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Construction and characterisation of a Semliki Forest virus vector based rubella prototype vaccine

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A thesis submitted to the University of Dublin, Trinity College for the degree of

Doctor of Philosophy

by

Sara Jane Callagy

Department of Microbiology,
Moyne Institute of Preventive Medicine,
Trinity College, Dublin

October- 2007

Declaration

This thesis is submitted by the undersigned to the University of Dublin, Trinity College for the examination of Doctorate of Philosophy. The work herein is entirely my own work and has not been submitted as an exercise for a degree to any other university. The librarian of Trinity College Dublin has my permission to lend or copy this thesis upon request.

Sara Jane Callagy

To my family

Denis, Mary, Tim and Gareth

Summary

Alphavirus vaccines such as those based on the Semliki Forest virus (SFV) replicon have been widely studied as candidate vaccines. Following intramuscular injection, dispersal of the RNA occurs to the local lymph node only, persisting only for 7 days. Recombinant SFV (rSFV) particles are suicidal and undergo only one round of multiplication, killing the cells by apoptosis induction. Replication of RNA is entirely cytoplasmic thus there is no possibility of insertional mutagenesis. Vaccination against rubella is contra-indicated in pregnant women or those considering pregnancy because of the risk of infection of the developing foetus, and such women therefore remain at risk of infection with the wild-type virus and consequent congenital rubella syndrome in the infant after birth.

The RA27/3 rubella vaccine strain is the most widely used and the genome has been sequenced to allow study of the attenuating mutations, making this strain a good choice for cDNA cloning to construct a prototype recombinant vaccine. The rubella RA27/3 E2 and E1 envelope genes were cloned into the SFV10-Enhanced (SFV10Enh) vector. Recombinant SFV particles, rSFVE2E1, were made using helper RNA and titration of infectious units (I.U.) by immunofluorescence using antisera was carried out.

Female BALB/c mice were immunised intramuscularly (i.m.) with different combinations of 10^6 rSFV particles expressing the rubella RA27/3 E2E1, measles Edmonston H and mumps Enders HN antigens and sera collected prior to immunisation, on weeks 2, 4 (pre-boost), 6, 8, and 12. A four week boost was found to elicit higher titres than a two week boost. Mouse anti-RV serum whole IgG was detected by a limiting dilution enzyme linked immunosorbent assay (ELISA) and functional antibody titres were determined using a latex agglutination assay. Antibody isotyping indicated a T-helper 1 response predominated.

The first aim of this project was to construct and test in mice a prototype recombinant particle vector to express the E1 and E2 proteins of rubella virus, the main antigenic determinants, which would not consist of live virus but should induce good immunity. Since SFV vectors multiply well in mice, and induce good immunity, unlike wild-type measles, rubella and mumps viruses, the second aim was to use the SFV vector to test for immune interactions in mice when the 3 recombinant vaccines were administered at the same time and at equal dose. For this we used the attenuated measles Edmonston H antigen and the mumps Enders HN antigen and found that both together and separately they significantly reduced the anti-rubella immune response.

Aims

The MMR is a model multivalent vaccine for testing immune interactions of different components. The current MMR vaccine cannot be administered to infants with acquired maternal immunity and the rubella vaccine cannot be given to seronegative pregnant women. Recombinant DNA technology has the potential to abrogate possible side effects of the live attenuated virus vaccine strains.

The rationale behind this Ph.D. thesis was thus to:

- Construct and characterise a prototype SFV recombinant particle vector expressing the immunogenic E1 and E2 proteins of the rubella virus RA27/3 vaccine strain

- Test for immune interactions to three recombinant SFV vaccines expressing the rubella virus RA27/3 vaccine strain E2 and E1 proteins, the measles virus Edmonston vaccine strain H protein and the mumps virus Enders vaccine strain HN protein

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I would like to thank my supervisor, Prof. Greg Atkins for granting me the opportunity to undertake my studies in his laboratory. I am grateful for my learning experience as a graduate student and for his encouragement to broaden my horizons.

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I thank my parents, Denis and Mary and my brothers, Tim and Gareth; a*ltissima quaeque flumina minimo sono labi.*

Presentations

Construction and characterisation of a Semliki Forest virus vector based rubella prototype vaccine

Oral presentations:

Society for General Microbiology (SGM) meeting, University of Manchester, UK, 2007

SGM meeting, Heriot-Watt University, Edinburgh, Scotland, 2005

EU Collaboration SFVectors meetings in Estonia, Spain & Ireland, 2004-2006

Poster presentations:

6th National Congress of the Italian Society of Virology, Orvieto, Italy, 2006

NFID, 9th Annual Conference on Vaccine Research, Baltimore, Maryland, USA 2006

IRCSET Annual Symposium, Croke Park, Ireland, 2004 & 2005

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Abbreviations

a.a. - amino acid

Ag - antigen

ATCC - American type culture collection

APC - antigen presenting cell

AMI - antibody mediated immunity

Ab – antibodies

AIDS - acquired immunodeficiency disease syndrome

ATP - adenosine triphosphate

BHK - baby hamster kidney

bp - base pair

CAL- Calreticulin

CAT - chloramphenicol and acetyltransferase

CDC - Centers for Disease Control

cDNA – complementary DNA

CMI - cell-mediated immunity

CMV - cytomegalovirus

CNS - central nervous system

ConA - concanavalin A

CP – capsid protein

CPE - cytopathic effect

CRS – Congenital rubella syndrome

CTL - cytotoxic T-lymphocyte

CV - cytopathic vacuoles

DAPI - 4, -6, diamidino-2-phenylindole

dATP - deoxyadenosine triphosphate

days post-infection-dpi

DC - dendritic cell

DTH - delayed-type hypersensitivity

DEPC - diethylpyrocarbonate

DI – defective interfering

DMEM- Dulbecco's minimum essential medium

DNA - deoxyribonucleic acid

dNTP - deoxynucleoside triphosphates

ds- double-stranded

dsDNA - double-stranded DNA

dsRNA- double-stranded RNA

DTT - Dithiotreitol

EDTA- ethylenediaminetetra-acetic acid

EGFP - Enhanced green fluorescent

ELISA - enzyme linked immunosorbent assay

ER - endoplasmic reticulum

FBS - foetal bovine serum

FCS - foetal calf serum

FITC - fluorescein isothiocyanate

FMDV - foot-and-mouth disease virus

g - gravitational force

GM-CSF - granulocyte-macrophage colony-stimulating factor

H & E - Haematoxylin and Eosin

HBSS - Hank's balanced salts solution

HAI - hemagglutination inhibition

HEPES - N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid

H - helicase

HIV - human immunodeficiency virus

HLA - human leukocyte antigen

HPV - Human papilloma virus

HRP - horseradish peroxidase

HSP – Heat Shock Protein

i.m. - intramuscular

i.n. - intranasal

i.p. - intraperitoneal

i.t. - intratumoural

i.v. – intravenous

IFN - interferon

Ig - immunoglobin

IL – interleukin

IDDM - insulin-dependent diabetes

IPTG – isopropyl-beta-D-galactopyranoside

IU - infectious units

MMP - Matrix metalloproteinases

MV – measles virus

M - molar

MCS - multiple cloning site

MS – multiple sclerosis

MT - methyltransferase

MMR – measles, mumps and rubella

MHC - major histocompatibility complex

mRNA - messenger ribonucleic acid

MMLV - moloney murine leukaemia virus

MLR – Mixed lymphocyte reaction

MVA – Modified vaccinia virus ankara

NT - neutralization

NK - Natural killer cells

nsP - non-structural protein

NTR - nontranslated region

NO - nitric oxide

nt - nucleotide

one-way ANOVA – one-way analysis of variance

ORF – Open reading frame

p.i. - post-infection

PID - days post inoculation

PCL - Packaging cell lines

PCP - papain cleavage protease

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PBMC - peripheral blood mononuclear cells

PKR - protein kinase

PM – plasma membrane

PRP - progressive rubella panencephalitis

P - protease

r – recombinant

R - replicase

Rb - Retinoblastoma

RBC - red blood cell

RdRp - RNA-dependent RNA polymerase

RER- rough endoplasmic reticulum

RF – replicative form

RGI – Genotype I

RGII – Genotype II

RI – replicative intermediate

RLP – RV-like particle

RNA – ribonucleic acid

RNase – ribonuclease

RPV - Rinderpest virus

rpv – replication competent vector

RRV – Ross River virus

rpm - revolutions per minute

RT - reverse transcription

RT-PCR - reverse transcription PCR

s.c. - subcutaneously

SFV - Semliki Forest virus

SG – subgenomic

SIV – simian immunodeficiency virus

SINV - Sindbis virus

SL – stem loop

SLAM - signaling lymphocyte activation molecule

SP – synthetic peptide

spf - specific pathogen-free

TAP - Transporter associated with antigen processing

TGF- transforming growth factor

TLR – toll like receptor

TNF - Tumour necrosis factor

Tr cells - regulatory T cells

UTR - untranslated

VEEV - Venezuelan equine encephalitis virus

VEGFR - vascular endothelial growth factor receptor

wt – wild type

Chapter 1

General Introduction

1. INTRODUCTION

1.1. Rubella virus

1.1.1 Togaviruses

The *Togaviridae* (from the Latin toga, a Roman mantle or cloak) family of viruses consists of only two genera: *Alphavirus* and *Rubivirus* which are characterised by small, lipid enveloped, spherical particles with a single-stranded positive ribonucleic acid (RNA) genome (Schlesinger, 2001). Semliki Forest Virus (SFV) is a member of the alphavirus genus while Rubella virus (RV) is the sole member of the rubivirus genus and scrutiny of homologies indicates no straightforward evolutionary relatedness. SFV has provided valuable models for examining the synthesis, posttranslational modifications, and localization of membrane glycoproteins. SFV has been linked to human disease on only two occasions mainly causing myalgias and arthralgia (Griffin, 2001). SFV has thus been genetically engineered to express heterologous proteins and such attenuated vectors are being used to develop vaccines. RV is well known for its ability to cause disease in humans, the most severe form being congenital rubella syndrome (CRS). RV has a limited host range and is found only in humans, unlike SFV which also grows in insect cells. This makes RV a candidate for global eradication using the available live attenuated vaccines and potentially a recombinant genetic vaccine in the final stages of eradication (Atkins *et al.*, 2003).

1.1.2 History and Epidemiology

It has been suggested that RV originated by a complicated event involving recombination with progenitors of the current alphaviruses, human hepatitis E virus and perhaps plant viruses (Risco *et al.*, 2003). While there is only a single serotype of RV, there are two genotypes, RGI and RGII, which differ by 8-9% at the nucleotide level in the E1 glycoprotein gene (Hofmann *et al.*, 2003; Pugachev *et al.*, 1997a) (Figure 1.1). Significant antigenic differences do not exist between RGI and RGII viruses (Duncan *et al.*, 2000; Trudel, 1988; Zheng *et al.*, 2003). There are more than 60 isolated strains worldwide (Wang *et al.*, 2003). The JR23 strain is more virulent than other strains (Wang *et al.*, 2003). CNS involvement occurs in about 0.03% of all patients and clinical encephalitis usually occurs within one week. Genotype I contains 60 viruses from North America, Europe and Japan, comprising the majority of the available sequences from wild

type (wt) and vaccine strains (Hofmann *et al.*, 2003). Genotype II contains three viruses from China and India (Frey *et al.*, 1998). Antigenic drift does not appear to play a role in CRS. However two viruses isolated from chronic arthritis patients exhibited changes in important epitopes (Frey *et al.*, 1998).

The clinical manifestations of rubella were first recorded by two German physicians, de Bergan in 1752 and Orlow in 1758. Considered a derivative of measles, it was thus named German measles. The illness was documented as a distinct entity in 1814 by George de Maton and became known as "rotheln". The disease was then renamed rubella (from the latin for reddish things) in 1866 by Henry Veale, a British army surgeon (Lee *et al.*, 2000). The viral aetiology of the disease was documented in 1938 by Hiro and Tasaka. In 1941, Norman Gregg, an Australian ophthalmic surgeon, reported the devastating teratogenic effects of the virus. He discovered a link between the increase of congenital cataracts in newborn children and rubella infection during the mother's pregnancy. In 1962 the isolation in cell culture of the etiological agent of rubella was reported by Parkman *et al* in African green monkey kidney cells and Weller *et al* in human amnion cells by visible cytopathic effect (CPE) (Lee *et al.*, 2000).

1.1.3 Genome organization

The genome of RV is a single-stranded RNA of positive-polarity that is about 10 kb nucleotides in length and has a high GC content of 69% (Chantler *et al.*, 2001). The genomic RNA contains 2 long overlapping open reading frames (ORFs) that are separated by a 123 nucleotide untranslated (UTR) region (Suomalainen *et al.*). When the RA27/3 sequence was compared to that of the rubella wt M-33 and Therien strains there was a 1.0 to 2.8 % variation between the three strains at the nucleotide level and a 1.1 to 2.4 % difference at the amino acid (a.a.) level (Figure 1.1). The Therien and RA27/3 strains were more closely related at both the nucleotide and a.a. levels (Pugachev *et al.*, 2000a). However the sequence of the three strains from other geographic regions and isolated at different times may have revealed a greater degree of variation (Pugachev *et al.*, 2000a). The sequence of the RV 3' UTR varied by one to three nucleosides whereas the 3' UTR of alphaviruses can vary by up to 20 % and can contain large insertions/deletions. The striking low divergence of the RV genome may be due to the specificity of the viral antigens (Ags) whereby cross reactions caused by antibodies (Abs) or T cells induced by other viral infections may not prevent susceptible hosts from RV infection. Thus RV would not need to escape from heterologous immune responses (Hofmann *et al.*, 2003).

1.1.4 RV Infection

1.1.4.1 Postnatally acquired rubella

Postnatal infection with clinical symptoms occurs in approximately 50–67% of cases in infants and 85% in adults (Katow, 2004). The remaining cases are asymptomatically infected thus serodiagnosis is required for a confirmed diagnosis. The main symptoms include maculopapular rash, lymphadenopathy, fever, conjunctivitis and arthralgia. Characterised by a fever, a rash lasting about 3 days (it is often called the 'three-day measles') and lymphadenopathy, rubella is a mild disease occurring mainly in children (Katow, 2004). Infection with RV is via inhalation of aerosol with the virus infecting the cells in the upper-respiratory tract. Cell entry occurs like SFV by receptor-mediated endocytosis and an acid triggered fusion step (Yang *et al.*, 1998). Rubella spreads and replicates in the lymphoid tissue of the nasopharynx and upper-respiratory tract leading to a viraemia which causes systemic infection, involving many organs, including the placenta (Banatvala *et al.*, 2004). The virus spreads to the regional lymph nodes (Lee *et al.*, 2000). The first clinical manifestation of rubella is the appearance of a maculopapular rash on the face some 16 to 20 days after exposure which spreads over the trunk and later the extremities (Lee *et al.*, 2000). Other symptoms can include low-grade fever, lymphadenopathy, sore throat, and general malaise.

Postnatally acquired rubella is rarely linked with complications, apart from joint symptoms such as arthritis and arthralgia (Banatvala *et al.*, 2004). Complications of rubella infection less frequently include thromobocytopenia purpura, postinfectious encephalopathy or encephalomyelitis and rubella panencephalitis (Lee *et al.*, 2000). Three neurological syndromes can result from sequelae of RV infection including a postinfectious encephalitis following acute infection, a spectrum of neurological manifestations following congenital infection and progressive rubella panencephalitis (PRP) which is an extremely rare neurodegenerative disorder. The pathogenesis of these syndromes is not known. Virus invasion and replication in the brain accounts for the neurological lesions in CRS and PRP may be autoimmune in nature, possibly triggered by molecular mimicry between viral and host epitopes (Frey, 1997).

1.1.4.2 CRS

With the exception of Japan, Singapore and Taiwan, in most Asian countries rubella

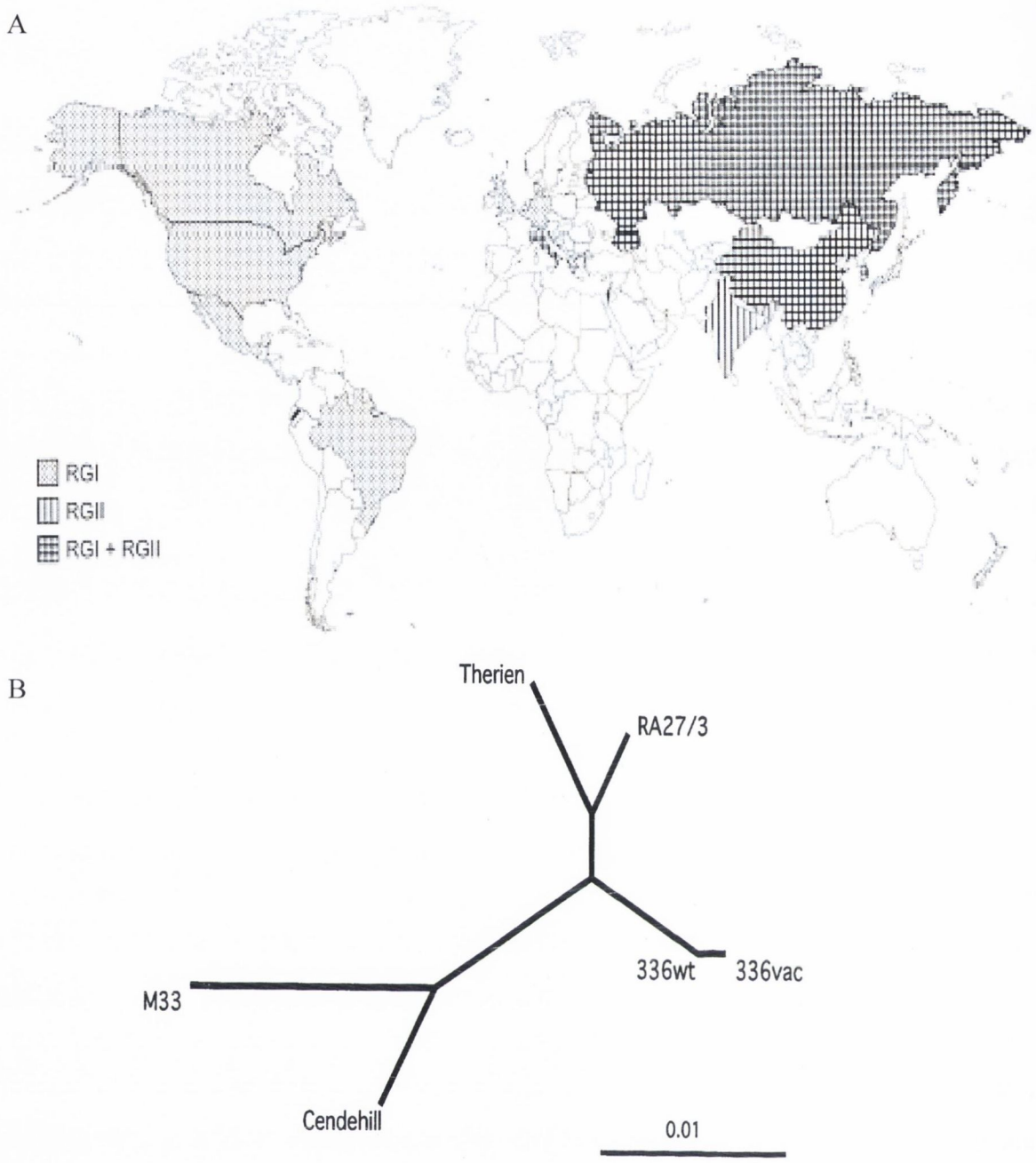


Figure 1.1 Geographical distribution and divergence of RV genotypes I and II.

A) Geographical distribution of RV genotypes I and II with 104 isolates. Countries with genotype I (RGI), genotype II (RGII) and genotypes I and II (RGI + RGII) are shown. Notice that all virus isolates in genotype II are found in the Asian continent except Italy (suspected to be imported) (Katow, 2004). B) The phylogenetic tree of six virus wt and vaccine strains was drawn using structural protein open reading frame (SP-ORF) sequences by the software CLUSTAL W, and is shown in non-root dendrograph. Bar, 0.01 nucleotide difference (Kakizawa *et al.*, 2001).

remains uncontrolled and the burden of diseases from CRS is high (Katow, 2004). If RV is acquired in the first trimester of pregnancy CRS can occur. This can result in serious foetal defects such as hearing loss, congenital heart defects, mental retardation, cataracts and glaucoma. Foetal damage results from a combination of rubella-virus-induced cellular damage and the effect of the virus on dividing cells. Placental infection occurs during maternal viraemia, resulting in focally distributed areas of necrosis in the epithelium of chorionic villae and in the endothelial cells (EC) of its capillaries (Tondury *et al.*, 1966) These cells seem to be desquamated into the lumen of vessels which suggests that RV is transported into the foetal circulation as infected endothelial cell emboli possibly resulting in infection and damage of foetal organs. During early pregnancy, foetal defence mechanisms are undeveloped, and a distinguishing feature of rubella embryopathy in early gestation is cellular necrosis in the absence of any inflammatory response (Banatvala *et al.*, 2004). The most severe pathology associated with RV, CRS, may be caused partly, by apoptotic cell death (Duncan *et al.*, 2000). Although apoptosis in the early stages of infection would restrict virus replication efficiency, apoptosis may actually assist the spread of progeny viruses to neighbouring cells (Hofmann *et al.*, 1999). Rubella embryopathy could conceivably be related to untimely and inappropriate induction of RV-associated apoptosis in embryonic and foetal tissue. The contribution of this apoptosis to pathogenicity *in vivo*, and the underlying mechanisms of the well-known defective organogenesis supposedly caused by mitotic inhibition, are still unclear (Hofmann *et al.*, 1999).

The specific pathway leading to teratogenicity remains to be elucidated (Lee *et al.*, 2000). There is evidence that RV infection may be connected with an inhibition of the development of organ precursor cells and that interference of actin assembly may be involved (Lee *et al.*, 2000). The virus does not affect major morphogenetic processes and it is not known what controls the spread of the virus in the early embryo (Webster, 1998). A variable region in non-structural protein (nsP) 1 (a.a. residues 697-800) has been identified and it is possible that this region contributes to the molecular basis of RV embryopathy. Phylogenetic analysis reveals a strong positive selection for this region (Hofmann *et al.*, 2003). Most sera from a study on newborns and infants with CRS contained no E2 protein-specific antibody and none of the CRS sera reacted well with C protein. However all natural immune sera tested recognised all three of the structural polypeptides (de Mazancourt *et al.*, 1986). Intrauterine exposure to E1 may result in selective immunological tolerance to the RV E1 protein and this may be a feature of RV persistence (Mauracher *et al.*, 1993).

1.1.4.3 Public Health policy and prevention

With the aim of preventing CRS, following the isolation of the virus in 1961, attenuated Rubella vaccine strains were initially developed to produce live rubella vaccines and placed in use in vaccination programs (Banatvala *et al.*, 2004). The rubella strain M-33 was attenuated in African green monkey kidney cells by Parkman *et al* to produce the first attenuated vaccine strain HPV-77 (Cabasso *et al.*, 1967). Thus live, attenuated RV vaccines, which have been used since 1969 to immunise infants and susceptible women of child-bearing age, have successfully reduced the incidence of infection (Ou *et al.*, 1994). Sweden introduced a two-dose programme of vaccination against measles, mumps and rubella (MMR) with a combined vaccine in 1982 and was the first country in the world to do so (Broliden *et al.*, 1998). The vaccination was carried out at the ages of 18 months and 12 years. Currently, the vaccine is administered universally at 15 months of age and again at 5-10 years in combination with the measles and mumps live, attenuated vaccines (Pougatcheva *et al.*, 1999). Finland is the first documented country to be free of indigenous measles (since 1996), mumps and rubella (Peltola *et al.*, 2000).

It has been suggested that, if rubella were combined with measles vaccine in all campaigns for the eradication of measles, rubella could also be eradicated for little extra cost (Pogue *et al.*, 1996) (Figure 1.2). Humans are believed to be the only natural host and reservoir for the virus further validating the possibility of eradication as in the case of polio (Chantler *et al.*, 2001). In 2000, measles was pronounced no longer endemic in the US. On March 21, 2005, the Centres for Disease Control (CDC) declared rubella (also known as German measles) eliminated in the US. The CDC began a rubella eradication program in 1989, and in the subsequent decade, only 117 cases were reported. In 2001, less than 100 US cases were reported, and in 2004, less than 10 cases surfaced, all of which were likely acquired in other countries and imported into the US. The Pan-American Health Organization has set a target to eradicate rubella from all of North and South America by 2010 (Bloom, 2005). Vaccination of infants only reduces infection of nonpregnant women by circulating rubella virus thus resulting in an increase in their susceptibility to residual exposures during later pregnancy. This paradoxical effect can be dealt with by a single mass vaccination of both sexes up to 39 years of age and a catch-up vaccination of children up to 15 years of age (Plotkin, 2006). To conserve the nonvirulence and stability of seed stock of the vaccine virus, which gained the license for manufacturing, introduction of the seed lot system was recommended by the WHO (Frey *et al.*, 1998). Under this system identification of the individual vaccine strain by nucleotide sequencing will be requested to

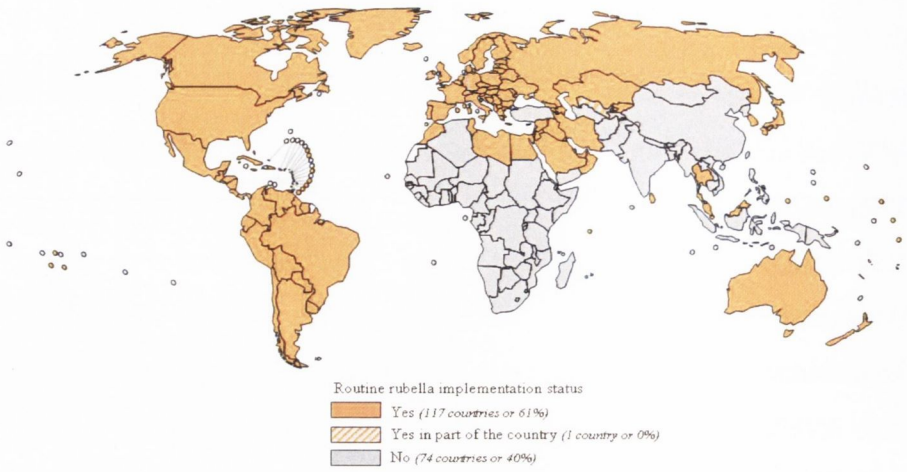
certify sequence conservation even after a limited number of passages.

1.1.4.4 Susceptibility to rubella infection

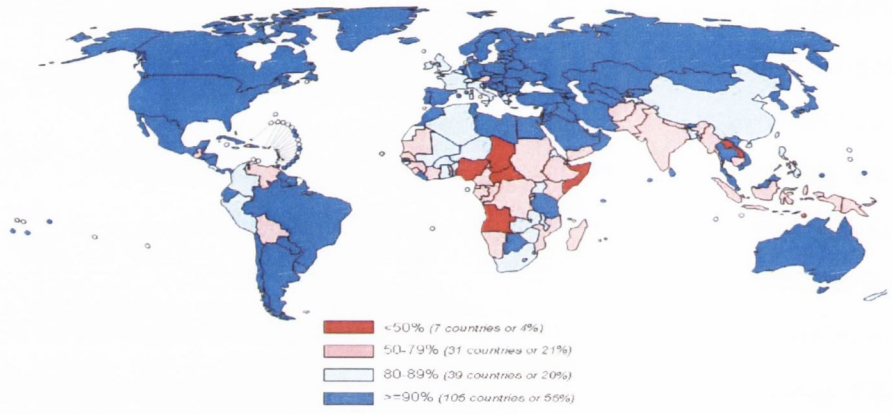
Serologic surveys have demonstrated that 6 to 25% of women of childbearing age could be RV seronegative and thus at risk of infection (Mitchell *et al.*, 1996). RV reinfection occurs more frequently in vaccinated than in naturally immune individuals and the increasing number of reports of rubella in individuals who were vaccinated in infancy denote RV vaccine failure. Such individuals may lack critical components of rubella immunity that would otherwise protect them from virus challenge and viraemia (Mitchell *et al.*, 1996). A US serologic survey of young women (mean age, 20 years) in 1993, revealed a rubella seronegativity rate of 10.1% despite MMR vaccination (Mitchell *et al.*, 1999). A previous study had found that 1/11 (9.1%) children had become seronegative within 15 years of receiving the live attenuated RA27/3 strain vaccine (Mitchell *et al.*, 1999). Long-term persistence of RV-vaccine induced cell-mediated immunity (CMI) is under debate (Mitchell *et al.*, 1999).

There are hormonal influences on RV-specific immunity, which might result in differential handling of RV (Mitchell, 1999b). This may partially explain why women are predisposed to adverse outcome of rubella infection and immunisation. There were no notable sex differences in the distribution of E1 specific antibodies, whereas more males produced E2 specific antibodies after vaccination and more females produced C specific antibodies (Mitchell, 1999b). There was a brisker onset of recall antibody mediated immunity (AMI) in males compared to females. A mathematical model of protection elicited by an immunisation with a single rubella dose suggests that, assuming 80% of 2-year old children would be vaccinated, 90% of vaccinees would develop protective antibodies and 1% per year would lose immunity, record low levels of CRS would result within 20 to 25 years. By then the population at risk would be so large that an importation of virus would cause an exponential increase in embryopathy, large enough to exceed the pre-vaccination level (Peltola *et al.*, 2000). Some adolescents and young adults remain susceptible either because they escaped immunisation in childhood or are primary vaccine failures (Bakshi *et al.*, 1990). Sub-Saharan Africa does not include rubella immunisation in a national program, even though measles immunisation is routine at 9 months of age (Lawn *et al.*, 2000). Nine rubella serosurveys have been conducted in West Africa, including one study in Ghana. Rubella susceptibility rates were reported between 10% and 32% for women of reproductive age. In fact the proportion of susceptible women was very

Countries using rubella vaccine in their routine national immunization system, 2005



Immunisation coverage with measles containing vaccines in infants, 2005



Countries using mumps vaccine in their routine national immunization system, 2005

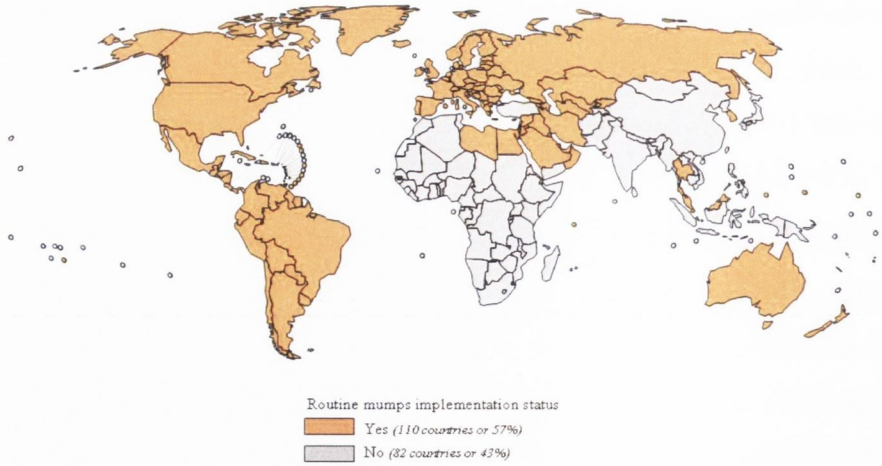


Figure 1.2 Immunisation coverage with rubella, measles & mumps vaccines in 2005

(http://www.who.int/immunisation_monitoring/diseases/rubella/en/)

similar to that in the prevaccine era in industrialised countries (Lawn *et al.*, 2000).

1.1.4.5 Diagnosis and treatment

Clinical diagnosis of rubella is unpredictable and laboratory confirmation vital (Banatvala *et al.*, 2004). In several parts of the tropics, alphaviruses and flaviviruses may induce rubella-like illnesses (Banatvala *et al.*, 2004). A Brazilian study shows that between 1994 and 1998, maculopapular rashes that were too complicated to differentiate clinically were induced by rubella, parvovirus B19, dengue, human herpesvirus 6, and measles, a number of which were circulating concomitantly. Parvovirus B19 can circulate concurrently with RV and although non-teratogenic, is related to a high incidence of miscarriage, usually in the second trimester and more often foetal hydrops (Banatvala *et al.*, 2004). Diagnosis is made by serological techniques such as detection of specific IgM by enzyme linked immunosorbent assay (ELISA), neutralising antibodies or using hemagglutination inhibition (HAI) assays (Banatvala *et al.*, 2004). IgG1 has been found to be the predominant antibody isotype in patient serum samples (59%) following the onset of symptoms due to rubella infection (Sarnesto *et al.*, 1985). The other antibodies present were IgM (23%), IgA (8%) and IgG3 (3%). Serological techniques are currently used to diagnose rubella, and the risk to the foetus is assessed by determining the gestational age at the time of maternal infection. When serological results are inconclusive, infection has occurred between 13-20 weeks gestation or when maternal re-infection is confirmed or suspected, a technique for prenatal diagnosis would be useful to assess the risk to the foetus (Johnstone *et al.*, 1996). The detection of rubella-specific immunoglobulin (Ig) M in foetal blood is obtained at cordocentesis and isolation of RV is by cell culture from samples such as chorionic villi or amniotic fluid. The former method is not reliable until 22 weeks of gestation and the latter is labour-intensive and time-consuming. A new technique to detect RV RNA in amniotic fluid and foetal blood is reverse transcription (RT) followed by PCR amplification (Bosma *et al.*, 1995; Tang *et al.*, 2003). A nested PCR has been employed on the E1 ORF, and less abundant non-structural (NS) protein coding sequences and a definitive result obtained within 24 h of receipt of a specimen, in contrast to virus isolation, which normally takes 3 to 4 weeks (Johnstone *et al.*, 1996). The HAI assay and neutralisation (NT) assay are used for detecting protecting antibodies to RV. It has been demonstrated that neutralising antibodies detected by neutralisation assays may not be useful in protecting from reinfection. The HAI assay is highly specific and at the moment it is considered the "gold standard" method for the determination of protective immunity to

RV (Cordoba *et al.*, 2000). The E1 protein contains 4 NT domains and the target sequence for HAI antibodies between a.a. residues 209-291. One NT domain between E1 residues 213 and 339 has been mapped which recognises both murine and human antibodies (Mitchell *et al.*, 1996). Studies indicate that after RV infection or vaccination, antibodies directed to E1 (residues 213 to 239) increase in parallel and correlate with the RV-specific antibody measured by ELISA, HAI and NT tests (Mitchell *et al.*, 1996). The antiviral activity of polysaccharides is generally exerted by carbohydrate polymers with a negative electric charge, which is usually provided by sulphate groups. It has been shown that scleroglucan, a neutral polysaccharide, can inhibit the replication of RV (Mastromarino *et al.*, 1997).

1.1.5 Life cycle of RV

1.1.5.1 Virus entry

Virus entry in to cells is believed to be by receptor mediated endocytosis, but the cellular receptor has not yet been identified (Petruzziello *et al.*, 1996). Membrane lipid molecules have been shown to have a role in the cell surface receptor for RV (Mastromarino *et al.*, 1990). The membrane glycoproteins are essential for binding to host cells and membrane fusion during infection (Bonnefoix *et al.*, 2003). The envelope protein E1 also plays a key role in binding to an unknown cell receptor, in fusion of the viral envelope with the host cell membrane (Hofmann *et al.*, 2003). Viruses such as SFV and RV entering by receptor mediated endocytosis use the endocytic machinery to travel within the cytoplasm and entry is by clathrin coated pit via late endosomes or lysosomes (Sodeik, 2000). This requires an intact cytoskeleton and the endocytic membrane system can manoeuvre through the dense actin cortex beneath the plasma membrane (PM). Endosomes can travel long distances and this is useful in neurons where the site of entry can be far away from the site of viral replication. Infection of the host cell results in the modification of lysosomes to produce enlarged structures filled with vesicles known as cytopathic vacuoles (CVs). Viral replication is endosomal and lysosomal, which is unique to togaviruses (Magliano *et al.*, 1998). CVs contain the viral "RNA-replication complexes" or RC, as confirmed by the presence of double-stranded (ds) RNA (Risco *et al.*, 2003). The endocytic lumen is biochemically different from the extracellular milieu in pH, proteolytic capacity and possibly also redox potential. Thus, this environment can 'prime' previously unstable virions for disassembly and genome uncoating – the obligatory prerequisites for

viral transcription and replication. SFV fuses with the endosome (Sodeik, 2000).

1.1.5.2 RV replication

The 40S RV genomic RNA provides the template for the synthesis of a complementary RNA which in turn acts as a template for the production of both 40S genomic and 24S subgenomic (SG) RNA (Magliano *et al.*, 1998) (Figure 1.3). A fully double stranded replicative form (RF) of 19-20S and a partially dsRNA replicative intermediate (RI) of 21S are also produced by this transcription process. RV negative strand RNA is synthesised preferentially *in cis* while positive strand RNA can be synthesised both *in cis* and *in trans* but with higher efficiency *in cis*. The genomic RNA provides the mRNA template for translation of the non-structural genes as a 200 kd precursor (Chantler *et al.*, 2001). Processing of p200 to the two mature products (p150 and p90) is carried out by an RV-associated protease residing in the C terminal region of p150. The processing of the RV non-structural proteins is crucial for virus replication. The nsP region from residue 920 to 1296 is necessary for trans-cleavage activity (Yao *et al.*, 1998). P200 is the principal replicase for negative strand RNA as are p150/p90 for positive strand RNA (Liang *et al.*, 2001). Uncleaved p200 could function in negative-strand RNA synthesis but the cleavage products p150 and p90 are required for efficient positive-strand RNA synthesis (Yao *et al.*, 1998).

In the rubella life cycle the progeny full-length positive-strand RNA has three possible fates. It may be used in the replication complexes as template for negative strand synthesis (full length) or alternatively undergo translation to yield the non-structural protein precursor. At later stages of infection it may be encapsidated as part of the assembly process. The mechanism by which these events are regulated is not yet understood (Chantler *et al.*, 2001). The 5' and 3' terminal RV RNA sequences contain stem loop (SL) structures thought to be involved in RV RNA translation and in replication. Only a few nucleotides at the extreme 5' end of the RV genome are essential for viability (Pugachev *et al.*, 1998a). Mutations that affect the two A residues at ntd 2 and 3 simultaneously are lethal. An inverted GC-rich repeat sequence located at the 3' end of the RV genomic RNA SL structure is necessary for initiation of negative strand RNA synthesis (Atreya *et al.*, 1995). The 3'-terminal structure of RV negative-strand RNA was found to share the same host protein-binding activity with similar structures in alphaviruses (Nakhasi *et al.*, 1991). The RV 5' end binding proteins are Ro/SSA-associated antigens and the 3' end binding protein is calreticulin (CAL) (Nakhasi *et al.*, 1994; Singh *et al.*, 1994).

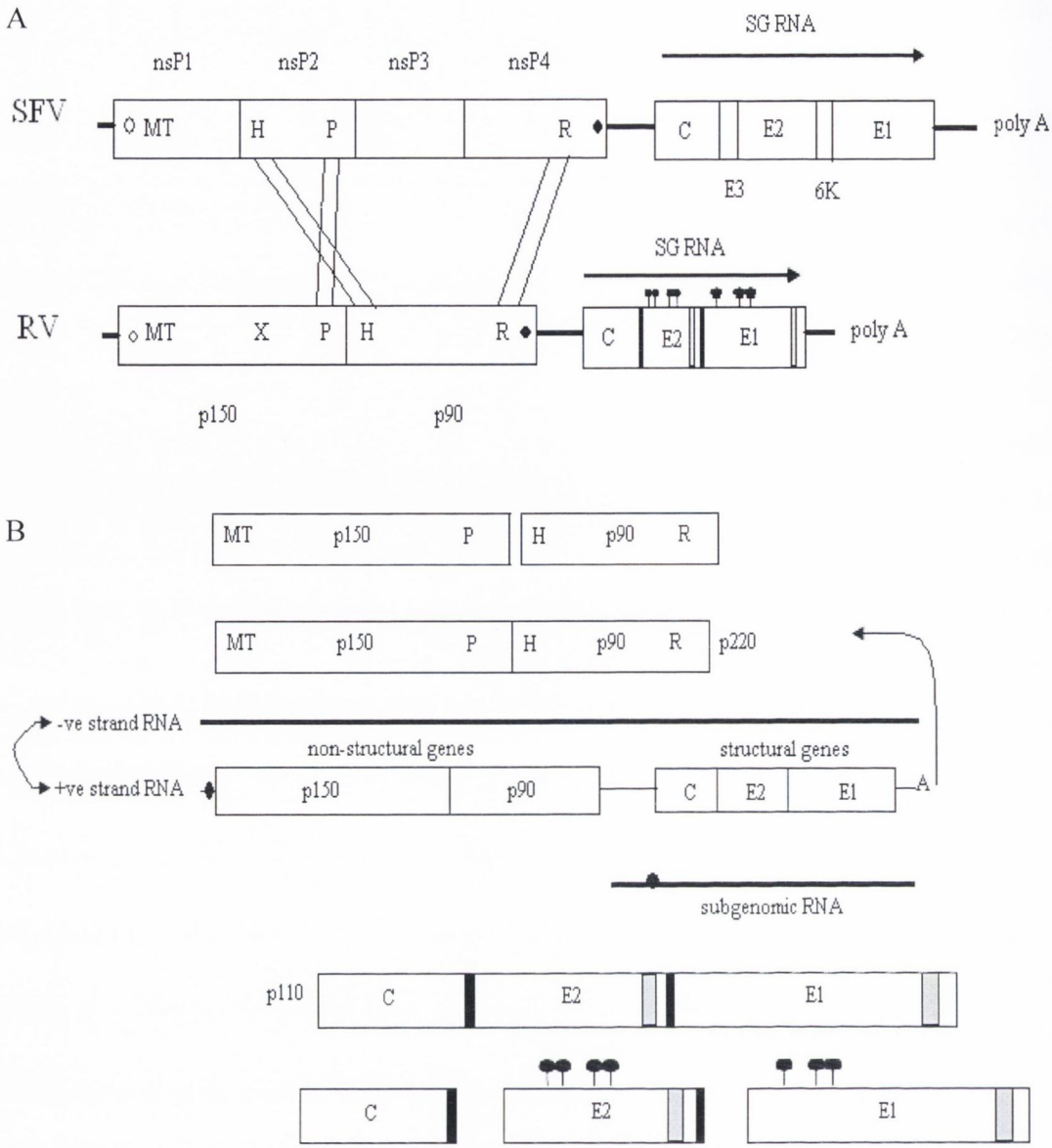


Figure 1.3 Organisation of the genomes of SFV and RV

A) The 5' two thirds of these genomes codes for the non-structural proteins (nsPs), and the 3' one third codes for the structural proteins, translated from SG RNAs. Untranslated regions are designated by *black lines*, and open reading frames, by *open boxes*. The viral RNAs have 5' terminal caps and 3' poly A tails. The *open* and *closed circles* at the 5' termini and the start of the SG RNA respectively indicate conserved sequences. The location of the a.a. motifs for helicases (H), replicases (R) and proteases (P) are indicated. Adapted from Schlesinger *et al.*, 2001. B) The transmembrane regions (grey) and signal sequences of E2 and E1 are indicated (black). Regions of the nonstructural proteins believed to encode enzymatic functions, including the methyltransferase (MT), protease (P), helicase (H) and replicase (R) are also shown.

Phosphorylated forms of CAL, a host protein which regulates Ca^{2+} storage, bind to the RV RNA 3' SL both *in vitro* and *in vivo*. CAL could support a productive infection of the virus by promoting viral RNA replication or translation, could target viral RNA for degradation or compartmentalise RV RNA in cellular organelles to avoid surveillance by the host immune system, leading to RV persistence (Atreya *et al.*, 1995). The interaction of CAL and the RV genome has been proposed to be related to viral pathogenesis, mainly arthritis (Chen *et al.*, 1999). CAL is classified as an autoantigen as antisera from patients suffering from autoimmune diseases, such as onchocercariasis, can cross react with CAL and it has less homology with other autoantigens. RV replicates to low titres in cell culture and does not shut off host cell macromolecular synthesis.

1.1.5.3 Nonstructural Gene Open Reading Frame (ORF)

The order of the functional motifs in the RV NSP-ORF is X-protease-helicase-replicase unlike that of the alphavirus NSP-ORF which is helicase-protease-X-replicase (Forng *et al.*, 1995). The X-domain located between residues 834 and 940 was found to be important for NS-pro *trans* cleavage activity. The order of conserved motifs for RV NSP i.e. methyltransferase-protease-helicase-polymerase differs from alphavirus NSP which is methyltransferase-helicase-protease-polymerase (Yao *et al.*, 1998). The inhibition of cell deoxyribonucleic acid (DNA) and RNA synthesis that occurs during alphavirus replication is thought to be due to nsP2. This function is associated with co-localisation to the nucleus. There is, however, a lack of RV non-structural protein that migrates to the nucleus and mediates inhibition (Forng *et al.*, 1995). A GDD motif indicative of replicase activity and a GK (S/T) motif indicative of helicase function have been mapped to the non-structural RV proteins (Chen, 1996). p90 has been proposed to be the RV RNA-dependent RNA polymerase (RdRp) (Yao *et al.*, 1998). The GDD motif proved critical for RV replication and p90 functions as RV RdRp as the catalytic subunit of the viral replicase required for the replication of all positive-strand RNA viruses (Yao *et al.*, 1998). The p90 nonstructural protein N-terminus contains sequence motifs characteristic of the helicase superfamily I as well as the conserved motifs of superfamily III RNA polymerase at its C terminal region (Pugachev *et al.*, 1997b). Thus the putative replicase activity has been assigned to NSP90 due to the presence of conserved viral replicase motifs (Atreya *et al.*, 1998).

NSP90 appears to interact with Retinoblastoma (Rb) and other members of the Rb family of proteins to support viral replication thus inhibiting cell proliferation and reportedly producing a mitotic inhibitor (Atreya *et al.*, 1998). The NsP90-Rb interaction

could perturb normal Rb function, which is to control normal cell growth (both cell proliferation and cell mass) by forming complexes with different transcription factors (TF). It is thought the manifestation of teratogenesis may stem from interactions between RV-encoded proteins and key cellular proteins. The Rb binding LXCXE motif is required for efficient virus replication and RV replication is reduced in cells lacking Rb (Forng *et al.*, 1999). There may be a common theme involving Rb in virus induction of teratogeny by DNA and RNA viruses (Forng *et al.*, 1999). The viral protease activity of RV resides in the C terminal domain of p150 and was thought to belong to the papain family like the alphavirus nsP2 protease and possibly the leader protease of the foot and mouth disease virus (Chen *et al.*, 1996). It is now thought that the RV NS protease is a novel virus metalloprotease rather than a papain cleavage protease (PCP) (Liu *et al.*, 1998). The RV NS protease domain mediates the cleavage of a polyprotein precursor into two mature products and cleavage occurs after translation of the NSP-ORF is complete (Chen *et al.*, 1996). Site directed mutagenesis of the Gly-1300 residue (the viral protease cleavage site) and of the Cys-1151 residue (one of the catalytic dyad residues of the viral protease) abolished p200 precursor cleavage and protease activity respectively (Yao *et al.*, 1998). The RV NS protease can function *in trans* like the alphavirus NS proteases (Liu *et al.*, 1998). The RV NS protease has been found to require the presence of divalent cations such as Zn^{2+}, Ca^{2+} and Co^{2+} to function efficiently. Fungizone, a polyene antibiotic, widely used in tissue culture systems and in clinical medicine was found to inhibit growth of RV in Vero cells but not measles and mumps viruses, suggesting impairment of virus specific host cell proteins (Umino *et al.*, 2001).

1.1.5.4 RV structural proteins and virion assembly

The only purpose of the SG RNA is to act as a template for the structural viral protein 110-kd precursor (Chantler *et al.*, 2001). The three structural proteins seem to travel together from the rough endoplasmic reticulum (RER) to the Golgi complex, where virion assembly takes place. The three structural proteins, capsid, E2, and E1 are produced by two signal peptidase-mediated cleavages within the polyprotein (Law *et al.*, 2001). The capsid protein interacts with the genomic RNA to form the electron-dense core of the virus particle. E2 and E1 are both type I membrane proteins (Hobman *et al.*, 1992). Evidence suggests that RV E1 plays a dominant role in membrane fusion and the E1 internal hydrophobic domain is thought to be involved (Qiu *et al.*, 2000). The envelope glycoprotein E1 exists as a heterodimer with the heavily glycosylated E2 to form the viral

spike that protrudes from the surface of the virion (Chantler *et al.*, 2001). Two hydrophobic consensus signal sequences precede the amino termini for each of the two membrane proteins and are necessary for directing translocation of the glycoproteins into the lumen of the endoplasmic reticulum (ER) (Marr *et al.*, 1991) (Figure 1.5). The E2 signal peptide then remains attached to the carboxy terminus of the capsid protein (CP) (Law *et al.*, 2001). This is unique to RV among the Togaviruses. When expressed together E2 and E1 are transported as a complex and targeted to the Golgi cisternae in several cell types and thus retained in the Golgi apparatus (Hobman *et al.*, 1992). In fact, formation of an E1-E2 heterodimer is necessary for the transport of E1 and E2 out of the ER lumen to the Golgi apparatus and PM (Qiu *et al.*, 2000). RV E1 glycoprotein, which does not complex with the E2 protein, is unable to be transported to the Golgi complex and accumulates in a novel pre-Golgi compartment in continuity with the RER (Hobman *et al.*, 1992). It has been shown that the transmembrane and cytoplasmic domains of E2 are required for the targeting of the E2E1 heterodimers to the sites of budding such as the Golgi apparatus (Lee *et al.*, 2000). In addition, it has been demonstrated that the signal sequence preceeding E2 can mediate translocation of the E2 protein in the absence of an intact E1 signal peptide and cleavage of the E2E1 polyprotein requires the E2 signal peptide (Oker-Blom *et al.*, 1990). Data suggest that, although neither glycoprotein contains a dominant intracellular retention signal, E2 and E1 are largely retained in the Golgi even when present as a transport-competent heterodimer. Both proteins are transported to the surface when they were expressed together from cDNA, although with low efficiencies in all cell lines (Baron *et al.*, 1992).

The time required for E2 and E1 to mature, leave the RER and become endo H resistant was quite long (t1/2= 1-1.5 h), presumably due to the folding and maturation of the proteins in a heterodimer complex. The rate-limiting step of the folding and transport of E2E1 heterodimers from the ER to the Golgi is the maturation of E1 in the ER and it is thought that E2 and E1 are transported from the ER to the Golgi in a COPII/COPI dependent manner (Law *et al.*, 2001). E2 has a Golgi retention signal that functions to retain the glycoprotein heterodimer at the Golgi (Law *et al.*, 2001). RV E2 has an unclear biological function although the protein appears to contain at least one viral neutralisation epitope and a strain-specific epitope (Qiu *et al.*, 1992a).

There is a lack of uniform processing of the glycans on the RV glycoproteins. Glycosylation influences the conformation of the proteins by promoting correct disulfide-bond formation and folding (Sanchez *et al.*, 1991). The asparagine-linked (N-linked) oligosaccharides in E1 are large, probably four-branched, showing a lectin binding pattern

and suggesting the complex type, with terminal Gal, GlcNAc and sialic acid (Lundstrom *et al.*, 1991) (Figure 1.4). N-linked glycosylation of E2 takes place following translocation. At least two stable intermediates are involved in the processing of RV E2 glycans, a 39-kDa high-mannose containing precursor and a 42-kDa form bearing some complex sugars (Qiu *et al.*, 1992a). The transport of E2 to the PM is inefficient, with only a small fraction of E2 destined for the cell surface, while the majority of E2 accumulates in the ER and the Golgi cisternae (Qiu *et al.*, 1992a). In fact, compared to alphavirus glycoprotein processing, that of RV occurs relatively slowly and the transport of the E2 and E1 glycoproteins to the PM is inefficient (Yang *et al.*, 1998). This heterodimer is also formed and transported to the surface when the two proteins are coexpressed from cloned complementary DNA (cDNA) (Baron *et al.*, 1992; Baron *et al.*, 1991). Although neither glycoprotein carries a dominant intracellular retention signal, E2 and E1 are largely retained in the Golgi when present as a competent heterodimer. In the absence of E2, the E1 protein was slowly converted to a high molecular weight aggregate (Baron *et al.*, 1991). The CPs are anchored to the cell membrane by their hydrophobic C termini containing the putative E2 signal peptide, with the N-terminal region inside the viral envelope (Chen *et al.*, 2004b). The E2 and E1 proteins remain cell associated when expressed in the absence of the CP.

The rubella capsid gene is unusually rich in C and G residues (G + C 72.8%) and there are regions of up to 45% C or 35% G residues. While codon usage is non-random, there is a strong preference for C and G residues in the third position (Takkinen *et al.*, 1988). RV CP interacts with an essential 29-nucleotide packaging signal RNA sequence (nucleotides 347 to 375) of viral genomic RNA, forming the Nucleocapsid (NC). The major RNA binding domain of RV CP has been located within these a.a. residues 28 to 56, but other regions, including the C terminus, might also be involved in enhancing the interaction (Law *et al.*, 2003; Wang *et al.*, 2002). This N-terminal region is hydrophilic and rich in prolines and arginines. The RV capsid, through its RNA binding domain is involved in packaging the genomic RNA into NCs (Beatch *et al.*, 2000). The C protein must engage in homo-oligomeric (capsid-capsid) interactions during NC formation and also interact with E2 and/or E1 during budding. Whereas alphavirus capsids are released into the cytosol after self-catalysed cleavage from the structural protein precursor, the RV capsid does not possess protease activity and remains largely membrane associated after a signal peptidase mediated cleavage from the E2E1 precursor (Beatch *et al.*, 2000). The NCs of alphaviruses form in the cytoplasm of infected cells well before budding occurs. However, RV NC assembly occurs on the surface of intracellular membranes and is coincident with

virus budding. A single serine at residue 46 within the RNA binding region is required for normal phosphorylation of the capsid and this is important in virus replication. It has been proposed that phosphorylation serves to negatively regulate binding of viral genomic RNA (Law *et al.*, 2003). The N terminal one third of the CP modulates the ratio of SG RNA to viral genome RNA synthesis when provided *in cis*. This effect could reflect a regulatory effect on the part of the virus to allow initial accumulation of SG RNA and translation of the virion proteins before genome RNA synthesis was maximised (Tzeng *et al.*, 2005b). Recombinants have been constructed using the SFV and SINV cDNAs in which the spike genes were exchanged (Smyth *et al.*, 1997). The SFV/SINV (spike) RNA directed efficient assembly of infectious virus which was analysed by expression thus demonstrating that chimeras can be functional. RV has evolved a strategy whereby one of its proteins is recruited to interact with and exploit the cellular defence machinery of p32 (also known as TAP) to its advantage (Mohan *et al.*, 2002). The RV capsid is a phosphoprotein and the p32 binding region is a.a. 45 to 89 which also contains the RNA binding domain. p32 may regulate the binding of genomic RNA to RV capsid by controlling its level of phosphorylation which may affect capsid oligomerisation and virus assembly. It is unclear whether the interaction between p32 and capsid is pro- or anti-apoptotic and a number of virus proteins have been shown to have both functions (Beatch *et al.*, 2000). The cellular p32 protein was originally identified as a Cq binding membrane protein. P32 binds to a highly conserved N-terminal domain of the RV capsid and the CP interactive region of p32 is its C-terminus (Mohan *et al.*, 2002). The interaction with the RV capsid is found to occur at the cytoplasmic face of the mitochondrial membrane. Mitochondria cluster around RV replication sites which are modified endosomes or lysosomes and are located in the perinuclear region (Beatch *et al.*, 2000). Recruitment of the mitochondria to RV replication sites could provide the energy required for virus replication (Lee *et al.*, 1996). The association of RV core particles with mitochondria appears unique within the family Togaviridae (Lee *et al.*, 1999).

The E1 protein of RV shows no significant homology with alphavirus El envelope proteins and there is no X-ray crystal structure analysis of RV E1 available (Atreya *et al.*, 1998; Hofmann *et al.*, 2000). There is actually no homology between the a.a. sequence of the RV structural proteins and the a.a. sequence of the alphavirus structural proteins (Frey *et al.*, 1988). RV virions assemble in association with Golgi membranes whereas alphaviruses assemble nucleocapsids in the cytoplasm which then bud through the PM (Risco *et al.*, 2003). RV buds from intracellular membranes while alphaviruses mature at the PM and unlike alphaviruses RV NC assembly occurs independently of membrane virus

budding (Risco *et al.*, 2003). Association between Golgi stacks and the CVs containing the viral RNA-replication complexes indicates a potential physical connection between the sites of viral replication and virion assembly that could mediate the transfer of replicated genomes to the assembly sites (Risco *et al.*, 2003). The virus particle has a diameter of about 60 nm (Banatvala *et al.*, 2004). The RV virion contains the RNA genome enclosed within an icosahedral capsid composed of multiple copies of the basic protein, C, of 33 kDa. Surrounding this quasispherical NC with cubic symmetry is a lipid bilayer of cell origins in which viral glycoproteins El (58 kDa) and E2 (42 to 47 kDa) are embedded (Banatvala *et al.*, 2004; Chaye *et al.*, 1992b). The expression of RV antigens has been demonstrated *in vivo* on the surface of lymphocytes, monocytes and granulocytes, and transiently on leucocytes, following both naturally acquired and vaccine-induced rubella infection (Cusi *et al.*, 1995a). These cells therefore harbour the virus *in vivo* perhaps supporting replication and there is evidence that RV can establish a persistent infection. RV-infected leucocytes may play a role in the pathogenesis of infection and could provide a site for long-term persistence of the virus and consequent adverse manifestations (Cusi *et al.*, 1995a). It is known that noninfectious subviral particles comprising one or more viral structural proteins can form in infected or transfected cells (Hobman *et al.*, 1994). It has been determined that the RV 40S genomic RNA is not required for efficient particle assembly (Hobman *et al.*, 1994). However formation of RV-like particles (RLPs) was dependent on the CP as these structures were not seen in CHO cells expressing solely E2 and E1 from a recombinant plasmid (Hobman *et al.*, 1994).

1.1.5.5 RV cytopathic effect

The genetic determinants of cell death have been mapped to the non-structural proteins (Pugachev *et al.*, 1997b). RV induced apoptosis was first described in 1998. Cytopathology and cell death in Vero cells were shown to be due to the induction of apoptosis (Pugachev *et al.*, 1998b). *In vitro*, RV induces cytopathic changes on several cell lines such as cell detachment from the monolayer and condensation of chromatin (Hofmann *et al.*, 1999). Variation has been observed in the apoptotic response to RV in cell culture and may indicate how selective organ damage occurs in a CRS foetus. It is hypothesised that apoptosis is a cellular response to limit viral spread. It is possible that particular cell types in the foetus are susceptible to RV-induced apoptosis (Duncan *et al.*, 1999). Characteristics of apoptotic cell death include a reduction in the cell volume and the endonuclease mediated cleavage of DNA into oligonucleosomal length fragments.

