Development of a candidate VP2-based subunit vaccine for African Horsesickness virus serotype 5

BY

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PHILIPIANS 4:13: **"I have strength for all things in Christ Who empowers me."**

\- THE AMPLIFIED BIBLE

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SUMMARY

DEVELOPMENT OF A CANDIDATE VP2-BASED SUBUNIT VACCINE FOR AFRICAN HORSESICKNESS VIRUS SEROTYPE 5

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African horsesickness (AHS) is a lethal disease of horses. The aetiological agent is African horsesickness virus (AHSV) (genus *Orbivirus;* family: *Reoviridae)*. Immunity to all nine serotypes is needed for full protection. The AHSV virion is composed of seven structural proteins and 10 dsRNA genes. The outer capsid protein, VP2, determines the serotype and elicits protective neutralising antibodies (NAbs).

The existing polyvalent attenuated vaccine has some drawbacks. The most important ones are the exclusion of serotypes 5 and 9. Also, vaccinated and naturally infected horses cannot be differentiated. This impedes the international movement of horses. Recombinant subunit vaccines should solve both problems. Previously, it was established that baculovirus-expressed AHSV VP2 induces NAbs and partial protection. The main aim of this investigation was to improve the level of protection and develop a prototype VP2-based AHSV-5 vaccine to supplement the current attenuated vaccine and pave the way for developing a VP2-based subunit vaccine that incorporates all nine AHSV serotypes.

Preliminary challenge experiments in horses indicated that AHSV-5 rVP2 provides incomplete protection against a lethal AHSV-5 challenge. Probable causes and solutions for this problem had to be sought. Analysis of the baculovirus-expressed AHSV-5 VP2 showed that most (*ca.* 90 %) is aggregated and that only the soluble part (*ca.* 10 %) elicits NAbs in guinea pigs and full protection in horses. Preliminary small-scale production studies indicated that solubility of the AHSV-5 rVP2 could be improved considerably by optimising *in vitro* infection conditions.

A significant finding was that the safety and efficacy of soluble AHSV-5 rVP2 is determined by the adjuvant used. Saponin-based adjuvants rendered the best results, albeit with dose-related side effects in horses. Saponin Q was tolerated best. These results for the first time implicated AHSV VP2 in stimulating a protective cell-mediated immunity. Based on these results, it is recommended that a candidate AHSV-5 VP2-based subunit vaccine, consisting of 50 µg rVP2 and 3.0 mg Saponin Q for the primary immunisation followed by a 50 µg rVP2 and 0.6 mg Saponin Q booster, be formulated for the purpose of field trials.

The knowledge generated during this study, combined with the recent cloning of the VP2 genes of all nine AHSV serotypes, provides a route for the development of a complete recombinant vaccine that will offer protection against all nine AHSV serotypes and could well free the restraint on the import and export of horses to and from South Africa.

OPSOMMING

DEVELOPMENT OF A CANDIDATE VP2-BASED SUBUNIT VACCINE FOR AFRICAN HORSESICKNESS VIRUS SEROTYPE 5

DEUR

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Perdesiekte (AHS) is 'n dodelike siekte van perde. Dit word veroorsaak deur die perdesiekte virus (PSV) van die genus *Orbivirus* en familie *Reoviridae*. 'n Perd moet immuniteit teen al nege serotipes toon vir volle beskerming. Die PSV virion is saamgestel uit sewe strukturele proteïene en 10 ddRNS gene.

Die geattenueerde polivalente entstof wat tans teen perdesiekte gebruik word toon sekere tekortkomings. Die belangrikste hiervan is die afwesigheid van serotipes 5 en 9 en die onvermoë om tussen natuurlik geïnfekteerde en geënte perde te onderskei. Hierdie beperkinge bemoeilik die internasionale vervoer van perde. Rekombinante subeenheid-entstowwe behoort 'n oplossing vir bovermelde probleme te bied. Voordat hierdie studie onderneem is, is alreeds bewys dat die buitedop PSV proteïen VP2, wat in die bakulovirus sisteem uitgedruk word, neutraliserende teenliggame en gedeeltelike beskerming bewerkstellig. Die hoofdoel van hierdie ondersoek was om beskerming te verbeter en om 'n prototipe VP2-gebaseerde PSV-5 entstof te ontwikkel om die huidige geattenueerde entstof aan te vul en sodoende

die fondasie te lê vir die ontwikkeling van 'n volledige VP2-gebaseerde PSV entstof, wat al nege serotipes insluit.

Voorlopige dagingseksperimente van perde het getoon dat PSV-5 rVP2 net gedeeltelike beskerming teen virulente PSV bied. Moontlike verklarings vir die swak beskerming moes dus gevind. Verdere ondersoek van die PSV-5 rVP2, uitgedruk in die bakulovirus sisteem, het aangedui dat die meeste VP2 (*ca.* 90 %) onoplosbaar is en dat slegs die oplosbare deel (*ca.* 10 %) neutraliserende teenliggame in marmotte en volledige beskerming in perde induseer.

'n Betekenisvolle bevinding van hierdie studie is dat die veiligheid en effektiwiteit van oplosbare PSV-5 rVP2 bepaal word deur die adjuvant wat saam met die rVP2 ingespuit word. Saponien-gebaseerde adjuvante het die beste beskerming gebied, maar perde het 'n dosis-verwante hipersensitiwiteit getoon. Die ondersoek het ook vir die eerste keer aangedui dat PSV VP2 dalk 'n beskermende sel-gemedieerde immuniteit kan induseer. Na aanleiding van bogenoemde bevinding word aanbeveel dat 'n kandidaat PSV-5 VP2-gebaseerde subeenheid-entstof geformuleer word. Die dosisse van bogenoemde entstof behoort 50 µg rVP2 en 3.0 mg Saponien Q vir die eerste inenting te wees, gevolg deur 'n versterkende inenting van 50 µg rVP2 en 0.6 mg Saponien Q.

Die bevindinge van hierdie studie, tesame met die onlangse klonering van die VP2 gene van al die PSV serotipes, baan die weg vir die ontwikkeling van 'n volledige rekombinante PSV entstof. Sodanige entstof sal na verwagting beskerming teen al nege PSV serotipes bied en die beperking op die in- en uitvoer van perde na en van Suid Afrika ophef.

ABBREVIATIONS

AcNPV	-	*Autographa californica* nuclear polyhedrosis virus
AHS	-	African horsesickness
AHSV	-	African horsesickness virus
AIV	-	avian influenza virus
$AlCl_3.6H_2O$	-	aluminium chloride-6-hydrate
AlOOH	-	aluminium oxyhydroxide
$AlPO_4$	-	aluminium phosphate
ATCC	-	American Type Culture Collection
ATP	-	adenosine triphosphate
B	-	booster
BEVS	-	baculovirus expression vector system
BHK	-	Baby hamster kidney
BTV	-	bluetongue virus
C	-	challenge
ca.	-	*circa* (approximately), ongeveer
C1	-	control 1
C2	-	control 2
°C	-	degrees celsius
CLPs	-	core-like particles
CMI	-	cell-mediated immunity
cm	-	centimetre
cm^2	-	square centimetre
CO_2	-	carbon dioxide
CPE	-	cytopathic effect
CPV	-	canine parvovirus
CSFV	-	classical swine fever virus
d	-	days
dpc	-	days *post* challenge
dsRNA	-	double-stranded ribonucleic acid
ddRNS	-	dubbeldraad ribonukle ensuur
E	-	envelope
E. coli	-	*Escherichia coli*

e.g.	-	*exempli gratia* (for example)
EHDV	-	epizootic haemorrhagic disease virus
ELISA	-	emzyme linked immunosorbent assay
EMEM	-	Eagle's minimum essential medium
ENSO	-	El Niño/Southern Oscillation
et al.	-	*et alii* (and others)
FCS	-	foetal calf serum
FeLV	-	feline leukaemia virus
Fig(s).	-	figure(s)
g	-	gram, gravitational force
h	-	hours
HP	-	haemagglutinin protein
I	-	primary immunisation
IB	-	inclusion body
IBs	-	inclusion bodies
IgE	-	immunoglobulin E
ISCOM	-	immunostimulating complex
L	-	large, litre
log	-	logarithm
M	-	molar
mA	-	milliamperes
ME	-	minimum essential
MEM	-	minimum essential medium
MHC	-	major histocompatibility complex
min	-	minutes
µg	-	microgram
µl	-	microliter
mg	-	milligram
ml	-	millilitre
MOI	-	multiplicity of infection
mRNA	-	messenger ribonucleic acid
MW	-	molecular weight
N	-	negative
NAb	-	neutralising antibody

NAbs	-	neutralising antibodies
Na_2HPO_4	-	di-sodium hydrogen phosphate
no.	-	number
nos.	-	numbers
NS	-	non-structural
OBP	-	Onderstepoort Biological Products
OIE	-	Office International des Epizooties
OVI	-	Onderstepoort Veterinary Institute
P	-	passage
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PFU	-	plaque forming units
p.i.	-	*post* infection
PSB	-	protein solvent buffer
PSV	-	perdesiekte virus
rBac-5	-	recombinant baculovirus expressing AHSV-5 VP2
rVP2	-	recombinant viral protein 2
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
RSA	-	Republic of South Africa
s	-	seconds
S	-	segment
SA	-	South Africa
SDS	-	sodium dodecyl sulphate
Sf	-	*Spodoptera frugiperda*
spp.	-	species
ssRNA	-	single-stranded ribonucleic acid
$TCID_{50}$	-	50 % tissue culture infectious dose
Th1	-	T-helper 1
Th2	-	T-helper 2
TM	-	trade mark
TOH	-	time of harvest
TOI	-	time of infection
V	-	volts

w/v	-	weight per volume
VIBs	-	viral inclusion bodies
VLPs	-	virus-like particles
VP	-	viral protein

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ETHICAL CLEARANCE

All experiments on animals were performed with ethical clearance from the ethical committee of the Onderstepoort Veterinary Institute in accordance with "The National Code for Animal Use in Research, Education, Diagnosis and Testing of drugs and Related Substances in South Africa".

CHAPTER 1

Introduction and Literature Review

1.1 HISTORY OF AFRICAN HORSESICKNESS

African horsesickness (AHS) is a devastating disease of horses. In fully susceptible horses it has a mortality rate of more than 95 %. AHS has been designated an A-list disease by the Office International des Epizooties (OIE) (Coetzer and Eramus, 1994). The disease was first historically documented in an Arabian script in the year 1327-28. A Father Monclaro also mentioned the disease in his account of the journey of Francisco Barreto to East Africa during 1569 (Henning, 1956).

When the first settlers arrived in South Africa in 1652, African horsesickness virus (AHSV) was already present in indigenous reservoir hosts. Records of the Dutch East India Company state that as early as 1719 nearly 1700 horses succumbed to the dreaded "perreziekte" or "paardenziekte". Particularly severe losses to the disease were recorded during the years 1780, 1801, 1839, 1854, 1862, 1891 and subsequently. The most virulent outbreak was the year of 1854-5 when mortalities amounted to 70 000, comprising more than 40 % of the total horse population. The Voortrekkers also suffered severe losses through horsesickness and explorers like Livingstone (1857) were forced to travel on foot or on the back of an ox (Henning, 1956). Keeping in mind that travelling on horseback was the main means of conveyance during that time, the economical impact of these outbreaks was quite severe. Due to this fact, the British Army decided that the severe losses of horses hampered development in southern Africa and that something had to be done. A veterinary surgeon, Joshua A. Nunn FRCVS of the Royal Artillery was appointed in 1886 to investigate the disease. He concluded AHS was caused by a microbe, that looked like anthrax, and that climatic changes played an important role in causing outbreaks (Gutche, 1979).

Outbreaks in non-endemic areas also had devastating consequences. During 1959 – 1960, the disease spread from Iran to West Pakistan, Afghanistan, Cyprus, Iraq, Syria, Lebanon and Jordan. An estimated 300 000 *Equidae* were destroyed during this outbreak (Maurer and McCully, 1963). The most recent epizootic started in September 1987 in a Safari park near Madrid, Spain. Initially 146

horses died, but a year later there was another outbreak in the province of Cadiz and 156 horses died. Eighteen thousand animals were then vaccinated. In July 1989, another outbreak was reported in northern Spain. This time the disease spread to Portugal and Morocco and 110 animals died and 900 were destroyed (Rodriguez, Hooghuis and Casta o, 1992). The outbreak was caused by serotype 4 and was attributed to the importation of 10 viraemic zebra from Namibia (Lubroth, 1988).

Once the filterability of the virus through Berkefeld and Chamberland filters (M'Fadyean, 1900; Nocard, 1901; Theiler, 1901 and Sieber 1911) had been proven, research into the development of a vaccine against this disease was initiated. Theiler (1906, 1915, 1921) was the first researcher who realised that immunised horses contracted disease when challenged with a heterologous AHSV strain. McIntosh (1958) classified seven distinct immunological types of AHSV and Howell (1962) added two more. Horses have to be immune to all nine serotypes to be protected against disease. The development of an attenuated polyvalent vaccine and the obligatory vaccination of horses, greatly reduced the incidence of AHS in South Africa. However, this vaccine lacks two serotypes, namely serotypes 5 and 9, and does not enable one to differentiate between vaccinated and naturally infected horses. The latter drawback hampers the international movement of horses greatly. For the horse industry it is important to develop a vaccine for AHSV that will allow this. Presently, international vaccine development concentrates increasingly on the development of recombinant vaccines, which do not contain the live organism.

1.2 AFRICAN HORSESICKNESS: CLASSIFICATION, TRANSMISSION, EPIDEMIOLOGY AND PROTEIN CODING ASSIGNMENT

1.2.1 CAUSATIVE AGENT AND CLASSIFICATION

The African horsesickness virus (AHSV) which causes AHS has a dsRNA genome, consisting of 10 genome segments. It has an icosahedral capsid, and belongs to the *Orbivirus* genus of the family *Reoviridae*, (Oellermann, 1970; Bremer, 1976; Bremer, Huismans and van Dijk, 1990). Other virus hosts in this family include vertebrates, arthropods and plants. Nineteen groups of orbiviruses are distinguished and most are arthropod borne (Calisher and Mertens, 1998; Roy, 2001). Many of the orbiviruses cause serious diseases of livestock and a few cause febrile illnesses in humans (Calisher and Mertens, 1998).

1.2.2 TRANSMISSION AND EPIDEMIOLOGY

Mules and donkeys are much less susceptible to AHSV than horses, while zebras are very resistant and act as reservoirs (Davies and Lund, 1974; Erasmus *et al.*, 1978 and Coetzer and Erasmus, 1994). Alexander *et al.* (1995) suggested that a diversity of African carnivore species might act as a field reservoir for the virus, but was contradicted by Braverman and Chizov-Ginzburg (1996) when they could not detect canine blood in blood meals from *Culicoides* spp.

Most countries in sub-Saharan Africa are endemic to AHS (Coetzer and Erasmus, 1994; House, 1993; Mellor, 1993; Mellor, Boorman and Baylis, 2000). Outbreaks occur periodically beyond these borders, but until recently the virus could not maintain itself outside these areas for more than two to three consecutive years. Explanations for this phenomenon include the absence of a long-term vertebrate reservoir and the absence or seasonal incidence of vector species. However, the recent Mediterranean outbreak, from 1987 to 1991, necessitated a reassessment of the situation (Mellor, 1993; Mellor, Boorman and Baylis, 2000).

AHSV is transmitted to susceptible animals by blood-sucking *Culicoides* midges that become infected by feeding on animals during the febrile and viraemic stages of infection (Du Toit, 1944). The main vector implicated in transmission is *Culicoides imicola* (Meiswinkel, 1998), but *Culicoides bolitinos* has also been indicated in recent outbreaks in South Africa (Meiswinkel, Baylis and Labuschagne, 2000). Although other insects, like mosquitoes have also been indicated (Mirchamsy *et al.*, 1970), none have been conclusively shown as playing a role under natural conditions (Coetzer and Erasmus, 1994).

