to test this hypothesis.

Although encouraging initial results were obtained in terms of determining the conditions required to cause expression in the skin of cattle, and in proving both *in vitro* and *in vivo* that the constructs were functioning and capable of eliciting an

immune response, several factors led to the work being stopped. Firstly, during the course of these studies it became apparent that registration of a DNA vaccine in Indonesia was going to be difficult; there was no precedent there, and very few precedents in any country. Secondly, it was felt that as adequate supplies of high-quality antigen were not available to screen vaccinated animals for humoral and CMI responses, that any attempts to further optimise the vaccine constructs by co-administration of cytokines or incorporation of individual epitopes such as the major homology region (MHR) of CA as a marker epitope would be frustrated by lack of an adequate assay to quantify levels of response. The levels of Tat expressed in *cos7* cells was low, requiring cells from 12 x 25 cm^2 flasks to purify sufficient protein for one small Western blot. Purification of viral antigen in Indonesia would therefore be difficult due to problems in maintaining equipment, and development of eukaryotic expression systems for all of the proteins of interest in Indonesia was considered a very difficult challenge. In the light of these obstacles, the work on DNA vaccines for JDV was discontinued.

Chapter 4

Development of a model for the kinetics of Jembrana disease virus replication and clearance during acute infection.

Summary.

This chapter describes the development of a simple model to describe the progression of the acute phase of JDV in a manner that allows meaningful comparison of data obtained from different animals, as all parameters showed large animal-to-animal variation.

Introduction.

Infection with JDV causes an acute febrile response lasting between 5-12 days and animals characteristically develop anorexia, leucopenia, oral erosions and enlargement of superficial lymph nodes (Soesanto *et al*., 1990). High levels of infectious virus were detected (10^8 ID_{50}/ml plasma) during the febrile period, reducing to 10^5 ID_{50}/ml on the first day after the febrile period and further declining to about 10 ID_{50}/ml at 42 and 72 days after infection (Soeharsono *et al*., 1995b). The leucopenia was principally due to a loss of lymphocytes and most of the haematological changes occurred during the febrile period and are well documented. Immunosuppression during infection is characterised by a delayed seroconversion to viral proteins, until approximately 6 weeks after infection. Surviving animals are resistant to reinfection, retain infectious virus for at least 2 years and do not appear to suffer relapses (Soeharsono *et al*., 1990). Attempts to cultivate JDV in continuous cell lines *in vitro* have so far been unsuccessful in Indonesia, although evidence of limited persistence in cultured peripheral blood mononuclear cells (PBMC) has been reported (Kertayadnya *et al*., 1993).

Quantification of lentiviruses without using traditional cell culture techniques is now well established and surrogate assays such as measurement of HIV-1 p24 CA protein, (Lombardi *et al.*, 1994; Kashiwase *et al.*, 1997) viral reverse transcriptase (RT) activity (Yamamoto *et al*., 1996), and quantification of the amount of viral genomic

RNA or integrated DNA in tissues or plasma by quantitative PCR are all currently used. Comparison of a capture ELISA for JDV CA protein that has been used to monitor JDV CA protein levels at the point of disease control and a recently developed quantitative RT-PCR (qRT-PCR) method for viral RNA in plasma has shown good correlation (Stewart *et al*., 2005). The qRT-PCR method is more sensitive, allowing a more clear analysis of the dynamics of JDV replication.

Most lentiviruses have relatively labile genomes which are prone to variation and the development of quasispecies is common (Kyaw-Tanner and Robinson, 1996; Zheng *et al.*, 1997; Baccam *et al.*, 2003). The complete genome of JDV_{TAB} (Chadwick *et al*., 1995b), has been compared in a recent analysis to JDV *gag*, *pol* and *env* sequences from isolates obtained from different timepoints and geographical regions. JDV proviral DNA, unlike the other lentiviruses, shows a remarkably high level of nucleotide conservation, particularly between isolates from the same region taken at different times (Desport, 2007). However, another strain obtained from an affected animal in a different region, Pulukan, of Bali in 2001, termed JDV_{PUL}, showed some divergence in the *env* sequences.

In order to describe any effect of candidate vaccines on the kinetics of viral replication and clearance during JD, in addition to those seen on the febrile response and leucopaenia in experimentally infected controls, a model for typical JDV infections was required. An analysis of the kinetics of virus replication in experimentally infected Bali cattle was conducted and the results of these experimental infections and an analysis of the results are described in this chapter. Observed deviations from the model in 20% of experimentally infected cases are described and the implications for diagnosis and control of the disease are discussed.

Materials and methods.

Experimental cattle.

Female Bali cattle between 6 and 12 months of age were purchased from Nusa Penida, an island off the coast of Bali that is considered free of JD, and transported to BPPH V in Denpasar, Bali. The animals were housed together in a fly-proof building, and fed a diet of elephant grass and concentrate *ad libitum.* Animals were housed for

1 week prior to experiments and were tested using antibody ELISA (Hartaningsih *et al*., 1994) to ensure that they were JDV seronegative.

JDV infectious doses.

2 levels of challenge dose were used, either "low" an estimated $4x10^2$ ID_{50}, or "high", an estimated 1-2 $x10^3$ ID_{50}. The ID_{50} for JDV was determined previously by titration in animals (Soeharsono *et al*., 1990) and these workers found that the titre of infectious JDV in animals on the second day of the febrile reaction was about 10^8 ID_{50}/ml, and this was correlated to levels of circulating viral CA protein by an antigen capture ELISA and qRT-PCR (Stewart *et al.*, 2005). In these experiments, the approximate ID_{50}/ml in the plasma of a donor animal on the second day of fever which had been previously infected with a stored (-80°C) spleen suspension containing the desired virus strain was therefore determined by antigen capture ELISA in Bali prior to preparation of the infection dose. The correct infection dose of virus was then obtained by appropriate dilution, in DMEM, of the donor animal plasma to give a final volume of 2 ml. This was administered by intravenous injection into the jugular vein of each animal as shown in Table 4.1. Confirmation of the viral titre in the donor animal plasma was carried out retrospectively by qRT-PCR, as detailed below.

Table 4.1 Estimated and actual infection doses of each strain of JDV for Trials II-V.

Trial	Donor	Plasma viraemia level in donor determined by ELISA (ID_{50})	Infectious dose administered to cattle (ID_{50})	Infectious dose determined by qRT-PCR
II	**CB 65**	$1.5 x10^8$	$1 x 10^3$	Not determined
III	**CB 143**	$8 x 10^8$	$4 x 10^2$	$1.01 x 10^3$
IV	**Not known**	$1.6 x 10^7$	$1 x 10^3$	Not determined
V	**CB 115**	$1 x 10^8$	$1 x 10^3$	$6.4 x 10^2$

Sampling regimes in infected cattle.

Clinical signs were monitored post-infection, and some animals were killed and necropsied at various times post recovery. Rectal temperatures were taken daily for 21 days post infection. Blood samples were collected into EDTA for preparation of plasma and estimation of leucocyte concentration, and serum samples were prepared

by centrifugation from clotted blood taken into tubes without EDTA. All plasma and serum samples were stored at -80^{0}C until analysed.

Viral RNA extraction.

Plasma samples were clarified by centrifugation (8,000 *g*, 5 min) prior to extraction of RNA. Total RNA from either 140 µl (Trial II, IV and V) or 280 µl (Trial III) of thawed plasma from each sample time-point was extracted using a QIAmp Viral RNA mini kit (QIAGEN) following the manufacturer's instructions, with a final elution volume of 60 µl in buffer EB. Extracted RNA was stored at –80°C until required.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR).

All primer and probe sequences were derived from the previously published JDV_{TAB} sequence; Genbank accession No. U21603 (Chadwick *et al*., 1995b). The primer set (Invitrogen) utilised is shown in Table 4.2. These primers were designed to amplify a 121 bp fragment of the JDV *gag* gene.

Table 4.2 Primers and probe for qRT-PCR.

Primer.	Sequence.
JDV gag1f	5'-GGGAGACCCGTCAGATGTGGA-3'
JDV gag1r	5'-TGGGAAGCATGGACAATCAG-3'
Probe	FAM-5'-CCCACAACTTAGAAAGAACTTCCCCGCTG-3'-BHQ-1

The fluorogenic probe (Geneworks) was labelled at the 5' end with the reporter dye, 6-carboxyfluorescein, and at the 3' end with the Black Hole-2 quencher dye. qRT:PCR reactions were performed initially in an ABI 7700 Sequence Detection System (PerkinElmer), and subsequently in a Rotor-Gene 3000 (Corbett Research). Initially, an Access RT-PCR kit (Promega) was used to make a reaction master mix consisting of final concentrations of:0.5X AMV/T*fl* reaction buffer, 0.8 mM of each dNTP, 2 mM $MgCl_2$, 100 nM of each primer, 0.1 µM fluorogenic probe, 0.2 U/rxn RNAseIn (Promega), 0.4% (w/v) Triton X-100, 2 mM DTT, 0.1 U/µl AMV-RT and 0.1 U/µl T*fl* DNA polymerase in a final volume of 23 µl, in 100µl tubes (Axygen) in a 76 well rotor. 2 µl of extracted total RNA were added to each tube, and each sample

was assayed in duplicate, with fluorescence excited at a wavelength of 470 nm and detected at a wavelength of 510 nm. The one-step RT-PCR protocol consisted of an RT step at 48°C for 45 min, a 3 min inactivation step at 95°C , followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 60°C for 30 seconds (Stewart *et al*., 2005). Fluorescence data was collected in the annealing and extension steps of the PCR cycle.

An improvement on this assay was introduced in the analysis of the RNA samples obtained from the animals in Trial V. For these assays, a premixed 2X mastermix, (Iscript, BioRad) was used, with the addition of both primers and fluorogenic probe to a final concentration of 100 nM as above, and ultrapure water added to give a final 1X concentration. MMLV reverse transcriptase was used in this assay following the manufacturer's instructions. 1 µl of RNA sample was added to 9 µl of master mix in 100 µl tubes (Axygen) and the one-step RT-PCR protocol consisted, in this case, of an RT step of 50°C for 10 min, a 5 min inactivation step at 95°C with a 2 second pause at 92°C to prevent thermal overshoot , followed by 40 cycles of 92°C for 2 seconds, 95°C for 15 seconds, 58°C for 30 seconds and 60°C for 30 seconds.

A standard curve was generated in each run by amplification of duplicate serial 10-fold dilutions of a plasmid, pCR2.1 (Invitrogen) containing nucleotides 19-2881 of the sequence of the JDV_{TAB} isolate, encompassing the target amplicon. Copy numbers of the plasmid added to each standard reaction were calculated, with 1 copy of plasmid equivalent to 1 viral genome. Estimation of the relative efficiency of the RT step was performed by including a standard RNA sample in each run, and blanks (no probe, no template, no RT, template no RT) were included in each run. The results obtained from the Prism 7700 were analysed with Sequence Detector 1.9.1 software, and for the later experiments, the Rotor-Gene proprietary software, Version 6.0, was used.

Calculations.

The rate of increase in viral titre(γ) was calculated for each sampling interval as $(\ln Titre_{Tn}-\ln Titre_{Tn-1})/(T_n-T_{-1})$, where T was time in hours post-infection. The

doubling time or clearance half-life for each sampling interval was calculated as $\ln(2)/\gamma$, expressed in h.

Total leucocyte concentrations were expressed as the percentage decrease normalised to the concentration on the day of infection.

Fever score was used as a measure of the severity of the febrile period, and calculated as the area under the body temperature curve, but above the 39.5°C cutoff line.

Statistical analysis.

The mean results for each measure in each phase were compared with Student's 2-sample t-test .

Data condensation.

The phases of the progression of acute JD were defined in terms of the rate of increase and decrease of the viral titre in the plasma of infected animals, and are shown below. The mean of data obtained for each parameter measured during the experiment was calculated for the period of each phase, and these were then compared for statistical differences using Student's 2-sample t-test .

Table 4.3 Definitions of the phases of virus load during JD in animals experimentally infected with JDV_{PUL}.

Phase.	Name	Definition.
1	**Lag**	No virus detected in consecutive samples PI
2	**Initial appearance**	Doubling time of viral titre in plasma <10 h.
3	**Initial plateau**	Doubling time of increases to >10 h
4	**Second growth phase**	Doubling time <10 h.
5	**Plateau**	Doubling time or half-life >10 h.
6	**Resolution**	Begins when half-life first <10 h (>10hs later).
7	**Post recovery**	24 h immediately following "recovery".

Results.

Typical febrile responses to infection with JDV $_{TAB}$ and JDV $_{PUL}$.

A range of responses to infection were seen (Figure 4.1) and these are summarised in Table 4.3. In the majority of experimentally infected animals, the clinical disease followed a typical course: an incubation period of several days, followed by a febrile period with a range of clinical signs, resolving as body temperature returned to normal. The animals either recovered, or died shortly after the febrile period. Apart from this typical disease profile, 2 other patterns of disease were also seen (described below). The temperature profiles from 17 mock-vaccinated Bali cattle experimentally infected with JDV during 4 separate infection experiments were analysed to determine the range of values that are normally observed during the acute phase of JDV infection. The mean temperatures post-infection were calculated for the normal responders for each of the 4 trials, as the virus strains used and doses varied between trials. The mean incubation period before the onset of fever in both of the groups that were infected with JDV $_{TAB}$ (Trials II and III) was 7 days, even though the animals in Trial III received a lower infection dose, and the animals infected with JDV$_{PUL}$ developed fever an average of 1.2 days later.

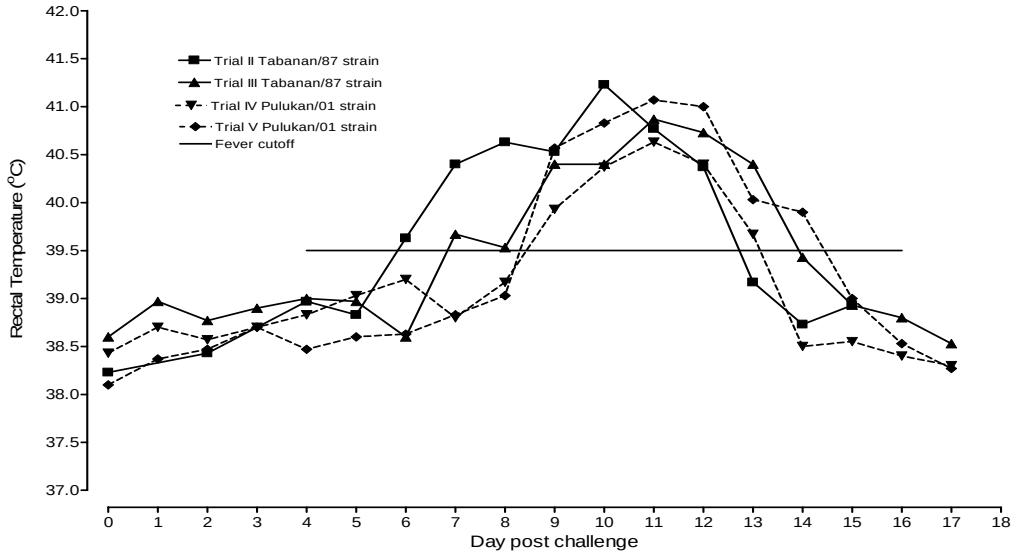


Figure 4.1 Mean daily rectal temperatures of groups of cattle following infection with 2 strains of JDV (JDV$_{TAB}$ and JDV$_{PUL}$).

Table 4.4 Summary of clinical and virological observations in 12 cattle experimentally infected with 2 strains of JDV.

Strain of JDV and dose	Animal	Peak viraemia (days post-infection)	Infection to onset of fever (days)	Infection to peak fever (days)	Infection to end of febrile period (days)	Duration of febrile period (days)	Fever score *	Outcome of infection
JDV_{TAB} 1000 ID_{50}	**CB 61**	10	7	11	13	6	5.7	Survived
	CB 62	11	7	11	13	6	6.1	Survived
	CB 63	8	6	11	13	7	9.9	Survived
JDV_{TAB} 400 ID_{50}	**CB 83**	12	7	11	14	7	5.5	Survived
	CB 85	11	9	11	15	6	5.6	Survived
	CB 86	12	7	12	15	8	6.8	Survived
	Mean	**10.67**	**7.17**	**11.17**	**13.83**	**6.67**	**6.60**	
JDV_{PUL} 1000 ID_{50}	**CB 108**	10	5	12	14	9	9.4	Survived
	CB 110	11	10	12	13	3	2.2	Survived
	CB 135	13	9	11	16	7	8.2	Survived
	CB 137	12	9	11	15	6	7.3	Survived
	CB 138	10	9	12	15	5	4.5	Survived
	Mean	**11.20**	**8.40**	**11.00**	**14.60**	**6.00**	**6.32**	
	CB 109	11	9	11	12	3	2.2	Died

*** Fever score defined as area under body temperature curve above the 39°C cutoff line.**

Table 4.5 Viraemia (genome copies/ml plasma) at various stages of the febrile response in animals that survived the infection. Onset of fever onset was determined as the first day after infection when rectal temperature exceeded 39.5 and end of febrile period was defined as the last day where rectal temperature exceeded 39.5°C.

Strain of JDV and dose	Animal	At onset of febrile period (log_{10})	At peak viraemia (log_{10})	At end of febrile period (log_{10})
JDV_{TAB} 1000 ID_{50}	**CB 61**	11.81	12.22	9.77
	CB 62	11.09	11.97	11.17
	CB 63	10.56	11.29	6.81
JDV_{TAB} 400 ID_{50}	**CB 83**	10.08	11.76	9.82
	CB 85	9.15	10.63	6.53
	CB 86	10.33	11.11	7.48
	Mean	**10.50**	**11.50**	**8.60**
JDV_{PUL} 1000 ID_{50}	**CB 108**	6.28	11.76	8.39
	CB 110	9.95	10.63	
	CB 135	9.74	11.11	9.64
	CB 137	9.57	10.41	7.61
	CB 138	8.96	10.34	8.14
	Mean	**8.90**	**10.85**	**8.44**

Mean viraemia at the onset of fever was lower for typical responders infected with JDV_{PUL} than in those infected with JDV_{TAB}. The peak viraemia, and that at resolution of fever, was also lower in these animals. The numbers for viraemia at onset and at peak were significantly lower ($p=0.02$ and $p=0.05$ respectively) when log_{10} values were compared.

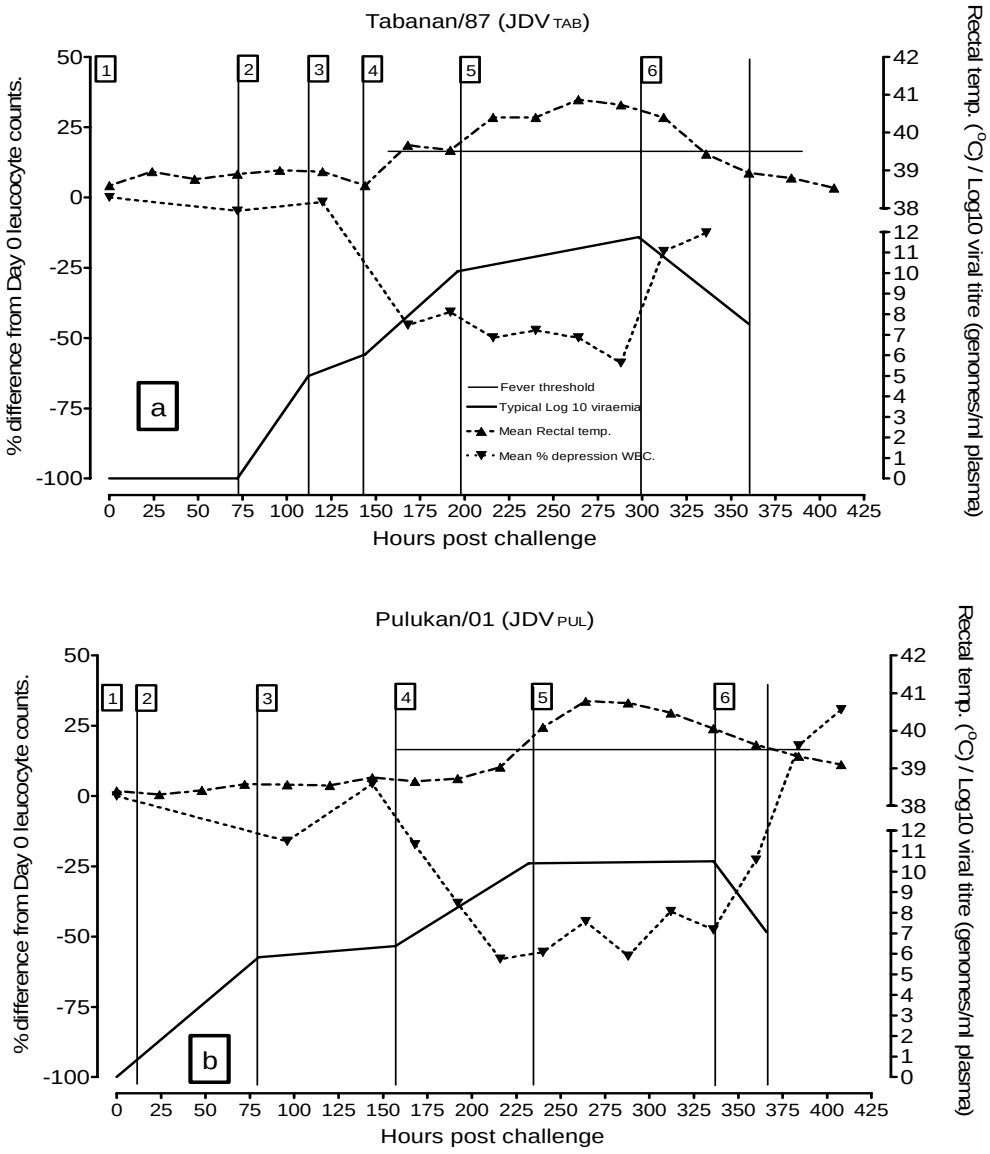


Figure 4.2 Differences in the clinical parameters (febrile response and leucopenia) and virus load in animals infected with 2 strains of JDV that survived the infection. Phases of the acute disease are labelled 1-6 and defined as described below.

The occurrence and duration of the phases, and the means of the values of the observed and calculated measures for each phase could then be compared between strains with a simple 2-sample t-test . Figure 4.2 shows schematic illustrations of the identified phases of acute JD following infection with 2 different strains of virus.