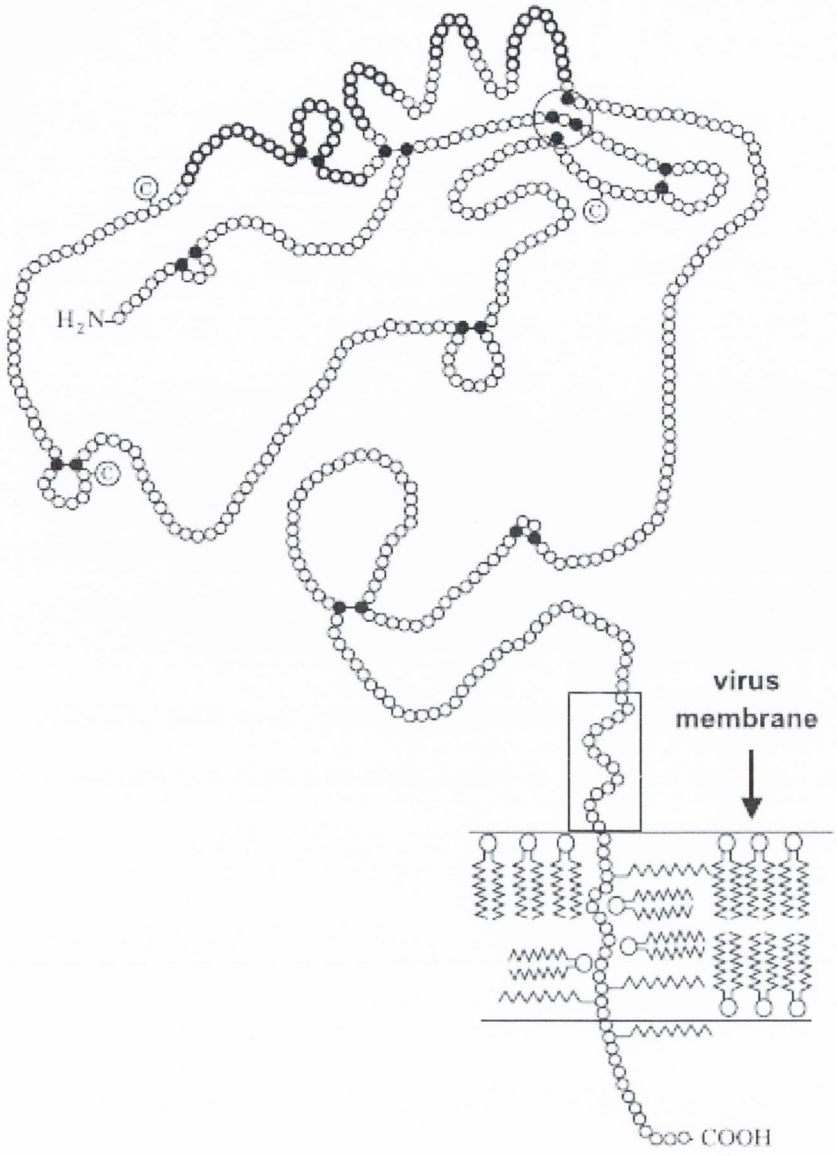


Figure 1.4 A model of the E1 protein of RV

The model includes all known linear neutralising domains (bold circles), disulfide bridges, N-bound glycosylation side chains (C) and a predicted transmembrane segment with acylated fatty acid chains. A predicted helical segment is enclosed by a rectangle. The disulfide bonding between cysteine residues in the encircled region has not been determined with certainty (Gros *et al.*, 1997).

Apoptosis also results in a loss of membrane phospholipids asymmetry which results in the exposure of phosphatidylserine (PS) at the surface of the cell (Hofmann *et al.*, 1999). In permissive cell cultures, the CPE of RV has been shown to be due to caspase-dependent apoptosis (Cooray *et al.*, 2005). The attenuated strain RA27/3 has also been shown to induce apoptosis (Domegan *et al.*, 2002). Studies to show that RV induces

apoptosis were carried out in susceptible cell lines and human cell lines that display RV-induced CPE have not been established. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is used by many cell types for inhibition of apoptosis and cellular survival. Actually the induction of cell survival downstream of PI3K via activation of Akt and upregulation of GSK-3β phosphorylation occurs concurrently with activation of the apoptotic cascade, suggesting a delicate balance between cell survival and apoptosis during RV infection. It is unclear whether this is controlled by the virus or is simply a cellular response although apoptosis is likely to enable the spread of RV (Cooray, 2004).

Apoptosis is likely to facilitate the spread of RV, as there is a lack of cell lysis (Cooray, 2004). PI3K–Akt signalling appears to assist virus replication of the non-transforming RV. Following the production of viral progeny, virus particle budding and release is then facilitated by virus induction of apoptosis (Cooray, 2004). In contrast, inhibition of the Ras-Raf-MEKERK pathway impaired RV replication and growth thus reducing RV-induced apoptosis and suggested that the normal cellular growth is required for efficient virus production (Cooray *et al.*, 2005). It has been suggested that the accumulation of viral components is necessary for apoptosis induction (Duncan *et al.*, 1999). It has been shown that expression of the RV structural proteins, in the absence of virus infection, induces apoptotic cell death at a rate similar to RV infection (Duncan *et al.*, 2000). The capsid by itself can account for nearly all of the apoptotic effect. The capsid polypeptide must be fused to a membrane anchoring sequence to have the cell death effect. The anchor may be the natural E2 signal peptide or a heterologous membrane anchoring domain. However the E2 signal peptide itself does not induce apoptosis (Duncan *et al.*, 2000). A p53 dependent pathway has been implicated in RV apoptosis (Megyeri *et al.*, 1999). The TO-336 vaccine strain was used to show triggering of apoptosis in Vero cells and RK13 cells. Human embryonic fibroblasts proved resistant to apoptosis. The transcription factor, p53, is a major mediator of either growth arrest or apoptosis. P53 activates several downstream target genes including the pro-apoptotic bax and p21.

1.2 MMR Immune correlates of protection

1.2.1 Immunogenic determinants of RV

The three structural genes, the capsid, E2 and E1, are the main immunogenic determinants of rubella infection. Immunity to RV has been defined using hemagglutination inhibition titres of ≥1:8, NT titres of ≥1:8 or ≥1:16 and enzyme immunoassay (EIA) levels of ≥10 IU/ml based on comparisons to the World Health

Organisation (WHO) rubella IgG standard serum (Chaye *et al.*, 1992b; Mitchell *et al.*, 1999). The El glycoprotein is the most antigenic of the three structural proteins in eliciting both cellular and humoral responses. The E1 molecule contains domains that are involved in virus attachment to the surface of red blood cells (hemagglutination) and the initiation of infection. The fine antigenic structure of E1 has been characterised and HAI and neutralisation sites have been localised to $E1_{245}$-$E1_{285}$, with three critical regions for antibody binding ($E1_{245}$-$E1_{251}$, $E1_{260}$-$E1_{266}$, and $E1_{275}$-$E1_{284}$) (Chantler *et al.*, 2001). The E2E1 sequence contains one CpG motif in the optimal immunostimulatory complex which has been shown to enhance the immune response induced by DNA vaccines (Pougatcheva *et al.*, 1999).

The contribution of these additional motifs to the immune response observed is unknown. Results suggest that pre-existing neutralising antibodies against a certain epitope could be more important as a memory response after natural infection than after vaccination and could explain why reinfection occurs more frequently in vaccinated than in naturally immune individuals (Cordoba *et al.*, 2000). A HLA DR3-specific epitope and a HLA DR4-specific epitope have been identified in the rubella E1 protein (a.a. 254-266 and 272-285 respectively). Virus protection is mainly elicited by neutralising anti-E1 glycoprotein antibodies (Perrenoud *et al.*, 2004). However, a T cell dependent immune response has been identified as important in immunity to RV and it has been indicated that the E1 glycoprotein contains both T and B cell epitopes which may be necessary for human immune response induction (Perrenoud *et al.*, 2004).

E2 contains only one weak neutralising domain as identified by the use of neutralising antibodies (Giessauf *et al.*, 2004). The synthetic peptide SP15 (E1 a.a.s 208 to 239), present in the Therien and RA27/3 strains has been shown to induce antibodies with neutralising and hemagglutination inhibition activity in mice and rabbits (Cordoba *et al.*, 2000). The E1 carbohydrate moieties are easily removed on E1 but not on E2, suggesting that E2 epitopes may be buried under El in the El-E2 complexes and thus are not easily accessible to the immune system of the host (Chaye *et al.*, 1992b). While anti-E2 glycoprotein antibodies disappear within months in humans and do not undergo affinity maturation, antibodies to the E1 glycoprotein persist for decades and over a period of a few months progressively increase their affinity (Perrenoud *et al.*, 2004). Antigenic competition between the E1 and E2 glycoproteins may reflect the reduced levels of humoral and cellular immune responses against the E2 glycoprotein (Chaye *et al.*, 1992b). In natural influenza infection, suppression of the anti-neuraminidase response is due to antigenic competition between competing immunogens resulting in an immune response

directed predominantly against the H antigen (Chaye *et al.*, 1992b). While antibodies to E1 predominate following most RV infections, antibodies to E2 are relatively more abundant in CRS as opposed to other forms of RV infection (Chaye *et al.*, 1992b). The E2 B cell epitopes may overlap the T cell epitopes and this is evident in individuals with CRS, in whom both cellular and humoral responses to E2 are elevated (Chaye *et al.*, 1992b). The frequency and magnitude of cellular responses to E2 SPs were somewhat lower (Mitchell *et al.*, 1993a). Patients with CRS, those in whom RV immunisation failed and certain patients with rubella associated arthritis may have specific defects in immune recognition of the E1 protein (Mitchell *et al.*, 1993b).

RV T cell epitopes have been identified by lymphoproliferation and others shown to be capable of inducing cytotoxic class I or class II MHC-restricted T cell clones (Chantler *et al.*, 2001). The functional significance in protective immunity, however, of immunologic recognition of lymphoproliferative or cytotoxic T-cell epitopes is poorly defined (Chantler *et al.*, 2001). Recombinant proteins expressing overlapping sequences of the RV capsid were used to identify domains of this protein that contained cell-mediated immunodominant sequences (Lovett *et al.*, 1993). RV-specific T cells likely act as either helper T-cells in activating a protective (virus neutralising) antibody response or as CTLs which limit initial viral replication, preventing virus spread to other tissue sites (Mitchell *et al.*, 1999). The RV capsid is the major target of MHC class I restricted lysis but contains both MHC class I and MHC class II restricted epitopes (Wolinsky *et al.*, 1991). Thus the capsid has been identified as a target of RV-specific CTLs and two major CTL epitopes defined: one within C9 to C22 which is A2 restricted and one within C11 to C29 which is in fact presented by multiple class I molecules (Wolinsky *et al.*, 1991).

The high frequency of insulin-dependent diabetes (IDDM) in children with congenital rubella suggests that the infectious agent and in particular the capsid may trigger the autoimmune process (Karounos *et al.*, 1993). Molecular evidence suggests that complexes of viral proteins and cellular protein can challenge self tolerance and RV 5' (+) SL RNA has been shown to interact with La autoantigen (Pogue *et al.*, 1996). IDDM is considered an autoimmune disease that results from destruction of insulin secreting β-cells in the pancreatic islets. A monoclonal antibody (mAb), C9, which recognises a defined domain within the CP of RV, was found to react with extracts from a rat p-cell tumour and normal rat and human islets. An immunogenic epitope on the RV CP mimicked by a similar structure on a β-cell protein, the 52 KDa islet antigen, suggests that RV may sensitise susceptible individuals for an autoimmune response to β cell antigen and contribute to β destruction in IDDM (Karounos *et al.*, 1993). A T cell line (19KW) from a

patient with IDDM that reacts to purified 52 KDa islet protein has been established (Sobel *et al.*, 1997). Thus the RV E1 glycoprotein has proven to be a better immunogen than RV E2 or C and IgG responses have been found to be directed mainly to the E1 glycoprotein (Chaye *et al.*, 1992b).

Five distinct immunoreactive regions were identified in RV E1 protein which stimulated cellular responses in 29-83% of the subjects tested, spanning residues (11-39), (154-179), (199-239), (226-277), and (389-412). Two synthetic peptides (SPs), E1 (213-239) and E1 (258-277) which contained previously identified virus neutralising antibody domains, reacted with serum antibodies and also stimulated lymphoproliferation. These E1 sequences must thus contain linked or overlapping B- and T-cell antigenic sites. T-cell mapping studies using SPs have revealed overlapping cytotoxic T-lymphocyte (CTL) and NT antibody epitopes between E1 residues 273-285 and other studies have demonstrated T-cell proliferative activity may be induced by peptides which contain previously mapped NT antibody domains on E1 and E2 suggesting a mechanism for helper T-cell and B-cell interactions (Mitchell *et al.*, 1999). The proximity of B and T cell epitopes on E2 and E1 suggests that viral immunity elicits B-cell and MHC class II-restricted T-cell repertoires focused about common antigenic sites (McCarthy *et al.*, 1993).

During T-cell and B-cell cooperation for antibody production the B cell selects the T cell with which to cooperate and this is postulated to take place at the level of antigen processing and presentation by the B cells. A paratope of an Ig binds to a region on the antigen thus possibly protecting it from enzymatic degradation during processing and thus this region will be presented intact. Therefore, B-cell epitopes may overlap those of T-cell epitopes (Chaye *et al.*, 1992b). T-cell epitopes set apart from B-cell recognition sites are also likely involved in summoning help for antibody production (Mitchell *et al.*, 1999). Identification of immunodominant helper T-cell determinants could aid in identification of nearby B-cell determinants within relatively short, accessible sequences of viral structural proteins and vice versa, thus facilitating the rational design of new synthetic vaccines against RV and other agents (McCarthy *et al.*, 1993).

Studies of cell-mediated immunity towards RV to date have primarily focused on the depression of cell-mediated immunity following natural infection or vaccination (Chaye *et al.*, 1992b). Specific T-lymphocytes are also activated by RV infection or vaccination with live attenuated RV vaccines (Mitchell *et al.*, 1999). The role of RV-specific T-cells in protective immunity or tissue damage is unknown. A gap in the RV-specific T-cell receptor (TcR) repertoire could lead to failure to provide help for protective antibody responses (Mitchell *et al.*, 1999).

1.2.1.1 Measles and mumps viruses

MV replicates in tracheal and bronchial epithelial cells in the respiratory tract during the incubation period and from there infection spreads to the draining lymph nodes (Ohgimoto *et al.*, 2001). A source of virus for spread to other tissues is thought to be MV replication in lymphoid or reticuloendothelial giant cells (Warthin-Finkeldy cells). There are approximately 30 million cases of measles and 1 million deaths associated with measles per year worldwide (Tatsuo *et al.*, 2001). On the basis of phylogenetic analysis of morbilliviruses, it is hypothesized that when cattle were domesticated, they transferred a morbillivirus, a progenitor of modern Rinderpest virus (RPV), to humans, which finally evolved into MV. Among all viral proteins, the H is the least conserved among canine distemper virus (CDV) and MV (Tatsuo *et al.*, 2001). In 2000 there was an outbreak of measles in Dublin resulting in the deaths of three children as a result of a vaccine-preventable illness (McBrien *et al.*, 2003).

1.2.1.2 Measles and mumps immunogenic determinants

MV neutralising Abs, the main correlates of protection, are primarily directed to the Hemagglutinin (H) glycoprotein (Capozzo *et al.*, 2006). The H-neuraminidase (HN) protein is the major target for the humoral immune response upon mumps virus infection (Cusi *et al.*, 2001b). The HN protein is involved in the initial process of viral infection by attaching to the cellular sialic acid receptors (Kashiwagi *et al.*, 1999). The HN and F proteins are involved cooperatively in the next infection step of membrane fusion and viral penetration (Kashiwagi *et al.*, 1999). Epitopes involved in the neutralising antibody response have been identified on the HN protein (Cusi *et al.*, 2001b).

Thus, the main target of neutralising and protective antibodies in humans and in animal models is the MV H protein (El Kasmi *et al.*, 2000). Such antibodies are mainly directed against conformational epitopes (El Kasmi *et al.*, 2000). Virus infected cells have been shown to express two types of H protein with different extents of glycosylation (Ogura *et al.*, 2000). These two forms of H protein (78 kDa and 74 kDa) are present in cells infected with Edmonston cDNA expressed from a vector. Only the mature 78 kDa protein, a completely glycosylated form of the 74 kDa protein is expressed on the cell surface of infected cells (Ogura *et al.*, 2000). The H protein functions in attachment to the human host cellular receptors CD46 and CD150 (signalling lymphocyte activation molecule (SLAM)) (Moss *et al.*, 2006). CD46 is a ubiquitous complement regulatory

molecule expressed on all nucleated cells in humans and in Old World monkeys (Moss *et al.*, 2006; Schneider *et al.*, 2000). The affinity of Edmonston H is high for the constitutively expressed CD46 (Ohgimoto *et al.*, 2001). The attachment of H is mediated by an interaction with the F protein and subsequent fusion of the viral envelope with the host cell membrane (Moss *et al.*, 2006). The MV H protein is an important determinant of MV tropism for both lymphocytes and DC (Ohgimoto *et al.*, 2001).

A single a.a. at position 481 Asn/Tyr (H 481 NY) in the H protein determines whether the virus can utilise CD46 (Erlenhofer *et al.*, 2002). The binding sites for SLAM and CD46 are distinct as this a.a. alteration has no effect on the usage of SLAM as a receptor (Erlenhofer *et al.*, 2002). The wt and vaccine MV strains use SLAM as a receptor for virus uptake and syncitium formation (Erlenhofer *et al.*, 2002). Mainly vaccine strains are able to use both CD46 and SLAM. In fact MV can use SLAMs of nonhost species as receptors, albeit at reduced efficiencies (Tatsuo *et al.*, 2001). MV wt strains induce expression of their cellular receptor, CD150, through Toll-like receptor (TLR)-2 in monocytes (Bieback *et al.*, 2002).

1.2.1.3 Measles virus and immunosuppression

The three main human immunosuppressive viral diseases are attributed to MV, human immunodeficiency virus (HIV) and cytomegalovirus (CMV) (Vidalain *et al.*, 2001). Immunesuppression associated with morbillivirus infections may affect the mortality rate by allowing secondary bacterial infections that are lethal to the host to thrive (Heaney *et al.*, 2002b). Professional APC such as DC are susceptible to MV infection and this has been shown through the use of vaccine strains (Fugier-Vivier *et al.*, 1997; Ohgimoto *et al.*, 2001). MV-infected DC are likely to undergo rapid cell death either by fusion or by apoptosis (Ohgimoto *et al.*, 2001). MV is the only virus that has been implicated in DC apoptosis (Servet-Delprat *et al.*, 2000a). The manner and effect of this apoptosis has not been elucidated. Replication in DC seems to be important and modification of CD40 signalling by MV infection may be the major mechanism which induces MV immunosuppression (Servet-Delprat *et al.*, 2000b). Recombinant wt MV H protein exhibited a tropism for DC when expressed *in vitro* and for secondary lymphoid tissues *in vivo* (Schneider-Schaulies *et al.*, 2006). It has been suggested that *in vitro* MV induced type I IFN would impair differentiation of DC from their precursors (Hahm *et al.*, 2005). DCs are the only professional APCs able to prime naïve T lymphocytes (Fugier-Vivier *et al.*, 1997). It has been proposed that the mechanism of MV-induced immunosuppression

may be due to the interaction of uninfected lymphocytes with MV H and/or fusion proteins expressed on the surface of an infected APC leading to the APC's delivery of a negative transmembrane signal to the responding T cells which then arrests their proliferation. The ligands on the uninfected T cells are known (Fugier-Vivier *et al.*, 1997). A dominant inhibitory signal is delivered to T cells by the MV glycoproteins on the surface of DC thus overcoming positive signals by co-stimulatory molecules promoted by maturation factors released from infected DC (Klagge *et al.*, 2000). The interaction of MV with DCs is thought to be central to MV-induced immunosuppression, although direct infection of these cells *in vivo* has not yet been shown (Klagge *et al.*, 2004).

The MV glycoprotein complex, consisting of the H and the proteolytically activated F1/2 heterodimer is capable of signalling to T cells *in vitro* and *in vivo* and can cause T cell arrest *in vitro* (Schneider-Schaulies *et al.*, 2006). For MV, contact dependent T-cell arrest requires co-expression of the H protein and this arrest does not depend on the known MV entry receptors. MV wt F/H proteins can arrest proliferation of murine T cells which do not express either of the two known H-protein binding receptors, CD46 and CD150 (Schneider-Schaulies *et al.*, 2006). CD46 and CD150 specific antibodies do not prevent silencing of human T cells and both molecules have been found to promote rather than inhibit TCR signalling (Schneider-Schaulies *et al.*, 2006). During immunosuppression MV is cleared rapidly from the host but lymphoproliferative responses to mitogen recall antigens are suppressed for up to several months after infection. Interestingly both B and T subsets of leukocytes are infected but not the $CD4^+/CD8^+$ ratio. MV infection interferes with the differentiation and specialisation of lymphocyte functions but does not alter those already established (Heaney *et al.*, 2002a). Viruses evade control by the adaptive immune response by silencing T cells and T-cell silencing in order to spread (for MV) and/or to establish and maintain chronic infections (for retroviruses). The importance of the two strategies for individual viral infections may be linked directly to the nature of the infection process (Schneider-Schaulies *et al.*, 2006). In acute, self limiting infections where viral proteins are produced in large amounts, the T cell silencing strategy may predominate. This involves the manufacture of proteins to dampen APC and T cell activation rapidly rather than relying on the time consuming induction of a regulatory T (Tr) cell population that needs to be activated and expanded (Schneider-Schaulies *et al.*, 2006).

MV induces a prolonged immunosuppression even when there is no detectable virus present (Vidalain *et al.*, 2001). Lymphopenia has been shown to be caused mostly by cytolysis of non-infected cells. *In vivo* the cytolysis mechanism, now believed to be the main mechanism of immunosuppression remains to be elucidated (Vidalain *et al.*, 2001).

The initial effective Th1 response carries out viral clearance while the Th2 response later supports the development of MV specific-antibody (Moss *et al.*, 2004). Down regulation of IL-12 production by monocytes occurs *in vitro* despite the fact that only a small proportion of cells are predominantly infected (Moss *et al.*, 2004). The complex of both Edmonston MV glycoproteins, F and H, has been implicated in triggering MV-induced suppression of mitogen-dependent proliferation (Schlender *et al.*, 1996). The effect was not observed using a recombinant Edmonston MV in which F and H were replaced with vesicular stomatitis virus G glycoprotein or (*ii*) when either of these proteins was expressed alone (Schlender *et al.*, 1996). Later work contradicted that of Yanagi and Schlender. Steineur *et al* found that UV-treated Vero cells infected with a vaccinia recombinant encoding MV-H and F, coexpressed H and F at the cell surface but were inefficient at blocking DC/T mixed lymphocyte reaction (MLR). This suggested that although H and F may act as adhesion molecules and allow interaction of a small number of infected Langerhans cells with T cells (or uninfected DC), other molecules, possibly upregulated by MV infection, may be responsible for transducing the inhibitory signal (Steineur *et al.*, 1998).

Wt MV H protein, but not vaccine strain H protein activates cells via both human and murine TLR-2 (Bieback *et al.*, 2002). This property is abolished by mutation of a single a.a., asparagine, at position 481 to tyrosine which is important for interaction with the CD46 receptor (Bieback *et al.*, 2002). This mutation is found in attenuated vaccine strains. TLR-2 is responsible for recognition of gram positive bacteria, bacterial lipoproteins and lipoteichoic acid (Bieback *et al.*, 2002). Loss of the capability to activate TLR-2 may be considered as an attenuation marker (Bieback *et al.*, 2002). Regulation of TLR signalling may play an important role for viral pathogenicity and induction of immunosuppression by MV wild-type strains (Bieback *et al.*, 2002). This TLR-2 signalling by MV on APC may induce tolerance to subsequent activation by bacterial cell wall components (Bieback *et al.*, 2002). This could be important in the sensitivity to opportunistic infections associated with acute cases of measles (Bieback *et al.*, 2002). The Edmonston vaccine strain was only found to inhibit lymphocyte proliferation in cotton rats when 1,000 fold more vaccine virus was used as compared to wt virus (Pfeuffer *et al.*, 2003).

1.2.2 Rubella Vaccine History

Many current human vaccines were developed more than 30 yrs ago on an empirical basis and are based on killed and attenuated viruses with little knowledge of the

protective antigens or the mechanism of protective immunity (Mills, 2002). Eight live attenuated rubella RUB vaccines were initially developed to prevent CRS (Pugachev *et al.*, 2000a). Ideally these strains had to be safe, stably attenuated, and protective. Six of these vaccines are in current use: the RA27/3 vaccine (the single rubella vaccine, Ervevax, manufactured by Glaxosmithkline (GSK) Biologicals and Meruvax, manufactured by Merck & Co Inc) which was developed in the U.S. and is utilised worldwide and the BRDII vaccine developed and used in China along with the RA27/3 vaccine (Zheng *et al.*, 2003). There are five vaccine strains manufactured in Japan; Matsuura, TO-336, Takahashi, Matsuba and DCRB19, although the latter is currently not used (Frey *et al.*, 1998). The sequence in the E1 region of each of the five strains is clearly different from that of the RA27/3 RV vaccine (Frey *et al.*, 1998).

Attenuation of the Japanese vaccines was achieved by serial passage of each wild-type progenitor virus at 35°C or less, and these vaccine viruses exhibit a small plaque, temperature-sensitive phenotype (Kakizawa *et al.*, 2001). The sequences of the genomes in the TO-336 vaccine strain (TO-336vac) of RV and its wild progenitor virus (TO-336wt) have been determined and compared with each other, the first study of its kind (Kakizawa *et al.*, 2001). Of the ten a.a. substitutions, eight were in the NSP-ORF and two were in the E1 gene of the SP-ORF. When the TO-336 sequence is compared with four other strains, Therien and M33 (wt viruses), and RA27/3 and Cendehill (vaccine viruses), the mutations responsible for attenuation appear to differ with each vaccine strain (Kakizawa *et al.*, 2001). The Cendehill specific mutations thought to be determinants of joint cell growth are located in two regions, the 5 nontranslated (NTR) region and in a region encoding the carboxy terminal region of the p150 which extends into the helicase domain of p90 (Wolinsky *et al.*, 1991). The HPV-77 vaccine strain was also developed in the U.S. and the Cendehill strain was developed in Europe but the accepted vaccine strain remains RA27/3 based on its effective immunogenicity and lack of adverse reactions in children (Pugachev *et al.*, 2000a). It induces an immune response most comparable to that following natural rubella infection (Best *et al.*, 2003). This strain was derived from a rubella-infected foetal kidney isolate and was attenuated by 4 passages in human embryonic kidney cells and 17 to 25 passages in WI-38 fibroblast cells (Chantler *et al.*, 2001).

Although the full sequences of the RA27/3 and Cendehill vaccine strains have been reported, the wt parent virus and intermediate attenuating passages for neither of these vaccines is available making direct attenuation studies impossible (Pugachev *et al.*, 2000a). The RA27/3 genome sequence however could allow for comparison with other wt strains and identification of attenuating mutations, which makes this strain a good choice for the

prototype vaccine (Pugachev *et al.*, 2000a). Understanding the determinants of attenuation is important and may explain why the RA27/3 vaccine has been associated with transient chronic arthritis in adult women and the Japanese vaccines have not. Although TO-336vac was found superior to RA27/3 in frequency of joint symptoms RA27/3 was superior to TO-336vac in antibody durability. The replication of RA27/3 (Meruvax II) and Cendehill (Cendevax) is highly restricted in joint cells and both of these strains show limited ability to penetrate and persist in the organ cultures. However, wt strains and HPV77/DE5 vaccine grow to high titre in lymphoreticular cells and chondrocytes. This biologic variation has been associated with differences in E1 and E2. Local viral replication may play a role in the pathogenesis of rubella-associated arthritis (Miki *et al.*, 1992).

Attenuation of the Cendehill strain of RV has not destroyed its ability to induce apoptosis (Cooray *et al.*, 2003). However attenuated strains of RV appear to pose little threat to the developing foetus (Cooray *et al.*, 2003). It is possible that virus attenuation may affect other biological properties, such as replication rate or virus assembly and release, which would thus limit the teratogenic effects during infection in pregnancy. In a comparison of RA27/3 with the Therien wild type strain, there was one nucleotide mutation in the 5' UTR and 17 a.a. mutations as attenuating candidates (Kakizawa *et al.*, 2001). The number of mutations would probably be smaller and more specific could the RA27/3 vaccine strain be compared to its progenitor virus directly. As vaccination does not result in congenital abnormalities when accidentally given in early pregnancy, comparisons of vaccine strains with wt strains may reveal key motifs associated with teratogenicity (Lee *et al.*, 2000). No antigenic differences have been observed among virus strains confirming that rubella vaccines will protect against circulating strains (Best *et al.*, 1992).

1.2.3 MMR Vaccine

In most parts of the world, until 1992, the most widely used MMR vaccines were M-M-R II produced by Merck & Co Inc and Pluserix produced by (GSK) Biologicals (Lee *et al.*, 2002). M-M-R II vaccine contains the Enders Edmonston measles strain, the Jeryl Lynn mumps strain and the RA27/3 rubella strain. The Urabe Am9 vaccine, contained in Pluserix, was withdrawn from use following a high incidence of adverse CNS events such as aseptic meningitis. The GSK MMR vaccine (Priorix) was thus developed which is a trivalent live attenuated MMR vaccine containing the Schwarz measles strain, the RIT 4385 mumps strain and the Wistar RA27/3 rubella strain (Wellington *et al.*, 2003). The RIT 4385 mumps vaccine is derived from the JL5 component of the Jeryl Lynn mumps

vaccine strain and is licensed in Europe (Carbone *et al.*, 2001). The GSK MMR causes fewer injection-site adverse effects such as pain, swelling and redness than Merck MMR (Wellington *et al.*, 2003). Both trivalent vaccines elicited an equivalent immunogenicity when used at 15 months of age. Priorix is administered subcutaneously but it may also be given intramuscularly.

MV was first isolated from the blood of David Edmonston in 1954 by John Enders and Thomas Peebles (Moss *et al.*, 2006). Both the Schwarz (SW) and Edmonston-Zagreb (EZ) vaccine strains are derived from the original Edmonston strain, differing only in their passage levels and cell substrate - chick embryo fibroblasts and WI-38, respectively. EZ was significantly more immunogenic than SW in infants with maternal antibodies, and produced seroconversions in high frequencies (Bennett *et al.*, 1999). Maternal antibodies interfere with the immune responses following immunisation of children with measles vaccine up to 12 months after birth (Obeid *et al.*, 1994).

In 1990, the World Health Organization recommended only high-titre EZ vaccine for routine use at 6 months of age in developing countries where risk of measles deaths made it unwise to wait until later ages to vaccinate (Bennett *et al.*, 1999). Measles vaccine has also been recommended in subgroups of HIV-1 children without severe immunosuppression (Azzari *et al.*, 2005). The SW vaccine tends to induce higher antibody titres than EZ in children with little or no maternal antibodies and may be more useful for routine immunisation at 15 months of age (Bennett *et al.*, 1999). Measles peak incidence has shifted to infants <12 months old (Gans *et al.*, 2004). Measles vaccination elicits T cell responses in infants as young as 6 months old which may prime the humoral response to a second dose and could have benefits in developed and developing countries (Gans *et al.*, 2004). There is a need for a vaccine which can be used early in life, and if it is a vectored vaccine, maternal antibodies to the vaccine should be absent (Cardoso *et al.*, 1996).

Vaccine viruses differ at most by 0.3% from the Edmonston wild-type strain and up to 7% for the H gene. The MV Edmonston genome is very stable and reversion to pathogenicity has never been observed with this vaccine (Lorin *et al.*, 2004). In fact, Edmonston-derived live attenuated MV strains differ from the Edmonston wild-type isolate by at least 45 coding mutations, making reversion almost impossible (Parks *et al.*, 2001). The mumps Rubini vaccine strain has been found to confer insufficient protection in children (Cruz Rojo *et al.*, 2003). The MMR vaccine administered by the aerosol route has been shown to elicit a superior response than the subcutaneous route in one study in Mexico where seroconversion was demonstrated for all three components of the vaccine despite the presence of existing antibodies (Fernandez de Castro *et al.*, 2005).

1.2.4 Vaccine adverse reactions and contraindications

There are a higher rate of adverse effects in females following MMR vaccination than in males, irrespective of the humoral response (Shohat *et al.*, 2000). The perceived adverse effects of the MMR vaccine could in theory be attributed to its composition as a mixture of three live replicating viruses all of which were derived by uncontrolled attenuation through passage in cell culture and/or embryonated eggs, and the molecular basis for this attenuation is unknown (Atkins *et al.*, 2003). It should be possible to construct nonreplicating MMR vaccines that stimulate immunity in a more controlled manner and for which biosafety parameters could be more easily manipulated (Atkins *et al.*, 2003). An adverse event can be said to be caused by a vaccine if it is specifically associated with a laboratory finding or a clinical syndrome (Chen *et al.*, 1998).

Documented adverse events caused by component vaccines are fever in up to 15% and rash in up to 5% of measles vaccine recipients, low grade fever and parotitis in up to 0.7% of mumps vaccine recipients (Robertson *et al.*, 1988). Failure rates of 3 to 14% have been shown in efficacy and immunogenicity studies with the Edmonston vaccine strain (Ovsyannikova *et al.*, 2007a). There are medical concerns such as primary vaccine failure and arthritis associated with rubella vaccination (Ou *et al.*, 1994). Subprotective levels of rubella antibody may exist in 4 to 23% of vaccinees 6 to 16 years after rubella vaccination (Ovsyannikova *et al.*, 2007a). These perceived problems with the rubella vaccine could derive from its composition as a live attenuated, replicating virus (Atkins *et al.*, 2003). It may be desirable to improve the biosafety of the single rubella vaccine by the development of a non-replicating vaccine that will stimulate efficient immunity and protection.

Between 10% and 40% of adult rubella seronegative women vaccinated with the RA27/3 vaccine develop acute, usually transient, arthritis or arthralgia (Chantler *et al.*, 2001; Tingle *et al.*, 1985b). Rubella vaccine is associated with lymphadenopathy (up to 9% of recipients), and possibly the rare chronic arthropathy (Jefferson *et al.*, 2003; Robertson *et al.*, 1988). RV vaccine has also been associated with Guillan Barre syndrome, and optic neuritis (a neurological condition) (Shoenfeld *et al.*, 2000). Thus present rubella vaccine preparations are reported to cause symptoms such as joint pain, acute arthritis and meningitis (Robinson *et al.*, 1995). As rubella is a live attenuated vaccine, it should not be given to women who are pregnant or to immunocompromised patients, with the exception of HIV positive individuals (Best *et al.*, 2003). Vaccination is also recommended for unvaccinated and/or seronegative adolescents and adults, thus a nonreplicating rubella vaccine would be of additional use in the case of women in early pregnancy or considering

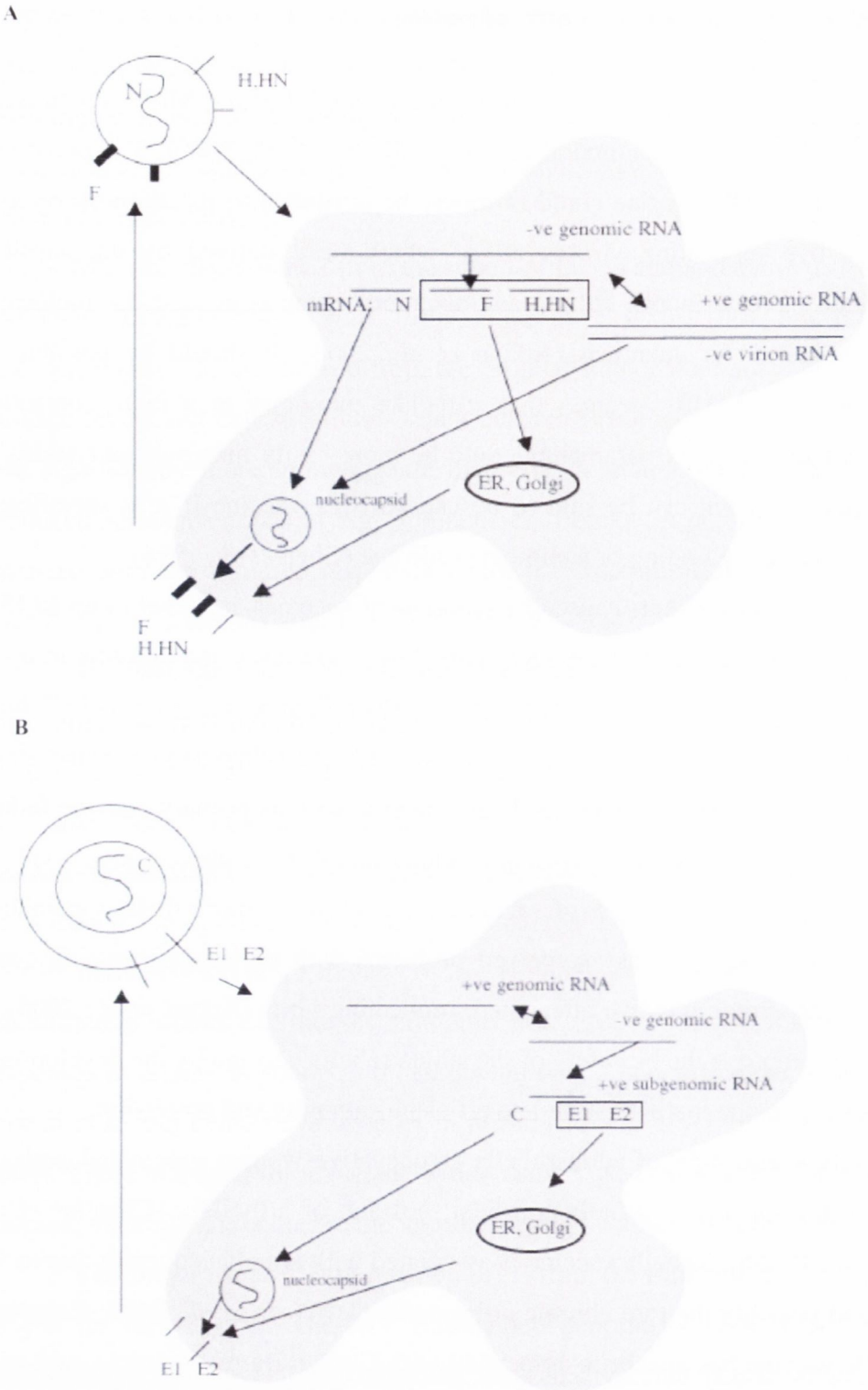


Figure 1.5 Synthesis of the main MMR antigens

(A) Synthesis of the measles H, or H-neuraminidase (mumps, HN), F (fusion) and N (NC) antigens of the measles and mumps viruses. (B) Synthesis of the two envelope proteins (E1 and E2) and CP of RV (Atkins *et al.*, 2003)

pregnancy, who are found to be seronegative (Pougatcheva *et al.*, 1999). Failed rubella immunisations to RA27/3 in adults may be more important than previously acknowledged in view of altered patterns of virus-specific immunity and the associations of this failure with the rubella carrier state (Tingle *et al.*, 1985a).

Whether or not these adverse effects are substantiated, with recent improvements in vaccine technology, there is a case for replacement of the current live attenuated single rubella vaccine with a genetically engineered non-replicating vaccine (Atkins *et al.*, 2003). Despite reduced virulence of the live attenuated rubella vaccine, vertical transmission from mother to foetus is not prevented. In one case of periconceptional rubella RA27/3 vaccination in a seronegative woman there was evidence of long-term persistent foetal infection (Hofmann *et al.*, 2000). The effect of the intrauterine infection was however benign with no defects found in the baby and cessation of virus shedding by five months of age. Adverse effects cannot be completely prevented from occurring as long as live virus vaccines are used widely. The unknown risk of developing late onset symptoms such as juvenile diabetes mellitus type 1 is a problem associated with intrauterine infection and may be the result of virus mediated lysis of insulin producing beta cells on a background of genetic susceptibility to autoimmunity (Hofmann *et al.*, 2000). There is however no causal relationship between vaccines administered to infants and adults and increased risk of diabetes (Best *et al.*, 2003). Similarly no causal relationship has been found between the MMR vaccine and autism (Best *et al.*, 2003).

A virus-induced immune response could also cause an autoimmune reaction responsible for demyelination as a C antigen motif and a sequence of the myelin basic protein have been found to be similar (Cusi *et al.*, 1999). This was first described in a 23 year old RA27/3 vaccinee and later shown in mice. E2 (a.a.s 244-263) which induced the highest level of response among the E2 SPs tested in human adult subjects, was of interest due to previous reports of sequence homology of this RV region with human myelin and its potential immunopathogenic role in demyelinating autoimmune diseases (Mitchell *et al.*, 1993a). Fever occurring subsequent to measles vaccination is related to the replication of the live attenuated vaccine virus and subcutaneous injection of an attenuated measles strain can result in respiratory excretion of this virus (Morfin *et al.*, 2002). This raises the question of a possible transmission of a measles vaccine strain, as well as the consequences of infection by such a strain, particularly in immunocompromised children and such patients who are at risk of developing serious measles diseases. Viral culture and molecular biology techniques could be useful to investigate vaccine virus behaviour and evaluate the incidence of viral excretion after MMR vaccination (Morfin *et al.*, 2002).

1.2.5 Infectious cDNA virus clones

RV is a positive-stranded RNA virus, for which infectious cDNA clones are available. To develop a RV vector that would be acceptable as a vaccine vector, an infectious cDNA clone of the RA27/3 vaccine virus was constructed (Pugachev et al., 2000a). The genomic DNA sequence of the RA27/3 vaccine strain was determined in 1997 (Pugachev et al., 1997a). Genome-length cDNA clones have been developed for Sindbis virus (SINV), Venezuelan equine encephalitis virus (VEEV), Ross River virus (RRV), and SFV (Wang et al., 1994). An infectious cDNA clone allows for synthesis of infectious *in vitro* transcripts from a plasmid containing a cDNA copy of a viral genome positioned adjacent to an RNA polymerase promoter (Pugachev et al., 2000a). The SINV and SFV genome length cDNA clones have been developed into broad host range vectors for high level expression of foreign proteins in eukaryotic cells (Wang et al., 1994). An infectious cDNA clone equivalent to the anti-genome of the Edmonston strain of measles virus (MV) and a procedure to rescue the corresponding virus have been established (Radecke et al., 1995). This cDNA has been adapted to create a vector to express foreign genes, can accommodate up to 5 kb of foreign DNA and is genetically very stable (Lorin et al., 2004). Recombinant MV expressing the HIV-1 89.6 envelope glycoprotein raised neutralising antibody and specific T lymphocyte responses against HIV in mice and macaques including those with a pre-existing anti-MV immunity (Lorin et al., 2004). MV replicates entirely in the cytoplasm, ruling out the possibility of integration in host DNA (Lorin et al., 2004). Measles Edmonston virus has been genetically modified to allow entry via cell surface molecules other than its receptor CD46 with the aim of using this in cytoreductive tumour therapy (Schneider et al., 2000).

A genome length cDNA clone was constructed for the Therien RV strain in recent years and has allowed for the analysis of the molecular biology of this positive polarity virus (Wang *et al.*, 1994). This plasmid (Robo102) contained a complete copy of the RV wt Therien strain genomic RNA (Wang *et al.*, 1994). One attempt was made to develop such a clone into an expression vector by insertion of a second promoter for the SG RNA species at the end of the viral genome. However, this construct proved unstable (Pugachev *et al.*, 2000b). The specific infectivity of this transcript had been increased (~ 5 plaques/10 μg of transcript to 10^4 plaques/μg), by replacing regions based on the w-Therien strain of RV with the variant f-Therien (Pugachev *et al.*, 1997b). The genetic determinants that mediate cytopathogenicity were also mapped to the nonstructural proteins. An RV replicon in which a puromycin resistance gene replaced most of the SP-ORF and generated stable,

replicon-containing cell lines following antibiotic selection was used for deletion mapping of the 3' conserved sequence element (CSE), including the region overlapping the 3' end of the SP-ORF (Chen *et al.*, 2004a). The development of RV replicons makes possible the generation from the RV replicons of suicide vectors which cannot spread from cell to cell for delivery and expression of foreign genes is a possibility. However such a system will require an efficient packaging systems for the replicons (Chen *et al.*, 2004a). Immunisation with a RV vector based on the live attenuated vaccine has been attempted with the dual aim of inducing immunity to RV and the heterologous genes being expressed. The initial RV vector was constructed using the wt Therien RV infectious clone Robo302 whereby the SG promoter was duplicated, resulting in a virus that synthesised two SG RNAs (Tzeng *et al.*, 2001). To mimic RV defective-interfering (DI) RNAs an RV replicon was constructed by replacing the 3' proximal structural protein ORF (SP-ORF) in Robo402, an RV infectious cDNA clone with a reporter gene (GFP) (Tzeng *et al.*, 2001). Only DI RNAs containing the NS-ORF are selected for, thus demonstrating its importance. A deletion in the Robo402 NS-ORF showed that RV could dispense with 10% of its genome and still replicate. Despite this deletion the RVrep/GFP was able to express GFP in the presence of standard helper RV and spread to other cells. This feature has now been developed as a diagnostic tool whereby rescue of reporter gene expression by NotI replicons occurred when coinfection was done with clinical specimens containing RV (Tzeng *et al.*, 2005a). Thus the native SP-ORF is translated from one SG RNA and the foreign gene is translated from another. The additional SG promoter was placed in between the two ORFs. As the RV SG promoter had not been mapped at this time, a region consisting of the 3' terminal 126 ntds of the NSP-ORF and the entire 120-ntd noncoding region between the NSP and SP ORFs was used. A multiple cloning site (MCS) was placed between the SG promoters for insertion of foreign genes. The plasmid was termed Robo302 and the reporter genes chloramphenicol and acetyltransferase (CAT) and green fluorescent protein were used to test expression (Tzeng *et al.*, 2005a). CAT expression by a SIN vector was approximately 7.5 times greater than CAT expression by the dsRobo vector. Expression by the CAT vector increased 1.8 fold by 6 days. However, GFP expression was unstable and this phenomenon has been described before in other double SG alphavirus vectors. It was thought that homologous recombination between the two SG promoters had caused the loss of GFP expression. To eliminate the possibility of homologous recombination in the RV vector, an internal ribosome entry site (IRES) element was incorporated into the RV expression vector in place of the second SG promoter. EGFP expression was tested in this vector and this siRobo vector exhibited greater stability of foreign gene expression. This

study contributed to knowledge of RV replication strategy and to the discovery of the SG promoter (Tzeng *et al.*, 2002). It showed that an IRES element can function in the capacity of SG RNA, however the latter is preferred.

1.2.6 Animal models

There are no reliable models in small animals for the study of clinically symptomatic rubella infection due to the poor replication of RV in lab animals (Perrenoud *et al.*, 2004). All testing of any vaccine prototype must first be carried out in a small animal model to obtain preliminary data before proceeding to primate models. However, a variety of laboratory animals (including subhuman primates) can be asymptomatically infected with RV, develop viraemia, shed virus in respiratory secretions and produce humoral responses in a manner that parallels events in humans (Chantler et al., 2001). Mice cannot be used as a challenge model because they do not develop overt disease, but both humoral and cell-mediated responses to infection can be detected after intravenous, intrasplenic or intramuscular injection in mice of different haplotypes (Banatvala et al., 2004; Cusi et al., 1995). Although RV has been cultured and identified *in vitro*, the teratogenic effect of the virus *in vivo* and the pathological effects in various animal species have not been well established (Cusi et al., 1995). The study of host factors responsible for elimination of RV in infants with persistent congenital rubella infection has been hindered by difficulties in reproducing pathological effects in animal models (Cusi et al., 1995). A prototype new animal model using the mouse has been developed with the aim of providing further information in order to evaluate novel recombinant and/or synthetic vaccines that will protect against RV (Cusi et al., 1995). It has been found that RV can infect mouse PBM cells thus reaching all the organs transported by the blood, and this new animal model aimed to provide information, using mice, on the duration of the viraemic phase. In this study, the immunogenicity of the lysate of insect cells infected by a recombinant baculovirus expressing E2 and C proteins of RV and the RA27/3 rubella vaccine were tested in a mouse model (Cusi *et al.*, 1995). After a booster injection at 2 weeks, the mice were challenged with live RV and the blood analysed by RT-PCR post challenge. In the RV challenge mouse studies, BALB/c mice immunised with RA27/3 vaccine were the only ones able to inhibit the challenge virus replication. It seems that the E2 and C proteins alone, are not sufficient to protect animals against RV infection and that E1 derived from RA27/3 vaccine likely represents the predominant antigen to which lymphocyte proliferative and antibody responses are developed (Cusi *et al.*, 1995).

1.2.7 MMR prototype vaccine studies

DNA vaccines for measles, mumps, and rubella viruses have been constructed and tested in animal models but are not highly immunogenic (Atkins *et al.*, 2003). A new rubella vaccine should minimise postvaccination joint involvement in adult women and be administrable to seronegative pregnant women who are at risk for contracting rubella during pregnancy (Frey, 1994). A prototype vaccine should have increased biosafety that would be useful in final stages of eradication and act as a support against reintroduction of infection. In humans, virus neutralisation antibodies and cell-mediated immune responses such as $CD4^+$ and $CD8^+$ CTLs are essential in the elimination of RV during infection. Rubella virus-like particles (VLPs) have been investigated as an alternative to conventional attenuated RV vaccines since these particles contain all of the RV structural proteins and are non-replicating (Qiu *et al.*, 1994). There is preliminary data to suggest that animals immunised with RLPs develop neutralising and hemagglutinin-inhibiting antibodies (Hobman *et al.*, 1994). Secretion of RV antigen by stably transfected cells is more economical than propagation of RV in culture (Hobman *et al.*, 1994). rE1 glycoprotein has elicited neutralising anti-RV antibodies in BALB/c mice and boosted *in vivo* the production of human anti-RV antibodies in SCID mice implanted with tonsil fragments from RV immune donors (Perrenoud *et al.*, 2004).

One of the strategies to address the problem of measles mortality in developing countries is to develop a new generation of measles vaccines that can be administered to infants who are too young to receive the current licensed measles vaccine (Song *et al.*, 2005). Characterisation of the immune response to a genetic measles vaccine is important because a formalin-inactivated whole-virus vaccine licensed and used in the 1960s sometimes predisposed vaccinated children to the development of severe atypical measles syndrome when they were then exposed to wild-type MV (Song *et al.*, 2005). This is thought to have resulted from aberrant prior priming that lead to low-avidity antibodies with high complement-fixing capacity which favoured the deposition of immune complexes (Song *et al.*, 2005). This occurrence of atypical measles was previously thought to be due to a lack of antibodies against the F protein, which allowed more virus replication because of uninhibited cell-to-cell fusion. The lack of manifestations of atypical measles in monkeys immunised only with the H protein proved that the absence of antibodies to F after vaccination was not enough to elicit atypical measles after exposure to wt MV (Polack *et al.*, 2000).

In mice, SIN-H has been shown to induce high titre, dose dependent antibody after

a single vaccination and in macaques MV neutralising antibody and IFN-gamma memory T cells were induced. Most monkeys were protected from rash following MV challenge. After challenge, IFN-gamma secreting CD4+ and CD8+ T cells appeared in vaccinated monkeys with peaks 1 and 3-4 months post challenge (Pan *et al.*, 2005). Newborn mice vaccinated with the licensed live-attenuated measles vaccine preferentially elicited a Th2 type response with high IgG1 and IL-5 (Capozzo *et al.*, 2006). The immune responses elicited by SINV replicon-based DNA vaccines encoding MV-H (pMSIN-H) glycoprotein in neonatal mice born to naive and measles-immune mothers have been investigated (Capozzo *et al.*, 2006). pMSIN-H elicited strong Th1 type cell-mediated immunity, measured by T cell proliferation and IFN-γ production, that was unaffected by maternal Abs (Capozzo *et al.*, 2006). Abs elicited by pMSIN-H persisted longer in circulation than those induced by H and fusion glycoproteins (pMSINH-FdU), which fell shortly after the boost. The Abs elicited in response to pMSIN-H showed sustained avidity maturation despite the presence of maternal Abs (Capozzo *et al.*, 2006). Mice immunised with SINV-based DNA replicon plasmids encoding both MV-H and MV-F induced anti-measles antibody responses, that were at least twofold lower than those in mice immunised with pMSIN-H alone (Song *et al.*, 2005). Coimmunising with two DNA vaccines encoding MV-H and MV-F slowed MV-specific CTL responses and decreased plaque reduction neutralising (PRN) antibodies compared to responses elicited by MV-H (Polack *et al.*, 2000).

Good protective immunity against MV is provided by vaccination with F and H proteins in cotton rats (Schlereth *et al.*, 2000a). A recombinant vesicular stomatitis virus (VSV) expressing the MV H (VSV-H) induces titres to MV in the cotton rat model despite the presence of MV-specific antibodies (Schlereth *et al.*, 2000b). As MV H is not a functional part of the VSV-H envelope, MV specific antibodies only slightly inhibit VSV-H replication *in vitro* and this dissociation of function and antigenicity is probably key to the induction of a neutralising antibody in the presence of maternal antibody (Schlereth *et al.*, 2000b). Immunisation of cotton rats via a mucosal surface with another vesicular stomatitis virus expressing the MV H induced seroconversion in the presence of maternal antibodies and subsequent protection (Niewiesk, 2001). Oral delivery of purified tobacco derived MV-H protein has been investigated in a murine model (Webster *et al.*, 2005). MV-H protein was subsequently expressed in transgenic lettuce and found to be immunogenic in mice via the oral route (Webster *et al.*, 2006). When H + F-liposomes and H + F-Iscoms were used as a primary immunogen in a rat model, they did not induce an immune anergy, unlike soluble H + F (Bakouche *et al.*, 1987). Vaccination of BALB/c

(H2d) mice with H and F, but not NP vaccinia virus recombinant constructs completely protected the animals against a lethal MV challenge (Wild *et al.*, 1992). The H recombinant also protected CBA (H2k) mice whereas the F recombinant did so poorly. These results demonstrate a host related restriction of the immune response to particular antigens (Wild *et al.*, 1992). Although a modified vaccinia virus ankara (MVA) vector encoding the F and H proteins was slightly less effective in inducing MV-neutralising antibodies in macaques in the absence of passively transferred antibodies than the currently licensed vaccine, it proved more effective in the presence of such antibodies (Stittelaar *et al.*, 2000). Such a construct could serve as an alternative to the current vaccine for infants with maternally acquired MV-neutralising antibodies and for adults with waning vaccine induced immunity (Stittelaar *et al.*, 2000). A murine hypersensitivity model has also been established to study the mechanism of MV induced immunosuppression (Marie et al., 2001). Four live attenuated mumps virus vaccines have been shown to induce humoral and cell mediated responses in mice immunised intranasally and intramuscularly (Cusi *et al.*, 2001a). The mumps V protein has been shown to target Stat1 or Stat2 for degradation (Gotoh *et al.*, 2001).

1.3 SFV

SFV was first isolated from a pool of 130 female mosquitoes (*Aedes abnormalis* Theobald) which were caught in Bwamba, Uganda in 1942. Three African strains and one South African strain of SFV have been characterised by quantitative virulence markers in mice, guinea pigs and rabbits; allowing for the study of heterogeneity of strain populations and its influence on the expression of virulence and the mechanism of modification by passage (Bradish et al., 1971). SFV is an enveloped positive single-stranded RNA virus that has been utilised in the development of a vector system. Construction of an infectious cDNA clone of SFV, derived from the prototype strain has facilitated the analysis of the molecular basis of SFV virulence (Liljestrom, 1991a). This full-length cDNA clone of SFV can also be used as a template for the production of infectious runoff transcripts, and presents a method for an extremely efficient RNA transfection system based on electroporation (Liljestrom, 1991a). In the European Union, SFV is classified as a Biosafety Level 2 virus (E. C. Council Directive 93}88}EEC, 1993) (Atkins et al., 1999). The NC and spikes of the membrane are organized according to T = 4 icosahedral symmetry and the geometry of the SFV envelope surface lattice, the shape of the trimeric spikes and their arrangement on the lipid bilayer have been reconstructed to 3.5 nm

resolution (Barth et al., 1997; Vogel et al., 1986) (Figure 1.6). The NC contains the viral RNA genome in complex with 240 copies of the CP which are arranged into 30 hexameric and 12 pentameric capsomers (Barth et al., 1997; Vogel et al., 1986). This NC structure contains an equal number of copies of protein heterodimer embedded in the membrane (Garoff et al., 1994). Thus there are 80 spikes in the membrane, each of which consists of three copies of a heterodimeric membrane protein complex (Barth et al., 1992). These oligomers are involved in the entry of the virus through receptor binding and membrane fusion activity (Garoff et al., 1994).

1.3.1 Life cycle of SFV

1.3.1.1 Viral entry

The best studied viruses, with respect to entry and uncoating, are SFV and influenza A (Dick *et al.*, 1996). SFV may follow a similar mechanism of penetration and uncoating to influenza (Dick *et al.*, 1996). SFV uses acid-triggered membrane fusion to infect host cells and this fusion with cellular membranes is mediated by the viral spike proteins (Qiu *et al.*, 2000). The E1 protein is vital for the low pH-induced pore formation by SFV spikes and may play a crucial role in the penetration and uncoating process of SFV (Dick *et al.*, 1996). It is widely accepted that the enveloped SFV requires the clathrin-dependent endocytic pathway for entry of viruses into host cells (De Tulleo *et al.*, 1998). SFV was thought to enter a cell solely through the MHC Class I receptor (Balcarova *et al.*, 1981). It is now known that this is not the only mechanism for entry (Oldstone *et al.*, 1980). SFV enters the cell via endocytosis and triggered by the acidic cellular conditions, the spike proteins undergo a conformational change leading to pore formation across the viral membrane, exposure of the putative fusion peptide located in the E1 protein (Lys-79 to Asp-97) and consequent fusion of the viral and endosomal membranes (Dick *et al.*, 1996) (Figure 1.9). Pore formation allows protons to cross the viral membrane and trigger structural changes within the NC, perhaps priming the NC for the uncoating process (Dick *et al.*, 1996). The possibility of the involvement of the minor membrane component, the 6K has been excluded (Dick *et al.*, 1996). Sindbis and the non-human rhinovirus 14 (HRV 14) depend on the clathrin pathway to enter cells whereas the non-enveloped poliovirus does not. SFV matures by budding at the PM of infected cells and enters uninfected ones by a membrane fusion process in the endosomes directed by the p62/E2-E1 membrane protein heterodimer of the virus (Barth *et al.*, 1997). Understanding of these processes

involved in viral entry and passage into the cytoplasm have furthered the development of new strategies for vaccines (Patterson *et al.*, 1986).

1.3.1.2 Viral RNA replication

The synthesis and processing of SFV-specific nonstructural proteins (nsP1-4) *in vivo* and *in vitro* was determined by peptide mapping experiments with temperature-sensitive mutants (Lehtovaara *et al.*, 1980). SFV, a relatively simple virus, encodes only nine functional proteins of unique sequence, three of which are structural proteins (Atkins *et al.*, 1999). The genome is divided such that the replicase is encoded by one ORF on the genomic RNA, while the structural proteins are encoded in a second ORF, on a separate SG RNA species (Berglund *et al.*, 1996) (Figure 1.7). The viral nonstructural proteins and viral RNAs are implicated in the inhibition of host cell protein synthesis and this involves the limiting of the level of initiation factors such as eIFB required for translation of viral RNA which usurp the requirements of the host cell (Frolov *et al.*, 1994). The viral nonstructural proteins are translated from the messenger genomic RNA which also serves as the template for synthesis of the complementary minus strand (Griffin, 2001). Both new genomic RNA and SG RNA are synthesised from the minus strand (Griffin, 2001). Translation is initiated at a 5' AUG site and proceeds until encountering three termination codons just downstream of the start of the SG RNA sequences.