Availability of the *Culicoides* vectors limits the geographical distribution and seasonal incidence of AHSV. The ecological elements normally associated with large foci of *C. imicola* (i.e. moisture-retentive clay soils, a higher rainfall and/or irrigation of pastures) are the main factors determining the incidence of the disease (Mellor, 1993; Meiswinkel, 1998). Outbreaks of epizootics are likely mediated by the combination of rainfall and drought brought to South Africa by the warm, El Niño, phase of the El Niño/Southern Oscillation (ENSO) (Baylis, Mellor and Meiswinkel, 1999).

The lowveld of the Limpopo Province and Mpumalanga are the only endemic areas for AHS in South Africa. Usually the disease makes its first appearance in these areas during or after spring. Southward spreading (Fig 1.1) of the disease is facilitated by the movement of infected horses, climatic conditions and breeding of the vectors. Most (75 %) of the zebra in South Africa occur in the Kruger National Park. Since it is known that zebra can act as a reservoir of the virus (Erasmus *et al.*, 1978), it

is speculated that the endemic state of the disease is maintained by these animals in the northern parts of South Africa. Spreading of the virus within one season (one season- being the period between spring and fall when AHS occurs and which coincides with the active period of the insect vectors of *Culicoides* species) is terminated by the first frosts in May. At the start of a new season, the disease again first appears in the endemic area (Bosman, Brückner and Faul, 1995).

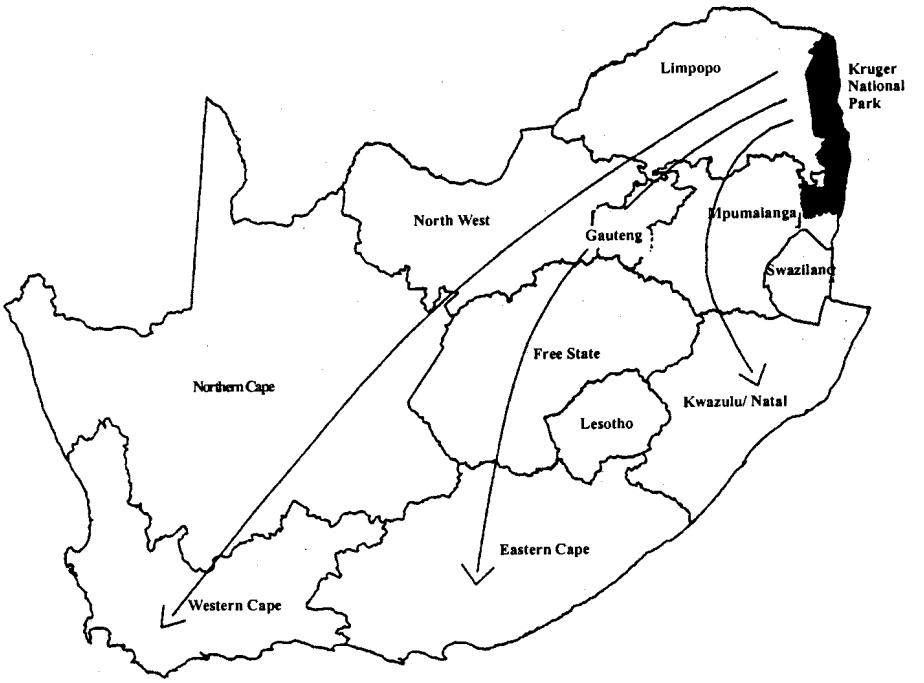

Fig. 1.1 Possible infection routes of AHS in South Africa (Bosman, Brückner and Faul, 1995).

1.2.3 CLINICAL MANIFESTATION OF AHS

Four clinical forms of AHS in horses are recognised (Theiler, 1921, 1930):

1. The mild horsesickness fever form is characterised by a febrile period of approximately five days and full recovery. Horses partially resistant to AHS and zebras and donkeys mostly display this form.

2. The pulmonary or acute form, also called the "dunkop" form, is characterised by the absence of subcutaneous swelling of the head. Mortality can be as high as 95 % with a clinical course of between five to seven days. Affected animals suffer from severe rapid distressed respiration before death and die by literally drowning in their own fluids. Sometimes a seemingly healthy animal may suddenly become inactive and lazy and develop acute dyspnoea from which it might die (Henning, 1956). Typical necropsy lesions are severe pulmonary oedema and hydrothorax (Coetzer and Erasmus, 1994). Laegreid *et al.* (1992)

have shown the presence of virus in the pulmonary microvascular endothelial cells of horses experimentally infected with AHSV. This may partially explain the development of pulmonary oedema in infected horses.

3. Characteristically horses with the cardiac or "dikkop" form develop subcutaneous swelling of the neck and head, particularly the supraorbital fossae and eyelids, with a clinical course of between five to 15 days. Mortality may range between 50 to 90 %. Necropsy lesions include oedema of subcutaneous tissues, oedema of the intermascular fascia (particularly the neck), hydropericardium and occasional necrosis of the myocardium, especially the papillary muscles (Coetzer and Erasmus, 1994). The "dikkop" form usually dominates milder outbreaks (Henning, 1956).

4. The acute or mixed form is the most common form of the disease and is a mixed form of the clinical signs and pathological lesions of the cardiac and pulmonary forms. Mortality ranges from 50 to 90 % (Coetzer and Erasmus, 1994).

The rigid distinction between the four forms is not fully justifiable and most cases are of the mixed form (Coetzer and Erasmus, 1994). The virulence phenotype of the inoculated virus determines the form of disease (Laegreid *et al.,* 1993). The isolation of more than one serotype from a naturally infected animal has never been reported (Coetzer and Erasmus, 1994).

1.2.4 MORPHOLOGY OF AHSV VIRUS PARTICLES

AHS virions consist of seven structural proteins that surround 10 dsRNA genome segments. These will be discussed in more detail below.

The morphology of AHSV is largely the same as that of BTV (Fig. 1.2), the prototype orbivirus. In 1972 Verwoerd and co-workers showed that the BTV virion is comprised of seven polypeptides that constitute the capsid and that two of these polypeptides (VP2 and VP5) are present as a protein layer surrounding the capsid. Nowadays it is known that the seven structural proteins of both BTV and AHSV virions are organised in three concentric layers. The innermost components are the three minor proteins (VP1, VP4 and VP6) and the ten segments of the double-stranded RNA genome (dsRNA). VP3 surrounds these components and together with VP1, VP4, VP6 and the 10 ds RNA genes constitute the subcore. The subcore is encapsidated by 260 trimers of the VP7 protein to form the core particle (Grimes *et al.*, 1998; Roy and Sutton, 1998; Stuart *et al.*, 1998; Roy, 2001). The outer capsid, which is composed of VP2 and VP5, surrounds the core (Verwoerd *et al.*, 1972).

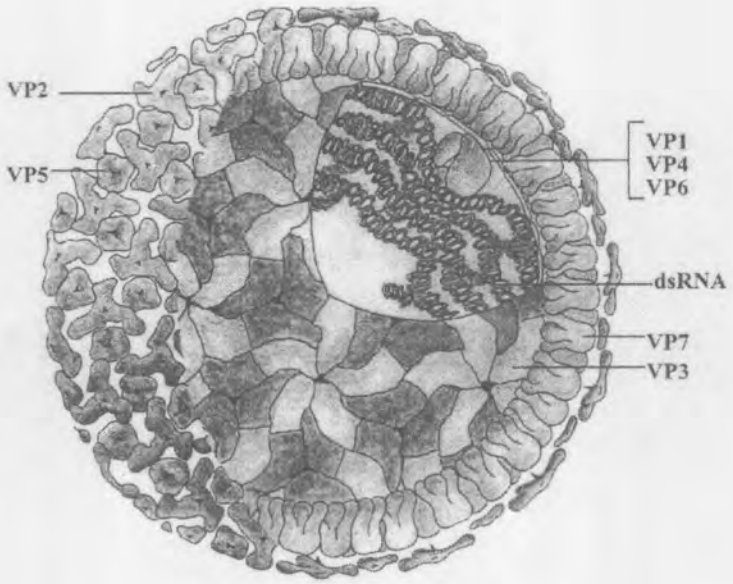

Fig. 1.2 Schematic diagram of the BTV particle (Roy, 2001).

1.2.5 GENOME CODING ASSIGNMENT

The AHSV genome segments are numbered S1-10 in order of migration during polyacrylamide gel electrophoresis. The mobility of the segments vary between serotypes (Bremer, 1976; Bremer, Huismans and van Dijk, 1990 and Grubman and Lewis, 1992). According to Grubman and Lewis (1992), the AHSV genome encodes one minor (VP1) and four major structural proteins (VP2, VP3, VP5 and VP7), three major (NS1, NS2 and NS3) and two minor non-structural proteins (NS4 and NS4A). Two minor core proteins (VP4 and VP6) are also expressed (Bremer, 1976; Grubman and Lewis, 1992). Although Grubman and Lewis (1992) showed that segment 10 (S10) encodes NS4 and NS4A, Van Staden and Huismans (1991) found the proteins encoded by this genome similar to NS3 and NS3A encoded by the S10 genomes of BTV and epizootic haemorrhagic disease virus (EHDV). The majority of the segments synthesise only one protein. Some investigators reported that segment 6 of AHSV encodes VP5 and a truncated form of VP5 (Grubman and Lewis, 1992; Martínez-Torrecuadrada *et al.*, 1994). However, Du Plessis and Nel (1997) observed only one authentic protein band when AHSV-9 VP5 was expressed in the baculovirus expression system. Table 1.1 summarises the coding assignment of each genome segment. A more comprehensive discussion follows.

Table 1.1 **Coding assignment of the AHSV genome segments with the location and putative functions of the encoded proteins**

Genome segment	Proteins	Location	Putative function
S1	VP1	core	RNA polymerase
S2	VP2	outer shell	Serotype specific antigen, neutralisation, hemagglutination, virus entry, cell attachment
S3	VP3	core	Scaffold for VP7, interaction with viral genome
S4	VP4	core	Enzyme activities associated with RNA capping
S5	NS1	non-structural	Tubule formation
S6	VP5	outer shell	Membrane destabilisation of host cells
S7	VP7	core	Group-specific antigen
S8	NS2	non-structural	Associated with VIBs, phosphorylated, binds ssRNA, might recruit mRNA into VIBs
S9	VP6	core	Helicase
S10	NS3/NS3A	non-structural	Mediates release of virus particles from infected cells

1.2.6 AHSV STRUCTURAL PROTEINS

1.2.6.1 The outer structural proteins VP2 and VP5

It has been shown for BTV and AHSV that VP2 is encoded by genome segment S2 and that it is the most variable of the viral proteins. VP2 determines serotype specificity in BTV and AHSV (Huismans and Erasmus, 1981; Kahlon, Sugiyama and Roy, 1983; Bremer, Huismans and van Dijk, 1990 and Roy *et al.*, 1990a) and elicits neutralising antibodies that confer protection against a homologous virus challenge (Huismans *et al.*, 1987; Inumaru and Roy, 1987; Burrage *et al.*, 1993; Martínez-Torrecuadrada *et al.*, 1994; Martínez-Torrecuadrada and Casal, 1995; Martínez-Torrecuadrada *et al.* 1996; Roy *et al.*, 1996; Stone-Marchat *et al.*, 1996). Due to its serotype-specificity, the VP2 gene of AHSV has successfully been used as a probe for serotyping isolates (Koekemoer *et al.*, 2000). It has been shown for BTV that the neutralising epitopes of VP2 are conformation dependent (White and Eaton, 1990). Hassan and Roy (1999) found that the majority of the BTV VP2 protein forms a dimer and to a lesser extent trimers. It was also shown that VP2 of BTV possesses hemagglutinin activity (Cowley and Gorman, 1987; Hassan and Roy, 1999) and is responsible for cell attachment and virus entry into mammalian cells (Hassan and Roy, 1999).

It was recently found that BTV-10 VP5 can induce cytotoxicity by permeabilising mammalian and *Culicoides* insect cells. These studies suggest that although VP5 binds to the cell surface, it is not responsible for virion internalisation, but may have a role in membrane destabilisation required for core access to the cytoplasm (Hassan *et al.*, 2001). Martínez-Torrecuadrada *et al.* (1999) showed that, in contrast to BTV VP5 (Marshall and Roy, 1990), recombinant VP5 of AHSV-4 can elicit a significant neutralising response, albeit at much lower titres than induced by VP2. It was also concluded, that, like for VP2, the N-terminus of VP5 seems the most immunodominant region and may be the most accessible to antibodies due to its surface exposure. Loudon *et al.* (1991) successfully assembled heterologous virus-like particles (VLPs) of BTV by co-infection of baculovirus recombinants expressing VP2, VP3, VP5 and VP7. They concluded that the successful assembly of an outer capsid consisting of VP2 and VP5 proteins of different serotypes indicates that, although these proteins vary significantly, their overall structures are relatively similar.

1.2.6.2 The major core proteins: VP3 and VP7

BTV VP3 forms an inner scaffold for the deposition of VP7 in the core. VP3 also interacts with the three minor proteins (Grimes *et al.*, 1998). The high degree of conservation of VP3 among BTV serotypes and other orbiviruses indicates that it plays a particular role in virus capsid morphology and the organisation of the virus genome. This phenomenon is also true for AHSV and EHDV (Inumaru, Ghiasi and Roy, 1987; Iwata, Yamagawa and Roy, 1992 and Maree, Durbach and Huismans, 1998).

VP7 is a group-reactive antigen. BTV-10 VP7 was found to react with antibodies raised to different BTV serotypes and to a much lower level with antibodies raised to AHSV and EHDV, indicating that it possesses group-specific determinants (Oldfield *et al.*, 1990). The same phenomenon was demonstrated for VP7 of AHSV-4: it reacts with antisera from all nine AHSV serotypes and at a much lower level with BTV antisera (Chuma *et al.*, 1992). Due to its group-specificity this protein can be used for the detection of AHSV by making use of different ELISA techniques (du Plessis, van Wyngaardt and Bremer, 1990; Chuma *et al.*, 1992; Laviada *et al.*, 1992; Wade-Evans *et al.*, 1993; Bremer, du Plessis and van Dijk, 1994; House *et al.*, 1996 and du Plessis *et al.*, 1999).

The word "orbis" means sphere and refers to the round capsomers that are formed by VP7 on the surface of the core of orbiviruses (Grimes *et al.*, 1998). BTV VP7 is the first capsid protein from a virus of the *Reoviridae* family that has been crystallised. The structure analysis revealed that VP7 forms trimers in solution and when crystallised (Basak, Stuart and Roy, 1992). The structures of BTV-10 and AHSV-4 VP7 proteins are similar, suggesting that the structures of VP7 proteins of all

orbiviruses are highly conserved (Roy, 2001). When co-expressed, AHSV and BTV VP3 and VP7 assemble into core-like particles (Maree, Durbach and Huismans, 1998 and French and Roy, 1990).

1.2.6.3 The minor core proteins: VP1, VP4 and VP6

The minor core proteins have enzyme activities required for replication and transcription.

Typical motifs associated with RNA polymerase activity have been identified in the VP1 amino acid sequences for BTV (Koonin, Gorbalenya and Chumakov, 1989) and AHSV (Vreede and Huismans, 1998). Ramadevi, Kochan and Roy (1999) showed that BTV VP1 synthesised dsRNA from a ssRNA template *in vitro*.

Highly purified BTV VP4 possesses various enzyme activities that are associated with RNA capping (Ramadevi *et al.*, 1998).

Baculovirus-expressed BTV VP6 acts as a helicase that hydrolyses adenosine triphosphate (ATP) in the presence of divalent cations (Staubner *et al.*, 1997). This attribute might allow VP6 to participate in mRNA transcription and virus assembly by interacting with the ten RNA segments (Roy *et al.*, 1990b).