Phase 1, a lag phase where no virus was detectable in samples taken up to 144 h post-infection, was seen in 2/3 of the animals that responded typically to infection with 400 ID_{50} of the JDV_{TAB} strain (Figure 4.2a and Tables 4.6 and 4.7). In contrast, all animals inoculated with an estimated 1000 ID_{50} of JDV_{PUL} had a detectable plasma virus load at the first sampling time-point (Figure 4.2b). During Phase 2, a rapid increase in the numbers of RNA genome copies/ml in plasma occurred in both groups. In 2 out of 3 animals infected with JDV_{TAB} this phase was extended out to the start of Phase 5, with no slowing of the rate of increase in viral numbers, Phase 3, as seen in all animals infected with JDV_{PUL}. The initial appearance of viral RNA in the plasma was accompanied by a slight decrease in leucocyte counts, more marked in JDV_{PUL}, and a transient slight but significant ($p=0.019$) increase in rectal temperature, at day 3-4 post infection, in animals infected with JDV_{TAB}. The numbers of viral genomes/ml plasma increased faster in those animals infected with JDV_{TAB} than with JDV_{PUL}, where a decrease in virus load was seen during Phase 3 in 2 out of 4 animals. These animals then tended to have a longer Phase 4 than JDV_{TAB} infected animals, reaching a lower maximum titre. In JDV_{PUL} infected animals, the lower peak virus load was largely due to a slower increase in numbers or actual decline seen in Phase 3, as although the rate of increase in the virus load during Phase 4 was slower, the duration of this phase was longer.

The body temperature slowly increased during Phase 4, and all animals became febrile, defined as a rectal temperature of 39.5°C or over, as the plasma viraemia increased. Animals infected with JDV_{PUL} became pyrexic at a lower plasma viral titre than those infected with JDV_{TAB}. The clinically febrile period encompassed Phase 5: a plateau phase, with maximum and constant viral titres in plasma suggesting the rate of viral replication was in equilibrium with the rate of clearance. This phase was also characterised by continued fever and low leucocyte counts. This plateau phase was longer in JDV_{PUL} infected animals, but was followed by a shorter Phase 6, as defined

by a return to a rectal temperature of less than 39.5°C. The lower maximum viraemia reached meant that the animals returned to normal more rapidly despite clearance half-lives being longer in these animals. As viral titre started to spontaneously decrease in Phase 6, leucocyte counts rapidly increased to the levels seen prior to challenge, or higher, and body temperatures returned to normal. The only animal that died after infection with JDV in these experiments, CB 109, showed a decrease in body temperature but a large increase in viral titre with no recovery of the leucocyte counts (Table 4.7). Phase 7 was the 24 h period immediately following recovery from clinically elevated rectal temperture.

Table 4.6 Comparison of summary of clinical and virological response in animals surviving infection with JDV $_{TAB}$ or JDV $_{PUL}$ (Viraemia expressed as log_{10} viral genomes/ml).

Parameter	Strain of JDV	Phase						
		1	2	3	4	5	6	7
Rectal temperature (°C)	JDV $_{PUL}$		38.4	38.8	39.5	40.7	39.8	38.8
	JDV $_{TAB}$	38.9	39.1	38.8	39.3	40.5	39.9	38.9
Leucocyte count (% of value at day 0)	JDV $_{PUL}$		98.6	90.2	67.8	45.3	60.9	98.2
	JDV $_{TAB}$	95.1	76.5	104.5	73.6	49.4	67.2	
Plasma viraemia (log10 genomes/ml)	JDV $_{PUL}$		3.3	4.5	7.6	10.0	9.4	7.9
	JDV $_{TAB}$	0	4.8	5.5	9.0	10.9	9.5	7.9
Growth rate (γ)	JDV $_{PUL}$		0.14	0.02	0.13	0.03	-0.12	-0.11
	JDV $_{TAB}$	NA	0.15	0.05	0.12	0.02	-0.11	-0.15
Doubling time/ clearance half-life (h)	JDV $_{PUL}$		4.89	-22.04	5.52	33.38	-7.82	-7.91
	JDV $_{TAB}$	NA	4.50	13.70	5.88	69.40	-6.38	-6.42
Duration (h)	JDV $_{PUL}$		62.4	72	96	102	24	24
	JDV $_{TAB}$	96	104	48	72	80	64	24

Table 4.7 Summary of clinical and virological responses in animal CB 109 infected with the Pulukan/01 strain that died 12 days after infection (Viraemia expressed as log_{10} viral genomes/ml).

Parameter	Phase.						
	1	2	3	4	5	6	7
Temperature (°C)		38.6	38.8	39.5	40.0		
WBC (% day 0)		100.00	71.63	63.49	61.71		
Plasma viraemia (log 10)		5.39	6.89	9.90	11.06		
Growth rate (γ)		0.148	0.086	0.148	0.132		
Doubling time/half-life (h)		4.67	8.05	4.69	5.26		
Duration (h)		72	120	24	72		

Table 4.8 Results of comparison, using a 2 sample t-test , of the mean of measures of parameters for each phase of the clinical and virological response in surviving groups after infection with JDV_{TAB} and JDV_{PUL}.

Parameter	Differences in values
Growth rate	No significant differences in any phase
Doubling time/half-life	No significant differences in any phase
Plasma viraemia	No significant differences in any phase
Body temperature	Phase 2: JDV_{TAB} infected animals significantly higher (p=0.019).
Duration of phase	Phase 2: JDV_{TAB} infected animals significantly shorter (p=0.016). Phase 6: JDV_{TAB} infected animals significantly longer (p=0.004).

Atypical febrile responses to infection with JDV_{TAB} and JDV_{PUL}.

6 animals did not respond as expected to infection. 4 of these, CB 64, CB84, CB134 and CB 136 did not develop a typical febrile response during the period of analysis, with no febrile response in 2 animals (CB 84 and CB 136) and only a transient mild febrile response with a maximum temperature of 39.7°C seen after 4 (CB134) and 16 days (CB64) (Figure 4.3 and Table 4.9). Previously these may have been discounted as failed infections but the presence of viral RNA in plasma indicated that replication of virus had occurred in these animals (Table 4.10). 2 patterns of viral replication were seen in these animals: CB 64, CB 84 and CB 134 exhibited an undulating viral titre increasing to a maximum titre of about 10^8 genomes/ml; CB 136 showed a more typical viraemic pattern (Figure 4.3) and 2 animals, CB 109 and CB 111, had

elevated body temperatures for an extended period before the onset of a typical febrile response, with virus present but not increasing in titre during this period (Figure 4.3).

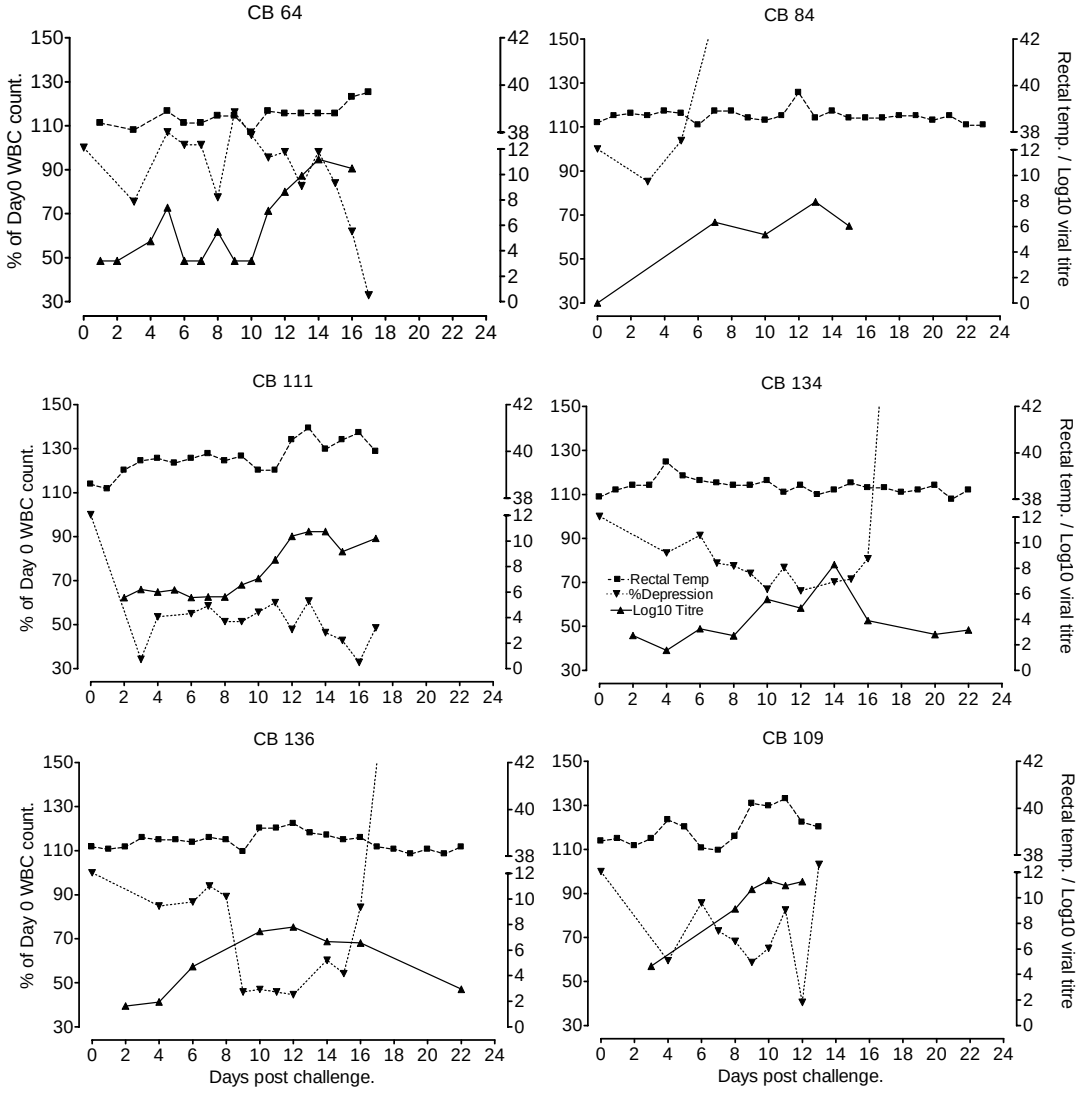


Figure 4.3 The clinical and virological responses in animals that responded atypically to infection and 1 animal (CB 109) that died.

Table 4.9 Observed parameters in animals that did not respond to infection as expected.

Strain of JDV and dose	Animal	Peak viraemia (days post-infection)	Infection to onset of fever (days)	Infection to peak fever (days)	Infection to end of febrile period (days)	Duration of febrile period (days)	Fever score *	Outcome of infection
JDV_{TAB} 1000 ID_{50}	**CB 64**	14	15	16	17	2	0.2	Survived
JDV_{TAB} 400 ID_{50}	**CB 84**	13	NA	NA	NA	NA	0	Survived
JDV_{PUL} 1000 ID_{50}	**CB 111**	3 and 14	3 and 12	7 and 13	10	7 and 6	7.2	Died
	CB134	14	4	4	5	1	0.1	Survived
	CB 136	12	N/A	N/A	N/A	N/A	0	Survived

NA denotes not applicable as no febrile response was detected.

Table 4.10 Viraemia (genomes/ml plasma) at relevant fever setpoints in animals that responded atypically.

Animal.	Viraemia at onset of fever	Peak JDV RNA (copies/ml plasma)
CB 64	3.0×10^{10}	1.5×10^{11}
CB 84	N/A	8.6×10^{7}
CB 111 (biphasic)	1.6×10^{6} (2.3×10^{10})	1.6×10^{6} (3.0×10^{10})
CB134	3.75×10^{1}	1.95×10^{8}
CB 136	N/A	6.47×10^{7}

Discussion.

It is evident that experimental infection with JDV results in a broad spectrum of related responses, ranging from apparent failure of infection to the death of the animal. This broad range means that it is difficult to define the response, and this is necessary to determine the efficacy of a vaccine. Clinical disease in 70% of the experimentally challenged animals followed a typical progression, with symptoms developing as the plasma virus load increased, and resolving with clearance of the virus, which even during the course of the experiment in some cases reached undetectable levels. Virus is still present, however, for a long period at low levels after apparent clinical recovery, as blood taken from recovered animal was found to be infectious with a titre of approximately 10 ID_{50} 9 months after infection (Soeharsono *et al*., 1995b).

The progress of a typical acute episode, as measured by rectal temperature, could be correlated to the plasma virus load. However, at set points in the disease process, determined using rectal temperature as a correlate of infection, such as the beginning and end of the febrile period, considerable variation was found in the levels of the plasma virus load in animals infected with JDV_{PUL} or JDV_{TAB}. This high level of animal to animal variation, and in particular the difference in incubation period before onset of the febrile period, would normally make it difficult to compare the effects of infection with 2 viral isolates using conventional statistics. To address this problem, in this thesis a simple model was developed that better describes the progress of acute JD. This model uses differences in the calculated doubling time and rates of clearance of virus in plasma, to divide the acute period of disease into several phases, defined in Table 4.3. These were identified consistently throughout each infection with the JDV_{PUL} strain of JDV, and it was decided to use this as the exemplar of a typical JDV infection. To examine differences in viral strains and subsequently the effects of vaccination, using data obtained from qRT-PCR assays for levels of JDV RNA in the plasma, data condensation of the other measures taken during the experiment was required to allow simple comparison of the effects of infection with the 2 strains of virus.

The proposed model is similar to that used by several groups in attempting to predict from the severity of the initial acute disease, the eventual progression to an AIDS-like state in animals and humans, and also to examine the effects of intervention with drugs or vaccination on viral load (Perelson *et al.*, 1996; Bonhoeffer *et al.*, 1997; Nowak *et al.*, 1997; Stafford *et al.*, 2000). One of the limitations of this type of analysis is that the levels of viral RNA in plasma only represents overspill of virus from the main sites of replication. Large numbers of JDV infected cells have been reported in the spleen and lymph nodes during the acute phase of the disease (Chadwick *et al.*, 1998), and it is likely that the levels in plasma are probably only a proxy for the actual overall viral load at any point in the disease. However, quantification of plasma viral RNA is the only measurement available to easily and directly examine the effects of strain difference or vaccination on the progress of the disease.

The differences in the plasma virus load in the early stages of JD seen after infection with the 2 strains of JDV, could be due to both host and virus related factors. These could include factors such as the retention of virus in the tissues, differences in the rates of viral replication and viral infectivity between strains, or differences in the ability of the early innate immune response, before the development of an adaptive response, to control virus.

The differences in the length of time between viral challenge and onset of clinical disease is closely correlated to the number of infectious virus particles used in the challenge dose (Soeharsono *et al.*, 1990). Initial experiments on transmission of JDV established that the plasma of animals in the second day of the febrile period contained 10^8 ID_{50} infectious doses / ml. These results were obtained by titration in animals. Quantification by RT-qPCR of the levels of virus in the plasma of animals at the same stage of fever as those used in the initial experiments consistently gave considerably higher virus numbers. This was expected as the numbers of virus particles capable of causing infection is estimated to be 1% or lower of the total particles present. The use of RT-qPCR routinely to calculate the challenge dose routinely was not possible in Indonesia, so a capture ELISA for JDV CA was developed, and as this showed good correlation with the results obtained by RT-

qPCR was utilised to determine the challenge dose, with results confirmed later by RT-qPCR (Stewart *et al.*, 2005). This approach allowed the numbers of infectious virus particles administered to be determined to within a log of the value desired.

The reasons for the difference in the disease process induced by JDV_{TAB} and JDV_{PUL} are not known but small differences in sequences between these 2 strains of JDV may be sufficient to cause differences in the phenotype. Preliminary sequence analysis has shown that JDV_{PUL} is genetically similar to JDV_{TAB} but differs in the *vif* (S.Peterson, personal communication), *env* and LTR sequences. In MVV, single mutations in the gene encoding CA and in *vif* together have been shown to confer a highly pathogenic phenotype on an apathogenic infectious clone (Gudmundsson *et al.*, 2005). JDV_{PUL} also has a 7 base insertion in the U3 region of the LTR compared to JDV_{TAB} (Setiyaningsih, 2006; Desport, 2007). Differences in this region of the LTR increased replication capacity of EIAV *in vitro* (Madden and Shih, 1996). JD has similarities in aetiology to EIAV, in that the same organs are implicated as sites of tissue replication, and that both diseases are ultimately successfully immunologically controlled by host reponses, although the transient episodes of viraemia declining in severity over time characteristic of EIAV are not reported in cattle after recovery from the acute stage of JD.

Animals infected with JDV_{TAB} do not have detectable virus for several days post infection, in contrast to JDV_{PUL} where virus was detectable at an earlier stage. The actual moment of detectable levels of virus appearing in the plasma was not always accurately determined, as insufficient samples were taken in the first few days post-infection, and it is likely that all the animals would have had a definable initial lag phase if sufficient samples had been taken. The time of initiation of Phase 2 would also have been influenced by too long an interval between the early sampling times, and this was addressed in later experiments. The start and end of Phase 3, and subsequent phases, were more accurately identified as more samples were taken at this stage of the infection.

The site of initial replication of JDV is not known, although virus is present in the spleen on the first day of fever (Chadwick *et al.*, 1998). The splenomegaly

characteristic of the disease (Dharma *et al.*, 1991) indicates that initial infection is originally blood-borne, and as antigens, such as viruses, arriving by this route are trapped by professional antigen presenting cells in the spleen it is probable that this is site of initial replication (Janeway, 2001). Initial rates of replication may differ due to the LTR insertions mentioned above, or perhaps the ability of the viral strains to disseminate may differ. The sudden appearance of virus in the blood probably represents rapid release from another body compartment, probably the spleen. The total white cell count over these initial days post-infection declined slightly in both groups, possibly as infected cells are removed from the circulation by the spleen, and this first round of replication must give rise to the virus seen in the initial appearance. The tropism of JDV has not been confirmed, but in common with all other lentiviruses is likely to be either lymphocytes or macrophages (Chadwick *et al.*, 1998).

The plasma virus load in JDV_{TAB} infected animals rose rapidly from first detection whereas in animals infected with JDV_{PUL} there was a significantly longer initial plateau phase (Phase3). This may indicate retention of virus in tissue compartments, or possibly an earlier and more effective innate immune response to JDV_{PUL}, leading to pyrexia at a lower viral titre in the plasma, due to release of inflammatory mediators from macrophages, neutrophils and activated lymphocytes. Phase 4 was associated with a rapid increase in plasma viral titre, and a corresponding leucopaenia which was previously shown to be due to lymphopaenia and neutropaenia (Soesanto *et al.*, 1990). The febrile onset occurred during this phase, and is associated with a marked proliferative response in the T cell areas of the spleen (Dharma *et al.*, 1994). This lymphoproliferative response would provide an increased number of target cells for the virus if the tropism is for T-cells, allowing unrestrained viral replication to the high levels seen at the peak of viraemia. The observation that the plasma virus load in JDV_{TAB} infections was consistently higher (up to 100-fold) than that with JDV_{PUL}, despite there being no difference in the time post-infection, suggests that immune activation occurs at a lower plasma viral load in animals infected with JDV_{PUL}. The earlier response seems to limit viral replication, resulting in lower plasma virus load and a lesser febrile response. The leucopenia during Phases 2 and early Phase 3 as

cells are recruited to the spleen in the immediate period post-infection was greater when animals were infected with JDV_{PUL} than with JDV_{TAB} (Figure 4.2). The rebound increase of peripheral leucocytes later in this phase also indicated greater cell proliferation in response to infection in these animals, than in JDV_{TAB} infected animals. The peak viraemic period during JDV_{PUL} infection was only ~0.5 log lower than JDV_{TAB}, indicating that growth and release continues during Phase 5 for longer with this strain. Phase 5, where the plasma virus load plateaus, presumably as rates of increase match clearance rates, may represent saturation of the system as all available target cells are depleted and the innate immune system starts to alter the balance in favour of viral clearance, or may be the result of an efficient CMI response being rapidly induced. As control of viral replication is established, and the target cell population is depleted, the levels of virus fall quickly, in Phase 6, and the leucopaenia resolves, with rapid increase to higher than original levels of leucocytes. In this phase there must be some immune control mechanism to limit resurgence of virus as the immune system returns to normal, and a very low viral setpoint is established, the duration of which is not known.The significantly longer Phase 6 caused by slower clearance rates of JDV_{TAB} meant that numbers of virus at clinical recovery were similar.

Despite the apparent presence of a marginally more effective early response to JDV_{PUL} over the entire series of infection experiments using these 2 strains, higher mortalities occurred in animals challenged with $JDV_{PUL,}$ surprisingly given the lower mean maximum temperatures and viraemia characteristic of disease caused by this strain. The requirement of lentiviruses for terminally differentiated cells as sites of replication means that induction of an early response that is ineffective can potentiate the severity of disease by increasing the numbers of potential target cells, and CB 109, infected with $JDV_{PUL,}$ which died after a short low fever, illustrated this. This animal had entirely failed to control viral replication, with no decrease of circulating virus seen although it had apparently recovered as fever abated. Total depletion of target cells responsible for cytokine release and pyrexia could account for this. Experimentally infected animals normally have a case fatality rate of about 17% (Soesanto *et al.*, 1990). In a control group of 4 or 5 animals this represents a mortality

of less than 1 animal per group which explains the death of only 1 normally responding cow after infection with the JDV_{PUL} strain.

Over the entire series of experiments atypical responses were seen in control animals infected with both strains of virus, and these responses fell into 2 categories. Some animals with no febrile response (Figure 4.3) showed an apparently typical pattern of viral replication, with an increase to a plateau followed by spontaneous clearance but with maximum levels of virus somewhat lower than in animals showing clinical signs e.g CB136. Similar responses have been seen in African green monkeys challenged with SIV_{AGM}, where virus replicates to a high level without immune activation and the associated pathology (Broussard *et al.*, 2001) and in captive sooty mangabeys naturally infected with SIV_{SM} (Rey-Cuille *et al.*, 1998). Other cows showed an undulating viraemia during the acute phase, with no accompanying febrile response. Studies in at-risk humans report that between 10% and 60% of patients infected with HIV-1 do not experience a primary acute disease, although in studies including control groups the incidence of acute disease is higher, from 81% to 87% (Kahn and Walker, 1998). Mock vaccinated cats in FIV vaccine trials also show a small proportion that are resistant to infection (Verschoor *et al.*, 1996).

During the recovery phase of JD the earliest detectable immune responses are against the JDV CA protein (Hartaningsih *et al.*, 1994). The typical responders in these trials had detectable antibodies between 41-58 days post infection and a peak response at 122-125 days post infection (Personal communication, J. Lewis; data not shown). Sampling of 2 responding animals in Trial V was discontinued before the full response was developed as these animals were used in a further vaccine trial. The atypical responders from these groups did not develop antibody responses to JDV CA that were detectable using the JDV antibody ELISA (Table 4.11), although weak responses were observed by Western blot (Personal communication, J. Lewis; data not shown). No febrile response was seen during viraemia and very low levels of antibody to JDV proteins were detected. The failure of these animals to seroconvert strongly to the presence of highly antigenic CA protein is interesting. Suppression of the immune system by proteins expressed from endogenous retroviruses has been reported (Larsson and Andersson, 1998). Dual infection with other

immunosuppressive viruses may be a factor, some studies have shown cattle infected with both BIV and bovine leukaemia virus (BLV) are immunosupressed (Hidalgo and Bonilla, 1996), although this is not reported by other investigators (Flaming *et al.*, 1997; Isaacson *et al.*, 1998).