The viral SG RNA is transcribed from the genome-length complementary (minus) strand from an internal promoter (Frolov *et al.*, 1994). In the alphaviruses, synthesis of the SG RNA is directed by a short 25-nt long sequence which is located immediately upstream from the SG start site and is known as the SG promoter (Pugachev *et al.*, 2000a). All of the structural proteins are synthesised from a common coding unit on the 26S (4.1-kb) mRNA viral subgenome in the order C-p62-6K-E1 (Barth *et al.*, 1997; Liljestrom *et al.*, 1991). Defective interfering (DI) viruses have been observed in nearly every animal virus system studied, including SFV and they are heterogenous particularly in respect of their interference properties (Barrett *et al.*, 1984a). DI SFV were found to have a 16% decrease in mean diameter when compared to SFV by electron microscopy (Barrett *et al.*, 1984b). The proteolytic cleavages at the nsP1/2 and nsP2/3 sites are catalysed by the proteinase moiety of nsP2 (Takkinen *et al.*, 1991). Cleavage at the nsP3/4 sites is independent and nsP4 is produced by nascent cleavage of the growing P1234, catalysed by its own proteinase activity. The SFV RNA packaging signal is thought to lie between nucleotides 720 and 777 (White *et al.*, 1998). The role of the nonstructural polyprotein, of cleavage

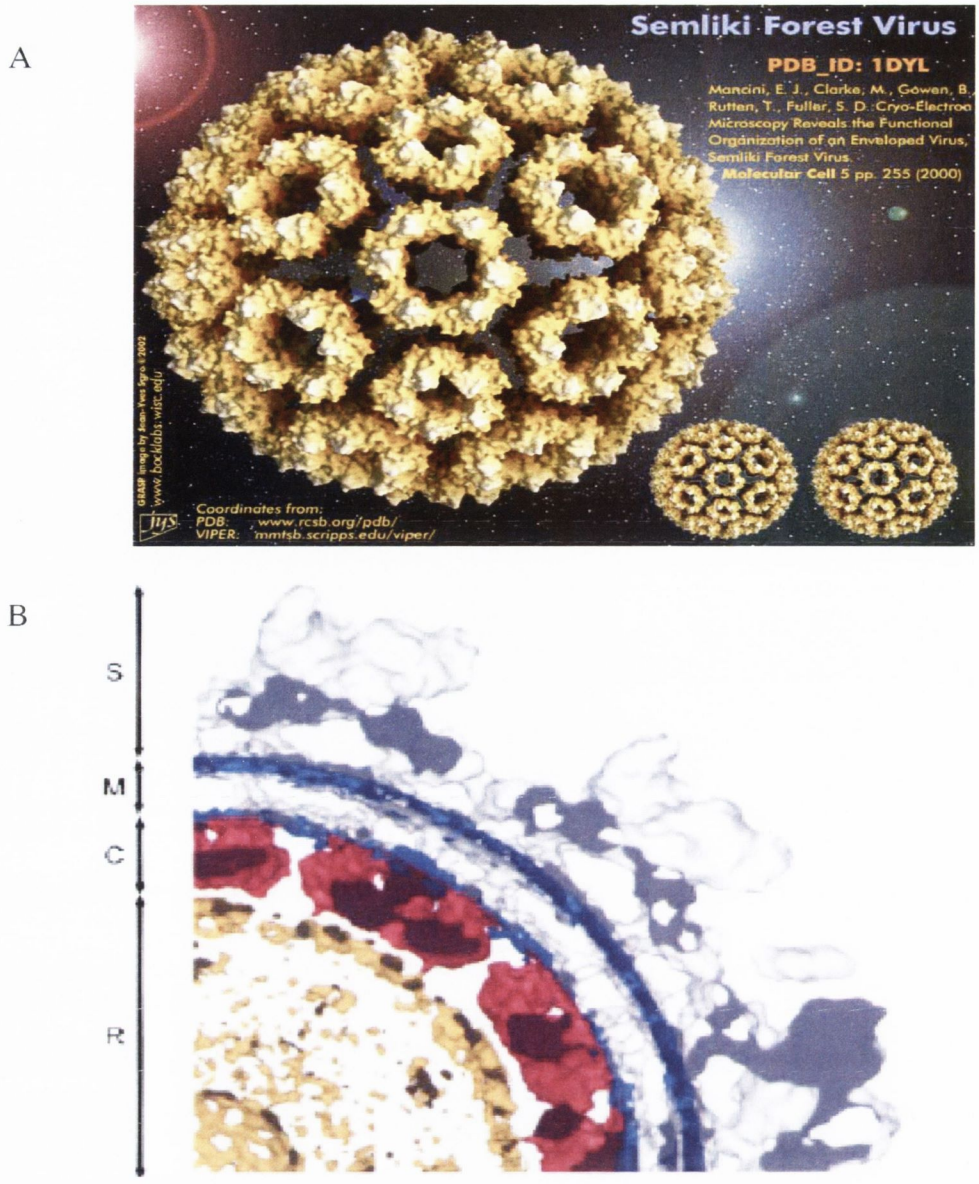


Figure 1.6 SFV structure

A) The 240 copies of the C protein monomer are arranged in a T=4 lattice with pentamer–hexamer clustering. B) The internal organization is revealed by a central section of the virus surface view. The RNA (R) (shown in yellow) is enclosed in an icosahedral NC made of 240 copies of C protein (C) (shown in red). The NC is enveloped by a lipid bilayer (M) (inner and outer leaflets shown in blue). The lipid bilayer is covered and penetrated by 80 glycoprotein spikes (S) (shown in white) (Mancini *et al.*, 2000).

intermediates and of mature proteins in the synthesis of SFV RNA has been studied using mutants (Hwang *et al.*, 2004).

The nsP1 protein has been implicated in the synthesis of the minus strand and also has both guanine-7 methyltransferase and guanyltransferase activities. The conserved

methyltransferase/capping enzyme domain lies in the N-terminal half of nsP1, the N-terminal part of which binds to S-adenosylmethionine (AdoMet) during capping reactions (Tuittila *et al.*, 2003). An active nonstructural protease in the C terminal of nsP2 is required for RNA synthesis and is responsible for all three cleavages in the nonstructural polyprotein (Hwang *et al.*, 2004). The nsP2 protein is involved in regulation of minus strand RNA synthesis and in the initiation of SG RNA synthesis (Griffin, 2001). The nsP2 protein is also an RNA helicase with NTPase activity and has the 5' triphosphate activity needed for the initiation of RNA-capping reactions (Tuittila *et al.*, 2003). It may be involved in the inhibition of host cell protein synthesis in addition. A single mutation in the multifunctional RNA replicase protein can limit virus spread in the brain, demonstrating the importance for pathogenesis of events linked with RNA replication on nsP2 nuclear transport (Fazakerley *et al.*, 2002). Cleavage between nsP3 and the viral RNA polymerase, nsP4, is essential for SFV RNA synthesis (Hwang *et al.*, 2004).

The nsP3 protein is thought to be involved in the generation of CVs (Tuittila *et al.*, 2003). This protein plays a role in SG 26S and negative-strand RNA synthesis and is also thought to function with nsP1 to anchor replication complexes to cell membrane structures (Galbraith *et al.*, 2006). It has been established that deletions in the nsP3 hypervariable domain attenuate virulence after peripheral inoculation and also reduce virulence after intranasal inoculation in mice (Galbraith *et al.*, 2006). The avirulent rA774 SFV strain has an opal termination codon close to the 5' end of the nsP3 (a.a. 469) whereas SFV4 has an arginine residue at this position (Tuittila *et al.*, 2003). The role of this arginine is critical for conversion to neurovirulence of the rA774 strain in conjunction with two nsP3 mutations out of a possible 8 a.a. mutations. However virulence determinants of SFV are distributed over a wide region of the nonstructural genes (Tuittila *et al.*, 2003). The nsP4 protein is considered to be the RNA polymerase of the virus. GC+ oligodendrocytes and A2B5+/GFAP+ fibrous astrocytes, are more readily infected by SFV A7 than other brain cell types including the protoplasmic astrocytes (Bruce *et al.*, 1984).

1.3.1.3 Structural proteins and virus assembly

SFV directs the synthesis of the transmembrane proteins p62, 6K, and E1, all of which are made in equimolar amounts from a polyprotein precursor molecule (Lusa *et al.*, 1991). The structural proteins are cleaved cotranslationally by the serine protease activity of C (33 kDa) and by the signal peptidase activity of the ER membrane (Barth *et al.*, 1997). The 6K (6 kDa) protein functions as a signal sequence for E1 (50 kDa) and the E1 protein

has been shown to control the NC-binding activity of p62/E2 (62 kDa) (Barth *et al.*, 1997). The deletion of the 6K protein results in a dramatic reduction in virus release, suggesting that the protein exerts its function late in the assembly pathway, possibly during virus budding (Liljestrom, 1991a). This small membrane protein provides the signal sequence for El translocation (Liljestrom, 1991a). It has also been suggested that the 6K protein functions in the stability of the wt fusion-active spike conformation (McInerney *et al.*, 2004). A hydrophobic peptide in the C-terminal region of the 6K protein can direct the translocation of the E1 protein as well as that of a heterologous protein. This peptide is also required for E1 translocation in the context of the complete structural polyprotein and a C-terminal region of the p62 protein functions as a signal sequence for the 6K protein. Thus two internal cleavable signal sequences are involved in the generation of the SFV membrane proteins (Liljestrom *et al.*, 1991).

The transport of the SFV glycoproteins from the trans-Golgi network (TGN) to the PM has been dissected (de Curtis *et al.*, 1988). In the TGN proteins destined for different locations are included into separate transport vesicles (de Curtis *et al.*, 1988). Proteolytic cleavage of the SFV p62 protein into the E2 and E3 polypeptide chains occurs before arrival to the PM (de Curtis *et al.*, 1988). Control steps preventing production of incomplete particles in SFV infected cells include i) the linkage of E1 transport out of the ER and complex formation with p62 ii) the dependence of NC formation on genome encapsidation and iii) the spike-NC interaction that drives the budding reaction (Barth *et al.*, 1997). In Sindbis, sequences downstream of the initiating codon for the CP enhanced translation of the mRNA in infected cells, but had the opposite effect in uninfected cells (Frolov *et al.*, 1994). An autoprotease model has been suggested for co-translational cleavage of SINV CPs (Aliperti *et al.*, 1978). The C protein of RV does not possess autoprotease activity (Clarke *et al.*, 1987). In SINV, residues 97 to 106 of the NC are important in the recognition of the genomic RNA packaging signal thus dictating specificity in the encapsidation reaction *in vivo* (Owen *et al.*, 1996).

The 6K protein associates with the p62E1 complex in the ER and is transported with the complex to the cell surface where during virus budding, E1 and p62 (now the mature E2 protein) are incorporated into new virions whereas the 6K is mostly excluded (Lusa *et al.*, 1991). A spike deletion variant and a CP deletion variant are budding-negative when expressed separately but can easily complement each other when transfected into the same cell demonstrating that enveloped viruses use different budding strategies (Garoff *et al.*, 1994). One of the budding strategies depends on a NC-spike interaction as exemplified by SFV and another one is based on a direct core-lipid bilayer interaction which is the

case with retroviruses. The SFV E2 protein is a class II viral fusion protein and is involved in fusion of the enveloped virus with the cell membrane. During infection with SFV, the E2 interacts with receptors on the target cell PM resulting in the uptake of virus in clathrin-coated vesicles (Zhang *et al.*, 2005). The SFV spike comprises three copies of a membrane protein heterodimer (Barth *et al.*, 1995). The acidic environment of the endosome promotes dissociation of the E2/E1 heterodimer allowing the free E1 to insert into the target membrane following homotrimerisation (Zhang *et al.*, 2005). Thus cleavage of p62 creates a low pH environment which surrounds incoming virus in endosomes inducing dissociation of the p62E1 heterodimeric structure which is followed by a rearrangement of E1 subunits into fusion active homotrimers (Garoff *et al.*, 1994). Data suggests that the heterodimers are probably complexed together into the trimeric structures (spikes), at the PM to expose a multivalent binding site for the NC and thereby drive efficient virus budding. The SFV p62 protein contains an uncleaved 16 residue signal sequence peptide at its NH_2-terminal region which becomes glycosylated during synthesis and translocation of the polypeptide into the lumen of the ER but does not complete the transfer process (Garoff *et al.*, 1990). The two subunits of the spike heterodimer (p62 and E1) are synthesised sequentially from a common mRNA together with the capsid and are preferentially derived from the same mRNA translation product (heterodimerisation *in cis*) (Barth *et al.*, 1995).

1.3.1.4 SFV and pathogenesis

Virulent SFV4 and avirulent A7 strains of SFV cause cell death by apoptosis in BHK cells and oligodendrocytes in glial cell cultures and by necrosis in cerebellar neuron cultures (Atkins, 1983; Atkins *et al.*, 1982b; Atkins *et al.*, 1985; Glasgow *et al.*, 1997). SINV, encoding two polyproteins, appears to thrive in apoptotic cells perhaps in part because its replication cycle is completed prior to cell death. SINV primarily targets neurons and SINV-induced apoptosis in mouse brains and spinal cords has been found to correlate with mortality (Lewis *et al.*, 1996). Following extraneural inoculation in the mouse, SFV is efficiently neuroinvasive and crosses the blood-brain barrier to initiate perivascular foci of infection in neurons and oligodendrocytes, resulting in mild encephalitis to fatal panencephalitis (Atkins *et al.*, 1982a). Whereas SFV infections of the developing nervous system are always highly destructive and are generally fatal, those of the mature nervous system can result in persistent infection with no apparent cell loss (Fazakerley, 2004).

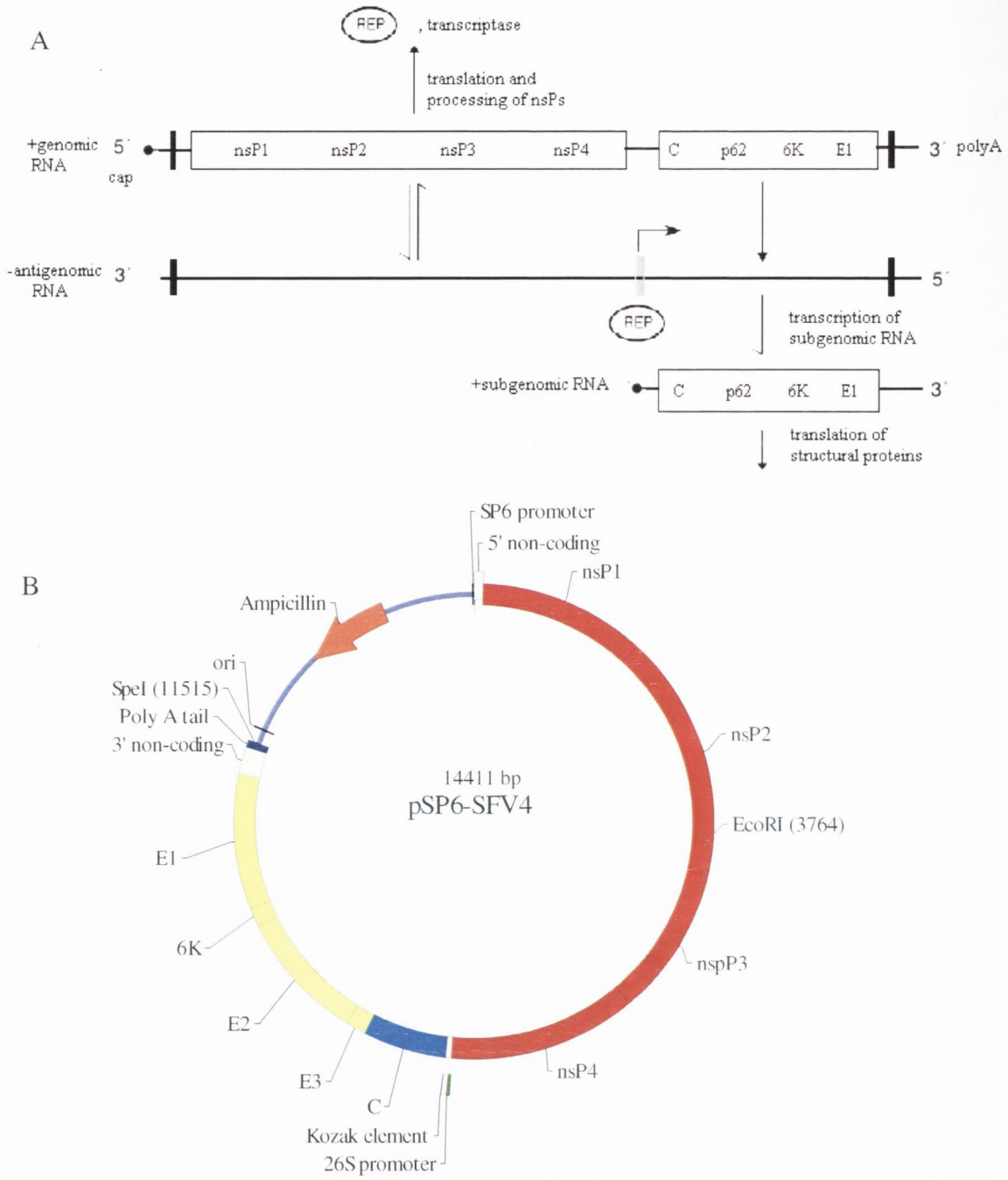


Figure 1.7 SFV life cycle and infectious clone pSP6-SFV4

A) The nsPs are translated from the first two thirds of the (+) strand genomic RNA which serves as the mRNA for translation of the nsPs. The nsPs are subsequently processed into four proteins involved in replication and transcription. The genomic RNAs and the SG 26S RNA are synthesised from the (-) strand complementary strand. B) The SFV genome (11445 nt) has been cloned under the control of the SP6 promoter. To produce infectious virus, the plasmid is linearised with Spe1, then transcribed with SP6 polymerase to produce infectious RNA, which is then transfected into BHK cells by electroporation.

Certain SFV infections result in changes in the normal levels of some CNS neurotransmitters, which persist after infectious virus can no longer be detected and could be a contributing factor in some neurological diseases (Barrett *et al.*, 1986). Matrix metalloproteinases (MMPs) are a group of endoproteinases that can degrade protein components of the extracellular matrix including collagen, fibronectin and laminin and thus have important regulatory roles in bone remodelling and angiogenesis. However, overproduction of MMPs can lead to breakdown of the blood–brain barrier (Keogh *et al.*, 2003b). It has been shown that MMP-2 and MMP-9 expression is induced in the brains of mice infected intranasally (i.n.) with SFV-A7. There is also increased MMP expression in active lesions of multiple sclerosis (MS) in humans and they have been implicated in various diseases including Alzheimer's and cancer. Treatment of mice with the panMMP inhibitor GM6001 ameliorated the development of SFV-induced neuropathological lesions via an effect on the integrity of the blood–brain barrier (Keogh *et al.*, 2003b).

Wt SFV infects the neurons and glial cells of mice resulting in the demyelination of the central nervous system (CNS) (Smyth *et al.*, 1990). Immune-mediated demyelination, which follows infection with avirulent strains, is induced by phagocytosis of myelin debris from infected oligodendrocytes, and the presentation of antigens derived from such debris to T-helper lymphocytes (Atkins *et al.*, 1990). Animal studies indicate that the virulence of SFV strains is controlled by rapidity of multiplication in the CNS, leading to a lethal threshold of damage, rather than differential cell tropism or cell death mechanisms.

1.3.1.5 SFV and the immune response

Four viruses with non-segmented genomes have been used as models of neurovirulence and demyelinating disease: JHM coronavirus, Theiler's virus, SINV and SFV. The construction of infectious clones for the latter three viruses has allowed analysis of sequences involved in the determination of neuropathogenesis, through the construction of chimeric viruses and site specific mutagenesis (Atkins *et al.*, 1994). M9-SFV infection induces long-term expression of pro-inflammatory cytokines in the CNS of SJL mice which is not associated with persistence of the virus genome and may be a relevant model for the pathogenesis of MS in man (Donnelly *et al.*, 1997). N-Acetylethyleneimine (AEI) was used to inactivate the avirulent SFV A774 strain and SFV specific IgG was produced equal to that of mice given live virus (Amor *et al.*, 1986). The mice were protected against the lethal SFV L10 strain. Restriction of the replication of the A7(74) strain of SFV in mice has been shown to be age-related. The focal restricted infection in the CNS of adult

mice infected with SFV A7(74) is independent of particular immune responses such as IgM antibodies which clear the viraemia (Amor *et al.*, 1996). IgG antibodies clear infectious virus but cells positive for viral RNA remain and these may normally be cleared by T cell responses which are damaging and give rise to lesions of demyelination (Amor *et al.*, 1996). Temperature sensitive mutants of A7 SFV virus have been found to have altered pathogenicity for mouse embryos with multiplication arrested in one mutant (Hearne *et al.*, 1987). Other temperature sensitive mutants induced skin and skeletal defects in foetal mice resulting from the tropism of the virus for mesenchymal cells involved in the development of such tissue (Mabruk *et al.*, 1988).

The immune-mediated demyelination induced by avirulent strains may be triggered by apoptosis of oligodendrocytes. SFV4 and A7 share similar cell tropisms for the murine CNS, but differ in the severity and rate of development of cytolytic damage (Balluz *et al.*, 1993). Mononuclear cell infiltrates and cytokine profiles have been characterised in the CNS of mice following infection with the demyelinating A7(74) strain of SFV, an important viral model of MS (Morris *et al.*, 1997). Mononuclear cell infiltrates in the CNS, predominantly $CD8^+$ lymphocytes, were first observed at 3 days and were maximal during clearance of infectious virus and macrophage/microglia and B lymphocytes were most numerous during the subsequent phase of demyelination (Morris *et al.*, 1997).

$CD4^+$ T-lymphocytes were observed at low levels throughout infection. MHC class II, IL-1 α, IL-2, IL-2R, TNF-α and IL-6 were strongly upregulated in the CNS of SFV-infected mice. Whereas IL-1 α, IL-1 β, IL-10, and TGF-β 1 were observed on day 3 following infection, GMCSF, IL-2 and TNF-α were first apparent at day 7 when the cellular infiltration in the CNS was most intense. In contrast IFN-γ and IL-6 were first observed on day 10 prior to the demyelination phase of disease, thus no clear bias of either a Th1 or Th2 response was observed in the CNS during infection (Morris *et al.*, 1997). Cytokines in the lesions of demyelination suggest a role in the pathogenesis of myelin damage (Morris *et al.*, 1997). Mutants of SFV strain Lio showing altered virulence in weanling mice allowed for the characterisation of virulence (Barrett *et al.*, 1980). In immunocompetent mice the development of lesions and demyelination may be a result of an immunopathological response to virus infection which is related to the presence of thymus derived lymphocytes (Jagelman *et al.*, 1978).

Epitopes have been identified on the SFV E2 and E1 glycoproteins which are associated with inducing hemagglutinating and neutralising antibodies and elicit protection in recipient mice against lethal challenge (Boere *et al.*, 1984). Attempts were made to develop formalin inactivated SFV vaccine and a passive agglutination method developed

to test potency (Hubschle *et al.*, 1980). A vaccine against SFV consisting of a monoclonal anti-idiotypic antibody cross-linked to a protein, which contains virus-specific T-helper cell epitopes significantly prevented virus multiplication in BALB/c mice (Kraaijeveld *et al.*, 1992). A memory T cell response could not be detected, however, favouring a booster regimen in future. Other monoclonal anti-idiotypic antibodies induced against a SFV-neutralising mAb, have been investigated with regard to their vaccine potential (Oosterlaken *et al.*, 1992). Subunit vaccines, containing the SFV spike glycoproteins in three different forms, have been prepared: detergent-solubilised monomers, detergent- and lipid-free octamers, and virosomes in which the spike proteins are reconstituted into phospholipid vesicles (Balcarova *et al.*, 1981). The multimeric forms induced high humoral antibody titres in mice whereas the monomeric form was much less immunogenic (Balcarova *et al.*, 1981). An active type-I interferon system prevents virus spread in extraneural tissues (Fazakerley, 2002).

Certain DI SFV preparations have been shown to protect the majority of mice from lethal challenge. No signs of disease were apparent and the DI virus was not detectable in the brain or olfactory lobes (Barrett *et al.*, 1984e). An equivalent amount of non-infectious virus antigen did not change the course of disease demonstrating the prophylactic activity of DI virus (Barrett *et al.*, 1984c). The efficiency with which DI SFV prevent the SFV-induced lethal encephalitis is dependent on the mouse strain, the amount of DI and the amount of infectious SFV (Barrett *et al.*, 1984d).

Two short linear peptides designated H (a.a positions 227-243) and L (a.a positions 297-310), 17 and 14 a.a.s long, on the SFV E2-envelope polypeptide are shown to be involved in the protection of mice against lethal challenge with SFV and the effectiveness of other peptides has been investigated (Grosfeld *et al.*, 1991; Snijders *et al.*, 1991). One potential T-helper cell epitope of SFV has been identified between a.a.s 137 and 151 of the SFV E2 membrane protein on the basis of its ability to induce delayed-type hypersensitivity (DTH) in mice (Snijders *et al.*, 1992b). In conjunction with a linear B-cell epitope of E2, this DTH-inducing region provided protection in 70-100% of peptide immunised mice after challenge with virulent SFV (Snijders *et al.*, 1992b). The peptide induced antibodies did not however neutralise SFV *in vitro*. Further studies on this protective effect demonstrated that B-cell rather than T-cell memory played a major role (Snijders *et al.*, 1992a). Both reduced and non-reduced E1 and E2 glycoprotein preparations induced protection against lethal SFV challenge (Truyen *et al.*, 1991).

1.4 Alphavirus vectors

Alphaviral vectors have been constructed for SINV, VEEV and SFV, with two general approaches employed. In the first instance the viral structural genes were replaced with a heterologous gene and in the second, a duplicate SG mRNA promoter was used to drive the expression of the foreign genes (Berglund et al., 1996). The advantage of using RNA is its rapid degradation in cells and no risk of integration of foreign genetic information into the host cell genome (Berglund et al., 1996). For SFV, three strategies for *in vitro* and *in vivo* gene-delivery have been developed, the main one relying on the packaging of recombinant vectors into suicidal viral particles and infection of target cells (Karlsson et al., 2004). The other two methods are based on direct transfection of target cells, either by using naked DNA encoding the SFV replicon placed downstream of an RNA polymerase II dependent promoter, or by using *in vitro* transcribed RNA encoding the SFV replicon. Thus these vectors can be used for gene delivery as naked RNA or DNA (Smerdou et al., 1999). Delivery of the self-replicating SFV vector into target cells is the end result for all three approaches, with expression of foreign genes being driven from a highly efficient viral SG promoter (Karlsson et al., 2004). Tissue/cell specific infection has been achieved in SIN vectors by the introduction of an IgG-binding domain of Protein A into one of the spike proteins of SIN, thus enabling efficient targeting of infection to human lymphoblastoid cells (Lundstrom, 1999). Mutant SFV vectors demonstrating lower and more physiological expression levels and non-cytopathogenic vectors have also been developed for various applications within receptor research with implications for drug screening of recombinant proteins (Lundstrom, 2002b; Lundstrom et al., 2001). Tumour vaccines have been designed whereby injection of SFV vectors as naked RNA molecules, DNA plasmids or recombinant particles have been tested for both therapeutic and prophylactic efficacy (Lundstrom, 2002a). SIN vector particles, which are not known to be lymphotropic, have been developed to increase their potency and the tropism re-directed for efficient infection of dendritic cells (DCs), both *in vitro* and *in vivo* (Polo et al., 2000). SINV and SFV are established models for studies in molecular and cell biology and VEEV, although a human pathogen is now becoming a potentially valuable vaccine vector (Schlesinger, 2001). Both SINV and SFV vectors are serving as tools for fundamental studies in biology such as development in insects and analysis of protein functions in neuronal cells (Schlesinger, 2001). The initial immunogenicity evaluations with a HIV p55Gag antigen in a VEErep/SINenv replicon particle chimera indicate potent induction of cellular immune responses. Such an approach could result in increased *in vivo* potency,

ease of production, and overall safety (Perri et al., 2003). Major advantages of the live virus vector over the purified approach are that the antigen is replicating and that it is presented to the immune system in its naïve conformation (Mills, 2002). Because the antigen is synthesised in the host, it will be properly folded and glycosylated and can gain entry to the endogenous route of antigen processing in the APCs thus allowing induction of functionally important antibodies and class I restricted T cells, respectively (Mills, 2002).

1.4.1 The SFV vector system

Following the construction of the first full-length infectious clone of SFV, a vector system was developed which induces high-level transient expression of cloned genes in transfected cells (Smerdou, 1999) (Figures 1.7 & 1.8). Recombinant alphavirus RNA can be synthesised *in vitro* from plasmids containing the alphavirus replicon under the control of a prokaryotic promoter such as SP6 or T7 and probably induces protective immune responses *in vivo* due to the high level of expression of the recombinant antigen in the transfected cells (Smerdou *et al.*, 1999). The SG RNA of SFV is translated to high levels in infected cells which was one of the attractions for developing SFV as an expression vector (Frolov *et al.*, 1996). Replicon (self replicating) SFV vectors have been constructed where a heterologous foreign gene replaces the viral structural genes. The capsid and spike (envelope) proteins of the structural polyprotein are encoded by two defective, packaging-deficient 'helper' plasmids. Recombinant RNA containing the foreign gene is packaged into infectious virus particles in BHK-21 cells by using co-electroporation with these helper RNA molecules (Berglund *et al.*, 1999; Leitner *et al.*, 2003). Such helper plasmids allow replicons to be assembled into particles under conditions in which the helper itself is not packaged (Griffin, 2001). Specifically, these helper vectors have been developed to carry the viral structural genes but they lack the replicase genes including the genomic packaging signal (Tubulekas, 1997). SFV infects a broad host range of cell types, there is no pre-existing immunity against the vector in the majority of individuals, RNA replication is cytoplasmic and vector structural proteins are not expressed (Atkins *et al.*, 2000). Thus, rSFV can be used in repeated immunisations as vector structural proteins are not expressed (Colmenero *et al.*, 2001). Such particles, while infectious, are self limiting and produce nsPs as well as genomic and SG RNAs, but in the absence of structural proteins, do not form new particles (Griffin, 2001). The insertion of a hairpin structure at a particular location downstream of the initiating AUG seems to have evolved to allow alphaviruses to enhance translation of their mRNA, and consequently produce high levels of structural

proteins for virus assembly (Frolov *et al.*, 1996). SFV vectors incorporating this RNA increase heterologous protein synthesis to the level of wt polyprotein (Sjoberg *et al.*, 1994). The autoprotease activity of the CP has also been abolished by mutation further increasing the biosafety of the system. In the pSFV10Enhanced (pSFV10Enh) particle vector a translational enhancer containing the first 34 a.a.s of the capsid gene has been engineered in front of the MCS (Hanke *et al.*, 2003). This putative hairpin structure is crucial for 10 fold enhancement of reporter gene translation (Sjoberg *et al.*, 1996). A sequence coding for the foot-and-mouth disease virus (FMDV) 2A autoprotease has also been inserted in frame between the capsid translational enhancer and the MCS, which allows cotranslational removal of the enhancer sequence.

Thus the particle vector system consists of a self-replicating and self-transcribing RNA molecule that relies only on the host cell machinery for protein translation. When administered as a vaccine to express heterologous antigens, these rSFV particles are suicidal and undergo only one round of multiplication and kill the infected cells (Liljestrom, 1991b). Alphavirus infection occurs via receptor-mediated endocytosis but naked RNA can also initiate infection when introduced into the cytoplasm (Liljestrom, 1991b). The SFV expression vectors, lacking the structural genes, are classified as Biosafety Level 2 in the EU and USA (Federal Register, 1993) (Atkins *et al.*, 1999). The construction of a conditional lethal helper system has now largely overcome biosafety problems associated with potential alphaviral infection of humans, and should further increase the utility of these types of vector in animal cell systems (Liljestrom, 1994). Extensive analysis has failed to demonstrate the presence of wild-type viruses formed from SFV particles and at least two recombination events and one reversion are required to generate replication-competent virus particles thus emphasising the biosafety of the two-helper system (Hewson, 1999). In fact this strategy has been shown to increase the biosafety of the rSFV system by considerably reducing the probability of replication competent virus (rpv) generation to lower than 10^{-13} (Colmenero *et al.*, 2001). To increase the safety and efficacy of the VEEV vaccine, previously defined attenuating mutations were included in the replicon and/or its helper, thus preventing any viable recombinant virus from initiating a virulent infection (Pushko *et al.*, 1997). A second approach, similar to the one used for the SFV vector system, used a bipartite helper to supply structural proteins for packaging of the replicon into particles, thus requiring at least two recombination events for the generation of viable virus (Pushko *et al.*, 1997). The T- and B-cell responses elicited by antigen expressing viral vector alone or in boosting following a naked DNA prime are both broader and of a higher magnitude than following

immunisation with DNA alone (Karlsson *et al.*, 2003). The suicidal vectors replicate inside the target cell, mimicking an authentic virus infection but there is no spread of the progeny virus in the vaccine which results in the activation of innate anti-viral responses in the antigen-expressing cell (Karlsson *et al.*, 2003). Packaging cell lines (PCLs) can be used to produce alphavirus vector particle stocks that are free from contaminating replication-competent virus and could facilitate large scale vector production (Polo *et al.*, 1999). The PCLs are stably transformed with inducible SINV structural protein expression cassettes and these proteins are only expressed in response to input vector and consequent synthesis of vector-encoded replicase proteins (Polo *et al.*, 1999). SFV vaccine particles adsorb rapidly to cells at the injection site and are known to induce p53-independent apoptosis late in infection by expression of the nonstructural region of the virus genome and therefore probably not resulting in damage to cellular DNA (Atkins *et al.*, 2000; Glasgow *et al.*, 1998). Intramuscular inoculation resulted in the detection of lacZ-RNA in local lymph nodes and at the site of injection (quadriceps) (Colmenero *et al.*, 2001). Splenocytes from mice injected intramuscularly developed slightly higher levels of antigen-specific cytotoxicity compared with mice immunised subcutaneously (Colmenero *et al.*, 2001). Occasional myofiber necrosis is visible from 6 hours to 3 days post inoculation (PID) and increases from PID 7-14, disappearing by PID 28. RNA is mainly detected from 6 hrs to three days post inoculation and myofibers expressing enhanced green fluorescent protein (EGFP) were highest at PID 3 (Atkins *et al.*, 2000). Persistence of SFV RNA particles up to 7 days at the injection site of mice is short term and detection is transient in secondary lymphoid organs with no dissemination to distal sites when compared to a standard naked DNA vector or an SFV DNA RNA vector (Atkins *et al.*, 2000). The routes of injection, namely intravenous (i.v.), intramuscular (i.m.) and subcutaneous (s.c.) injection of rSFV particles have been related to the degree of CTL responses and frequency of specific T cells detected by MHC-I tetramers (Colmenero *et al.*, 2001). There was a higher accumulation of antigen-specific T cells in the spleen after immunisation, in the following order i.v. > i.m. > s.c. (Colmenero *et al.*, 2001). Following i.v. injection, rSFV-RNA was distributed to a variety of different tissues, while it was confined locally after i.m. and s.c. injections. All three routes of immunisation generated significant levels of antigen-specific CTL responses, the distribution of which correlated with the *in vivo* distribution pattern of rSFV (Colmenero *et al.*, 2001). The highest frequency of antigen-specific CTLs was in the spleen or local lymph node and was dependent on injection route. Increased numbers of specific CD8+ T cells were also evident. Major targets for rSFV are non-lymphoid cells resident at the injection site rather than DCs (Huckriede *et al.*, 2004).

1.4.2 Potential applications of the SFV vector system

The SFV vector has yielded promising results in terms of $CD4^+$ T-cell and CTL induction as well as in mixed-modality immunisation strategies combining DNA with SFV or SFV with poxvirus vectors (Girard *et al.*, 1999; Tubulekas, 1997). When SFV DNA vaccines were compared to traditional DNA vaccines, considerably lesser amounts of DNA were needed to obtain comparable levels of humoral or cellular immune responses in mice (Girard *et al.*, 1999). SFV replicons induce strong immune responses; mice immunised with an anti-HIVA vaccine showed strong T cell mediated responses and memory responses that lasted for at least 6 months (Hanke *et al.*, 2003). rSFV has been used for immunisation of macaques, which resulted in immune responses against the envelope protein of HIV-1 and simian immunodeficiency virus (SIV) (Hanke *et al.*, 2003). Data suggests that rSFV may be a useful component of a viral vector prime-protein boost regimen aimed at stimulating both cell-mediated immune responses and neutralising antibodies against HIV-1 (Forsell *et al.*, 2005). SFV vectors have been used to functionally express both the HIV-1 envelope precursor gp160 and the cleaved external portion gp120 (Paul *et al.*, 1993). A linear peptide sequence of the predominant neutralising domain or V3 loop of HIV-1 was used to induce neutralising antibodies in BALB/c mice in conjunction with an SFV H2-d restricted T cell epitope and various adjuvants (Fernandez *et al.*, 1998). SFV RNA vectors have been used to immunise the envelope protein gp160 of HIV-1IIIB in cynomolgus macaques and three out of four vaccinated monkeys had no demonstrable viral antigenemia and a low viral load (Berglund *et al.*, 1997).

The immune responses elicited by vaccination with a MVA vector expressing SIV-macJ5 genes (env, gag–pol, nef, rev and tat) given alone were compared with that of a prime-boost regimen in which a SFV vector expressing the same SIV genes (SFV–SIVmac) was combined with the MVA–SIV vaccine (Nilsson *et al.*, 2001). Stronger humoral and cellular immune responses were elicited with the prime-boost regimen than with vaccination with MVA–SIVmac alone although none of the cynomolgus macaques were protected against a rigorous heterologous mucosal SIV challenge (Nilsson *et al.*, 2001). SFV particles containing SIV gp 160 have been used to vaccinate and protect pigtail macaques against lethal challenge with SIV. RSFV retrovirus particles with functional protease and reverse transcriptase activity indicate a potential use for retroviruses in gene therapy (Li *et al.*, 1996).

It has been demonstrated that SFV recombinants can result in clearance of Respiratory Syncitial virus (RSV) without enhancing the RSV-associated disease (Chen *et*

al., 2002). Targeting of antigen expression (intracellular versus secreted) may also be an important factor to consider in the design of nucleic acid vaccines. Immunisation of mice with SFV plasmid vectors expressing an RSV vaccine candidate elicited significant serum anti-BBG2Na IgG responses in the mice receiving the plasmid encoding the secreted version of BBG2Na and not the intracellular version (Andersson *et al.*, 2000). The SFV induced Th1 response was relevant for RSV where a Th2 type immune response, elicited after formaldehyde inactivated RSV vaccination, is associated with immunopathology and more severe lung disease after infection (Fleeton *et al.*, 2001). Resistance to RSV without disease enhancement was achieved with rSFV recombinants encoding the RSV fusion (F) and attachment (G) proteins and administered via the nasal route (Chen *et al.*, 2002).

Mice immunised with rSFV encoding Influenza A nucleoprotein (NP) or *E. coli* LacZ developed long-lasting antigen specific IgG levels and cytotoxic T cell memory was induced. These protective immune responses persisted for over one year and were achieved by different immunisation routes. Development of immune responses against the vector itself were shown not to inhibit boost responses by subsequent immunisations with rSFV (Berglund *et al.*, 1999). Intramuscular immunisation of mice with suicide particles expressing the influenza nucleoprotein showed that a strong, class I-restricted CTL cell response can be obtained using only 100 infectious units (Zhou *et al.*, 1994). Recombinant naked RNAs encoding the HA of influenza (FLU) or F envelope protein of RSV have been injected intramuscularly into mice and evoked significant protection against challenge virus (Fleeton *et al.*, 2001). While the antibody titres induced were not as high as those elicited when mice were immunised with rSFV particles or rSFV DNA coding for the same antigens, they were sufficient to afford protective immunity. Mice vaccinated intraperitoneally (i.p.) with SFV particles expressing the Louping Ill virus prME and/or rSFV-NS1 genes were significantly protected from lethal i.p. challenge by two strains of LIV (Fleeton *et al.*, 1999). None of the vaccinated mice that survived challenge exhibited CNS pathology (Fleeton *et al.*, 1999). Antibodies produced in mice following rSFV immunisation against the prME and MS1 antigens were primarily of the IgG2a subtype, indicating that a T-helper 1 immune response was induced (Fleeton *et al.*, 1999). In sheep, the rSFV vaccine offered protection against subcutaneous challenge with LIV, and partial protection following intranasal administration of LIV (Morris-Downes *et al.*, 2001).

Louping ill virus (LIV) infection of mice has also been used as a model to evaluate the protective efficacy of SFV-based vaccines in comparison to a standard naked DNA vaccine and a commercial formaldehyde inactivated vaccine (Fleeton *et al.*, 2000). Protection was provided by rSFV particles against neuronal degeneration and encephalitis

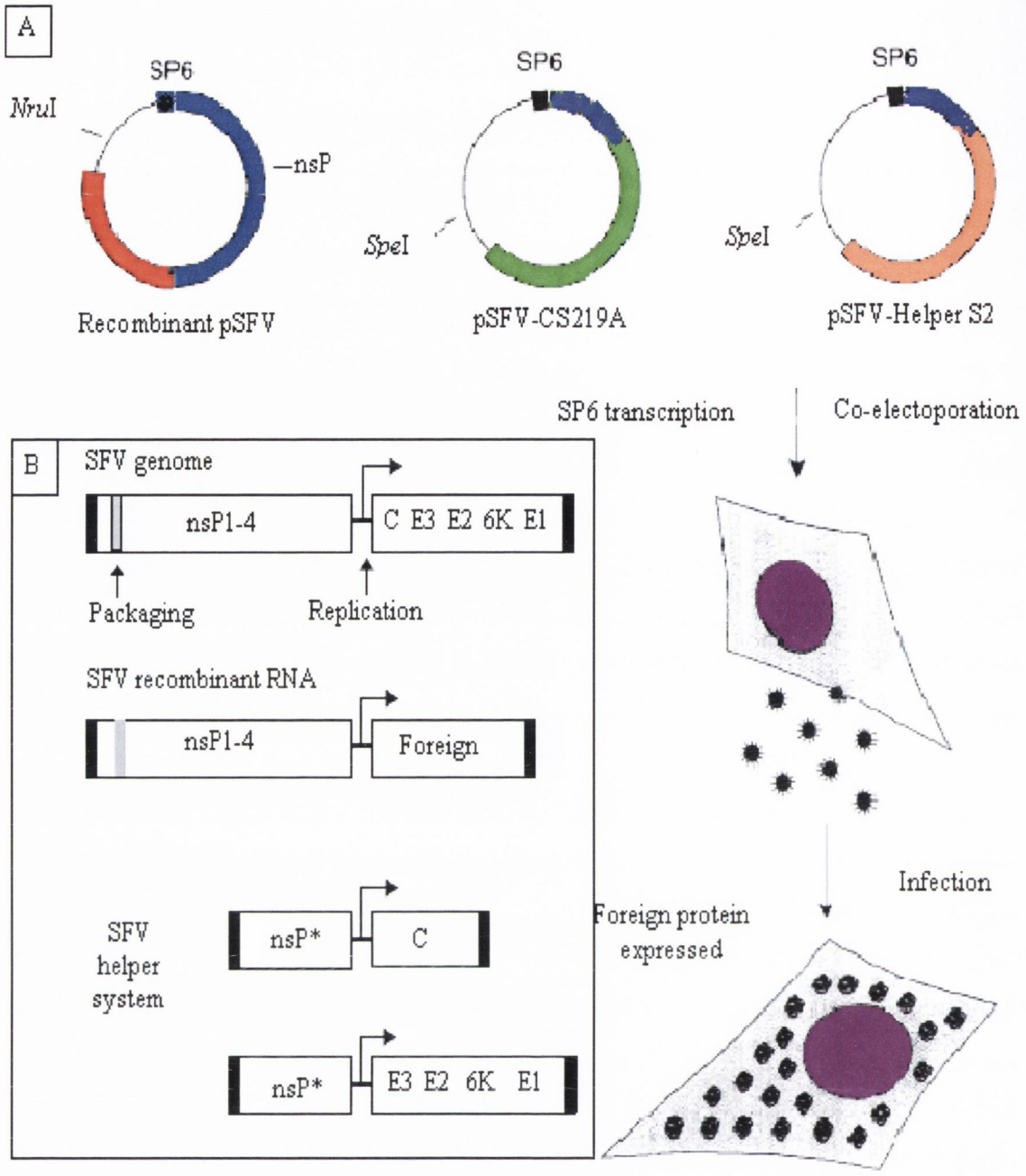


Figure 1.8 Production of rSFV-Virus like particles (rSFV-VLPs)

A) The recombinant pSFV, p-helper CS219A and p-helper S2 plasmids are invitrotranscribed and the RNA is electroporated into sBHK cells. The rSFV particles are harvested and titrated by infection of cells. B) The organization of the wt SFV genome is shown at the top. As the helper RNA lacks the sequence needed for its packaging into NCs, only recombinant RNAs should be packaged. This helper variant was also constructed with a mutation in the gene encoding the viral spike protein such that its product cannot undergo normal proteolytic processing to activate viral entry functions (Berglund *et al.*, 1993).

induced by two of the three challenge strains, and partial protection was provided against the highly virulent strain, whereas the other vaccines tested gave lower levels of partial protection (Fleeton *et al.*, 2000). This protection was afforded by vaccination by both the intraperitoneal and intramuscular routes. The data for Louping Ill was reinforced in a further study whereby two i.m. naked RNA rSFV-PrME immunisations afforded protection against virus challenge in 70% of mice.

The SFV replicon system in fact combines a wide choice of animal cell hosts (Liljestrom, 1991b). Immunisation of 1-day-old specific pathogen free (spf) chicks with rSFV particles encoding the protective VP2 protein or the VP2:VP4:VP3 polyprotein of infectious bursal disease virus (IBDV) proteins resulted in specific antibodies being elicited in all birds, neutralising antibodies being induced in some but not all birds (Phenix *et al.*, 2001). The first report of SFV particles used in a transgenic mouse model were in the case of a hepatitis C virus (HCV) NS3 based rSFV candidate vaccine which induced similar immune responses to an NS3 DNA-based vaccine (Brinster *et al.*, 2002). This contrasted with reports for other viral SFV based vaccines where the SFV particles stimulated superior immune responses than corresponding DNA-base vaccines (Brand *et al.*, 1998; Fleeton *et al.*, 2000). In the cases of two viruses, Murray Valley encephalitis virus and hantaviruses, recombinant alphavirus particles failed to protect animals from a virus challenge, while DNA-based vaccines had a protective effect (Colombage *et al.*, 1998; Kamrud *et al.*, 1999). The SFV replicon system has been used to develop an immunoassay based on MV recombinant H protein to test immunity against measles with high specificity and sensitivity resulting (Bouche *et al.*, 1998). Infectious pancreatic necrosis virus (IPNV) recombinant proteins produced after infection of the salmonid cell line CHSE-214 with such rSFV retained their antigenicity and resulted in the formation of IPNV-like particles, which were similar in size and morphology to IPNV (McKenna *et al.*, 2001). The similarity between the genomes of salmon pancreas disease virus (SPDV) and SFV makes it probable that SPDV can be developed as an alphavirus vector similar to that based on SFV (McKenna *et al.*, 2001). SFV-mediated expression in fish cells is dependent on cell type and temperature and SFV expression of the protective VP2 antigen of infectious pancreatic necrosis virus has been achieved *in vitro* (Phenix *et al.*, 2000). Immunisation of an experimental murine model with rSFV particles encoding a fusion protein of human papillomavirus 16 (HPV) E6 and E7 efficiently induced CTL activity and CTL memory resulting in a potent therapeutic anti-tumour effect (Daemen *et al.*, 2003).

The feasibility of employing the SFV expression system as a CNS vector has been investigated (Jerusalmi, 2003). Following intranasal administration of SFV particles

expressing EGFP, the viral RNA could be detected in the olfactory mucosa only, whereas fluorescence was detected in axons in the olfactory bulb. This demonstrated that only the expressed protein was present and that the i.n. route had potential for non-invasive protein delivery to the CNS. When administered via the i.n. route an IL-10 expressing SFV vector was found to ameliorate disease in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Jerusalmi, 2003). The duration of expression was found to be dependent on the half life of the protein. The nonpathogenic properties of the nonintegrating SFV strain A7(74) have been used to construct a replication-proficient expression vector to noninvasively target the CNS for short term heterologous gene expression (Vaha-Koskela *et al.*, 2003). Mice infected intraperitoneally with the recombinant virus remained completely asymptomatic but showed randomly distributed CNS expression of EGFP as evidenced by immunohistochemistry. The SFV patchy A7(74) infection of the adult CNS is cleared within 10 days. After immunisation in BALB/c mice, the pseudorabies glycoprotein C-specific ELISA antibodies and neutralising antibodies induced were relatively lower than those obtained in mice immunised with a conventional plasmid DNA vaccine (Xiao *et al.*, 2004). However, mice immunised with the suicidal SFV vaccine could confer more efficient protection than the plasmid DNA vaccine when challenged 4 weeks after secondary immunisation. The suicidal SFV vaccine induced stronger lymphocyte proliferative responses and higher levels of IFN-gamma, suggesting the SFV vaccine could induce an enhanced Th1-type immune response (Xiao *et al.*, 2004). Interleukin (IL)-12-defective mice have exhibited severe neuronal necrosis but not SFV demyelination when infected with SFV compared to wt mice and this correlated with higher virus titres in the brain. In IL-4-defective mice the severity of demyelination and neuronal depletion was reduced. This correlated with reduced brain virus titres and enhanced SFV-specific IFN-γ production, indicating that type 1 T cells play a role in the control of A7-SFV replication but not directly in SFV-induced pathology and demyelination in the CNS (Keogh *et al.*, 2002; Keogh *et al.*, 2003a). IL-12 is produced mainly by macrophages and DCs in response to viral components and in the CNS, this cytokine is produced locally by microglia and is regulated by astrocytes (Keogh *et al.*, 2002).

Elucidating the mechanisms of apoptosis induction by SFV may lead to the development of SFV vectors as agents to treat cancer by apoptosis induction (Atkins *et al.*, 1999). Recombinant alphavirus RNA replicons may facilitate gene therapy of cancer and demonstrate promise for safe tumour-killing and tumour-specific immune responses (Yamanaka, 2004). The induction of apoptotic cell death of transfected cells *in vivo* is

required for the increased effectiveness of replicase based vaccines (Leitner *et al.*, 2004). Apoptotic death of the virus infected cell involves RNase L and the RNA dependent protein kinase (PKR), which are responsible for degradation of mRNA and inhibition of protein translation respectively (Leitner *et al.*, 2004). Apoptosis could also result from leakage of cytochrome *c* from mitochondria and subsequent activation of caspase 3 (Lee *et al.*, 1994). Replicase-based DNA vaccines have proven to be superior to DNA vaccines in varied pre-clinical murine models of infectious disease and cancer (Leitner *et al.*, 2004). DNA vaccines have not been licensed by the Food and Drug Administration (FDA) due to the fear of viral DNA integrating into the host chromosome, leading to mutation or abnormalities which play an important role in autoimmune disease.

Replicase-based vector uptake and antigen expression correlate with the level of apoptosis induced and mediation of apoptosis appears to be through dsRNA production (Leitner *et al.*, 2004). The mechanism for apoptosis by DNA plasmids is not clear. Infection-induced apoptosis could function to enhance CTL cross-priming of antigen by antigen presenting cells (APCs) in lymphoid organs (Colmenero *et al.*, 2001). Thus this replicase based nucleic acid modulated host cell apoptosis by the delivery of a strong adjuvant signal coupled with a vaccine while avoiding the harmful side effects associated with strong conventional adjuvants (Leitner *et al.*, 2004). This apoptosis effect may contribute to the generation of a specific immune response caused by uptake of antigen-loaded apoptotic bodies by DCs and consequent activation of a specific response through cross-presentation (Karlsson *et al.*, 2003).

Both SFV particles expressing EGFP and Bax induced apoptosis in a rat prostate adenocarcinoma cell line, thus overcoming the Bcl-2 mediated cell death inhibitory effects of the cells. Both types of particles were shown to inhibit tumour growth *in vivo*, when injected into solid tumours grown as xenographs in nude mice (Fleeton *et al.*, 2001). Thus expression of the Bax gene by the SFV vector enhanced its cytopathic and anti-tumour potential and in particular counteracted the action of the anti-apoptotic Bcl-2 gene without delay. Thus SFV vectors have potential for cancer therapy, particularly those tumours such as prostate cancer that are resistant to chemotherapy, radiotherapy or therapy by other vectors (Fleeton *et al.*, 2001). A replicase-based DNA vaccine encoding a non-mutated self-antigen has been examined to see whether it could be used to break tolerance and prevent B16 melanoma (Leitner *et al.*, 2003). This novel, naked DNA vaccine encoding an alphavirus replicon (self replicating mRNA) and the self/tumour antigen tyrosinase-related protein-1 can break tolerance and provide immunity to melanoma in C57BL/6 mice (Leitner *et al.*, 2003). Pre-immunisation with DCs pulsed with the same type of cDNA as

in the tumour by a self-replicating SFV RNA vector has been shown to protect mice from a malignant glioma tumour challenge, and therapeutic immunisation prolonged the survival of mice with established tumours (Yamanaka *et al.*, 2001b).

The SFV induced apoptosis in DCs and their death facilitated the uptake of apoptotic cells by other DCs, thus providing a potential mechanism for enhanced immunogenicity (Yamanaka *et al.*, 2001b). Apoptotic bodies formed from primary target cells are eventually endocytosed by DCs and it has been shown that human DC efficiently present antigens derived from apoptotic cells and stimulate class l-restricted $CD8^+$ CTLs (Girard *et al.*, 1999). DCs also have the capacity to translate genetic material from phagocytosed apoptotic bodies, which potentially stimulates antigen presentation further. The use of IL-18 and IL-12 in DC-based immunotherapy for malignant glioma has also proven beneficial in mice (Yamanaka *et al.*, 2002a). DC based immunotherapy with rSFV-cDNA, IL-12 and IL-18, produced significant antitumour effects against the growth of murine glioma cells *in vivo* and induced specific antitumour immunity, coinciding with increased IFN-gamma production. The antitumour effects of this combined therapy are mediated by CD4+ and CD8+ T cells and by natural killer (NK) cells (Yamanaka *et al.*, 2002a). Intratumourally injected DCs that have been transiently transduced with IL-12 do not require pulsing of a source of tumour antigens to induce tumour regression (Yamanaka *et al.*, 2002b). Local delivery of DCs pulsed with IL-18 bound by SFV combined with the systemic administration of IL-12 enhanced the induction of the T helper type 1 response from tumour-specific CD4+ and CD8+ T cells and NK cells as well as antitumour immunity (Yamanaka *et al.*, 2003). Intratumoural (i.t.) injection of SFV particles has resulted in tumour regression and in addition encapsulation of these particles into liposomes has generated highly efficient targeting to tumours. Intratumoural administration of high titre enhanced particles expressing biologically functional IL-12 to treat implanted K-BALB tumours in BALB/c mice resulted in complete tumour regression (Chikkanna-Gowda *et al.*, 2005). Furthermore, inhibition of primary tumour growth and lung metastases of a metastatic (4T1) tumour model indicated the potential of such particles as an efficient antitumour therapeutic agent (Chikkanna-Gowda *et al.*, 2005). Experiments in mice demonstrated that immunisation with the enhanced SFV vector expressing a fusion protein of the HPV16 E6 and E7 oncoproteins resulted in prevention of tumour outgrowth and subsequent protection against tumour re-challenge (Daemen *et al.*, 2002). In further studies with this model, regression and complete elimination of established tumours was achieved. Furthermore, long-term, high level CTL activity of up to 340 days was found to accompany the anti-tumour response and resulted in CTL memory formation (Daemen *et*

al., 2003). Mice vaccinated with rSFV expressing a tumour rejection antigen P815 generated strong CTL responses and were protected against P815 mastocytoma challenge (Colmenero *et al.*, 1999). In fact both antigen-specific (P815A) and cytokine (IL-12) mediated immunotherapy with rSFV vectors inhibit P815 tumour progression (Colmenero *et al.*, 2002).

Antiangiogenic therapy for a malignant brain tumour using SFV carrying the Endostatin gene was investigated to improve therapeutic efficacy and results indicated that this treatment inhibited the angiogenesis with established tumours in mice (Yamanaka *et al.*, 2001a). Significant inhibition of angiogenesis, tumour growth and metastatic spread in mice was demonstrated with rSFV particles expressing murine vascular endothelial growth factor receptor-2 (VEGFR-2). This demonstrates that immunisation with such rSFV particles can break immune tolerance to this self antigen (Lyons *et al.*, 2007). SFV vectors have also been used for production of retrovirus-like particles (Lundstrom, 2003). Thus compared with common gene vaccines, the SFV based recombinant genetic vaccine has the best immunological effect, providing new strategies for clinical genetic therapy of tumours (Ni *et al.*, 2004). Mutations in ras genes are involved in approximately 30% of all human malignancies. Ras proteins are involved in cellular differentiation, proliferation and survival by acting as binary switches at nodes in signal transduction pathways which become constitutively active by point mutations (Smyth *et al.*, 2005). Kras is the most common and is overexpressed in a murine tumour cell line, Kbalb, inducing fast growing syngeneic tumours in BALB/c mice. rSFV-p62-6k was shown to inhibit tumour growth and this anti-tumour response was enhanced by prior immunisation of mice with rSFV-p62-6k VLPs (Smyth *et al.*, 2005).

Functional assays on recombinant proteins are possible until approximately 24 hours post-infection and thus a multitude of nuclear, cytoplasmic, membrane-associated and secreted proteins have been expressed from SFV vectors in a variety of different cell lines and primary cell cultures (Lundstrom, 1999). Site-directed mutagenesis of the SFV SG 26S promoter has down-regulated the heterologous gene expression and noncytopathogenic and temperature–sensitive mutants have been developed by point mutations in the viral nonstructural nsP2 and nsP4 genes (Lundstrom *et al.*, 2001). These vectors do not show the typical shut down of host cell protein synthesis after SFV infections allowing for a substantially prolonged survival of host cells and the study of proteins at more physiological expression levels (Lundstrom *et al.*, 2001). The interaction of receptor and G protein subunits in neurons has been particularly studied due to the 80-90% SFV infectivity in these cells (Lundstrom, 1999). SFV vectors are attractive for

neurobiological studies as they exhibit a strong preference of expression in neuronal cells in primary cell cultures, in organotypic hippocampal slices and *in vivo* (Lundstrom, 2002b). Expression of the G protein-coupled receptors, Human neurokinin-2 and dopamine D3, has been successfully studied in several cell lines using the SFV expression system (Lundstrom *et al.*, 1995). Two types of ligand-gated ion channels, mouse serotonin 5-HT3 receptor and rat and human purinoreceptor P2x subtypes, have also been effectively expressed with the SFV expression system in various cell lines (Lundstrom *et al.*, 1997).

1.5 The anti-viral immune response

Type 1 (α/β) Interferon (IFN) is induced after alphavirus infection of experimental animals and most probably humans (Griffin, 2001). The formation of dsRNA, which is not found in mammalian cells, appears to be the necessary step in replication for IFN induction (Janeway *et al.*, 2005). IFNs induce a state of resistance to viral replication in all cells and secondly induce increased synthesis of MHC class 1 expression in virus infected cells making them more susceptible to killing by CD8 CTLs. Thirdly; they activate NK cells which kill infected cells specifically. The primary source of IFN after peripheral inoculation may be macrophages or DCs in lymphatic tissue. *In vitro* production of IFN follows the initial release of virus from infected cells by 2 to 3 hours. Double stranded RNA is also recognised as a distinct pattern by TLR-3. Type I interferon (IFN-α and IFN-β) is secreted by virus-infected cells whereas immune, type II, or γ-interferon (IFN-γ) is secreted by thymus-derived (T) cells under certain conditions of activation and by NK cells (Boehm *et al.*, 1997). Over 200 genes are now known to be regulated by IFN-γ (Boehm *et al.*, 1997). It is now generally accepted that activation of naive T cells requires antigen presentation by professional APCs, in particular DCs (Huckriede *et al.*, 2004). The mechanism of antigen processing and presentation involved in immunisation with SFV vectors has been elucidated (Huckriede *et al.*, 2004) (Figure 1.9). DCs are not a primary target for the recombinant virus particles. The recombinant virus particles could primarily transfect other cell populations which could serve as a source of apoptotic bodies containing substantial amounts of the expressed antigen when they undergo apoptosis (Huckriede *et al.*, 2004). DCs have been shown to take up apoptotic bodies and efficiently present the enclosed antigens on MHC class I molecules in a process of so-called cross-priming. DCs present the relevant antigenic peptides on their surface in the context of MHC class I molecules and once activated provide co-stimulatory activity by surface molecules like CD40, CD80, CD86 (Huckriede *et al.*, 2004).

NK cells are large granular lymphocytes which make up about 10% of the lymphocyte population and are found mainly in peripheral blood (Gardiner, 1999). NK cells can most likely defend an organism against viral invasion using several different strategies to discriminate between normal and aberrant cells. One function of NK cells, according to the 'missing self' hypothesis, is to recognise and eliminate cells that fail to express self major histocompatibility complex (MHC) class I molecules (Ljunggren *et al.*, 1990). NK cells have a major role to play in innate immunity to viruses. Antibody production by B cells is dependent on help from T cells: Th1 cells for intracellular viruses and bacteria and Th2 cells for extracellular and toxin producing bacteria (Mills, 2002). A mechanism that promotes cross priming during viral infections has been proposed. Murine $CD8\alpha^+$ s are activated by double-stranded (ds) RNA present in virally infected cells but absent from uninfected cells. DCs are then activated by phagocytosing infected material and subsequent signalling through the dsRNA receptor, TLR-3. TLR-3 may have evolved to permit cross priming of CTLs against virus that does not infect DCs. SFV has only limited ability to infect $CD8\alpha^+$ DCs *in vitro* and does not induce $CD8\alpha^+$ DC activation when added as free viral particles. Thus viruses that fail to activate DCs when present as free viral particles can stimulate DCs when present as virally infected cells (Schulz *et al.*, 2005). Human TLR-7 and TLR-8 recognise ssRNA and can directly and indirectly (through lysosomal signalling) activate NK cells which produce IFN-γ (Hart *et al.*, 2005).