1.2.7 AHSV NON-STRUCTURAL PROTEINS

AHSV and BTV have three non-structural proteins: NS1, NS2 and NS3/NS3A. The putative functions of these proteins are discussed below.

The formation of tubules in infected cells characterises the replication of orbiviruses (Huismans and Els, 1979). Studies done on BTV and AHSV tubule formation, indicate that they are composed of the NS1 protein. (Urakawa and Roy, 1988; Maree and Huismans, 1997; van Staden et al., 1998). NS1 is synthesised more abundantly than any other BTV protein (Huismans and Verwoerd, 1973; Huismans, 1979). The NS1 gene displays a high degree of conservation for both BTV (Hwang *et al.*, 1993) and AHSV (Mizukoshi *et al.*, 1992 and Maree and Huismans, 1997) and may be used as a group-specific diagnostic reagent. BTV-10 NS1 has a low content of charged amino acids but is rich in cysteine (Hwang *et al.*, 1993) which indicates that it has a highly ordered structure. Analysis of deletion and site-directed mutants of BTV-10 NS1 indicated that certain cysteines and both termini are important for tubule formation (Monastyrskaya *et al.*, 1994). Recombinant chimeric NS1 tubules from BTV can

be used as vaccine delivery systems for multiple epitopes due to their high degree of immunogenicity (Mikhailov *et al.*, 1996; Gosh, Borca and Roy, 2002 and Gosh *et al.*, 2002).

BTV non-structural protein NS2, is associated with viral inclusion bodies (VIBs) in infected cells (Thomas, Booth and Roy, 1990). It is also the only virus-specific phosphorylated protein that is present in infected cells. BTV NS2 binds ssRNA, but not dsRNA (Huismans, van Dijk and Bauskin, 1987). It is believed that NS2 is involved in recruiting mRNA species into VIBs during assembly of virus components (Roy, 2001).

In contrast to the high expression levels of BTV NS1 and NS2, the two smallest non-structural BTV proteins, NS3 and NS3A are synthesised in minute amounts in infected cells (Huismans, 1979). In both BTV (Huismans, 1979 and van Dijk and Huismans, 1988) and AHSV (van Staden and Huismans, 1991), two in-phase overlapping reading frames on genome segment S10 encode these two proteins. NS3 proteins of different AHSV serotypes are not highly conserved (van Staden and Huismans, 1991 and Martin *et al.*, 1998). It has been shown for AHSV NS3 that the variation in the NS3 genome sequence influences the virulence characteristics of the progeny virus strain (Martin *et al.*, 1998). Hyatt, Zhao and Roy (1993) found that, by making use of the baculovirus expression system, NS3/NS3A determines the budding and release of VLPs from infected cells. Stoltz *et al.* (1996) also illustrated the same phenomenon for AHSV NS3. Recombinant AHSV NS3/NS3A has been investigated as antigen to distinguish between horses vaccinated with inactivated vaccine and naturally infected ones. Antibodies against NS3/NS3A of AHSV-4 were detected in horses suffering from replicating AHSV-4 but not in the sera of horses vaccinated with inactivated purified AHSV-4 vaccine (Laviada *et al.*, 1995). However, Idrissi Bougrine *et al.* (1999) contradicted these findings when they showed that NS3 is not sufficiently eliminated from the inactivated AHSV-4 vaccine.

1.3 AHSV VACCINE DEVELOPMENT

1.3.1 ATTENUATED POLYVALENT VACCINES

Research on effective vaccination against AHS has always been in line with international vaccine development trends. It started in the early 1900s, when Theiler (1908, 1909) found that simultaneous injection of a virulent AHSV strain and homologous serum, obtained in horses by hyperimmunisation, vaccinated mules. This immunisation regime was widely used during the early 1900s (Galambos, 1999). In 1931 Goodpasture showed that virus could be grown on embryonated hens eggs (Galambos, 1999). Shortly thereafter, it was shown that AHSV could be attenuated by serial intracerebral passage in mice (Nieschulz, 1932, Alexander, 1933 and Alexander and du Toit,

1934). The first attenuated AHS vaccines were passaged 100 times intracerebrally in mice and were used for many decades in South Africa. These vaccines consisted of six of the nine serotypes (1, 2, 3, 4, 5, 6) (van Dijk, 1998). However, this attenuation method was very tedious and sometimes caused encephalitis and loss of immunogenicity in vaccinated horses (Erasmus, 1978).

In 1949 Enders, Weller and Robbins successfully cultivated poliomyelitis virus on human embryonic tissues. Successful cultivation of AHSV in tissue culture followed suit (Erasmus, 1963a, 1964). The discovery that plaque size correlates with pathogenicity of AHS in Vero cells (Erasmus, 1978), paved the way for the development of a tissue culture-derived polyvalent AHS vaccine (van Dijk, 1998). Large plaques (4.0-6.0 mm) were found to be non-pathogenic for horses (Erasmus, 1978). An attenuated polyvalent vaccine that was grown in cell culture was developed in South Africa. This vaccine contained three of the cell-culture-attenuated strains that displayed large plaques (serotypes 4, 7 and 8). The remaining serotypes included in the vaccine were the mouse brain attenuated strains of serotypes 1, 2, 3, 5 and 6, which were subsequently propagated in cell cultures. This vaccine, which consisted of two polyvalent vaccine combinations (combination 1: serotypes 1, 3, 4 and 5; combination 2: serotypes 2, 6, 7 and 8), was sold in South Africa until 1990. Production was then discontinued since the vaccine was implicated in the development of non-fatal encephalitis and chorioretinitis in four laboratory workers, who were involved in packaging of the vaccine (Van der Meyden *et al.*, 1992).

The current polyvalent attenuated vaccine, produced and widely used in South Africa is composed of large plaque strains attenuated and propagated in cell culture. The vaccine consists of two vials: vial 1 contains serotypes 1, 3 and 4 and vial 2 contains serotypes 2, 6, 7 and 8. The two vaccines must be administered 28 days apart and horses should be rested after each vaccination (Information Pamphlet Onderstepoort Biological Products; House *et al.*, 1992). Serotype 5 is excluded from the vaccine due to attenuation difficulties (van Dijk, 1998). This situation creates a gap in immunological protection of the horse population in southern Africa. Serotype 9 has never been included, since serotype 6 provides cross protection. Also serotype 9 is rarely implicated in outbreaks in southern Africa (van Dijk, 1998). According to the OIE Manual of Standards for Diagnostic Tests and Vaccines (2000), immunity is known to persist for at least four years. However, annual vaccination for at least three to four years (van Dijk, 1998) is advised, since possible interference between the individual serotypes can occur (OIE Manual of Standards for Diagnostic Tests and Vaccines, 2000). Monovalent vaccines are also produced on request. Vaccination with monovalent vaccines stimulates practically a life-long immunity (OIE Manual of Standards for Diagnostic Tests and Vaccines, 2000).

Development and use of the attenuated vaccine has lead to drastic decreases in the number of horses that die during horse sickness outbreaks in South Africa. However, some concerns regarding the use of this vaccine exist. These include the following: true polyvalent immunity is only acquired after three to four immunisations. Repeated immunisation of ten times or more might lead to an unresponsiveness or hypersensitivity. Such horses may also contract peracute horsesickness, often from serotypes included in the vaccine (Erasmus, 1978). Since live attenuated viruses need to replicate in the host to elicit immunity, foals born to immune mares can only be immunised after they have lost their passive immunity (van Dijk, 1998). Simultaneous administration of several vaccine strains may result in incomplete immunity due to interference during vaccine virus replication (Roy *et al.*, 1996). Back-mutation or reassortment with wild-type virus might lead to revergence to virulence. Incomplete attenuation may occur, resulting in disease. Since horses also have to be rested after vaccination, working and racing horses are often not vaccinated due to time and money considerations. The frequent international movement and foreign trade of horses is hampered by the problem that it is currently not possible to distinguish vaccinated from naturally infected horses (Van Dijk, 1998).

1.3.2 MONOVALENT INACTIVATED VACCINES

The development of inactivated vaccines against AHSV has also been investigated. The advantage of inactivated vaccines is that they cannot revert to virulence, recombine with other viruses and do not have the potential to infect insect vectors (House *et al.*, 1994). However, some infectious virus in the vaccine might not be inactivated, causing vaccine failure (Hassanain *et al.*, 1990).

In 1933 Du Toit, Alexander and Neitz first reported vaccination of mules by using an inactivated formalised virus. In 1966 Ozawa and Bahrami also showed the feasibility of a formalised inactivated AHSV-9 vaccine. However, this process was not commercialised until the 1987-1990 AHSV epizootic in Spain, when a commercial inactivated vaccine for AHSV-4 was developed and used experimentally in Spain and Morocco (House, 1998). When nine ponies were vaccinated with this vaccine and challenged four weeks later, three ponies developed a febrile response and one showed reddened oral mucosa and congested conjunctiva. All nine vaccinated ponies survived the challenge whereas the three unvaccinated controls died. Seven of the nine ponies developed an anamnestic antibody response, indicating virus replication. A transient low-level viraemia developed in one pony (House *et al.*, 1992). Hassanain (1992) also tested an inactivated vaccine for AHSV-9, using binary ethyleneimine as inactivant and found this vaccine to be an efficient immunogen. Although these vaccines have been proven successful, production of mainly serotype 4 inactivated vaccine

(Dubourget, *et al.*, 1992, OIE Manual of Standards for Diagnostic Tests and Vaccines, 2000) is done on a limited scale and mainly on request (van Dijk, 1998).

1.3.3 RECOMBINANT SUBUNIT AHSV VACCINES

In 1984, McAleer and colleagues developed the first human recombinant vaccine against hepatitis B. Research in this field experienced a boom thereafter, also in veterinary vaccinology. Developmental and basic research regarding the development of a recombinant subunit vaccine for AHSV has been done for many years at the Onderstepoort Veterinary Institute (OVI). It is generally accepted that recombinant subunit vaccines will be applied as the next generation AHSV vaccines. The major advantages of these vaccines are the exclusion of the time consumption and dangers associated with the attenuation process, the possibility to introduce diagnostic tests that will distinguish between vaccinated and naturally infected horses (Van Dijk, 1998) and safety. Horses will no longer receive live AHSV virus that excludes the possibility of infecting the vector host and alleviates the need to rest the horses after vaccination. As baculoviruses (the expression system commonly used for orbivirus protein expression) are not infectious for humans and animals (Murphy *et al.*, 1997), the risk for personnel involved in the manufacturing process of these vaccines is eliminated. The attenuated and inactivated AHSV vaccines currently in use are manufactured by passaging the virus in roller cultures of Vero or BHK-21 (baby hamster kidney) cells (O.I.E . Manual of Standards for Diagnostic Tests and Vaccines, 2000). Incubation of *Spodoptera frugiperda* (Sf) cells, used for propagation of baculoviruses, can easily be scaled up to fermenters, rendering vaccine production less labour intensive.

Huismans *et al.* (1987) paved the way for the development of recombinant subunit vaccines against orbiviruses by showing that purified BTV VP2 induces a protective immune response in sheep. The ability of the baculovirus genome to incorporate and express large amounts of foreign DNA makes this system suitable for the simultaneous expression of dual and multiple orbivirus genes (Roy and Sutton, 1998). In 1990 Roy and associates administered baculovirus-expressed BTV proteins in different combinations to sheep. They found that a dose of ca. 50 µg rVP2 protected some, but not all sheep. However, two successive injections of 100 µg rVP2 rendered complete protection. When ca. 20 µg rVP5 was added, only 50 µg quantities of rVP2 were necessary to render complete protection. The enhancement of immunity was ascribed to the synergistic effect of the proteins, which improves presentation of neutralising epitopes. Addition of other BTV proteins (VP1, VP3, VP6, VP7, NS1, NS2 and NS3) did not enhance antibody titres nor the level of protection.

Proof of principle of the efficiency of an AHSV vaccine consisting only of VP2 has already been established. Stone-Marchat *et al.* (1996) showed that vaccinia-expressed VP2 of AHSV-4 fully protected horses against disease and viraemia. Increased neutralising and ELISA antibody titres after challenge indicated limited replication of virus in immunised horses. This response was considered similar to that conferred by existing vaccines. Roy *et al.* (1996) also showed that baculovirus-expressed AHSV-4 VP2 provided protection against virulent AHSV challenge. However, a preliminary study (see Chapter 2) indicated that baculovirus-expressed AHSV-5 VP2 could not protect horses sufficiently against challenge.

Martínez-Torrecuadrada *et al.* (1996) investigated the influence of VP5 and VP7 from AHSV-4 on the immune response elicited by VP2. They found that when horses were immunised with the purified proteins (VP2 alone, VP2 + VP5, VP2 + VP5 + VP7), only the mixture of VP2 with VP5 and VP7 conferred protection. When the VP2 was used in cellular extracts, especially with unpurified VP5, the neutralising antibody (NAb) titres increased significantly. Although addition of VP5 and VP7 can enhance immunogenicity, this laboratory found that expression of these proteins with VP2 lowers VP2 production significantly (S Maree, personal communication).

In 1992 Roy, French and Erasmus demonstrated the protective efficacy of recombinant baculovirus-expressed virus-like particles (VLPs) of BTV-10, consisting of VP2, VP5, VP3 and VP7. The results they obtained indicated that these particles are highly immunogenic. As little as 10 μg VLPs, in conjunction with the appropriate adjuvant, renders complete protection until four months after vaccination. They attributed the lower dose of VLPs needed to elicit immunogenic protection to the correct presentation of VP2 epitopes due to its association with the other structural proteins. Maree *et al.* (1998) also reported the expression of AHSV-9 core-like particles (CLPs) in the baculovirus system, consisting of AHSV-9 VP3 and VP7. Although, production of AHSV VLPs has been achieved the yield was too low for use in a vaccine (S Maree, personal communication).

1.4 THE ROLE OF ADJUVANTS IN THE DEVELOPMENT OF RECOMBINANT SUBUNIT VACCINES

1.4.1 DEFINITION AND CLASSIFICATION OF ADJUVANTS

One of the major disadvantages associated with recombinant protein vaccines is that they are poorly immunogenic when administered alone. Therefore, incorporation of a compatible adjuvant in such vaccine should also be tested. Classification of an effective adjuvant may also reduce vaccine costs

by reducing the dose of antigen and can help overcome antigen competition in combination vaccines (Singh and O'Hagan, 1999).

An adjuvant is defined as a substance that "non-specifically enhances the immune response to an antigen" (Roitt, Brostoff and Male, 1989). There are various reasons why one would attempt to enhance the immune response. They include: improvement of the immunogenicity of weak antigens; enhancement of the speed and duration of the immune response; modulation of antibody avidity, specificity, isotype or subclass distribution; stimulation of cell-mediated immunity; induction of mucosal immunity and enhancement of immune responses in immunodeficient individuals. The most effective adjuvant for a given antigen will to a large extent depend on the type of immune response required for protective immunity (Singh and O'Hagan, 1999).

Three broad classes of adjuvants are distinguished: 1) active immunostimulants that increase specific immune response to an antigen; 2) carriers or immunogenic proteins that provide T-cell help to a conjugated hapten antigen and 3) vehicle type adjuvants which serve as a matrix for antigen and provide immune stimulation (Kensil, 1996). It is important to remember that adjuvants are physiologically foreign to the body and therefore potentially toxic. They can mediate their effects by increasing cellular infiltration, inflammation and trafficking to the injection site. This hyperactivation of the immune response may lead to adverse side-effects (Gupta *et al.*, 1993). A balance, therefore, has to be found between toxicity and adjuvanticity.

1.4.2 ADJUVANTS TESTED IN THIS STUDY

1.4.2.1 Montanide ISA-50 (Seppic, Paris)

This adjuvant is classified as an oil emulsion. These adjuvants stimulate depot formation at the injection site and slow release of the antigen with stimulation of the antibody-producing cells (Gupta *et al.*, 1993). Due to the infiltration by leucocytes, inflammation at the injection site is inevitable (Allison, 1997).