All animals were given a large challenge dose, to ensure as far as possible that infection would occur. A perhaps more realistic approach, of repeated small challenges to establish infection (McDermott *et al.*, 2004), could not be implemented due to time and cost restraints. Producing a standard infection dose (Table 1), was difficult as there is no culture system or infectious clone for JDV, and storage of purified virus in liquid nitrogen is not possible for extended periods in Bali. The protocol used to produce the virus for experimental infection involved passage in a donor animal, with live virus infectious doses being prepared on the second day of fever (Kertayadnya *et al.*, 1993). There is a possibility of enhancing pathogenicity of the infection strain by selecting for virus that has evaded immune control by this method. The virus commonly used in infection experiments in macaques, $SHIV_{89.6P,}$ has developed extreme pathogenicity through serial passage as it has lost its dual receptor requirement, needing only CXCR4 to infect CD4 T-cells (Feinberg and Moore, 2002). Cats challenged with FIV obtained during the acute phase of disease in a donor animal developed fatal disease more rapidly than when virus was prepared from the same donor cat during the chronic phase of disease (Diehl *et al.*, 1995). However, JDV transmission is postulated to be via an insect vector, suggesting that transmission would be most likely to occur during the acute phase of the disease when viral titres are high. Therefore, taking the infectious dose at the height of fever is likely to at least reflect the viral population at the moment of natural transmission, even if the dose is unrealistically high to ensure infection within the timescale of the experiment.

In conclusion, the divison of the disease into these phases, and the condensation of the data from each phase allowed statistical comparisons to be made between measures taken in different phases from the 2 infection groups. Subtle differences between the diseases seen post-infection with 2 strains could be identified. Further work with larger numbers of animals, and more frequent sampling throughout the

immediate period post infection and up to the point where virus becomes undetectable in all animals will shed more light on possible variations in the pathogenicity of different strains of JDV.

Chapter 5

The effect of an inactivated viral vaccine on plasma viral load during experimentally induced Jembrana disease.

Summary.

In this chapter the effects of vaccination with a tissue derived, inactivated viral preparation on viral growth and clearance during the acute phase of experimentally induced JD in Bali cattle are described.

Introduction.

The most successful vaccines against the animal lentiviruses are those using whole attenuated, or inactivated virus preparations. At least some measure of protection has been induced with inactivated viral preparations in cats against feline immunodeficiency disease (Yamamoto *et al.*, 1993; Hosie *et al.*, 2000), in horses against equine infectious anaemia (Issel *et al.*, 1992), and against intravaginal challenge with $SHIV_{89.6}$ in macaques mucosally immunised with inactivated $SHIV_{89.6}$ (Ambrose *et al.*, 2003). However, potentiation of lentiviral disease following vaccination is occasionally seen in goats vaccinated against CAEV (Russo *et al.*, 1993).

An inactivated viral vaccine against JDV has been developed in Bali, Indonesia. It consists of detergent inactivated virus in homogenised spleen tissue taken from an experimentally infected donor animal at the peak of viraemia (Hartaningsih *et al.*, 2001). However, widespread prophylactic vaccination regimes are not employed in Indonesia, as the expense of producing the vaccine is high, and there are fears of spreading not only JDV, but other pathogens, if inactivation should fail. The efficacy of this vaccine has been determined previously by analysis of clinical symptoms and pathological signs and has been shown to reduce the febrile period and severity of disease after challenge with live virus (Hartaningsih *et al.*, 2001). Advances in the technology of qRT:PCR have allowed accurate quantitation of the levels of circulating lentiviral RNA in plasma after infection, with low limits of detection and a wide dynamic range compared with p24 ELISAs (Hofmann-Lehmann *et al.*, 2000;

Klein *et al.*, 2003). The recent development of a qRT:PCR assay in our laboratory for the detection of JDV RNA in plasma (Stewart *et al.*, 2005), has facilitated a much closer examination of the effects of different vaccination regimes on the rate of viral replication and clearance.

Materials and methods.

Experimental cattle.

Experimental cattle were purchased and maintained as described previously in Chapter 4.

Vaccine.

The vaccine was prepared as previously described (Hartaningsih *et al.*, 2001). Briefly, the capsule was removed from the spleen of an animal experimentally infected with the Pulukan strain of JDV, and was homogenised at a ratio of 10% w/v in Medium 199 (ICN) at 4^0C. Virus was inactivated by addition of Triton-X 100 to a final concentration of 1% v/v and subsequent stirring for 30 mins at room temperature. The antigen preparation was mixed and homogenised with a mineral oil adjuvant (MOA) at a ratio of 2:1 MOA:antigen. 2 vaccinations, each of 1 ml, were administered intramuscularly into the neck of the cattle, 56 and 28 days before challenge. Control animals were given 3 x 1 ml doses of GST **(**1 mg/ml) homogenised in an equal volume of IFA. Post vaccination, the cattle were examined daily for any sign of clinical disease. Rectal temperatures were taken, and the absence of a febrile response was used to confirm the complete inactivation of JDV in the vaccine.

JDV challenge dose.

All animals in this experiment were challenged with an estimated $4x10^2$ 50% ID_{50} of the JDV_{TAB} strain of JDV, determined by ELISA as previously described in Chapter 4. Confirmation of the viral titre in the plasma of the donor animal was performed retrospectively by a quantitative RT-PCR as described below.

Samples.

The sampling regime for the animals was as described in Chapter 4.

Viral RNA extraction.

The method was as described in Chapter 4, using 280 µl samples of plasma instead of 140 µl.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR).

qRT-PCR was performed as described in Chapter 4, with the master mix made using the Access RT-PCR kit from Promega, and 2 µl samples of RNA.

Production of biotinylated proteins for use as Western blot antigens.

To produce antigens that would be recognised only by antibody to the vaccine protein in sera from experimental cattle, and not by those raised to the GST fusion partner, the same genes as were cloned into the pGEX-6P expression vector (Chapter 6) were also ligated into the PinPoint Xa-1T-tailed vector (Promega) for the expression of biotinylated proteins in *E. coli*. The vector is supplied with all 3 possible reading frames to suit cloning of any amplicon with an overhanging A, as added by *taq* polymerase during amplification, and genes of interest were cloned in-frame with the N-terminal biotinylatable tag. The resulting recombinant plasmids were transformed by heat-shock into JM109 competent *E. coli*. A 200 µl volume of recovered transformed cells were plated on LB agar plates supplemented with 100 µg/ml ampicillin and incubated (37˚C, overnight). The colonies were screened for transformants with inserts in the correct orientation by directional PCR (Chapter 3) using gene specific forward primers and SP6 reverse vector primers. Thermocycling conditions were standard, with a T_m of 50˚C. Positive clones were verified by sequencing as described in Chapter 3. Protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM to log-phase cultures of bacterial clones in LB broth, supplemented with 100 µg/ml ampicillin and biotin at a concentration of 2 µM. The expression of the proteins was monitored by PAGE and a Western blot technique using an alkaline phosphatase labelled streptavidin probe and WesternBlue chromogenic substrate (Promega).

Seroconversion of vaccinated animals.

Prior to challenge, the serum of all the animals were tested by Western blot for the presence of antibodies to JDV viral proteins, as described in Chapter 4, using viral antigens expressed as described above.

Calculations.

Doubling times, fever score and statistical analysis were performed as described in Chapter 4.

Results.

All animals vaccinated with the inactivated JDV vaccine had seroconverted, prior to challenge, to the viral CA and MA proteins, when assayed by Western blot. Only 1 had detectable antibody to SU, and none had detectable antibodies to viral TM or Tat. No control animals had pre-existing antibodies to JDV (Table 5.1).

Table 5.1 Seroconversion to JDV proteins prior to challenge, as determined by Western immunoblot.

JDV protein antigen	JDV vaccinated animals				Mock vaccinated animals			
	CB 75	CB 76	CB 77	CB 78	CB 83	CB 84	CB 85	CB 86
CA	+	+	+	+	–	–	–	–
MA	+	±	±	+	–	–	–	–
SU	–	–	±	–	–	–	–	–
TM	–	–	–	–	–	–	–	–
Tat	–	–	–	–	–	–	–	–

± denotes weak response

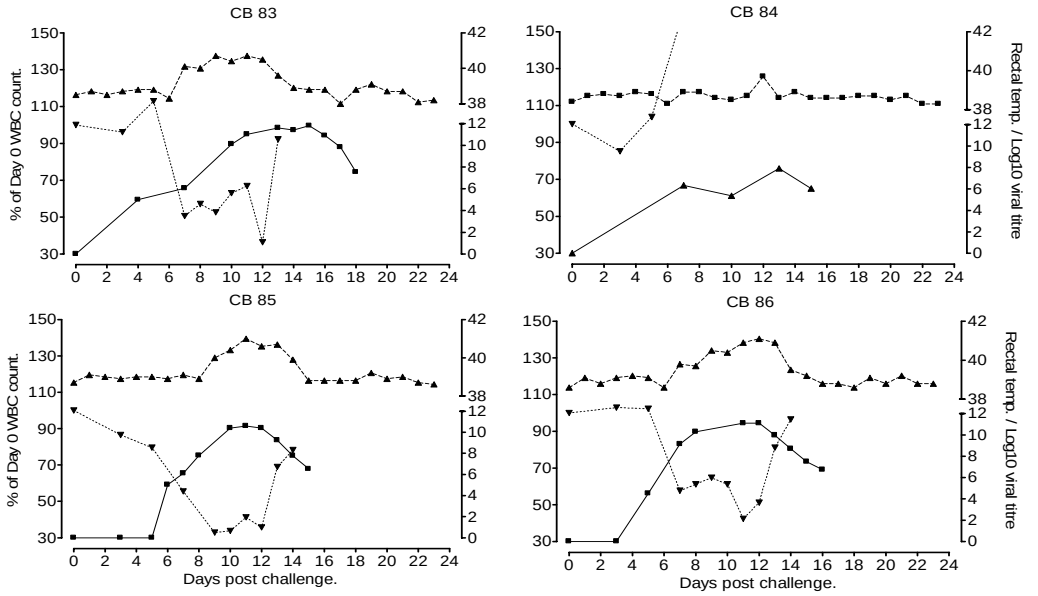
Challenge dose.

The challenge dose estimated by ELISA was 400 ID_{50} but by qRT-PCR was determined to be 1.1 $x10^3$ ID_{50}.

Duration and severity of febrile response.

3 of the 4 animals in the control group developed a febrile response (a rectal temperature of $\geq 39.5^0$C) within 7-8 days of infection; the other animal in the control group did not develop a febrile response. In the vaccinated group, 2 of the 4 cattle developed a febrile response after a similar period, but animal CB76 developed fever 18 days post-infection, and a further animal did not develop a febrile response. The absence of a febrile response in some infected animals was previously observed in Chapter 4, and these animals were considered as atypical responders.

Of the animals that developed a febrile response, the mean duration of fever was significantly shorter ($p=0.05$) in the vaccinated group (5.3 days) than the control group (7 days). The results for each animal are shown graphically in Figures 5.1 and 5.2, and summarised in Table 5.2.



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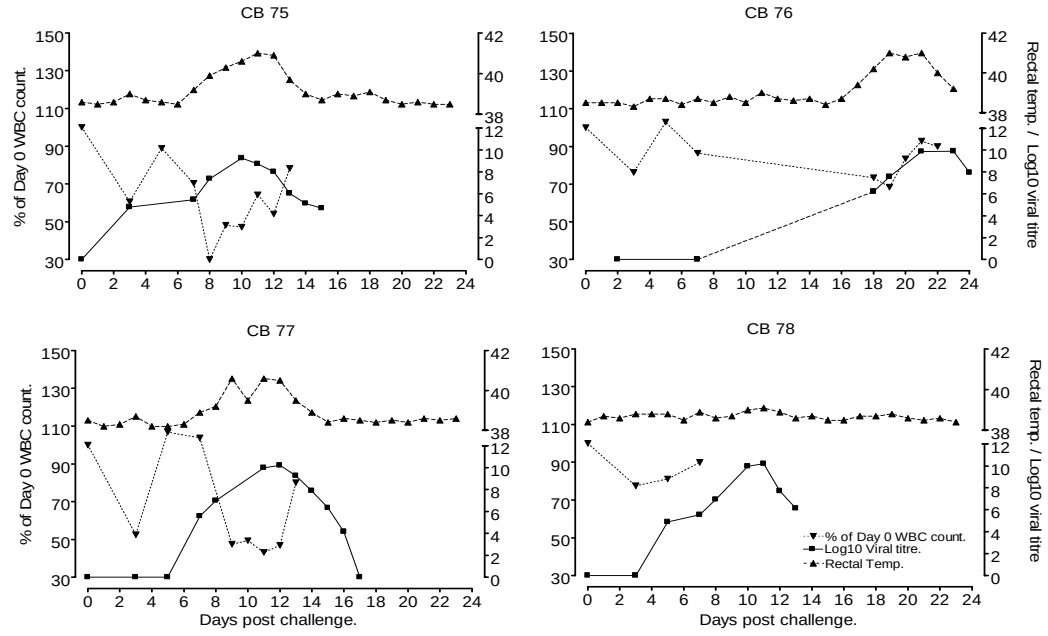


B

Figure 5.1 (A) Clinical response of individual mock vaccinated cattle experimentally infected with JDV, showing typical (CB 83, 85, 86) and atypical responses (CB 84).
(B) Clinical response of individual vaccinated cattle experimentally infected with JDV, showing typical (CB 75, 76,77) and atypical responses (CB78).

Table 5.2 Clinical parameters in mock vaccinated and vaccinated cattle experimentally infected with JDV. Animals that did not develop clinical changes (atypical responders) have been excluded from statistical calculations

Group	Animal	Peak viraemia (days post infection)	Time from infection to onset of fever (days)	Time from infection to peak fever (days)	Time from infection to end of febrile period (days)	Duration of febrile period (days)	Fever score (Area under curve)	Duration of leucopenia (days)
Control	**CB 83**	12	7	11	14	7	5.5	1
	CB 85	11	9	11	15	6	5.6	0
	CB 86	12	7	12	15	8	6.8	3
	Mean	**11.67**	**7.67**	**11.33**	**14.67**	**7.00**	**5.97**	**2**
Atypical	**CB 84**	13	12	12	12	1	0.4	-
Vaccinated	**CB 75**	9	8	10	14	6	5.4	1
	CB 76	20	18	19	23	5	5.5	0
	CB 77	10	9	9	14	5	3.2	4
	Mean	**13.00**	**11.67**	**12.67**	**17.00**	**5.33**	**4.70**	**1.66**
Atypical	**CB 78**	22	-	-	-	-	-	ND

Table 5.3 Plasma virus load determined by qRT-PCR at set points in the disease process (figures in italics inferred using calculated doubling times).

Group	Animal.	Viraemia at onset of fever.	Peak viraemia	Viraemia at end of febrile period
Control	**CB 83**	$1.21x10^{10}$	$5.79x10^{11}$	$6.62x10^{9}$
	CB 85	$7.12x10^{8}$	$4.26x10^{10}$	$3.41x10^{6}$
	CB 86	$2.14x10^{10}$	$1.27x10^{11}$	$3.04x10^{7}$
	Mean	**$1.14x10^{10}$**	**$2.5x10^{11}$**	**$2.22x10^{9}$**
Atypical	**CB 84**		$8.57x10^{7}$	
Vaccinated	**CB 75**	$2.34x10^{7}$	$1.79\ x10^{9}$	$1.29x10^{5}$
	CB 76	*$3.38x10^{8}$*	$7.91\ x10^{9}$	$4.44x10^{4}$
	CB 77	*$1.11x10^{8}$*	$1.63\ x10^{10}$	$2.11x10^{6}$
	Mean	**$1.57x10^{8}$**	**$8.67x10^{9}$**	**$7.61x10^{5}$**
Atypical	**CB 78**	$9.36x10^{9}$	$1.47x10^{10}$	$5.16x10^{7}$

Table 5.4 Statistical analysis of significant differences between observed parameters.

Measure.	Differences.
Duration of fever.	Control significantly longer (p=0.033)
Viraemia at onset.	Controls higher (approaching significance; p=0.06)

Duration and severity of leucopenia.

White cell counts for the typical responders in the vaccinated group declined sharply immediately after challenge, followed by a transient recovery to levels similar to before infection. This recovery was not sustained, and a moderate leucopenia followed. The typical responders in the control group did not show a comparable initial decrease and rebound in white cell numbers, and the duration of the leucopenia tended to be longer, although using the definition previously applied (<4000 WBC/µl) there was no difference between the groups. The results for each animal are shown graphically in Figures 5.1.

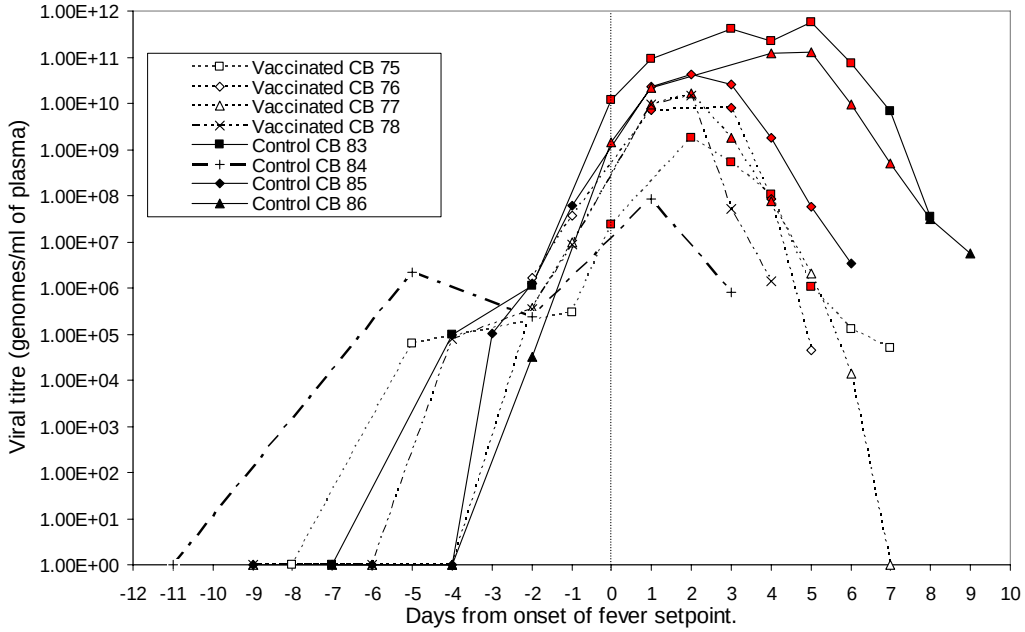


Figure 5.2 Direct comparison of viral plasma titres (genomes/ml aligned to day 0 of fever setpoint) from individual vaccinated and control animals. (Red filled points indicate that sample was taken on a day when animal was febrile, dotted lines indicate viral load in vaccinated animals, solid lines indicate data from control animals).

All infected animals in both the vaccinated and mock vaccinated groups had detectable levels of circulating viral RNA in the plasma over the period of the experiment, even in those animals with no febrile response. The control animal that did not develop a febrile response had an undulating plasma virus load, peaking at 10^8 virus genomes/ml. The vaccinated animal that did not develop a febrile response

also showed a plasma virus load typical of that detected in febrile animals, with a peak of 10^{10} genomes/ml. The peak viraemia in all of the vaccinated animals was less than that of those control animals that developed a febrile response. In the vaccinated animals, peak plasma virus loads coincided with either the day of onset of fever or the next day, then fell rapidly, whereas higher levels of plasma virus persisted in the febrile controls for several days, in 2 animals (CB83, CB86) not peaking until 6 days after onset of fever (Figure 5.2).

Modelling of disease.

Utilising the method described in Chapter 4, the disease period for each animal was divided into phases, utilising the rates of increase and decrease of viral RNA in the plasma to define the phases. The phases of the disease process, based on the rates of increase and decrease of viral RNA in the plasma, for each group are shown in Figure 5.3. The rectal temperature and leucocyte counts are means for each day of the experiment. The mean viraemia line represented condensed data from each phase.

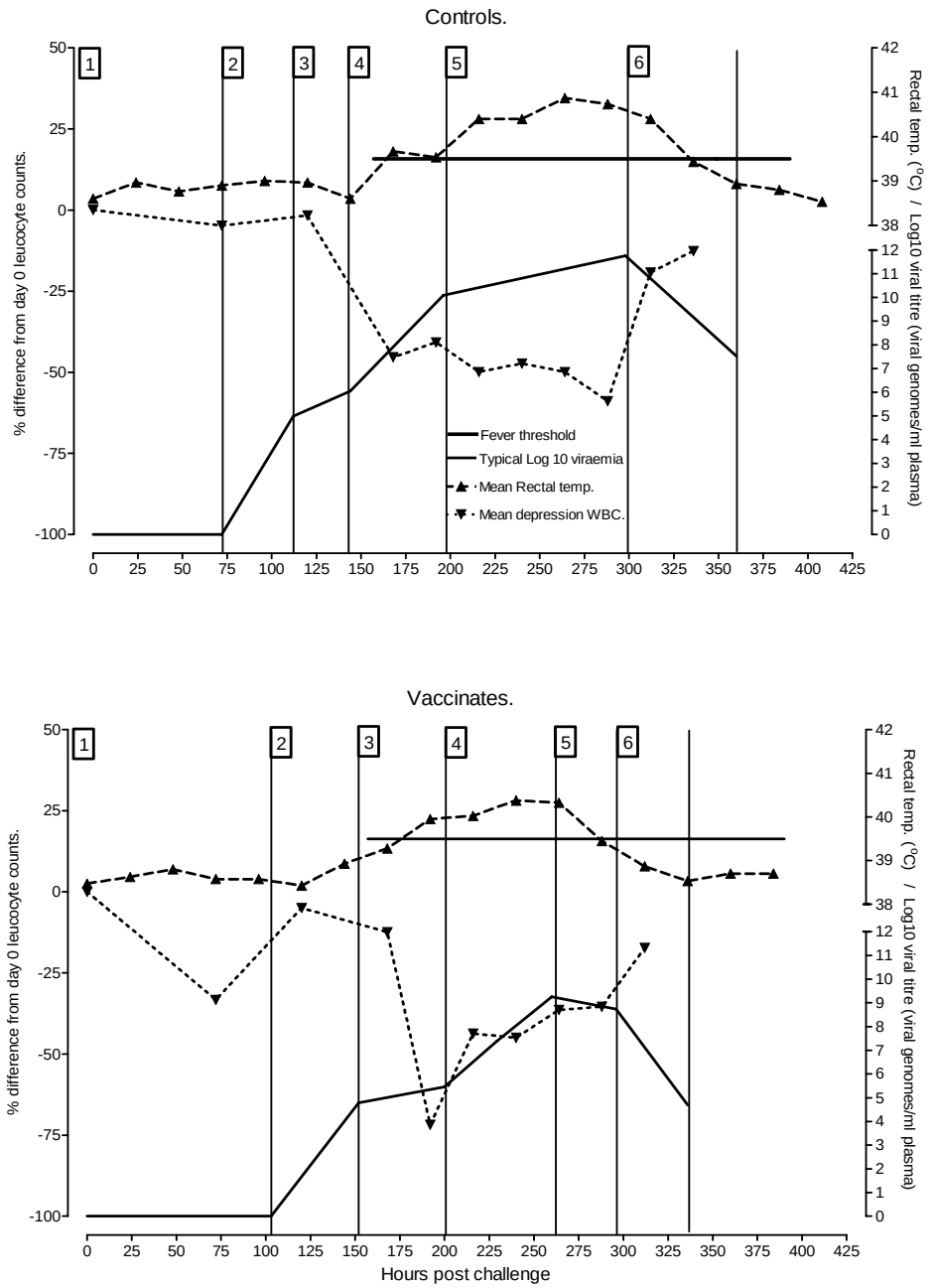


Figure 5.3 Schematic representation of condensed viraemia data, with mean daily temperature and WBC counts for control and vaccinated groups.