Upregulation of expression of class I and class II MHC molecules on the cell surface was one of the first biological effects of the interferons noted. The class II pathway is constitutively active only in professional antigen presenting cells (that is, DCs and their immediate precursors and B cells) and is regulated by a single IFN-γ inducible transcription factor CIITA (class II transactivator) (Boehm *et al.*, 1997). The class II antigen-presentation pathway relies on the presence of peptides in the MIIC lysosomal/endosomal compartment. The antiviral actions of IFN-γ have been ascribed mainly to the transcriptional induction of three genes, specifically double-stranded RNA activated protein kinase (PKR), 20-50 oligoadenylate synthetase (2–5A synthetase) and dsRNA specific adenosine deaminase (dsRAD) (Boehm *et al.*, 1997). It activates macrophages, Th1 cells and NK cells and upregulates MHC class II molecules. IFN-γ also induces nitric oxide (NO) synthase in macrophages, which generates large amounts of NO, a gaseous radical synthesised from L-arginine which has been shown to inhibit viral replication in the early stages of infection (Keogh *et al.*, 2003a). NO can furthermore suppress Th1 cell development via the suppression of IL-2 gene expression.

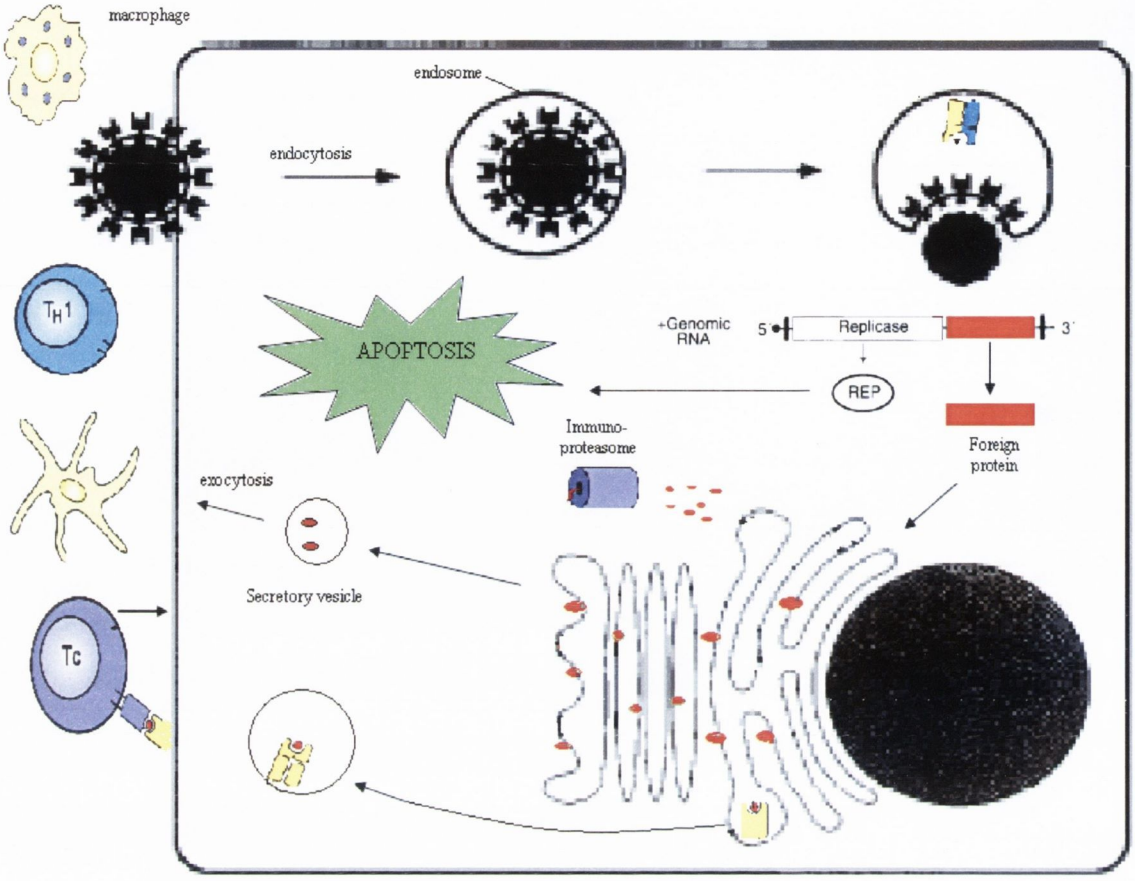


Figure 1.9 Events following rSFV entry into a host cell

The virus infects the cell by receptor-mediated endocytosis. The transmembrane spike glycoprotein of the virus catalyses fusion between the viral and endosomal membranes when the pH in the endosome drops, delivering the NC into the cytoplasm. The genomic RNA is freed from the NC following its disruption and functions as an mRNA (positive-strand RNA) that translates into the viral replicase. The replicase produces new full-length genomic RNAs (via a negative-strand mirror template) as well as a SG RNA species that encodes the recombinant proteins. The CP enhancer cleaves itself from the nascent protein which is translocated into the ER. Peptides that bind to MHC class II molecules are generated in acidified endocytic vesicles. The foreign protein is returned to the cytosol by retrograde translocation and degraded by the proteasome. The resulting peptides are transported back into the lumen of the ER via TAP and loaded onto MHC I molecules. CTLs, DCs, NKs and macrophages are all involved in generating an immune response to the foreign protein.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Cell lines and virus stocks

The Baby Hamster Kidney 21 (BHK-21) cell line and the RA27/3 RV vaccine strain were purchased from the American Type Culture Collection (ATCC) (Maryland, U.S.A). The BHK cell line, sBHK, was a gift from Prof. P. Liljestrom, (Microbiology and Tumourbiology Centre, Karolinska Institute, Stockholm, Sweden) and the Green African Monkey Kidney (Vero) cell line was a gift from Prof. L. Cosby (Department of Medicine, Queens University, Belfast, Northern Ireland). Ervevax vaccine (monovalent RA27/3 RV vaccine) was a gift from GlaxoSmithKline. Glasgow BHK medium with L-Glutamine, RPMI-1640 medium, Dulbecco's Minimum Essential medium with HEPES (DMEM)-HEPES, Hank's Balanced Salt Solution (HBSS), newborn calf serum (NCS), foetal bovine serum (FBS), tryptose phosphate broth, HEPES, trypsin-EDTA, 100 mM sodium pyruvate and non-essential a.a.s were from Gibco BRL (UK). Bovine Albumin Fraction V Solution (7.5%), Penecillin/Streptomycin (5000 units/ml, 5000 μg/ml) solution, 200 mM L-Glutamine, Penecillin/Streptomycin (10,000 units/ml, 10 mg/ml), 2-Mercaptoethanol were also purchased from Gibco-Life Technologies. The Dulbecco's phosphate buffered saline (Ca-, Mg-) was from Invitrogen (UK) and the 75 cm^2 and 150 cm^2 cell culture flasks were from Iwaki, Japan.

2.1.2 Expression Vectors

The SFV Expression vectors, pSFV10-Enhanced (pSFV10Enh) (Figure 2.1), pSFV-helper-S2 and pSFV-CS219A vectors (Figure 2.2) were a gift from Prof. P. Liljestrom (Microbiology and Tumourbiology centre, Karolinska Institute, Stockholm, Sweden). The Litmus28 plasmid (Figure 2.1) was purchased from New England Biolabs (NEB) (Massachusetts, U.S.A.). The pSFV10H and pSFV10EnhHN plasmids were constructed by Dr Barbara Kelly and Dr Marina Fleeton.

2.1.3 Infectious RA27/3 cDNA clone

The pucRA, RA27/3 infectious cDNA, clone was a gift from Dr. T. K. Frey (Department of Biology, Georgia State University, Atlanta, Georgia, U.S.A.).

2.1.4 Molecular Biology Reagents

The Wizard SV Gel and PCR Clean-Up System, Recombinant RNasin Ribonuclease Inhibitor, deoxynucleoside triphosphates (dNTPs) mixture, $MgCl_2$, nuclease-free water and 10X loading dye were obtained from Promega (Wisconsin, U.S.A.). The high (5X) concentration T4 DNA Ligase and molecular weight DNA markers were from New England Biolabs (NEB) (Massachusetts, U.S.A.). First strand cDNA synthesis from the RNA was carried out using the Superscript III First-Strand Synthesis System which was purchased from Invitrogen Life Technologies. The Triplemaster PCR System was purchased from Eppendorf AG (Hamburg, Germany). XL10-Gold Ultracompetent cells (Stratagene), DH5α (NEB) and *E. coli* K12 ER2925 (NEB) were used in the transformation reactions. The SP6 RNA POLYMERASE, Cloned $FPLC_{pure}$ and the mRNA cap analog, $m^7G(5')ppp(5')G$, Sodium for the Invitrotranscription reaction were obtained from Amersham Biosciences (Uppsala, Sweden). 100 mM DTT was from Promega. The RNA Isolator, TRI Reagent, was obtained from Molecular Research Centre. Nucleotide extraction kits, mini-prep and midi-prep plasmid purification kits and gene amplification primers were purchased from Qiagen Ltd. (West Sussex, UK) as were the primers (Table 2.1). The restriction enzymes (and corresponding buffers) *BamH*I, *Bcl*I, *Dra*III, *EcoR*V, *EcoR*I, *Hind*III, *Nru*I, *Pst*I, *Sgf*I, *Spe*I were purchased from NEB.

2.1.5 Equipment

For centrifugation during the Virus-like particle (VLP) production, a Sorvall RC 5C plus centrifuge with a SS-28 rotor and a Beckman L8-M were used. A SW40Ti rotor including swing buckets and ultracentrifuge tubes were from Beckman Coulter Instruments Inc. (CA, USA). Bacterial cultures were centrifuged in a refrigerated tabletop centrifuge from IEC Micromax. Electroporation cuvettes (4 mm) were purchased from Apollo (USA). Fluorescence microscopy was carried out with a Nikon Eclipse E400 Epifluorescence microscope. The Nikon filters used for fluorescence detection were the GFP filter at 460-500 nm, the DAPI filter at 340-380 nm and the G2A filter at 510-560 nm. ELISA plates were rinsed on a MultiWash II washer from Tri Continent, and absorbances read using a Multiskan RC reader from Thermo Labsystems. Pipettes and micropipettes were from Gilson. Further equipment that was used includes: GeneQuant DNA/RNA spectrophotometer (Thermo Labsystems), PCR machine (Hybaid), water bath (Memmert), BioRad Gel Doc 2000 and accompanying Multi-Analyst (version 1.1) software, heating

block (Grants instruments Ltd., Cambridge, England), BioRad Gene pulser Xcell, haemocytometer (Neubauer-Blaubrand, Germany), Vortex-2-Gene (Scientific Industries), Chemical fumehood (Chemical Systems Control Ltd. Ireland), multispeed refrigerated centrifuge (Medical Supply Ireland), a pH meter (Denver Instruments, USA), a MICROFLOW Biological Safety cabinet (AGB Scientific, UK).

Table 2.1 RA27/3 E1 and E2 PCR and sequencing primers

Primer Pair	Sense	Position	Sequence (5'-3')	PCR/Nested PCR (NPCR) Sequencing (S)	Product Size-bp
E2ExtF	+	7292-7311	ACCACCGAGCGCATTGAGAC	PCR	1150
E2ExtR	-	8423-8442	AAGCCCTCGCACGAGACATC	PCR	
E2IntF	+	7343-7351	TCGAGC**AAGCTT**TTCGGTGCC	NPCR	914
E2IntR	-	8221-8242	GTTGTACCC**CTGCAG**GACGACC	NPCR	
AP1			GAACTGGTCGTCCTTAC	S	
Ap2			GTAAGGACGACCAGTTC	S	
L28AP1			ATTTTTGTGATGCTCGTC	S	
E2AP2			CAGCATCACCGAAACGC	S	
L28AP3			AAGTTGGGTAACGCCAG	S	
E2AP4			GTCGTAGTGGCTTTGCG	S	
E1ExtF	+	8148-8167	TCCCGTGGGTCCTGATATTC	PCR	1589
E1ExtR	-	9718-9737	TAGTGGGCTAGTGCGGGTTT	PCR	
E1IntF	+	8223-1936	TCGTC**CTGCAG**GGGTACAAC	NPCR	1445
E1IntR	-	9683-9703	CAT**ACTAGT**CTAGCGCGGCGC	NPCR	
E1SP1			ATGCAGGTCGTGGGAGTGTTC	S	
E1SP2			TACGTCCAGCACCCTCACAAG	S	
E1SP3			CACTGGCCCACGATGGTC	S	
E1SP4			GTGACCGGGTGCTACCAG	S	
E1SP5			CGTGTGTGTGGTGACGACAC	S	
E1SP6			CACTGGAGCCTGCATTTG	S	

2.1.6 Mice

Specific pathogen free (spf) mice (Harlan, UK) were maintained in accordance with the principles outlined in S1 17/94 European Communities regulations, 1994, for care and use of laboratory animals. Halothane was obtained from Vet Drugs, Rhône Mérieux, syringes (1.0 ml and 0.5 ml microfine insulin) and needles (18G) were from Becton Dickinson (Le pont de Claix, France). 6-8 week-old BALB/c, 3 week-old outbred Swiss, 6-8 week-old C57BL/6 and 6-8 week-old A/J mice were obtained from Harlan, UK. Mice were kept in a barrier system with 12 h per day of artificial light and food and water *ad libitum*.

2.1.7 Antibodies

Normal goat, rabbit and rat serum were purchased from Sigma Aldrich (U.S.A.). Rubella E1 Monoclonal Antibody (MAb 1-6) was purchased from Viral Antigens Inc. (Tennessee, U.S.A.). The Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP antibody, for the RV ELISA and the polyclonal rabbit anti-mouse Immunoglobulins/FITC were obtained from DakoCytomation (Denmark). Purified Rat biotin conjugated Anti-Mouse IgG_1 Monoclonal antibody, Purified Rat biotin conjugated Anti-Mouse IgG_{2a} Monoclonal antibody, Streptavidin-Horseradish Peroxidase (SAv-HRP) conjugate were from BD Biosciences Pharmingen (U.S.A.).

2.1.8 Histological and Pathological studies

Haematoxylin Harris, eosin aqueous solution, dichromate eosin and DPX solutions were from BDH Ltd. UK.

2.1.9 T cell proliferation

Cell strainers (40 μm and 70 μm) were purchased from Falcon and the Red Blood Cell (RBC) lysis buffer was purchased from eBioscience (California, U.S.A.). OptEIA™ Set Mouse IFN-γ and OptEIA™ Set Mouse IL-5 cytokine ELISA kits were from BD BioSciences. Recombinant E2 protein (a.a.s 31-105) and E1 mosaic protein (a.a.s 157-176, 374-390, 213-239) were from BioSpacific, USA.

2.1.10 Antibody Assays

ELISA plates coated with antigen from the HPVV strain of RV were purchased from Viral Antigens, Inc. The OptEIA mouse IFN-γ ELISA set was obtained from BD Biosciences Pharmingen (U.S.A.). The ELISA substrate was 3,3',5'5-Tetramethylbenzidine (TMB) from Sigma (U.S.A.). The Rubagen latex agglutination assay kit was obtained from BIOKIT (Barcelona, Spain).

2.1.11 Miscellaneous

Sucrose was from BDH Ltd (Poole, UK). Trypan blue, Tween 20, Tween 80 and ampicillin were from Sigma (USA). Phenol: chloroform and agarose were from Promega. TNE buffer: 50 mM Tris, 0.1 M NaCl (Sigma Aldrich) and 1 mM EDTA (Promega) were dissolved in 800 ml distilled water, pH adjusted to 7.4 and volume to 1 litre. Phosphate buffered-saline (PBS) and 7.5% BSA were from Gibco, $MgCl_2$ and glycerol were from BDH, UK. The 5-bromo-4-chloro-3-indoyly-β-D-galactopyranoside (X-gal) was from Biosynth AG (Switzerland). Isopropyl-beta-D-thiogalactopyranoside (IPTG) was from Sigma. The suppliers of other materials were as follows: Ethidium bromide (BioRad, USA), eppendorf tubes (Axygen, USA), TBE (Promega, USA) agarose (Roche, Germany), blue/orange-loading dye (Promega, USA), cryotubes, maxi 96 well dishes and TC microwell 96 well plates (Nunc, Denmark), SW-28 ultra centrifuge-tubes (Ultra-clear, Beckman), Tris, chloroform and isopropanol (BDH laboratory, UK), ethanol (Merck, Germany), Vectachield DAPI Hardset mountant (Vector laboratories, USA), Luria-Bertani broth (Oxoid, UK), flat-bottomed 96 wells ELISA plate (NUNC, Denmark), 70μm cell strainer (Falcon), dimethylsulfoxide (DMSO) (Sigma-Aldrich). The Edmonston measles and Enders mumps coated ELISA plates were from Virion-Serion (Germany).

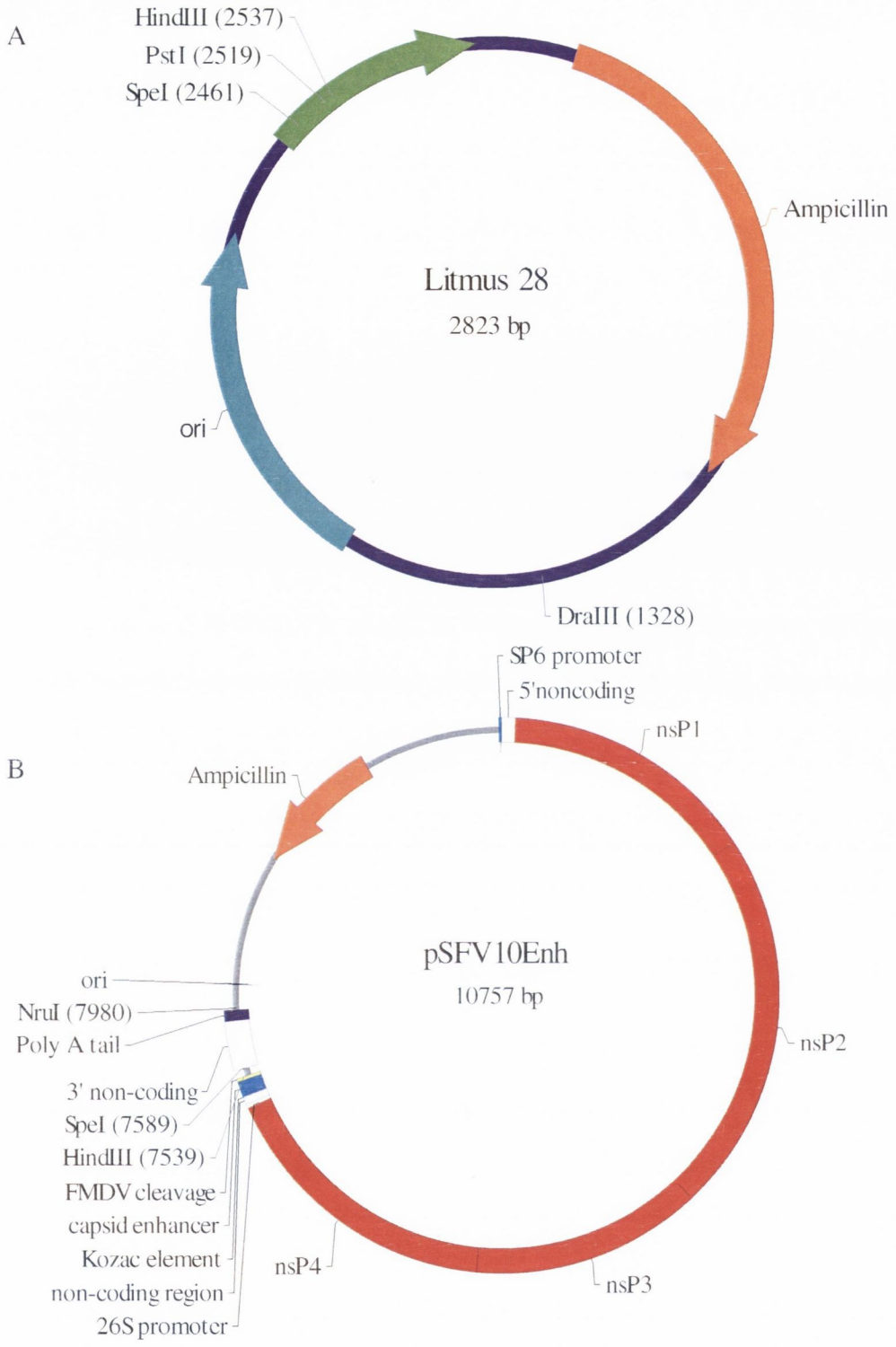


Figure 2.1 L28 and pSFV expression vectors for directional cloning of E2 and E1

Map of the Litmus 28 plasmid (A) and pSFV10Enh (B) expression vectors featuring sites used in the MCS for directional cloning of the E2 and E1 genes as well as the *Nru*I linearisation site and the regions that improve expression from the SFV vector.

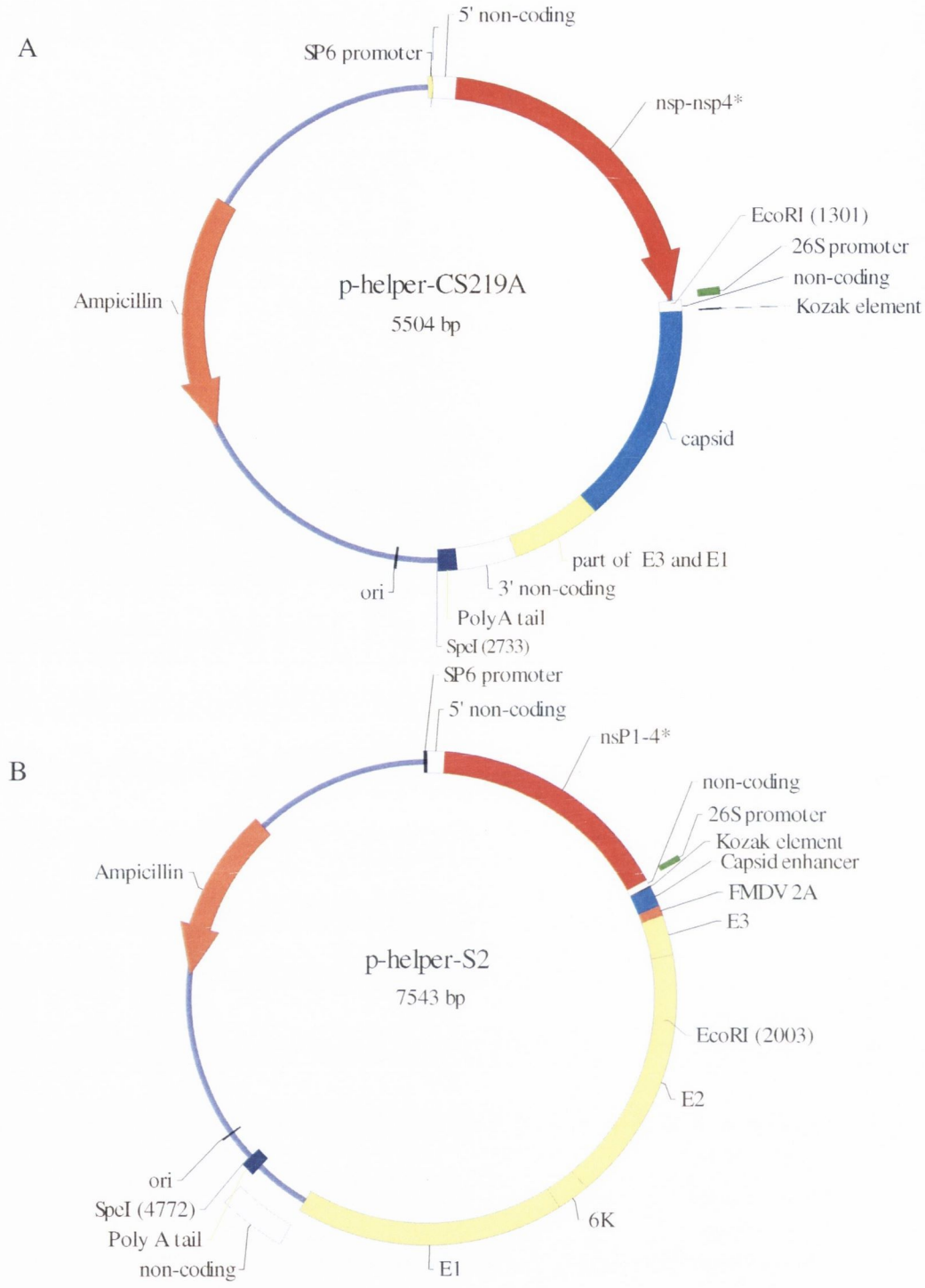


Figure 2.2 SFV helper vectors

The helper capsid (A) and helper spike (B) plasmids were used in the production of rSFVEnh-E2E1 particles following linearisation at the *Spe*I site and invitrotranscription with Sp6 Polymerase.

2.2 METHODS

2.2.1 Cell culture

African Green Monkey Kidney (Vero) cells (QUB) were propagated in DMEM-Hepes supplemented with 5% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-Glutamine and maintained at 37 °C or 35 °C in a humidified atmosphere of 5% CO_2 in 75 cm^2 or 150 cm^2 cell culture flasks. Baby Hamster Kidney (BHK-21) cells and sBHK cells were propagated in BHK medium supplemented with 5% (v/v) FBS, 5% (v/v) tryptose phosphate broth, 2% (v/v) 1M HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine. Splenocytes were grown in RPMI-1640 containing 10% (v/v) FBS, 10 mM HEPES, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 10 mM sodium pyruvate, 0.8% non-essential a.a.s, 50 µM β-mercaptoethanol and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in 96 well round bottom plates. Cell stocks were prepared by slow freezing (at the rate of -1°C/ min) to -70 °C in medium containing 10 % DMSO and subsequently stored in liquid nitrogen (-235 °C). Confluent monolayers of BHK-21, sBHK and Vero cells were detached using Trypsin-EDTA. These confluent monolayers were washed with Dulbecco's phosphate buffered saline without calcium, magnesium and sodium bicarbonate (PBS-) and incubated with 0.05% Trypsin, 5.3 mM EDTA at 37 °C until cell detachment occurred. To terminate trypsinisation, specific culture medium was added to the flask. The BHK-21 cells were subcultured routinely at a ratio of 1:3, the sBHK at a ratio of 1:4 and the Vero cells at a ratio of 1:6 depending on confluency. Cell lines were passaged a maximum of 12 times.

2.2.2 Virus growth

The ATCC RA27/3 virus was grown in Vero cells and RNA was isolated when a CPE was evident by rounding of cells and the appearance of apoptotic bodies (5 days post infection).

2.2.3 Cloning of rSFV10Enh-E2E1

For cloning the RA27/3 envelope genes E2 and E1 into the SFV10Enh vector a strategy was designed using the Litmus 28 plasmid and the unique restriction sites in the MCS of both vectors (Figure 2.1).

2.2.3.1 Total RNA extraction from ATCC RA27/3 infected Vero cells

RNA was isolated from an RA27/3 infected monolayer of Vero cells using 1 ml of TRI Reagent per T75 cm^2 flask. The monolayer was homogenised using a cell scraper. The homogenised sample was removed to a microcentrifuge tube and incubated at room temperature for 5 min to permit the complete dissociation of the nucleoprotein complexes. Phase separation was achieved by the addition of 200 µl of chloroform per 1 ml of TRI Reagent. The samples were covered tightly and shaken gently but thoroughly for 15 sec, to allow complete emulsification, and incubated at room temperature for 15 min. The resulting mixture was centrifuged at 14,000 revolutions per minute (rpm) for 15 min at 4 °C. Following centrifugation, the mixture separated into a lower red, phenol chloroform phase, an interphase and the colourless upper aqueous phase.

The aqueous phase was transferred to a fresh tube and the RNA was precipitated by mixing with 500 µl of isopropanol. The samples were stored at room temperature for 10 min and then centrifuged at 14,000 rpm for 10 min at 4 °C. The RNA precipitate formed a gel like pellet on the side and bottom of the tube and the supernatant was discarded. The pellet was washed once with 1 ml of 75% ethanol by gentle inversion of the tube and centrifugation at 14,000 rpm for 10 min at 4 °C. As much of the 75% ethanol as possible was removed and the pellet was air dried for 20 min. To dissolve the pellet, 100 µl of preheated nuclease free water was used and the sample incubated at 60 °C for 10 min. Aliquots of the RNA were stored at -70 °C. Negative control RNA was isolated from concurrently uninfected Vero cells. The RNA concentration was measured on a DNA/RNA spectrophotometer and 1 µl of the extracted RNA was analysed on a 0.6% (*wt/v*) agarose gel with a 1 kb molecular weight marker.

2.2.3.2 Isolation of RA27/3 E2 and E1 genes by RT-PCR

The moloney murine leukaemia virus (MMLV) based reverse transcriptase kit, Superscript III was used to synthesise cDNA as per the manufacturer's instructions. Prior to the RT, the RNA was heated to 95 °C for 1 min and then kept on ice to prevent reformation of RNA secondary structure. RT reactions were performed in 50 µl reaction volumes in 0.5 ml microcentrifuge tubes containing 10µl total RNA and the reaction components at a final concentration of 0.04 µM gene specific primer (E1 reverse primer), 0.5 mM dNTP mix (containing 0.5 mM each of dATP, dGTP, dCTP and dTTP), 1X RT buffer, 5 mM $MgCl_2$, 0.01 M dithiotreitol (DTT), 0.8 U RNaseOUT, 4 U Superscript III RT

and diethylpyrocarbonate (DEPC) treated water. The RNA, primer and dNTP mixture was incubated at 65 °C for 5 min before the addition of the rest of the components. The RT reaction was incubated at 55 °C for 50 min and 85 °C for 5 min. RNase H (0.04U) was added after first strand synthesis and the reaction was further incubated at 37 °C for 20 min to remove any RNA bound to cDNA in RNA-DNA hybrids. The controls for the RT procedure included samples with either no RNA or no RT added. The cDNA was analysed by agarose gel electrophoresis and used immediately in PCR reactions or stored at -20 °C until required. One µl of the cDNA along with the negative controls was analysed on a 0.8% TBE agarose gel stained with ethidium bromide. A 30 ml 0.8% TBE agarose gel containing 3 µl 10 mg/ml ethidium bromide was prepared and 1 µl of each cDNA sample was mixed with 9 µl of TBE buffer and 2 µl 6X blue/orange loading dye. Samples were loaded in 12 µl amounts and run at 80 mA with 10 µl 1 kb molecular weight marker (5 µl 1 kb marker, 17 µl loading dye and 78 µl TBE). Gels were run for approximately 60 min and then visualized using a BioRad Gel Doc 2000 and corresponding Multi-Analyst (version 1.1) software.

2.2.3.3. PCR amplification of RA27/3 cDNA

A nested PCR using the proofreading TripleMaster Enzyme System was utilised as per the manufacturer's instructions. The 5X Taqmaster, PCR enhancer, was used to increase the yield of the E2 gene PCR reaction. The 50 µl PCR reaction mix was comprised of 1X Tuning Buffer, 2.5 mM Mg^{2+}, 0.2 µM primers and 13 µl (50ng) DNA, 1X Taqmaster Enhancer, 500 µM dNTP mix and 0.04 U/µl Triplemaster Polymerase Mix. A 2 µl aliquot from the first round PCR was used in the nested PCR. Samples were incubated at 98 °C for 5 min, followed by 40 cycles of 98 °C for 10 s, 57 or 59 °C for 10 s (depending on primer pair annealing temperature), 72 °C for 3 min and a final extension of 72 °C for 2 min. The PCR products were analysed by agarose gel electrophoresis as described in section 2.2.3.2

2.2.3.4 Analysis of the amplified RA27/3 E2 & E1 genes

The products of separate RT-PCR reactions were purified using the Wizard SV Gel and PCR Clean-Up System. An equal volume of Membrane Binding Solution was added to each PCR reaction. The prepared PCR product was transferred to an individual SV Minicolumn assembly and incubated for 1 min at room temperature. The SV Minicolumn

assembly was centrifuged (10,000 g, 1 min). The liquid in the collection tube was discarded and the column was washed by adding 700 µl of membrane wash solution, previously diluted with 95% ethanol and centrifuged (10,000 g, 1 min). The collection tube was emptied and 500 µl of Membrane Wash Solution added followed by centrifugation (10,000 g, 5 min). The collection tube was emptied and the column assembly recentrifuged for 1 min. The SV Minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease free water applied directly to the centre of the column. The column was incubated at room temperature for 1 min and centrifuged (10,000 g, 1 min). The concentration of the pooled, purified PCR product was estimated on an agarose gel by comparing 1 µl of sample DNA with 0.5 µg of a 1 kb molecular weight marker as per section 2.2.3.2.

The purified PCR amplified products of both the E2 and E1 genes were analysed by unique restriction digestion and observed for their characteristic digested band size on an agarose gel as described in section 2.2.3.2. The *Dra*III enzyme was used for the analysis of the E2 gene whereas *Nhe*I and *BamH*I were used for the analysis of the E2 gene (Figure 2.3). To test for the presence of the E2 gene insert by restriction digestion, 1 µl of DNA was digested for 2 h at 37 °C with *Dra*III enzyme in the presence of 1X BSA and 1X NEBuffer 3 in a total volume of 20 µl. The digested products for each DNA sample were analysed on a 0.8% agarose gel by comparison with a 1 kb DNA ladder. To test for the presence of the E1 gene by restriction digestion, 1 µl of DNA was digested for 1 h at 37 °C with *BamH*I enzyme in the presence of 1X BSA and 1X NEBUnique buffer or *Nhe*I in the presence of 1X BSA and 1X NEBuffer 2, both reactions in a total volume of 20 µl. The digested products for each sample were analysed on a 0.8% agarose gel by comparison with a 1kb DNA ladder. After confirming the specific E2 and E1 genes by restriction digestion, each 49 µl of purified PCR amplified DNA was mixed with 8 µl of loading dye, run on a 0.8% (w/v) agarose gel and placed on a UV transilluminator. The bands of the correct size DNA fragments of E2 and E1 in the gel were excised using a clean sharp blade and purified using the Wizard® SV Gel and PCR Clean-Up System. The excised gel bands were weighed and mixed with Membrane Binding Solution at a ratio of 10 µl of solution per 10 mg of agarose gel slice. The mixture was vortexed and incubated at 50-65 °C for 10 min or until the gel slice dissolved completely (the tubes were vortexed every few min). Once the agarose gel was completely dissolved, the gel mixture was transferred to an SV Minicolumn assembly and following a 1 min incubation at room temperature, centrifuged at 10,000 x g for 1 min. The liquid was discarded from the collection tube, the column washed by the addition of 700 µl of the Membrane Wash solution, previously diluted with

781 accaccgagc gcattgagac ccgctcggcg cgccatcctt cgagcatccg cttcggtgcc

841 ccccaggcct tccttgccgg gctcttgctc gccgcggtcg ccgttggcac cgcgcgcgcc

901 gggctccagc cccgcgttga tatggcggca ccccctatgc cgccgcagcc cccccgtgcg

961 cacgggcagc attacggcca ccaccaccat cagctgccgt tcctcgggca cgacggccat

1021 catggcggca ccttgcgcgt cggccagcat caccgaaacg ccagcgacgt gctgcccggc

1081 cactggctcc aaggcggctg gggttgctac aacctgagcg actggcacca gggcactcat

1141 gtctgtcaca ccaagcacat ggacttttgg tgtgtggagc acgaccgacc gccgcccgcg

1201 accccgacgt ctctcaccac cgcggcgaac tccacgaccg ccgccacccc cgccactgcg

1261 ccggcccctc gccacgccgg cctcaatgac agctgcggcg gcttcttgtc tgggtgcggg

1321 ccgatgcgcc tgcgccacgg cgctgacacc cggtgcggtc ggttgatctg cgggctgtct

1381 accaccgccc agtacccgcc tacccggttt ggctatgcta tgcggtgggg ccttcccccc

1441 tgggaactgg tcgtccttac cgcccgcccc gaagacggct ggacttgtcg tggcgtgccc

1501 gcccacccag gcacccgctg ccccgaactg gtgagcccca tgggacgcgc gacttgctcc

1561 ccagcctcgg ccctctggct cgccacagcg aacgcgctgt ctcttgacca cgcgttcgcg

1621 gccttcgtcc tgctggtccc gtgggtcctg atattcatgg tgtgccgccg cgcctgtcgc

1681 cgccgcggcg ccgccgccgc cctcaccgcg gtcgtcctgc aggggtacaa cccccccgcc

EI

1741 tatggcgagg aggctttcac ctacctctgc actgcaccgg ggtgcgccac tcaagcacct

1801 gtccccgtgc gcctcgctgg cgtccgcttt gagtccaaga ttgtggacgg cggctgcttt

1861 gccccatggg acctcgaggc cactggagcc tgcatttgcg agatccccac tgatgtctcg

1921 tgcgagggct tgggggcctg ggtacccaca gcccccttgcg cgcgcatctg gaatggcaca

1981 cagcgcgcgt gcaccttctg ggctgtcaac gcctactcct ctggcgggta cgcgcagctg

2041 gcctcttact tcaaccctgg cggcagctac tacaagcagt accaccctac cgcgtgcgag

2101 gttcaacctg ccttcggaca cagcgacgcg gcctgctggg gcttccccac cgacaccgtg

2161 atgagcgtgt tcgccctcgc tagctacgtc cagcaccctc acaagaccgt ccgggtcaag

2221 tttcatacag agactaggac tgtctggcaa ctctccgttg ctggcgtgtc gtgcaacgtc

2281 accactgaac acccgttctg caacacgccg cacggacaac tcgaggtcca ggtcctgcct

2341 gaccctgggg acctggttga gtacattatg aaccacaccg gcaatcagca gtcccggtgg

2401 ggcctcggga gcccgaattg ccatggcccc gattgggcct ccccggtttg ccaacgccat

2461 tcccctgact gctcgcggct tgtgggggtc acgccagagc gtccccggct gcgcctggtc

2521 gacgccgacg acccccctgct gcgcactgcc cctgggcccg gcgaggtgtg ggtcacgcct

2581 gtcataggct ctcaggcgcg caagtgcgga ctccacatac gcgctggacc gtacggccat

2641 gctaccgtcg aaatgcccga gtggatccac gcccactcta ccagcgaccc ctggcaccca

2701 ccgggccccct tgcggttgaa gttcaagaca gttcgcccgg tggccctgcc acgcgcgtta

2761 gcgccacccc gcaatgtgcg tgtgaccggg tgctaccagt gcggtacccc cgcgctggtg

2821 gaaggccttg ccccgggggg agggaattgc catctcaccg tcaatggcga ggatgtcggc

2881 gccttccccc ctgggaagtt cgtcaccgcc gccctcctca acactccccc gccctaccaa

2941 gtcagctgcg ggggcgagag cgatcgcgcg agcgcgcggg tcattgaccc cgccgcgcaa

3001 tcgtttaccg gcgtggtgta tggcacacac accactgctg tgtcggagac ccggcagacc

3061 tgggcggagt gggctgctgc ccattggtgg cagctcactc tgggcgccat ttgcgccctc

3121 ctacttgctg gcttactcgc ttgctgtgcc aaatgcttgt actacttgcg cggcgctata

3181 gcgccgcgct ag

Figure 2.3 The sequences of the coding regions of the RA27/3 E2 and E1 genes

This sequence includes the E2 signal sequence (69 bases) located at the C-terminal of the C protein (bold). The coding region of E2 is 846 bp long (codes for 282 a.a.s). The forward primer was designed to include the first 9 nucleotides of the E2 signal sequence coding region and a *Hind*III restriction site. The reverse primer for the E2 gene was designed to incorporate 7 nucleotides surrounding the *Pst*I site at 943 bp in blue. The external forward primer for the E1 gene was located on either side of this natural *Pst*I site. The E1 coding region is 1443 bp long and codes for 481 a.a.s. The reverse primer on the E1 gene was designed to incorporate the last 12 nucleotides of the gene and a *Spe*I site. The restriction sites *Dra*III in red, *BamH*I in green and *Nhe*I in orange on the coding sequences of the genes were used for analysis of the PCR amplified E2 and E1 genes. The *Bcl*I site in purple and *Sgf*I site in yellow were used for correction of the E2 T cell epitope by ligation with a fragment from the pucRA RA27/3 infectious clone.

95% ethanol, to the SV Minicolumn and centrifuged as before. The wash was repeated with 500 µl of Membrane Wash Solution and the SV Minicolumn centrifuged for 5 min at 10,000 x g. The collection tube was emptied and the column assembly was recentrifuged for 1 min. The SV Mini column was transferred to a clean 1.7 ml microcentrifuge tube and 25 µl added directly to the centre of the column. Following a 1 min incubation the column was centrifuged for 1 min at 10,000 x g. The concentration of the purified E1 and E2 DNA was estimated on a gel by comparing 1 µl of sample DNA with 0.5 µg of 1 kb DNA ladder as per section 2.2.3.2.

2.2.3.5 Transformation

2.2.3.5.1 Preparation of competent *E. coli* DH5α cells

Cells used for transformation of rSFV10Enh and 'helper' SFV plasmids were *Escherichia coli* (*E. coli*) strain DH5α. An *E. coli* culture was grown up overnight in a 2 ml volume of L-broth at 37 °C. A 400 ml amount of L-broth was inoculated with the 2 ml overnight culture in a 2 Litre baffled flask and incubated at 37 °C until the cells reached the mid-log phase of growth (OD600nm of 0.4 - 0.5). This took between 3 and 4 h. The culture was then chilled on ice for 1 h and decanted into two 250 ml Sorvall bottles and spun in the GSA rotor at 4000 rpm for 10 min at 4 °C. The pellets were gently resuspended

in 2 x 15 ml of ice-cold 100 mM $MgCl_2$ and the cells spun as before. The cells were gently resuspended in 2 x 50 ml of ice cold 100 mM $CaCl_2$ and placed on ice for 1 h. The cells were pelleted as above and resuspended in 2 x 10 ml ice cold 100 mM $CaCl_2$. Ice-cold 80% glycerol was gradually added with swirling to give a 10% final solution (2 x 1.5 ml). The cell-glycerol suspension was aliquoted into 0.1 ml amounts into sterile eppendorfs on ice and snap-frozen in liquid nitrogen before storage at -70 °C.

2.2.3.6 Transformation of *E. coli* DH5α cells

Competent DH5α cells were transformed with pSFV10Enh, Litmus 28 plasmid and the 'helper' plasmids separately, by incubating 200 µl of competent cells with 1 µl of each plasmid DNA for 1 h on ice (mixed every 10 min). The cells were transformed by heat shock at 42 °C for 2 min and placed on ice for a further 10 min. Transformed cells were plated onto ampicillin L-agar plates containing 1mg/ml ampicillin. The plates were incubated upside down overnight at 37 °C and observed for colony growth.

2.2.3.7 Screening of plasmid DNA from the transformed colonies

A number of isolated colonies of plasmids were inoculated in separate 5 ml LB medium aliquots containing ampicillin (100 µg/ml). The cell cultures were incubated for 18 h at 37 °C with shaking at 300 rpm. The starter culture was used to inoculate 150 ml LB broth for high copy plasmid L28 and 250 ml LB broth for low copy plasmid SFV10Enh; and the plasmids, including the low copy 'helper' plasmids, were grown at 37 °C for 18 h with shaking at 300 rpm. The bacterial cells were harvested by centrifugation at 6,000 g for 15 min at 4 °C in a Sorvall. The pellets were pooled and resuspended in 6 ml Buffer P1. To the suspension, 6 ml of P2 Buffer was added and lysed by gentle inversion six times and incubation at room temperature for 5 min. To the lysate, 6 ml of chilled buffer P3 was added and mixed by inversion six times. The lysate was poured into the barrel of the QIAfilter Cartridge and incubated for 10 min at room temperature. The cell lysate was filtered through the HiSpeed Midi Tip which had been equilibrated with 4 ml QBT. The cell lysate was allowed to enter the resin by gravity flow and the Tip was washed with 20 ml Buffer QC.

The DNA was eluted into a 20 ml universal and precipitated by addition of 3.5 ml room-temperature isopropanol, mixing and incubation at RT for 5 min. The eluate/isopropanol was filtered through a QIAprecipitator Midi Module using constant

pressure and discarded. The DNA was washed by filtering 2 ml 70% ethanol through the syringe. The membrane was dried by pressing air through the QIAprecipitator twice and the nozzle dried with absorbent paper to prevent ethanol carryover. The QIAprecipitator was attached to a 5 ml syringe and the DNA was eluted into a 1.7 ml eppendorf collection tube using 500 µl of nuclease free water. This step was repeated with the eluate to ensure that the maximum amount of DNA was solubilised and recovered from the QIAprecipitator. The DNA was analysed by UV Spectrophotometry and by agarose gel electrophoresis as described previously.

2.2.3.8 Preparation of Litmus28 and E2E1 gene inserts for cloning

The L28 plasmid and the E2 PCR product were digested at the unique *Hind*III and *Pst*I restriction sites and cleaned using a Qiaquick nucleotide removal kit protocol. This protocol ensured removal of enzymes, salts and unincorporated nucleotides. The same process was repeated with *Pst*I and *Spe*I digested E1 PCR product and L28 plasmid. Five volumes of Buffer PN were added to 1 volume of the reaction sample and mixed. Therefore 250 µl of Buffer PN was added to a 50 µl reaction sample. To bind the DNA, the sample was applied to a QIAquick spin column in a collection tube and centrifuged for 1 min at 6000 rpm. The flow-through was discarded and the QIAquick column placed back into the same tube. The QIAquick column was washed by the addition of 750 µl of Buffer PE (96-100% ethanol added) and centrifugation for 1 min at 6000 rpm. The flow-through was discarded and the QIAquick column was placed back in the same tube and centrifuged for an additional 1 min at 13,000 rpm to remove residual Buffer PE. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl of H_2O was added to the centre of the QIAquick membrane, let stand at room temperature for 1 min and the column centrifuged for 1 min at 13,000 rpm.

2.2.3.9 DNA ligation

The E2 and E1 genes were ligated separately to the L28 plasmid. The gel extraction Wizard SV Gel and PCR purification kit was used when the E2E1 genes were cut out of the L28-E2E1 vector to purify the genes and separate them from the L28 backbone as in section 2.2.3.4. The ligation reactions contained 50 ng of vector DNA with a three molar excess of insert, 2 µl of 10x ligase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin) and 1 µl (20 U) of 5X

high concentration T4 DNA ligase. The mixture was mixed, incubated at 16 °C overnight and either used immediately in a transformation or stored at -20 °C. A control ligation was carried out using only L28 plasmid or pSFV10Enh vector DNA.

2.2.3.10 Transformation of XL10-Gold Ultracompetent cells

XL10-Gold cells were transformed with ligation preparations of L28E2, L28E1 and L28E2E1 in separate reactions. Briefly, 14 ml BD falcon round bottom polypropylene tubes were pre-chilled on ice. The NZY^+ broth (containing filter-sterilised 1 M MgCl2, 1 M $MgSO_4$ and 20% glucose) was preheated to 42 °C and the ultracompetent cells thawed on ice. When thawed the cells were mixed gently and 100 µl aliquoted into each pre-chilled tube. To each aliquot of cells 4 µl of β-Mercaptoethanol mix was added. The tubes were swirled gently and then incubated on ice for 10 min, swirling gently every 2 min. The entire ligation mix was added to one aliquot of cells. The tubes were swirled gently and then incubated on ice for 30 min. The tubes were heat-pulsed in a 42 °C water bath for 30 seconds and incubated on ice for 2 min. Preheated NZY^+ broth was added and the tubes incubated at 37 °C for 1 h with shaking at 225-250 rpm. The transformation mixture (200 µl) was plated on LB agar plates coated with 100 µl of 10 mM IPTG and 100 µl 2% X-gal for colour screening. The plates were incubated at 37 °C overnight and colonies were subsequently screened.

2.2.3.11 Screening of the plasmid DNA from the transformed colonies

After ligation and transformation, colonies were screened initially using the blue-white colour screening whereby colonies containing plasmids with inserts were white and colonies without inserts were blue. A number of isolated colonies containing recombinant plasmids were used to inoculate 5 ml aliquots of L-broth containing ampicillin (100 µg/ml). The cell cultures were grown for 18 h with shaking at 37 °C. This culture was used to purify the plasmid using the Qiagen Miniprep Plasmid Purification Kit that is based on the modified alkaline lysis protocol.

Cells were harvested by centrifugation (1000 g, 10 min, 4 °C). Each pellet was resuspended in 250 µl of buffer P1 (10 mM EDTA, 100 µg/ml RNase A, 50 mM Tris-HCL, pH 8.0) and then transferred to a 1.7 ml eppendorf tube. Cells were lysed by the addition of 250 µl of buffer P2 (200mM NaOH, 0.8% (*w/v*) SDS) by inverting the tube 5-6 times. Buffer N3 (350 µl) was added and mixed gently prior to centrifugation (10,000 g,

10 min). The supernatant was applied to a QIAprep column and further centrifuged (10,000 g, 10 min). The column was then washed with 500 µl of buffer PB, to remove all trace of nuclease activity. The column was again washed with 750 µl of buffer PE, and centrifuged twice (10,000 g, 1 min). Each plasmid DNA from the different transformed colonies was screened for the presence of insert (E2 and/or E1) by restriction digestion analysis.

To test for the presence of the E2 gene insert by digestion, 1 µl of DNA was digested for 2 h at 37 °C with *Dra*III enzyme in the presence of 1X BSA and 1X NEBuffer 3. The digested products for each DNA sample were analysed on a 0.8% agarose gel by comparison with a 1kb DNA ladder. To test for the presence of the E1 gene by digestion 1 µl of DNA was digested for 1 h at 37 °C with *BamH*I enzyme in the presence of 1X BSA and 1X NEBUnique buffer. The digested products for each DNA sample were analysed on a 0.8% agarose gel by comparison with a 1 kb DNA molecular weight marker.

2.2.3.12 Sequencing of rubella genes in Litmus 28 plasmid

All of the sequencing was carried out by Fusion Antibodies Ltd., Belfast and sequencing primers were designed using the Sigma Genosys DNA calculator. As the E2 and E1 gene combined size is greater than 2 kb, it was necessary to include multiple primers for the efficient amplification of the insert. Oligonucleotide primers for sequencing the insert were designed to amplify the insert along with a small portion of the vector from both ends of the insert. The same primers that were used for cDNA amplification of E2 and E1 were employed for sequencing purposes. Chromas 2.23 files were analysed with Blastn (NCBI database). The nucleotide sequence files were translated to a.a. sequences using the Baylor College of Medicine Sequence Utilities tool. Nucleotide and a.a. sequences were aligned and analysed using the Clustal W multiple sequence alignment software.

2.2.4 Correction of E2 T-cell epitope mutation

2.2.4.1 Preparation of competent *E. coli* ER2925 cells

A few isolated colonies of *E. coli* K12 were inoculated in 2 ml of Luria-Bertani (LB) broth without any antibiotics and grown at 37 °C for 18 h with shaking at 200 rpm. The 2 ml overnight culture was used to inoculate 200 ml of L-broth in a 2 L baffled flask. The cell cultures were grown to mid log phase at 37 °C (OD at 600 nm of 0.4 to 0.5), with

shaking at 200 rpm. The cells were chilled on ice for 1 h. The culture was decanted into four 50 ml falcon tubes and spun at 4000 rpm for 10 min at 4 °C. The pellets were gently resuspended in two 15 ml amounts of ice cold 100 mM $MgCL_2$ and the cell suspension was further centrifuged (4000 rpm, 10 min, 4 °C). The cells were then gently resuspended in two separate 50 ml amounts of ice cold 100 mM $CaCl_2$ and placed on ice for 1 h. The cells were pelleted (4000 rpm, 10 min, 4 °C) and resuspended in two separate 10 ml volumes of ice cold 100 mM $CaCl_2$. Ice cold 80% glycerol (1.5 ml per 10 ml) was gradually added to give a 10% final solution. The cell-glycerol suspension was aliquoted in 100 µl amounts in sterile prechilled microcentrifuge tubes. The tubes were snap-frozen in liquid nitrogen and placed at -70 °C until required.

2.2.4.2 Transformation of *E. coli* ER2925 cells

The pucRA infectious cDNA clone (sequence unpublished) and the L28 plasmid were transformed into *E. coli* K12 ER2925 cells which are Dam^- and Dcm^- and yielded DNA which could be cut with Dam or Dcm- sensitive restriction enzymes.

2.2.4.3 Preparation of L28E2E1, SFV10Enh and pucRA

The RA27/3 E2E1 unique sites *Bcl*I and *AsiS*I (*Sgf*I isoschizomer) were chosen due to their absence in L28. The K12 pucRA sites were double digested with *EcoR*V (4214 bp) and *EcoR*I (linearisation site) to eliminate the other *Bcl*I and *Sgf*I sites (2336 bp and 9476 bp respectively). The resulting fragment was double digested with *Bcl*I and *Sgf*I and this product was transformed into the *Bcl*I and *Sgf*I cut L28E2E1. The corrected E2 and E1 genes were then digested at the *Hind*III and *Spe*I sites and inserted into pSFV10Enh as per section 2.2.3.9 (Figure 2.4).

2.2.4.4 Screening for recombinant pucRA-pSFV10Enh-E2E1 clones from the transformed colonies by restriction digestion

At each step of the cloning process, restriction digestion was used to monitor insertion of fragments by digestion with restriction enzymes unique to the 1359 bp fragment such as *BamH*I. Double digestion was carried out using the *Hind*III and *Spe*I sites and the pSFV10Enh-E2E1 with the pucRA 1359 bp fragment was sequenced as previously described in section 2.2.3.12.

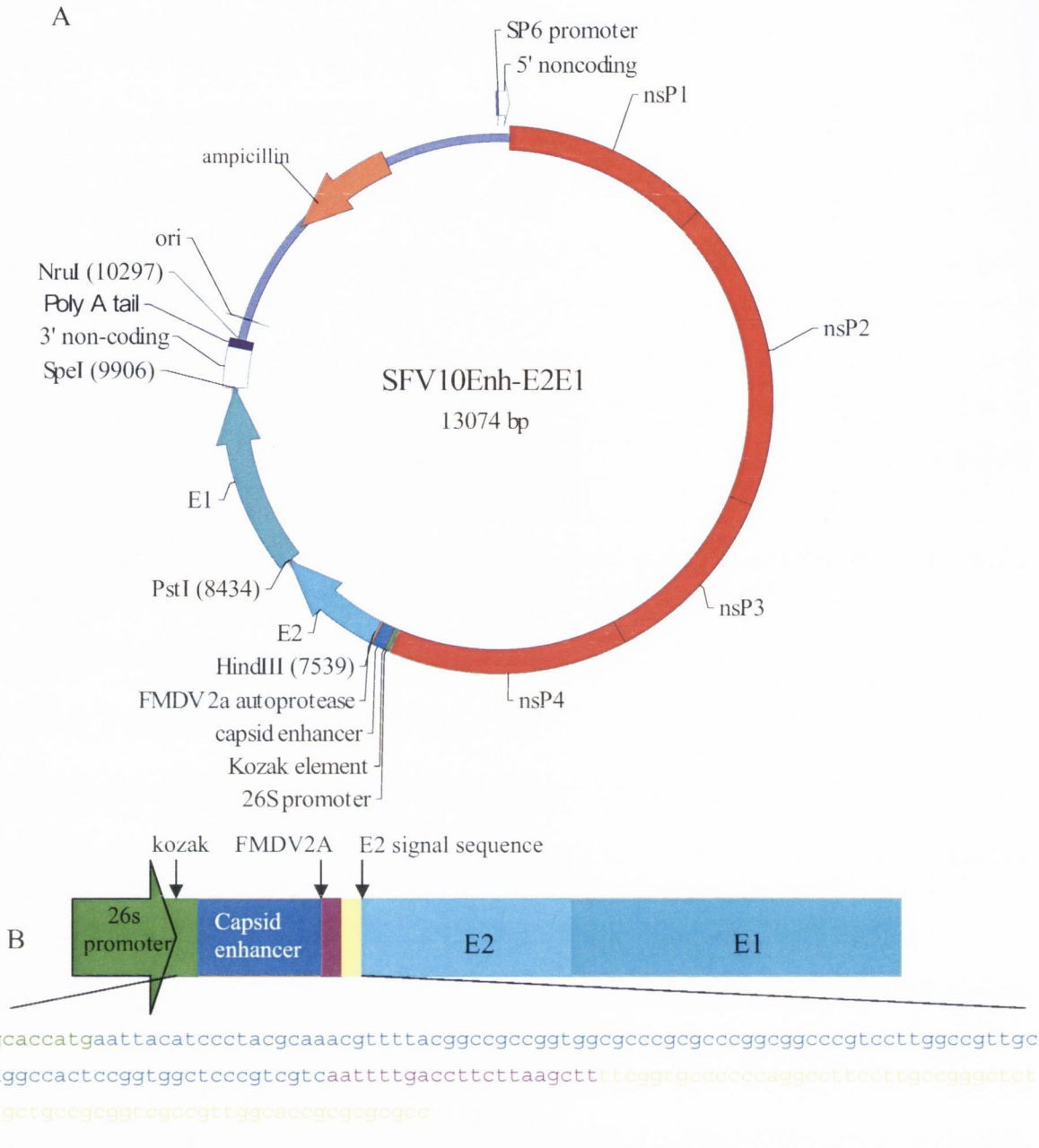


Figure 2.4 The SFV10Enh vector containing the RA27/3 E2 and E1 genes

A) To construct pSFV10Enh-E2E1, the E2 and E1 genes were inserted into Litmus 28 at the *Hind*III, *Pst*I & *Spe*I sites to produce L28-E2E1. A 1359 bp region of the E2 T cell epitope was corrected with a fragment from the pucRA infectious cDNA clone. Then, the E2E1 genes were cut at the *Hind*III and *Spe*I sites and cloned into the pSFV10Enh vector. B) The kozak sequence, first methionine (green) and the 99 nucleotides of the 32 a.a. capsid enhancer protein element (dark blue) are shown. The E2 gene signal sequence (yellow) is inserted at a *Hind*III site in the Foot and Mouth Disease virus (FMDV) 2A autoprotease nucleotide sequence (purple). Cleavage of E2 is mediated by host cell signal peptidase.

2.2.5 RSF virus-like particle (VLP) production

2.2.5.1 SFV vectors

The SFV split-helper system, comprising the packaging vectors pSFV-HelperS2 (encoding the envelope proteins) and pSFV-CS219A (encoding the CP) was employed together with the constructed pSFV10Enh-E2E1 to produce rSFV10Enh-E2E1 VLPs.

2.2.5.2 Preparation of recombinant and helper SFV plasmids

Competent DH5α cells were transformed with pSFV10Enh-E2E1, pSFV-Helper S2 and pSFV-CS219A separately as outlined in section 2.2.3.6. Transformed pure single colonies were then grown in LB-broth separately for each plasmid DNA. Each of the plasmid DNAs were purified from *E. coli* DH5α cells using the QIAGEN Miniprep Plasmid Purification kit as outlined in section 2.2.3.11. Plasmid concentrations were estimated using a DNA/RNA spectrophotometer and visualized by gel electrophoresis as described in 2.2.3.2.

2.2.5.3 Linearisation of plasmid DNA for *in vitro* transcription

In order to synthesise both helper and rSFV viral RNA, each plasmid was linearised using a unique restriction enzyme. The *Spe*I site was used for the helper SFV plasmids (Figure 2.2) and the *Nru*I site was used for the pSFV10Enh-E2E1 plasmid (Figure 2.4). A total of 10 µg plasmid DNA was linearised in a 100 µl volume containing 10 µl NEBuffer 2 or *Nru*I NEBUnique buffer (50 mM NaCl, 10 mM Tris-HCL, 10 mM $MgCl_2$, 1mM DTT, pH 7.9), and 20 U *Spe*I or *Nru*I. Following 16-18 h digestion in a water bath at 37 °C, cut and uncut plasmids were visualised by agarose gel electrophoresis along with 0.2 µg of a λ *Hind*III molecular weight marker and a 1 kb marker as described in section 2.2.3.2. Linearised plasmids were then purified using a Qiagen nucleotide extraction kit and resuspended in a final volume of 50 µl sterile nuclease free water as described in 2.2.3.8. The concentrations of the linearised plasmid DNA were estimated on a 0.8% agarose by comparing band intensities with those of 0.2 µg Lambda *Hind*III DNA and 1 kb molecular weight markers as outlined in section 2.2.3.2.