1.4.2.2 Aluminum phosphate ($AlPO_4$)

Aluminum salts have been widely used in human and veterinary vaccines since 1930 and have an excellent safety record (Cox and Coulter, 1997). Two formulations are used in commercial vaccines namely aluminum oxyhydroxide (AlOOH), commonly referred to as aluminum hydroxide or aluminum hydroxyphosphate ($Al(OH)_x(PO_4)_y$), commonly referred to as aluminum phosphate

(HogenEsch, 2002). Two methods are generally used to prepare vaccines containing these adjuvants. In the one method, a solution of aluminum salt is added to the antigen to form precipitate of protein aluminate. However, these alum-precipitated vaccines are highly variable in composition and are not commonly used anymore. In the other method of preparation the solution of antigen is added to preformed precipitates of above mentioned aluminum compounds (Nicklas, 1992; Gupta *et al.*, 1993).

It is postulated that aluminum salts stimulate immunity mainly by depot formation at the injection site. The slow release of adjuvant then prolongs the time of interaction between antigen and antigen-presenting cells (Gupta *et al.*, 1993). However, recent work suggests that the adjuvant effect might rather be stimulated by retention of the antigen at the injection site due to adsorption to the aluminum compounds. This retention at a high concentration for a sufficient period of time may allow uptake and presentation of antigen presenting cells. These adjuvants mainly stimulate a type 2 (Th2) immune response without enhancing the type 1 (Th1) immune response, which render them less suitable for intracellular bacteria and parasites for which antibodies alone render insufficient protection (HogenEsch, 2002). One major disadvantage of aluminum-based adjuvants is their tendency to stimulate strong IgE responses (Gupta *et al.*, 1993; Cox and Coulter, 1997). The induction of antigen-specific IgE responses may predispose susceptible individuals to allergic reactions against vaccine components (HogenEsch, 2002).

1.4.2.3 Saponin-based adjuvants

Saponins are plant-derived sterol glycosides or triterpene glycosides that have found widespread use in commercial veterinary vaccines as potent alternatives to aluminum hydroxide with less local reactogenicity than oil in water formulations. Saponin can be included as crude extracts, or as the better-defined Quil A (Kensil, 1996). They are surface-active agents that can cause hemolysis of red blood cells *in vitro*, a property that does not appear to correlate with adjuvant activity (Singh and O'Hagan, 1999). The impurity of saponin adjuvants hampers interpretation of immunogenic results, because these may be due to components that constitute only a small part of the composition (Kensil, 1996).

The adjuvant activity of saponin is attributed to its binding to cell membrane-bound cholesterol (Glauert, Dingle and Lucy, 1962; Bomford, 1982; Scott, Goss-Sampson and Bomford 1985). Retention of the antigen at the subcutaneous injection site is prolonged and the amount reaching the spleen is increased. This action corresponds with a depot type of adjuvant action and a prolonged stimulation of the immune system. The prolonged retention may also contribute to the haemolytic effect and acute inflammation observed at the injection site (Scott *et al.*, 1985). A pure fraction of

Quil A (QS 21) was shown to induce class I MHC antigen-restricted cytotoxic T-lymphocytes, inducing mainly a Th1-type response and antibodies of the IgG2a isotype (Wu *et al.*, 1994; Kensil, 1996).

1.5 EXPRESSION OF AHSV VP2 PROTEINS

1.5.1 BACULOVIRUS EXPRESSION

The baculovirus expression system is commonly used for expression of AHSV proteins. One of the reasons for this system's popularity is that it is an eukaryotic expression system. It thus supports many eukaryotic protein modification and transport mechanisms. The viral genome can accommodate large inserts of foreign DNA, which facilitates cloning of multiple proteins in one vector (Murphy *et al.*, 1997).

The lytic *Autographa californica* nuclear polyhedrosis virus (AcNPV) is the baculovirus type mostly used for foreign protein expression. In the literature it is mostly referred to as "baculovirus". Two types of viral progeny, namely non-occluded virus (extracellular) and occluded (polyhedra-derived) viruses are released from 18 to 72 h *post* infection (p.i.) or until the cell lyses. Occlusion bodies are mostly composed of polyhedrin protein that is dispensable for virus survival and propagation in tissue culture. Furthermore, polyhedrin protein constitutes more than half of the total cellular protein late in the infectious cycle. Viruses lacking this gene are distinguished from normal viruses by their plaque morphology. Recombinant baculoviruses are mostly generated by replacement of the polyhedrin gene with a foreign gene through homologous recombination (Murphy *et al.*, 1997).

The AHSV-5 VP2 gene that was cloned in this laboratory was expressed by using the BAC-to-BAC™ (Life Technologies) baculovirus expression system (Vreede *et al.*, 1998). This system utilises site-specific transposition and a baculovirus shuttle vector or bacmid that can replicate in *Escherichia coli* and also infect lepidopteran cells. The bacmid contains a mini-F replicon that ensures autonomous replication and stable segregation of plasmids at low copy number, a kanamycin resistance marker and a target site for the bacterial transposon. All these features are inserted into the polyhedrin locus of AcNPV. The transposition functions are supplied by a helper plasmid. The baculovirus promoter controls expression of the foreign gene when the resulting composite bacmid is introduced into insect cells (Luckow *et al.*, 1993). Since selection of recombinant DNA is already done in *E. coli*, the selection time needed to identify recombinant virus, is greatly reduced.

1.5.1.1 Factors determining recombinant protein yield obtained in the baculovirus expression system

Factors that impact on the yield of recombinant protein(s) in the baculovirus expression system include the insect host cell line, the multiplicity of infection (MOI), time of infection (TOI) and the medium used to incubate the insect host cells. A more detailed discussion is given below.

Cell line

Several insect cell lines can be used as hosts for recombinant protein expression by baculoviruses. The most popular cell lines are derived from the fall army worm (*Spodoptera frugiperda*). The cell line IPLB-SF-21 (Sf-21) was derived from ovarial tissue of *S. frugiperda*. The cell line mostly used for baculovirus expression, Sf-9, is a clonal isolate from Sf-21. (Lier, Vlak and Tramper, 1992). Cell lines derived from the cabbage looper (*Trichoplusia ni*) are also commonly used and include TN-368 and BTI-TN5BI-4 (High Five) cells. It has been shown that Sf-9 cells can be used for preparation of high titre virus stocks and High Five cells for production of high levels recombinant protein (Schlaeger *et al.*, 1995). Expression levels of different recombinant proteins differ in different cells. Therefore, different cell lines should be tested for expression of a given protein. However, other factors, that will be discussed later, should also be considered when a cell line is chosen.

MOI/TOI

Various studies indicate that the growth phase of the insect cells, cell density (also referred to as time of infection (TOI)), and multiplicity of infection (MOI) of the baculovirus influence the final product yield. The MOI is defined as the number of plaque forming units (PFU) per cell that is added at the time of infection (Licari and Bailey, 1991). Usually a MOI of >5 is used for recombinant protein production. With this mode of infection an almost similar cell yield than at the TOI is obtained since cell growth is immediately halted due to synchronous infection. However, recent research indicates that infection with a low MOI can increase recombinant protein production. Only a fraction of cells is initially infected when using a low MOI. Thus, non-infected cells will continue to multiply and will only be infected by virus progeny released from surrounding infected cells. A cell yield much higher than at the time of infection may therefore be achieved (Wong *et al.*, 1995). Thus, optimal protein production with a low MOI is ensured by a lower cell density at TOI than the expected cell yield (Wong *et al.*, 1995). Reid *et al.* (1995) states that the TOI/MOI must be such that early cell death or overgrowth is avoided. However, the product yield may decrease significantly when cultures are infected later than a certain optimal TOI. Cells are usually infected in the early or mid-exponential growth phase. High product yield can still be obtained in the late exponential phase if the medium is changed (Power *et al.*, 1994). The time of harvest (TOH) also influences product yield, especially

when low MOIs are used for infection (Reid *et al.*,1995). Marteijn *et al.* (2000) found that there is a relation between maximum growth rate and inoculation density. Lower inoculation densities cause a decline in maximum growth rate. The medium and cell line used determines this decline. Furthermore, to ensure high virus and protein production the passage number of the cells from initiation should not be too high (Maruniak, 1996). Ultimately it is required that the cells are held in a healthy physiological state, free of oxygen and nutrient limitations to ensure a high product yield (Kioukia *et al.*, 1995).

Medium

The medium used for insect cell maintenance should be optimised for each cell line and virus (Maruniak, 1996). Most media are based on the original Grace's medium to which mostly 10 % foetal calf serum (FCS) is added. This concentration can be lowered to 1 % without affecting cell growth. However, a higher concentration is necessary for infection. Some suggested functions for serum include supply of nutrients like lipids and growth factors and protection of cells against toxic compounds and mechanical forces (Van Lier, Vlak and Tramper, 1992). Different serum-free media are also available. The use of serum-free media is preferred above serum-containing media due to a reduction in production costs and safety. Serum-free media are considered to be free of mammalian viruses and prions.

1.5.1.2 Aggregation of heterologous expressed recombinant proteins

One of the major advantages of the BEVS lies in the high levels of heterologous protein expression. However, in some instances, required *post*-translational modifications may fail to keep pace with the very high levels of expression (O'Reilly *et al.*, 1994), which may lead to aggregation, misfolding and limited solubility of polypeptide chains (Speed et al., 1996; Yon, 1996; Wigley et al., 2001).

This phenomenon has also been shown for viral capsid proteins. The major capsid protein of African swine fever virus (ASFV), p73, failed to fold and aggregated when it was expressed in cells. Murphy *et al.* (1990) showed that the glycosylation and solubility of HIV envelope proteins, expressed in the BEVS, decreased markedly from 24 to 72 h after infection. Assembly with scaffold proteins or host- and virus-specific chaperones usually aids functional viral protein folding (Cobbold *et al.*, 2001). The absence of the required chaperones in insect cells might favour incorrect protein folding of the expressed viral protein.

Other factors that influence the solubility of a protein are the net charge, hydrophobicity and secondary structure. Aggregation is generally favoured by mutations that bring the net charge closer

to neutrality. The hydrophobicity and β-sheet propensity of the expressed protein also determines its aggregation rate (Chiti *et al.*, 2002).

Thus, expression of biologically active proteins in the BEVS is determined by many factors that should be optimised for each protein to ensure functionality.

1.5.1.3 Other veterinary vaccines developed by using the baculovirus expression system

Although there is a great need for baculovirus-expressed recombinant subunit veterinary vaccines, not much work is published on this subject. This might be due to a tendency to protect technology.

In 1992 the potential of a recombinant vaccine for canine parvovirus (CPV), consisting of the baculovirus-expressed structural protein VP2 (the major component of CPV capsids), was demonstrated in dogs (De Turiso *et al.*, 1992).

A subunit vaccine against feline leukaemia virus (FeLV) has also been developed. This vaccine consists of the major envelope glycoprotein (gp70) which is expressed in *Escherichia coli* (Clark *et al.*, 1991; Kensil *et al.*, 1991 and Marciani *et al.*, 1991).

In 2001 a vaccine for Hong Kong bird flu (H5N1) was produced within eight weeks (Wrotnowski, 2001). This vaccine was based on the principle that baculovirus-expressed avian influenza virus (AIV) H5 haemagglutinin protein (HP) provides protection from clinical signs and death in chickens after lethal challenge by human-origin HP H5N1 Hong Kong strains 156/97 and 483/97 (Swayne *et al.*, 2001).

A baculovirus-expressed veterinary subunit marker vaccine is also being developed against classical swine fever virus (CSFV). It was shown that the envelope glycoprotein E2 expressed in insect cells protects swine from classical swine fever (Hulst *et al.*, 1993; van Rijn *et al.*, 1996). Bouma *et al.* (1999) also demonstrated that such a vaccine, prepared in water-oil-water emulsion was stable for at least 18 months, when stored at 4°C. However, Klinkenberg *et al.* (2002) showed that maternal-derived immunity might lessen protection against virus transmission after six months.

1.6 AIMS OF THIS STUDY

AHS is a devastating disease of horses and outbreaks can cause huge economic losses. Vaccination of horses is now obligatory in South Africa, where an attenuated polyvalent vaccine is currently produced that excludes serotypes 5 and 9 and does not guarantee complete protection of foals within one AHS season. The development of a subunit vaccine will keep AHSV vaccine development on the forefront of veterinary vaccinology, while simultaneously addressing the concerns about the current vaccine. However, since there are nine serotypes of AHSV and horses have to be immune to all nine serotypes to be protected against disease, a set of nine rVP2s, one for each serotype, will be needed for a complete subunit vaccine repertoire.

Although the principle that the outer capsid protein VP2 of AHSV-4 can elicit a protective immune response has already been established (Martínez-Torrecuadrada, Díaz-Laviada and Roy, 1996; Stone-Marchat *et al.*, 1996; Roy *et al.*, 1996), no further research has been done on the development, formulation and large-scale production of recombinant subunit vaccines for AHS. The main focus of the work presented in this thesis is to develop a baculovirus-expressed VP2-based recombinant subunit vaccine for AHSV serotype 5 (AHSV-5) to prototype stage. The findings and technologies developed here will form the foundation to be used in the upscaling of the AHSV-5 recombinant vaccine. As a first step it is envisaged that AHSV-5 rVP2 will be included in the current vaccine. Ultimately a complete AHSV recombinant vaccine repertoire should become available.

Three specific aims were identified for this study:

1) To characterise the AHSV-5 rVP2, establish whether aggregation and misfolding occurs and to determine to what extent this impacts on the biological activity of rVP2 (Chapter 2).

2) To establish a vaccine formulation that will optimise the immunogenicity of the AHSV-5 rVP2. Factors that will be investigated are biological active rVP2 concentration and type of adjuvant (Chapter 3).

3) To determine whether the vaccine formulation, established in 2, will provide protection against disease in horses for a period of at least one season, i.e. about eight months (Chapter 4).

CHAPTER 2

The influence of aggregation on the immunogenicity of the baculovirus-expressed AHSV-5 VP2 (rVP2)

Part of the work presented in this chapter has been published under the following title: du Plessis M, Cloete M. Aitchison H & van Dijk, AA 1998. Protein aggregation complicates the development of baculovirus-expressed African horsesickness virus serotype 5 subunit vaccines. Onderstepoort Journal of Veterinary Research, 65: 321-329.

2.1 INTRODUCTION

The current live attenuated AHSV is not complete, since it does not contain serotypes 5 and 9. Therefore, a baculovirus-expressed AHSV-5 VP2 was first tested in this laboratory for its ability to elicit a protective immune response in horses. Horses were immunised twice with 50 µg baculovirus-expressed AHSV-5 VP2 and once with 150 µg. However, only partial protection was achieved. The challenged animals developed severe AHS clinical signs such as high fever and swollen supraorbital fossae and petechiae of the ventral aspect of their tongues (results published in above publication). This was in contrast to the work of Martínez-Torrecuadrada *et al.* (1996), Roy *et al.* (1996) and Stone-Marchat *et al.* (1996) who showed that AHSV-4 rVP2 can provide complete protection against challenge in horses.

The first goal of this study was to find the reason for the poor protection induced by rAHSV5-VP2. Misfolding and aggregation could be an explanation. It is commonly known that overexpression of recombinant proteins in foreign hosts can lead to aggregation of the foreign protein. This phenomenon results in the formation of biologically inactive proteins (Wigley *et al.*, 2001; Speed, Wang and King, 1996; Yon, 1996). The formation of insoluble recombinant complexes in insect cells by using the baculovirus expression system has also been reported (Katagiri and Ingham, 2002; Yokoyama *et al*, 2000; Betenbaugh *et al*, 1996; O'Reilly, Miller and Luckow, 1994).