Table 5.5 Condensed data from each phase for control and vaccinated groups.

Parameter	Group	Phase						
		1	2	3	4	5	6	7
Rectal temperature (°C)	**Control**	38.9	39.0	38.7	39.0	40.5	39.6	38.9
	Vaccinated	38.5	38.9	38.7	39.5	40.2	39.7	38.8
Leucocyte count (% Day 0)	**Control**	95.1	85.6	133.0	73.6	49.4	67.2	
	Vaccinated	89.4	78.8	84.5	69.7	61.0	71.4	
Log_{10} plasma virus load (genomes/ml)	**Control**	0	4.38	5.68	7.84	10.91	8.90	7.89
	Vaccinated	0	3.49	4.48	7.57	9.65	7.81	5.69
Growth/ clearance rate (γ)	**Control**		0.137	0.010	0.100	0.019	-0.105	-0.146
	Vaccinated	0	0.120	0.091	0.087	-0.053	-0.174	-0.096
Doubling time/ clearance half-life (h)	**Control**	0	5.38	-4.42	7.16	69.40	-6.69	-6.42
	Vaccinated	0.00	3.89	25.96	5.55	123.39	-4.36	-8.33
Duration of phases (h)	**Control**	96	120	60	72	80	66	24
	Vaccinated	104	80	136	72	36	48	24

Growth rate is a measure of the rate of increase or decrease in viral titre, essentially the gradient of the titre curve in each phase, doubling time is derived from this, and is the calculated time for viral numbers/ml to double at that growth rate.

Table 5.6 Significant results, obtained by statistical analysis, using an unpaired t-test to compare the condensed data.

Parameter	Statistical significance
Rectal temperature	Phase 1: Controls significantly higher (p=0.014)
Leucocyte count	Phase 2: Controls higher count, approaching significance (p=0.06)
Log_{10} plasma virus load	Phase 5: Controls significantly higher (p=0.02) Phase 6: Controls significantly higher (p=0.05)
Growth / clearance rate	Phase 2: Controls higher, approaching significance (p=0.06) Phase 5: Controls significantly higher (p=0.05) Phase 6: Controls lower, approaching significance (p=0.07)
Doubling time / clearance half-life	Phase 6: Control half-life longer, approaching significance (p=0.06).
Phase duration	Phase 5: Control significantly longer (p=0.02)

Control animals had significantly (p=0.014) higher rectal temperatures and a less marked reduction in total leucocyte counts immediately post-infection than the vaccinated animals. A higher viral growth rate in the control animals seen in Phase 2 (p=0.06) and Phase 5 (p=0.05), combined with slower clearance rates during Phase 6 gave significantly higher viral loads for a longer duration during Phases 5 and 6 in this group (Table 5.6).

Discussion.

Because of the acute nature of JD, with about 20% case fatality rates at this stage with no recurrence, vaccination is aimed at amelioration of the acute phase of the disease (Hartaningsih *et al.*, 2001), to decrease the duration and severity of the viraemia thus reducing the chances of transmission.

This current study differs in some regards from previous studies using the same vaccine preparation, where 4/10 vaccinated animals had no febrile response or leucopenia after challenge (Hartaningsih *et al.*, 2001). In these previous reports, non-vaccinated animals were used as controls, whereas in the current experiments the control animals were vaccinated with an unrelated protein (GST) in a mineral oil adjuvant. An ideal control would have been vaccination with uninfected spleen homogenate. However, the development of a febrile response 7-8 days after infection, the depletion of leucocytes, and development and duration of viraemia in these mock vaccinated animals was similar to that seen previously in non-vaccinated animals experimentally infected with a similar infectious dose of virus (Soeharsono *et al*., 1995b), confirming that the mock-vaccination had no effect on disease progression.

In previous experiments (Hartaningsih *et al.*, 2001), indirect indicators of the severity of the viral infection, such as changes in mortality rates, severity of leucopenia, duration of the febrile period, and the length of the incubation period were used to assess vaccine efficacy and to describe any amelioration of the disease process. The most significant difference between the methodology employed in the current experiments and that used previously was the development of a qRT-PCR assay to quantify viral load (Stewart *et al.*, 2005). This procedure was incorporated to try to determine if the apparent protection of animals seen in the earlier experiments was due to suppression of virus replication, and perhaps the induction of a sterilising immunity, or if other mechanisms were involved. Additionally, the production of recombinant proteins, and use in Western blot analysis, allowed assessment of the antibody responses induced by the inactivated vaccine preparation.

The use of the inactivated viral vaccine has been demonstrated to induce an antibody responses to several viral proteins (Hartaningsih *et al.*, 2001) and the nature of this

response was demonstrated by the use of various recombinant viral protein antigens (Table 5.1). A response to SU was not detected in a majority of vaccinated animals but whether this was due to a lack of a response or an inability to detect the response with the recombinant protein antigens is unknown. Antigenicity of the SU could have been affected by the detergent used to inactivate the virus, or the use in the Western blots of a truncated recombinant antigen possibly lacking epitopes recognised by sera raised to native protein. The absence of a detectable response to Tat was not unexpected, as Tat is reported to be highly expressed early in infection only (Setiyaningsih, personal communication) and may not be present at high levels in the vaccine preparation prepared from spleen tissue taken on the second day of the febrile period. Experience in this laboratory is that naturally recovered animals have a weak or undetectable antibody response to Tat, indicating weak antigenicity of this JDV protein, at least for the humoral immune system (Setiyaningsih, personal communication).

3 different patterns of infection were seen in cattle infected with JDV in the current experiment. A normal pattern of disease, where infection was followed by an acute disease process with a marked febrile response and concomitant viraemia, was seen in 6/8 animals; this was typical of the JD infections reported by Soesanto *et al* (1990). A second pattern of limited viral replication without a febrile response was seen in 1 of the 8 animals, and a third pattern of infection was seen in another of the 8 animals where there were high levels of circulating virus with no febrile response. Similar varying patterns of viral replication have been seen in SIV-infected macaques in vaccine trials, with varying patterns apparently unrelated to any effect of the vaccines used (Waisman *et al.*, 1996; Silvera *et al.*, 2002). For clarity, animals with a normal pattern of infection will be discussed first.

The most obvious indicator of protection is a decrease in the duration of the febrile response. The mock-vaccinated control animals had a significantly longer febrile period than the 3 responder vaccinated animals, and a shorter incubation period before the onset of fever. Quantification of the plasma virus load in responder animals showed that, as expected, the duration of the febrile period correlated with the plasma virus load, with elevated temperatures occurring only when the plasma

virus load was high (Figure 5.1). However, there was a trend for the vaccinated animals to become febrile at a lower viral titre than the control mock-vaccinated animals (Figure 5.2), although a failure to collect plasma RNA samples on the day of onset of fever for 2 vaccinated animals prevented confirmation of this. The development of fever at lower viral titres and the rebound in peripheral blood leucocyte counts in the vaccinated group indicated a primed CMI response, as inflammatory mediators leading to fever in viral infections include chemokines induced by T lymphocyte-produced interferon gamma (IFNγ). In lentiviral infections it is known that immune activation favours viral replication (Fauci *et al.*, 1991; Poli and Fauci, 1993). Thus there is a balance between rate of viral production and rate of immune mediated viral clearance in a lentiviral infection The vaccinated animals also had markedly slower rates of increase of the plasma virus load, and lower body temperatures over the first 7 days post-infection than the controls ($p=0.06$, Table 5.6), which might have been associated with the presence of specific antibody to JDV as a consequence of the vaccination. Opsonisation of virus by circulating antibody could promote phagocytosis of virus by macrophages via Fc receptor-mediated uptake, or infected cells coated with antibody may be removed by natural killer cells. This would have the effect of removing the replication-enhancing effects of IFNγ earlier in the progression of the infection, decreasing sequestration of T-cells to the secondary lymphoid tissue and so lessening the destruction of the germinal centres seen in unmodified JD. This would have the further effect of allowing earlier immune reconstitution, and subsequent development of an antibody response post challenge. Vaccinated animals showed improved preservation of immune function post challenge, maintaining or increasing antibody levels to viral proteins. Control animals showed no induction of antibodies for an extended period post challenge (personal communication, J.Lewis). Antibody does not seem to be involved in the resolution of natural infections, as circulating antibodies to JDV components are not detectable until at least 8 weeks post-infection (Hartaningsih *et al.*, 1994) coinciding with recovery of the lymphoid architecture (Dharma *et al.*, 1994).

Peak plasma virus loads were significantly higher in control animals during Phases 5 and 6 and the duration of the high levels of viraemia in Phase 5 was significantly

longer in these animals (Table 5.6), indicating vaccination had an effect on the kinetics of virus replication. Although the vaccinated animals had apparently recovered after a shorter fever than the controls, circulating virus was still present on the day of recovery, although the titres were far lower than in the control animals at this setpoint. The febrile response in one control animal, CB 83, resolved while viral titre was still very high. Failure to maintain a strong response may be due to the immunosuppressive nature of the disease, in that depletion of effector cells by the viral load prevents maintenance of response and hence a longer high level viraemia. Pro-inflammatory cytokines have been shown to be associated with viral replication *in vivo* (Poli *et al.*, 1999; Decrion *et al.*, 2005), and immune activation enhances viral replication in chimpanzees infected with HIV-1 (Fultz *et al.*, 1992). In this trial, following failure to establish sterilising immunity and thus prevent establishment of an infection, the induction of a vigorous and successful immune response will be accompanied by fever. In this case there will be a balance between the effectiveness of viral clearance, at what level of virus the response is induced, and the intensity of that response and associated inflammation. The trend for a response to be induced by lower levels of virus in the vaccinated animals highlights the balance between effective immune activation and potentiation of viral replication.

A classical indicator of infection with JDV is the precipitous drop in circulating leucocytes as the febrile response develops. Although the precise cellular tropism of the virus is unknown, infected cells appear earliest in the spleen (Chadwick *et al.*, 1998). Presumably, antigens that are introduced to the host directly via the blood are taken up by antigen presenting cells in the spleen and the recruitment of naïve T cells and other lymphocytes would contribute to the loss of these cells from the circulation and the ensuing enlargement of this organ. Vaccinated animals showed a sharp decrease in total leucocyte counts 3 days post-infection, corresponding to a slight transient rise in body temperature, which was more marked in the animals with the more severe leucopenia. Previous studies have shown that the leucopenia in experimentally infected non-vaccinated animals was due mainly to a severe lymphopenia, with mild neutropenia (Soesanto *et al.*, 1990). Possibly the initial fall in leucocyte counts reflects earlier clearance of infected cells in the peripheral blood by

antibody dependent cellular cytotoxicity (ADCC) or a potentiation of initial infection of monocytes by Fc-mediated uptake of antibody coated virus. Increased trafficking of activated lymphocytes to the lymph nodes could also account for the initial decrease in peripheral blood lymphocytes. Transient increases in JDV titre have been noted in other experiments around 3 days post-infection (unpublished observation). Unfortunately, it was not possible to perform cell proliferation assays to directly measure specific cell responses in this experiment. It would be useful to examine cell population subsets early in infection before virus is detectable to determine differences in the profile between vaccinates and controls, to gain an insight into the very early pathogenesis of JD.

One of the most interesting observations during these experiments was that JDV infection in naïve animals did not necessarily cause a febrile response. 2 animals, CB84 and CB78 (1 from each group), did not develop fever. Failure of challenge was suspected with the control animal, and protection was hoped for in the vaccinated animal. qRT-PCR revealed that both animals had circulating virus. The control animal, CB84, had a lower level of virus replication than the other animals and also exhibited a sharp increase in leucocyte counts at the point where the other animals showed the most rapid decrease, just before the leucocyte examinations were discontinued in this animal. The vaccinated animal, CB 78 had a comparable viraemia to the other members of the group, with only a slight contemporary body temperature rise. As the target cells of JDV are suspected to be both macrophages and lymphocytes (Dharma *et al.*, 1991; Dharma *et al.*, 1994), activated anti-JDV T-cells in vaccinated animals may present a different pool of potential host cells in these animals, leading to productive infection, but not necessarily acute clinical disease.

Without further work to identify the target cell type for JD, and to explore the role of antibody, complement, and the cytokine milieu in the immediate post-infection period, as well as the role of the genetics of the cattle in determing susceptibilty to JDV, it is impossible to know the reasons why some animals tolerate high viral loads while others succumb to acute disease or even die.

In conclusion, despite the problems inherent in attempting immunological control of lentivirus infections, it is apparent that vaccination of Bali cattle with inactivated virus not only reduced the duration of the acute clinical signs of infection but also reduced the magnitude and duration of viraemia when they were subsequently infected with JDV. This should result in reduced mortality, and a reduction in the opportunity for transmission of the virus which should facilitate the control of JD.

Chapter 6

Evaluation of recombinant Jembrana disease virus Tat and capsid proteins as vaccines for control of Jembrana disease.

Summary.

In this chapter, the use of a pGEX-6-P system (Pharmacia) to express JDV proteins in *E. coli* is described. The proteins were expressed as insoluble inclusion bodies and methods for purification and solubilisation of the proteins are described. 3 vaccine trials are described where Tat and CA protein vaccines were tested in Bali cattle, individually and in combination. After vaccination 3 times at approximately fourteen day intervals, the efficacy of the vaccine was tested by subsequent challenge of the vaccinated cattle 14-21 days later with JDV, and assessment of the effects of vaccination on resultant clinical signs and plasma virus load. A strong antibody response to the administered CA and Tat proteins occurred. There was evidence of ameliorisation of the disease process in animals vaccinated with a combination of CA and Tat, which resulted in lower plasma virus loads in the early stages and at the peak of the acute disease process, and lower levels of fever and leucopenia. Vaccine proteins administered with incomplete Freund's adjuvant were more efficacious than vaccines administered with QuilA.

Introduction.

Attempts to control JD in Indonesia include the use of a detergent-inactivated tissue-derived whole virus vaccine which has been shown (Chapter 5) to reduce the febrile period and viral load in experimentally vaccinated and challenged animals (Hartaningsih *et al.*, 2001). Because such a vaccine can only be produced on a limited and non-commercial scale, is expensive and might contain adventitious agents, the use of subunit virus proteins produced by recombinant DNA technology has been investigated.

Vaccination with recombinant protein expressed in *Escherichia coli* has induced protection against diseases such as foot and mouth disease (Goeddel *et al.*, 1979) and influenza (Neirynck *et al.*, 1999) but attempts to induce a protective immune response

to lentiviruses by vaccination with recombinant structural proteins have met with varying degrees of success, from partial protection against homologous FIV challenge in cats vaccinated with SU expressed in bacteria (Leutenegger *et al.*, 1998) to enhancement of disease in animals vaccinated with baculovirus expressed glycoproteins (Wang *et al.*, 1994; Siebelink *et al.*, 1995; Raabe *et al.*, 1998).

Several recombinant proteins were trialled as potential vaccines for the control of JD and the results are reported in this chapter. Only results from cattle vaccinated with recombinant GST fusions to the CA, Tat and MA proteins in various combinations are presented; other proteins including SU and TM were trialled but without detectable efficacy and these results have not been included.

The CA protein has been trialled by various groups as a means of induction of a protective immune response to lentiviruses (Caley *et al.*, 1997; Steger *et al.*, 1999; Fraser *et al.*, 2002; Soutullo *et al.*, 2005). It is also known that Bali cattle vaccinated against JDV with the tissue-derived whole virus vaccine, and naturally recovered animals, also develop strong antibody responses to CA (Desport *et al.*, 2005) which has at least 3 separate antigenic domains, one of which is the highly conserved major homology region (Grund *et al.*, 1994), and would be expected to cross-react antigenically with a broad spectrum of virus strains.

MA was included as analysis of antibody responses to JDV proteins in cattle that had been vaccinated with the inactivated viral vaccine (Chapter 4) showed a strong response to MA that correlated with a lack of fever in one animal (J. Lewis, personal communication). The genes for these 2 proteins are sequential and in-frame with each other (Chadwick *et al*., 1995b) and it was decided to trial this protein but expressed together with CA as a single fusion, reducing purification costs.

Tat has been proposed as a suitable candidate for use as a vaccine in many lentiviral infections. The protein is multifunctional, and as well as its primary function as a *trans*activator essential for viral replication, Tat is also released into the milieu around infected cells (Frankel and Pabo, 1988; Verhoef *et al.*, 1996) and can enter uninfected cells by a non-endocytotic pathway, directly through the cell membrane (Tyagi *et al.*, 2001). The subsequent upregulation of viral receptors (Huang *et al.*,

1998) and down regulation of MHC increases the susceptibility of the cell to infection and decreases the likelihood of its recognition by the immune system (Pugliese and Gioannini, 1993). A vaccine inducing a neutralising antibody response to Tat, presents the possibility of interfering with these effects of exogenous Tat, potentially decreasing the severity of JD.

Materials and methods.

Expression of GST fusion proteins.

Proteins were expressed from JDV genes ligated into the pGEX-6-P system (Pharmacia); proteins expressed in this bacterial system are N-terminally fused to glutathione-S-transferase (GST) to aid in purification and identification. The constructs were prepared as previously described (Burkala *et al.*, 1998) and contained the full-length JDV *cap* gene and exon1 of the JDV *tat* gene. The gene coding for MA and CA was amplified by standard PCR methods from plasmid clone#139 with the primers in Table 6.1. A Tm of 54˚C for the first 4 cycles was used followed by 25 cycles with a T_m of 60˚C. The resulting PCR amplicon was digested with BamH1 and short fragments of DNA and contaminating enzyme removed with a PCR cleanup kit (QIAGEN). The purified fragments were ligated into BamH1-digested pGEX 6P1 using standard methods (Sambrook, 1989). Positive clones were identified by directional PCR as described in Chapter 3, and a glycerol stock of a culture of the clone was prepared. Proteins were expressed from log-phase cultures of transformed Top10 *E. coli* in 2YT broth supplemented with 100 µg/ml ampicillin and incubated at 37˚C with vigorous shaking. Protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM when the cultures reached an OD_{600} of 0.6, except for the expression of GST/Tat where induction was initiated at late log phase, when an OD_{600} of 0.9 had been attained. After induction, cultures were grown to stationary phase, and bacterial pellets harvested from 400 ml amounts of culture by centrifugation (8000 *g*, 20 min, 4˚C). Pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% v/v glycerol) and frozen at -80˚C until required.

Table 6.1 Primers used to amplify entire matrix/capsid region of the JDV genome

Primer	Sequence
398JMABamfor	5'-GACCGGATCCGGACGTTTCCAGTCGTTGG-3'
JCAPBamrev,	5'-GCAGGGATCCCAAGAATTGCATCTTCTGTTTAC-3'

Purification of GST fusion proteins.

Tat used in Trial I, a preliminary trial not reported here, was purified from pelleted bacteria lysed by freeze/thawing (5 cycles of -186˚C to +42˚C). The lysate was centrifuged (18,000 *g*, 3 min) and the pellet was washed 5 times by resuspension in wash buffer, (50 mM Na_2HPO_4, 300 mM NaCl, 2 mM EDTA, 10% glycerol, 2% Triton X), mixing with a rotating mixer at 4˚C for 30 min, followed by centrifugation (18,000 *g*, 3 min). The final washed pellet was resuspended in solubilisation buffer A, (6 M urea, 50 mM Na_2HPO_4, 1 mM EDTA, 1 mM mercaptoethanol, 20% glycerol) and rotated (4˚C, overnight). After a final centrifugation, the supernate was analysed for levels of Tat protein by PAGE and Western immunoblotting as described in Chapter 3. Further development of the method subsequently employed a proprietary extraction buffer, B-PER (Pierce) in place of the wash buffer. The final stage of preparation was exhaustive dialysis against PBS, using "Snakeskin" dialysis tubing with a 10kDa molecular weight cutoff (Pierce).

In Trials II and III (Table 6.2), the proteins were purified as for Trial I, but instead of using dialysis to concentrate them, the refolded solutions were ultrafiltered in an pressure filtration cell (Amicon), utilising a 10 kDa cutoff membrane (Ultracell; Amicon) pressurised with nitrogen (70 psi) to give a final volume of 1/20 of the original solution.

In Trials IV and V, protein inclusion bodies were initially purified by repeated washing by centrifugation as above but instead of solubilising, refolding and concentrating as in Trials I, II and III, the inclusion bodies were solubilised in as small a volume as possible of solubilisation buffer C (2 M urea, 2 M Tris pH12, 0.2 mM dithiothreitol).

Preparation of GST for mock vaccination of control cattle.

Bacteria transformed with the pGEX plasmid with no insert were grown in 2YT supplemented with ampicillin (100 µg/ml) and induced with IPTG (final concentration 0.1 mM) at a culture OD_{600} of 0.6. After 4 h of further growth, the bacteria were harvested by centrifugation (18,000 *g*, 3 min), washed in PBS by centrifugation and resuspended in 50 µl of lysis buffer/ml of original culture, and lysed by freezing and thawing as detailed above. After clarification of the lysate by centrifugation, the supernatant was passed 3 times through a column of glutathione / Sepharose resin (0.2 ml volume, equilibrated with PBS). Bound GST was displaced with 10 column volumes of 30 mM glutathione in PBS and was soluble.

Quantification of proteins.

Densitometry was used to quantify the levels of protein in the vaccine preparations. Appropriate dilutions of each preparation were denatured in loading buffer (100 mM Tris-HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS containing 0.4 ml mercaptoethanol and 0.2 ml of bromophenol blue) and electrophoresed in 10% resolving polyacrylamide gels with 4% stacking gels under reducing conditions in a Mini Protean system (Bio-Rad). A range of concentrations between 50 ng and 1 µg/lane of reference proteins BSA (66 kDa) and lysozyme (16 kDa) were also run in parallel lanes on each gel, and where required, molecular weight markers (BioRad PrecisionPlus, 100 kDa, prestained) were included in 1 lane of the gel. The gels were stained with Coomassie brilliant blue (0.05% bromophenol blue in 45% v/v methanol, 45% water 10 % glacial acetic acid, rocking, overnight, RT) and immersed in destain (45% v/v methanol, 45% water, 10 % glacial acetic acid) until the gel background was clear. After washing and rehydrating in distilled water, the gels were imaged on a ProExpress Imaging System (PerkinElmer), and the resulting high resolution images subjected to image analysis with ProPic software (PerkinElmer). Pixel density mapping allowed the amounts of protein present in each non-saturated band on the imaged gel to be calculated. The amounts of the vaccine proteins present were then calculated from a standard curve of the various concentrations of the reference proteins included in each gel. The concentration was determined as the

mean of the concentration obtained using the standard curve for each reference protein.