2.2.5.4 *In vitro* SP6 RNA transcription

A 1.5 µg amount of each linearised plasmid DNA was used as template in a 50 µl SP6 Polymerase RNA transcription reaction containing SP6 buffer (40 mM HEPES-KOH pH 7.4. 6 mM MgOAc, 2mM Spermidine-HCL), 1 mM $m^7G(5')ppp(5')G$, sodium (CAP), 5 mM DTT, 1 mM each rATP, rCTP, rUTP, 500 µM rGTP, 60 U rRNasin ribonuclease inhibitor and 34 U SP6 RNA Polymerase in nuclease free water. This reaction was carried out in 1.5 ml eppendorf tubes separately for each DNA sample. The tubes were vortexed, centrifuged briefly and incubated at 37 °C for 1 h 50 min on a heating block. Each of the RNA transcripts of rSFV10Enh-E2E1, rSFV-Helper S2 and rSFV-CS219A were analysed by running 1 µl on a 0.6% (w/v) agarose gel as described in section 2.2.3.2. *In vitro* transcription of the pSFV10Enh-E2E1, pSFV-Helper S2 and pSFV-CS219A plasmids was carried out in a 300 µl volume. For each stock of rSFV10Enh-E2E1 or SFV10Enh VLPs, three 150 cm^2 flasks of confluent Swedish-BHK cells were prepared.

2.2.5.5 Co-electroporation of plasmid RNA

For each high titre batch of rSFV10Enh-E2E1 particles, 9 µg SFV10Enh-E2E1 RNA, helper spike RNA and helper capsid RNA were *in vitro* transcribed and electroporated into sBHK cells. Three 150 cm^2 tissue culture flasks of about 90% confluent Swedish-BHK cells were propagated and trypsinised as described in 2.2.1. The cells were resuspended in 10 ml of fresh BHK medium. Cells were centrifuged at 400 x g for 10 min at room temperature and the pellets were gently resuspended in 10 ml PBS and spun again. The cells of all the confluent flasks were resuspended finally in 3.9 ml of PBS and kept on ice. The *in vitro*-transcribed RNA reactions were mixed with this 3.9 ml cell suspension to give a total volume of 4.8 ml which was divided among six, 4 mm electroporation cuvettes (800 µl/cuvette). A total of six electroporations were carried out in quick succession for each batch of VLP stock using a gene pulser. The Biorad genepulser Xcell settings were as follows: 850 Volts (V) with a 4 msec pulse length for 2 pulses at a 5 sec pulse interval. The cell suspensions of all six electroporations were mixed with 100 ml of fresh BHK medium and divided into two 150 cm^2 flasks (50 ml/flask). After a 36-40 h incubation at 33 °C in a humidified atmosphere with 5% CO_2 the medium containing viral particles was clarified three times by centrifugation at 4 °C in a Sorvall SS34 rotor for 15 min at 18,000 rpm and placed on ice.

2.2.5.6 Harvesting and purification of high titre rSFV particles

Particles were concentrated by ultracentrifugation through a 20% (w/v) sucrose cushion in a Beckman SW28 rotor at 25,000 rpm for 2 h 15 min at 4 °C. The clarified supernatant was aliquoted into three SW-28 ultracentrifuge tubes with 5 ml of 20% sucrose (in TNE buffer) as a cushion beneath the virus supernatant. The tubes were placed into SW28 ultracentrifuge buckets and all the tubes were balanced before ultracentrifugation. Following ultracentrifugation, the supernatant was immediately removed, the sides of the tubes dried with sterile cotton tips, and each pellet was resuspended with 300 µl 1X TNE buffer (50 mM Tris-HCL, pH 7.4, 100 mM NaCl, 0.1 mM EDTA) per tube and incubated on ice for 2 h. The pellet was resuspended with gentle pipetting and the virus like particles (rSFV10Enh-E2E1 or rSFV10Enh) were collected. A further 200 µl was added to the first tube and used to rinse out the remaining two tubes to give a final volume of 1 ml. The resuspended VLPs were aliquoted in 100 µl into cryotubes and were snap frozen using liquid nitrogen before storage at -70 °C. Virus pellets were resuspended in pre-chilled 1X TNE buffer and aliquots were snap frozen in liquid N_2 and stored at –70 °C until use.

2.2.5.7 High titre rSFV particle titration by immunofluorescence

To determine the titre of the stocks, sBHK cells were grown to confluency on 22 x 22 mm glass coverslips in a 6 well plate. Serial dilutions (1 µl, 0.1 µl, 0.01 µl, 0.001 µl and 0.0001 µl) of virus stock were made using a total volume of 500 µl for infection. The cells were infected with 500 µl of virus dilutions for 1 h at 37 °C by rocking every 10 min. To each well, 2.5 ml fresh BHK medium was added and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. After 16-18 h immunofluorescence (IF) was carried out. The cells were washed twice with PBS (Ca+, Mg+). The cells were then fixed in 2 ml of 4% paraformaldehyde (PFA) in PBS without Ca or Mg (PBS^-) for 20-30 min. The PFA was removed and following two washes in PBS^-, the cells were incubated in acetic acid:ethanol (1:2) at -20 °C for 5-10 min. The cells were blocked with 50 µl of 1.5% normal rabbit serum in PBS^- on slides covered in parafilm for 30 min. The coverslips were then immediately incubated with monoclonal mouse anti-RV E1 antibody (1:2000 dilution) for 60 min at 37 °C. Following two 5 min washes with PBS-Tween (0.05% Tween 80) and two 5 min washes with PBS^-, the coverslips were incubated with 50 µl secondary antibody (FITC conjugated rabbit anti-mouse secondary antibody) for 30 min at RT. The cells on the coverslips were mounted on slides with 4, -6, diamidino-2-phenylindole (DAPI)

mountant and examined using a fluorescent microscope. The average number of positive cells from 20 random fields at a magnification of 40x was used to calculate VLP titres and expressed as infectious units (IU) per ml. Photos were taken using Act1 software in conjunction with a Nikon camera attached to the fluorescent microscope.

2.2.6 *In vivo* studies

2.2.6.1 Determination of mouse model

Groups of six, 8 week old, BALB/c, 3 week old outbred Swiss, 6-8 week old C57BL/6 and 6-8 week old A/J mice were used for *in vivo* experimentation to determine the most suitable mouse model, which were maintained as described in section 2.1.6. The mice were inoculated, either intraperitoneally (i.p.) or intramuscularly (i.m.), with 10^3 $TCID_{50}$ of Ervevax (monovalent RA27/3 RV vaccine) and the mice were boosted at 2 weeks post initial immunisation. All mice were checked daily for clinical signs or discomfort following i.m. injection.

2.2.6.2.1 Humoral immune response to Ervevax, rSFVE2E1; rSFVE2E1, rSFVH plus rSFVHN

Groups of ten mice were immunised i.m. with Ervevax (50 µl of 10^3 $TCID_{50}$), rSFVE2E1 and the combined rSFVE2E1, rSFVH plus rSFVHN vaccine constructs (50 µl of 10^6 infectious units (i.u.) of each construct/mouse), and groups of control mice were immunised with TNE buffer alone and 10^6 rSFV without cloned gene. All mice were boosted with an equivalent dose at two weeks post initial immunisation. This experiment was repeated with a four week boost instead of a two week boost regimen as described in section 2.2.6.2.2.

2.2.6.2.2 Humoral immune response to rSFVE2E1; rSFVE2E1, rSFVH plus rSFVHN

Groups of ten mice were immunised i.m. with rSFVE2E1 and the combined rSFVE2E1, rSFVH plus rSFVHN vaccine constructs (50 µl of 10^6 infectious units (i.u.) of each construct/mouse) and groups of control mice were immunised with TNE buffer alone and 10^6 rSFV without cloned gene. All mice were boosted with an equivalent dose at four weeks post initial immunisation.

2.2.6.3 Humoral immune response to rSFVE2E1; rSFVE2E1, rSFVH plus rSFVHN; rSFVE2E1 plus rSFVH and rSFVE2E1 plus rSFVHN

In an independent experiment, groups of ten, 6-8 week old female BALB/c mice were immunised with monovalent rSFVE2E1, divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN. A mixture of rSFVE2E1 plus rSFV (no cloned gene), rSFV (no cloned gene) alone and TNE buffer alone were also included as controls. The dose of each construct was again 10^6 i.u. given i.m., except that of the vector without cloned gene which was also given at 2 x 10^6 i.u., with the rSFVE2E1 construct, to mimic the trivalent vaccine. The divalent constructs were also supplemented with a dose of rSFV (no cloned gene). All mice were boosted 4 weeks post initial immunisation.

2.2.6.4 Cell mediated immune response to rSFVE2E1; rSFVE2E1, rSFVH plus rSFVHN; rSFVE2E1 plus rSFVH and rSFVE2E1 plus rSFVHN

Groups of six, 6-8 week old BALB/c female mice were immunised with monovalent rSFVE2E1, divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN. A mixture of rSFVE2E1 plus rSFV (no cloned gene), rSFV (no cloned gene) alone and TNE buffer alone were also included as controls. The dose of each construct was again 10^6 i.u. given i.m., except that of the vector without cloned gene which was also given at 2 x 10^6 i.u., with the rSFVE2E1 construct, to mimic the trivalent vaccine. Spleens were harvested from these mice 1 week post initial immunisation.

2.2.7 Immunological studies

2.2.7.1 Harvesting of serum, muscle and spleens from immunised mice

Mice immunised with Ervevax and boosted at two weeks were euthanised by halothane overdose at 1, 2 and 4 weeks post initial inoculation. Blood was obtained by cardiac puncture and severing of the hepatic artery using aseptic technique to open the abdomen. A pipette was used to harvest blood from the mice into a 1.7 ml eppendorf tube, which was subsequently incubated at 37 °C for 1 h and 4 °C for 2 h. The clotted blood was

centrifuged at 400 g for 10 min at 4 °C and the serum was decanted and aliquoted before storage at -20 °C.

Sera from mice immunised with VLPs and relevant controls and boosted at 4 weeks was obtained from blood samples collected at the junction of the facial, submandibular veins using the Golden rod technique and processed individually as above. Spleens for T cell assays were removed from mice euthanised with halothane 7 days post immunisation, whereby the abdomen was opened aseptically and the spleen excised using a sharp clean blade.

2.2.7.2 RV antibody titration

ELISAS were performed to assay RV antibodies in the serum from the different mouse strains boosted at 2 weeks. Doubling dilutions of mouse serum were added to the plate and incubated at RT for 1 h, followed by a rabbit anti mouse HRP antibody (1:200) for 30 min, 3,3',5,5' Tetramethylbenzidine (TMB) liquid substrate for 2 min and 1M H_2SO_4 to stop the reaction. Absorbance was detected at 450 nm using an ELISA plate reader. Doubling dilutions of the E1 monoclonal antibody were used to create a standard curve from which antibody values (ng/ml) were interpolated through linear regression. Controls for this assay involved wells that received no serum or secondary antibody.

Mouse anti-RV serum IgG was detected by a limiting dilution ELISA for groups boosted at 4 weeks. Doubling dilutions of mouse serum were added to IgG rubella ELISA plates and incubated at room temperature for 1 h, followed by a polyclonal rabbit anti-mouse immunoglobulins/HRP conjugated antibody (1:1000) for 1 h, TMB liquid substrate for 30 min and 1M H_2SO_4 was used to stop the reaction. Absorbance was detected at 450 nm using an ELISA plate reader. The same procedure was followed for the measles and mumps ELISAs. The rubella ELISA plates were coated at a concentration of 3.125 μg/ml, the measles plates at a concentration of 7.5 μg/ml and the mumps plates at 4 μg/ml. Controls for this assay involved serum from rSFV10Enh-Empty particles and 1X TNE immunised mice and wells that received no serum. Cut-off titres were calculated as the dilution giving an OD exceeding 0.025 above the mean of the control sera +2SD.

2.2.7.3 Detection of functional antibodies by latex agglutination assay

The quantitative rubagen latex particle agglutination test was employed as per the manufacturer's instructions to visualise and quantify the antigen-antibody reaction. Sera

were initially diluted 1:5 as specified by the assay protocol in dilution buffer provided with the assay and subsequent two fold dilutions were made in the same buffer. When the latex reagent is mixed with human serum, if the serum contains approximately more than 10 IU/ml of RV antibodies, a clear agglutination will appear, which corresponds to a protective antibody level (1:10) against RV with this assay. The highest dilution exhibiting detectable agglutination was used as the antibody titre. The sensitivity of rubagen correlates perfectly with that of the HAI test.

2.2.7.4 Splenocyte T cell proliferation: interferon-γ and IL-5 assays

Spleens were harvested from six replicate mice immunised, as described in section 2.2.6.4. and collected in HBSS on ice. The tissue in HBSS was passed through a 70 μm cell strainer into a 50 ml Falcon tube with the rubber end of a syringe plunger. The cells were spun down at 1500 rpm for 10 min at 4 °C and the supernatant discarded. The cells were flicked and 5 ml of 1x red blood cell (RBC) lysis buffer was added and incubated for 2 min at room temperature. The tubes were topped up with HBSS and spun as before, twice. The supernatant was discarded and the cells were resuspended in 5 ml RPMI-1640 medium containing 10% heat inactivated FBS, 20 mM HEPES buffer, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.8% sodium pyruvate, 0.8% non-essential a.a.s and 50 μm B-Mercaptoethanol. The cells in the suspension were counted by trypan blue exclusion assay using a hemocytometer as follows: from the 5 ml cell suspension a 1:100 dilution was made and 90 μl of this dilution was removed and added to 10 μl of trypan blue. Of this mixture, 10 μl was loaded into the gap between the hemocytometer and the coverslip. The clear, viable (trypan blue excluding cells) distinguished from those of dead (blue) and red blood (dark) cells were counted in all the four squares and the average number of viable cells was calculated. Wells of a 24 well plate were seeded with 5 x 10^5 splenocytes per well in a total volume of 1 ml medium. Triplicate wells were incubated with a total of 1 μg, 5 μg and 20 μg, respectively, of the combined rubella E2 and E1 recombinant proteins. Triplicate wells containing splenocytes from each group were incubated with the positive control Concanavalin A (ConA) (1 μg/ml) and a negative control consisted of splenocytes incubated in medium only. The plates were incubated at 37 °C for 72 h and 300 μl aliquots of the supernatants of each sample stored at -20 °C for further analysis.

Supernatant samples were tested for the presence of cytokines by use of a capture ELISA specific for mouse IL-5 or IFN-γ. Wells of a 96 well plate were coated with 100 μl/well of capture antibody; diluted 1:250 in coating buffer (0.1 M Sodium Carbonate, pH

9.5) sealed and incubated overnight at 4 °C. Wells were washed 3 times with 300 µl/well of wash buffer (PBS, with 0.05% Tween-20) and plates blocked for 1 h at RT with 200 µl/well of assay diluent (PBS with 10% FBS). Wells were again washed 3 times with wash buffer, sealed and incubated for 2 h at RT with separate aliquots of 100 µl/well of standard (serial dilutions in assay diluent), samples or controls. Plates were again washed as before, but with 10 total washes. A 100 µl amount of TMB substrate was added, and plates incubated at RT in the dark for 30 min, before addition of 50 µl stop solution (2N H_2SO_4). Absorbances were read at 450 nm within 30 minutes of adding the stop solution. Mean concentrations (pg/ml) were determined for duplicate samples from the standard curve using log-log regression analysis.

2.2.8 Histopathology

2.2.8.1 Sampling and processing of muscle samples

For general pathology and immuno-histochemical analysis, mice immunised with Ervevax were sampled in replicates of six on days 7, 14 and 28 (two weeks post boost). Mice were sacrificed by halothane. Muscles were excised, placed in 10% formol saline and processed for pathological studies. Sections of sampled muscles were prepared for general pathology by Ms. Alex Whelan-Buckley (Research Assistant, Department of Veterinary Pathology, University College Dublin (UCD)). Muscle samples were dehydrated through a series of graded alcohol washes as follows: 50% (v/v) alcohol for 1 h, 70% (v/v) alcohol for 1 h and 90% (v/v) alcohol for 1 h, before 2 x 40 min absolute alcohol washes. Samples were then immersed for 1 h in an absolute alcohol and xylene (1:1) solution before 3 x 40 min xylene washes. This was followed by 4 x 40 min immersions on paraffin wax and subsequent mounting onto blocks. For routine histology and immunohistochemistry, 3 µm sections were cut using a microtome and dried overnight at 37 °C in an oven.

2.2.8.2 Haematoxylin and eosin staining for routine histology

Sections of paraffin embedded muscles were examined using the haematoxylin and eosin (H & E) staining method. Dried sections were dewaxed with 3 x 10 min washes in 100% xylene and consequently rehydrated through 100%, 95%, 70% ethanol for 5 min each followed by distilled water. Sections were then placed in Harris' haematoxylin for 10 min, rinsed under running tap water until cleared, differentiated in 0.8% acid alcohol and

'blued-up' by dipping three times in 3% ammonia water. Following a wash under running tap water, sections were counterstained in 0.8% dichromate eosin for 2 min and washed under running tap water for a further 5 min or until cleared. Stained sections were then dehydrated and mounted using DPX by covering the section with a coverslip without air bubbles. H & E stained slides were prepared from three separate sections of each muscle to provide representative pathological observations.

2.2.8.3 Histology

Histological examination was carried out by Professor Brian Sheahan (Department of Veterinary Pathology, UCD) and the muscle samples were read blind by light microscopy and lesions were graded on a scale of 0-5. Relevant histological details were noted such as: myofiber necrosis and mineralization, myofiber regeneration, indicated by sarcolemmal nuclear proliferation (SNP) and inflammation of interstitial fat deposits (steatitis). No abnormalities were detected in muscle samples from control uninjected muscle.

2.2.9 Statistical analysis

Normal probability plots were constructed to evaluate the normality of the data to make distributional assumptions whereby the data was plotted against theoretical quantiles of a Normal distribution. The data were log transformed to normality to remove skewness where necessary, resulting in correct Normal probability plots and similar standard deviations among samples (Altman 2000). The mean of the log data was back-transformed to yield the geometric mean and a corresponding 95% confidence interval of the estimate. Thus Normality and equal variance were verified. The statistical significance of the differences in antibody titres, IFN-γ and IL-5 titres among the different experimental immunisation groups was analysed by the parametric one-way analysis of variance (one-way ANOVA). The Bonferroni correction post test was used to compare the Probability (P) value of each group with other groups. The IgG2a and IgG1 data was analysed using a Two-way ANOVA and a paired *t* test to determine the IgG2a:IgG1 titre ratios. All the statistical analyses were performed using the GraphPad Prism 4 program and a P value less than 0.05 was considered significant.

Chapter 3

Cloning & *in vitro* characterisation of an SFV based RA27/3-E2E1 vector

3.1 Introduction

Replication-deficient SFV vectors have demonstrated transient expression in many different mammalian cell types and have been used in a variety of candidate vaccines. The RA27/3 RV genome has been sequenced and found to contain 9762 nucleotides (ntds) exclusive of the 3' poly A tract (GenBank Accession Number L78917) (Pugachev *et al.*, 1997a). The RA27/3 structural protein coding sequence had also previously been determined from cDNA clones (ntds 6307-9762, GenBank Accession #X14781) (Nakhasi *et al.*, 1989). These two sequences were found to differ at 46 out of the 3456 residues that they have in common (1.3%). The high G + C content of the RV genome may have contributed to cloning and sequencing discrepancies. The complete coding sequence of the virion envelope protein, E1 has been determined (Therien strain) (Frey *et al.*, 1986). The sequences of the C protein and E2 protein were subsequently determined. There was a higher degree of change in the RA27/3 strain than in the HPV77 strain when they were compared (Frey *et al.*, 1998) to the wt Therien rubella strain which might have resulted in the RA27/3 strain being a safer and more effective vaccine than the HPV77 strain (Atreya *et al.*, 1998).

According to the signal hypothesis, translocation of secretory and membrane proteins into the ER is mediated by a hydrophobic signal peptide which, in the case of E1, was found to lie in the 69 carboxyl-terminal a.a.s of the E2 protein. This sequence is necessary for the insertion of El into the ER and incorporates a sequence of 20 hydrophobic a.a.s preceding E1, likely to be necessary for this function (Hobman *et al.*, 1992; Hobman *et al.*, 1993). The E2 signal sequence consists of a 21-residue stretch of uncharged, mainly nonpolar a.a.s which confers membrane association on the CP (Suomalainen *et al.*, 1990). The E2 signal peptide serves as a membrane anchor for the CP and this domain is required for RV glycoprotein-dependent localisation of the CP to the juxtanuclear region and subsequent virus assembly at the Golgi complex (Law *et al.*, 2001). Cleavage between C and E2 proteins appears to be mediated by signal peptidase which cleaves after the E2 signal sequence (Suomalainen *et al.*, 1990) (Figure 3.1). RV is different to the alphaviruses in this regard where the C protein autoproteolytically cleaves itself from the polyprotein and the signal sequence of the following membrane protein (p62) remains part of that protein (Suomalainen *et al.*, 1990). The low level of immune response to E2 in RV infection may be due to the inaccessibility of the polypeptide chain to the host's immune system because of masking by glycosylation (Chaye *et al.*, 1992b). It has been proposed that the elevated level of immune response to E2 is a direct

consequence of persistent infection in individuals with CRS (Chaye *et al.*, 1992b). Slow release of the viral antigens, due to persistent infection may lead to continual immunostimulation and an increased immune response to antigens that are not seen by the immune system in the case of rapidly cleared viral infections. RV infections have been linked to arthritis, diabetes and encephalitis and perhaps in susceptible individuals vigorous immune responses directed against E2 and or CP may result in autoimmunity as an indirect consequence of persistent infection (Chaye *et al.*, 1992b).

Figure 3.1 The C-E2 junction and a.a. sequence of the RV structural polyprotein. The E2 signal sequence, a stretch of hydrophobic and noncharged a.a.s, is underlined. The carboxy termini of C and C_{-ss} are also indicated (Suomalainen *et al.*, 1990).

For molecular epidemiology, the nucleotide sequence of the RV E1 gene (encoding the H) has been routinely used (Frey *et al.*, 1998). The nucleotide sequence of the region of the RV genome which encodes a.a.s 195-296 of the E1 glycoprotein (E1-195-296) has been determined from a panel of 22 RVs obtained from Europe, USA and Asia between 1963 and 1995 (Bosma *et al.*, 1996). E1-195-296 contains neutralising and hemagglutinating determinants, and may represent a major antigenic domain. The nucleotide sequence divergence of the 22 RVs compared to the Therien strain sequence ranged from 0.65-7.14% but the majority of the ntd changes did not affect E1-195-296 a.a. sequence (Bosma *et al.*, 1996). This chapter describes how the E2 and E1 genes of the RA27/3 vaccine strain were cloned into the enhanced SFV vector (SFV10Enh) and a region of the E2 T cell epitope corrected with a fragment of the pucRA infectious cDNA RA27/3 clone. The changes in the E2 C terminus were in an E2 T cell epitope but also in the E1 signal sequence which directs the translation of membrane associated and secreted proteins into the lumen of the ER. The RV capsid gene has not be cloned into the prototype vaccine as the RNA binding domain of RV is located within a.a. residues 28 to 56 and an interaction with the SFV non-structural region could not be ruled out (Chen *et al.*, 2004b). The RA27/3 strain used to construct this prototype vaccine is the most widely used vaccine strain. Following production of recombinant virus like particles for the study, the expression of the rSFV10Enh-E2E1 construct was assessed in sBHK cells.

3.2 Results

3.2.1 Cloning strategy

This chapter details the cloning of the ATCC RA27/3 genes into the pSFV10Enh vector and the strategy is outlined in Figure 3.2.

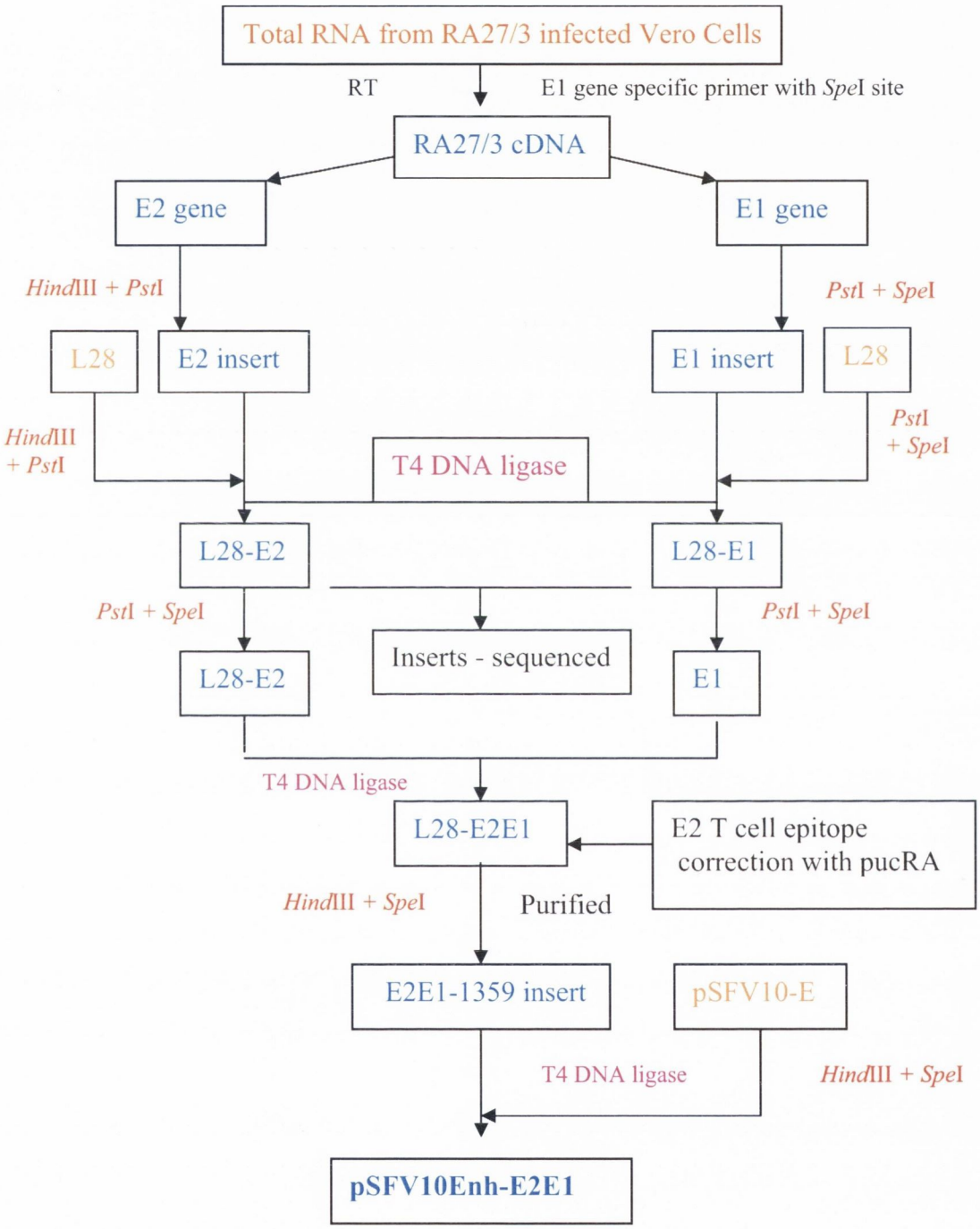


Figure 3.2 Schematic representation of the pSFV10Enh-E2E1 cloning strategy

3.2.2 Isolation of total RNA and detection of RA27/3 RNA

Vero cells were infected with the RA27/3 virus (ATCC) and mock infected with PBS for one to five days. RNA was harvested from flasks of RA27/3 and mock infected Vero cells and the RNA was compared by agarose gel electrophoresis. Detection of RV was carried out by RT-PCR using the avian myeloblastosis virus (AMV) reverse transcriptase and primers designed to detect a 185 bp T-cell epitope region in the E1 gene (Bosma *et al.*, 1995). The extent of the RA27/3 infection of Vero cells was thus compared by agarose gel electrophoresis (Figure 3.3). The amount of RNA in the RA27/3 infected Vero cells increased over time as measured by RT-PCR until five days post infection when the CPE had visibly decreased the cell density considerably.

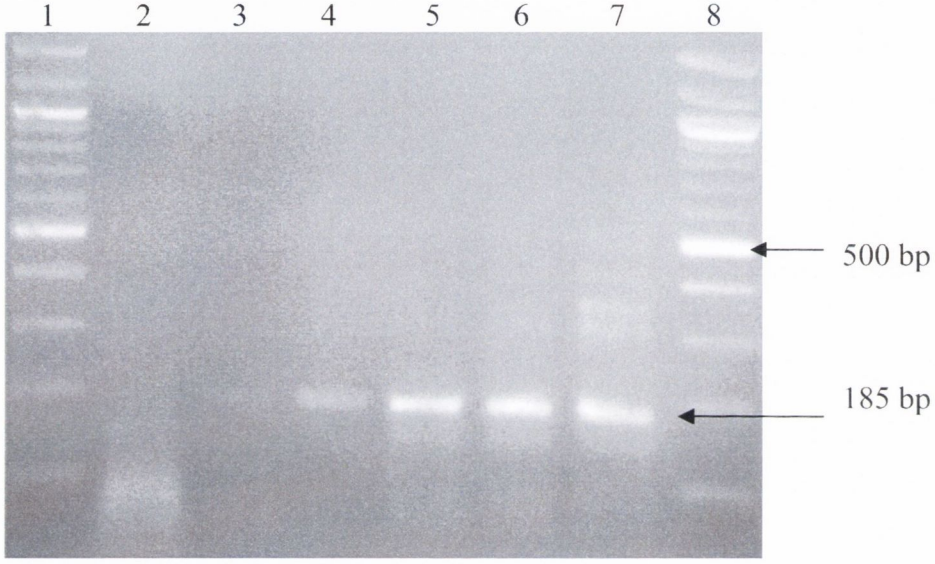


Figure 3.3 Gel analysis of PCR amplified 185 bp region of an E1 fragment

Total RNA was isolated from Vero cells infected with RA27/3 (ATCC) at one to five days pi and converted to cDNA. The integrity of this RNA was checked by RT-PCR using primers specific for actin (not shown). The PCR amplified E1 fragment was analysed by electrophoresis of a 10 µl aliquot on a 1.0 % (w/v) agarose gel. The RA27/3 RNA increased over time.

Lane 1; 0.5 µg of 100 bp DNA ladder. Lane 2; mock infected control. Lane 3; 24 h PCR product. Lane 4; 48 h PCR product. Lane5; 72 h PCR product. Lane 6; 96 h PCR product. Lane 7; 120 h PCR product. Lane 8; 0.5 µg of 100 bp DNA ladder.

3.2.3 Construction of Litmus 28-E2E1

External and internal primers (Table 2.1) were used in a separate nested PCR of the E2 and E1 genes as displayed in Figure 3.4 and the genes cloned into Litmus 28 (Figure 3.5).

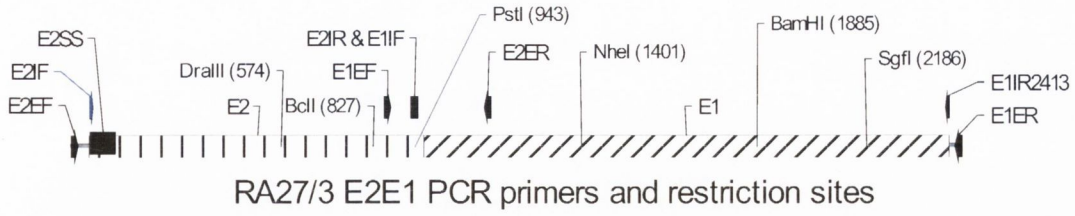


Figure 3.4 Diagrammatic representation of PCR strategy for E2 and E1 genes

The E2 signal sequence and unique restriction sites used in the cloning strategy are shown.

Figure 3.5 Strategy for cloning of the E2 & E1 genes into the Litmus 28 vector

3.2.3.1 Construction of Litmus28-E2

The RA27/3 E2 cDNA was synthesised from total RNA of ATCC RA27/3 infected Vero cells using MMLV RT. Triplemaster Taq polymerase amplified E2 with its signal sequence (915 bp) (Figure 3.6) was cloned into *Hind*III and *Pst*I digested Litmus 28 (Figure 3.7).

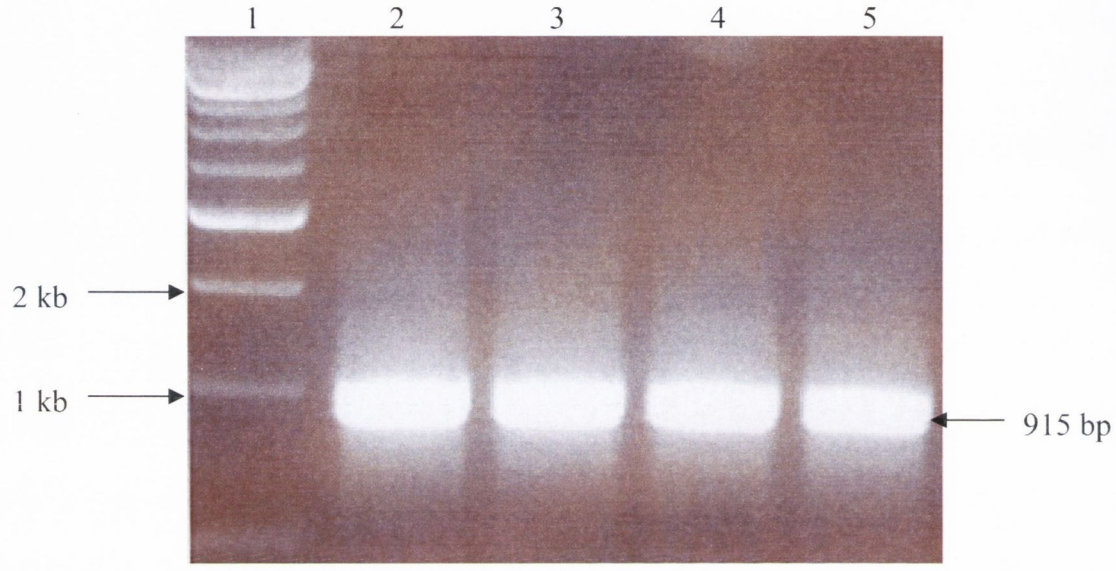


Figure 3.6 Analysis of RT-PCR amplified RA27/3 E2 gene

Total RNA was isolated from Vero cells 5 days post infection (dpi) with RA27/3 and cDNA synthesised using MMLV-RT. A nested PCR was performed on E2 first round PCR products and a 1 µl aliquot was visualized on a 1.0% (w/v) agarose gel.

Lane 1; 0.5 µg of a 500bp DNA ladder. Lanes 2-5; E2 gene PCR product: 915 bp.

PCR amplified E2 in Litmus 28 was analysed for insertion by *Dra*III digestion on an agarose gel (Figure 3.7) A unique restriction site *Dra*III in the E2 gene (Figure 2.3) at position 574 was selected to analyse the insertion of the gene in Litmus 28. The presence and correct insertion of the E2 gene in Litmus-28 after *Dra*III digestion produced two fragments of 2007 bp and 1712 bp due to the *Dra*III site at position 1326 of the Litmus 28 plasmid.

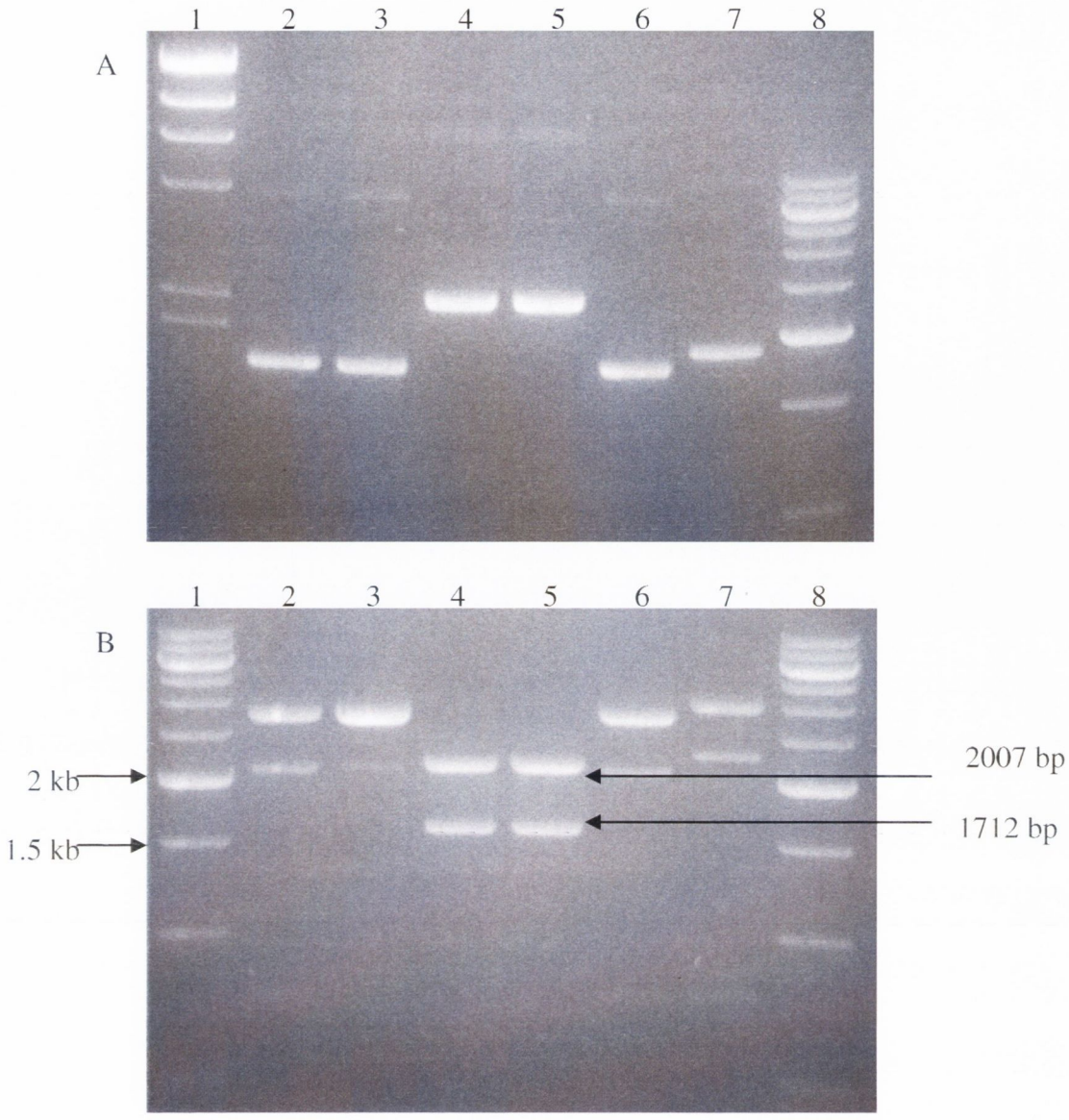


Figure 3.7 Screening for L28-E2 clones by restriction analysis

A 1.0 μg amount of the Litmus28-E2 construct was digested using *Dra*III. 1.0 μl of the digested product was analysed on a 1% agarose gel.

A) Lane 1; λ *Hind*III DNA molecular weight marker. Lanes 2-7; L28-E2 plasmid DNA for screening. Lane 8; 0.5 μg of a 1 kb molecular weight marker.

B) Lanes 1 and 8; 0.5 μg of a 1kb molecular weight marker. Lanes 2-7; *Dra*III digested potential Litmus 28-E2 positive clones: the samples in Lanes 4 and 5 were found to contain the E2 gene whereas those in Lanes 2, 3, 6 and 7 did not contain the E2 insertion.

3.2.3.2 Construction of Litmus28-E1 and L28-E2E1

The RA27/3 E1 cDNA was also synthesised from total RNA of ATCC RA27/3 infected Vero cells using MMLV-RT. PCR amplified E1 with signal sequence (1443 bp) (Figure 3.8) was simultaneously cloned into *Pst*I and *Spe*I digested L28-E2 and L28 to get L28-E1 (Figure 3.9) and L28-E2E1 (Figure 3.10).

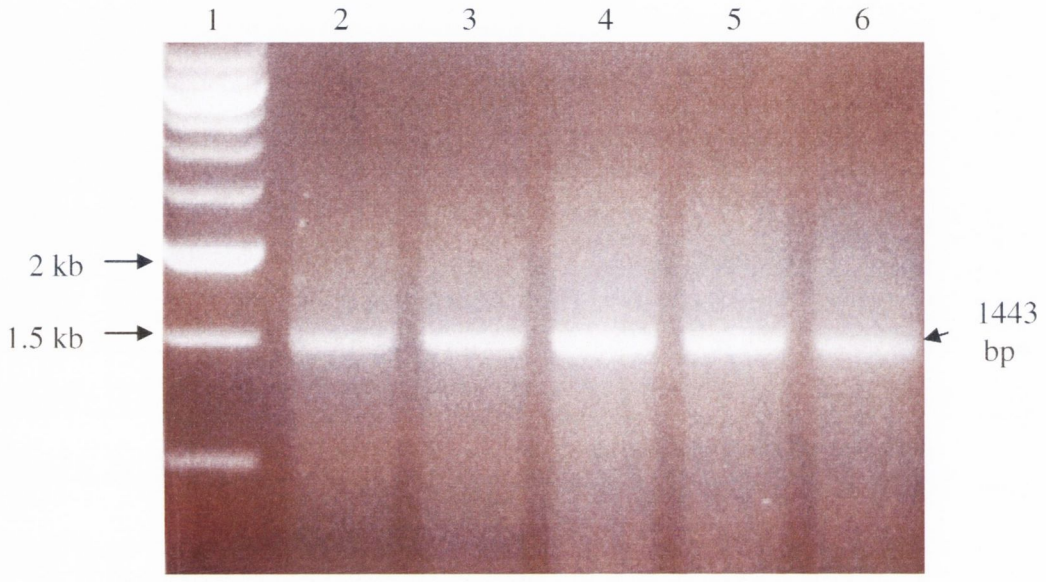


Figure 3.8 Gel analysis of PCR amplified RA27/3 E1 gene

Total RNA was isolated from RA27/3 Vero cells 5 dpi and cDNA synthesised using a MMLV reverse transcriptase. A nested PCR on E1 first round PCR products with E1IntF and E1IntR specific primers using Triplemaster taq polymerase for G-C rich templates was analysed by electrophoresis. A 1 µl aliquot was visualized on a 1.0% (w/v) agarose gel.

Lane 1; 1kb DNA ladder. Lanes 2-5; E1 gene PCR product: 1443 bp.

The L28-E1 constructs were analysed to confirm insertion using *Nhe*I digestion (Figure 3.9). The insertion of the E1 gene into Litmus 28 was analysed by restriction digestion of a site unique to E1, *Nhe*I (Figure 2.3). A 1.0 µg amount of the L28-E1 construct was thus digested with *Nhe*I. The L28-E2E1 constructs were analysed by double digestion with *Dra*III and *BamH*I, a restriction site unique to E1 (Figure 2.3 & 3.10).

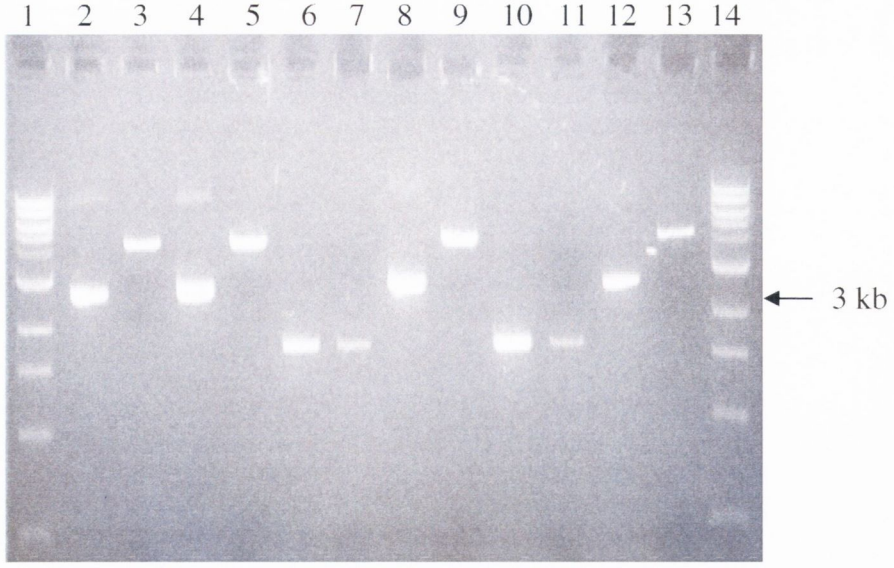


Figure 3.9 Screening for L28-E1 clones by restriction analysis with *Nhe*I

Lanes 1 & 14; 0.5 µg of a 1 kb marker. Lanes 2, 4, 6, 8, 12; uncut plasmid mini preparations. Lanes 3, 5, 9, 13; linearised L28-E1. Lanes 7 & 11; L28 plasmid without E1 insert.

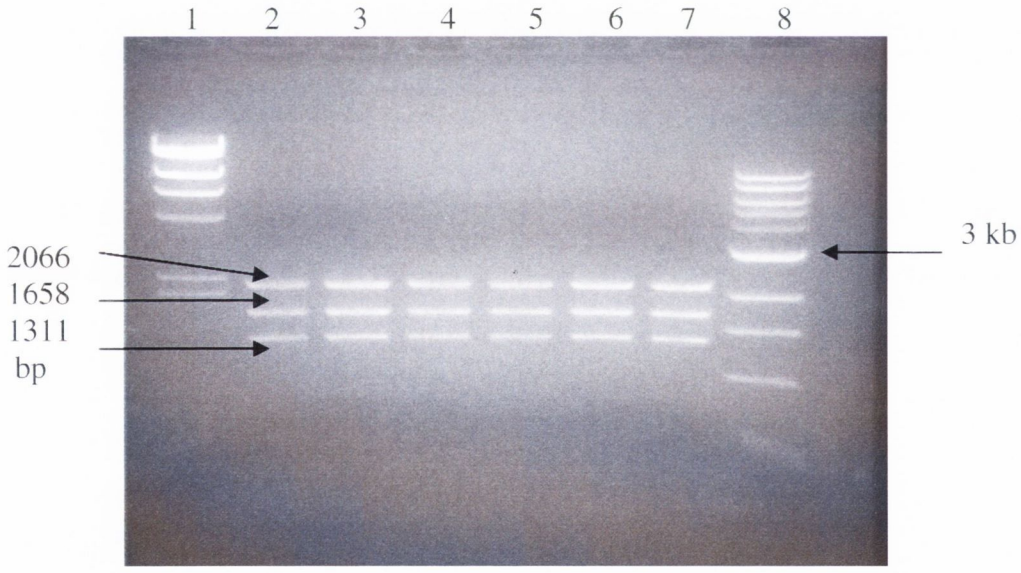


Figure 3.10 Screening of L28-E2E1 clones by restriction analysis with *Dra*III & *BamH*I

Lane 1; λ *Hind*III DNA marker. Lanes 2-7; L28-E2E1 positive clones. Lane 8; 0.5 µg of 1 kb molecular weight marker.

3.2.4 Sequencing of Litmus28-E2E1 construct

The sequencing reactions were carried out using primers shown in Figure 3.11 as well as primers specific to the Litmus28 vector designed to sequence the ends of the genes (Table 2.1). Four L28-E2E1 clones were sent for sequencing, however only one was sequenced fully. The GC rich content of the RA27/3 genome contributed to difficulties with the sequencing requiring many regions to be resequenced to obtain the full sequence. Thus only the clone with the most sequence homology to the published sequences was sequenced completely.

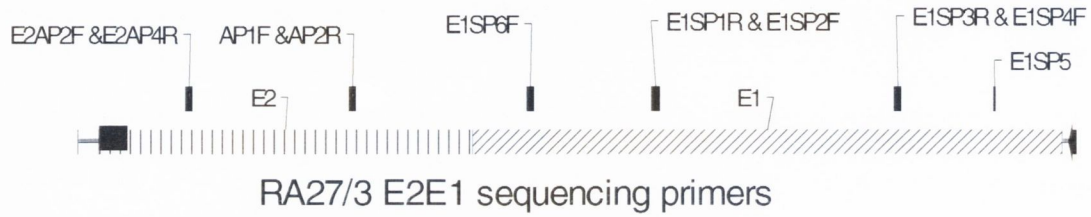


Figure 3.11 Diagrammatic representation of the position of the sequencing primers

Primers were also used on the L28 and SFV10Enh vectors to sequence the 5' and 3' termini of the E2 and E1 genes.

Clustal W sequence protein alignment was used to compare the published RA27/3 genomic sequences; GenBank #L78917 (Pugachev *et al.*, 1997a), from Meruvax II vaccine (Merck Research Laboratories) and GenBank #X14871 (Nakhasi *et al.*, 1989), from the Merck RA27/3 strain; to the L28-E2E1 clone. There were seven amino acid differences which are highlighted in red between the two published sequences and L28-E2E1 (Figure 3.12). There were three amino acid differences between L78917 and L28-E2E1 however these differences were not seen with the X14871 sequence (highlighted in dark purple). There were a further ten amino acid differences between the L78917 and the X14871 sequence. The defined T cell epitopes depicted in blue: a.a.s; 15-35, 440-414 and 520-531 were unchanged with the exception of residues 276 and 447. The antibody binding domains are depicted in orange and remained intact. The E2 signal sequence is highlighted in light purple. The L28-E2E1 clone with the most sequence homology to the published RA27/3 sequence (GenBank L78917) and the RA27/3 infectious cDNA clone, pucRA, were transformed in *E. coli* K12 cells (Figure 3.12).

```
L78917          RLLRMPVRGLDGDSAPLPPHTTERIETRSARHPWRIRFGAPQAFLAGLLLAAVAVGTARA 300
L28E2E1         ------------------------------------FGAPQAFLAGLLLAAVAVGTARA 23
X14871          RLLRMPVRGLDGDTAPLSPHTTERIETRSARHPSSIRFGAPQAFLAGLLLAAVAVGTARA 300
                                                    ************************

L78917          GLQPRADMAAPPTLPQPPRAHGQHYGHHHHQLPFLGHDGHHGGTLRVGQHHRNASDVLPG 360
L28E2E1         GLQPRADMAAPPTLPQPPRAHGQHYGHHHHQLPFLGHDGHHGGTLRVGRHHRNASDVLPG 83
X14871          GLQPRVDMAAPPMPPQPPRAHGQHYGHHHHQLPFLGHDGHHGGTLRVGQHHRNASDVLPG 360
                *****.******  ********************************:************

L78917          HWLQGGWGCYNLSDWHQGTHVCHTKHMDFWCVEHDRPPPATPTPLTTAANSTTAATPATA 420
L28E2E1         HWLQGGWGCYDLSDWHQGTHVCHTKHMDFWCVEHDRPPPATPTPLTTAANSTTAATPATA 143
X14871          HWLQGGWGCYNLSDWHQGTHVCHTKHMDFWCVEHDRPPPATPTSLTTAANSTTAATPATA 420
                **********:**********************************.****************

L78917          PAPCHAGLNDSCGGFLSGCGPMRLRHGADTRCGRLICGLSTTAQYPPTRFGCAMRWGLPP 480
L28E2E1         PAPCHAGLNDSCGGFLSGCGPMRLRHGADTRCGRLICGLSTTAQYPPTRFGCAMRWGLPP 203
X14871          PAPCHAGLNDSCGGFLSGCGPMRLRHGADTRCGRLICGLSTTAQYPPTRFGYAMRWGLPP 480
                ************************************************** ********

L78917          WELVVLTARPEDGWTCRGVPAHPGTRCPELVSPMGRATCSPASALWLATANALSLDHALA 540
L28E2E1         WELVVLTARPEDGWTCRGVPAHPGTRCPELVSPMGRATCSPASALWLATANALSLDHALV 263
X14871          WELVVLTARPEDGWTCRGVPAHPGTRCPELVSPMGRATCSPASALWLATANALSLDHAFA 540
                ************************************************************:.

L78917          AFVLLVPWVLIFMVCRRTCRRRGAAAALTAVVLQGYNPPAYGEEAFTYLCTAPGCATQAP 600
L28E2E1         AFVLLVPWVLIFIVCRRACRRRGAAAALAAVVLQGYNPPAYGEEAFTYLCTAPGCATQAP 323
X14871          AFVLLVPWVLIFMVCRRACRRRGAAAALTAVVLQGYNPPAYGEEAFTYLCTAPGCATQAP 600
                ************:****:**********:******************************

L78917          VPVRLAGVRFESKIVDGGCFAPWDLEATGACICEIPTDVSCEGLGAWVPTAPCARIWNGT 660
L28E2E1         VPVRLAGVRFESKIVDGGCFAPWDLEATGACICEIPTDVSCEGLGAWVPTAPCARIWNGT 383
X14871          VPVRLAGVRFESKIVDGGCFAPWDLEATGACICEIPTDVSCEGLGAWVPTAPCARIWNGT 660
                ************************************************************

L78917          QRACTFWAVNAYSSGGYAQLASYFNPGGSYYKQYHPTACEVEPAFGHSDAACWGFPTDTV 720
L28E2E1         QRACTFWAVNAYSSGGYAQLASYFNPGGSYYKQYHPTACEVEPAFGHSDAACWGFPTDTV 443
X14871          QRACTFWAVNAYSSGGYAQLASYFNPGGSYYKQYHPTACEVQPAFGHSDAACWGFPTDTV 720
                ****************************************:*******************

L78917          MSVFALASYVQHPHKTVRVKFHTETRTVWQLSVAGVSCNVTTEHPFCNTPHGQLEVQVPP 780
L28E2E1         MSVSALASYVQHPHKTVRVKFHTETRTVWQLSVAGVSCNVTTEHPFCNTPHGQLEVQVPP 503
X14871          MSVFALASYVQHPHKTVRVKFHTETRTVWQLSVAGVSCNVTTEHPFCNTPHGQLEVQVLP 780
                *** ************************************************** * 

L78917          DPGDLVEYIMNHTGNQQSRWGLGSPNCHGPDWASPVCQRHSPDCSRLVGATPERPRLRLV 840
L28E2E1         DPGDLVEYIMNHTGNQQSRWGLGSPNCHGPDWASPVCQRHSPDCSRLVGATPERPRLRLV 563
X14871          DPGDLVEYIMNHTGNQQSRWGLGSPNCHGPDWASPVCQRHSPDCSRLVGVTPERPRLRLV 840
                ************************************************.**********

L78917          DADDPLLRTAPGPGEVWVTPVIGSQARKCGLHIRAGPYGHATVEMPEWIHAHTTSDPWHP 900
L28E2E1         DADDPLLRTAPGPGEVWVTPVIGSQARKCGLHIRAGPYGHATVEMPEWIHAHTTSDPWHP 623
X14871          DADDPLLRTAPGPGEVWVTPVIGSQARKCGLHIRAGPYGHATVEMPEWIHAHSTSDPWHP 900
                ************************************************:*******

L78917          PGPLGLKFKTVRPVALPRTLAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDLG 960
L28E2E1         PGPLGLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDAG 683
X14871          PGPLRLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDVG 960
                **** *************:************************************ * 

L78917          AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 1020
L28E2E1         AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 743
X14871          AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 1020
                ************************************************************

L78917          WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRGAIAPR 1063
L28E2E1         WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRSAIAPR 786
X14871          WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRGAIAPR 1063
                ************************************.*****
```

Figure 3.12 Sequence analysis of L28-E2E1

The L28-E2E1 amino acid sequence was aligned with the two RA27/3 sequences (GenBank #L78917 & #X1487) using Clustal W software (Thompson *et al.*, 1994).

3.2.5 Correction of E2 T cell epitope sequence

The nucleotide changes in L28-E2E1 were corrected as detailed in section 2.2.4.3. and shown schematically in Figure 3.13.

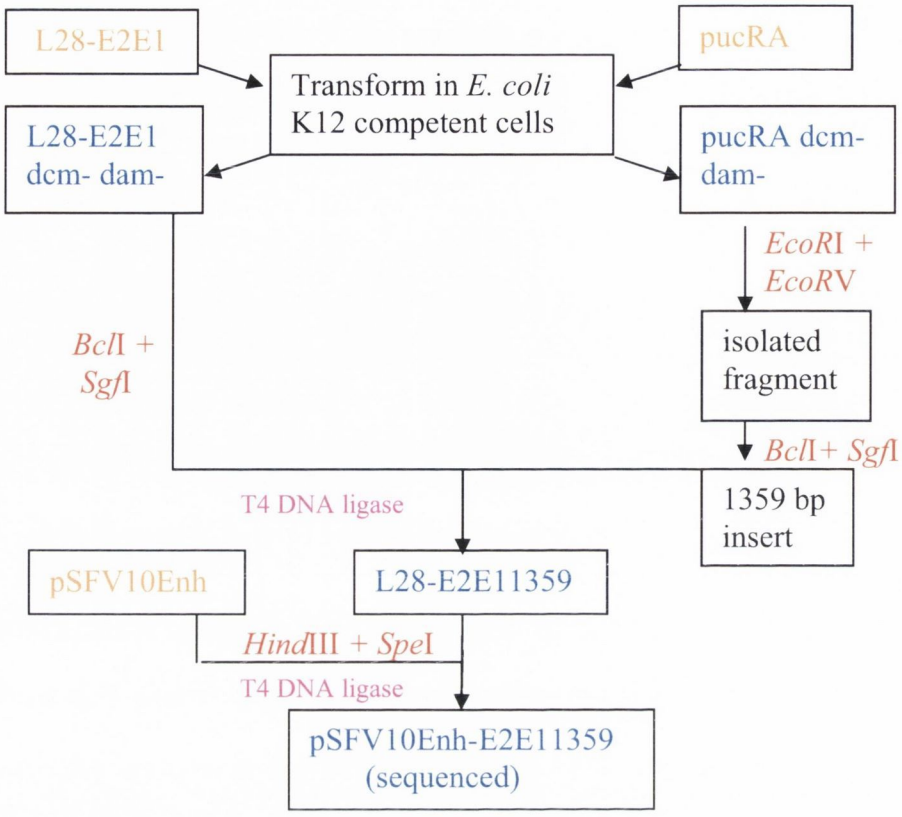


Figure 3.13 Schematic representation of E2 T cell epitope correction

As the pucRA sequence is unpublished, the L78917 sequence was used as a guideline for the location of restriction sites on pucRA (Figure 3.14). The pucRa plasmid was digested with *EcoR*1 and *EcoR*V (Figure 3.15A). The *EcoR*I and *EcoR*V fragment was then digested with *Sgf*I and *Asi*SI (Figure 3.15B) and cloned into the *Sgf*I and *Asi*SI digested L28-E2E1 clone (Figure 3.16). Insertion of this 1359 bp fragment was confirmed by digestion of the unique restriction site *BamH*I on the E1 gene (Figure 3.16).

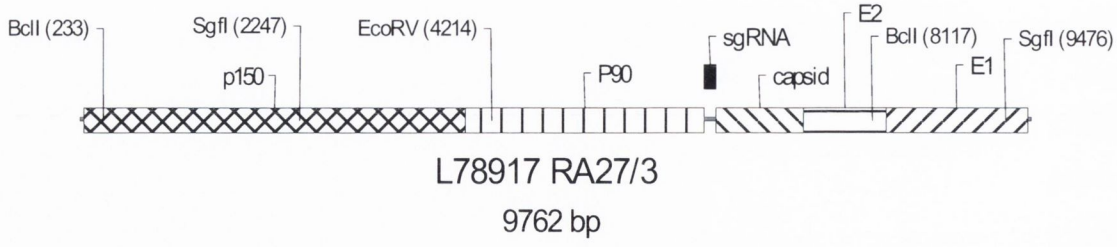


Figure 3.14 Diagrammatic representation of E2 T cell epitope correction

The L78917 RA27/3 vaccine strain sequence and the restriction sites employed to correct the L28-E2E1 T cell epitope region are represented.