Thus, the hypothesis that there are two forms of AHSV-5 rVP2 namely a soluble biologically active conformation and an aggregative form, which is not biologically active, will have to be tested. The experimental approach will be to investigate the ratio of soluble and insoluble aggregated rVP2 using

low speed centrifugation. Generally, the neutralising antibody titre of a horse to a specific AHS serotype reflects its immunity to that serotype. Guinea pigs have been shown to serve as an excellent small animal model to identify vaccine preparations that induce a protective immune response based on the AHSV-specific neutralising antibodies that are induced (Erasmus, 1963b). To test the immunogenicity guinea pigs were immunised with aggregated and soluble forms of rVP2 and the NAb titres elicited were determined.

2.2 MATERIALS AND METHODS

2.2.1 Sf9 cell cultivation

Sf9 cells (American Type Culture Collection, ATCC CRL 1711) were cultivated at 27 °C in Grace's insect medium (Highveld Biologicals, South Africa) supplemented with 10 % FCS, which had been inactivated at 56 °C for 30 min (Highveld Biologicals, South Africa), and a commercial penicillin/streptomycin/fungizone mixture (Highveld Biologicals, South Africa). The cells were grown either as monolayers as described by O' Reilly, Miller and Luckow (1994) or in shaker cultures at 100 rpm. Shaker cultures also contained 0.1 % (w/v) Pluronic F-68 (SIGMA-Aldrich Co. Ltd.) and were maintained in one quarter of the volume of the flask. Cell densities were maintained between 0.2 x 10^6 cells/ml and 2.5 x 10^6 cells/ml, determined by making use of a haemocytometer.

2.2.2 Cloning of the AHSV-5 VP2 gene

Cloning of the AHSV-5 VP2 gene and construction of a recombinant baculovirus expressing the AHSV-5 VP2 gene were done previously in this laboratory (Vreede *et al.*, 1998).

2.2.3 Preparation of recombinant baculovirus stocks expressing AHSV-5 VP2

Recombinant baculoviruses, expressing AHSV-5 VP2 (rBac-5) were prepared by making use of the BAC-TO-BAC™ Baculovirus Expression System (Life Technologies). Volumes of 100 µl from the transfection overlays were used to infect 3.0 x 10^6 cells in 5.0 ml medium in 25 cm^2 tissue culture flasks. The infected cells were incubated for 72 h at 27 °C and the medium overlay was collected by centrifugation at 1000 x g for 5.0 min. The obtained baculovirus stock was referred to as passage 1 virus stock, which was kept in the dark at 4.0 °C. Thereafter, 100 µl volumes from the passage 1 stock was used to infect 1.0 x 10^7 cells in 75 cm^2 tissue culture flasks in 20 ml complete medium. The medium was again collected after 72 h incubation and the obtained virus was referred to as passage 2 virus stock. After the passage 2 stocks were filter-sterilised through a 0.22 µm filter, their titres were

determined. The passage 2 stocks were then used to infect 250 ml suspension Sf9 cell cultures, counting 1.0 - 2.5 x 10^6 cells/ml and grown in 1 L Erlenmeyer flasks, at a MOI of 0.1. Infection was done by collection of the cells in 20 ml Grace's medium without FCS after centrifugation at 1000 x g for 5.0 min. Virus was added and the cells were gently rocked in 100 ml Erlenmeyer flasks for 2.0 h at room temperature. Thereafter, the infected cells were transferred to 1 L Erlenmeyer flasks and medium with 10 % inactivated FCS was added to render a final volume of 250 ml. After 72 h incubation incubation at 27 ^{0}C, the cells were centrifuged in the same manner and the medium containing the virus was collected. The collected virus suspension, referred to as passage 3 virus stock (P3), was titrated and used as the working stock to prepare rVP2.

2.2.4 Titration of passage 2 and passage 3 recombinant baculovirus stocks

Sf9 cells were seeded in 3.0 ml volumes in six well tissue-culture dishes in Grace's insect medium without FCS at a density of 1.0 x 10^6 cells/well. The cells were left on the work bench to attach for about one h, while the virus dilutions were prepared in Grace's medium without FCS. Dilutions of 10^{-1} to 10^{-8} were prepared in duplicate in 1200 µl volumes in Eppendorf tubes. The medium was decanted from the seeded cells and replaced with 500 µl volumes of only the 10^{-3} – 10^{-8} dilutions. The cells were then very gently rocked for 2.0 h at room temperature. Meanwhile, a 2.0 % agarose mixture (SeaKem® ME agarose, FMC BioProducts, USA) was diluted to 0.5 % in complete Grace's insect medium. The infection mixtures were gently removed with a pipette from the infected cells and replaced with 2.0 ml per well of the cooled agarose mixtures. The cells were then incubated in an air-tight container for four days at 27 °C. On the fourth day, 1.0 ml filtre sterilised neutral red, containing 100 µg/ml neutral red (prepared from a 3.0 mg/ml solution supplied by GIBCO BRL, Scotland), in complete Grace's insect medium, was added to each well. The plates were left for 3.0 to 5.0 h. The neutral red was decanted and the plates were incubated overnight at 27 °C. The next day the titres of the stocks were determined. The P3 titres were generally in the order of 1.0 x 10^8 pfu/ml.

2.2.5 Preparation of aggregated and soluble baculovirus-expressed AHSV-5 VP2

Sf9 cells were grown as suspension cultures as described earlier. When the cells had reached a density of 1.0 to 2.0 x 10^6 cells/ml, they were infected at a MOI of 2.0 with P3 baculovirus stock expressing AHSV-5 VP2 and incubated for 40 to 48 h at 27 ^{0}C. The cells were harvested by centrifugation at 1000 x g for 5.0 min at 15 ^{0}C and washed three times in decreasing volumes of 100 ml, 50 ml and 20 ml of phosphate buffered saline (PBS) (137 mM NaCl; 2.7 mM KCl; 4.3 mM $Na_2HPO_4.2H_2O$; 1.4 mM KH_2PO_4). Finally, the cells were resuspended in PBS at a concentration of 1.0 x 10^8 cells/ ml and stored at -70 ^{0}C. Frozen cells were lysed by three cycles of freezing and thawing of crude cell lysates.

Thawing was done at 37 °C in a waterbath, followed by freezing at –70 °C. To separate soluble and insoluble AHSV-5 rVP2, low speed centrifugation of infected Sf9 cell lysates at 4000 x g for 15 min was performed. The supernatant, containing the soluble fraction was diluted three times in 1.0 x protein solvent buffer (PSB), prepared from 2.0 x PSB [10 ml solution containing 2.5 ml 0.5 M Tris (pH 8.0); 4.0 ml 10 % SDS; 2.0 ml glycerol; 1.0 ml 2-mercaptoethanol; 0.5 ml H_2O] and resuspended. The aggregate, containing the insoluble fraction was diluted six times in 1.0 x PSB. Following protein separation by SDS-PAGE gel electrophoresis, AHSV-5 rVP2 quantification was done by comparison of the supernatants and pellets from known amounts of Sf9 cells infected with rBac-5 to known amounts of marker protein by making use of the LumiAnalyst™ (Roche) computer software programme.

2.2.6 SDS-PAGE gel electrophoresis

Samples were heated for 5.0 min in boiling water and sonicated for 15 min. Electrophoresis was done in 12 % discontinuous polyacrylamide gels at 120 V in a 7.0 x 10 cm Hoefer® Mighty Small™ (SA Scientific Products) electrophoresis unit according to the method of Laemmli (1970). The gels were stained according to the method of Wong *et al.* (2000). Briefly, each gel was placed in approximately 100 ml Fairbanks A solution, containing 0.05 % Coomassie® Brilliant Blue (MERCK, Germany), 25 % isopropanol (MERCK) and 10 % acetic acid (Associated Chemical Enterprises c.c. RSA) and heated in a microwave oven until boiling. Thereafter, it was cooled to room temperature with gentle shaking for approximately 5.0 min, followed by washing in distilled H_2O. The gel was then placed in approximately 100 ml Fairbanks D solution, containing 10 % acetic acid and heated for 90 s in a microwave oven. It was again cooled to room temperature and depending on the resolution of the obtained protein bands, destaining with Fairbanks D was repeated.

2.2.7 Immunisation and bleeding of guinea pigs

Female guinea pigs, supplied by Onderstepoort Biological Products (OBP) (Onderstepoort, SA) were used. The animals were approximately three months old and were kept in wire cages in an enclosed room with controlled temperature of 15 to 18 °C and fed a pelleted diet and water enriched with 200mg/L ascorbic acid. The animals were immunised intra-peritoneally and boosted twice on days 28 and 56 with the rVP2 preparations, containing approximately 50 µg rVP2. The first inoculum contained 1.0 ml ISA-50 Seppic as adjuvant with 50 µg rVP2, prepared to 1.0 ml in PBS to render a final volume of 2.0 ml. The boosters were done in 1.0 ml inocula, containing 0.5 ml ISA-50 Seppic adjuvant and 0.5 ml PBS containing 50 µg rVP2. Bleeding was done by cardiac puncture under

anaesthesia 14 and 28 d after the second booster to allow for individual variation between animals to reach their peak antibody response. Serum samples were prepared by allowing coagulation of red blood cells at 37 °C for one h, followed by low speed centrifugation at 2000 x g at 4 °C for 15 min. The serum fractions were then aliquoted and frozen at –20 °C.

2.2.8 Plaque reduction neutralisation assay to determine neutralising antibodies in guinea pigs

The method of Huismans and Erasmus (1981) had to be adapted to obtain clearly distinguishable plaques. The following procedure rendered clearly distinguishable plaques and repeatable results:

2.2.8.1 Maintenance and cultivation of Vero cells

Vero cells (American Type Culture Collection, ATCC CCL 81) were incubated at 37 °C in a CO_2 incubator and maintained in MEM (Minimum Essential Medium Earl's Base), supplied by Highveld Biologicals, South Africa, containing 5.0 % FCS (Highveld Biologicals, South Africa) and a commercial streptomycin/penicillin/fungizone mixture (Highveld Biologicals, South Africa). The cells were passaged when they were confluent, which was generally after every three to four days.

2.2.8.2 Preparation of plates and serum dilutions

Vero cells were passaged onto 24 well tissue culture plates such that the cells formed a confluent monolayer the following day. To ensure this, 10 % FCS was added to the medium. Serum dilutions were done in Eppendorf tubes. Shortly, 0.5 ml in MEM without FCS was added to all but the first tube, which received 0.95 ml. A 1:20 to 1:409690 dilution series of the serum was then prepared by making a 1:20 dilution in the first tube. The contents were mixed by using a 1.0 ml Gilson pipette with at least four strokes of the plunger. Thereafter, 0.5 ml of the first dilution was transferred to the next tube. This procedure was repeated for all the tubes in the row and the 0.5 ml from the last tube was discarded. An AHSV-5 dilution (diluted in MEM without FCS to render 100 PFU per 500 µl) was added in 0.5 ml volumes to each tube and the mixture was incubated at 37 °C for 30 min. The medium was decanted from the prepared plates and 0.4 ml of the virus/serum mixtures was added to each well. This was done in duplicate for each dilution. Controls included uninfected cells, cells infected with 0.5 ml diluted virus and serum from a naïve horse. The plates were then incubated for 2.0 h at 37 °C. Thereafter, a 2.0 % mixture of low melting agarose (SeaKem® ME agarose, FMC BioProducts, USA) was diluted to 1.0 % in MEM containing 10 % FCS. Volumes of 0.4 ml of this agarose mixture were then added to each well to render a final FCS concentration of 2.5 %. The plates were incubated at 37 °C in a CO_2 incubator for five days and monitored daily.

2.2.8.3 Staining of the cells

Five days p.i, the cells were stained by adding 200 µl of a 100 µg/ml neutral red filter-sterilised solution (prepared from a 3.0 mg/ml solution, SIGMA-Aldrich. Co. Ltd) to each well. After 3.0 to 5.0 h incubation at 37 °C, the neutral red stain was removed and the cells were further incubated overnight. The neutralisation titres were expressed as the reciprocal of the serum dilution causing a 50 % reduction in the number of plaques.

2.3 RESULTS

As was explained under section 2.1, horses that were initially immunised with the AHSV-5 rVP2 were poorly protected against disease. Therefore, the first step in the further development of a VP2-based recombinant subunit vaccine for AHSV was to identify the reason(s) for the poor protection. To test the hypothesis that AHSV-5 rVP2 has two conformations, namely a soluble form and an aggregated form; the first experiment applied low speed centrifugation (4000 x g) to the AHSV-5 rVP2-expressing cell lysates to separate the aggregates from the more soluble material. The results obtained from SDS-PAGE analysis, allowed the determination of how much of the AHSV-5 rVP2 was expressed in the soluble form and whether solubility determined the biological activity of the rVP2.

2.3.1 SDS-PAGE analysis of aggregated and soluble fractions of baculovirus-expressed AHSV-5 VP2

Quantification of the electrophoresis bands was done by image capturing of white light transillumination of the Coomassie® brilliant blue stained gel and visual comparison of the amounts of rVP2 to known concentrations of commercially available markers (Fig. 2.1, lanes 1 and 2). Quantification showed that only about 10 % of the expressed AHSV-5 VP2 in the cell lysates of recombinant baculovirus-infected Sf9 cells was soluble (Fig. 2.1, lanes 3 and 4).

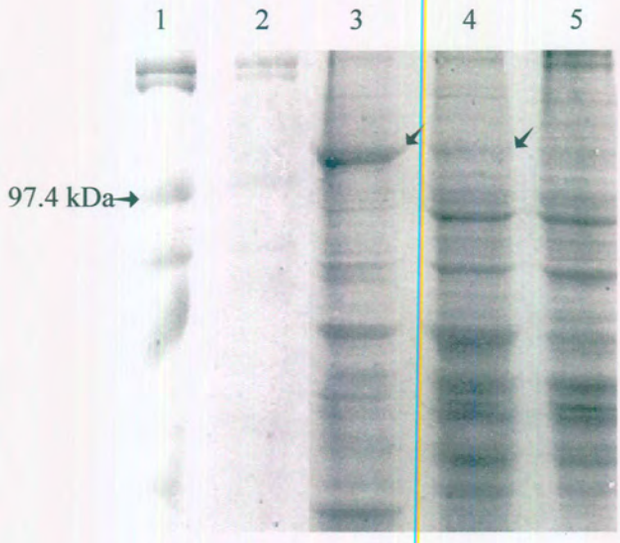

Fig. 2.1 Coomassie® brilliant blue stained 12% SDS-PAGE analysis of aggregated and soluble baculovirus-expressed AHSV5 VP2 obtained from comparable amounts of lysed Sf9 cells infected with rBac-5. Lane 1: 5.0 μg Rainbow™ molecular mass marker proteins; lane 2: 1.0 μg Rainbow™ molecular mass marker proteins; lane 3: aggregated fraction of crude lysate of recombinant AHSV-5 VP2 baculovirus-infected Sf9 cells; lane 4: soluble fraction of crude lysate of recombinant AHSV-5 VP2 baculovirus-infected Sf9 cells; lane 5: crude lysate of uninfected Sf9 cells. Arrows indicate the position of the rVP2.

2.3.2 Determination of the immunogenicity of the soluble and aggregated forms of baculovirus-expressed AHSV-5 VP2

To establish whether the low percentage of solubility for the rVP2 played a role in the poor protection observed in horses, the soluble and aggregated fractions were separated and injected into guinea pigs. The guinea pigs were divided into two groups of ten guinea pigs each. One group was immunised with the soluble rVP2 and the other group with the aggregated fraction, representing an equal amount of rVP2 estimated from the result in Fig. 2.1.

Seven of the ten guinea pigs immunised with soluble rVP2 developed high neutralising antibody tires of ≥ 80 (Table 2.1). Of these, five had antibody titres of 620 and higher. In contrast, of the ten guinea pigs injected with the aggregated VP2, only one developed a good neutralising antibody titre. In general, there was no significant difference in neutralising antibody titre between sera prepared from blood taken on day 14 and day 28 after the booster, as is characteristic of sampling during the plateau phase of the immune response.