Vaccine trials.

3 vaccine trials were conducted using 4 or 5 animals per vaccine group as detailed in Table 6.2, with mock vaccinated control animals in each trial. After vaccination, the animals were infected with either JDV_{TAB} or JDV_{PUL} at the doses and times shown in Table 6.2. The method of infection and the sampling regime after infection was as previously described in Chapter 5. All animals were tested for antibody to the vaccine protein prior to challenge by Western blot as previously described in Chapter 3. The plasma viral load was determined as previously described in Chapter 4.

Analysis of the results of the effect of vaccination on clinical parameters and viral load were made as described previously (Chapter 4) by dividing the acute disease resulting from infection into 7 phases and utilising an unpaired t-test to compare the mean values in each phase.

An aliquot of RNA extracted from the plasma of animal CB137 from Trial V, 12 days PI, was included in each qRT-PCR assay of the virus load, as a positive control and to determine the repeatability of the assay.

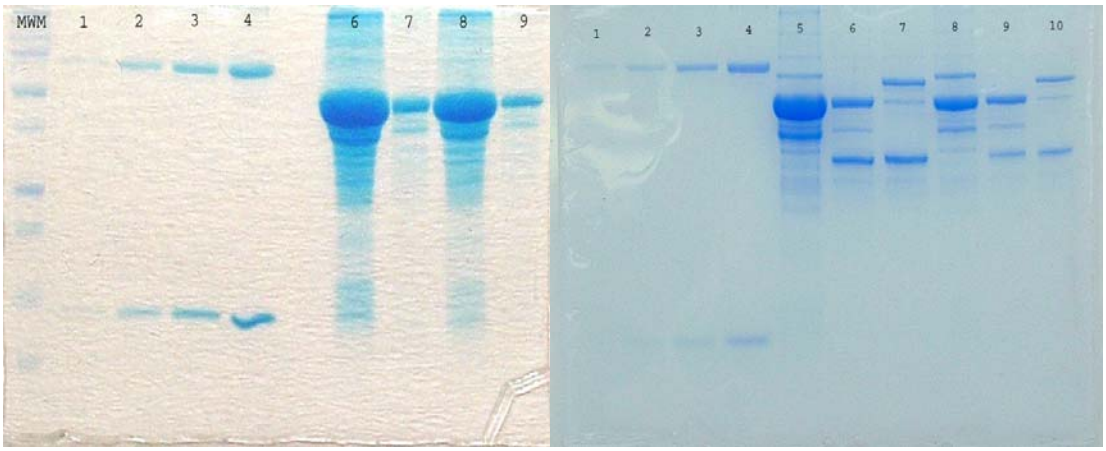
Table 6.2 Details of the vaccination regimes, vaccine antigens used and challenge doses used in 3 vaccine trials to determine the efficacy as vaccines of various recombinant Tat, CA and MA proteins.

Trial	Cattle per group	Antigen	Adjuvant	Protein per dose	Vaccination times (days after initial vaccination)			Time of challenge (days after final vaccination)	Challenge strain (and estimated dose)
					1	2	3		
III	4	Tat	IFA	2 mg	0	14	35	32	JDV_{TAB} (400 ID_{50})
	4	CA							
	4	GST							
IV	4	Tat + CA	IFA	2 mg each protein	0	14	28	21	JDV_{PUL} (1,000 ID_{50})
	4	Tat + CA	QuilA						
	4	No vaccination							
V	5	Tat + CA	IFA	2 mg each protein	0	14	28	23	JDV_{PUL} (1,000 ID_{50})
	5	Tat + MA/CA							
	5	No vaccination							

Results.

Protein expression and purification.

All the GST-tagged proteins used as vaccines were expressed as insoluble proteins that accumulated within inclusion bodies. These were partially purified by differential centrifugation and exhaustive washing to remove soluble contaminating proteins. Subsequent solubilisation of the inclusion bodies in a small volume of 6 M urea resulted in a concentrated protein solution that was quantified by densitometry. Typical examples of these SDS-PAGE gels are shown in Figure 6.1. After high resolution imaging, the pixel density of each imaged band was measured and used to create a 3-D image of that band, which was analysed by the ProPic^{TM} software to give a volume. Plotting of the calculated band volumes against the known concentrations of the reference proteins BSA and lysozyme gave a calibration curve, which was then used to quantify the recombinant protein concentration. The caluclated purity of each final recombinant protein product used in the vaccine trials is shown in Table 6.3.



A **B**

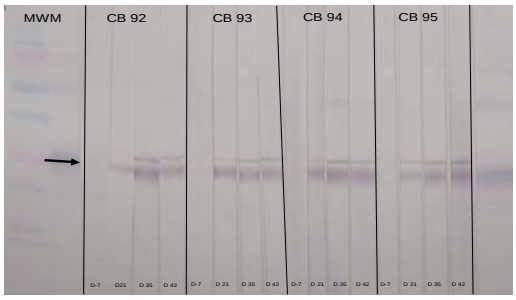
Figure 6.1 A) Typical gel for densitometric quantification of CA protein used for preparation of the vaccine used in Trial V. Gel contains, from left; MWM (BioRad), lane 1;100 ng of BSA and lysozyme, lane 2; 250 ng BSA and lysozyme, lane 3; 500 ng of BSA and lysozyme, lane 4; 1,000 ng BSA and lysozyme, lane 5; empty, lane 6 CA preparation 1, lane 7; 1/10 dilution of CA preparation 1, lane 8 CA preparation 2. lane 9; 1/10 dilution of CA preparation 2.
B) Typical gel for final quantification of protein content of several vaccine preparations. Lane 1 to 4, increasing amounts of BSA and lysozyme as in a), lane 5; CA+SU, lane 6; Tat+CA, lane 7;Tat+MA/CA (5 µl of 1/10 dilution of protein preparations) lane 8; CA+SU, lane 9; Tat+CA, lane 10;Tat+MA/CA (equivalent of 0.5 µl final vaccine preparations loaded/well).

Table 6.2 Final purity of proteins used in vaccine preparations for vaccine Trials III, IV and V. The percentage purity was calculated by densitometric quantification of the electrophoresed proteins from a standard curve derived from reference protein standards using ProPic™ (PerkinElmer) software.

Protein	Trial III	Trial IV	Trial V
GST-Tat	~81.5%	~76%	~88%
GST-CA	~73.4%	~89%	~94%
GST-MA/CA	N/A	N/A	~89%
GST	~100%	~100%	~100%

Seroconversion to recombinant JDV proteins in vaccinated animals.

All cattle developed antibody detectable by Western blotting to the recombinant virus proteins prior to the third vaccine dose, before challenge with infectious virus, but in many cases prior to the second vaccine dose (as shown in Table 6.14 for cattle in Trial 5). Examples of the seroconversion to the vaccine proteins during Trial IV detected by Western immunoblotting are shown in Figure 6.4.



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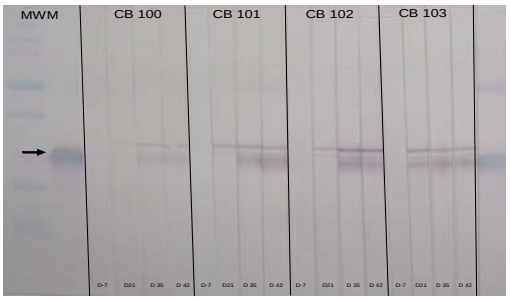

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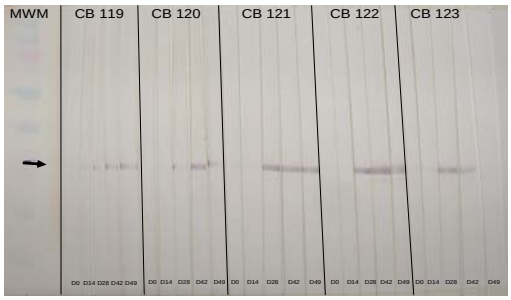

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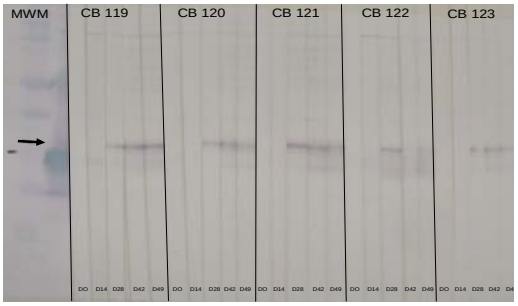

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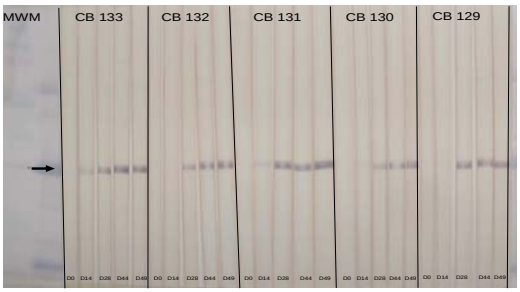


E

Figure 6.4 Examples of Western immunoblots demonstrating seroconversion to protein antigens used as vaccines in groups of cattle as shown in Table 6.1. Standard molecular weight markers are shown on left in each immunoblot, and the relevant antigen is indicated by an arrow.
(A) Response to Tat-IFA vaccine in Trial IV; 4 cattle and 4 lanes per animal with sera collected 7, 21, 35 and 42 days (the day of challenge) after the initial vaccination, indicating the presence of antibody in all 4 cattle commencing 21 days after the initial vaccination.
(B) Response to Tat-QuilA in Trial IV; 4 animals and 4 lanes per animal with sera collected 7, 21, 35 and 42 days (the day of challenge) after the initial vaccination, indicating the presence of antibody in only 1 animal 21 days after the initial vaccination but in all 4 animals commencing 35 days after the initial vaccination.
(C) Response to CA in group of 5 cattle vaccinated with combination of Tat and CA antigens during Trial V; 5 cattle and 5 lanes per animal with sera collected 0, 14, 28, 42 and 49 days (the day of challenge) after the initial vaccination, indicating the presence of antibody at 14 days in 2 cattle and at 28 days in all cattle
(D) Response to Tat in group of 5 cattle vaccinated with combination of Tat and CA antigens during Trial V; 4 animals and 5 lanes per animal with sera collected 0, 14, 28, 42 and 49 days (the day of challenge) after the initial vaccination, indicating the presence of antibody commencing at 28 days in all cattle.
(E) Response to MA in group of 5 cattle vaccinated with combination of Tat, MA and CA antigens during Trial V; 4 cattle and 5 lanes per animal with sera collected 0, 14, 28, 42 and 49 days (the day of challenge) after the initial vaccination, indicating the presence of antibody commencing at 14 days in 2 cattle and all cattle 28 days after the initial vaccination.

Repeatability of quantitative RT-PCR assay for viral plasma RNA.

An aliquot of RNA extracted from the plasma of animal CB 137, 12 days PI was included in each run during assays of the viral load from Trial V, as a positive control and to determine the repeatability of the assay. The standard deviation of the mean for the $\log_{10}$ transformed load determined in each of 12 assays was 0.119.

Vaccine trial with Tat and CA (Trial III).

Seroconversion to recombinant JDV proteins in vaccinated animals.

All cattle developed detectable antibody to the administered recombinant proteins prior to challenge with infectious virus (Table 6.4).

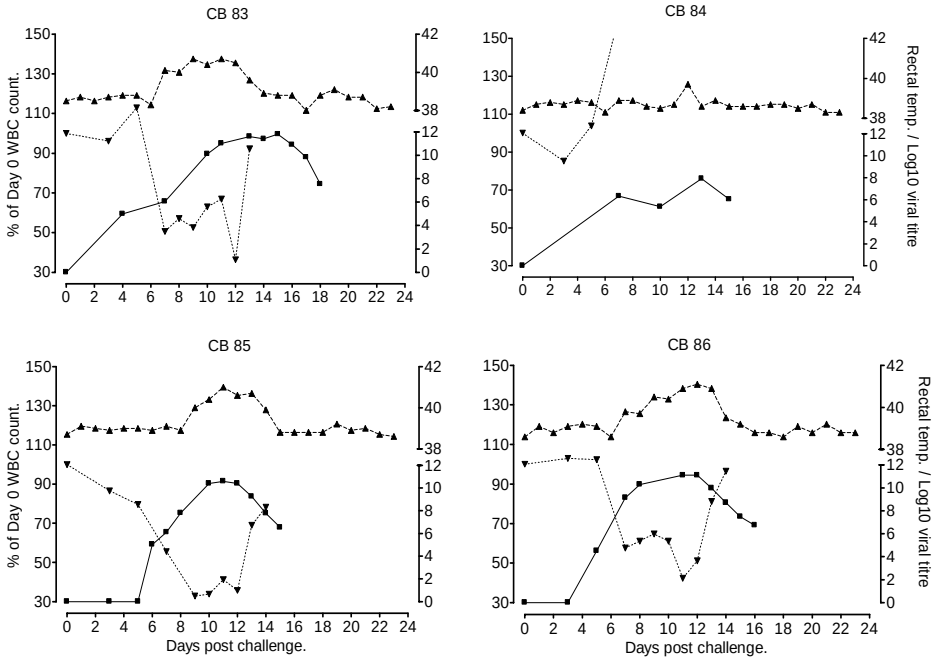
Table 6.4 Seroconversion of animals to administered proteins prior to challenge

Cow	WB Antigen	Day 0	Day 67 (Challenge)
CB 67	Tat (pin)	-	+
CB 68		-	+
CB 69		-	+
CB 70		-	+
CB 79	CA (pin)	-	+
CB 80		-	+
CB 81		-	+
CB 82		-	+
CB 83	GST	-	+
CB 84		-	+
CB 85		-	+
CB 86		-	+

The observed clinical parameters after infection with 400 ID_{50} of JDV_{TAB} during Trial III, after cattle were vaccinated 3 times, with Tat adjuvanted with IFA, are shown in Table 6.5. The only significant differences between the observed parameters in the vaccinated and control groups (Table 6.6) were in the duration of the incubation period that was significantly ($p=0.013$) shorter in the control group.

Table 6.5 Observed clinical parameters during Trial III in cattle vaccinated with Tat (IFA) CA (IFA) and in control animals mock vaccinated with GST (IFA) (as described in Table 6.1) following challenge with 400 ID_{50} of JDV_{TAB}.

Vaccine (adjuvant)	Animal	Time from infection to peak plasma viral load (days)	Duration of febrile period (days)	Time from infection to onset of febrile period (days)	Time from infection to peak of febrile period (days)	Time from infection to end of febrile period (days)	Maximum rectal temp. (°C) during acute disease	Fever score	Outcome
Tat (IFA)	**CB 67**	11	0	N/A	N/A	N/A	39.0	0	Died (d.11)
	CB 68	12	6	9	11	11	41.0	6.1	Survived
	CB 69	11	7	8	11	15	41.1	7.4	Survived
	CB 70	8	0	N/A	N/A	N/A	39.1	0	Died (d.9)
CA (IFA)	**CB 79**	8	2	10	10	12	40.1	1	Survived
	CB 80	12	5	9	12	14	41.4	6.4	Survived
	CB 81	11	6	9	11	15	41.3	6.7	Survived
	CB 82	12	5	9	11	14	41.2	5.4	Survived
GST (IFA)	**CB 83**	12	7	7	10	14	40.7	5.5	Survived
	CB 84	13	N/A	N/A	N/A	N/A	38.9	0	Survived
	CB 85	11	6	7	11	15	41.0	5.6	Survived
	CB 86	12	8	7	12	15	41.1	6.8	Survived



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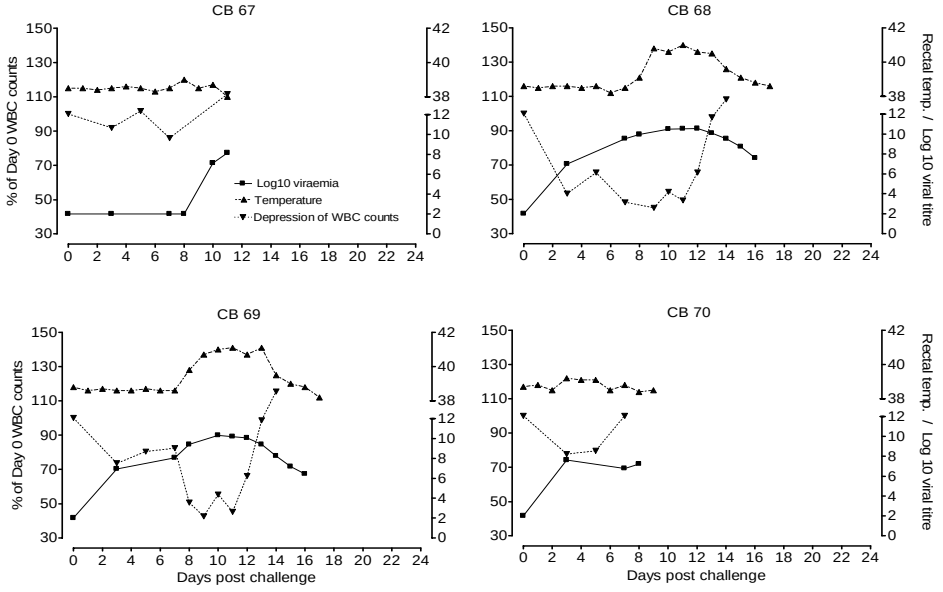


B

Figure 6.5a Graphic depiction of clinical parameters (rectal temperatures [°C], total leucocyte count as percentage of day 0 values, and log_{10} plasma viral load [genomes/ml]) in individual cattle in Trial III after 3 vaccinations with (A) GST (IFA) and (B) Tat (IFA), and challenge with 1,000 ID_{50} of JDV_{PUL}.

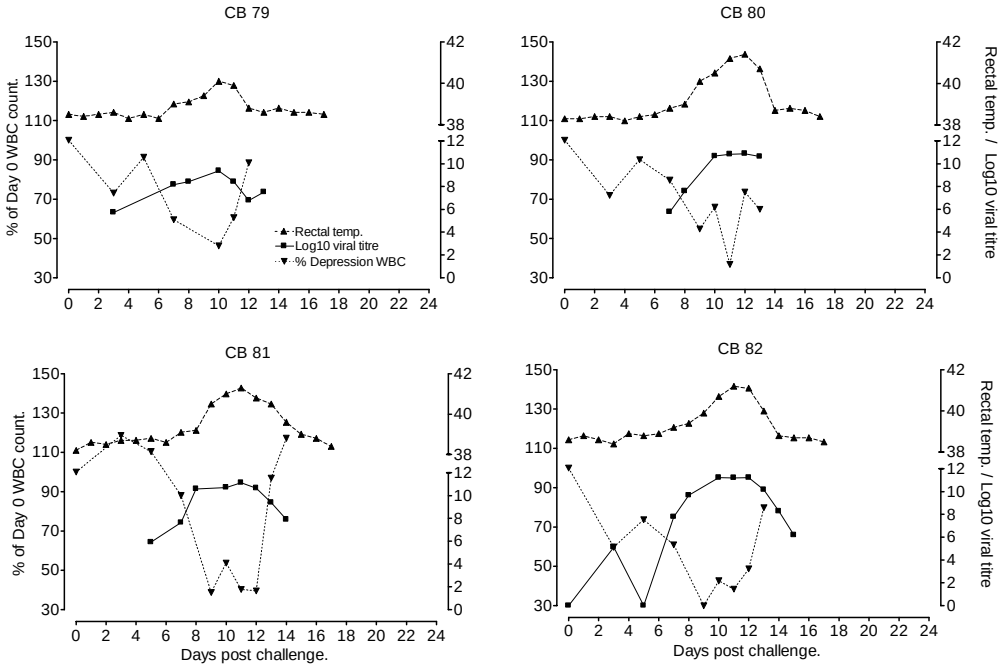


Figure 6.5b Graphic depiction of clinical parameters (rectal temperatures, total leucocyte count as percentage of day 0 values, and log_{10} plasma viral load) in individual cattle in Trial III after 3 vaccinations with CA/IFA, and challenge with 1,000 ID_{50} of JDV_{PUL}.

Table 6.6 Significant differences between the measured responses (shown in Table 6.5) to JDV challenge in Tat (IFA) and CA (IFA) vaccinated and GST vaccinated control animals in Trial III.

Observed measure	Significant differences between groups
Time to onset of febrile period	Significantly shorter (p=0.013) in control than in Tat (IFA) vaccinated group
	Significantly shorter (p=0.0003) in control than in CA (IFA) vaccinated group
Duration of febrile period	Significantly longer (p=0.039) in controls than in CA (IFA) group

The acute disease was divided into 7 phases (Table 6.7) and an unpaired t-test was used to compare the mean values for each measure in each phase (Table 6.8).

Table 6.7 Summary of condensed data for each measure during each of the 7 phases following viral challenge in Trial III.