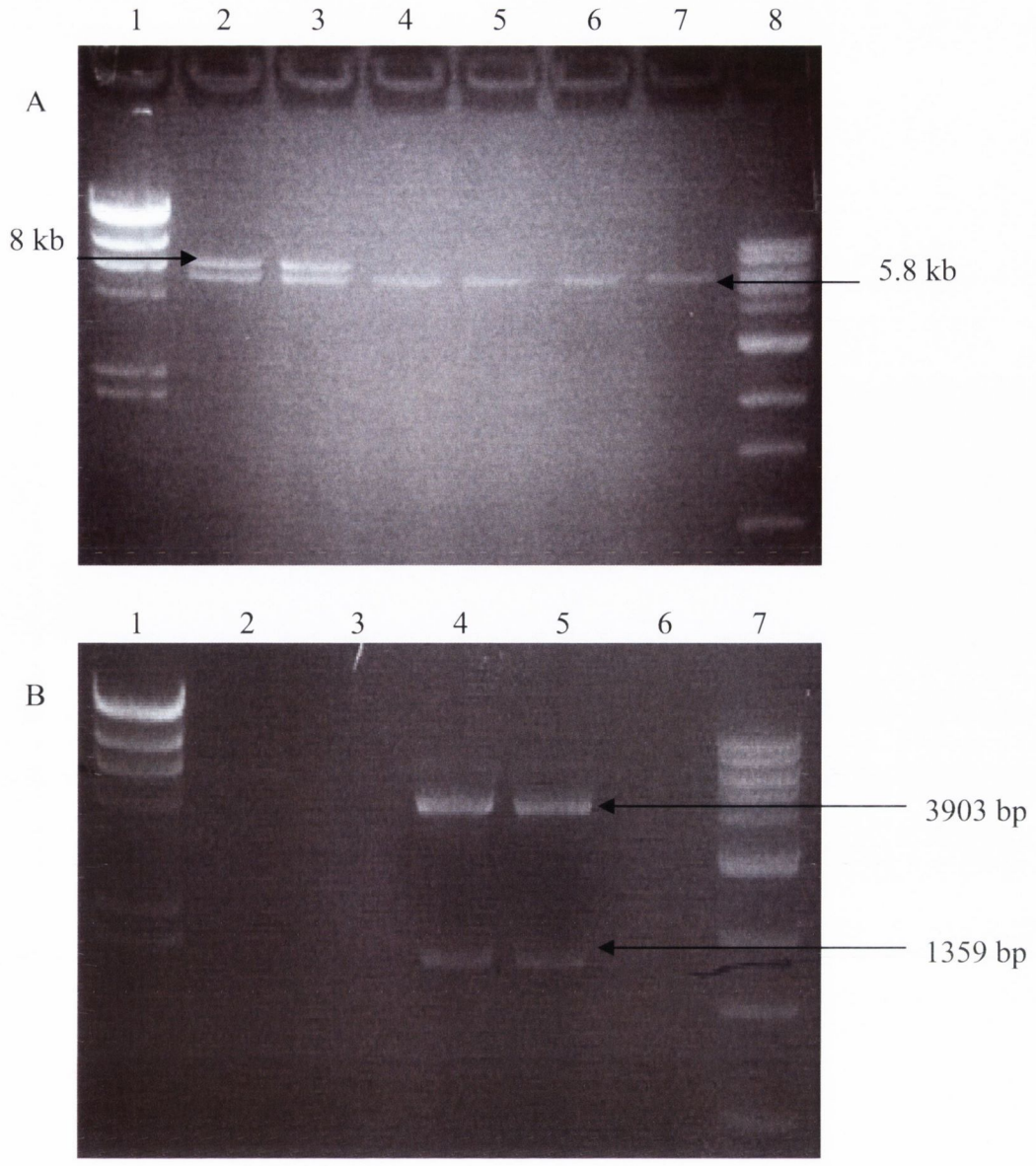


Figure 3.15 Preparation of pucRA for cloning into Litmus28-E2E1

The pucRA plasmid double digested with *EcoR*V and *EcoR*I followed by sequential digestion with *Sgf*I and *Bcl*I.

A) Agarose gel electrophoresis of the pucRA double digestion.

Lane 1; λ *Hind*III DNA marker. Lanes 2 & 3; pucRA *EcoR*V & *EcoR*I digest products showing residual pucRA DNA. Lanes 4-7; gel extracted and purified 5.8 kb product. Lane 8; 0.5 μg of 1 kb molecular weight marker.

B) pucRA 5.8 kb fragment digested with *Sgf*I and *Bcl*I.

Lane 1; λ *Hind*III DNA marker. Lanes 4 & 5; 3903 bp fragment and 1359 bp fragment of interest. Lane 7; 0.5 μg of 1 kb molecular weight marker.

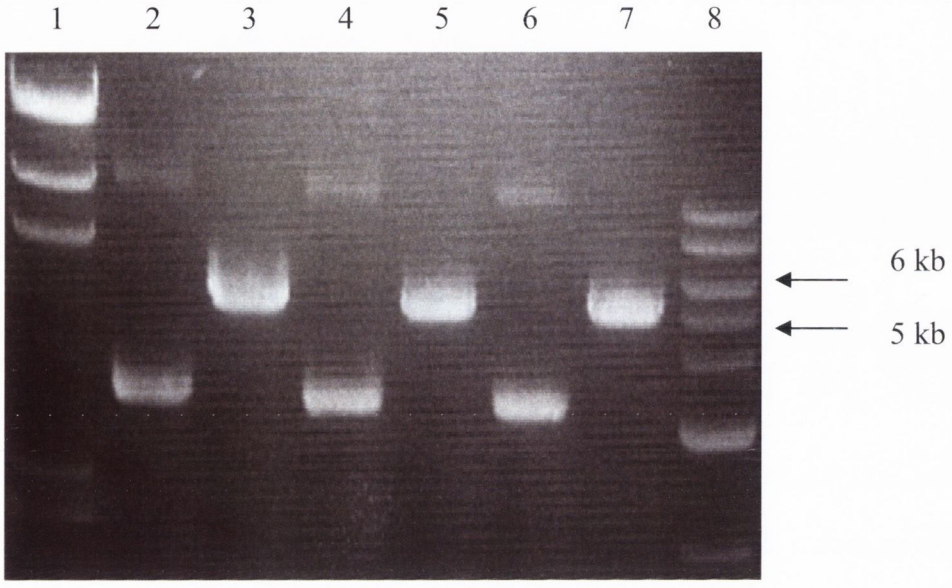


Figure 3.16 Screening for L28-E2E1-1359 clones by restriction analysis

The L28-E2E1 plasmid was digested at the *Bcl*I and *Sgf*I sites and the pucRA 1359 bp fragment ligated into it. The subsequent plasmid mini preparations were screened for inserts by linearising the clones with a *BamH*I restriction site particular to E1.

Lane 1; λ *Hind*III DNA marker. Lanes; 2, 4 & 5 uncut DNA. Lanes; 3, 5 & 7, corresponding *BamH*I cut DNA. Lane 8; 0.5 μg of 1 kb molecular weight marker.

3.2.5.1 Construction of pSFV10Enh-E2E1

The E2E1 gene fragment was excised from the corrected L28-E2E1 clone and cloned into pSFV10Enh at the *Hind*III and *Spe*I sites. Insertion of corrected E2 and E1 genes into pSFV10Enh was confirmed by digestion of the unique restriction site *BamH*I on the E1 gene and by double digestion of the cloned fragment with the *Hind*III and *Spe*I restriction enzymes (Figure 3.17).

3.2.6 Sequencing of pSFV10Enh-E2E1 construct

The pSFV10Enh-E2E1 construct and the pucRA plasmid E2 and E1 region were sequenced using the primers seen in Figure 3.11 and the a.a. sequences aligned with that of the published RA27/3 genomic sequence (GenBank # L78917), the Japanese vaccine strain, TO-336 (GenBank # AB047329), detailed in section 1.2.2 and the L28-E2E1 sequence (Figure 3.18). Sequence data was analysed at the nucleotide and protein level with close attention given to the T cell and B cell epitope conserved sequences of importance in immunological recognition. Of the nine a.a. differences in the L28E2E1 clone, compared to L78917 and highlighted in red, six have been rectified. The defined T cell epitopes depicted in blue (a.a.s; 15-35, 440-414 and 520-531) are now unchanged in pSFV10Enh-E2E1. A fourth T cell epitope lies from a.a. residues 579-680 which also overlaps the HAI and neutralisation sites, $E1_{575-581}$ and $E1_{590-599}$, highlighted in orange. Another critical region for antibody binding $E1_{560-566}$ remains unchanged (Dominguez *et al.*, 1990). The purple region denotes the E2 signal sequence. None of the three changes are in the region where candidate attenuating mutations of the RA27/3 sequence are hypothesized (Pugachev *et al.*, 2000a). A stretch of seven a.a.s including five arginine residues (R-R-A-C-R-R-R) were affected and subsequently corrected (Qiu *et al.*, 1994). This area is located before the putative signal peptide sequence of E1 and after the putative transmembrane anchor domain of E2 and functions to maintain the proper configuration of that region for access of cellular signal peptidase (Qiu *et al.*, 1994). E2 can reach the cell surface alone whereas E1 requires E2 for transport from the Golgi apparatus to the cell surface.

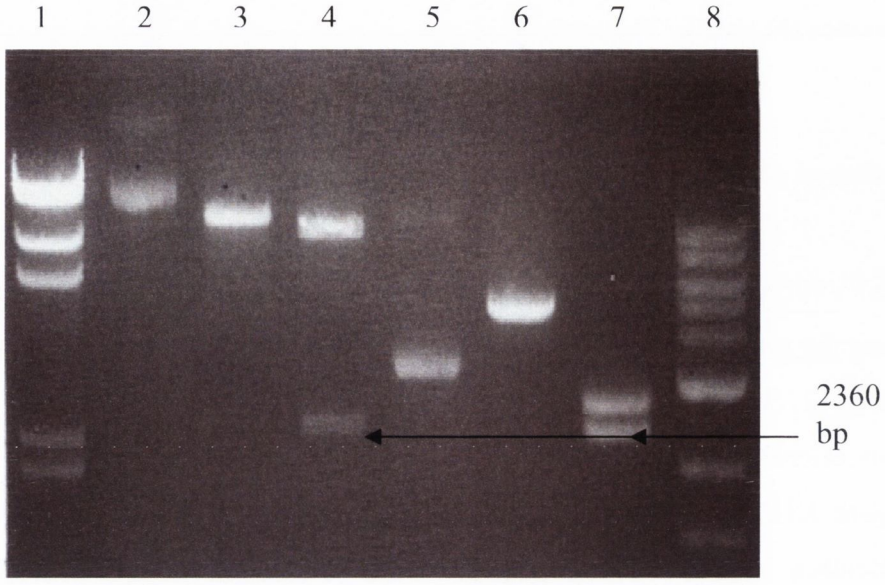


Figure 3.17 Restriction analysis of pSFV10Enh-E2E1

The L28-E2E11359 clone was digested with *Hind*III and *Spe*I and the E2E1 genes containing the pucRA fragment were cloned into the pSFV10Enh plasmid. The resulting clones were verified by restriction analysis using the unique E1 *BamH*I site and by excising the insert at the *Hind*III and *Spe*I sites. The pSFV10Enh-E2E1 DNA was cut with *BamH*I and double digested with *Hind*III and *Spe*I to verify successful insertion.

Lane 1; λ *Hind*III DNA marker. Lane 2; mini prep of uncut pSFV10Enh-E2E1 DNA. Lane 3; *BamH*I cut pSFV10Enh-E2E1 DNA. Lane 4; *Hind*III and *Spe*I pSFV10Enh-E2E1 digested positive clone. Lane 5; mini prep of uncut L28-E2E11359 DNA. Lane 6; *BamH*I cut L28-E2E11359 DNA. Lane7; *Hind*III and *Spe*I L28-E2E11359 digested positive clone. Lane 8; 0.5 μg of 1 kb molecular weight marker.

Figure 3.18 Sequence analysis of pSFV10Enh-E2E1 (*continued*)

```
L78917          PGPLGLKFKTVRPVALPRTLAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDLG 683
pucRA           PGPLGLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDVG 683
AB047329        PGPLGLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTLNGEDVG 960
SFVE2E1         PGPLGLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDVG 683
L28E2E1         PGPLGLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGGNCHLTVNGEDAG 683
                ******************:**********************************:**** *

L78917          AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 743
pucRA           AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 743
AB047329        AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 1020
SFVE2E1         AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 743
L28E2E1         AFPPGKFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQT 743
                ************************************************************

                                                  small → nucleophilic
L78917          WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRGAIAPR 786
pucRA           WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRGAIAPR 786
AB047329        WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRGAIAPR 1063
SFVE2E1         WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRSAIAPR 786
L28E2E1         WAEWAAAHWWQLTLGAICALLLAGLLACCAKCLYYLRSAIAPR 786
                ************************************.*****
```

Figure 3.18 Sequence analysis of pSFV10Enh-E2E1 by comparison with RV vaccine strain sequences

Clustal W sequence protein alignment comparing the pSFV10Enh-E2E1 clone to the published RA27/3 genomic sequence (GenBank: L78917), a Japanese vaccine strain (GenBank: AB047329), the pucRA E2E1 sequence from Fusion Antibodies and the L28-E2E1 clone sequence.

3.2.7 *In vitro* transcription

Helper and recombinant (rSFV10Enh-E2E1) plasmids were linearised prior to transcription using the unique *Nru*I restriction site on the recombinant SFV10Enh-E2E1 plasmid (Figure 3.19) and the *Spe*I site found on helper SFV plasmids (Figure 3.20) and analysed on a gel. These linearised helper and recombinant (rSFV10Enh-E2E1) plasmids were *in vitro* transcribed and analysed on a 0.6% agarose gel (Figure 3.21).

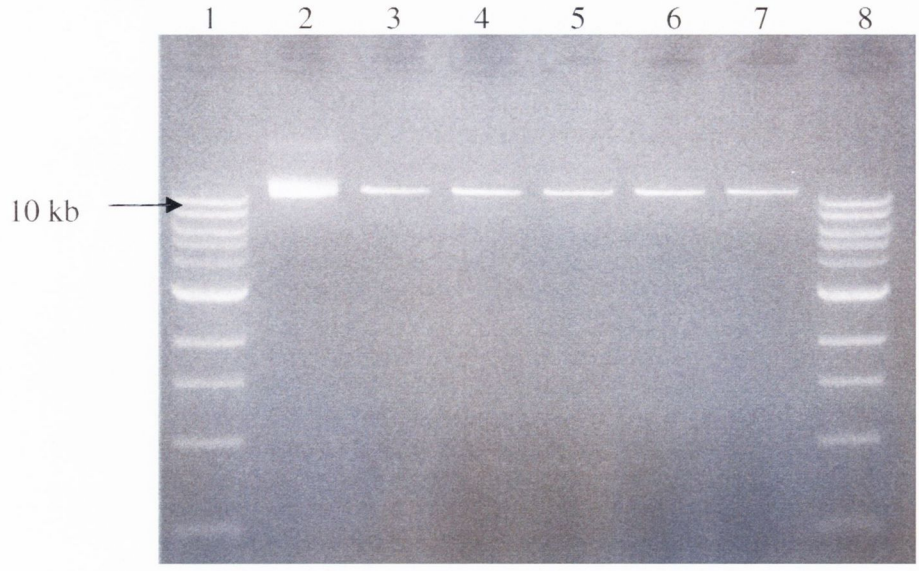


Figure 3.19 Linearised recombinant pSFV10Enh-E2E1 plasmid

Recombinant (rSFV10Enh-E2E1) plasmids were linearised prior to transcription using the unique restriction site *Nru*I on recombinant SFV10Enh-E2E1 plasmids. Linearised plasmid concentrations were calculated using the band intensity from the gel used for analysis and the amount of DNA needed for transcription was thus calculated.

Lanes 1 and 8; 0.5 ug of 1 kb DNA molecular weight marker. Lane 2; undigested pSFV10Enh-E2E1. Lanes 3-7; 1 µl of linearised pSFV10Enh-E2E1 DNA.

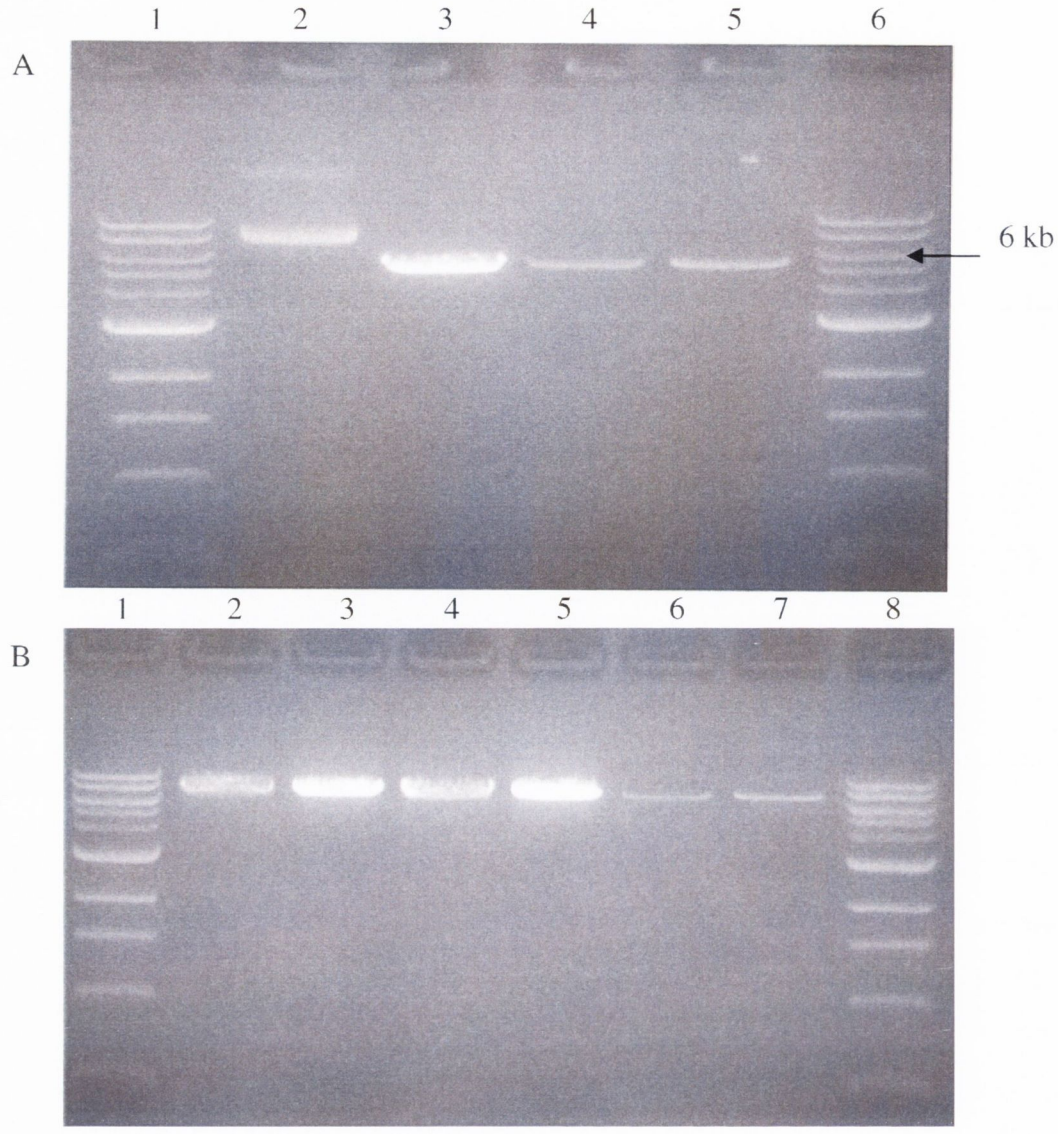


Figure 3.20 Linearised helper SFV plasmids

Helper plasmids were linearised prior to transcription using the unique restriction site *Spe*I found on helper SFV plasmids. Linearised plasmid concentrations were calculated using the DNA marker band intensity from the gel used for analysis and the amount of DNA needed for transcription was thus calculated.

A) Lanes 1 and 6; 0.5 ug of 1 kb DNA molecular weight marker. Lane 2; 1 µl of undigested pSFV-Helper CS219A DNA control. Lanes 3 to 5; 1 µl of digested pSFV-Helper CS219A DNA.

B) Lanes 1 and 8; 0.5 ug of 1 kb DNA molecular weight marker. Lanes 2 to 7; 1 µl of digested pSFV-Helper S2 DNA.

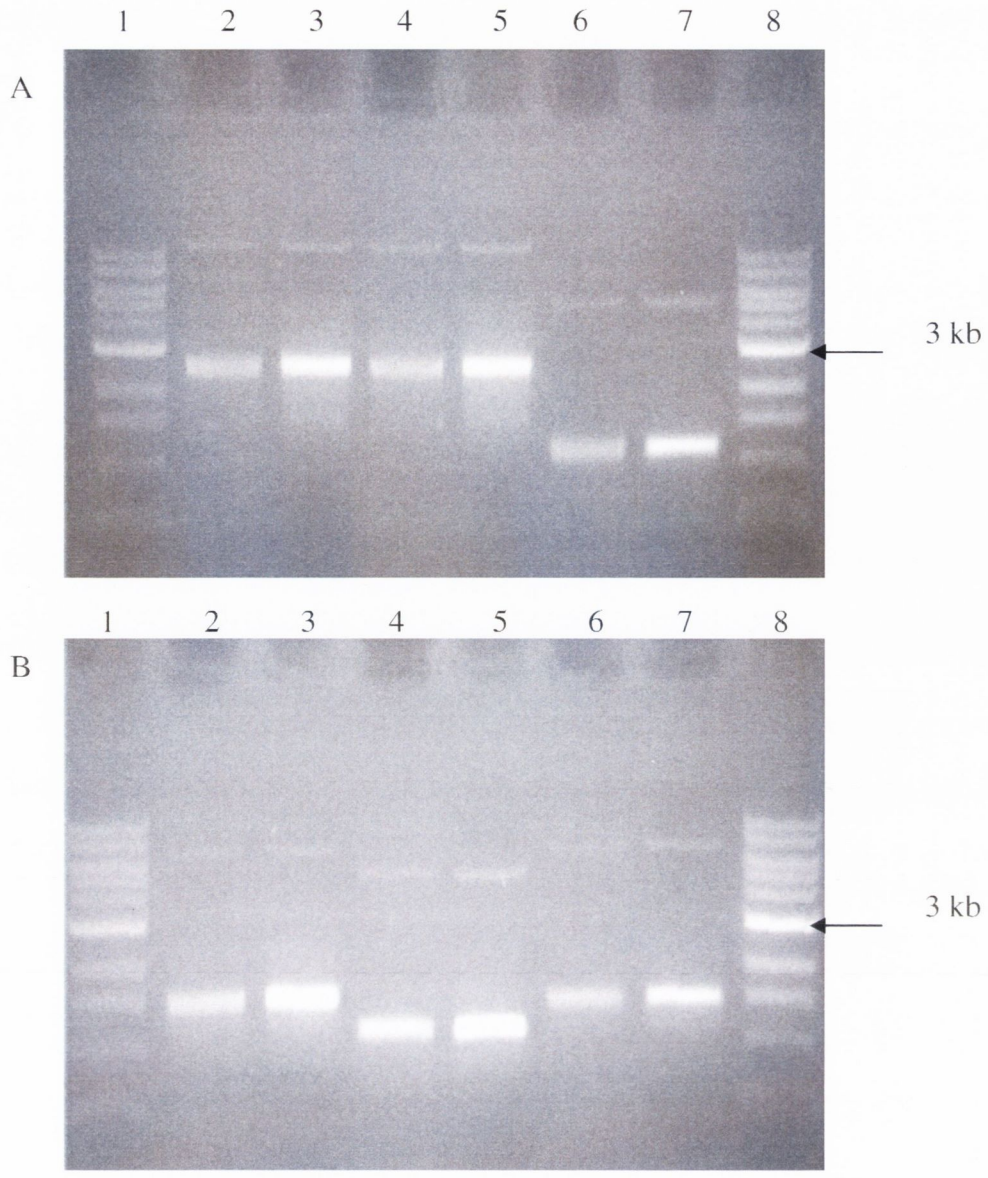


Figure 3.21 *In vitro* SP6 RNA transcription of helper and recombinant plasmids

Helper and recombinant (pSFV10Enh-E2E1) plasmids were *in vitro* transcribed and analysed on a 0.8% agarose gel stained with ethidium bromide. The concentration of the *in vitro* transcribed RNAs was approximately estimated by considering the staining intensity of the RNA bands.

A) Lanes 1 and 8; 0.5 ug of 1 kb DNA molecular weight marker. Lanes 2 and 4; 0.25 µl each of rSFV10Enh-E2E1 RNA. Lanes 3 and 5; 0.5 µl each of rSFV10Enh-E2E1 RNA. Lanes 6 & 7; 0.25 µl and 0.5 µl each of rSFV-Helper CS219A RNA.

B) Lanes 1 & 8; 0.5 ug of 1 kb DNA molecular weight marker. Lanes 2 & 6; 0.25 µl each of rSFV-Helper S2 RNA. Lanes 3 & 7; 0.5 µl each of rSFV-Helper S2 RNA. Lanes 4 & 5; 0.25 µl and 0.5 µl each of rSFV-Helper CS219A RNA.

3.2.8 Expression of rSFV10Enh-E2E1 particles *in vitro*

The expression of the rSFV10Enh-E2E1 particles was measured qualitatively in sBHK cells (Figure 3.22). This was carried out by immunofluorescent staining of the E1 protein and examination of the infected cells using microscopy. To check for conformation different epitope specific monoclonal antibodies could have been custom-designed to bind to the regions of the sequence that differed from the published sequences. Western blot analysis could have been used to check for size differences between the N-glycosylated E2 protein and the cloned E2 protein with the amino acid change at the N-linked glycosylation site. Not all N-linked glycosylation sites are used by viruses and this could have been used to determine based on size differences whether the site at residue 94 on the L28E2E1 clone was essential.

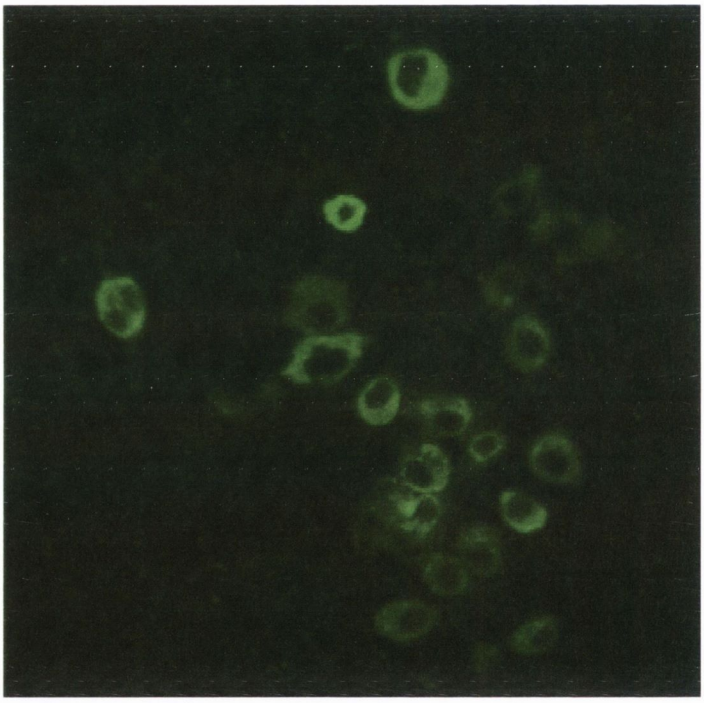


Figure 3.22 Qualitative analysis of E1 expression in Swedish BHK cells by immunofluorescence

The expression of E1 was qualitatively examined by immunofluorescence (IF) staining of sBHK cells infected by VLPs of rSFV10Enh-E2E1. IF was absent in mock infected cells (not shown). Original magnification of 40X.

3.3. Discussion

Current knowledge indicates that this is the first time that the RA27/3 E2 and E1 structural genes have been cloned and expressed in an SFV vector with a view to assessing the immunogenicity of the two RA27/3 envelope genes. A nested PCR strategy was decided on to avoid complications arising in the reaction due to secondary structures in the template. The external oligonucleotides (R2 and R7) were initially used with Taq polymerase to produce a 185-bp PCR product of a region of E1 from AMV reverse transcribed cDNA and thus to determine infection of Vero cells with RA27/3 RV (Bosma *et al.*, 1995). This RT-PCR method accurately detected four different RV isolates and four vaccine strains and is being developed for prenatal diagnosis of congenital rubella infection (Bosma *et al.*, 1995). Thus a nested PCR method was subsequently employed and the MMLV RT was used as per the literature (Eggerding *et al.*, 1991). In these RT-PCR reactions E2 and E1 specific primers were used in conjunction with the MMLV RT and the Triplemaster PCR system to yield E2 and E1 DNA. The cDNA synthesis method was changed to incorporate an RT kit using a version of MMLV RT that is engineered to reduce RNase H activity and provide increased thermal stability. The MMLV RT is an RNA-dependent polymerase that can be used in cDNA synthesis producing long messenger RNA templates. In fact it is the preferred reverse transcriptase for long mRNA templates because the RNase H activity of MMLV RT is weaker than the commonly used AMV RT. A nested PCR was preferable as no template was amplified using the primers based around the 5' and 3' end of each gene.

The RV genome is difficult to amplify due to the high concentration of GC bases and the potential for SL formation and competition of dNTPs. The temperatures that are associated with destabilising these secondary structures are above 95 °C and to maintain the stability and fidelity of the Triplemaster enzyme, a 5X Taqmaster Enhancer was employed. The use of DMSO, a G-C destabilising co-solvent was discontinued due its negative effect on the ability of the taq polymerase to proofread DNA. The nested PCR reduced background amplification and improved the efficiency of amplification. The external primer amplification of a long sequence, including a region just outside the target gene, during the first round of PCR increased the amount of target for the second round of amplification with the internal primers. Non target bands were suppressed or eliminated with nested primers, since the internal and external primers did not bind to the same sites (Eggerding *et al.*, 1991). Sequencing of the G-C rich RA27/3 template was problematic, requiring many regions to be re-sequenced due to interference from GC nucleotides in the

readout. Examples of these regions are a stretch of GC rich nucleotides at the 3' end of the E2 gene and the 5'end of the E1 gene. Translation of the sequence into protein, using the Baylor college of Medicine Sequence Utilities facility, has demonstrated that there were nine a.a. changes in total in the proteins when compared to the published RA27/3 genomic sequence (GenBank Accession #L78917). Two changes arose within a defined E2 T cell epitope (a.a. 276, isoleucine [I] to methionine [M] and a.a. 281, alanine [A] to threonine [T]) and no changes occurred in any of the three antibody binding regions. The change in the E1 signal sequence was at a.a. 292, alanine [A] to a threonine [T]. These changes and three more in undefined regions of the sequence were rectified by insertion of a 1359 bp fragment of the pucRA RA27/3 infectious cDNA clone as confirmed by sequence analysis. The polymerases that catalyze RNA replication and reverse transcription have minimal proof reading activities and may have had an effect on the RA27/3 genome sequence extracted from the infected Vero cells (Chantler *et al.*, 2001). Triplemaster polymerase with 2.3×10^{-6} fidelity was used instead of Taq polymerase which has 12.5×10^{-6} fidelity. The SFV replicase error rate has not been measured.

None of the changes represent a reversion to the wt sequence (GenBank: NC_001545) (Dominguez *et al.*, 1990). Of the remaining three changes, one is in an N-glycosylation site in the E2 protein (a.a. 94, asparagine [N] to aspartic acid [D]) and the other is adjacent to an N-glycosylation site at a.a. 72 at a residue perhaps involved in the formation of disulfide bonds (a.a. 72, cysteine [Q] to arginine [R]). Cysteine contains a sulfhydryl group (-SH) and this sulfur containing side chain is hydrophobic and highly reactive (Stryer, 1995). Cysteine plays a role in some proteins by forming disulfide links. Arginine is a basic amino acid, which is positively charged at neutral pH and has a very polar side chain which renders it hydrophilic (Stryer, 1995). Thus this change may have disrupted the protein folding. As this region does not contain any known conformational epitopes, this change may not affect presentation of the E2 protein to the immune system. Asparagine-linked (N-linked) carbohydrate moieties, often present on membrane bound and secreted proteins, can be removed in the cytosol by an enzyme reaction. The reaction changes the asparagine residue into aspartic acid and this diagnostic sequence can be seen in some peptides presented by MHC I molecules (Janeway *et al.*, 2005). Thus this residue change may confer an advantage in terms of antigen presentation of the E2 protein. Aspartic acid contains two acidic side chains and is usually called aspartate to emphasise that its side chain is nearly always negatively charged at physiological pH (Stryer, 1995). Asparagine is the uncharged derivative of aspartate and contains a terminal amide group in place of a carboxylate one.

The El and E2 proteins contain N-linked oligosaccharide as a result of their passage through the ER and Golgi apparatus (Hobman *et al.*, 1992). It has generally been accepted that glycosylation is essential to aid proteins in attaining and maintaining correct tertiary structure and undergoing intracellular transport and this process in E2 slows the movement of the E2 from the ER to the Golgi apparatus (Qiu *et al.*, 1992a). Slower glycan processing, lower stability and aberrant disulfide bonding of the mutant proteins are the result of removal of glycosylation sites from the E2 and E1 proteins (Qiu *et al.*, 1992a; Qiu *et al.*, 1992b). However studies suggest that RV E2 protein is a poor T cell Ag in healthy patients which may be due to this protein's high degree of glycosylation with both N- and O-linked sugars (Mauracher *et al.*, 1993). It has been shown that unglycosylated E1 expressed *in vivo* is still antigenic (Hobman *et al.*, 1992). Hemagglutinating (HA) and virus-neutralising (VN) epitopes of RVs are recognised by antibody whether or not the epitopes are glycsosylated (Chaye *et al.*, 1992a).

The third change is in the cytoplasmic tail (glycine [G] to serine [S]) and all are surrounded by a mixed a.a. profile. Glycine is the simplest a.a., having just one hydrogen atom as its side chain and is unique in being optically inactive. Serine contains aliphatic hydroxyl groups making it hydrophilic and reactive. The cytoplasmic domain of E1 is formed by a stretch of 13 a.a.s and this is preceded by a putative E1 transmembrane domain of 22 residues (Yao *et al.*, 1999). It has been postulated that the residues YYLRG in the E1 cytoplasmic tail constitute a domain that may interact with other proteins and be involved in virus release (Yao *et al.*, 2000). The E1 cytoplasmic domain may compel virus budding by interactions with NCs and interaction of C with the cytoplasmic tail of E1 is thought to play a role in RV virion assembly (Hobman *et al.*, 1994). Thus once the E2-E1 heterodimer is formed in the ER it is transported to the Golgi complex where RV virus buds through cellular membranes (Yao *et al.*, 1998). In BHK cells, the primary budding site for RV is at the Golgi membrane whereas in Vero cells, budding occurs at the PM. This function would not be necessary in terms of an SFV particle expressing rubella proteins where RV virions are not produced. In contrast the N-terminal region of the SFV E2 appears to be of importance in alphaviruses as a cytoplasmic tail (Yao *et al.*, 1999). Of note, the source of the vaccine virus differs in the case of each strain, from ATCC RA27/3 virus for the pSFV10Enh-E2E1 clone to Meruvax II RA27/3 for the L78917 sequence. Thus it is entirely possible that these sequences were different from the outset of these experiments. The RA27/3 vaccine virus was also passaged in Vero cells and RV defective-interfering (DI) RNAs may have been generated which contained deletions in the SP-ORF (Chen *et al.*, 2004a).

Chapter 4

Humoral responses to Ervevax vaccine & SFV based MMR constructs

4.1 Introduction

The variability of humoral and cellular immune responses modulated by human leukocyte antigen (HLA) genes is a significant factor in the protective effect of rubella vaccines in humans (Ovsyannikova *et al.*, 2004b). Results suggest that polymorphisms in HLA-B and HLA-C genes may influence the immunogenicity of the rubella vaccine and identification of such HLA-restricted responses would further work to identify epitopes that could be utilised in the design of rubella vaccines in genetically polymorphic populations (Ovsyannikova *et al.*, 2004b). An association of HLA genotype with incidence of joint reaction has been found (Ou *et al.*, 1998). This may allow the prediction of individuals who have the genetic susceptibility to develop complications and opens the way to selective immunisation of this target group with a more attenuated vaccine (Knipe *et al.*, 2001). HLA alleles have been found to have important associations with measles antibody seropositivity and this has implications for studies on MV antigen processing and presentation in seronegative and hyperseropositive individuals (Ovsyannikova *et al.*, 2004a). There are very few papers in the literature on assessment of immunity to RV in different mice strains (Ou *et al.*, 1994). In contrast to other CNS-tropic viruses, RV does not induce obvious clinical symptoms in BALB/c mice (Wang *et al.*, 2003). Mice cannot be used as a challenge model because they do not develop overt disease, but both humoral and cell-mediated responses to infection can be detected after intravenous, intrasplenic or intramuscular injection in mice of different haplotypes (Pougatcheva *et al.*, 1999).

Reports indicate a clear superiority in immunogenicity of bicistronic constructs rather than two coadministered plasmids (Song *et al.*, 2005). It has however been shown that coadministration of two separate DNA vaccines by i.m. immunisation can result in coexpression of both antigens in the same myocytes and stimulation of immune responses to both antigens (Song *et al.*, 2005). In this study the RA27/3 rubella E2E1, Edmonston measles H and Enders mumps HN antigens were injected intramuscularly by co-administration of SFV plasmids expressing the genes. The immune responses were assessed by ELISA and a latex agglutination assay. In central Europe the hemagglutination inhibition (HAI) test is the most extensively used method for determination of RV immune status (Giessauf *et al.*, 2004). The latex agglutination assay used in this study is routinely used in human serodiagnostic screening and a positive reaction using undiluted serum is considered immune (Pougatcheva *et al.*, 1999). This chapter thus describes the selection of the BALB/c mouse model and the RV-specific immune responses induced by the rSFV clones expressing the RA27/3 rubella, Edmonston measles and Enders mumps antigens.

4.2 Results

4.2.1 Determination of route of administration and mouse model with Ervevax.

The null hypothesis for this pilot study was that there would be no difference in mean antibody titres to Ervevax in the four different strains of mice, immunised as described in section 2.2.6.1 and illustrated in Figure 4.1.

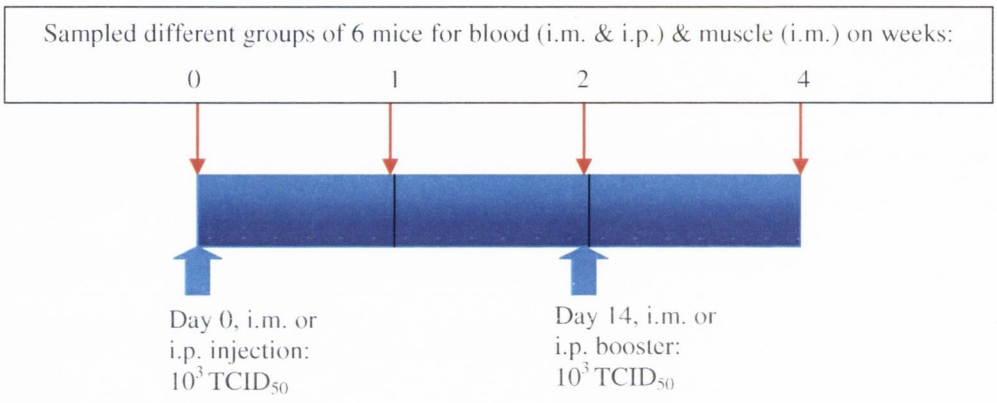


Figure 4.1 Strategy for *in vivo* experiments examining the immune response to Ervevax

Ervevax administered by the i.m. route elicited immune responses across the range of strains and the differences in titres were not significant (Figure 4.2). When the Bonferroni correction was applied to the i.m. results, at 28 days, the titres elicited in Outbred Swiss mice were no more significant than those in BALB/c mice. Thus as BALB/c mice are an inbred strain with a defined haplotype further studies were carried out in BALB/c mice using the i.m. route. When immunised with Ervevax i.p., the Outbred Swiss strain developed the highest mean antibody titre. This difference was significant as analysed by one-way ANOVA (Figure 4.3). The sera in this experiment were analysed with an ELISA designed to detect E1 specific antibodies using a standard curve derived from doubling dilutions of an E1 specific monoclonal antibody.

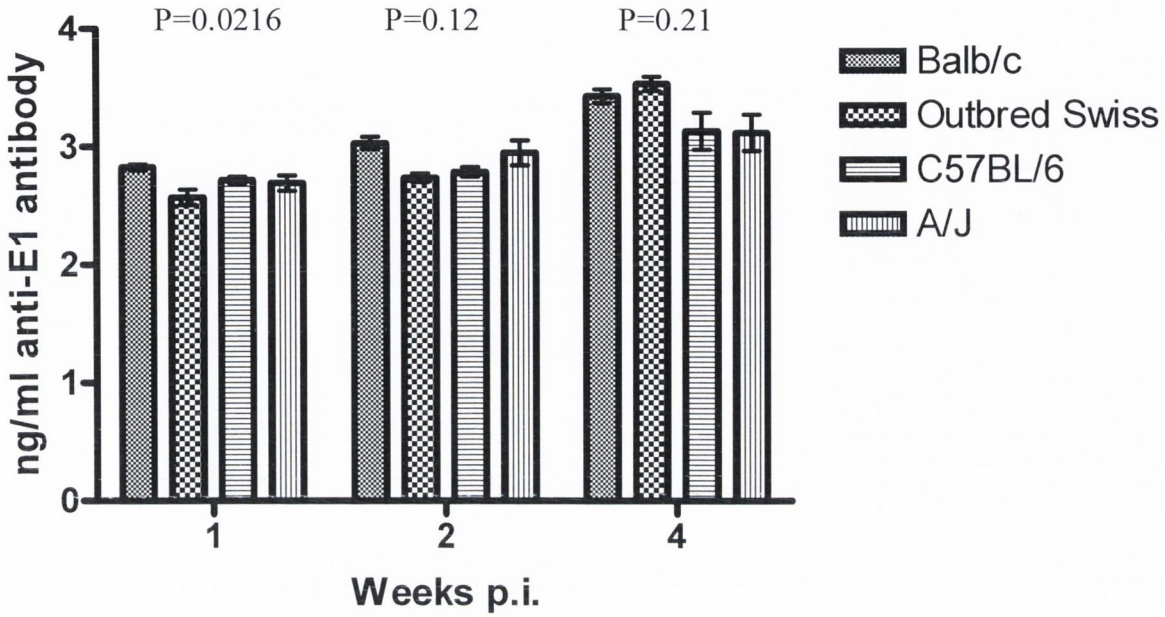


Figure 4.2 Humoral immune response to i.m. administered Ervevax in mice with defined and undefined MHC backgrounds

RV E1-specific antibody titres (ng/ml) elicited by Ervevax in BALB/c, Outbred Swiss, C57BL/6 and A/J mice (n=6) at 1 and 2 weeks post initial immunisation and 2 weeks post boost (4 weeks) when administered i.m. Bars represent the unpaired $\log_{10}$ mean antibody titres and the standard error of the mean is shown as a two sided error bar. There were no significant differences between titres in the different strains at 4 weeks (P=0.21, one-way ANOVA).

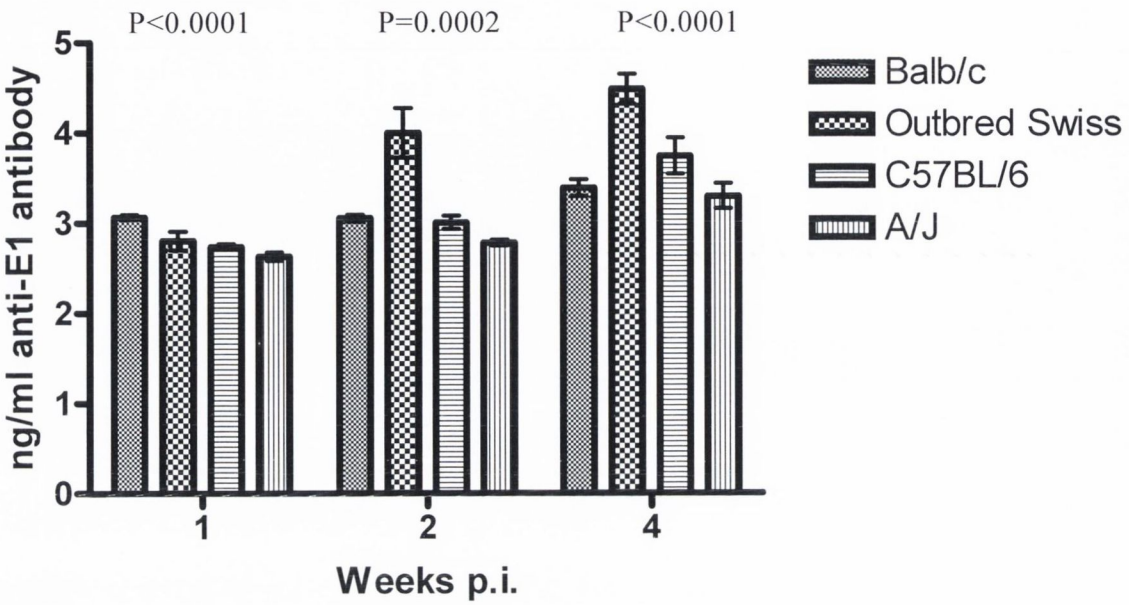


Figure 4.3 Humoral immune response to i.p. administered Ervevax in mice with defined and undefined MHC backgrounds

RV E1-specific antibody titres (ng/ml) elicited by Ervevax in BALB/c, Outbred Swiss, C57BL/6 and A/J mice (n=6) at 1 and 2 weeks post initial immunisation and 2 weeks post boost (4 weeks) when administered i.p. Bars represent the unpaired $\log_{10}$ mean antibody titres and the standard error of the mean is shown as a two sided error bar. The booster injection significantly increased the titres in all mice. However when administered i.p. Ervevax elicited the highest titres in Outbred Swiss mice pre and post boost as determined by the Bonferroni post test.

4.2.2 Histology of muscle tissue

Lesions in all four mouse strains were prominent 7 days post Ervevax immunisation and most had resolved at 28 days post immunisation (14 days post boost). Myofiber necrosis and mineralisation were prominent at 7 days post vaccination (Table 3, Figures 4.4 & 4.5). Myofiber regeneration, indicated by sarcolemmal nuclear proliferation (SNP), was most active at 7 days post vaccination and was almost complete at 28 days. Inflammation of interstitial fat deposits (steatitis) was seen only in mice sampled at day 7 post vaccination. No abnormalities were detected in muscle samples from control muscle.

Table 3. Tabular representation of the mean pathology scores for 6 mice per group

Strain	Days post prime	Pathology
BALB/c	7	SNP[a], myofiber necrosis[b], 3+[c]
Outbred Swiss	7	SNP, steatitis[d], 3+
C57Bl/6	7	SNP, 3+
A/J	7	SNP, myofiber necrosis, steatitis, 3+
BALB/c	14	SNP, 2+
Outbred Swiss	14	SNP, 2+
C57Bl/6	14	SNP, 1+
A/J	14	SNP, myofiber necrosis, 2+
BALB/c	28 (14 days post boost)	SNP, myofiber necrosis, 1+
Outbred Swiss	28 (14 days post boost)	SNP, 1+
C57Bl/6	28 (14 days post boost)	SNP, myofiber necrosis, 1+
A/J	28 (14 days post boost)	SNP, 1+

[a] SNP denotes Sarcolemmal nuclear proliferation, indicative of muscle regeneration

[b] Myofiber necrosis denotes loss of myofiber striations which are formed by sarcomeres

[c] Lesions were graded on a scale of 0-5, grade 5 indicating the most severe pathology

[d] Steatitis denotes inflammation of fat cells

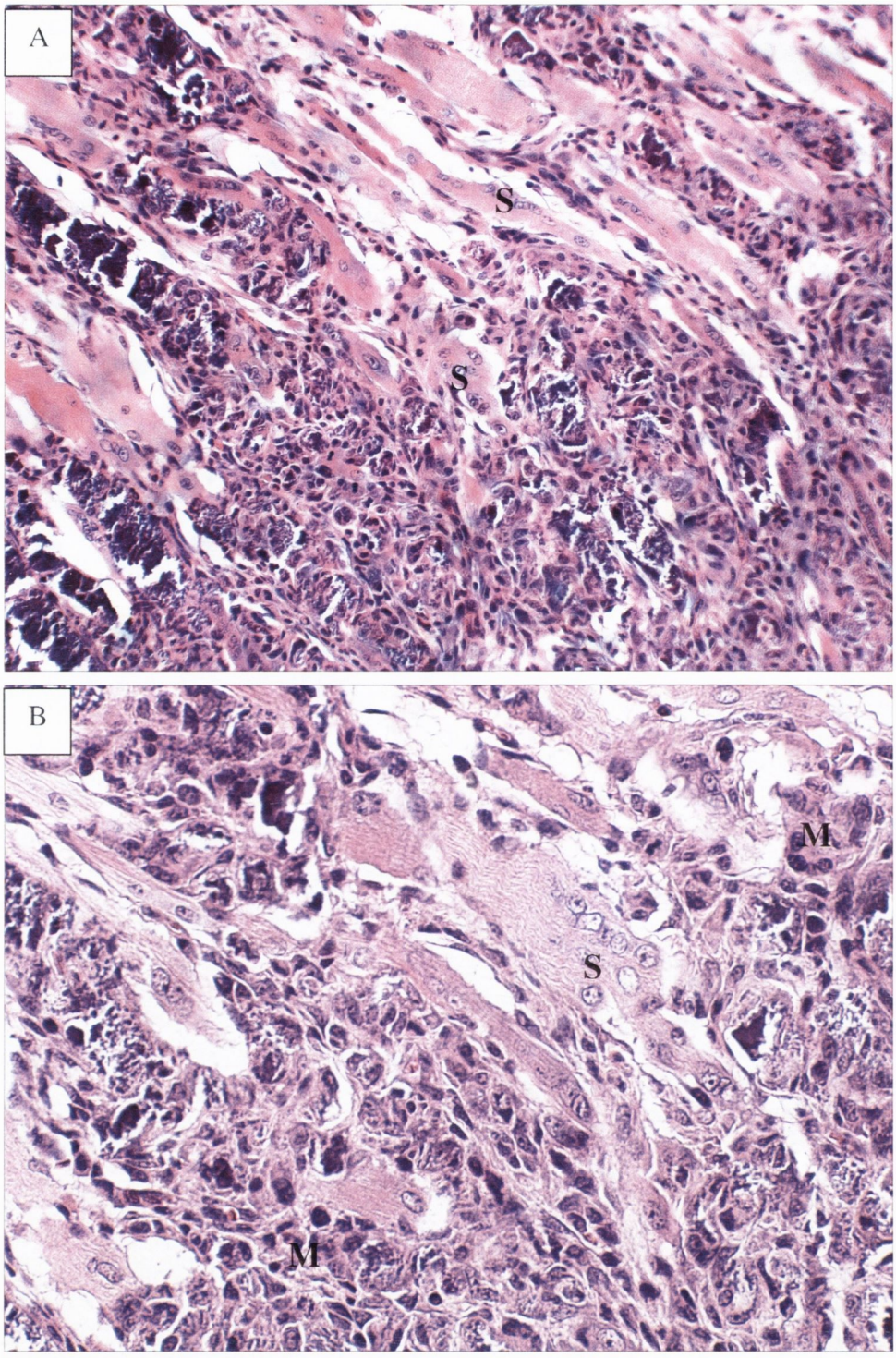


Figure 4.4 Histological changes at sites of intramuscular inoculation in BALB/c mice

(A) 7 dpi. Myofiber necrosis, mineralisation and macrophages (M). Surviving myofibers are atrophic and show sarcolemmal nuclear proliferation (S) indicative of regeneration. H&E, x20. (B) Higher magnification of (A). H&E, x40.

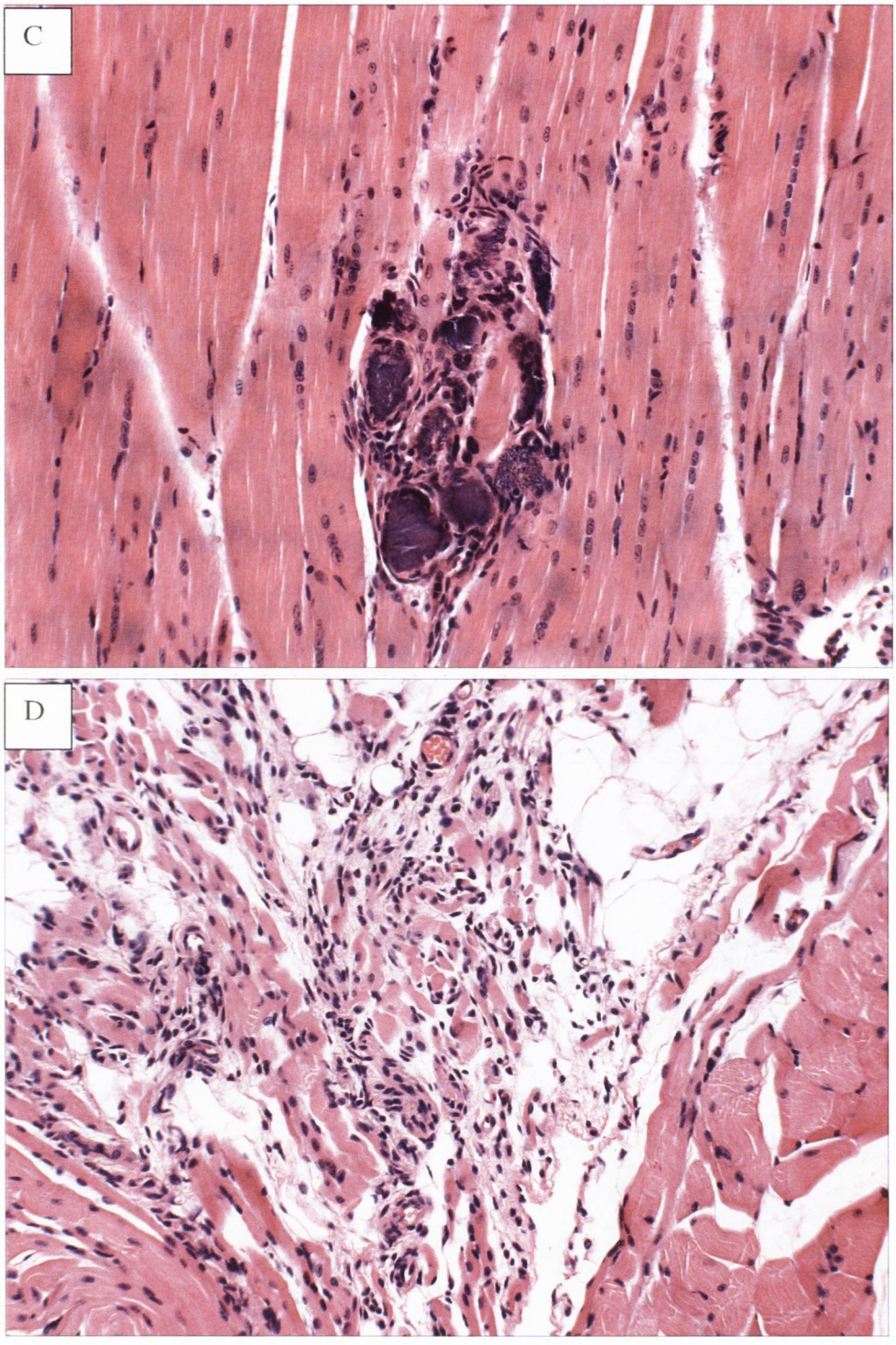


Figure 4.5 Histological changes at sites of intramuscular inoculation in BALB/c mice

(C) 14 dpi. Localised myofiber mineralisation. H&E, x20. (D) 28 dpi. Myofiber atrophy and fibrosis. Leucocytic infiltrates are scanty and mineralisation is absent. H&E, x20.

4.2.3 Humoral immune responses of primed BALB/c mice to monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN when boosted at two weeks.

Groups of ten mice were immunised as described in section 2.2.6.2.1 and illustrated in Figure 4.6. Unpaired antibody titres were determined by a limiting dilution ELISA (section 2.2.7.2) performed according to the literature (Berglund *et al.*, 1999) and a latex agglutination assay (section 2.2.7.3). The rSFV particles expressing the measles Edmonston H antigen and the mumps Enders HN antigen were gifts from Dr Marina Fleeton and Dr Barbara Kelly (section 2.1.2).

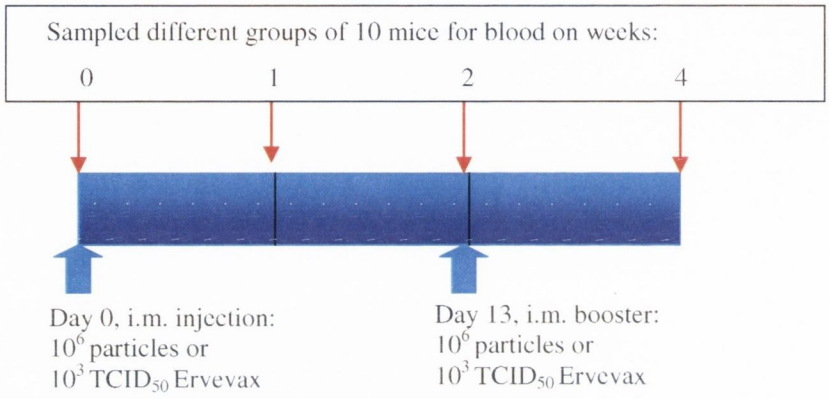


Figure 4.6 Strategy for examining the *in vivo* immune response to monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN after a two week boost

A booster immunisation at two weeks resulted in an increase in whole IgG RV-specific titres as evident by the difference in mean titres in weeks two and four ($P<0.001$) (Figure 4.7). The anti-E2E1 whole IgG ELISA titre of mice immunised and boosted with rSFV10Enh-E2E1 (E2E1) as demonstrated in Figure 4.8 was significantly higher than that induced by rSFV particles expressing the RA27/3 RV E2E1, Edmonston MV H and Enders Mumps HN antigens (E2E1, H, HN) ($P=0.01$). This result was confirmed and in fact more pronounced by data from the same mice using the latex agglutination assay (Figure 4.9) ($P<0.001$). No agglutination was observed in the serum of mice immunised and boosted at two weeks with 1000 $TCID_{50}$ Ervevax vaccine (Figure 4.9). RV is known to replicate poorly in animals other than its human host and perhaps a higher dose would have been necessary to induce functional antibodies.

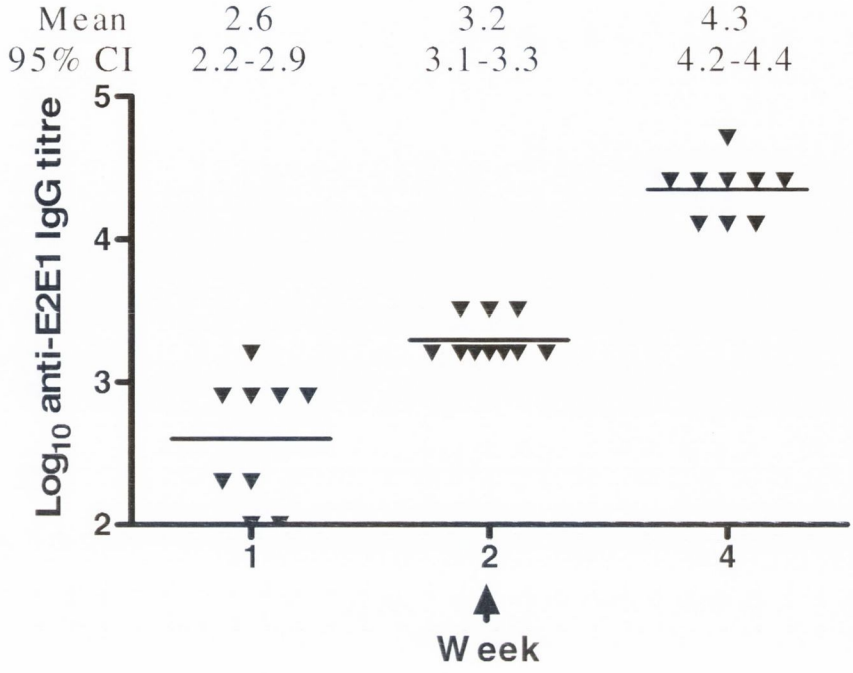


Figure 4.7 Scatter plot of the serum anti-RA27/3E2E1 IgG responses in BALB/c mice immunised with the monovalent rSFVE2E1

Paired data points indicate the reciprocal cut-off titre for individual mice as determined by a two fold limiting dilution ELISA. The data were analysed by one-way ANOVA with the Bonferroni multiple comparison. The vertical arrow indicates the boost regimen. Log_{10} mean titres and 95% confidence intervals are shown.