These results implied that baculovirus-expressed AHSV-5 rVP2 has to be in the soluble form to be biologically active in guinea pigs.

Table 2.1 **Neutralising antibody titres induced in guinea pigs by inoculation with soluble and aggregated preparations of baculovirus-expressed AHSV-5 VP2**

Antigen	Guinea pig number	Neutralising antibody titres	
		Day 14 *post* 2nd booster	Day 28 *post* 2nd booster
Soluble rVP2	1	1280	1280
	2	2560	1280
	3	160	160
	4	80	40
	5	<20	<20
	6	5120	5120
	7	<20	<20
	8	5120	-[1]
	9	20	80
	10	640	-[1]
Aggregated rVP2	11	<20	<20
	12	<20	<20
	13	<20	<20
	14	320	640
	15	<20	<20
	16	<20	<20
	17	<20	<20
	18	<20	<20
	19	<20	<20
	20	<20	<20

[1]Guinea pig died after day 14 *post* 2nd booster bleed

2.4 DISCUSSION

Baculovirus-expressed AHSV-5 VP2 in crude cell lysates (which failed to fully protect horses against challenge in earlier experiments) exist mainly (~ 90 %) in the aggregated state. When 10 guinea pigs were immunised with the insoluble, aggregated fraction, only one developed a neutralising antibody titre. In contrast, seven of the 10 guinea pigs that were immunised with an equivalent rVP2 dose of the soluble fraction developed significant neutralising antibody titres. This implies that a ten-fold correction factor should be brought into consideration, when vaccinating horses with whole cell lysates of rVP2. Thus in the preliminary challenge experiment (results not shown), the initial dose of 50 μg rVP2 that was used to immunise the horses, only contained 5.0 μg immunogenic rVP2, and the second booster only 15 μg. As the vaccination was found to provide insufficient protection, it could thus be that the doses of immunogenic rVP2 were too low to achieve full protection.

The results obtained in this experiment are not uncommon in modern biotechnology, since protein misfolding and aggregation are common phenomena (Wigley *et al.*, 2000; Speed, Wang and King, 1996; Yon, 1996). Many factors, all of which yet have to be investigated, may contribute to aggregation. These include conditions such as protein concentration, pH, temperature, ionic strength and redox environment (Yon, 1996). Differences in *post*-translational modifications of the expressed protein in the baculovirus expression system compared to the protein in its natural environment may also play a role. Overexpression of the desired protein (which is often seen) may result in failure of the cell to keep pace with the required *post*-translational modifications. Several steps can be taken to rectify this, including usage of promoters that are active earlier in infection and the earlier harvesting of cells (O'Reilly, Miller and Luckow, 1994). Other factors that might also play a role include MOI and cell density or TOI.

The finding that only soluble baculovirus-expressed AHSV-5 VP2 induced neutralising antibodies in guinea pigs, adds a new dimension to the development of AHSV VP2s as subunit vaccines. Further investigation is needed to keep formation of insoluble rVP2 aggregates to a minimum and to find optimum conditions for producing VP2 in a form capable of inducing neutralising antibodies. Ensuring correct protein folding to induce protective immunity will thus be crucial for the further development and eventual commercial viability of VP2-based recombinant subunit vaccines for AHS. Approaches to achieve this will be further investigated in Chapter 5. The next step was to test the hypothesis that soluble rVP2 could induce humoral immunity and protection against challenge in horses.

CHAPTER 3

The effect of different adjuvants on the immunogenicity of soluble baculovirus-expressed AHSV-5 VP2

The work presented in this chapter has been published under the following title: Scanlen M, Paweska JT, Verschoor JA & van Dijk AA 2002. The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine is determined by adjuvant. Vaccine, 20: 1079-1088.

3.1 INTRODUCTION

Others and we have shown that baculovirus- and vaccinia virus-expressed AHSV VP2 can protect horses against a lethal challenge. However, the level of protection induced in horses following immunisation with the first generation of baculovirus-expressed AHSV VP2s was very variable (Martínez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Stone-Marchat *et al.*, 1996). The data presented in Chapter 2 identified one major problem: that baculovirus-expressed AHSV-5 VP2 is highly aggregative and that only the soluble form induces neutralising antibodies in guinea pigs.

Traditional vaccines are heterogeneous and contain many epitopes that can provide additional T-cell help or function as adjuvants. One of the major disadvantages associated with recombinant protein vaccines is that they are poorly immunogenic when administered alone. Therefore, development of such vaccines should also include testing compatible adjuvants that are safe and potent. Adjuvants may also reduce vaccine costs by reducing the dose of antigen and can help overcome antigen competition in combination vaccines (Singh and O'Hagan, 1999). An adjuvant is defined as a substance that non-specifically enhances the immune response to an antigen (Roitt, Brostoff and Male, 1989). The most effective adjuvant for a given antigen will to a large extent depend on the type of immune response required for protective immunity (Singh and O'Hagan, 1999). When choosing an adjuvant, it is important to remember that adjuvants are physiologically foreign to the body. They can mediate their effects by increasing cellular infiltration, inflammation and trafficking to the injection site. The hyperactivation of the immune response may be accompanied by adverse side-effects (Gupta *et al.*, 1993). A balance therefore has to be found between toxicity and adjuvanticity.

In the early, unpublished pilot experiment that was done to test the immunogenicity of AHSV5-rVP2 in horses, Montanide ISA-50 was used as adjuvant. However, adverse reactions in horses were observed at the injection site after administering AHSV-5 rVP2 preparations emulsified in the adjuvant Montanide ISA-50 (unpublished data: M Scanlen and AA van Dijk). It is generally known, albeit not formally

published, that horses are quite sensitive to oil-based adjuvants. The rational for the initial use of the oil-based Montanide ISA-50 as adjuvant in horses stemmed from the success of this adjuvant with the first generation of baculovirus-expressed bluetongue virus VP2-based recombinant vaccine candidates in sheep (Roy *et al.*, 1992). Bluetongue virus (BTV) is the prototype *Orbivirus* and shares many biological and structural characteristics with AHSV.

The purpose of this investigation was threefold: Firstly, to confirm the hypothesis that baculovirus-expressed AHSV-5 VP2 has to be soluble to be biologically active and induce a protective immune response in horses; secondly, to identify a safe and effective adjuvant to be used with rVP2 in horses; and thirdly to investigate whether it would be possible to lower the protective dose of rVP2 from 50 to 10 µg. The experimental approach was to immunise three groups of horses with the adjuvants Montanide ISA-50, Aluminium phosphate ($AlPO_4$) and the saponin-based adjuvant Quil-A. Two doses, namely 10 and 50 µg, of rVP2 were tested with these adjuvants.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of soluble baculovirus-expressed AHSV-5 VP2

Soluble baculovirus-expressed AHSV-5 VP2 was prepared as described under section 2.2.5.

3.2.2 Immunisation of horses

All the animals were immunised subcutaneously at the side of the neck. The first immunisation was done on the left side and the second one on the right side. The inocula, containing soluble recombinant AHSV-5 VP2, were prepared immediately before use and mixed with the appropriate adjuvant as follows:

Montanide ISA-50: Each horse was inoculated with 50 µg rVP2 in 1.5 ml phosphate buffered saline (PBS), emulsified in an equal volume (1.5 ml) of Montanide ISA-50 (Seppic, Paris) adjuvant.

Aluminium phosphate: $AlPO_4$ was prepared by adding 2.0 M Na_2HPO_4 dropwise to 100 ml 0.025 M $AlCl_3.6H_2O$ while stirring continuously for 15 min. The mixture was then centrifuged at 400 x g in a benchtop centrifuge. The resulting supernatant was discarded and the sediment was washed and centrifuged three times as before. The final sediment was resuspended in an equal volume of saline; steam sterilised and stored at 4.0 ^{0}C. Each horse received a 3.0 ml volume consisting of rVP2 in 600 µl sterile PBS emulsified with 2.4 ml (3.0 mg) $AlPO_4$ adjuvant.

Quil A Saponin: A 5.0 mg/ml solution of Quil A (Superfos Biosector A/S, Denmark) was prepared in water and filter sterilised though a 0.22 micron filter. Each horse was immunised with a 3.0 ml inoculum containing rVP2 in PBS (1.4 ml) emulsified with 3.0 mg Quil A (600 µl of the 5.0 mg/ml Quil A solution).

Saponin Q: A 3.0 % (3.0 g/100 ml) Saponin Q (Dr H. Schmittmann GMBH Chemische Fabrik, Germany) solution, prepared in non-pyrogenic distilled water and filter sterilised though a 0.22 micron filter, was diluted to 0.6 mg/ml in sterile distilled water. Each horse was inoculated with a 3.0 ml volume consisting of rVP2 in 2.0 ml PBS emulsified with 1.0 ml of the diluted adjuvant.

3.2.3 Challenge virus

Challenge material for the first experiment comprised of Vero cell culture supernatant fluid containing $10^{6.7}$ $TCID_{50}$/ml AHSV-5. Horses were challenged by subcutaneous inoculation at the side of the neck with 2.0 ml of this Vero cell suspension. For the second experiment, horses were challenged similarly with a 2.0 ml inoculum, containing homogenised spleen supernatant with a 10^6 $TCID_{50}$/ml AHSV-5 titre. All the horses were monitored daily for clinical signs of AHS for a 21 day period after challenge. Rectal temperatures of the animals were taken twice daily and blood was collected daily for two weeks and then every two days for a minimum of two more weeks after challenge to monitor viraemia and prepare serum.

3.2.4 Plaque-reduction neutralisation assay

Plaque-reduction-neutralisation was performed as described in section 2.2.8.

3.2.5 Determination of viraemia

Two ml of heparinised blood from each individual collection was transferred into sterile tubes, centrifuged at 800 x g for 10 min and the plasma discarded. The collected blood cells were washed three times with sterile PBS after which the PBS was discarded and the cells were lysed using sterile distilled water. The volume of distilled water was equal to the volume of plasma. The lysed red blood cell suspension was stabilised by addition of double concentrated sterile PBS. Infectivity titrations were carried out in tissue culture grade 96-well microtitre plates as described by Venter *et al.* (1999). Undiluted volumes of 100 µl and ten-fold dilutions of each individual red blood cell lysate were inoculated in quadruplicate into plate wells, to which 100 µl of a Vero cell suspension in EMEM medium containing 4 x 10^5 cells/ ml was added. The inoculated microplates were incubated at 37 ^{0}C in a CO_2 incubator and examined microscopically for cytopathic effects for 12 days *post* inoculation. Infectivity end-points were expressed as $TCID_{50}$/ml of blood (Karber, 1931).

3.3 RESULTS

Young (one to five years old) AHS susceptible horses of different sex (see Tables 3.2 and 3.3), originating from an AHS-free area in the high-lying eastern Free State region of South Africa, were used. Horses that did not have any demonstrable ELISA antibodies to AHSV VP7 were selected. The horses were stabled

overnight during the immunisation period and were transferred to isolation stables one week prior to challenge after their ELISA antibody titres had been confirmed as still negative to AHSV VP7. They were kept in isolation during the three weeks challenge period and for one month thereafter. Animals were immunised with the adjuvant and antigen doses indicated in Table 3.1. Booster immunisations were given in the first experiment on day 28 (Table 3.2) and in the second experiment (Table 3.3) on day 49.

Table 3.1 **Immunisation strategies of Experiments 1 and 2 to determine the effect of the type and amount of adjuvant and the dose of soluble AHSV-5 rVP2 on the protective efficacy of an AHSV-5 VP2-based recombinant vaccine**

Experiment no	[1]Group no	Horse numbers	Adjuvant(s)	Adjuvant dose	Soluble rVP2 dose
1	1	1, 2, 3	[2]I. ISA-50 [3]B.ISA-50	1.5 ml 1.5 ml	50 µg 50 µg
	2	4, 5, 6	I. $AlPO_4$ B. $AlPO_4$	3.0 mg 3.0 mg	50 µg 50 µg
	3	7, 8	I. Quil A B. Quil A	3.0 mg 3.0 mg	50 µg 50 µg
	Control	9	none	none	none
2	4	11, 12	I. Quil A B. Saponin Q	3.0 mg 0.6 mg	10 µg 10 µg
		13, 14	I. Quil A B. Saponin Q	3.0 mg 0.6 mg	50 µg 50 µg
	5	15, 16	I. Quil A B. $AlPO_4$	3.0 mg 3.0mg	10 µg 10 µg
		17, 18	I. Quil A B. $AlPO_4$	3.0 mg 3.0 mg	50 µg 50 µg
	Control	10	none	none	none

[1]Group numbers were allocated according to adjuvant(s) administered

[2]Primary immunisation

[3]Booster immunisation

3.3.1 Challenge experiment 1 (Groups 1-3): *Comparison of protection in horses immunised twice with 50 µg soluble baculovirus-expressed AHSV-5 VP2 administered with different adjuvants*

The aim of the first experiment was twofold. The first goal was to establish whether the hypothesis that baculovirus-expressed AHSV-5 VP2 must be soluble to be biologically active and induce a protective immune response is correct (Chapter 2). In view of the sensitivity of horses to oil-based adjuvants such as Montanide ISA-50, the second goal was to evaluate two other adjuvants that are regularly used in veterinary vaccines, Quil A saponin and $AlPO_4$, for efficacy and safety when used with the soluble AHSV-5 rVP2. Eight horses were immunised with the rVP2 doses and adjuvants indicated in Table 3.1. One control (no. 9) did not receive any antigen or adjuvant. The immunised animals were boosted on day 28 (Table 3.2). All nine horses were challenged on day 112. The control horse (no. 9) developed typical clinical signs of AHS including petechia on the tongue and eyelids, supraorbital oedema, laboured respiration and fever from 4.0-7.0 days post challenge (dpc) and died on 7.0 dpc, confirming lethality of the challenge virus.

Two of the three horses in Group 1 (nos. 1 and 2) immunised with 50 µg rVP2 with Montanide ISA-50 as adjuvant developed NAb titres after the booster (Table 3.2). The highest titres reached by horse nos. 1 and 2 were 640 and 1280 respectively, which had dropped to 80 on the day of challenge. Horse no. 3 never developed a demonstrable NAb titre. When challenged, the two animals that developed NAb titres proved to be partially protected and horse no. 3 died 8.0 dpc. All three animals developed fever and clinical signs of AHS (Fig. 3.1). $TCID_{50}$ virus titrations of heparinised blood from all the horses, indicated detectable viraemia for three to eight days with horse nos. 1 and 3 showing high titres (Table 3.4). The two horses that survived both had a large anamnestic response, indicating that their immunity was not sufficient to suppress viral replication (Table 3.2). Thus, immunising horses with 50 µg soluble rVP2 in Montanide ISA-50 induces only partial protection against challenge as is reflected by development of clinical signs and viraemia.

When $AlPO_4$ was used as adjuvant (Group 2) the horses were also only partially protected when challenged and developed clinical signs and viraemia. Although none of the three horses developed demonstrable NAb titres in response to immunisation (Table 3.2), two (nos. 4 and 5), survived the challenge, while one (no. 6) died on 7.0 dpc. All three horses developed fever and clinical signs of AHS from 4.0 – 7.0 dpc (Fig. 3.1) and became viraemic after challenge. The $\log_{10}$ viraemia values of horse no. 6 were very high from 3.0 – 7.0 dpc, with the maximum of 7.5 recorded on the 5th dpc. Horse nos. 4 and 5 had a much lower viraemia, which lasted for 7.0 days for horse no. 4 (3.0 - 10 dpc) and 6.0 days for horse no. 5 (4 -10 dpc) (Table 3.4). Both surviving horses developed huge anamnestic responses after challenge (Table 3.2). Thus, when soluble rVP2 was administered with $AlPO_4$, only partial protection against challenge was achieved as is reflected by the presence of clinical signs and viraemia.