Clinical parameter	Vaccine group	Phase						
		1	2	3	4	5	6	7
Temperature (°C)	Control	38.93	39.08	38.77	39.30	40.55	39.95	38.93
	Tat (IFA)		38.62	38.55	39.20	40.41	39.73	39.05
	CA (IFA)	38.42	38.61	38.63	39.02	40.19	39.37	
Leucocyte count (% of day 0)	Control	-4.91	-23.48	4.55	-26.43	-50.6	-32.82	
	Tat (IFA)		-18.25	-32.71	-33.44	-36.6	-7.71	
	CA (IFA)	-2.34	-6.34	-19.67	-21.63	-43.39	-22.8	
Log_{10} plasma viral load (genome copies/ml)	Control	0.00	4.78	5.52	9.03	10.91	9.54	7.89
	Tat (IFA)		6.67	8.52	9.12	10.15	9.24	8.09
	CA (IFA)		5.29	6.37	8.04	10.30	8.99	
Viral doubling time / clearance half-life (h)	Control	0.00	4.50	13.70	5.88	69.40	-6.38	-6.42
	Tat (IFA)		3.11	18.64	5.38	103.68	-7.83	-7.99
	CA (IFA)		8.01	12.93	7.08	160.76	-9.50	
Phase duration (h)	Control	96	104	48	72	80	64	24
	Tat (IFA)		72	96	24	144	36	24
	CA (IFA)	72	64	72	40	120	24	

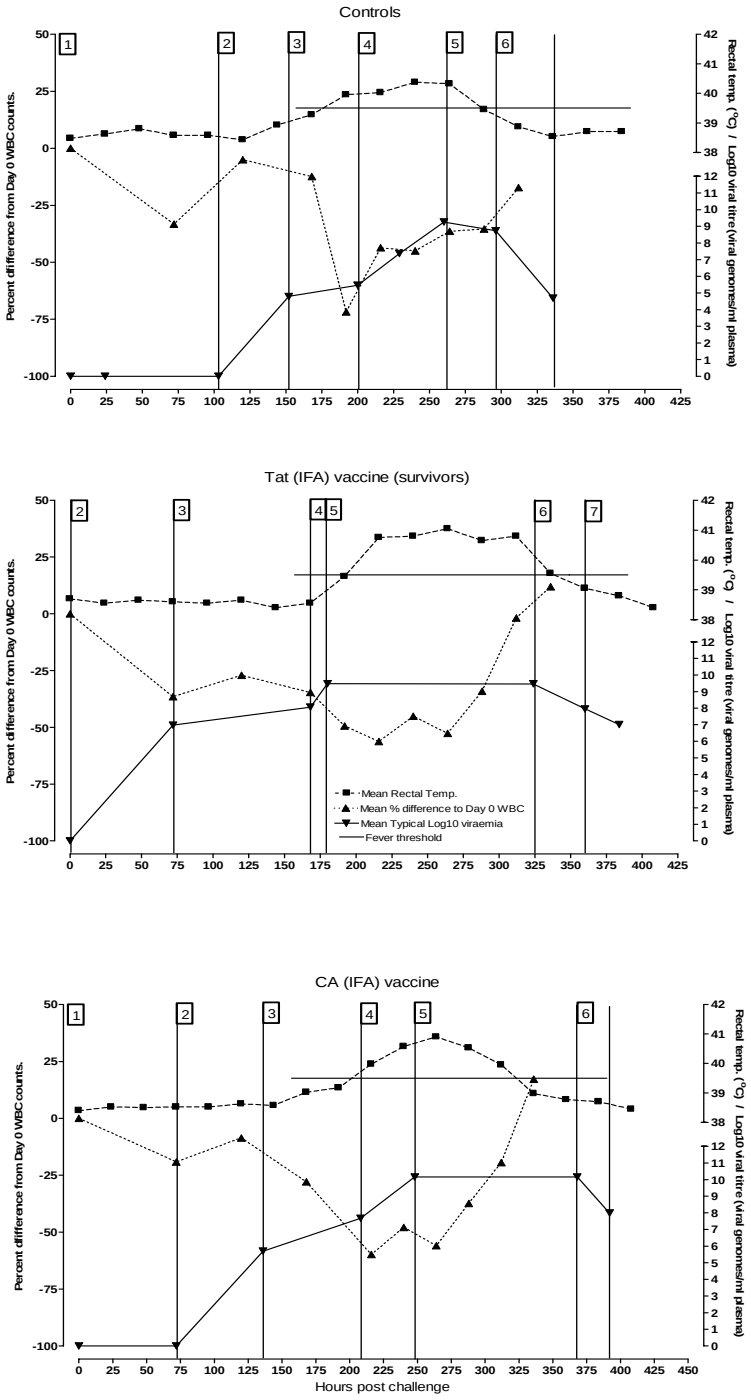


Figure 6.6 Graphic depiction of clinical parameters in cattle in Trial III after immunisation with either GST (IFA) (a control mock vaccination), CA (IFA) or Tat (IFA), and subsequent challenge with 1,000 ID_{50} of JDV_{PUL}. The results shown are a mean of measures from the 4 animals in each group at each phase of the acute disease process (as defined in Chapter 4).

Table 6.8 Significant differences between the measures in each phase (shown in Table 6.7) to JDV infection in surviving Tat vaccinated, CA vaccinated and control animals in Trial III.

Measure	Compared groups	Significant differences in measures by phase
Rectal temperature	**CA (IFA) v. controls**	Phase 1: Significantly higher (p=0.01) in controls Phase 2: Significantly higher (p=0.06) in controls Phase 6: Significantly higher (p=0.02) in controls
Depression of leucocyte count	**Tat (IFA) v. controls**	Phase 6: significantly greater (p=0.003) in controls
Viral titre doubling time / clearance half life	**Tat (IFA) v. controls**	Phase 2: significantly longer (p=0.0007) in controls
	CA (IFA) v. controls	Phase 2: significantly shorter (p=0.02) in controls Phase 6: significantly shorter (p=0.02) in controls
Duration of phases	**Tat (IFA) v. controls**	Phase 5: significantly shorter (p=0.051) in controls Phase 6: longer (approaching significance p=0.066) in controls.
	CA (IFA) v. controls	Phase 2: significantly longer (p=0.044) for controls Phase 5: significantly shorter (p=0.03) for controls Phase 6: significantly longer (p=0.015) for controls

Comparison of IFA and QuilA as adjuvants for vaccination with Tat (Trial IV).

Seroconversion to recombinant JDV proteins in vaccinated animals.

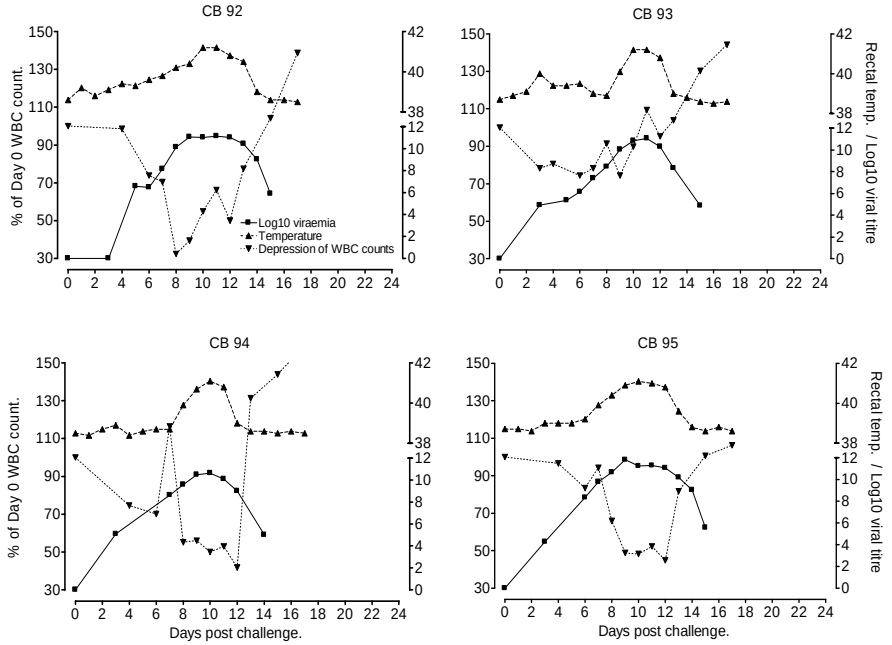
All cattle in the 2 vaccine groups developed detectable antibody to the administered recombinant proteins prior to challenge with infectious virus (Table 6.9). All animals vaccinated with the vaccine in IFA had detectable responses to Tat by day 21, and maintained strong responses up to the point of challenge. In contrast only 1 of 4 animals vaccinated with the vaccine in QuilA had a response by day 21, and the responses in this group at later timepoints prior to challenge were not as consistently strong as those in the group where IFA was used as an adjuvant.

Table 6.9 Differences in the seroconversion of cows to Tat when Tat+CA vaccine was administered using IFA or QuilA as adjuvant. (+/- indicates a barely detectable WB response, + is positive and ++ denotes strongly positive).

Cow	WB Antigen	Day -7	Day 21	Day 35	Day 42
CB 92		-	+/-	++	++
CB 93	**Tat+CA**	-	++	++	++
CB 94	**(IFA)**	-	++	++	++
CB 95		-	+	++	++
CB 100		-	-	+/-	+/-
CB 101	**Tat+CA**	-	-	+	++
CB 102	**(QuilA)**	-	-	++	+
CB 103		-	+/-	+	+

The clinical parameters observed after challenge with JDV for Trial IV where cattle were vaccinated 3 times with Tat administered using either IFA or QuilA as an adjuvant are shown in Table 6.10 and Figure 6.7 a, b, and c. It was apparent that the severity of the disease in those animals vaccinated with Tat adjuvanted with QuilA was increased compared with both control animals and those vaccinated with the same protein preparation administered with IFA. 3/4 animals in the Tat/QuilA group died during the febrile period, 2/4 in the control group and 0/4 in the group vaccinated with Tat/IFA. The control animals had detectable virus in the plasma sooner after challenge than the vaccinated groups, and had a shorter doubling time than both vaccinated groups during the initial appearance of virus in the plasma.

The significant difference between the observed parameters in the vaccinated and control groups are shown in Table 6.11. When the observed results for each group were compared with those of the control group with a t-test, both of the vaccinated groups show a more rapid progression to the peak of the fever, despite there being no difference in the time taken for viral titres to reach a maximum. Those animals vaccinated with QuilA as an adjuvant showed marked potentiation of the disease, as 3/4 animals died, and the mean maximum temperature for the group was significantly higher than the mean for the control group.



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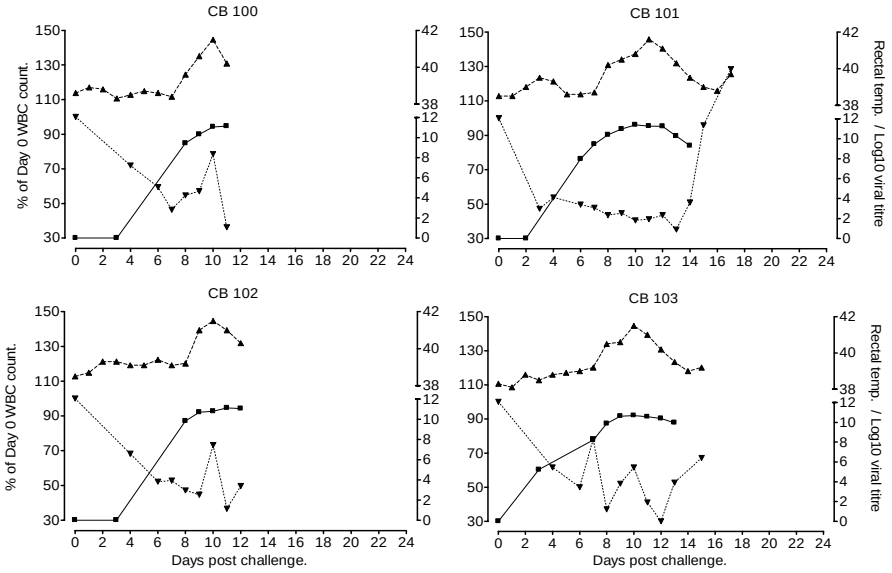

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Figure 6.7a Graphical depiction of measured clinical parameters (rectal temperatures [°C], total leucocyte count as percentage of day 0 values, and log_{10} plasma viral load [genomes/ml]) in individual cattle in Trial IV after vaccination with (A) Tat+CA (IFA) and (B) Tat+CA (QuilA) and challenge with 1,000 ID_{50} of JDV_{PUL}.

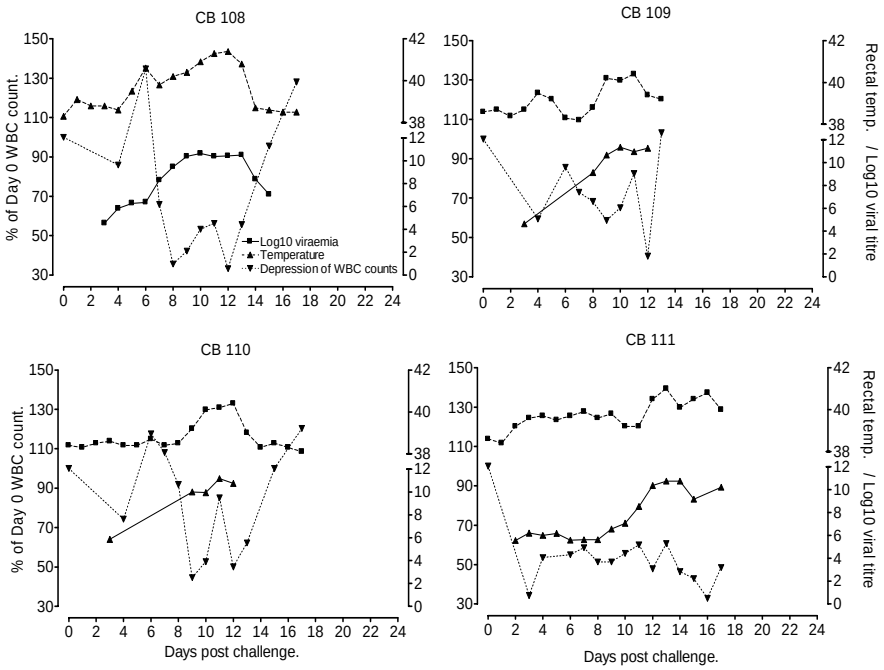

Figure 6.7b Graphical depiction of measured clinical parameters (rectal temperatures [°C], total leucocyte count as percentage of day 0 values, and log_{10} plasma viral load [genomes/ml]) in individual control cattle in Trial IV after challenge with 1,000 ID_{50} of JDV_{PUL}.

Table 6.10 Observed clinical parameters after challenge with 1,000 ID_{50} of JDV_{PUL} during Trial IV from cattle vaccinated with Tat+CA (IFA), Tat+CA (QuilA) and in non-vaccinated control animals (as described in Table 6.1).

Vaccine (adjuvant)	Animal	Time from challenge to peak plasma viral load (days)	Duration of febrile period (days)	Time from infection to onset of febrile period (days)	Time from infection to peak of febrile period (days)	Time from infection to end of febrile period (days)	Maximum rectal temp. (°C) during febrile period	Fever score	Outcome
Tat + CA (IFA)	**CB92**	11	8	6	10	14	41.2	7.7	Survived
	CB93	11	4	9	10	13	41.2	5.8	Survived
	CB94	10	4	8	10	12	41.1	4.5	Survived
	CB95	11	4	8	10	14	41.1	5.5	Survived
Tat + CA (QuilA)	**CB100**	11	4	8	10	dead	41.5	3.9	Died (d.11)
	CB101	11	7	8	11	15	41.6	7.7	Survived
	CB102	11	4	9	10	dead	41.5	5.8	Died (d.12)
	CB103	10	6	8	10	14	41.5	6.3	Died (d.15)
N/A	**CB108**	10	9	5	12	14	41.4	9.4	Survived
	CB109	10	3	9	11	12	40.4	2.2	Died (d.12)
	CB110	11	3	10	12	13	40.4	2.2	Survived
	CB111	14	6	12	13	17	41.0	7.2	Died (d.?)

Table 6.11 Significant differences between the measured responses (shown in Table 6.9) to JDV challenge in vaccinated and control animals in Trial IV.

Clinical parameters	Compared groups	Significant results
Days from challenge to peak febrile response	**Tat (IFA) v controls**	Significantly longer (p=0.001) in control than vaccinated group.
	Tat (QuilA) v controls	Significantly longer (p=0.005) in control than vaccinated group.
Maximum rectal temperature	**Tat (QuilA) v controls**	Significantly lower (p=0.012) in control than vaccinated group.

The acute disease was divided into 7 phases (Table 6.11, Figure 6.8) and an unpaired t-test was used to compare the mean values in each phase (Table 6.12).

Table 6.12 Summary of condensed data for each measure during each of the 7 phases following virus challenge in Trial IV.

Clinical parameter	Vaccine group	Phase						
		1	2	3	4	5	6	7
Temperature (°C)	Control		38.63	39.26	39.96	40.47	39.75	38.65
	Tat + CA (IFA)	39.10	39.03	39.73	39.88	40.96	39.93	38.80
	Tat + CA (QuilA)	38.83	39.29			40.74	39.60	38.90
Leucocyte count (% day 0)	Control		8.21	17.25	38.54	46.37	44.44	16.31
	Tat + CA (IFA)	0.00	4.66	22.72	33.10	35.30	27.24	1.19
	Tat + CA (QuilA)		2.35	17.25	38.54	49.07	44.44	4.44
Log_{10} plasma viral load (genome copies/ml)	Control		5.40	7.28	9.76	10.57	10.21	8.11
	Tat + CA (IFA)	0.00	6.42	7.57	10.56	10.97	10.33	8.70
	Tat + CA (QuilA)	0.00	10.17			10.94	10.80	9.30
Viral doubling time/ clearance half-life (h)	Control		3.84	36.23	6.84	402.80	3.41	5.45
	Tat + CA (IFA)	0.00	4.00	13.31	6.48	53.63	6.37	3.05
	Tat + CA (QuilA)		6.39			73.48	7.50	
Phase duration (h)	Control		72	112	84	96	24	24
	Tat + CA (IFA)	72	90	40	84	60	36	24
	Tat + CA (QuilA)	72	168			66	24	24
Survival	Control	2/4, 1 animal died in Phase 5, 1 2 days after "recovery".						
	Tat + CA (IFA)	4/4 survived.						
	Tat + CA (QuilA)	1/4, all 3 animals died in Phase 5.						

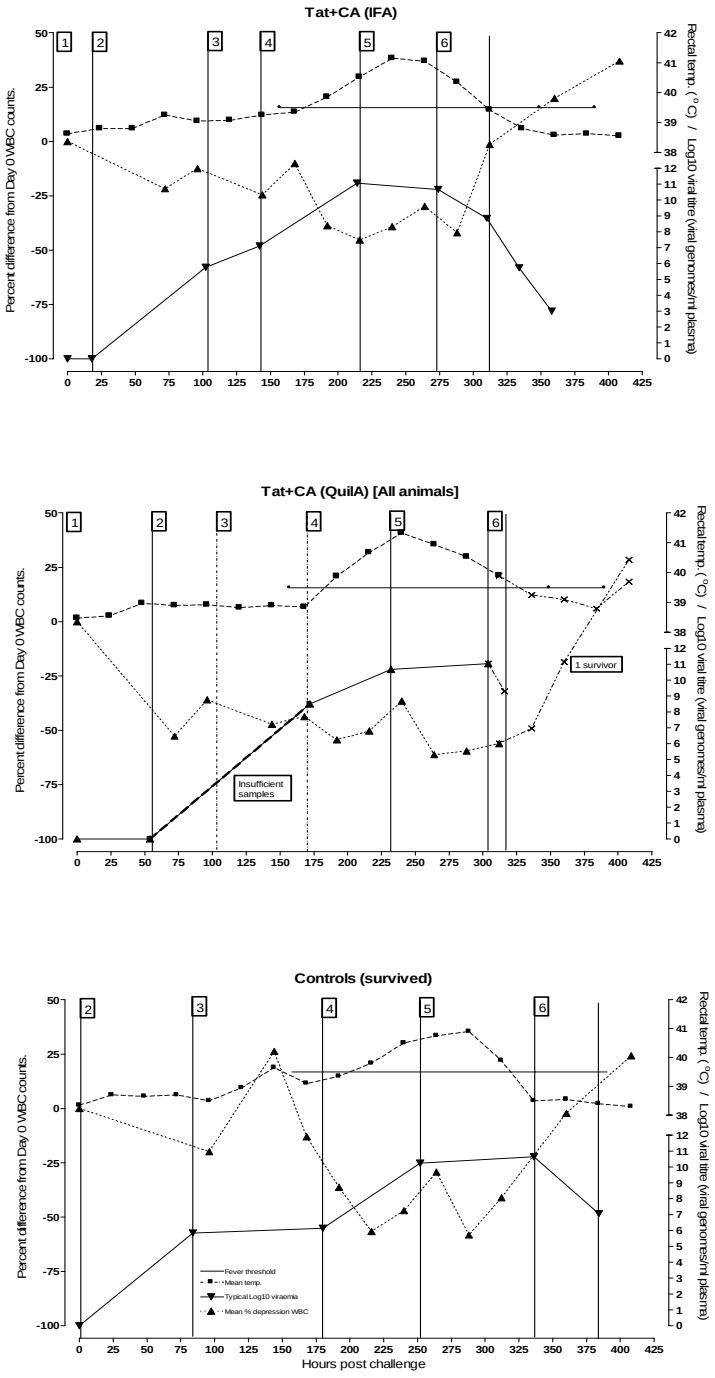

Figure 6.8 Graphical depiction of calculated parameters from cattle in Trial IV after vaccination with either Tat+CA (IFA) or Tat+CA (QuilA), or non-vaccinated controls and subsequent challenge with 1,000 ID_{50} of JDV_{PUL}. The results shown are a mean of 4 animals in each group (control group surviving animals only) at each phase of the acute disease process (as defined in Chapter 4).

Table 6.13 Significant differences during each phase of the disease process between the calculated parameters (shown in Table 6.12 and graphically in Figure 6.8) following JDV challenge of vaccinated and control animals in Trial IV.

Measure	Compared groups	Significant results.
Temperature	**Tat + CA (IFA) v. controls**	Phase 2: significantly lower (p=0.04) in control than vaccinated group Phase 5: significantly lower (p=0.05) in control than vaccinated group
	Tat + CA (QuilA) v. controls	Phase 2: significantly lower (p=0.0011) in control than vaccinated group
Depression of leucocyte count	**Tat + CA (QuilA) v. controls**	Phase 2: significantly less depressed (p=0.00015) in control than vaccinated group
Log_{10} plasma viral load	**Tat + CA (QuilA) v. controls**	Phase 2: significantly less ($p=1.14x10^{-6}$) in control than vaccinated group
Viral doubling time/ clearance half life	**Tat + CA (QuilA) v. controls**	Phase 2: significantly shorter (p=0.012) in control than vaccinated group
Phase duration	**Tat + CA (QuilA) v. controls**	Phase 2: significantly shorter (p=0.0044) in control than vaccinated group .

Comparison of Tat+CA, Tat+MA/CA as vaccines (Trial V).

Seroconversion to recombinant JDV proteins in vaccinated animals.

All cattle in the 3 vaccine groups developed detectable antibody to the administered recombinant proteins prior to challenge with infectious virus (Table 6.14). All animals administered the Tat vaccine had detectable responses to Tat by day 28, and maintained responses up to the point of challenge. The response to CA and MA (where administered) was detectable earlier, with 3/5 animals in the Tat + CA group showing a response by day 14, and 4/5 animals in the Tat + MA/CA group positive by the same timepoint.

Table 6.14 The seroconversion of cows to Tat, CA and MA when Tat+CA, or Tat+MA/CA vaccine was administered using IFA (+/- indicates a barely detectable WB response, + is positive and ++ denotes strongly positive).