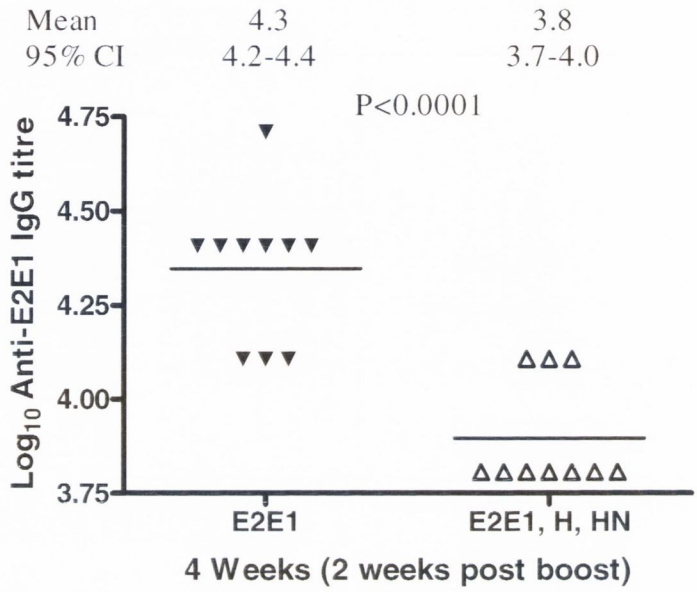


Figure 4.8 Scatter plot of the serum anti-RA27/3E2E1 IgG responses in BALB/c mice immunised with monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN

Data points indicate the reciprocal cut-off titre for individual mice as determined by a two fold limiting dilution ELISA starting from 1:50. The data were analysed by the Students *t* test and the titres elicited by monovalent rSFVE2E1 were higher than those elicited by trivalent rSFVE2E1, rSFVH plus rSFVHN. Log_{10} mean titres and 95% confidence intervals are shown.

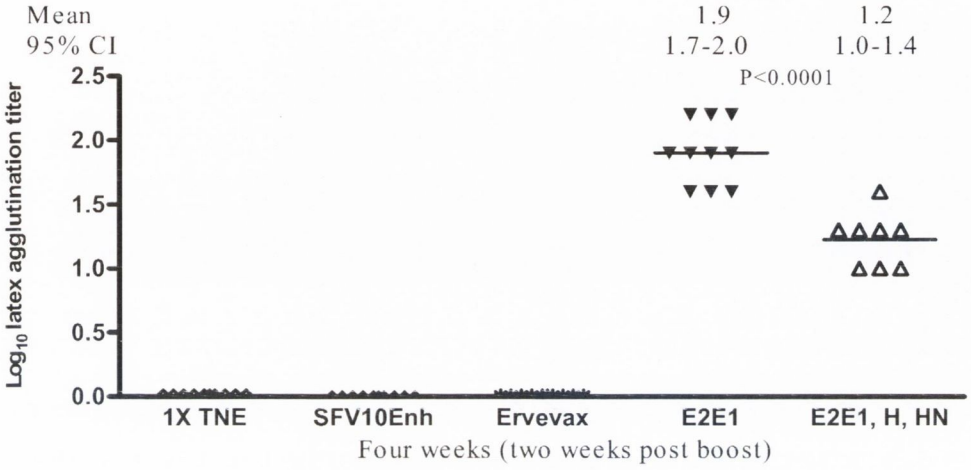


Figure 4.9 Functional antibody response induced by monovalent rSFVE2E1 and trivalent SFVE2E1, rSFVH plus rSFVHN after a 2 week boost

Reciprocal titres were determined using a latex agglutination assay and statistical analysis was carried out using the unpaired *t* test and the monovalent rSFVE2E1 construct (geometric mean, 79 [95% CI, 50-100]) was found to significantly elicit higher latex agglutination titres than the trivalent rSFVE2E1, rSFVH plus rSFVHN constructs (geometric mean, 16 [95% CI, 10-25]). Log_{10} mean titres and 95% confidence intervals are shown.

4.2.4 Humoral immune responses of primed BALB/c mice to monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN when boosted at four weeks

Groups of ten mice were immunised i.m. as described in section 2.2.6.2.2 and illustrated in Figure 4.10 with rSFVE2E1 and the combined rSFVE2E1, rSFVH and rSFVHN vaccine constructs (10^6 infectious units (i.u.) of each construct/mouse). Groups of control mice were immunised with TNE buffer (resuspension buffer) alone and 10^6 rSFV without cloned gene.

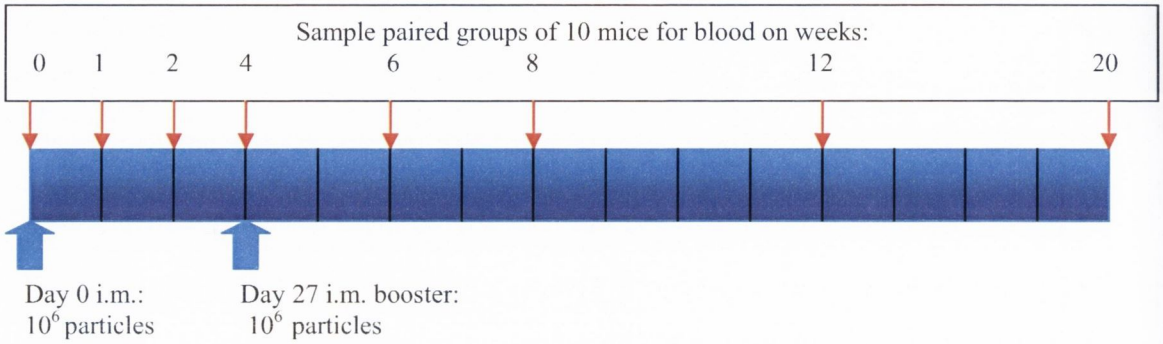


Figure 4.10 Strategy for examining the *in vivo* immune response to monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN after a four week boost

Paired antibody titres were determined by serial dilution using an RV specific IgG ELISA (Figure 4.11) and a latex agglutination assay (Figure 4.12). All mice developed RV specific antibodies pre- and post-boost as a result of the immunisation with rSFVE2E1 (geometric mean at 12 weeks, 72443 [95% CI, 55718-93972]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean at 12 weeks, 16904 [95% CI, 10471-27289]). No antibodies were developed in response to immunisation with TNE buffer or control rSFV particles. A similar result was obtained in the latex agglutination assay with the exception of the combined rSFVE2E1, rSFVH plus rSFVHN immunised group, where antibodies were only detected post boost. At two months post boost (12 weeks) the titres induced by rSFVE2E1 (geometric mean, 139 [95% CI 101-190]) were higher than those induced by the combined rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 30 [95% CI, 13-66]). Using both methods, titres in mice immunised with rSFVE2E1 were significantly higher than those immunised with trivalent rSFVE2E1, rSFVH plus rSFVHN, (**$P<0.001$ at all time points, Figure 4.11-4.12, and *$P<0.01$ at 4 weeks, Figure 4.11). There was an increase in post boost functional antibody titres in mice boosted at one month with rSFVE2E1 particles compared to those boosted at two weeks ($P=0.002$) (Figure 4.13). A specific humoral immune response was elicited against the H and HN antigens (Figure 4.14).

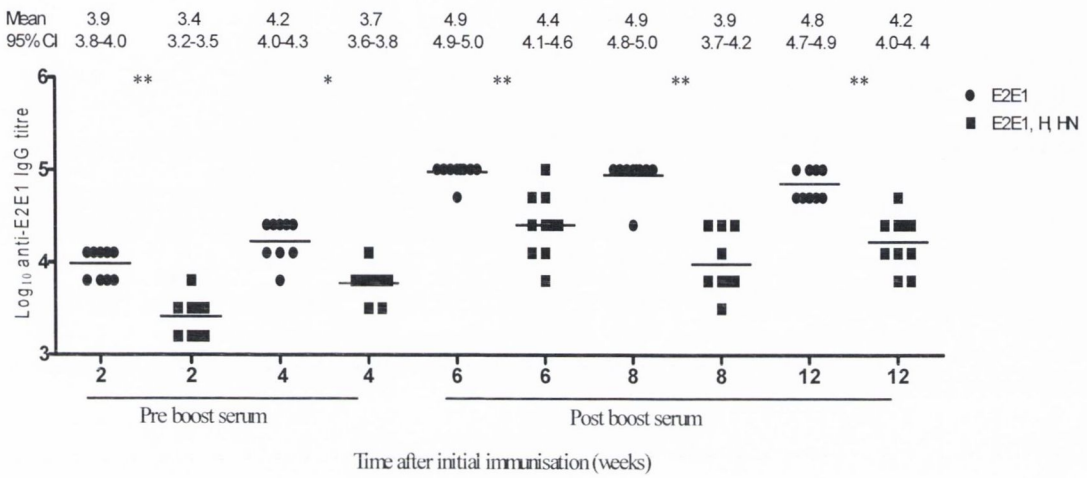


Figure 4.11 Antibody responses to rubella induced by monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN RNA vaccine constructs

Groups of ten, eight week old female BALB/c mice were immunised i.m. with monovalent rSFVE2E1; trivalent rSFVE2E1, rSFVH plus rSFVHN; rSFV10Enh (empty vector) and TNE buffer and boosted at four weeks. Sera were collected from all mice pre-immunisation, at weeks 2 and 4 (Day 27) following the initial immunisation and 6, 8 and 12 weeks post boost. Serum IgG antibody levels were determined as reciprocal cut-off titres by a limiting dilution ELISA assay. Paired data illustrate the antibody response over time and the average titre is represented by a horizontal bar. Log_{10} mean titres and 95% confidence intervals are shown and the residual standard deviation (s_{res}) for the one-way ANOVA was 0.2.

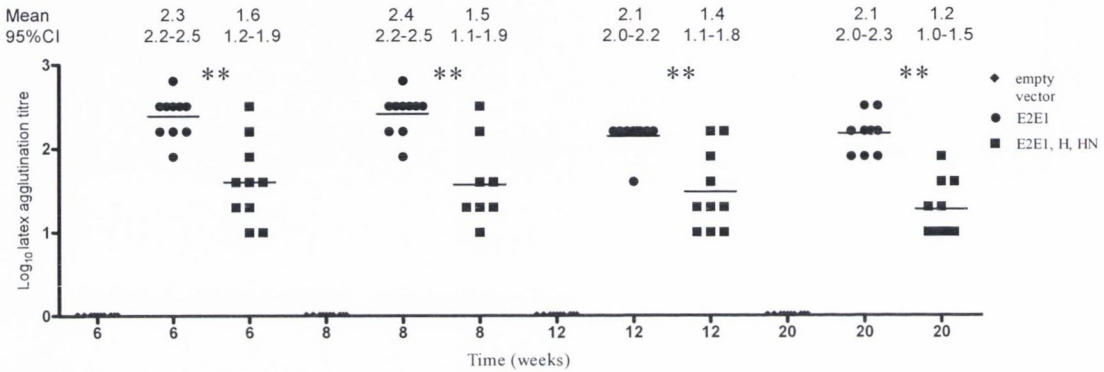


Figure 4.12 Functional antibody responses to rubella induced by monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN RNA vaccine constructs

The graph shows $\log_{10}$ transformed paired data illustrating post-boost serum latex agglutination titres indicating a similar pattern as the corresponding whole IgG titres in Figure 4.8 (**P<0.001). The $\log_{10}$ mean titres and 95% confidence intervals are shown for each group (n=10) and the residual standard deviation (s_{res}) for the one-way ANOVA was 0.28.

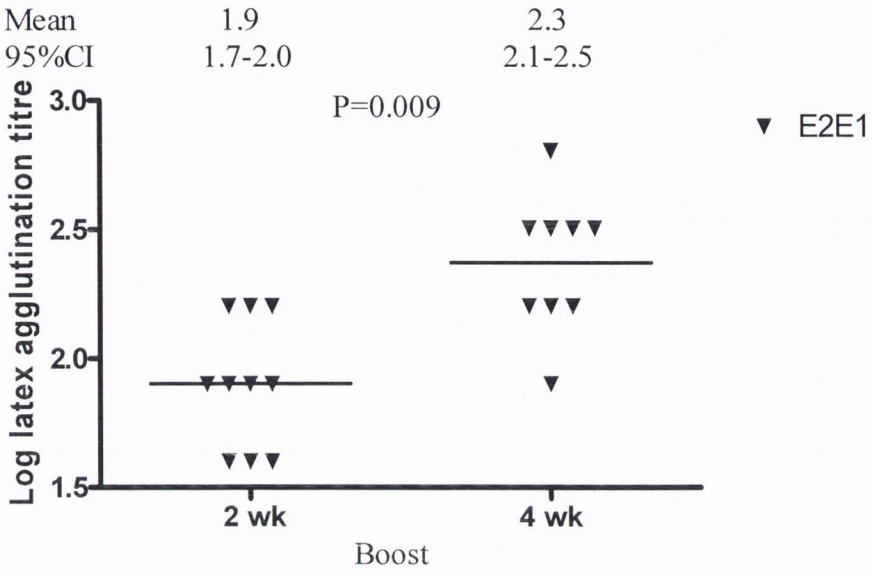


Figure 4.13 Effect of boost on mean latex agglutination antibody titres

A four week booster injection with rSFV10Enh-E2E1 particles (E2E1) resulted in significantly higher latex agglutination titres in the mice compared with those boosted at two weeks (Unpaired student *t* test, P=0.009). Log_{10} mean titres and 95% confidence intervals are shown.

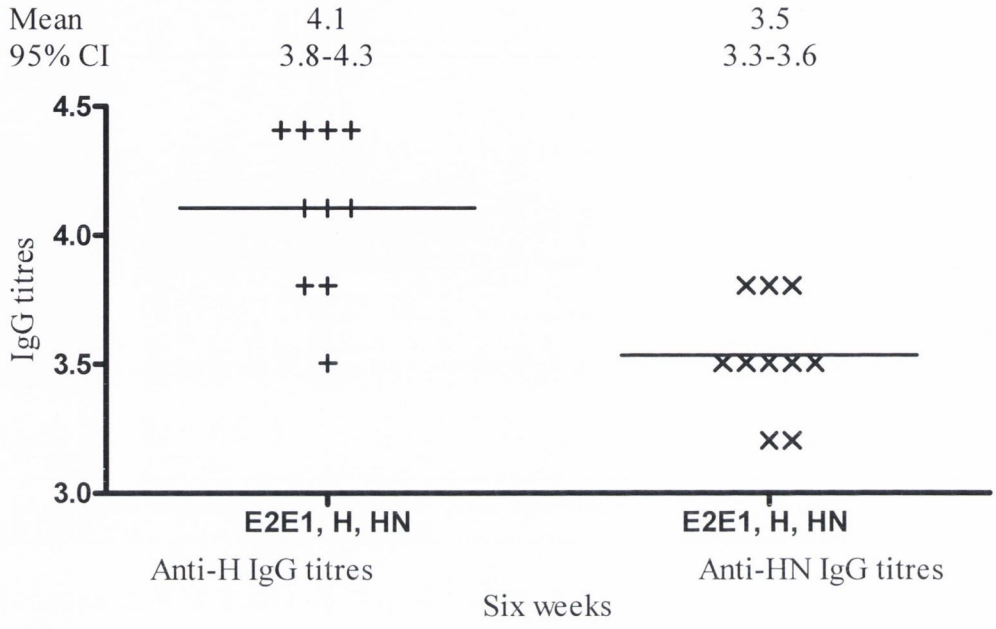


Figure 4.14 Six weeks (two weeks post boost) serum IgG antibody levels specific to Edmonston measles H and Enders mumps HN antigens

Paired data determined as reciprocal cut-off titres by a limiting dilution ELISA assay illustrate the antibody response over time and the average titre is represented by a horizontal bar. The anti-H IgG titres (geometric mean, 12589 [95% CI, 6309-19952]) were significantly higher than the anti-HN titres (geometric mean, 3162 [95% CI, 1995-3981]) (P=0.008, Unpaired *t* test). Log_{10} mean titres and 95% confidence intervals are shown.

4.3 Discussion

BALB/c mice have been used to test the immunogenicity of short peptides from E1 and E2, predicted to contain B cell epitopes (Robinson *et al.*, 1995). CD4+ T cells recognise foreign antigens as small peptides, not in their native form, that are presented as a complex with MHC class II molecules on the surface of antigen presenting cells (Ou *et al.*, 1994). The T cell response against rubella is ill-defined and the use of a genetically defined mouse model where the allotypic nature of the responding T cells and the target cells is known is advantageous (Ou *et al.*, 1994). Differences in T cell responses in mice may be explained by the fact that peptide recognition by T cells is restricted by particular MHC molecules on the antigen-presenting cell. T cell repertoires are selected depending on host MHC phenotype during T cell development. Different T cell responses to T cell epitopes have been observed in humans with different HLA phenotypes (Ou *et al.*, 1994). The processing of the rubella antigens is delayed through the ER and Golgi and they are targeted to the Golgi apparatus when compared to the measles H and mumps HN antigens which mature at the plasma membrane (Hobman *et al.*, 1993; Kuvamees *et al.*, 1994; Waxham *et al.*, 1986; Yamada *et al.*, 1988).

The first experiment (Section 4.2.1) determined the best mouse strain for assessment of immune responses to RV antigens to be the BALB/c strain. The null hypothesis being tested in this pilot study was that there would be no difference between the mean antibody titres for the immune responses elicited by the Ervevax vaccine as analysed by one-way ANOVA. The research hypothesis was that there would be a difference between the means of the antibody titres elicited by the vaccine. Normal probability plots were constructed to evaluate the normality of all of the data and to make distributional assumptions. Data found to be from a non-Normal distribution was log transformed to normality to remove skewness, resulting in correct Normal probability plots and similar standard deviations among samples (Altman, 1991). The mean of the log data was back-transformed to yield the geometric mean which as required was less than the mean of the raw data and similar to the median. Thus Normality and equal variance were verified and the parametric one-way ANOVA hypothesis test was used to analyse the log transformed data. The Bonferroni method was used to control the overall Type 1 error rate at no more than 5% to thus decrease the probability of obtaining false positive results in this analysis. An attempt was made to stain the muscle sections for rubella antigen using a goat polyclonal antibody however the background was too high and Ervevax was found in any event not to elicit functional antibodies in mice. The Ervevax was reconstituted in 50 µl for the i.m.

injections and 500 µl for the i.p. injections. It is possible that the reconstitution with a smaller volume than recommended by the manufacturer may have disrupted the pH and the ion balance thus inactivating the vaccine strain.

In a second experiment the measles H and mumps HN antigens were found to lower the immune response to the rubella antigens. This effect was investigated and the results presented in Chapter Five. The four week boost was found to result in higher titres post boost than a two week boost. The null hypothesis was that there would be no difference between the monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN RV-specific antibody titres. The alternative or research hypothesis was that there would be a difference in the mean antibody titre. As is evident from Figure 4.8 there was a consistently reduced antibody response in the BALB/c mice to the trivalent rSFVE2E1, rSFVH plus rSFVHN particles when compared to that elicited by the rSFVE2E1 particles. This difference was statistically significant as the P value, i.e. the probability of having observed this data (or more extreme data) when the null hypothesis is true, had a value of $P<0.001$. This effect was also observed in latex agglutination titres from the same mice as seen in post boost sera at two and four weeks.

The range of latex agglutination titres observed in our study (1:160 to 1:1280) are similar to those observed with a DNA vaccine prototype (Pougatcheva *et al.*, 1999). Antibodies induced in mice by this DNA vaccine were found to react primarily against the E1 protein (Pougatcheva *et al.*, 1999) A recombinant E1 glycoprotein has been shown to elicit neutralising antibodies in two animal models; BALB/c and SCID mice (Perrenoud *et al.*, 2004). Induction of low neutralising titres relative to latex agglutination appears to be a characteristic of the mouse system (Pougatcheva *et al.*, 1999). There is no pre-treatment of serum involved in the Rubagen method, minimal amounts of sera are needed and the results can be read visually after 8 min (Sever *et al.*, 1983). A study found both BALB/c mice; inoculated intramuscularly 5 times at 3-week intervals with 2 x 10^6 insect cells (in 400 µl) infected by a recombinant baculovirus (P2) expressing the RV E2 and C proteins and a group immunised with 3 single doses (1000 $TCID_{50}$) of RA27/3 rubella vaccine (Sclavo S.p.A., Siena, Italy); produced antibodies to the RV proteins. However, as the paper did not quote the ELISA titres, direct comparison to the data reported here is not possible (Cusi *et al.*, 1995).

Chapter 5

Effect of Edmonston H & Enders HN on anti-RV humoral response

5.1 Introduction

A competitive mechanism in BALB/c mice by which the anti-peptide component of the humoral response specifically suppresses generation of IgG responses to neighbouring determinants has been investigated and the target for interclonal B cell competition was found to not directly be Ag but, rather, limiting amounts of T cell help (Agarwal *et al.*, 1996). Studies of the BALB/c responses to peptide analogs revealed that the emergence of a dominant epitope was a consequence of active suppression of B cells directed against alternate determinants. A competitive process occurs in the early stages of the primary response, enforced by limiting amounts of Th cell help available (Agarwal *et al.*, 1996). The outcome of this process is positive selection of antibody subsets which correlate with overall higher avidity for epitope binding (Agarwal *et al.*, 1996). Thus evidence supports a role for Th cells in driving clonal selection during primary B cell responses (Agarwal *et al.*, 1996).

The cellular receptor for SFV is unknown but the E2 protein is thought to be important for entry unlike RV where E1 is thought to play a key role. The mumps virus and the measles virus receptors are well characterised. The receptors for these viruses have implications for their interference properties; however viral interference has also been shown to operate by other mechanisms. The combination of these viral antigens determines their immunosuppressive effects. In this study the H protein was cloned without the F protein with the aim of abrogating the immunosuppressive effect of these proteins. The H protein and SLAM interaction is important for vaccine design as the structure required for the interaction of morbillivirus H proteins may be well conserved among SLAMs of many different species despite sequence differences (Tatsuo *et al.*, 2001). Thus, other morbilliviruses may infect humans lacking adequate anti-MV immunity and this should be factored into MV eradication planning (Tatsuo *et al.*, 2001).

This chapter thus further describes the investigation of the RV-specific immune responses induced by the rSFV clones expressing the RA27/3 rubella, Edmonston measles and Enders mumps antigens. A control group consisting of one dose of rubella expressing SFV combined with two doses of empty vector is introduced to investigate whether an immune response to the vector is affecting the rubella immune response or whether homologous interference is occurring. The rubella antigens are also administered in combination with either measles or mumps antigens in an attempt to determine which antigen is mediating the reduction in the RV immune response. As rubella immunity is primarily antibody mediated the focus continues to be on the humoral response.

5.2 Results

5.2.1 Effect of measles H and mumps HN antigen expression on the anti-RV humoral response as measured by ELISA

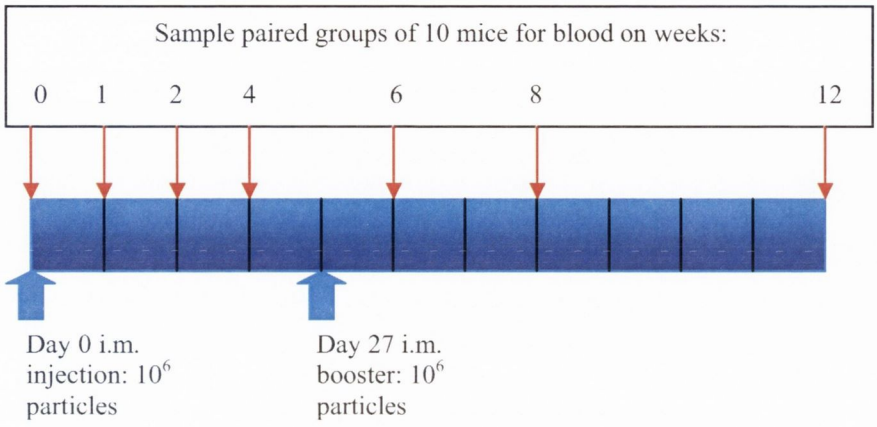


Figure 5.1 Strategy for examining the *in vivo* immune response to monovalent rSFVE2E1, divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN after a four week boost

In an experiment to repeat the results found in chapter four (Figures 4.11 & 4.12) and further elucidate, separately, the effect of the measles H and mumps HN antigens, groups of ten mice were immunised with monovalent rSFVE2E1, divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN. A mixture of rSFVE2E1 plus rSFV (no cloned gene), rSFV alone and TNE buffer alone were also included as controls. The immunisation regime is described in section 2.2.6.3 and illustrated in Figure 5.1. The booster injection at 4 weeks resulted in significantly boosted titres in all immunised groups as measured by ELISA (Figures 5.2A-5.6A.). The effect of the measles and mumps antigens on rubella immunity was assessed by IgG ELISA on days 14 (2 weeks), 27 (4 weeks), 42 (6 weeks), 56 (8 weeks) (Figures 5.7., 5.8., 5.9., 5.10). The dose of each construct was again 10^6 i.u. given i.m., except that of the vector without cloned gene which was also given at 2 x 10^6 i.u. with the rSFVE2E1 (E2E1, E) to mimic the trivalent vaccine. In fact this group of mice did not give a significantly different immune response to that produced by rSFVE2E1 alone as measured by antibody ELISA or a latex agglutination assay, indicating no effect of increased dose of the vector (Figure 5.8 & 5.11). The presence of one dose of particles containing no cloned gene also

had no effect on the reduction of the humoral immune response induced by rSFVE2E1 in the presence of rSFVH or rSFVHN as any reduction in these divalent groups was not to the same extent as in the trivalently immunised group at any of the time points.

RV-specific antibodies were detected as early as two weeks post priming where the RV specific immune response elicited by the rSFVE2E1 construct (geometric mean, 5571 [95% CI, 3531-8790]) was significantly higher than that elicited by the combined rSFVE2E1, rSFVH plus rSFVHN constructs (geometric mean, 1713 [95% CI, 993-2958]) ($P<0.01$) when measured by IgG ELISA (Figure 5.7). The other difference in mean titre was between the rSFVE2E1 plus rSFVH group (geometric mean, 2600 [95% CI, 1625-4159]), a difference which was not evident with the rSFVE2E1 plus rSFVHN group (geometric mean, 4528 [95% CI, 3184-6426]) ($P<0.01$) (Figure 5.7). In pre-boost (Day 27) sera the difference between the whole IgG antibody titres elicited by the rSFVE2E1 construct (geometric mean, 11142 [95% CI, 8147-15240]) and the combined rSFVE2E1, rSFVH plus rSFVHN constructs was marked (geometric mean, 2985 [95% CI, 1729-5152]) ($P<0.001$) (Figure 5.8). At this time mean antibody levels elicited by the rSFVE2E1 plus rSFVH construct (geometric mean, 3672 [95% CI, 1914-7063]) were also significantly lower than those elicited by the rSFVE2E1 construct ($P<0.01$) (Figure 5.8). The HN antigen had no effect at this point (geometric mean, 5199 [95% CI, 3459-7816]). When assayed two weeks post boost the sera from the trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 10,000 [95% CI, 6309.5-15848.93]); divalent rSFVE2E1 plus rSFVH (geometric mean, 25,118.8 [95% CI, 15,848.9-39810.7]) and divalent rSFVE2E1 plus rSFVHN (geometric mean, 15848.9 [95% CI, 10,000-19952.62]) immunised mice were found to contain significantly lower antibody titres than sera from mice immunised with monovalent rSFVE2E1 (geometric mean, 125,892 [95% CI, 79,432.8-158,489.3]), or the monovalent rSFVE2E1 (geometric mean, 100,000 [95%CI, 50,118-158,489]) control group ($P<0.001$) (Figure 5.9).

This was confirmed at 8 weeks (four weeks post-boost) when immune responses in mice immunised with monovalent rSFVE2E1 (geometric mean, 501187.23 [95% CI, 147910.83-316227.76]) were significantly higher than those immunised with divalent rSFVE2E1 plus rSFVH (geometric mean, 39810.71, [95% CI, 19952.6-79432.88]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 31622.77, [95% CI, 25118.86-39810.71] and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 12589.25 [95% CI, 7943.28-25118.86]) ($P<0.001$) (Figure 5.10). The result was confirmed by using Rubagen, a latex agglutination assay as outlined in section 5.2.2

5.2.2 Effect of measles H and mumps HN antigen expression on the anti-RV humoral response as measured by latex agglutination assay

The booster injection at 4 weeks resulted in significantly boosted titres in all immunised groups as measured by latex agglutination assay (Figures 5.2B-5.6B.). The effect of the measles and mumps antigens on rubella immunity was determined by latex agglutination on days 27, 42, 56 and 2 months post boost serum from the same sera analysed by ELISA. The latex agglutination pre-boost geometric mean titres demonstrate that the functional antibody titres elicited by the combined rSFVE2E1, rSFVH, plus rSFVHN constructs (none); the rSFVE2E1 plus rSFVH construct (geometric mean, 2.6 [95% CI, 1.0-6.7]) as well as the rSFVE2E1 plus rSFVHN (geometric mean, 2.1 [95% CI, 0.8-5.1]) were all significantly lower than those elicited by rSFVE2E1 alone (geometric mean, 28 [95% CI, 19.9-40.1]) ($P<0.001$) (Figure 5.11). When assayed two weeks post boost, the sera from the trivalent rSFVE2E1, rSFVH, plus rSFVHN (geometric mean, 49.2 [95% CI, 26.4-91.6]), divalent rSFVE2E1 plus rSFVH (geometric mean, 91.8 [95% CI, 42.6-198.1]) and divalent rSFVE2E1 plus rSFVHN (geometric mean, 52.7 [95% CI, 26-107.1]) immunised mice were once again found to contain significantly lower antibody titres than sera from mice immunised with monovalent rSFVE2E1 (geometric mean, 485 [95% CI, 319.1-736.2]) ($P<0.001$) (Figure 5.12). Consistent with this result, sera analysed four weeks post boost (8 weeks) from the divalent rSFVE2E1 plus rSFVH (geometric mean, 63.09 [95% CI, 25.11-125.89]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 39.81 [95% CI, 19.95-79.43]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 39.81 [95% CI, 15.84-79.43]) groups demonstrated lower latex agglutination titres than the monovalent rSFVE2E1 group (geometric mean, 398.1 [95% CI, 199.52-630.95]) ($P<0.001$) (Figure 5.13). In sera collected two months post boost (12 weeks), geometric mean latex agglutination titres in the mice immunised with monovalent rSFVE2E1 (184 [95% CI, 110, 307) had fallen but were still substantially higher than in the mice immunised with trivalent rSFVE2E1, rSFVH, plus rSFVHN, (27 [95% CI, 16-47]), divalent rSFVE2E1 plus rSFVH, (35 [95% CI, 19-64]) and divalent rSFVE2E1 plus rSFVHN (35 [95% CI, 17-70]) ($P<0.001$) (Figure 5.14). This data confirms that shown in chapter 4 (Figures 4.11 & 4.12), but in addition demonstrates that both the H and the HN antigens mediate this effect equally and that the two extra doses of SFV particles without cloned gene, (171 [95% CI, 105-280]) do not affect the anti-rubella functional antibody response (Figure 5.14).

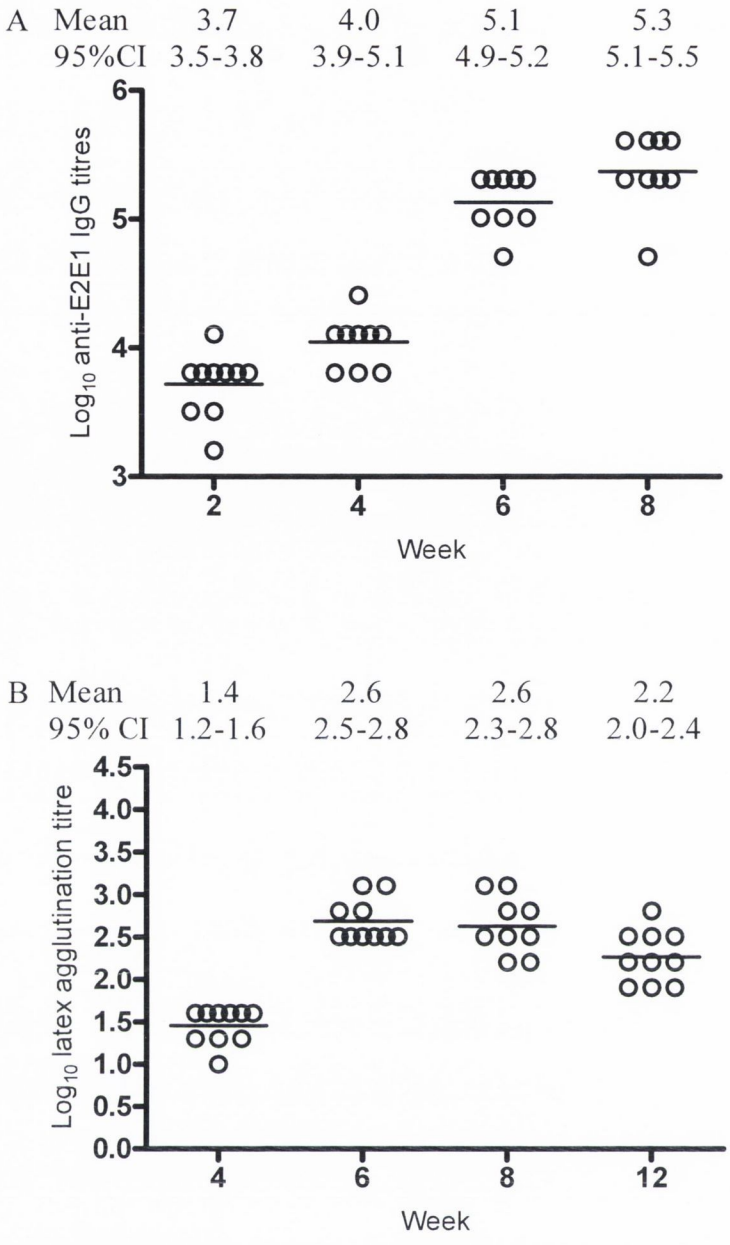


Figure 5.2 Effect of boost on rSFVE2E1 antibody titres

The IgG ELISA and latex agglutination titres to the rSFVE2E1 construct were analysed by one-way ANOVA and the post-boost titres at six weeks were found to be higher than the pre-boost titres at four weeks ($P<0.001$). There was no difference between IgG ELISA titres or latex agglutination titres at weeks 6 and 8 (A & B). However, the latex agglutination titres at 12 weeks were lower than those measured at 8 weeks ($P<0.05$) (B). Log_{10} mean titres and 95% confidence intervals are shown.

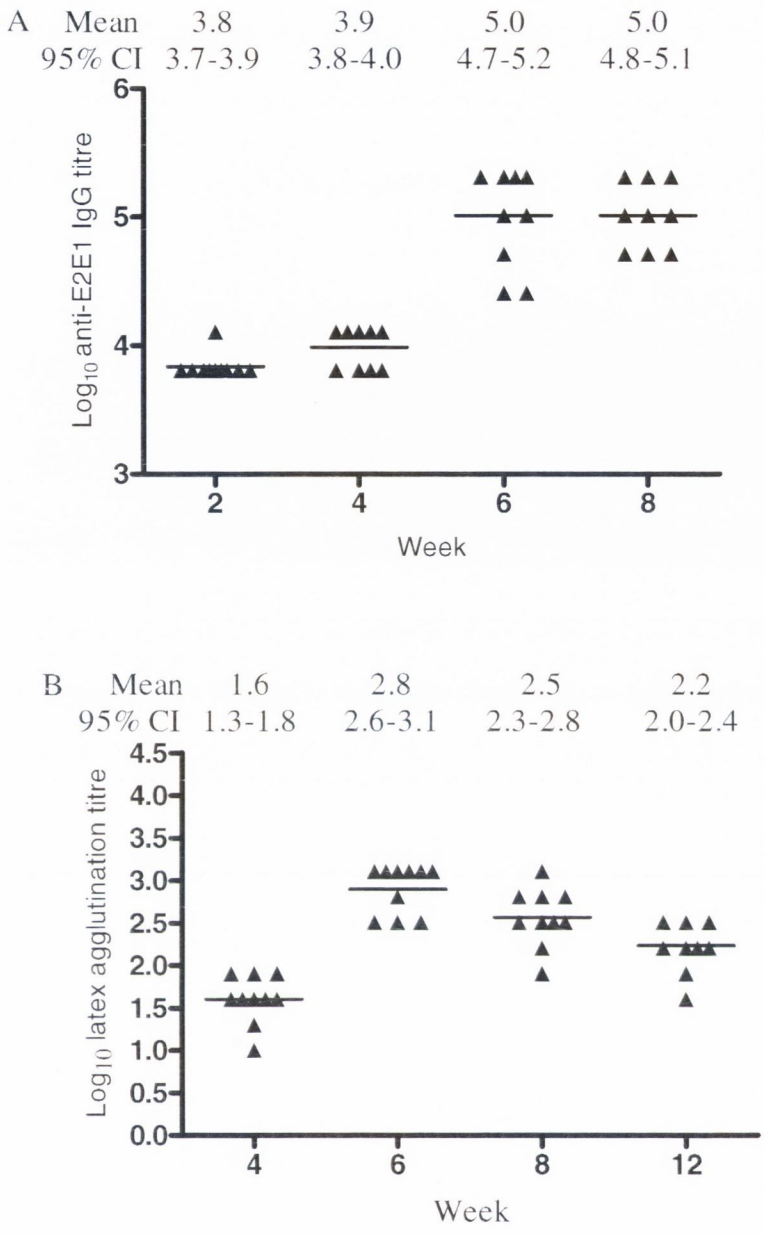


Figure 5.3 Effect of boost on rSFVE2E1 plus rSFV (no cloned gene) antibody titres

The boost injection with rSFVE2E1 plus rSFV (no cloned gene) (E2E1, E) resulted in an increase in antibody titres as measured by both methods ($P<0.001$) (A & B). There was no difference between titres at 6 and 8 weeks or 8 and 12 weeks ($P>0.05$). However the post boost titres at 6 weeks were more elevated than the 12 week titres ($P<0.001$). Log_{10} mean titres and 95% confidence intervals are shown.

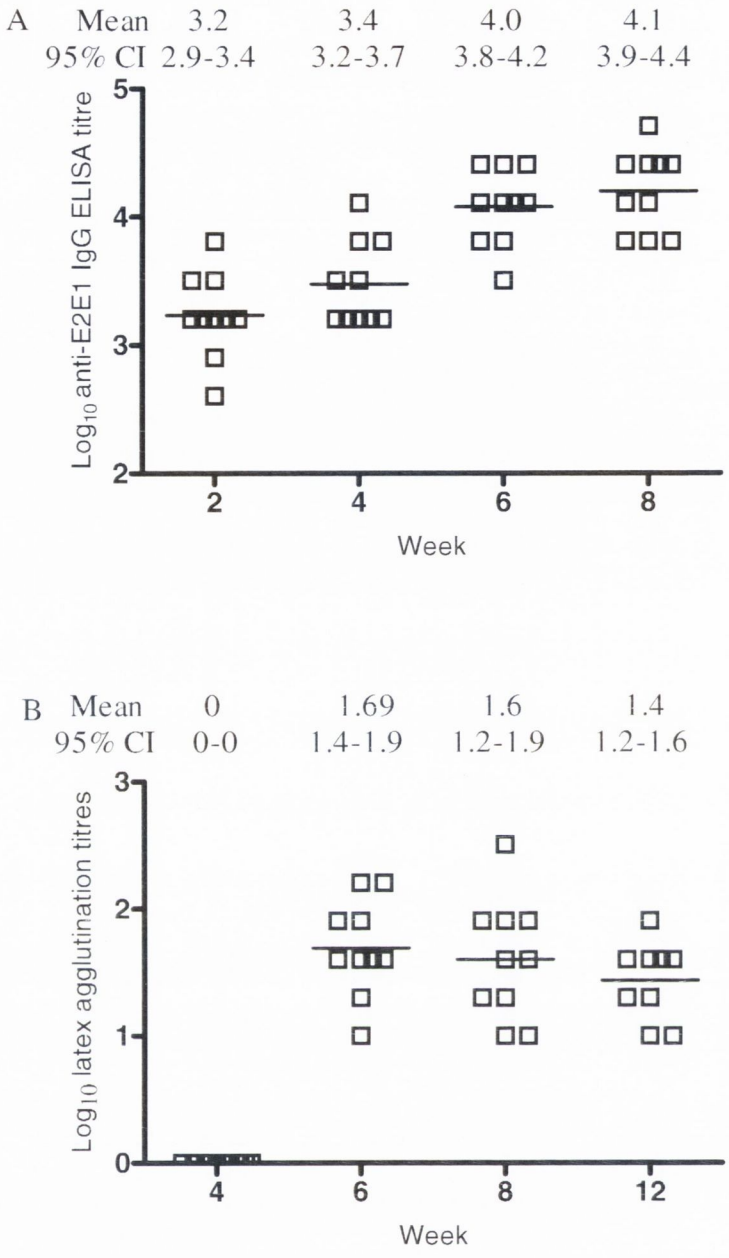


Figure 5.4 Effect of boost on rSFVE2E1, rSFVH plus rSFVHN antibody titres

The difference between pre and post-boost titres at four and six weeks respectively was less significant when measure by ELISA ($P<0.01$) (A) when compared to the latex agglutination titres ($P<0.001$) (B). There were no significant differences between mean titres post-boost as analysed by one-way ANOVA. There were notably no functional antibodies detected pre-boost in the mice immunised with the trivalent rSFVE2E1, rSFVH plus rSFVHN. Log_{10} mean titres and 95% confidence intervals are shown.

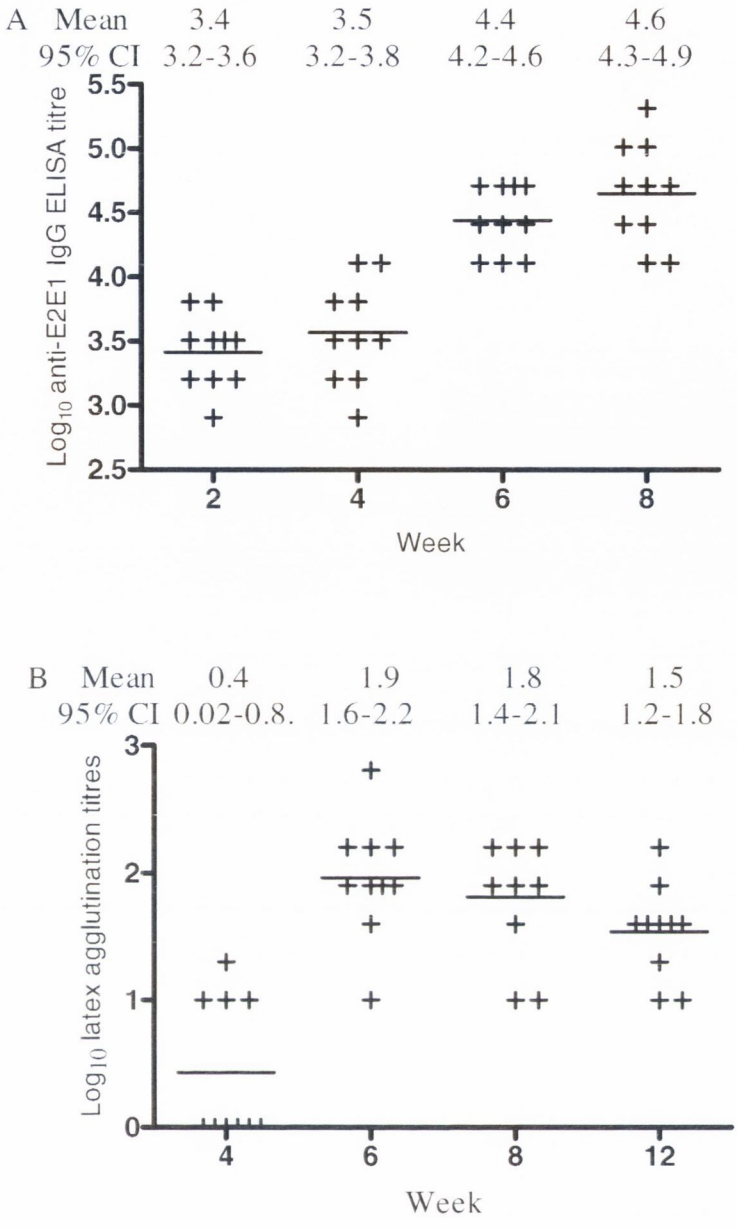


Figure 5.5 Effect of boost on rSFVE2E1 plus rSFVH antibody titres

The titres elicited by rSFVE2E1 plus rSFVH were boosted significantly as measured by both methods and analysed by one-way ANOVA ($P<0.001$). There was no significant decline in titres post boost determined by either method (A & B). Log_{10} mean titres and 95% confidence intervals are shown.

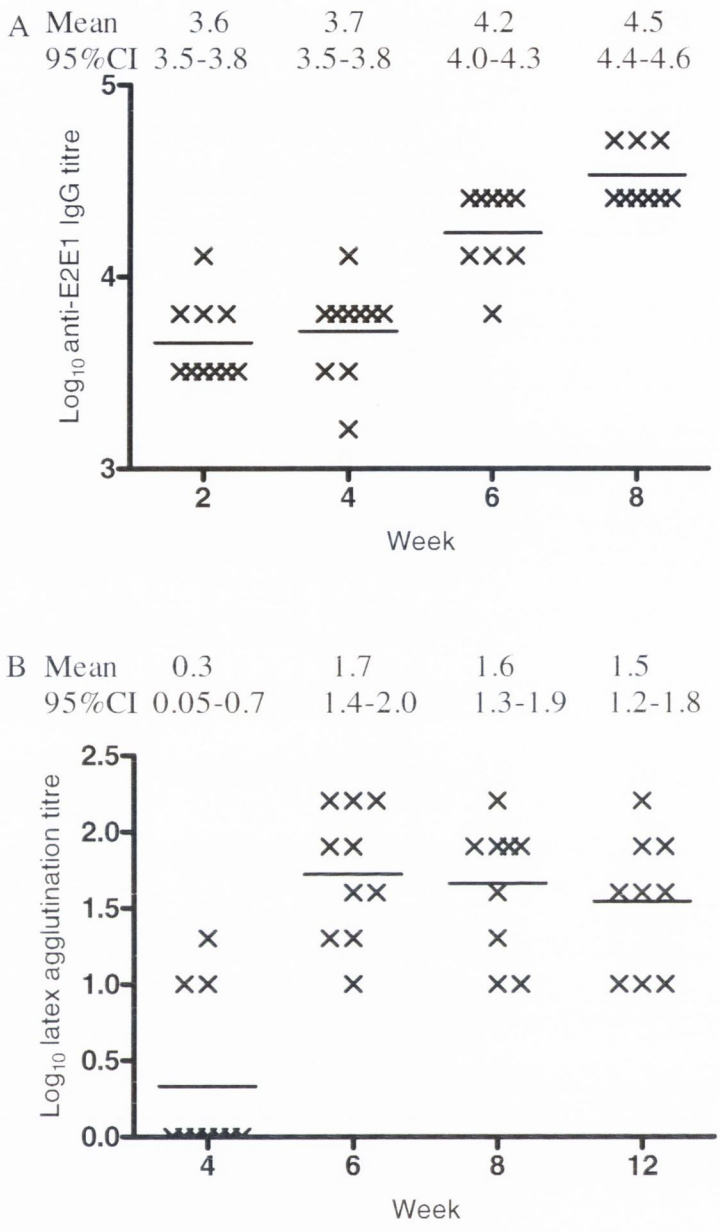


Figure 5.6 Effect of boost on rSFVE2E1 plus rSFVHN antibody titres

The titres elicited by rSFVE2E1 plus rSFVHN were boosted significantly as measured by both methods and analysed by one-way ANOVA ($P<0.001$). There was no significant decline in titres post boost determined by either method (A & B). Log_{10} mean titres and 95% confidence intervals are shown.

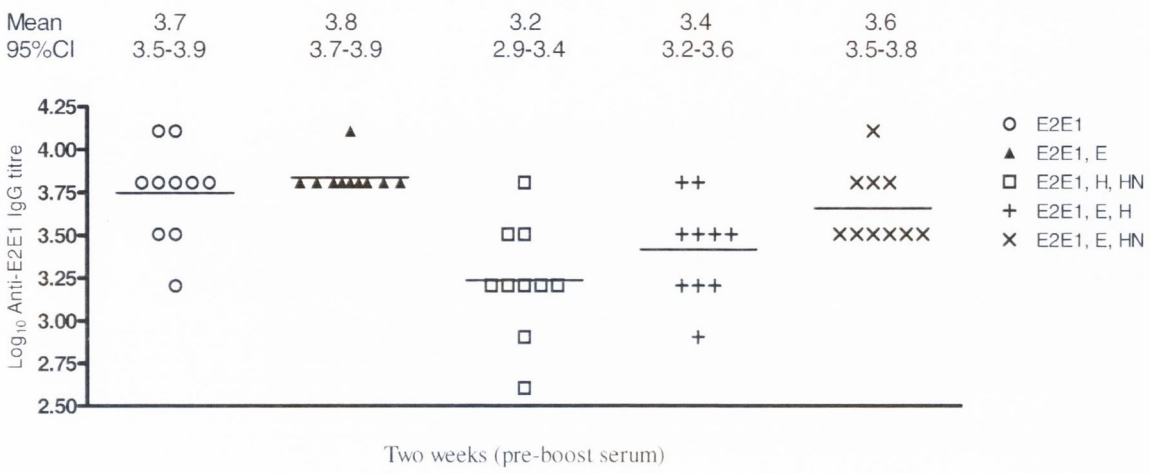


Figure 5.7 Post-prime humoral immune responses to rSFV vaccine constructs

Post prime whole antibody responses to monovalent rSFVE2E1 (geometric mean, 5011 [95% CI, 3162-7943]), divalent rSFVE2E1 plus rSFVH (geometric mean, 2511 [95% CI, 1584-3981], divalent rSFVE2E1 plus rSFVHN (geometric mean, 3981 [95% CI, 3162-6309]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 1584 [95% CI, 794-2511]) (n=10). As early as two weeks post prime anti-rubella specific IgG antibodies were detected in sera analysed by ELISA. The RV-specific antibody titres were significantly higher in the monovalent group compared to the trivalent group when analysed by one-way ANOVA ($P<0.001$) ($s_{res} = 0.24$). The divalent rSFVE2E1 plus rSFVH construct significantly reduced RV-specific pre-boost antibody titres ($P<0.01$) compared to monovalent rSFVE2E1 and rSFVE2E1 plus rSFV (no cloned gene) (geometric mean, 6309 [95% CI, 5011-7943]). Log_{10} mean titres and 95% confidence intervals are shown.

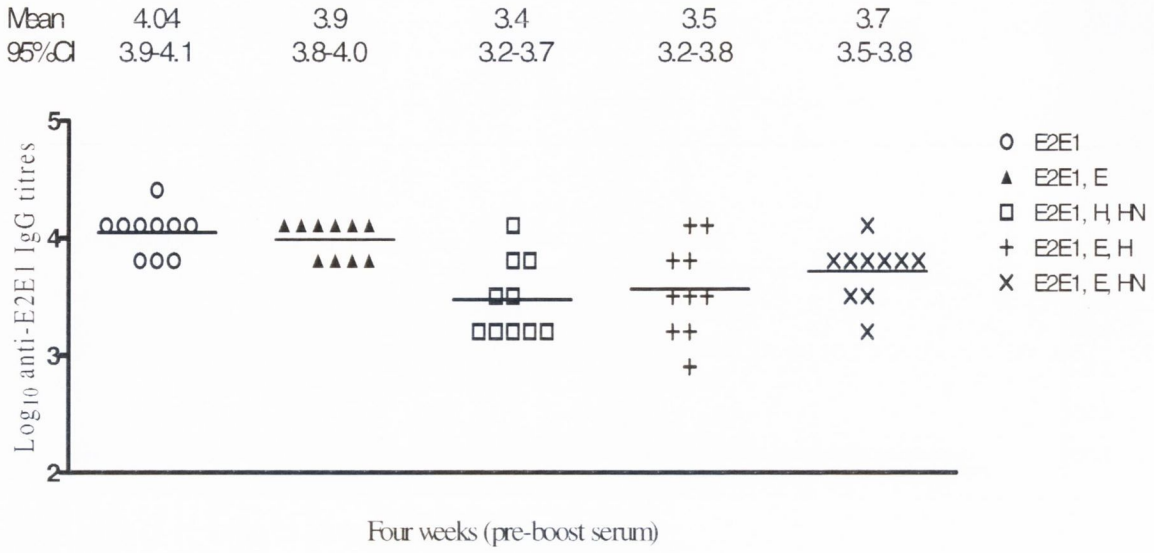


Figure 5.8 Pre-boost humoral immune responses to rSFV constructs

Pre-boost immune responses measured by whole IgG ELISA in mice immunised with monovalent rSFVE2E1 (geometric mean, 10964 [95% CI, 7943-12589]), divalent rSFVE2E1 plus rSFVH (geometric mean, 3162 [95% CI, 1584-6309], divalent rSFVE2E1 plus rSFVHN (geometric mean, 5011 [95% CI, 3162-6309]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 2511 [95% CI, 1584-5011]) (n=10). Pre-boost (4 weeks post prime) RV specific antibody titres were lowered in the trivalent immunised mice when compared to the monovalent immunised groups when analysed by one-way ANOVA ($P<0.001$) ($s_{res} = 0.26$). Specifically the rSFVH construct appeared to reduce RV-specific pre boost antibody titres ($P<0.01$) when compared to the titres induced by monovalent rSFVE2E1 and rSFVE2E1 plus rSFV (no cloned gene). The rSFVHN construct at this point did not have the same effect on titres. Log_{10} mean titres and 95% confidence intervals are shown.

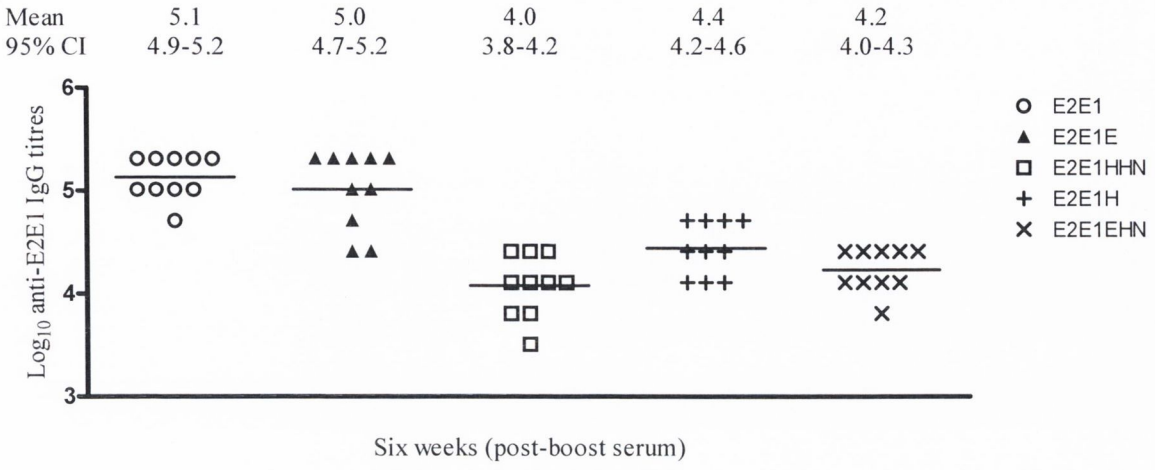


Figure 5.9 Two weeks post-boost humoral immune responses to rSFV constructs

Post-boost immune responses measured by whole IgG ELISA in mice immunised with monovalent rSFVE2E1 (geometric mean, 125892 [95% CI, 79,432.8-158,489.3]), divalent rSFVE2E1 plus rSFVH (geometric mean, 25118.8 [95% CI, 15,848.9-39810.7]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 15848.9 [95% CI, 10,000-19952.62]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 10,000 [95% CI, 6309.5-15848.93]) (n=10). At six weeks (two weeks post boost), the whole IgG RV-specific antibody titres were significantly higher in the monovalent groups compared to the trivalent and divalent groups when analysed by one-way ANOVA ($P<0.001$) [$s_{res}=.26$] Log_{10} mean titres and 95% confidence intervals are shown.

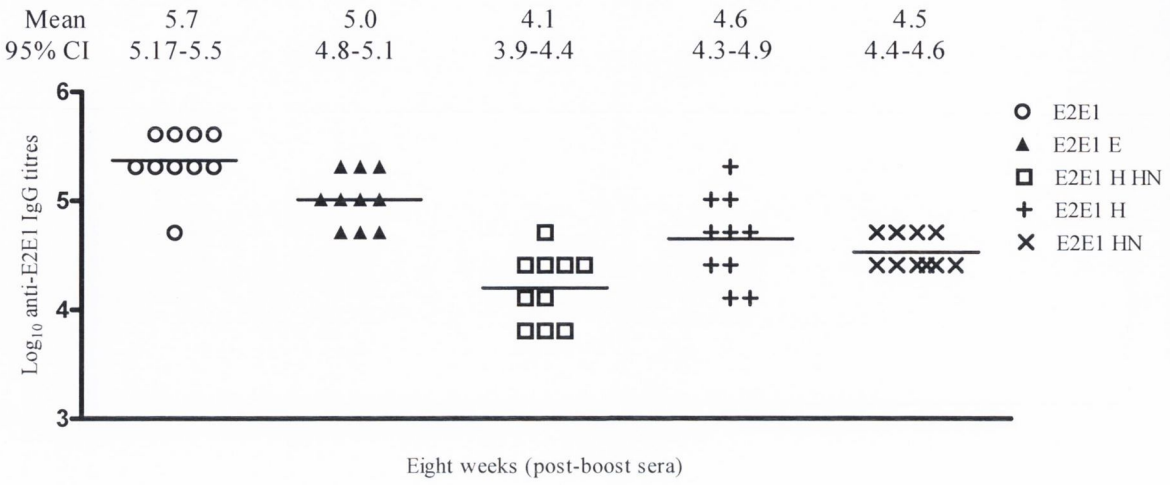


Figure 5.10 Four weeks post-boost humoral immune responses to rSFV constructs

Post-boost immune responses measured by whole IgG ELISA in mice immunised with monovalent rSFVE2E1 (geometric mean, 501187.23 [95% CI, 147910.83-316227.76]), divalent rSFVE2E1 plus rSFVH (geometric mean, 39810.71, [95% CI, 19952.6-79432.88]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 31622.77, [95% CI, 25118.86-39810.71] and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 12589.25 [95% CI, 7943.28-25118.86]) (n=10). At eight weeks (four weeks post boost), the RV-specific antibody titres were significantly higher in the monovalent group compared to the trivalent and divalent groups when analysed by one-way ANOVA ($P<0.001$) ($s_{res}=.28$). Log_{10} mean titres and 95% confidence intervals are shown.

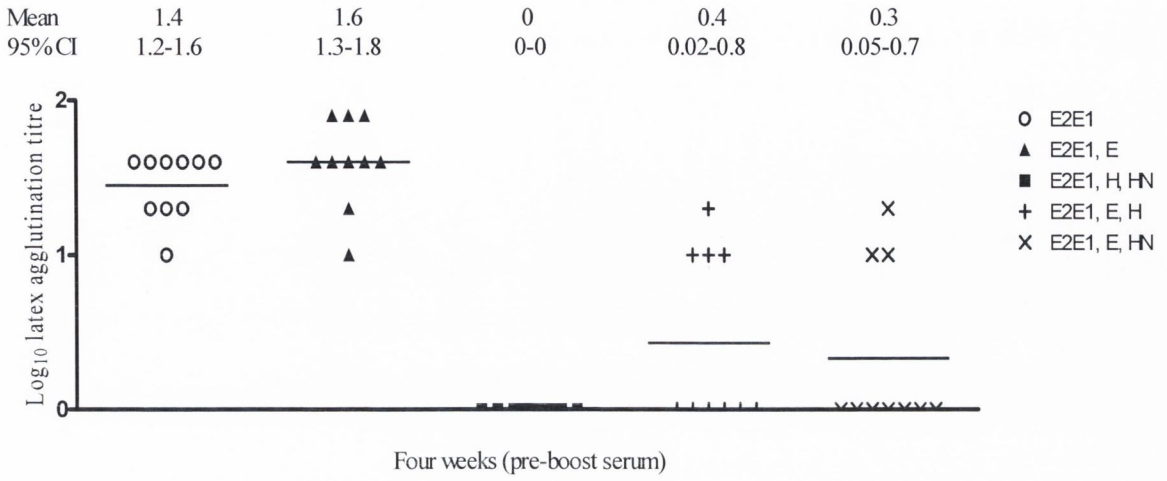


Figure 5.11 Pre-boost RV-specific latex agglutination titres elicited by rSFV constructs

Pre-boost functional antibody immune responses in mice immunised with monovalent rSFVE2E1 (geometric mean, 25 [95% CI, 15.8-39.8]), divalent rSFVE2E1 plus rSFVH (geometric mean, 2.5 [95% CI, 1.04-6.3]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 1.9 [95% CI, 1.12-5.01]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 0 [95% CI, 0-0]) (n=10). These differences seen in Figure 5.7 were confirmed by latex agglutination titres where titres in the trivalent and both divalent immunised groups were lower than the monovalent groups following one-way ANOVA analysis ($P<0.001$) ([s_{res}=.37]). Log_{10} mean titres and 95% confidence intervals are shown.