Full protection, as reflected by the complete absence of clinical signs and viraemia after challenge, was observed in both horses that were immunised with rVP2 emulsified in Quil A saponin (nos. 7 and 8, Group 3). They were the only horses in the experiment that developed a demonstrable NAb titre of 40 after the initial immunisation (Table 3.2). After boosting, the NAb titres of horse nos. 7 and 8 increased further to 10420 and on the day of challenge (day 112), it was still 10240 and 2560 respectively. These NAb titres were significantly higher than when rVP2 was administered in Montanide ISA-50 or $AlPO_4$. After challenge, neither of the horses developed a fever or any clinical sign of AHS (Fig. 3.1) and no viraemia could be detected (Table 3.4). They did, however, have an anamnestic response to challenge indicating that there was some virus replication (Table 3.2).

3.3.2 Challenge experiment 2 (Groups 4-5): *Comparison of protection in horses immunised with 10 µg and 50 µg doses of soluble baculovirus-expressed AHSV-5 VP2 and different doses and combinations of adjuvants*

Since there were only two horses in the well protected Group 3 (Quil A as adjuvant) in Experiment 1, a second experiment was done to confirm the results in four additional horses and to determine whether it would be possible to lower the protective dose of rVP2. Nine new horses were used. Eight horses were divided into two groups of four horses each. Originally the idea was to immunise them twice with 10 µg (horse nos. 11-16) or 50 µg soluble rVP2 (horse nos. 13-18), adjuvanted with 3.0 mg Quil A saponin and to evaluate the protective immune response by challenging. However, as all eight immunised horses developed severe inflammatory responses to Quil A saponin after immunisation, the outlay of this experiment was changed. Quil A saponin was not included in the booster injection, but two other adjuvants, namely Saponin Q and $AlPO_4$, were used instead (as explained later). The original two groups of four horses each were further divided in two subgroups of two horses each and boosted on day 49 as indicated in Table 3.1. All nine horses were challenged on day 133. The control horse (no. 10) developed typical clinical signs of AHS including petechia on the tongue and eyelids, supraorbital oedema, laboured respiration and fever from 4.0 dpc and died on 8.0 dpc, confirming lethality of the challenge virus. All eight immunised horses survived the challenge. Four were fully protected and four were partially protected. The 10 µg and 50 µg soluble rVP2 doses were equally protective.

The fully protected horses were the four that were boosted with vaccine containing Saponin Q as adjuvant (Group 4, nos. 11, 12, 13, 14; Table 3.1), irrespective of whether they received 10 µg or 50 µg rVP2. They all developed NAb titres by day 28 in response to the primary immunisation (Table 3.3). After boosting, the NAb titre increased further and peaked on day 105 (Table 3.3). Animals were monitored for clinical AHS signs for 18 days after the challenge. All four horses (nos. 11-14) were well protected. None developed a fever (Fig. 3.1), any other clinical signs of AHS or became viraemic (Table 3.4). All four did, however, mount an anamnestic response to the challenge (Table 3.3). Horse nos. 11 and 12, which received 10 µg rVP2, were just as well protected as nos. 13 and 14, which received 50 µg rVP2.

The four partially protected horses were those boosted with vaccine containing $AlPO_4$ as adjuvant (Group 5, nos. 15-18; Table 3.1). These horses also developed NAbs in response to the primary immunisation, which increased slightly after boosting. The NAb titres of these horses were lower than that of horses boosted with a vaccine containing Saponin Q as adjuvant (Group 4). When challenged, all four horses in Group 5 (nos. 15-18) survived, but were partially protected. They all developed fever (Fig. 3.1), clinical AHS signs for three days, 4-6 dpc, became viraemic (Table 3.4: average $\log_{10}$ value of 3.64) and had a huge anamnestic response, which dropped more slowly than those of Group 4 (Table 3.3).

3.3.3 Evaluation of tolerance of horses to different saponin preparations

In the first experiment horses were only monitored for hypersensitivity reactions to adjuvant after booster immunisations. All three horses which were injected with Montanide ISA-50 as adjuvant (nos. 1-3) developed large, sensitive swellings at the injection site within 24 h. None of the three horses for which $AlPO_4$ was used as adjuvant (nos. 4-6) had any adverse side-effects. Of the two that were immunised using Quil A as adjuvant (nos. 7 and 8), only one, no. 8, developed a small sensitive swelling at the injection site.

In the second experiment all the horses received 3.0 mg of a new batch of Quil A in the first immunisation and were monitored for adverse reactions twice daily. This time, all eight horses (nos. 11-18) developed large, well-defined and sensitive swellings at the injection site, which took seven days to subside. Therefore, inflammatory responses of a separate group of 12 horses to different doses of Quil A and other commercially available saponin preparations, Saponin Q and QA-21 (veterinary grade QS-21, Aquila Biopharmaceuticals, Inc.) were tested. The animals were subsequently boosted in the second experiment according to the outcome. Quil A was prepared as described in Materials and Methods and diluted to the desired concentration in sterile PBS in a 3.0 ml inoculum. Three horses were separately injected subcutaneously at the side of the neck with 3.0, 0.6 and 0.3 mg Quil A without antigen. Another group of three horses was inoculated similarly with Saponin Q without antigen. Six horses were evaluated likewise for hypersensitivity to QA-21 by injecting three groups of two horses each separately with 2.0, 1.0 and 0.5 mg QA-21. On the manufacturer's recommendation, a 1.0 mg/ml solution of QA-21 in water was prepared and filter sterilised though a 0.22 micron filter. The different concentrations of the QA-21 tested were also prepared by dilution of the stock solution in PBS to a 3.0 ml inoculum. The horses were monitored for seven days for adverse reactions at the injection site. The severity of reactions was scored as indicated in Table 3.5.

All the saponin preparations induced swellings, which were dose related, with the larger doses causing more severe swellings (Table 3.5). Reactions to Quil A (Fig. 3.2) were the worst and took the longest to subside. QA-21 caused intermediate reactions, while Saponin Q was the best tolerated. Horses injected with 0.6 mg or less Saponin Q developed very small swellings (Fig. 3.3), which disappeared completely

after 24 to 36 h (Table 3.5). Subsequently, the planning for the second vaccine experiment was adjusted and one group of horses (Table 3.11, Group 4) was boosted with vaccine containing 3.0 mg $AlPO_4$ as adjuvant and one group (Table 3.1, Group 5) with 0.6 mg Saponin Q as adjuvant. The results were already discussed above.

Table 3.2 **Neutralising antibody titres of horses immunised twice with 50 µg soluble baculovirus-expressed AHSV-5 VP2 administered with different adjuvants (Challenge experiment 1)**

			Days *post* first immunisation and challenge								
Group number and adjuvant	**rVP2 (µg)**	**Horse number + sex**	do [1]**I**	d28 [2]**B**	d56	d70	d112 [3]**C** ***0 dpc***	d126 ***14 dpc***	d154 ***42 dpc***	d168 ***56 dpc***	d182 ***70 dpc***
	2 x 50	**1 (F)**	<20	<20	40	640	80	>40960	>40960	10240	10240
GROUP 1:	2 x 50	**2 (F)**	<20	<20	1280	640	80	>40960	>40960	2560	1280
2 x ISA-50	2 x 50	**3 (M)**	<20	<20	<20	<20	<20	-	-	-	-
	2 x 50	**4 (F)**	<20	<20	<20	20	<20	>40960	>40960	10240	5120
GROUP 2:	2 x 50	**5 (F)**	<20	<20	<20	<20	<20	>40960	>40960	10240	10240
2 x $AlPO_4$	2 x 50	**6 (F)**	<20	<20	<20	<20	<20	-	-	-	-
GROUP 3:	2 x 50	**7 (F)**	<20	40	1280	10240	10240	>40960	>40960	1280	1280
2 x Quil A	2 x 50	**8 (F)**	<20	40	2560	10240	2560	>40960	>40960	1280	1280
Control	None	**9 (M)**	<20	<20	<20	<20	<20	-	-	-	-

[1]Primary immunisation

[2]Booster immunisation

[3]Challenge

Table 3.3 **Neutralising antibody titres of horses immunised twice with 10 or 50 μg doses soluble baculovirus-expressed AHSV-5 VP2 and different doses and combinations of adjuvants (Challenge experiment 2)**

			Days *post* first immunisation and challenge										
Group number and adjuvants	**rVP2 (μg)**	**Horse no. + sex**	do [1]**I**	d28	d49 [2]**B**	d77	d105	d133 [3]**C** ***0 dpc***	d147 ***14 dpc***	d175 ***42 dpc***	d203 ***70 dpc***	d231 ***98 dpc***	d259 ***126 dpc***
Control	-	**10 (M)**	<20	<20	<20	<20	<20	<20	-	-	-	-	-
GROUP 4:	2 x 10	**11 (M)**	<20	80	160	320	320	160	5120	2560	1280	640	160
	2 x 10	**12 (F)**	<20	320	320	640	1280	320	10240	2560	1280	640	320
I: Quil A	2 x 50	**13 (F)**	<20	40	20	160	160	80	40960	5120	1280	640	640
B: Saponin Q	2 x 50	**14 (F)**	<20	80	80	160	640	640	1280	1280	640	640	160
GROUP 5:	2 x 10	**15 (F)**	<20	20	20	40	40	40	40960	20480	10240	5120	640
	2 x 10	**16 (M)**	<20	80	40	40	80	40	40960	10240	1280	1280	640
I: Quil A	2 x 50	**17 (F)**	<20	-	20	40	80	80	>40960	10240	10240	10240	1280
B: $AlPO_4$	2 x 50	**18 (M)**	<20	20	20	80	80	40	40960	5120	2560	1280	1280

[1]Primary immunisation

[2]Booster immunisation

[3]Challenge

Table 3.4 **Viraemia (log_{10}) levels after challenge**

Experiment no.	Group no.	rVP2 (µg)	Horse no.	Days *post* challenge															
				d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13	d14	d15	d16
[a] 1	1	2 x 50	1	[b]N	N	N	0.80	4.00	4.75	3.75	0.80	0.80	0.80	0.80	N	N				
		2 x 50	2	N	N	N	N	N	N	0.80	N	N	0.80	0.80	N	N				
	2 x ISA-50	2 x 50	3	N	N	0.80	3.50	5.00	6.00	7.25	7.00	-	-	-	-	-				
	2	2 x 50	4	N	N	N	1.75	2.50	0.80	2.25	2.00	1.75	2.50	0.80	N	N				
		2 x 50	5	N	N	N	N	0.80	0.80	0.80	0.80	0.80	0.80	0.80	N	N				
	2 x $AlPO_4$	2 x 50	6	N	[c]0.80	0.80	4.75	6.75	7.50	6.75	7.00	-	-	-	-	-				
	3	2 x 50	7	N	N	N	N	N	N	N	N	N	N	N	N	N				
	2 x Quil A	2 x 50	8	N	N	N	N	N	N	N	N	N	N	N	N	N				
	Control	None	9	N	N	N	3.15	5.75	6.00	6.75	7.50	-	-	-	-	-				
2	**Control**	None	10	N	N	N	N	2.75	3.50	3.75	3.75	4.00	4.50	-	-	-	-	-	-	-
	4	2 x 10	11	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
		2 x 10	12	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	I: Quil A	2 x 50	13	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	B: Saponin Q	2 x 50	14	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	5	2 x 10	15	N	N	N	N	0.75	3.50	3.00	2.75	N	N	N	N	N	N	N	N	N
		2 x 10	16	N	N	N	N	3.15	3.37	3.75	N	N	N	N	N	N	N	N	N	N
	I: Quil A	2 x 50	17	N	N	N	N	0.75	3.15	3.50	N	N	N	N	N	N	N	N	N	N
	B: $AlPO_4$	2 x 50	18	N	N	N	N	2.50	3.50	2.75	N	N	N	N	N	N	N	N	N	N

[a]In Experiment 1 viraemia was only monitored up to 12 dpc.
[b]N = Negative. The minimum amount of virus that could be detected in our system is 0.75 $TCID_{50}$. A result is considered negative where no viraemia could be detected under these condition
s.
[c]Infectivity end-points are expressed as $TCID_{50}$/ml of blood.

Temperature profiles: Experiment 1

Temperature profiles: Experiment 2

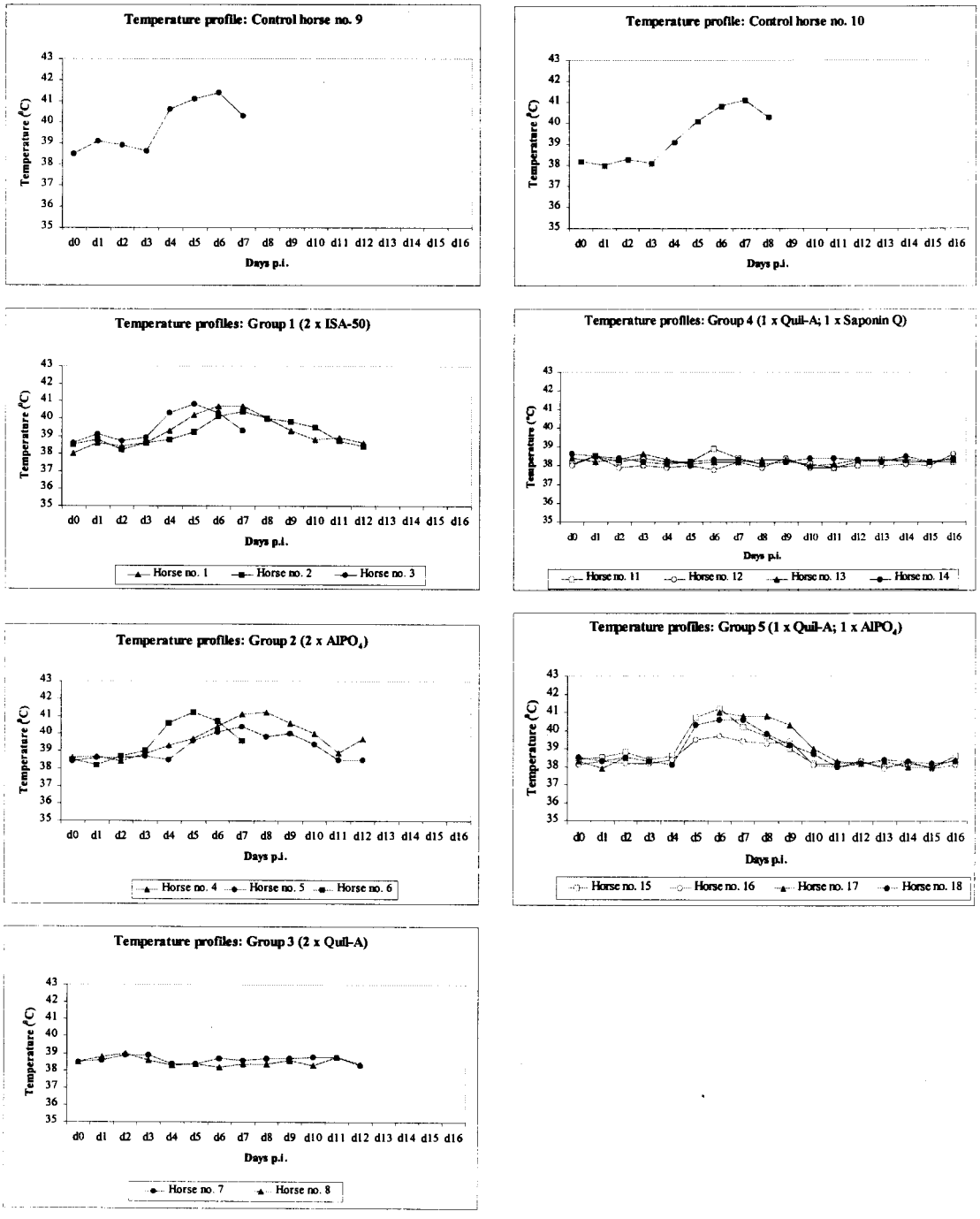

Fig. 3.1 **Temperature profiles after challenge of horses immunised with soluble AHSV-5 rVP2. In Experiment 1 (Groups 1 - 3), horses were immunised with 50 μg baculovirus-expressed AHSV-5 VP2 in combination with different adjuvants. In Experiment 2 (Groups 4 and 5), horses were immunised with either 10 μg or 50 μg baculovirus-expressed AHSV-5 VP2 and different doses and combinations of adjuvant. Two control horses (no. 9 and 10), which did not receive any antigen or adjuvant were also included. Temperatures were taken for 12 dpc in Experiment 1 and for 16 dpc in Experiment 2.**

Table 3.5 **Severity of inflammatory reactions observed in horses injected with different amounts of saponin-based adjuvants**

Adjuvant	Amount	*Sensitivity
Quil A	3.0 mg	+++
	0.6 mg	++
	0.3 mg	++
Saponin Q	3.0 mg	++
	0.6 mg	+
	0.3 mg	+
QS-21	2.0 mg	+++
	1.0 mg	++
	0.5 mg	++

***Quantification of scoring:**

+++ Very severe swellings of 6.0 - 20 cm diameter, which took 2 - 7 days to subside and were very sensitive.