Cow	WB Antigen	Day 0	Day 14	Day 28	Day 42	Day 49
CB119	Tat	-	-	+	++	++
	CA	-	+/-	+	++	++
CB120	Tat	-	-	+	++	++
	CA	-	-	+	++	+
CB121	Tat	-	-	+	++	++
	CA	-	-	++	++	++
CB122	Tat	-	-	++	+	+
	CA	-	+/-	+	++	++
CB123	Tat	-	-	+	+	+
	CA	-	+/-	++	++	ND
CB 129	Tat	-	-	+	+	+
	MA	-	+	++	++	++
	CA	-	+	++	++	++
CB 130	Tat	-	-	+	++	++
	MA	-	-	+	++	++
	CA	-	+/-	++	++	++
CB 131	Tat	-	-	+	++	++
	MA	-	+/-	++	++	++
	CA	-	+	+	++	+
CB 132	Tat	-	+/-	+	++	++
	MA	-	-	+	+	+
	CA	-	-	++	++	++
CB 133	Tat	-	+/-	+	++	++
	MA	-	-	++	++	++
	CA	-	+	+	+	ND

The measured clinical parameters in individual cattle vaccinated with a mixture of either Tat + CA or Tat + MA/CA compared to control animals are shown in Tables

6.13 and are shown schematically for each of the animals in the Tat+CA group (Figure 6.10), Tat+MA/CA group (Figure 6.11) and control group (Figure 6.12).

Table 6.15 Observed clinical parameters during Trial V from cattle vaccinated with Tat+CA (IFA), Tat+MA/CA (IFA) and from non-vaccinated control animals (as described in Table 6.2) following challenge with 1,000 ID_{50} of JDV_{PUL}.

Vaccine	Animal	Time from challenge to peak plasma viral load (days)	Duration of febrile period (days)	Time from infection to onset of febrile period (days)	Time from infection to peak of febrile period (days)	Time from infection to end of febrile period (days)	Maximum rectal temp. (°C) during acute disease	Fever score	Outcome
Tat+CA (IFA)	**CB119**	12	6	8	11	14	40.8	5.9	Survived
	CB120	11	5	9	10	14	41.3	5.4	Survived
	CB121	12	0 (1)	N/A (12)	12	13	39.0	0	Survived
	CB122	11	6	7	10	13	40.9	3.7	Survived
	CB123	11	2	11	11	13	40.5	1.3	Survived
Tat+MA/CA (IFA)	**CB129**	11	6	6	11	12	41.1	5.2	Died (d.12)
	CB130	11	5	10	10	15	41.3	5.2	Survived
	CB131	12	8	7	11	15	41.4	6.2	Survived
	CB132	11	7	7	11	14	41.2	6.1	Survived
	CB133	10	3	10	10	13	40.8	3.2	Survived
N/A	**CB134**	14	1	5	5	6	39.6	0.1	Survived
	CB135	13	7	9	11	16	41.7	8.2	Survived
	CB136	12	0 (4)	N/A (10)	12	14	39.4	0	Survived
	CB137	12	6	9	10	15	40.9	7.3	Survived
	CB138	10	5	9	11	15	40.6	4.5	Survived

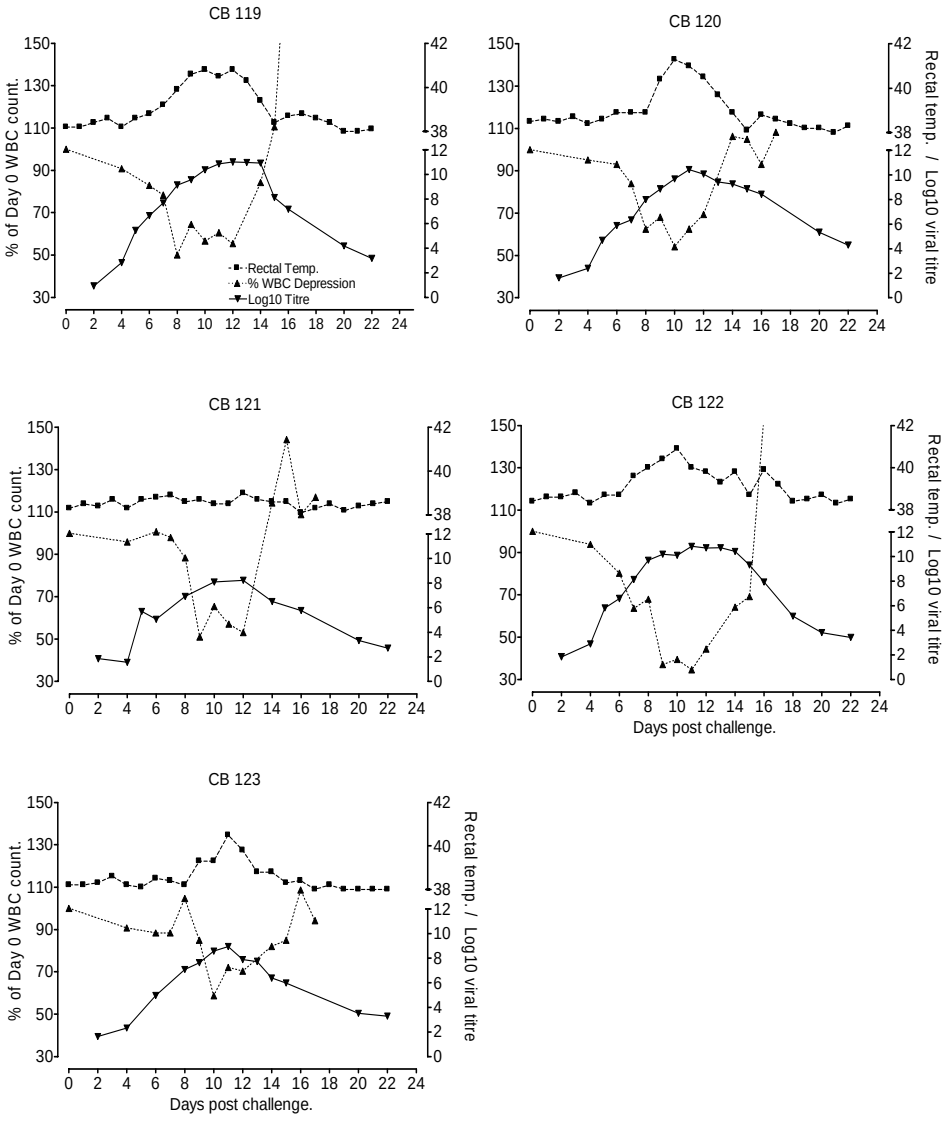

Figure 6.9a Graphical depiction of measured clinical parameters (rectal temperatures [°C], total leucocyte count as percentage of day 0 values, and log_{10} plasma viral load [genomes/ml]) in individual cattle in Trial V after vaccination with Tat+CA and challenge with 1,000 ID_{50} of JDV_{PUL}.

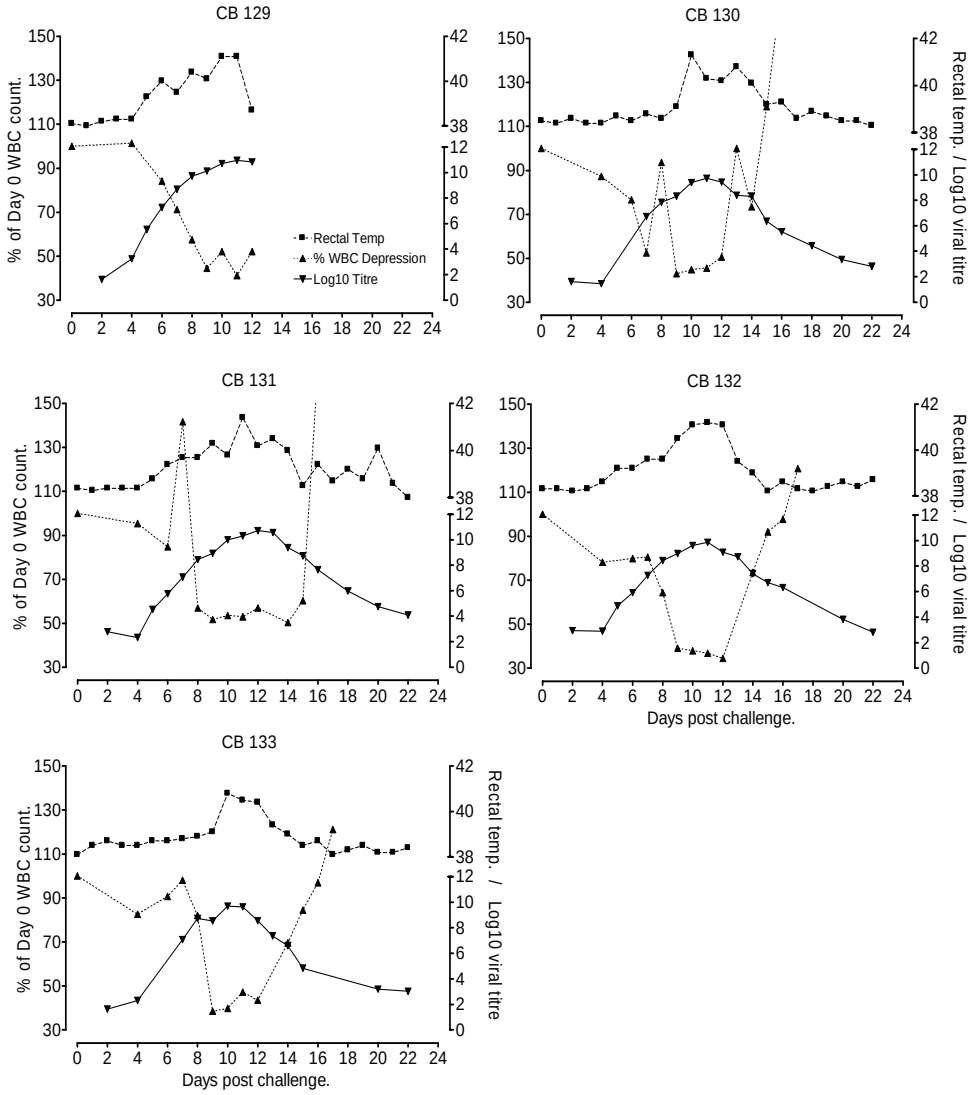

Figure 6.9b Graphical depiction of measured clinical parameters (rectal temperatures [˚C], total leucocyte count as percentage of day 0 values, and log_{10} plasma viral load [genomes/ml]) in individual cattle in Trial V after vaccination with Tat+MA/CA and challenge with 1,000 ID_{50} of JDV_{PUL}.

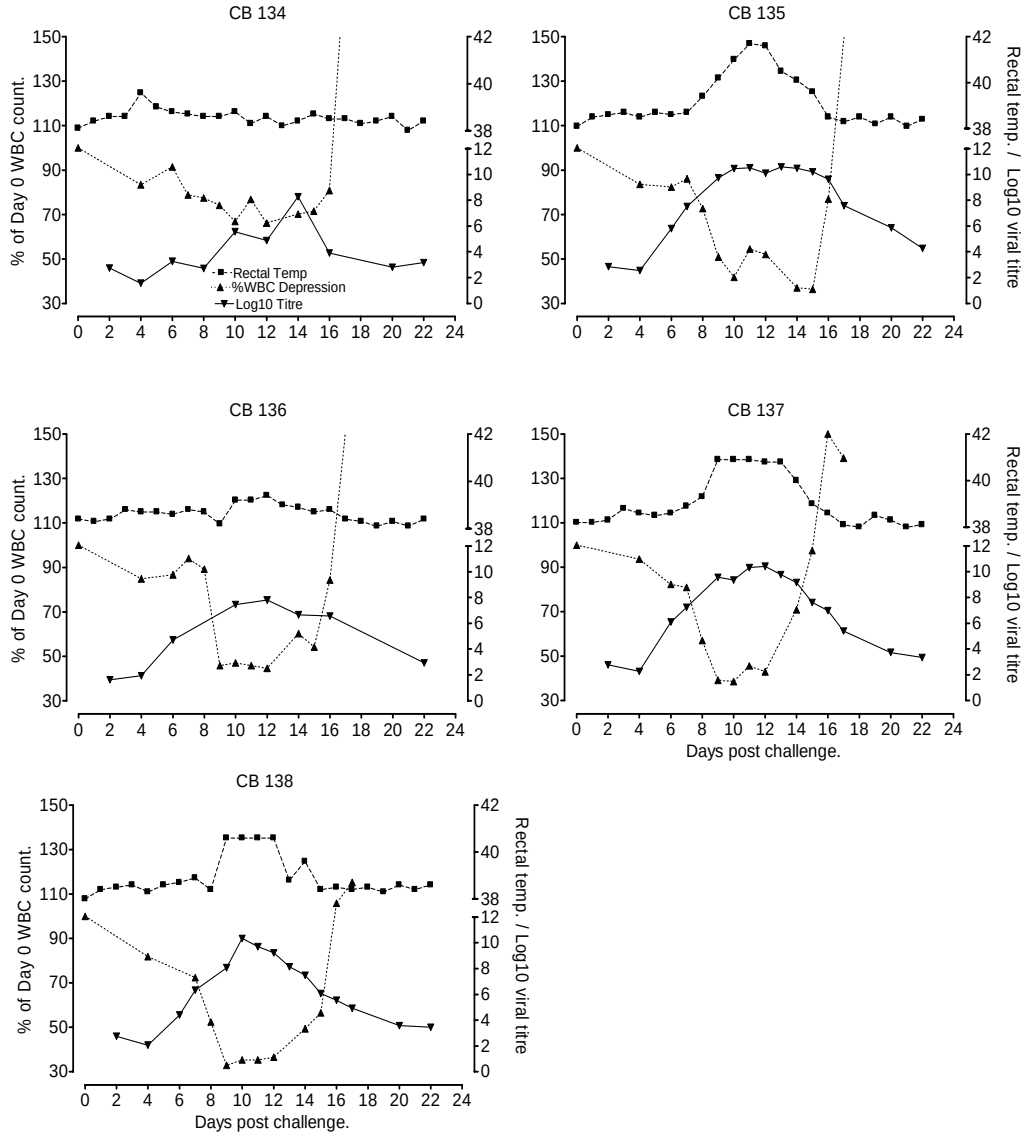

Figure 6.9c Graphical depiction of measured clinical parameters (rectal temperatures [°C], total leucocyte counts as percentage of day 0 values, and log_{10} plasma viral load [genomes/ml]) in individual non-vaccinated cattle in Trial V, following challenge with 1,000 ID_{50} of JDV_{PUL}.

The acute disease process within each group was divided into 7 phases (Table 6.14, Figure 6.13) and an unpaired t-test was used to compare the mean values of each measure during each phase (Table 6.15). Statistically significant differences occurred predominantly in Phases 1-5 during the early phases of the disease process.

Table 6.16 Summary of condensed data for each measure during each of the 7 phases following viral challenge during Trial V.

Measure	Vaccine group	Phase						
		1	2	3	4	5	6	7
Temperature (°C)	Control	NA	38.31	38.54	39.10	40.57	39.60	38.80
	Tat + CA	NA	38.43	38.44	38.68	39.58	39.70	38.86
	Tat + MACA	NA	38.39	38.45	38.94	40.17	39.67	39.00
Leucocyte count (% day 0)	Control	NA	0.00	-13.64	-33.09	-55.63	-39.68	-0.92
	Tat + CA	NA	0.00	-4.88	-13.05	-35.08	-28.78	-19.07
	Tat + MACA	NA	0.00	-14.13	-15.92	-48.16	-32.40	2.86
Log_{10} viraemia (genome copies /ml)	Control	NA	2.49	2.62	9.35	10.14	8.92	8.89
	Tat + CA	NA	1.46	2.18	8.41	10.28	8.53	10.07
	Tat + MACA	NA	2.30	2.55	7.90	9.82	9.58	7.91
Viral doubling time /clearance half-life (h)	Control	NA	5.18	33.83	5.28	26.06	-5.66	-8.73
	Tat + CA	NA	8.34	23.79	5.29	60.87	-27.62	-8.08
	Tat + MACA	NA	6.95	110.63	5.08	31.97	-6.06	-8.78
Phase duration (h)	Control	NA	48	48	120	88	24	24
	Tat + CA	NA	48	48	96	129.6	48	24
	Tat + MACA	NA	48	48	96	114	36	24

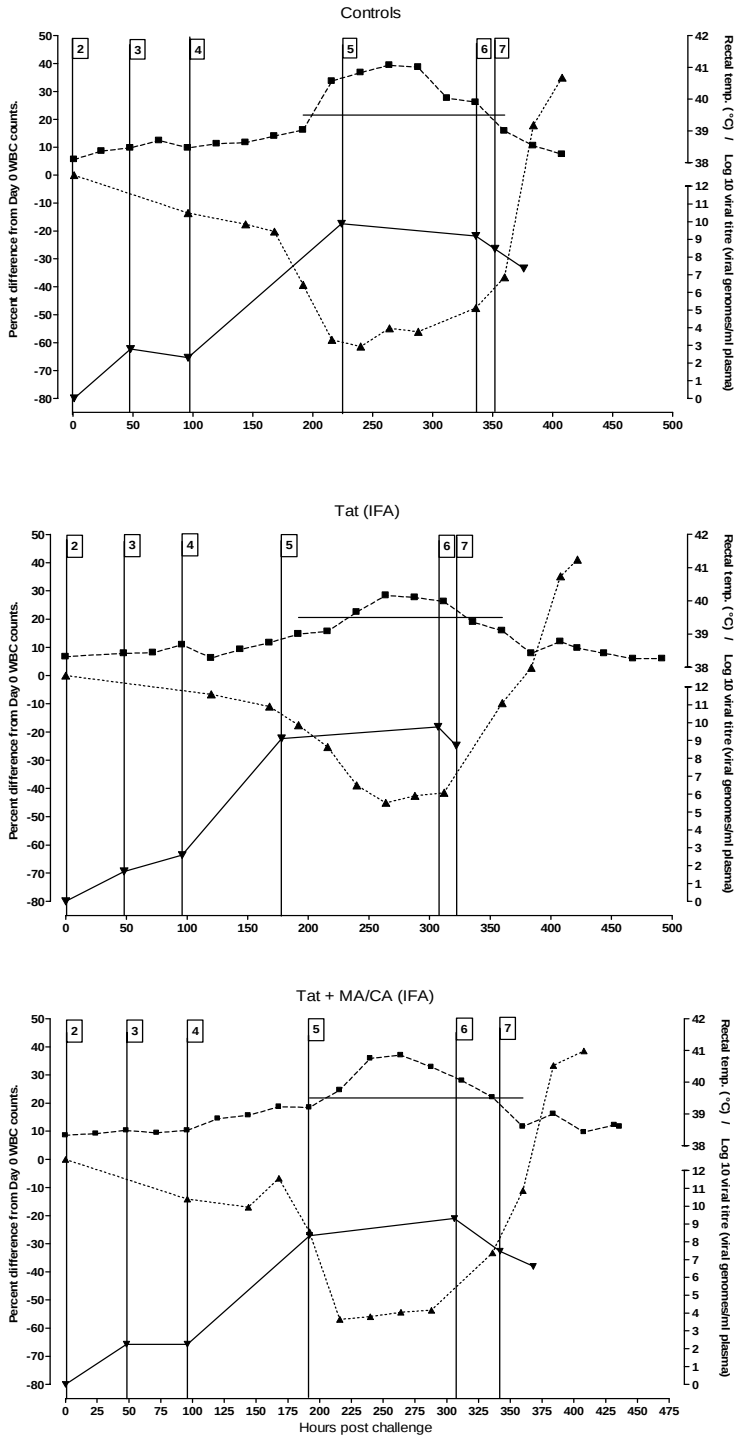

Figure 6.10 Graphical depiction of clinical parameters in cattle in Trial V after no vaccination (typical controls), or Tat+CA (IFA) or Tat+MA/CA (IFA) and subsequent challenge with 1,000 ID_{50} of JDV_{PUL}. The results shown are a mean of measures from the 5 animals in each group at each phase of the acute disease process (as defined in Chapter 4).

Table 6.17 Significant differences during each phase of the disease process between the calculated parameters (shown in Table 6.14 and graphically in Figure 6.15) following JDV challenge of vaccinated and control animals in Trial V.

Measure	Compared groups	Significant results
Temperature	**Tat+CA v. control**	Phase 4: significantly higher (p=0.029) in control than vaccinated group Phase 5: significantly higher (p=0.030) in control than vaccinated group
	Tat+MA/CA v. control	Phase 5: significantly higher (p=0.046) in control than vaccinated group
Depression of leucocyte count	**Tat+CA v. control**	Phase 3: significantly more depressed (p=0.020) in control than vaccinated group Phase 4: significantly more depressed (p=0.018) in control than vaccinated group Phase 5: significantly more depressed (p=0.029) in control than vaccinated group
	Tat+MA/CA v. control	Phase 4: significantly more depressed (p=0.033) in control than vaccinated group
Log_{10} plasma viral load	**Tat+CA v. control**	Phase 2: significantly higher ($p=1.73x10^{-5}$) in control than vaccinated group Phase 3: higher (approaching significance p=0.069) in control than vaccinated group Phase 4: significantly higher (p=0.029) in control than vaccinated group
	Tat+MA/CA v. control	Phase 4: significantly higher (p= 0.0004) in control than vaccinated group Phase 5: significantly higher (p=0.06) in control than vaccinated group
Viral doubling time / clearance half life	**Tat+CA v. control**	Phase 2: significantly shorter (p=0.00014) in control than vaccinated group
Phase duration	**Tat+MA/CA v. control**	Phase 4: significantly longer (p=0.046) in control than vaccinated group

Discussion.

Production of recombinant proteins in a prokaryotic expression system was chosen as the method of vaccine production as this was considered technically feasible in Indonesia. GST fusion proteins were used as vaccine proteins as several of the constructs had already been produced in earlier work in our laboratory (Burkala *et al.*, 1998) and high yields of protein were obtained. GST fusion vectors were easy to construct and there were many options for choosing the frame of insertion, strength of promoter and induction method. The pGEX-6P system (Amersham) was chosen for its standard induction technique utilising IPTG mediated de-repression of the *lac* operon, as this system gave flexibility in timing of induction, amount of inducer required and growth temperatures of the cultures. The use of GST as a fusion partner meant that soluble, or solubilised/refolded expressed proteins could be easily and cheaply purified and enzymatic cleavage of the GST fusion moiety was possible if required. There was also the added incidental advantage that vaccination with GST alone has been used to confer resistance to infection with *Schistosoma japonicum* in cattle (Wu *et al*., 2004). However, a number of the experimental cattle had pre-existing antibody to GST, prior to vaccination, presumably as a result of infection with *Schistosoma spp*. (results not presented). Although these responses were weak in comparison to the response to GST obtained after vaccination, their presence presented some difficulty in analysing the rapidity of response to the recombinant GST-fused vaccines.

Tat expressed as a GST fusion protein in *E. coli* accumulated as inclusion bodies in the bacterial cells. Such insoluble inclusion bodies in *E. coli* were first noted in bacteria growing in the presence of an amino acid analogue, appearing as an amorphous granule in the cell, consisting of misfolded proteins without any defined surrounding layer (Prouty *et al*., 1975). The CA-GST fusion protein was also deposited as inclusion bodies, but in this case about 50 % of this protein was expressed in a soluble form, detectable by Western blot in the centrifuged bacterial lysate supernate. The over-expression of the recombinant protein in bacterial expression systems is thought to outstrip the supply of chaperones to aid in the

folding process *in vivo*, leading to the deposition in inclusion bodies, especially where hydrophobic domains exist in the recombinant protein (Thomas and Baneyx, 1996) as in JDV Tat (Setiyaningsih, 2006). The formation of inclusion bodies when over-expressing proteins in bacterial expression systems has both advantages and disadvantages in respect of purification and retention of structure and activity of the expressed protein.