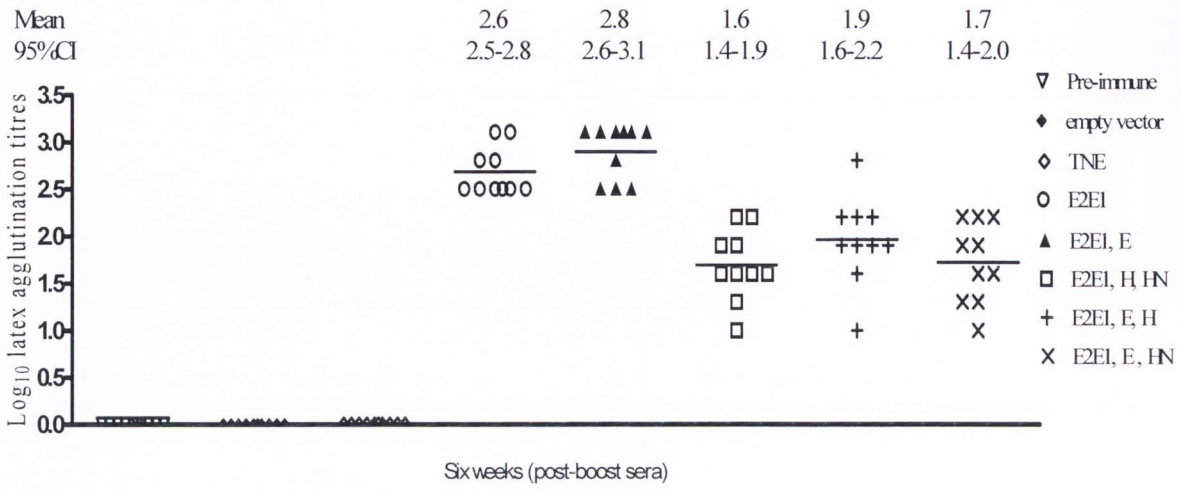


Figure 5.12 Two weeks post-boost RV-specific latex agglutination titres elicited by rSFV constructs

Post-boost immune responses in mice immunised with monovalent rSFVE2E1 (geometric mean, 398.1 [95% CI, 316.22-630.95]), divalent rSFVE2E1 plus rSFVH (geometric mean, 79.4 [95% CI, 39.8-158.4]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 50.1 [95% CI, 25.11-100]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 39.8 [95% CI, 25.11-79.4]) (n=10). At six weeks (two weeks post boost) the RV-specific antibody titres and latex agglutination titres were significantly higher in the monovalent groups compared to the trivalent and divalent groups when analysed by one-way ANOVA ($P<0.001$) ([$s_{res}=.28$]). Log_{10} mean titres and 95% confidence intervals are shown. No latex agglutination titres were detected in the control groups or in pre-immune sera.

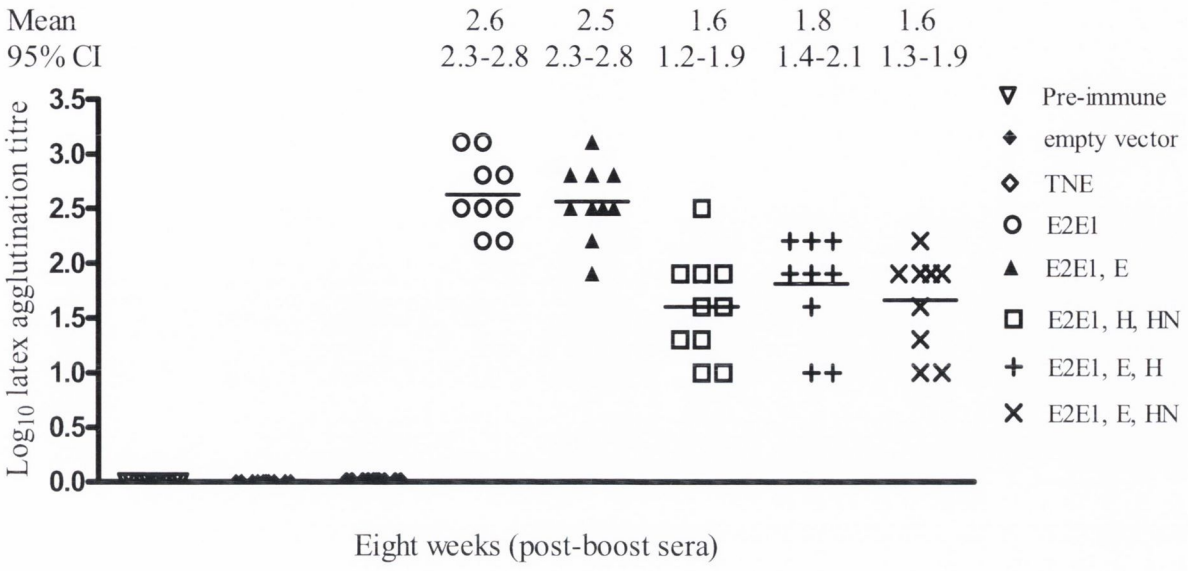


Figure 5.13 Four weeks post-boost RV-specific latex agglutination titres elicited by rSFV constructs

Post-boost immune responses in mice immunised with monovalent rSFVE2E1 (geometric mean, 398.1 [95% CI, 199.52-630.95]), divalent rSFVE2E1 plus rSFVH (geometric mean, 63.09 [95% CI, 25.11-125.89]), divalent rSFVE2E1 plus rSFVHN (geometric mean, 39.81 [95% CI, 19.95-79.43]) and trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 39.81 [95% CI, 15.84-79.43]) (n=10). At eight weeks (four weeks post boost) the latex agglutination titres were significantly higher in the monovalent group compared to the trivalent and divalent groups when analysed by one-way ANOVA ($P<0.001$) ($s_{res}=.31$). Log_{10} mean titres and 95% confidence intervals are shown.

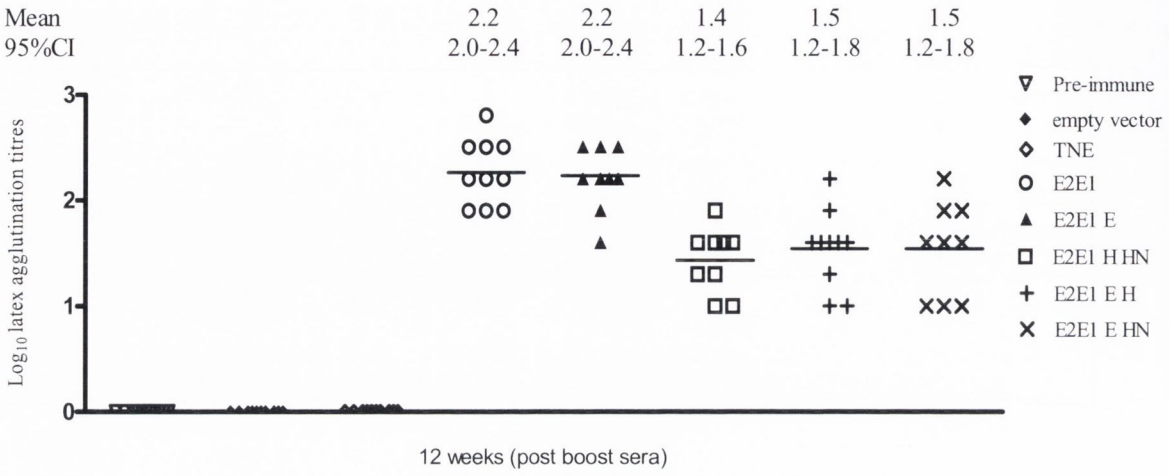


Figure 5.14 Longevity of anti-rubella latex agglutination titres to all rSFV constructs

Longevity of anti-rubella latex agglutination titres to all vaccine constructs. The difference in mean RV-specific antibody titres elicited by the monovalent (E2E1, geometric mean, 158.48 [95% CI, 100-251.18]), (E2E1, E, geometric mean, 158.48, [95% CI, 100-251.18]) and trivalent (E2E1, H, HN, geometric mean, 25.11 [95% CI, 15.84-39.81]) constructs is significant ($P<0.001$) ($n=10$). This reduction in immune response to the rubella E2 and E1 antigens is mediated equally by the measles H ($P<0.001$) (geometric mean, 31.62 [95% CI, 15.84-63.09]) and the mumps HN antigens ($P<0.001$) (geometric mean, 31.62, [95% CI, 15.84-63.09] (one-way ANOVA; $s_{res}=.27$). The $\log_{10}$ mean titres and 95% confidence intervals are shown.

5.2.3 Anti-measles and anti-mumps immune responses as measured by ELISA

All groups immunised with the H antigen developed MV specific IgG titres of 3-4 orders of magnitude and groups immunised with the HN antigen developed titres of 3 orders of magnitude at six weeks (Figure 5.15). Anti-measles specific IgG titres induced post-boost by trivalent rSFVE2E1, rSFVH plus rSFVHN (geometric mean, 12589 [95%CI, 7943-19952]) were significantly higher than the anti-mumps specific IgG titres induced by the trivalent constructs (geometric mean, 3162 [95%CI, 3162-3162]) (P=0.0001, unpaired *t* test). Anti-measles specific IgG titres induced by divalent rSFVE2E1 plus rSFVH (geometric mean, 6309 [95%CI, 3981-10,000]) were only marginally higher than the anti-mumps specific titres induced by divalent rSFVE2E1 plus rSFVHN (geometric mean, 3981 [95%CI, 2511-5011]) (P=0.04, unpaired *t* test).

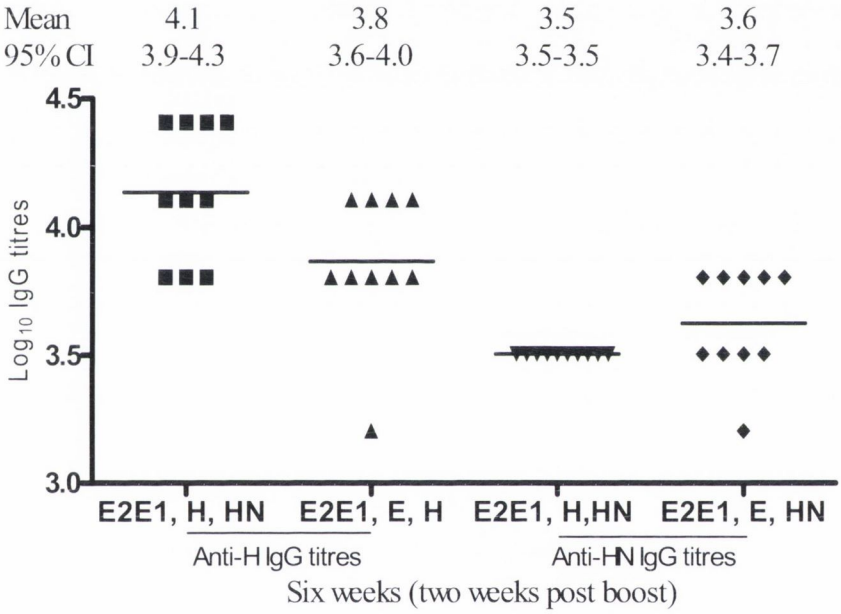


Figure 5.15 Whole antibody responses specific to measles and mumps SFV constructs

Six weeks (2 weeks post-boost) serum IgG antibody levels to Edmonston measles and Enders mumps were determined as reciprocal cut-off titres by a limiting dilution ELISA assay. Paired data illustrate the antibody response over time and the average titre is represented by a horizontal bar. Log_{10} mean titres and 95% confidence intervals are shown.

5.3 Discussion

This chapter demonstrates the reproducibility of the data found in Chapter four and also confirms a consistent inter-assay variability. The data are displayed initially by construct to convey the effect of the boost regimen and the longevity of the immune response as detected by two assays (Figures 5.2-5.6). In subsequent graphs the results are displayed at each time point to demonstrate the difference between the monovalently immunised group and the groups immunised with the divalent and trivalent constructs (Figures 5.7-5.14). Thus, the titres elicited by rSFVE2E1, rSFVE2E1 plus rSFV (no cloned gene), rSFVE2E1 plus rSFVH and rSFVE2E1 plus rSFVHN were boosted significantly as measured by the RV antibody ELISA and latex agglutination assay and analysed by one way ANOVA ($P<0.001$) (Figures 5.2, 5.3, 5.5, 5.6). The difference between pre- and post-boost titres elicited by the trivalent rSFVE2E1, rSFVH plus rSFVHN was more significant when measured by the latex agglutination assay ($P<0.001$) as compared to the ELISA result ($P<0.01$) (Figure 5.4). This demonstrates the value of more than one method of analysis. Interestingly there were no latex agglutination titres detected in sera at day 27 (pre-boost) of mice immunised with the trivalent rSFVE2E1, rSFVH plus rSFVHN. It appears that there is a statistically significant difference between titres elicited by single rubella immunisation of the mice and titres elicited by rubella immunisation in the presence of the measles and mumps antigens ($P<0.001$) (Figure 5.7-5.13). This effect of reduction of the anti-rubella whole IgG antibody titres and latex agglutination titres is mediated by both antigens.

The longevity of the immune response was measured up to two months post-boost (Figure 5.14). Although not as long a period as described in studies by Pougatcheva *et al.*, this result demonstrates potential for long lasting immunity in a murine model. There was no significant decline in post-boost titres of mice immunised with the combined rSFVE2E1, rSFVH plus rSFVHN constructs or indeed the divalent constructs ($P>0.05$, one way ANOVA). In the monovalent rSFVE2E1 immunised group, there were no significant differences between the whole IgG titres elicited to rSFVE2E1 at week 6 or 8. This result was confirmed by the latex agglutination titres. However the latex agglutination titres at 12 weeks were lower than those measured at 8 weeks ($P<0.05$). This was not the case in the group immunised with rSFVE2E1 (no cloned gene) where there was no difference in the immune response at 8 and 12 weeks as measured by both methods ($P>0.05$).

The measles and mumps specific antibody titres at six weeks in Figure 5.15 are not as high as the E2E1 specific antibody titres displayed in Figure 5.9 despite all antigens being

delivered at the same dose. This may be due to the fact that both are administered in combination with the E2 and E1 antigens. The titres would perhaps have been higher in groups immunised solely with rSFV particles expressing the Edmonston measles H antigen or the Enders mumps HN antigen and this work is ongoing. Nevertheless MV specific IgG titres of 3 orders of magnitude were in concordance with those elicited in BALB/c mice when immunised with a DNA vaccine encoding MV-H in a study by Song, Vindurampulle *et al* in 2005. Thus this study showed that at similar titres the H antigen mediated the reduction in RV immunity in the same murine model. The anti-measles specific titres elicited by trivalent rSFVE2E1, rSFVH plus rSFVHN were significantly higher than those elicited by the divalent rSFVE2E1 plus rSFVH as measured by ELISA (P=0.0378, unpaired t test). There were however no significant differences between the anti-mumps specific titres elicited by the trivalent rSFVE2E1, rSFVH plus rSFVHN or divalent rSFVE2E1 plus rSFVHN as measured by ELISA (P=0.0872, unpaired t test). Further whole IgG titres and neutralising antibody titres for the measles and mumps constructs would have proved useful to determine if there were any trends in these titres pre-and post-boost.

As antibody production is mediated by T helper cells, information on the antibody isotypes in the serum and cytokine levels induced from stimulated splenocytes further characterised this effect and these data are presented in the next chapter. There are significant differences between mouse and human immunology in both innate and adaptive immunity (Mestas *et al.*, 2004). Some aspects of human immunology can indeed not be modelled in mice. MHC II expression is absent on T cells and there is no constitutive expression of MHC II on EC or EC presentation of Ag to $CD4^+$ T cells. An adult mouse is thought to have a preimmune B cell repertoire of individual B cells bearing surface Ig $(sIg)^4$ receptors of at least 10^7 distinct specificities (Agarwal *et al.*, 1996). Upon encounter with a T-dependent Ag those subsets of B cells with receptors that are complementary to specific determinants on the Ag are activated, resulting in a low affinity, polyclonal, early stage primary response. Clonal selection is thought to occur at two levels, both of which are likely to be driven by competition for a limiting supply of Ag (Agarwal *et al.*, 1996). The first level involves competition between independent B cell clones resulting in the selection of B cells with high affinities for Ag (Agarwal *et al.*, 1996). The second is intraclonal and results in selection of those sIg variant B cells with the highest affinity for Ag (Agarwal *et al.*, 1996). A hypothesis has been proposed to account for interclonal selection in the early stages of a primary humoral response whereby loss of sIgD on resting B cell activation imposes an affinity barrier for continued recruitment by Ag, thus selecting for only the relatively high affinity B cells (George *et al.*, 1992).

The CD46 receptor is not expressed in mice except in the testis (Kerdiles *et al.*, 2006). SLAM is a self ligand glycoprotein which is expressed on immature thymocytes, activated T and B cells as well as mature DCs and macrophages in both humans and mice (Moss *et al.*, 2006; Ohno *et al.*, 2007). There are two isoforms of the murine SLAM transmembrane 1 protein, a murine orthologue of human SLAM, called mSLAM1 and mSLAM2, differing only in their 3' region (Castro *et al.*, 1999). The predicted a.a. sequences display 58 and 50 % similarity to human SLAM (Castro *et al.*, 1999). MV can infect various cultured cells, although with very low efficiency, via SLAM- and CD46-independent pathways (Yanagi *et al.*, 2006). MV Edmonston has been shown to bind to tyrosine kinase receptors and this is thought to perhaps increase the efficiency of secondary interactions with another, as yet unidentified cell membrane component (Schneider *et al.*, 2000). Wt MVs may use a cellular receptor other than the CD46 which has not yet been identified (Johnston *et al.*, 1999).

The mechanism of immunosuppression is inadequately understood but MV replication in leukocytes and MV-leukocyte interactions probably play a critical role (Fugier-Vivier *et al.*, 1997; Nagendra *et al.*, 1995). Leukocyte function-associated antigen 1 (LFA-1) is an integrin which is differentially expressed on the surface of leukocytes. Expression of this heterodimeric transmembrane protein, composed of an alpha chain (Cd11a) and a beta chain (CD18) has been reported to be necessary for the induction of many types of host immune responses (Nagendra *et al.*, 1995). The MV Edmonston-Zagreb vaccine strain is a modulator of LFA-1 expression. The MV H protein region encompassing a.a. site 116 (starting from the amino terminus) is responsible for the activation of LFA-1 (Nagendra *et al.*, 1995).

The concept of viral interference was introduced in 1942 when experimentally inactivated influenza B virus particles were found to interfere with the propagation of various viruses in chick embryo allantois (Henle *et al.*, 1943). Results suggested that infected cells produce some non-infectious interfering viral components which in turn induce interferon production in remaining uninfected cells which makes them resistant to superinfection. The interfering viral component was thought to be an incomplete or deficient hemagglutinating virus particle which is sedimentable by high speed centrifugation and neutralisable by antiviral serum (Henle, 1963). Cells made resistant by ultraviolet-inactivated Newcastle disease virus (NDV_{uv}) or interferon regain susceptibility to VSV superinfection after approximately 9 and 7 divisions respectively (Siminovitch *et al.*, 1957).

Chapter 6

Antibody isotypes & cell mediated responses elicited by rSFV constructs

6.1 Introduction

It has been established that the antibody isotype reflects the Th1/Th2 response and IFN-γ is associated with a Th1 response in cells and upregulates IgG2a production (Cardoso *et al.*, 1996). Th1 response cytokine secretion profiles promote cellular immunity and Th2 responses promote humoral immunity (Ovsyannikova *et al.*, 2007a). IFN-γ, identified 30 years ago as an antiviral agent is produced mainly by activated NK cells, activated T helper cells of the Th1 subset, and activated $CD8^+$ CTLs of the TC1 phenotype. Mouse as well as human IFN-γ is encoded by a single-copy gene, which generates a single 1.2-kb mRNA-species and a polypeptide of 166 residues with a cleaved hydrophobic signal sequence of 23 residues (Boehm *et al.*, 1997). Biologically active IFN-γ is a noncovalent 34-kDa homodimer and its signalling mechanism is through the JAK/STAT pathway. There is evidence for a dichotomy among $CD8^+$ cells TC1 and TC2 whereby Th1 cells secrete preferentially IFN-γ with other cytokines, and Th2 cells secrete preferentially IL-4 and IL-5 with other cytokines (Boehm *et al.*, 1997). IL-12, secreted in response to stimulation by DCs, macrophages and neutrophils, directly induces IFN-γ gene transcription and secretion in antigen-stimulated naive CD4C T cells, and IL-12 also stimulates IFN-γ secretion by NK cells as part of the activation process. While IL-12 is the primary determinant of Th1 differentiation, *in vitro* the presence of endogenously synthesised IFN-γ during the priming of naive $CD4^+$ T cells both accelerates and enhances the Th1-differentiating effects of IL-12 (Boehm *et al.*, 1997). The proinflammatory IFN-γ cytokine is a known inducer of HLA molecule surface expression and an enhancer of cell mediated immunity (Ovsyannikova *et al.*, 2007a). IFN-γ also plays a key role in T cell proliferation, differentiation and macrophage activation. Interferon has actually been correlated with establishing and maintaining a persistent infection with RV in different cell lines (Stanwick *et al.*, 1974). Viral infection results in endogenous processing and presentation of fragments of viral proteins to T-lymphocytes by MHC molecules (Cusi *et al.*, 1995). The structure of the MHC molecules that present peptides as well as the T-cell receptors influence the recognition of peptide antigens (Ou *et al.*, 1994). Different minimal T cell epitopes may be recognised by T cells from different MHC backgrounds and they may show some variation between species (Ou *et al.*, 1994). This chapter describes the indirect investigation of the T-helper phenotype on post-boost sera (2 weeks post-boost) as analysed in Chapter 5 and the cytokine profile of splenocytes stimulated with E2 and E1 recombinant proteins following immunisation with the same constructs as previously described.

6.2 Results

6.2.1 Antibody isotyping of monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN immune response

As an indirect measure of Th1 and Th2 immune responses induced by rSFV immunisation in Chapter 4, the six week (two weeks post boost) serum levels of the Ig isotypes IgG1 (Th2) and IgG2a (IgG3 in humans) (Th1) were titrated by two-fold limiting dilution ELISA. BALB/c mice immunised as detailed in section 2.2.6.2.2 with the monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN particles developed higher IgG2a anti-E2E1 titres than IgG1 anti-E2E1 titres suggesting a T-helper 1 immune response (Figure 6.1) ($P<0.001$, Two-way ANOVA). The immune response was lowered in mice co-immunised with rSFVE2E1, rSFVH plus rSFVHN particles (Figure 6.1).

6.2.2 Antibody isotyping of monovalent rSFVE2E1, trivalent rSFVE2E1, rSFVH plus rSFVHN, divalent rSFVE2E1 plus rSFVH and divalent rSFVE2E1 plus rSFVHN immune responses

A measure of IgG2a and IgG1 immune responses in six weeks (two weeks post boost) sera as detailed in chapter 5 was undertaken (Figure 6.2 & section 2.2.6.3). BALB/c mice immunised with monovalent rSFVE2E1; the control group rSFVE2E1 plus two doses of rSFV (no cloned gene) and divalent rSFVE2E1 plus rSFVH developed IgG1 titres of between 4 and 5 orders of magnitude. Mice immunised with trivalent rSFVE2E1, rSFVH plus rSFVHN and divalent rSFVE2E1 plus rSFVHN developed IgG1 titres of between 3 and 4 orders of magnitude. BALB/c mice immunised with monovalent rSFVE2E1 developed IgG2a titres of about 5.5 orders of magnitude while those immunised with the control group rSFVE2E1 plus two doses rSFV (no cloned gene); divalent rSFVE2E1 plus rSFVH and divalent rSFVE2E1 plus rSFVHN developed titres of between 5 and 5.5 orders of magnitude. BALB/c mice immunised with trivalent rSFVE2E1, rSFVH plus rSFVHN developed IgG2a titres of between 4 and 5 orders of magnitude. The anti-RV titres of IgG2a were found to be 0.5 of an order of magnitude higher in the mice immunised with rSFVE2E1 than those immunised with rSFVE2E1, rSFVH plus rSFVHN; rSFVE2E1 plus rSFVH or rSFVE2E1 plus rSFVHN ($P<0.001$, $P<0.01$ and $P<0.001$ respectively, one-way ANOVA) (Figure 6.2).

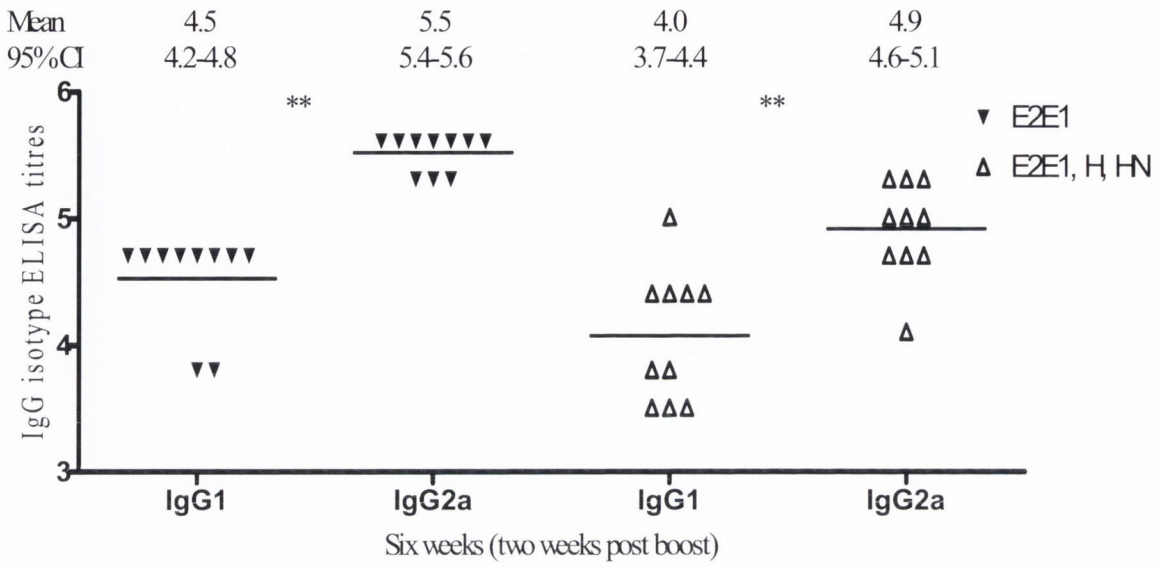


Figure 6.1 Antibody isotyping of six week (2 week post boost sera)

Analysis of serum univariate and bivariate IgG1 and IgG2a (IgG3 in humans) responses in BALB/c mice from Figures 4.8 and 4.9, immunised with monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs (n=10). The IgG2a titres were higher than the IgG1 titres at all time points (**P<0.001) (two-way ANOVA) which is indicative of a T helper 1 immune response. Log_{10} mean titres and 95% confidence intervals are displayed. The IgG2a/IgG1 ratios were calculated as the difference between the logarithms of the IgG2a and IgG1 titres and were determined using a paired *t* test as in Table 6.1.

Table 6.1 Table illustrating IgG2a/IgG1 ratios in six week (2 weeks post-boost) sera

Sera from mice immunised with monovalent rSFVE2E1 and trivalent rSFVE2E1, rSFVH plus rSFVHN and boosted at four weeks from Figure 6.1 were analysed for IgG2a and IgG1 isotypes by limiting dilution ELISA. The titres were log transformed and the difference between the logarithms of the IgG2a and IgG1 titres was back-transformed to yield the geometric mean ratio. The data suggested that the IgG2a titres were higher in all groups as determined by the paired *t* test.

Antigen	**E2E1**	**E2E1, H, HN**
P value from paired *t* test	**0.0002**	**0.0007**
Mean log difference	0.842	0.842
95% Confidence interval	0.525-1.161	0.4658-1.22
Geometric mean of ratio	**6.95**	**6.95**
95% Confidence interval	3.349-14.487	2.91-16.59

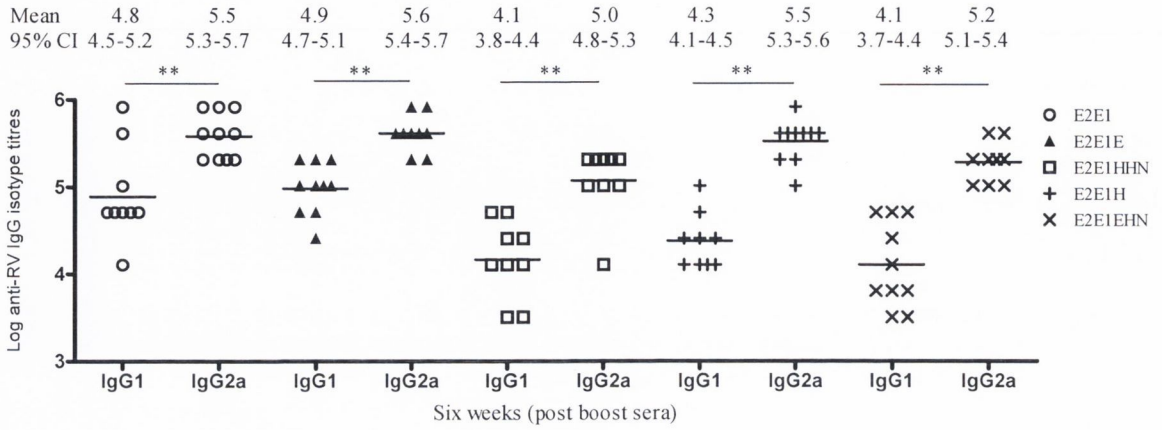


Figure 6.2 Antibody isotyping of six week (two week post boost) sera

Analysis of serum univariate and bivariate IgG1 and IgG2a (IgG3 in humans) responses in BALB/c mice from Figures 5.9 and 5.10, immunised with monovalent rSFVE2E1, divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs (n=10). The mean IgG2a titres were higher than the mean IgG1 titres in all groups (**P<0.001) (two-way ANOVA) which is indicative of a T helper 1 immune response. The RV-specific IgG2a titres were higher in the monovalent E2E1 immunised groups (E2E1, E2E1, E) compared to the trivalent E2E1, H, HN immunised group (P<0.01) but not compared to the divalent immunised groups (A; P>0.05) (one-way ANOVA, residual standard deviation = 0.3). Log_{10} mean titres and 95% Confidence intervals are shown. The IgG2a/IgG1 ratios are calculated as the difference between the logarithms of the IgG2a and IgG1 titres and were determined using a paired *t* test as in Table 6.2.

Table 6.2 Table illustrating IgG2a/IgG1 ratios in six week (2 week post boost) sera

Sera from mice as seen in Figure 6.2, immunised with monovalent rSFVE2E1, monovalent rSFVE2E1 plus rSFV (no cloned gene), divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs and boosted at four weeks were analysed for IgG2a and IgG1 isotypes by limiting dilution ELISA. The titres were log transformed and the difference between the logarithms of the IgG2a and IgG1 titres was back-transformed to yield the geometric mean ratio. The data suggested that the IgG2a titres were higher in all groups as determined by the paired *t* test.

Antigen	**E2E1**	**E2E1, E**	**E2E1, H, HN**	**E2E1, H**	**E2E1, HN**
P value	**0.0073**	**0.0002**	**0.0001**	**0.0001**	**0.0001**
Mean log difference	0.692	0.6322	0.903	1.144	1.174
95% CI	0.237-.14	0.395-0.869	0.582-1.224	0.899-1.388	0.775-1.573
Geometric mean ratio	**4.92**	**4.28**	**7.9**	**13.93**	**14.92**
95% CI	1.72-4.02	2.48-7.396	3.819-16.749	7.925-24.434	5.956-37.411

6.2.3 Measurement of cell mediated immunity by IFN-γ cytokine levels

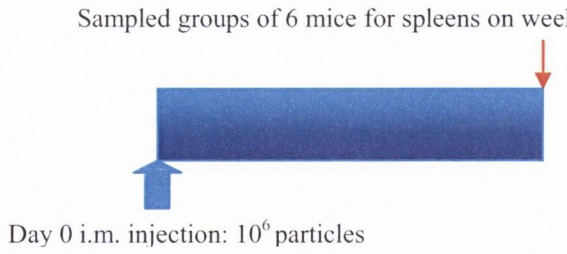


Figure 6.3 Immunisation regimens for cytokine analysis of splenocytes

Experiments involving measurement of the lymphoproliferative response to rubella antigen stimulation and interferon-gamma production by stimulated splenocytes proved insensitive (Figure 6.4). Splenocyte proliferation was induced with E2 (a.a.s 31-105) recombinant protein coupled with E1 (a.a.s 157-176, 374-390 and 213-239) recombinant mosaic protein and Con A, a positive control mitogen, seven days post prime as described in section 2.2.7.4. Mice were immunised as described in section 2.2.6.4 and illustrated in Figure 6.3. The supernatant harvested from the growth medium of the stimulated splenocytes was assayed using a cytokine ELISA to detect IFN-γ denoting a T helper 1 (Th1) response. In mice the IFN-γ cytokine, which acts as a macrophage activator, induces the production of IgG2a isotypes which corresponds with the Ig isotype data. Splenocytes stimulated with Con A released some IFN-γ which was higher than that released from splenocytes incubated with the E2E1 recombinant protein ($P<0.001$). The difference between these two groups was significant. There was no IFN-γ production from splenocytes incubated solely with growth medium as expected with the negative controls.

6.2.4 Measurement of cell mediated immunity by IL-5 cytokine levels

Splenocytes were assessed for IL-5 profiles by analysis of the supernatant of the splenocytes which had been stimulated as in section 2.2.7.4 with recombinant E2 and E1 RV proteins and Con A, a positive control mitogen, seven days post prime (section 2.2.6.4, Figure 6.3). Cytokine ELISAs performed on the growth medium of the stimulated splenocytes were used to detect IL-5 release which denotes a T helper 2 (Th2) response. In mice the IL-5 cytokine, required for B cell differentiation induces the production of the IgG1 isotype. IL-5 was only detected in the supernatant from the positive control, ConA stimulated splenocytes (Figure 6.5).

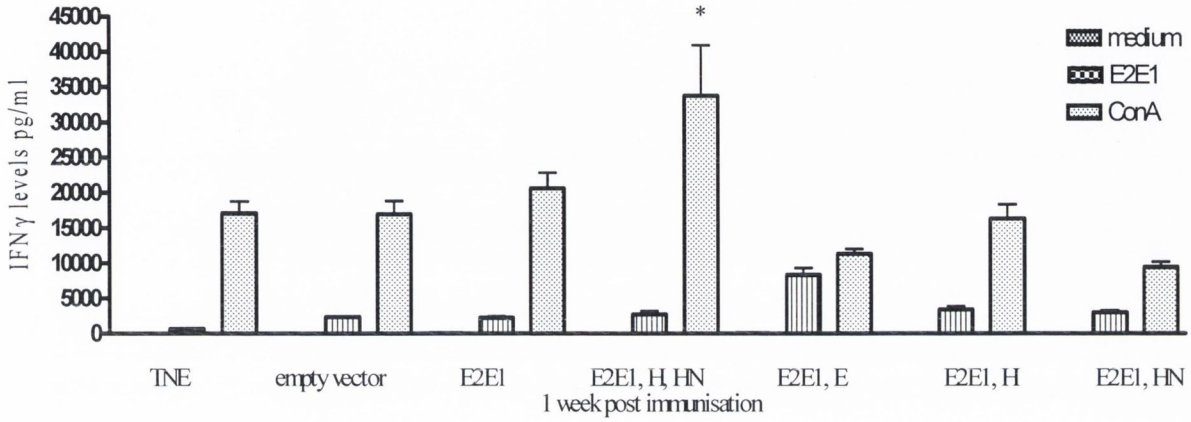


Figure 6.4 IFN-γ levels in BALB/c mice following particle administration

BALB/c mice (n=6) were immunised with monovalent rSFVE2E1, monovalent rSFVE2E1 plus rSFV (no cloned gene), divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs. Spleens were removed after 7 days and homogenized as described in section 2.2.7.1. Homogenised tissue samples were stimulated with the positive control ConA mitogen, E2E1 recombinant proteins and a negative control of growth medium and tested for the presence of IFN-γ by capture ELISA as described in section 2.2.7.4. The only significant amount of IFN-γ was found in E2E1, H, HN immunised splenocytes stimulated with ConA. There were no other detectable differences ($P>0.05$, one way ANOVA).

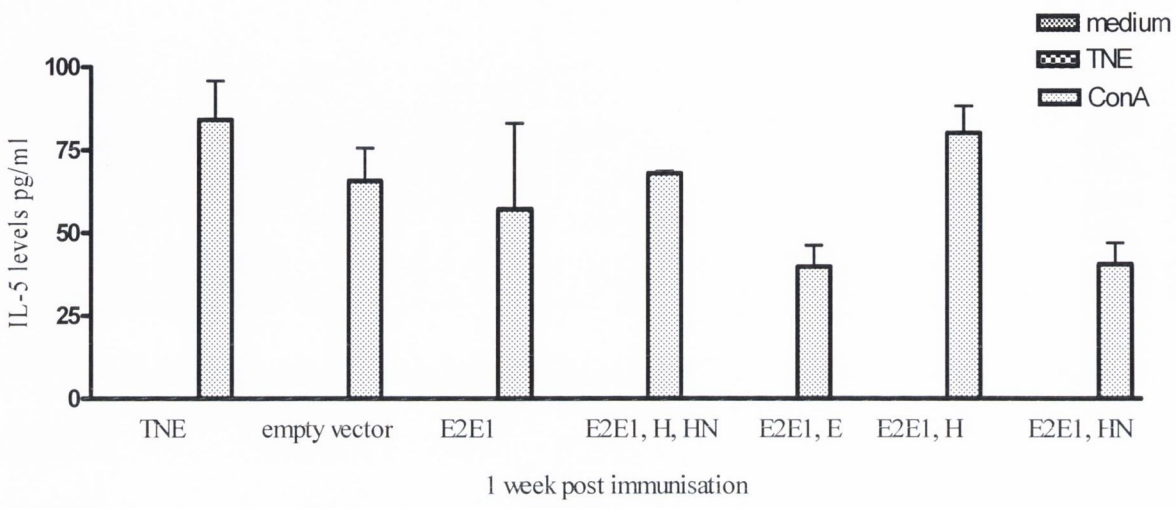


Figure 6.5 IL-5 levels in BALB/c mice following particle administration

BALB/c mice (n=6) were immunised with monovalent rSFVE2E1, monovalent rSFVE2E1 plus rSFV (no cloned gene), divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs. Spleens were removed after 7 days and homogenized as described in section 2.2.7.1. Homogenised tissue samples were stimulated with positive control ConA mitogen, E2E1 recombinant proteins and negative control growth medium and tested for the presence of IL-5 by capture ELISA as described in section 2.2.7.4.

6.3 Discussion

The null hypothesis of this study was that there would be no difference between the mean IgG2a and the mean IgG1 antibody isotype titres. However, the probability of having observed the data (or more extreme data) were this null hypothesis true is <0.05. Thus there was a statistically significant difference between the IgG2a and IgG1 titres elicited by the monovalent rSFVE2E1, monovalent rSFVE2E1 plus rSFV (no cloned gene), divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs. While the difference in mean titre was significant for each group, the ratios of anti-RV specific IgG2a to IgG1 antibodies were higher in groups immunised with the divalent rSFVE2E1 plus rSFVH, divalent rSFVE2E1 plus rSFVHN and trivalent rSFVE2E1, rSFVH plus rSFVHN vaccine constructs. There was thus no shift of antibodies to a Th2 response (IgG1) when the mice were immunised with the measles and mumps antigens. The ratios of IgG2a to IgG1 antibodies were in fact higher in mice immunised with rSFVE2E1 plus rSFVH and/or rSFVHN. While polarization of T cell populations and the Th1/Th2 paradigm is readily observed in mice, in the human immune system T cells of both phenotypes can often be generated simultaneously (Mestas *et al.*, 2004). The mean isotype titres were higher than the whole IgG titres and this may be explained by the biotin-streptavidin system used with the isotype secondary antibodies which may amplify the signal. It is worth noting that the IgG2a/IgG1 data does not reflect functional antibody isotypes. In murine models, the antibody isotype induced following intramuscular injection with measles H DNA is predominantly IgG2a which has the advantage that it can activate both the classical and the alternative pathways of complement fixation (Cardoso *et al.*, 1996). The cell mediated response to rubella virus is not well characterised in the mouse (Pougatcheva et al., 1999). There were negligible amounts of IFN-γ and no quantities of Il-5 produced by splenocytes stimulated with recombinant E2 and E1 proteins. T cell epitopes are found on all three RV structural proteins (Chantler *et al.*, 2001). The recombinant proteins used contain a major immunodominant T cell epitope of the E1 protein. However, as protection to RV infection is primarily antibody mediated, the focus remained on the Ig isotyping data. Rubella virus will have to be characterised in this regard to validate the use of mice as a model for pathogenesis studies or for more extensive testing of vaccine prototypes (particularly protection studies) (Pougatcheva *et al.*, 1999). IFN-γ is the main switch factor regulating IgG2a switching in the mouse and a minor regulator of switching to IgG3 (Boehm *et al.*, 1997). The Th1 response is believed to be stimulated mainly by the cytokines; IL-12, released

from monocytes, macrophages and DCs and IFN-γ, released by Th1 lymphocytes, NK cells and macrophages, where IFN-γ appears to make naïve CD4+ T cells more responsive to IL-12 (Berglund *et al.*, 1999). Measles vaccination can cause immune suppression characterised by IL-4 production and polarization of effector CD4+ T cells to produce T helper type 2 (Th2) cytokines. This skewing results in a deficient or low Th1 cellular immune response and increases susceptibility to co-infections with various other pathogens (Dhiman *et al.*, 2005). With an SFV induced immune response this effect could potentially be counteracted. Thus regardless of antigen expressed, the rSFV particles induced predominantly IgG2a (IgG3 in humans) antibodies which function in neutralisation, thereby indicating a possible Th1 type immune response. H specific T cell clones have been shown in murine cells. All the T cell clones recognised Ag in the context of H-2^d molecules and displayed a helper phenotype (Thy-1.2^+, $CD4^+$, $CD8^-$) (de Vries *et al.*, 1989). T cell clones produced IL-12 and IFN-γ in response to stimulation with UV-inactivated MV suggesting they belonged to a Th1-helper subset (de Vries *et al.*, 1989). Mouse lymphocytes have been shown to respond to defined regions of the H protein which differ according to mouse strain (Obeid *et al.*, 1993). The T cell repertoire for epitopes on the H protein was shown to be broader than that following immunisation with virus (Obeid *et al.*, 1993). In two strains of mice, one peptide was shown to be a dominant T cell epitope. TCR engagement with peptide-MHC is of central importance for the immune response of the host. In murine cells expressing CD46, after MV binding and fusion, H becomes associated with the PM and its ectodomain (the part of a transmembrane protein that projects out of the cell into the extracellular space) can reach the endosomal MHC-II but not the cytosolic MHC-I antigen presentation pathway (Cardoso *et al.*, 1996).

A vaccinia virus recombinant expressing the MV H proteins and administered intranasally has been shown to be immunogenic in mice (Etchart *et al.*, 1996). A CTL response was achieved by mucosal vaccination with a recombinant DNA plasmid encoding the H gene (Etchart *et al.*, 1997). Data suggests that the H proteins are targets for the measles CTL response (Jaye *et al.*, 1998). Another vaccinia based viral vector expressing measles H has been shown to elicit adult-like Th1 and CTL responses after injection, however despite the strong immunogenicity, antibody responses remained susceptible to inhibition by pre-existing measles antibodies (Kovarik *et al.*, 2001). In a rhesus macaque model a DNA H vaccine primed for a type 2 cytokine response with suppression of IL-12 and preferential production of IL-4 after challenge (Polack *et al.*, 2003).

Chapter 7

General Discussion

7.1 General Discussion

In this study the null hypothesis proposed was that the Edmonston H and Enders HN virion proteins would have no effect on the immune response in mice to the RA27/3 immunogenic E2 and E1 envelope proteins. In fact the H and HN virion proteins significantly reduced the immune response to the E2E1 proteins in BALB/c mice when administered together and separately. Since the derivation of the highly successful MMR attenuated vaccines, recombinant DNA technology has progressed. The live attenuated strain of RV currently cannot be recommended during pregnancy (despite the absence of adverse effects in babies born to mothers who were inadvertently vaccinated), acquired immunodeficiency disease syndrome (AIDS) children and children with other severe immunodeficiencies. Rubella non-immune women of childbearing age thus constitute a population at risk for CRS and those with immunodeficiencies can suffer severely from rubella infection (Perrenoud *et al.*, 2004). Thus, one of the main reasons for a non replicating vaccine would be to provide coverage in the case of a seronegative pregnant woman until the live attenuated vaccine could be administered postpartum (Pougatcheva et al., 1999). However, a recombinant nonreplicating vaccine could be particularly useful for these populations and in countries with suboptimal coverage, since it could be indiscriminately administered to at risk individuals (Perrenoud *et al.*, 2004).

The mouse is not a reliable animal model for the study of clinically symptomatic rubella infection and thus a challenge model was not pursued (Chantler et al., 2001). Nonetheless, this model provided a practical and economical initial base to begin assessing the immunogenicity of our SFV based constructs. Pre- and post-boost responses to the introduced RA27/3 E2E1 antigens are not inhibited by responses to the SFV carrier as demonstrated by equivalent responses to the rSFV-E2E1 expressing particles co-administered with two doses of empty particles. With the exception of small amounts of SFV viral replicase, no other vector proteins are produced which is an additional advantage when repeated immunisations with the same vector are needed (Zhou *et al.*, 1995). The humoral immune response following intravenous immunisation with rSFV-LacZ has been compared for doses of 10^2, 10^4 or 10^6 infectious units (I.U.) (Berglund *et al.*, 1999). Humoral immune responses were not detectable in mice immunised with one or two doses of 10^2 I.U. Mice that received two doses of 10^4 I.U. rSFV-LacZ displayed IgG at significant levels. One dose of 10^4 was insufficient to induce IgG titres. Immunisation with one or two doses of 10^6 rSFV-LacZ virus induced detectable IgG titres which were increased significantly following the second dose (Berglund *et al.*, 1999). Suicide SFV has been shown to induce cellular and

humoral anti-viral responses in two haplotype strains of mice, C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (Zhou *et al.*, 1995). As few as 100 particles induced a significant CTL response. In cell culture, a single cell may optimally produce up to 0.1 ng of protein which is not predicted *in vivo*, as probably not all administered particles will infect a cell. A dose of 100 I.U. could at best produce 1 ng of antigen *in vivo*, assuming only 10% of antigen production compared to the amount seen *in vitro*. Priming *in vivo* with 10^6 I.U. of SFVNP, however, gave the highest specific lysis of target cells expressing endogenous influenza viral Ag or exogenous influenza peptide. The latter dose was favoured over 10^4 and 10^2 recombinant viral particles although both of these doses also generated significant CTL responses (Zhou *et al.*, 1995).

Viral interference is described as the prevention of the replication of one virus by another and is a result of a number of different mechanisms, one of which may be contributing to the observed effect (Mahy, 1997). Virus attachment to cell receptors can be prevented by previous exposure to any virus which alters or destroys them. An example of this is homologous interference whereby a virus interferes with its own replication. However the use of two doses of SFV vector with no cloned gene did not reduce the rubella specific immune response in these experiments. Heterologous interference occurs between different virus species and is most frequently due to interferon production. However it can also be due to attachment interference or to some poorly understood mechanism of blocking of virus replication. Epitope dominance may be a factor in the immunosuppression described in the present study with a role being played by immune regulatory cells in selecting one type of immune response over another, but there is no evidence for this at present. The structural basis for immunodominance, whereby certain peptides within a large protein sequence are preferentially presented by the immune system during T cell recognition is partly a function of the peptide-MHC interaction (Nepom *et al.*, 1997). Concentrated investigation has centred on elucidation of the factors that determine antigenicity, immunogenicity, and immunodominance in antibody responses (Peters *et al.*, 2005). The presence (or absence) of helper T cells, and their phenotype, also has a dramatic influence on the type of B cell response observed and on the epitopes recognised (Peters *et al.*, 2005). T cells specific for a given epitope might limit the expansion of other T cell specificities by competition for cell-cell interactions, space or nutrients; or by rapidly eliminating or decreasing the pathogen concentration before other epitope specificities could be effectively stimulated (Peters *et al.*, 2005).

Only those peptides which bind MHC molecules in an individual are capable of being presented to T cells for recognition. This restricts the potential dominant recognition pattern to a few peptides from which additional determinants of immunodominance, at the level of T cell recognition are selected (Nepom *et al.*, 1997). Antigenic competition is more

often demonstrated in $CD8^+$ T cells where competition between T cells specific for responses to the same MHC/antigen are frequently observed and rarely observed for different MHC/antigen (Kedl *et al.*, 2003). Only competition between $CD4^+$ T cells for response to the same antigen has been observed. Data from other studies suggest that measles, mumps and rubella viral antigens may share common epitopes and most likely use a common antigen processing pathway (Ovsyannikova *et al.*, 2006). Prior immunisation against a subdominant antigen could dramatically reduce the response against a dominant antigen upon subsequent immunisation against both antigens (Kedl *et al.*, 2003). A subtype of T cells with immunosuppressive function and cytokine profiles distinct from either Th1 or Th2 cells, termed regulatory T (Tr) cells have been described (McGuirk *et al.*, 2002). Data suggest that Th1 and Tr1 cells circulate at the same time and that the Tr cells may be detectable prior to the generation of Th1 responses (McGuirk *et al.*, 2002). Some Tr1 cells exclusively secrete IL-10, type 3 T (Th3) cells secrete primarily TGF-β and $CD4^+CD25^+$ T cells inhibit immune responses through cell to cell contact. In addition recent studies have identified $CD8^+$ Tr cells which secrete either IL-10 or TGF-β (McGuirk *et al.*, 2002). Suppressive effects of Tr1 cell clones are reversed by neutralising IL-10. Th3 regulatory cells secrete high levels of TGF-β and may have a role in many aspects of immune regulation (McGuirk *et al.*, 2002). The primary function of Tr1 cells appears to be to suppress inflammatory or Th1-type protective responses and certain Tr1 cells can also suppress Th2 responses. It has been demonstrated that Tr1 cells recognise the same antigens or peptides on a pathogen as Th1 cells (McGuirk *et al.*, 2002). $CD4^+CD25^+$ Tr cells, $CD4^+CD25^-$ T cell population, $CD8^+$ cells and NK cells have also been shown to exert immunosuppressive function *in vitro* or *in vivo* (Mills *et al.*, 2004). Tr1 cells secrete high levels of IL-10 with or without IL-5, IL-13 or TGF-β but little or no IL-2, IL-4 and IFN-γ (Mills *et al.*, 2004). Tr1 or Th3 cells are generated from naïve T cells in the periphery after encounter with antigen and under the direction of DC with an activation status distinct from those that promote the differentiation of Th1 or Th2 cells (Mills *et al.*, 2004). DCs are one source of IL-10 that may promote the differentiation of Tr1 cells e.g. monocyte and NK t cells (Mills *et al.*, 2004). The demonstration that Tr cells can suppress the cytokine secretion and proliferation of both Th1 and Th2 cells indicates that certain pathogens may promote the induction of Tr cells in order to thwart protective immune responses (Mills, 2004; Mills *et al.*, 2004).

Antigen administered in high or repeated doses to primed animals can result in antigen-specific tolerance rather than an anamnestic response (Falk *et al.*, 2000). The suppression of responsiveness to high peptide doses is due to TCR downmodulation,

anergy and eventually death of the responding T cells (Falk *et al.*, 2000). TCR downregulation results from internalization of the entire TCR/Cd3 complex followed by its recycling or degradation and is due to antigenic stimulation, protein kinase C (PKC) activation or after CD3 ligation by mAb (Falk *et al.*, 2000). It's function appears to be to limit or adjust the T cell response to any given antigen and a strong antigenic stimulus often results in a reversible form of T cell tolerance called anergy (Falk *et al.*, 2000). TCR downregulation is correlated with the occupancy by an MHC peptide ligand and strong agonists lead to higher levels of down regulation while antagonists can completely inhibit down-regulation. Highly activated T cells attempt to expand but undergo apoptosis even faster, assisted perhaps by activation induced upregulation of apoptosis factors e.g. Fas and FasL (Falk *et al.*, 2000). TCR antagonism is characterised by selective inhibition of T cell responses by non-stimulatory antigen analogs. T cell anergy is characterised by lack of proliferation and lymphokine production. TCR antagonism and T cell tolerance are phenomena independent of each other (Alexander *et al.*, 1994). Anergic T cells can function as suppressor cells by inhibiting Ag presentation by DC (Bonnefoix *et al.*, 2003).

Vaccine failure has been reported with viral vaccines and an HLA-linked reason for the lack of response to a hepatitis viral vaccine has been suggested (Nepom *et al.*, 1997). Children who were homozygous for the HLA-DPB locus had higher mean rubella antibody levels compared with children who were heterozygous for the same locus (St. Sauver *et al.*, 2002). The association between HLA-DPB homozygosity and rubella antibodies is not observed with measles or mumps antibodies as well (St. Sauver *et al.*, 2002). While not all peptides which bind class II molecules induce immune responses, it is essential for peptides to bind class II molecules in order to be immunogenic (Nepom *et al.*, 1997). Given the genetic differences in HLA molecules, the existence of HLA allele-specific peptide binding provides a structural explanation of how a peptide may be immunodominant in one individual but not in another (Nepom *et al.*, 1997). In humans class I and class II HLA haplotypes have been associated with immune responses after 2 doses of MMR vaccine (Ovsyannikova *et al.*, 2006). In a previous study, significant associations were demonstrated between HLA class I genes and antibody levels after a single dose of measles vaccine. According to data, extinction of HLA associations with antibody levels follows a second dose of the MMR vaccine (Nepom *et al.*, 1997). Studies suggest that RV specific humoral and cellular immune responses in humans mediated by class II molecules may be more effective than those mediated by class I molecules (Ovsyannikova *et al.*, 2006). A 2 dose rubella vaccination regime may overcome the influence of HLA class I alleles on RV vaccine-induced antibody levels (Nepom *et al.*, 1997). Peptide vaccine design for

rubella has involved combinations of peptides which bind separately to multiple MHC molecules in an allele specific manner but together encompass multiple HLA specificities (Nepom *et al.*, 1997). Antibody levels to the MMR vaccine are not correlated. These viruses probably produce different antigenic peptides that may be presented differently by the same HLA genes. Thus it is likely that HLA associations with rubella antibodies (or lymphoproliferative levels) will differ from those with measles or mumps immune responses (St. Sauver *et al.*, 2002). The majority of HLA class I and class II molecules can be grouped in supertypes based on overlapping peptide binding sequence motifs and repertoires (Ovsyannikova *et al.*, 2007b). The use of HLA supertypes in a new MMR vaccine could provide antigenic peptides that will bind HLA alleles of a large proportion of the population (Ovsyannikova *et al.*, 2007b). Genetic associations between rubella vaccine immune responses and HLA supertypes have been found to be not as strong as those observed for measles and mumps (Ovsyannikova *et al.*, 2007b). A genetic association between humoral antibody level after measles vaccine and the HLA class II genes has been shown (Poland *et al.*, 2002). The alleles, DRB1 and DQA are associated with seronegativity (Poland *et al.*, 2002). In fact, an association has been made between antibody response and differential HLA class I and class II gene activation and may explain one potential mechanism underlying measles vaccine non response (Dhiman *et al.*, 2003a). Specifically a decrease in expression of HLA class I MICB molecule, HLA class 1-A and MHC complex class III heat shock protein (HSP) genes was demonstrated in seropositive individuals (Dhiman *et al.*, 2003a). However, studies suggest that TAP and DM gene polymorphisms do not influence antibody levels post measles vaccination (Dhiman *et al.*, 2003b). There are differences between the human and murine immune systems. Human EC can present antigen to CD4+ T cells as well as to CD8+ T cells as humans express MHC class I and II molecules whereas mouse EC express only MHC class II molecules (Mestas *et al.*, 2004).

The capacity of MV-infected monocytes to present different antigens to T lymphocytes has been tested. MV-infected peripheral blood mononuclear cells (PBMC) express up to 10-fold higher amounts of HLA-DR, -DP and -DQ than uninfected cells, thus wt MV-infected monocytes retain their antigen-presenting function (Leopardi *et al.*, 1993). The MV-infected monocytes were able to efficiently present MV antigens but the presentation of unrelated exogenous antigens to specific T lymphocytes was prevented (Leopardi *et al.*, 1993). A direct effect by wt MV on MHC class II expression might be exerted at the gene regulation level (Leopardi *et al.*, 1993). Wild type MV infected monocytes were thus found to present MV antigens but no unrelated antigens. Two reasons

were suggested. Firstly, this effect was thought to be due to competition between endogenously produced MV proteins and exogenous proteins at the presentation sites. Secondly, it was thought that MV infection perturbs antigen internalization and/or processing pathways within the cell. Thus, the blocking of responses to other recall antigens may result in an inhibition of the T cell-mediated response (Leopardi *et al.*, 1993). The antigen presenting function of wt MV-infected PBMC has been tested (Leopardi *et al.*, 1993). Uninfected or MV-infected PBMC were added to antigen-specific T cell cultures, and the proliferative response of T cells was assayed after further addition of rubella or tetanus toxoid antigens (Leopardi *et al.*, 1993). When rubella antigens were added to the respective antigen-specific T cell lines together with MV-infected PBMC, no stimulation of the T cells was found. In experiments with tetanus toxoid antigen, similar results were found. Results were similar when PBMC were added at 1, 24 or 48 h post-infection (Leopardi *et al.*, 1993).

For MV, coexpression of both the F and the H proteins is necessary and sufficient to induce immune suppression *in vitro* (Heaney *et al.*, 2002b). MV is rapidly cleared from the host, although lymphoproliferative responses to mitogens and recall antigens are suppressed for up to several months postinfection. The $CD4^+/CD8^+$ ratio is not altered despite both B and T subsets of leukocytes being affected (Heaney *et al.*, 2002b). It has been shown that immune suppression in the cotton rat model was due to cell cycle arrest, not apoptosis of the responder cell (RC) population (Heaney *et al.*, 2002b). Among the measles viral proteins only the N protein seems capable of inhibiting *in vitro* the synthesis of antibody and has been shown to block the proliferation of both non infected and activated T lymphocytes following interaction with the N receptor (Speziano *et al.*, 2004). It has been found that coexpression of both the F and the H glycoproteins on lymphocyte presenter cells (PC) were sufficient for induction of immunosuppression. This interaction of morbillivirus H and F proteins seems to involve some conformational change that is required to bind the as-yet-unidentified ligand responsible for initiating the process (Heaney *et al.*, 2002b). In conclusion, we have shown that a recombinant particle SFV vector inducing good immunity against the RV envelope proteins can be constructed using the SFV vector, which could possibly be used as an alternative to the present live virus vaccine after further testing and development. However, similar vectors expressing the H protein of MV and the HN protein of mumps virus suppress the immune response to the rubella vector in mice.

7.2 Future work and directions

The results of these experiments add to the body of literature on the interactions of the components of the MMR vaccine antigens. *In vitro* the epitope conformation of the regions with differences in three residues, when compared to the RA27/3 published sequence, could be analysed by immunofluorescence using custom-made antibodies. Indeed a western blot could determine differences in size between a glycosylated E2 protein and one without a carbohydrate moiety attached at the N-linked glycosylation site. This could determine whether the N-linked glycosylation site is utilised normally.

In vivo the immunisation schedule, the route of administration or the use of an adjuvant may influence the nature of the immune response generated. The codon usage of the GC rich RV is substantially different to normal eukaryotic cells and the SFV replicase machinery may consequently also have difficulty transcribing these genes. Further experimentation with other antigens is necessary to determine whether this is a specific effect of the measles and mumps proteins on the rubella protein, a property of the rubella protein itself, or a general immune suppressive or heterologous interference effect produced by measles H and mumps HN proteins. Administration of the vectors at different concentrations and in different muscles could perhaps determine whether this is a gene dosage or homologous interference effect, and the use of at least one other vector could determine whether it is an effect of antigen presentation by the SFV vector.

Chapter 8

References

8.1 References

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