The production of Tat and CA within inclusion bodies would ideally have led to research into methods of solubilisation and refolding of the proteins that would conserve their native conformation. It can be important to achieve correct refolding if specific activities of the protein, such as enzymatic or binding function, need to be retained, or if conformational epitopes need to be conserved. In these current studies, however, a decision was made to utilise and test simple purification methods that would be applicable to production in Indonesia. Solubilisation of the inclusion bodies was easily achieved in high pH buffers containing urea. Recombinant *Taenia ovis* protein vaccines administered as solubilised protein in a urea/dithiothreitol buffer were associated with an enhanced immune responses to the vaccine moiety of the fusion protein (Rothel *et al*., 1997) and the possibility of similar enhancement with JDV protein fused to GST caused this method of solubilisation to be adopted. The antibodies to GST seen in animals prior to vaccination described above made analysis of the possible differences in the relative strength of the responses to the vaccine protein and the fusion moiety described by these workers difficult.

A humoral response to the administered vaccine proteins was seen soon after administration of the second vaccine dose, normally 14 days apart. 3 vaccine doses at short intervals were given to ensure a strong response, with challenge only a short period after the final vaccine dose. The rapidity of the whole process was primarily to decrease costs of keeping the cattle indoors for an extended period in Indonesia. 2 vaccine doses more widely spaced may have had the same effect as 3 doses with a short interval between doses and the development of full vaccine efficacy, especially in induction of a CMI response to lentivirus vaccines, may not be attained for several months after the final boost (Hammond *et al.*, 1997; Craigo *et al.*, 2005). Further studies will be required to optimise the vaccination regime.

The vaccine proteins were quantified by densitometry rather than by a biochemical total protein assay as this enabled the purity of the protein and hence the actual amount of vaccine protein administered to be determined. The high quality of the imaging system used to do this allowed accurate quantification of the protein bands as long as the loading of the gel kept the amounts of protein to be stained within the linear range of Coomassie brilliant blue staining. Other dyes such as Sypro Ruby have a greater range of linearity but it was found that the protein of interest could be diluted into the linear range so enabling the use of the readily available Coomassie brilliant blue.

All animals seroconverted to the proteins used in the vaccines, as determined by Western blot using recombinant protein antigens expressed with the PinPoint system. Protein antigens produced by this system were used to give a different tag than the vaccine proteins, thus ensuring that seroconversion had occurred to the vaccine protein and not solely the GST fusion partner. It would arguably have been better to use native antigen obtained by purification of virus from the plasma of infected animals (Kertayadnya *et al*., 1993) to assess seroconversion. Native antigen may have possessed conformational epitopes not present on the bacterially expressed protein but this was unobtainable during these experiments. Although desirable, it was not possible to monitor CMI responses under the laboratory conditions available in Bali.

Three vaccine trials were reported in this chapter (Trials III, IV and V). It is admitted that our expertise in producing the proteins and assessing the efficacy of the response, and overcoming problems associated with the collection of samples and conducting the various assays developed during the course of these 3 trials. Other trials had been conducted, primarily examining potential role of glycosylated JDV envelope glycoproteins, without any identifiable efficacy. However, JDV Tat and CA proteins had been included and some efficacy was identified, leading to the 3 trials reported in this chapter.

A variable that was hard to control was the lack of a standard infectious inoculum of JDV. The practise in Indonesia has been to store infected spleen tissue at -80C and to use this to infect an animal, then collect plasma from the infected animal on the

second day of the resulting febrile response and use this as an inoculum (Soeharsono *et al.*, 1990). This is partly done to overcome the problems of continued temporary loss of electrical power resulting in poor freezer storage conditions. The titre of the virus was determined by antigen-capture ELISA (Stewart *et al.*, 2005) and the virus diluted appropriately so that it contained an estimated 1,000 ID_{50} for each inoculated animal. While this produced a reasonably accurate estimation of the number of virus particles, it did not discriminate between infectious and non-infectious virus particles, and it did not consider possible differences associated with passage of the virus through animals.

The first of the trials reported, Trial III, was an attempt to compare the effects of vaccination with Tat and CA but the significance of the results obtained was difficult to interpret. The death of 2 animals in the Tat vaccinated group in this trial occurred before the development of a febrile response or plasma virus load were seen and meant that numbers of the remaining cattle were too small for meaningful comparisons to be made. Although these deaths were obviously of concern, no reason could be determined for them, and this pattern of death was not seen in any other trials.

In Trial IV, the effect of combining Tat and CA into a single vaccine was investigated, as was the effect of comparing the adjuvants QuilA and IFA for this combination. 2 major observations were made: first, that vaccine administered using QuilA potentiated the disease when these animals were challenged with JDV post-vaccination; second, that Tat/IFA vaccination protected against challenge that was lethal for several animals in both the Tat/QuilA and control groups. The apparent stronger antibody response to Tat when it was adjuvanted with IFA may contribute to this protection, although it has been reported that Tat-neutralisation titres do not correlate with protection (Belliard *et al*., 2005). However these workers were investigating long term protection, not amelioration of the initial acute phase disease. The potentiation of infection associated with the use of QuilA as an adjuvant seen in Trial IV led to the deaths of 3/4 animals in this vaccinated group, compared with 0/4 in the group utilising IFA as an adjuvant, and only 2/4 in the control group also employing IFA as adjuvant. QuilA has been reported to induce a Th1 cytokine profile

in cats vaccinated with FIV (Stokes *et al*., 1999) and it is possible that a similar CMI response to JDV proteins administered with this adjuvant increased the number of target cells for the virus by altering co-receptor expression (Chen *et al*., 1999), thus potentiating the disease. In mice, however, QuilA induces a Th2 response (Victoratos *et al*., 1997). There was a weaker and delayed antibody response to Tat in the QuilA adjuvanted group compared with the IFA adjuvanted group, perhaps indicating a Th1 cytokine profile in these animals. Cytokine profiling by qRT-PCR as performed in SIV infected macaques (Hofmann-Lehmann *et al*., 2002), would shed light on the type of response engendered and whether a CMI response is potentially deleterious in the case of JD.

In Trial V, when combinations of Tat and CA were tested as vaccines, many more samples were taken and there was an increased precision of the methods used to monitor virus load that meant clear differences in the progress of the induced disease could be measured, resulting in greater confidence in the results obtained. In this trial, there was definite evidence of amelioration of the disease process in the vaccinated animals. They had lower plasma virus loads in the early stages and at the peak of the acute disease process, with lower levels of fever and leucopenia. The effect seen in Trial V, of apparent reduction in early viral replication, is in contrast to that seen when an inactivated tissue-derived whole virus vaccine was used (Chapter 5) when the predominant effect was an increase in the clearance rate of virus from the plasma.

Whether the protective effect of the vaccine in Trial V was due to the immune response to CA or Tat or a combination of both is unknown. Antibodies to Tat have been shown to neutralise the effects of extracellular Tat on HIV-1 replication and to correlate with protection of cynomolgus monkeys (Cafaro *et al.*, 1999; Maggiorella *et al.*, 2004) but not rhesus macaques (Silvera *et al*., 2002) from $SHIV_{89.6P}$ challenge. The differences in protection attained in these 2 primate types was attributed to the naturally lower replication and pathogenicity of $SHIV_{89.6P}$ in cynomolgus monkeys compared with rhesus macaques but there were differences in the vaccination regimes and routes of challenge. As in the case of the Bali cattle challenged with JDV, sterilising immunity was not achieved but in the $SHIV_{89.6P}$ trials in cynomolgus monkeys, vaccination did prevent progression to AIDS and in the vaccinated cattle it

caused amelioration of the acute phase disease and no mortality; any decrease in the severity of the disease process and reduction in case fatality rates would be worthwhile pursuing in the Indonesian cattle industry.

It is assumed that the protection seen in Trials IV and V was due to the effects of antibodies on extracellular Tat, decreasing early viral replication. Biologically active Tat can, unusually for a non-particulate antigen, be taken up by APC and induce a CTL response via the MHC class 1 pathway (Cafaro *et al*., 2000). However, the biological activity of the GST tagged Tat used in these trials was not tested by *in vivo trans*-activation studies, and cleavage of the GST tag from the protein prior to administration may have resulted in a more effective response. Simplicity of production methods was desired, and the expense of the enzymes required to cleave the proteins would probably prohibit scale-up of the potential vaccine in Indonesia.

It is possible that a longer period between the final vaccination of the cattle and challenge would allow development of a CTL response; delayed induction of an effective response has been found in trials of FIV vaccines (Hosie and Flynn, 1996). Combined with the probable effects of Tat antibody on the infectivity enhancing functions of the extracellular protein, a more clear cut level of protection might then be expected.

The core structural antigens, including CA, of lentiviruses are relatively conserved and contain several recognition determinants for CTL, and vaccination of macaques with VLPs containing CA decreased the viral load 10-100 fold following challenge with SIV_{mne} despite the lack of neutralising antibody, presumably due to induction of CMI responses (Polacino *et al.*, 1999). Bali cattle infected with JDV, or vaccinated with CA raise strong specific antibody responses (Hartaningsih *et al.*, 1994; Desport *et al.*, 2005), but the extent of any induction of CMI responses is not known. 1 animal in an early trial vaccinated with CA had a very short febrile episode, although it is possible that this was merely the result of an atypical response to infection, as seen in several animals in the susbsequent trials. Because of this, CA was included in the vaccine preparations, although any effect of administration was impossible to judge

without further research into the fine specificity of the immune response to the vaccines.

The inclusion of MA did not seem to have any marked effect on the efficacy of the vaccine, and in fact 1 animal in the group vaccinated with this protein died with an apparent uncontrolled viraemia. Although the structural MA protein has been shown to have pro-inflammatory effects in HIV-1 infection, which can be blocked by peptide directed antibody suggesting an extracellular function (De Francesco *et al*., 2002), high levels of antibodies to MA were correlated with delayed progression to AIDS (Lange *et al*., 1987).

In conclusion, although sterilising immunity was not achieved, detailed examination of the viral plasma loads, showed an effect of Tat-containing vaccines on the early stages of the acute phase of JD.

Chapter 7

General discussion

A vaccine for the control of JD has been used for several years in Indonesia (Hartaningsih *et al.*, 2001). The vaccine has been used on a small scale and particularly in areas where the disease has occurred for the first time (Hartaningsih, personal communication). While this vaccine provides a degree of protection (Hartaningsih *et al.*, 2001), it is prepared from the tissues of clinically affected cattle and there are significant problems of cost, the possible spread of adventitious agents, the difficulty of commercialising the vaccine and ethical considerations which have restricted its use and necessitated a search for an alternative method of vaccination. The aim of the studies reported in this thesis was to determine the feasibility of utilising DNA vaccines or recombinant protein vaccines as alternatives, with greater safety, perhaps greater efficacy and reduced cost than the tissue-derived vaccine. This thesis primarily focuses on the potential application of JDV Tat and CA proteins.

Many vaccines against lentivirus infection have been developed and tested. The most successful are attenuated live virus vaccines, with attenuation achieved either by laboratory adaptation by repeated serial passage or by genetic manipulation to remove virulence determinants. For JDV this type of vaccine is not yet possible as no culture method exists for the virus, despite repeated attempts to develop one. The possibility does exist to develop a heterologous vaccine using the closely related non-pathogenic BIV, which does replicate in cell cultures, and preliminary studies of this are underway in our laboratory. The only commercially available lentiviral vaccine is an inactivated whole virus preparation against several subtypes of FIV (Hohdatsu *et al.*, 1997; Pu *et al.*, 2005). Inactivated whole virus vaccines are also experimentally effective against other lentivirus infections (Carlson *et al.*, 1990; Issel *et al.*, 1992), with levels of antigen administered (Hartung *et al.*, 1992) and the length of time after vaccination prior to challenge being important in engendering an effective immune response (Montelaro *et al.*, 1998). Another factor that might be important in the development of an effective immune response is possible changes in efficacy due to mutation of virus over time following serial passage through animals. This cannot be

ruled out, although unusually for a lentivirus, JDV shows very little sequence variation between samples obtained from Bali over a period of 20 years (Desport *et al.,* 2007). Inactivated tissue-derived virus vaccines have been used in the past to control diseases such as foot and mouth (Balamurugan *et al.*, 2004) but were in this case supplanted by vaccine consisting of virus produced in cell culture. These vaccination methods involving the use of whole virus are not without problems of potential contamination with other infectious agents and research efforts have been directed to the development of sub-unit vaccines as an alternative. So, although a whole virus tissue-derived vaccines has been developed for JD (Hartaningsih *et al.*, 2001), in the absence of a cell culture method for replication of JDV, research on a sub-unit vaccine was initiated.

A number of problems needed to be considered and solved prior to the development of a successful vaccine, in addition to the development of the actual vaccine. A significant difficulty was how to effectively assess the efficacy of Jembrana disease vaccines: animals with Jembrana disease do not always look overtly ill and there is a low case mortality rate of around 17%. Efficacy has therefore been previously placed on assessing the severity of both the febrile response and associated leucopenia, the only clearly measurable clinical parameters. The problems associated with reliance on clinical signs to assess efficacy were exacerbated as it became apparent during the course of the studies reported in this thesis that at the dose rate used to infect cattle, between 400 and 1000 ID_{50} of virus, some animals failed to develop the typical febrile response that was thought to occur in all infected animals (Wilcox *et al.*, 1995). It was demonstrated, however, that some animals without febrile response were infected and had high titres of cell-free virus in the plasma, and these animals would perhaps have been previously considered unsuccessful attempts to produce infection. The reasons for the failure of these animals infected with 400 to 1000 ID_{50} of virus to develop a febrile response needs further investigation; it was not reported previously when much higher doses of virus were used to infect animals (Soeharsono *et al*., 1990). It was noted also that these non-febrile but infected animals failed to produce antibody to the highly immunogenic CA protein post-challenge (J Lewis, personal communication) suggesting it may be associated with a lack of activation of

immune cells. It is possible that a pre-existing strong Th2 response skewed the response to virus infection as has been reported in naturally asymptomatically SIV_{sm} infected mangabeys (Ansari *et al.*, 2003); this might occur in response to parasitic infections that prevent establishment of a Th1 cytokine milieu which normally favours viral replication and associated bystander immunopathology. Conversely, however, once infection has been established, an artificially induced Th2-biased response has been shown to enhance progression of disease in rhesus macaques infected with SIV, even in the presence of neutralising antibody induced by vaccination, as induction of CTL required to control viral replication is not as efficient (Boyer *et al.*, 2002).

The situation in the few animals that displayed no signs of disease despite the presence of a high titre of circulating virus in the blood, and failed to produce antibody to the highly immunogenic CA protein post-infection, is similar to simian lentivirus infections in their respective naturally occurring hosts. (Silvestri *et al.*, 2003; Ling *et al.*, 2004). Although JDV induces a more severe reaction in Bali cattle than in other cattle species, it is interesting to speculate that the lack of febrile response in cattle could reflect a tendency of JDV to adapt to Bali cattle over the years since the disease was first detected in this species. A possible avenue for further research would be to investigate the apparent rapidity of the co-evolution of Bali cattle and JDV to a state where the virus becomes commensal to the host. The closely related BIV does not cause any serious disease in *Bos taurus* and the rate that JDV is developing this relationship with *Bos javanicus* is of interest.

Infection of vaccinated cattle with 400 to 1,000 ID_{50} of virus by the intravenous route was likely to be a greater infectious dose than would be experienced by cattle under field conditions, and probably presented the immune system with an unrealistically high infectious dose. The high dose rate was used, however, to ensure infection. It is possible that lower challenge doses that mimic natural infections might have revealed a greater protective effect from vaccination. Different ways of administering the infectious dose, for instance by housing an infected donor animal with the vaccinated cattle, or using a conjunctival route of infection (Soeharsono *et al*., 1990) rather than

an intravenous route, or using a trickle challenge of low repeated doses mimicking spread by biting flies, could be tested in future.

During the course of the studies reported in this thesis, it was apparent that there were some differences in the pathogenicity of the 2 strains of virus that were used to induce JD in experimentally infected animals. The differences were more apparent when the disease model reported in Chapter 4 was utilised to compare the progression of the disease induced by the 2 viruses. Previous experimental infection studies of JD have primarily utilised the JDV_{TAB} strain which was first detected in 1987 (Kertayadnya *et al.*, 1993). The reason for the difference in pathogenicity of JDV_{TAB} and JDV_{PUL} are not known but JDV_{TAB} has been passaged in cattle many times over nearly 20 years and may have been genetically modified during this period, although as noted above the mutation rate appears low in JDV. Differences in pathogenicity and the disease induction rate between strains of EIAV have been reported to occur as a result of changes in the nucleotide sequences in the LTR region (Maury *et al.*, 1997; Cook *et al.*, 2003). This region has recently been found to be variable in JDV (Desport *et al.*, 2007), and might account for some of the differences in pathogenicity and disease induction observed during the course of these trials. Previously, it has been assumed that the pathogenicity of all strains of JDV was similar and the variation in virulence that has been detected might provide a future pathway to investigating the genetic basis of the pathogenicity of JDV. The lack of significant genetic variation between JDV isolates (Desport *et al*., 2007) bodes well for the efficacy of a vaccine against different strains in the field, as large differences in the level of protection following vaccination have been seen in horses challenged with genetically distinct heterologous or homologous virus (Issel *et al.*, 1992) and protection against heterologous FIV challenge in cats requires the use of a dual subtype vaccine (Hohdatsu *et al.*, 1997; Pu *et al.*, 2005).

The studies reported in this thesis have clearly indicated that any future efficacy testing should include an assessment of the virus load in the plasma of animals in vaccine trials, rather than relying on clinical observations to assess any amelioration of the disease, as infected asymptomatic animals still have the potential to spread disease. It was noted, however, that there was considerable variation in the rate of

progression of the disease in different animals, and the virus load, even in those receiving the same infectious inoculum. The development of a simple model in Chapter 4 to describe the initial acute phase of JDV infection provided a means of statistically comparing the effect of vaccination by subdividing the acute disease period into phases and comparing the progression of the disease in individual animals. Mathematical models have been developed to describe the effects of treatment on the progression of HIV infection to AIDS, and to predict the length of symptom-free infection following the initial acute phase of HIV infection, as well as the effects of vaccination on the progress of the AIDS epidemic (Wodarz and Nowak, 2002; Davenport *et al.*, 2004). Most models have focused on prediction of the long term outcome of HIV infection in humans or non-human primates but efforts have also been devoted to the initial acute phase of disease (Stafford *et al.*, 2000) despite the difficulty in obtaining daily samples from recently infected individuals to enable this. Unlike JDV infection, the acute phase of HIV infection often passes unnoticed and is not seen as the most serious manifestation of HIV infection, although intervention to decrease the severity of this phase has ramifications for the rapidity of progression to AIDS in humans (Blattner *et al.*, 2004) and non-human primates (Staprans *et al.*, 1999).

The type of immune response required to control the infection was not adequately assessed as it was not possible to undertake analysis of cell-mediated immune responses in the vaccinated cattle in Bali. Although the Tat/CA vaccine finally tested in Trial V (Chapter 6) was not directly compared with the effects of the inactivated viral vaccine (Chapter 3), it was apparent that treatment with the inactivated virus improved the rate of clearance of the virus compared with the Tat/CA vaccine. Conversely, the recombinant protein vaccine slowed the initial increase in plasma viraemia relative to the conventional vaccine, possibly due to an anti-Tat response decreasing the effects of extracellular Tat on bystander cells, as discussed in Chapter 6. Responses to Tat have not been detected after vaccination with the inactivated viral preparation (J Lewis, personal communication) possibly as this protein is expressed early in infection before the spleen of experimentally infected cattle is taken for preparation of vaccine. Large rebounds in the leucocyte populations post-challenge

suggest the possible presence of a large primed population of cells, possibly responsible for enhanced clearance of virus. Some efforts to vaccinate against animal lentivirus infections have potentiated disease (McGuire *et al.*, 1986; Karlas *et al.*, 1999) and it is possible in the studies reported in this thesis that the high number of mortalities, especially in Trial V where QuilA was investigated as an adjuvant, may have resulted from the induction of a strong CMI response providing a larger pool of susceptible target cells for virus replication. The type of response engendered by vaccination was important in these trials, with a balance between an effective response and a potentially exacerbating response apparently dependent on the adjuvant chosen. The inactivated viral vaccine appeared to have a significant effect in increasing the rate of clearance of plasma virus after the peak of viraemia. The most effective recombinant protein combination of Tat + CA in Trial V decreased the rate of appearance of viral genomes in the plasma in the earlier stages of the disease, prior to the peak viraemia. A vaccine regime consisting of inactivated JDV or BIV, coupled with recombinant Tat protein to exploit both arms of the immune system may be a fruitful avenue to explore in efforts to control JD.

Important guiding criteria for this project were that the technology required for vaccine production in Indonesia needed to be simple, and the cost of production had to be low. A number of potential DNA vaccine constructs were produced and expression of proteins from these constructs was achieved *in vitro* and the constructs are available if it was desired to continue investigations into these type of vaccines in the future. Producing virus vaccines is fraught with many difficulties but an important factor that is often overlooked is the importance of being able to license the vaccine. During the course of the studies reported in this thesis it became apparent that while DNA vaccines offered substantial advantages in terms of cost, ease of production and stability (Whalen, 1996a), serious doubts emerged as to whether such a vaccine would be licensed in Indonesia. Although several potential DNA vaccines for HIV and other diseases are in Phase 1 clinical trials (Brave *et al.*, 2005) or undergoing safety evaluation (Graham *et al.*, 2006; Sheets *et al.*, 2006), no precedents existed for their license in any country for use in any mammalian or avian species, and licensing such a vaccine in Indonesia was thought to be potentially difficult. It was this

conclusion that resulted in a decision to at least temporarily terminate these studies of potential DNA vaccination.

A bacterial expression system, with potential high yields of protein at a low cost was considered the most appropriate for use in Indonesia. Early studies, not reported in this thesis, indicated recombinant JDV SU and Tm proteins prepared in this system did not provide any protective efficacy and efforts were later directed to the use of CA and Tat. The failure of JDV SU and Tm to induce a protective immune response was possibly associated with the lack of post-translational modifications of the proteins in the bacterial system. However, a widely used feline leukaemia virus vaccine consisting of non-glycosylated SU produced in a bacterial expression system (Marciani *et al.*, 1991) is effective (Hofmann-Lehmann *et al.*, 1995), suggesting glycosylation of envelope proteins incorporated into a lentiviral vaccine may not necessarily be essential. Perhaps the choice of adjuvant could also be critically important for efficacy of these non-glycosylated envelope antigens.

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