++ Medium swellings of 3.0 - 12 cm diameter, which took 1 - 4 days to subside and was slightly to very sensitive.

+ Small, almost unnoticeable swellings of 3.0 - 6.0 cm diameter, which subsided after 24 - 36 h, with very mild to no sensitivity.

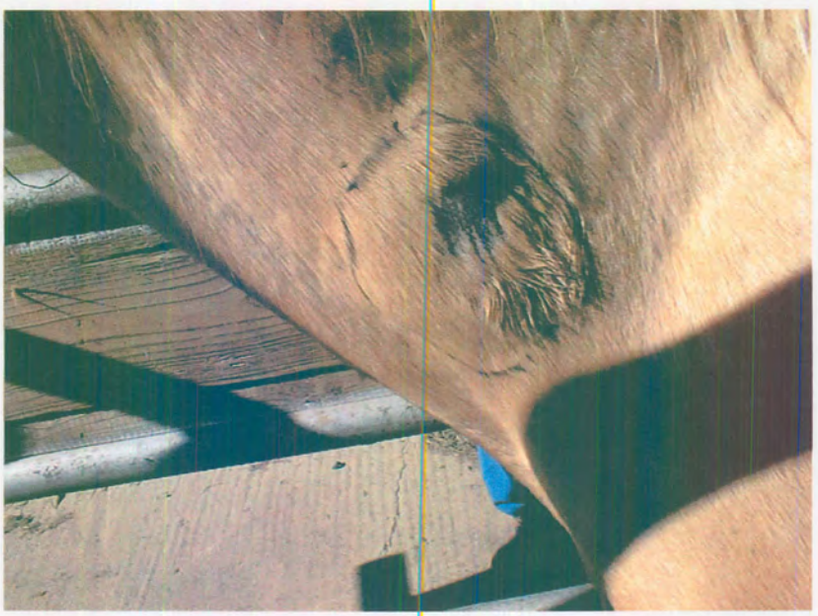

Fig 3.2 **Severe large swellings observed at the injection site when Quil A was used as adjuvant with the soluble baculovirus-expressed AHSV-5 VP2. Hair was shaven off to simplify observation.**

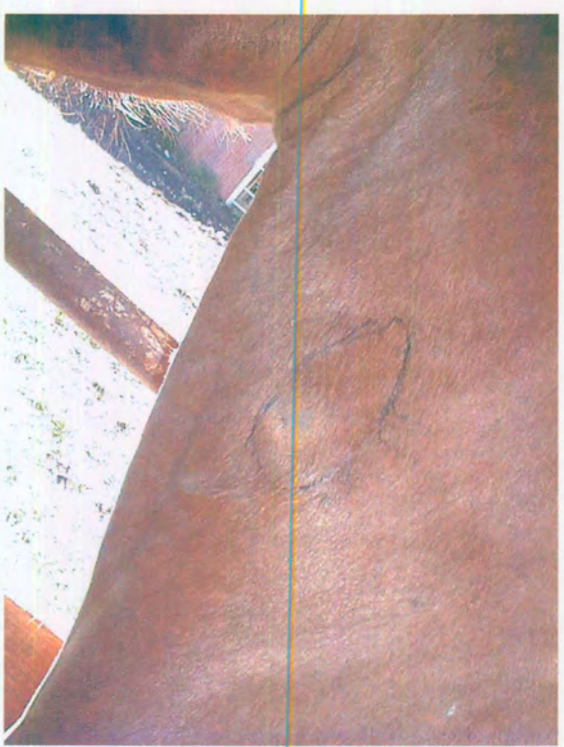

Fig 3.3 **Small to mild swellings observed at the injection site when Saponin Q was used as adjuvant with the soluble baculovirus-expressed AHSV-5 VP2.**

3.4 DISCUSSION

This is the first investigation that compared different adjuvants with regard to their effect on the level of protection elicited by immunising horses with soluble baculovirus-expressed AHSV VP2. The results established that AHSV rVP2 has to be soluble to be biological active and induce a protective immune response in horses and that the type of adjuvant used is crucial for inducing reliable protection. Administering soluble AHSV rVP2 with saponin adjuvants induced excellent protection to a lethal challenge, but there were adjuvant-dose related side effects, whereas $AlPO_4$ or Montanide ISA-50 evoked only partial immunity. Since saponin induces class I MHC antigen-restricted cytotoxic T lymphocytes (Wu *et al.*, 1994), the distinct advantage of saponin adjuvants compared to the oil-based ISA-50 or $AlPO_4$, suggests an important role for a Th1-type cell-mediated immunity (CMI) in protection against AHS. The results presented here, point to a direct role for AHSV VP2 in stimulating a protective cell-mediated immune response in addition to its well-documented ability to elicit a protective neutralising antibody-based humoral immune response. This assumption is supported by Romito *et al.* (1999) who found AHSV-specific CTLs in a horse immunised with a VP2 DNA vaccine.

Similar levels of protection were obtained with the 10 µg and 50 µg doses of soluble AHSV-5 rVP2. These results correlate with data published for AHSV-4 rVP2 where the NAb titres of horses that received 5.0 µg crude AHSV-4 rVP2 were not significantly lower than for horses that received larger amounts of rVP2 (Roy *et al.*, 1996). Our results reveal that the type of adjuvant is a very important factor impacting on protection in the 10 – 50 µg dose range. Adjuvant was also reported to be important with recombinant virus-like particle subunit vaccine candidates for two other viruses in the family *Reoviridae,* namely BTV and rotavirus. For BTV VLPs, Montanide ISA-50 significantly outperformed $Al(OH)_3$ and incomplete Freund's adjuvant, in eliciting protective immunity in sheep (Roy, French and Erasmus, 1992). Similarly, for rotavirus VLPs saponin QS-21 induced a balanced Th1/Th2 response which resulted in significantly higher antibody titres and complete protection in mice against challenge in comparison to $Al(OH)_3$ (Jiang, *et al.*, 1999).

Three adjuvants were tested in different combinations with 50 µg soluble baculovirus-expressed AHSV-5 VP2. Use of ISA-50 as adjuvant provided a reference point, since it gave the best results compared to other adjuvants in a BTV VLP vaccine trial in sheep (Roy, French and Erasmus, 1992). It was, therefore, used in our initial AHSV-5 rVP2 vaccine experiments. Our present investigation re-confirmed the sensitivity of horses for the oil-based Montanide ISA-50. This result however, is not uncommon for oil-based adjuvants. The mode of action of these adjuvants is attributed to depot formation at the injection site and slow release of the antigen with stimulation of the antibody-producing cells (Gupta *et al.*, 1993). Due to the infiltration by leucocytes, inflammation at the

injection site is inevitable (Allison, 1997). The result of this investigation that horses were only partially protected when soluble AHSV-5 rVP2 was administered with Montanide ISA-50, enables us to now rule Montanide ISA-50 out as a suitable adjuvant for AHSV recombinant vaccine formulations.

Although aluminum salts have an excellent safety record (Cox and Coulter 1997), it was found that $AlPO_4$ is also not a suitable adjuvant for our baculovirus-expressed VP2-based AHSV vaccine, since soluble AHSV-5 rVP2 emulsified in $AlPO_4$ failed to elicit a NAb response in horses and also induced only partial protection. Possible reasons for the partial protection by rVP2 in the presence of $AlPO_4$ could be the mode of action of $AlPO_4$ and/or that it does not induce the appropriate immune response needed for full protection. Adjuvanticity of aluminium salts relies on the conversion of soluble protein to a particulate form by adsorption to the surface of the precipitated aluminium salt (Skea and Barber, 1993), thus presenting the absorbed antigen in an insoluble form. Since, it has been previously shown that insoluble AHSV-5 rVP2 does not elicit a NAb response in guinea pigs (Chapter 2), the processing of soluble rVP2 and $AlPO_4$ could cause aggregation and thus explain the poor protection. Since $AlPO_4$ is known to induce an allergic Th2 type immune response (Cox and Coulter, 1997, HogenEsch, 2002), another reason for the partial protection could be that a Th2 response is not the major protective immune response to AHS. However, the fact that two of the three horses, that received $AlPO_4$ as adjuvant in the vaccine, survived the challenge without developing demonstrable humoral immunity does indicate some role for Th2 immunity in protection. This finding correlates with other published results implying that for AHSV recombinant vaccines there is no direct correlation between the presence of neutralising antibodies *in vitro* and protection *in vivo* (Martínez-Torrecuadrada, Díaz-Laviada and Roy, 1996 and Stone-Marchat *et al.*, 1996).

Three Saponin-based adjuvants were investigated. Quil A saponin was initially investigated, since it is widely used in veterinary vaccines (Kensil, 1996). In horses, it has been mostly used in the form of ISCOMS (immunostimulating complexes) (Sundquist, Lövgren and Morein, 1988; Mumford *et al.*, 1994). Although two injections of 50 µg soluble rVP2 emulsified in 3.0 mg Quil A induced excellent protection against challenge (Experiment 1), horses injected with Quil A from a new batch, developed large inflammatory swellings at the injection site (Experiment 2). The size of the swelling was dose related. Saponin QS-21, that is one of the four most predominant saponin fractions from *Quillaja saponaria* with relatively low toxicity (Kensil *et al.*, 1991), was also investigated. QS-21 formulations of rotavirus VLPs elicited higher antibody titres and better protection than $Al(OH)_3$ formulations (Jiang *et al.*, 1999). However, dose related swellings, similar to those observed with Quil A, were observed in horses. The third saponin preparation investigated was Saponin Q. The local reactions observed with Saponin Q were not as severe as with Quil A and QS-21. This result was unexpected, since Saponin Q is a very crude extract of *Quillaja* saponin. Although saponin-based adjuvants

induced side-effects in horses with AHSV-5 rVP2, the effects were not as severe as reported by Mirchamsy *et al.* (1972). These authors injected horses with 10 mg of a purified saponin preparation and inactivated AHSV vaccine. They reported that local reactions due to the adjuvant lasted for several weeks. This could also be due to the large dose of saponin that was used.

Due to our findings, horses in the second experiment that were originally injected with Quil A as adjuvant were either boosted with vaccine containing Saponin Q or $AlPO_4$. The rationale for boosting with $AlPO_4$ was to determine whether priming with Quil A would be sufficient to achieve protection. However, all the horses in Group 5 developed large inflammatory reactions at the site of injection after a booster injection with $AlPO_4$ as adjuvant. This could be due to the fact that they were originally injected with Quil A as adjuvant that primed the horses against rVP2, to give hypersensitivity to the antigen (rVP2) upon boosting. Horses that were boosted with vaccine containing 0.6 mg Saponin Q (Group 4), only developed slight swellings at the injection site. However, the NAb titres developed were lower than for Group 3, where 3.0 mg Quil A was administered twice as adjuvant. The adjuvant activity of saponin is attributed to binding of the adjuvant to cell membrane-bound cholesterol (Glauert, Dingle and Lucy, 1962; Bomford, 1982; Scott, Goss-Sampson and Bomford 1985). This action prolongs retention of the antigen at the subcutaneous injection site and increases the amount reaching the spleen. Retention of the antigen at the injection site corresponds with a depot type of adjuvant action and a prolonged stimulation of the immune system. The prolonged retention may also contribute to the haemolytic effect and acute inflammation observed at the injection site (Scott et al., 1985). Abolition of the inflammatory action of saponin by lower adjuvant doses reduced this phenomenon remarkably. The lower NAb titres could thus be attributed to the lower dose of adjuvant in the booster immunisations and the less severe inflammatory response elicited. Thus, reduction of saponin dose not only reduced the inflammatory response observed, but also the adjuvant activity. However, when saponin was injected twice with AHSV-5 rVP2, even at a lower dose in the booster immunisation, protection against a lethal viral challenge was achieved. Although animals developed anamnestic responses indicating that some virus replication still occurred, they were fully protected against clinical disease and demonstrable viraemia.

In order to understand the causal factor of the inflammatory adjuvant responses it will be necessary to analyse the composition of each of the saponin batches. The strength of the inflammatory response in horses suggests that the amount of Quil A could be lowered considerably and still achieve adjuvant effect. The booster injection with 0.6 mg Saponin Q compared to priming with 3.0 mg Quil A, is difficult to interpret because Saponin Q is a crude saponin of unknown potency and will have a different saponin profile compared to Quil A.

Thus, our results demonstrate that the efficacy and safety type of a recombinant VP2-based subunit AHSV vaccine is not only determined by the conformation of the rVP2, but also by the type of adjuvant used. Both doses of soluble rVP2 that were tested (10 μg and 50 μg) seemed equally protective. When horses were injected twice with saponin-based adjuvants in combination with the soluble rVP2, they were fully protected against clinical AHS disease and detectable viraemia. This finding proves that rVP2 alone is sufficient and no other recombinant AHSV structural proteins are needed to achieve reliable immunity to AHS. Our results are the first to differentiate between the effect of a possible Th1 inflammatory response and a Th2 allergic type response for AHS protection, with the former being the more important. Further investigations are needed to clarify the role of cell-mediated immunity in protection against AHS, to statistically assess the suitability of Saponin Q as adjuvant with our baculovirus-expressed AHSV-5 VP2 in larger numbers of horses and to establish whether a 10 μg rVP2 dose is enough to provide long term protection against AHSV challenge in horses.

CHAPTER 4

The level of protection and duration of immunity conferred by administering 10 g AHSV-5 baculovirus-expressed VP2 with Saponin Q as adjuvant

4.1 INTRODUCTION

In Chapter 3 it was shown that horses were not as sensitive to Saponin Q as to Quil-A. Previously when the horses received 3.0 mg Quil A in the primary immunisation and 0.6 mg Saponin Q in the second immunisation as adjuvants in a VP2-based recombinant subunit AHSV vaccine, complete protection against challenge was obtained. It was also found that there was no difference in protection induced by using either 10 or 50 µg rVP2 as antigen. However, several questions remained to be answered such as whether the same degree of protection could be obtained by using Saponin Q alone as adjuvant with 10 µg rVP2 and what the duration of protection with this particular immunisation regime is.

It is known that foals acquire passive colostral immunity from mares for three to six months after birth. The level of the acquired antibody titre correlates with the antibody titre in the serum of the mare. When foals are immunised with the live vaccine during this period and even for an undetermined period thereafter, no protective active immunity is obtained (Alexander and Mason, 1941). This phenomenon is due to the fact that the attenuated virus needs to replicate to confer immunity (van Dijk, 1998). Blackburn and Swanepoel (1988a; 1988b) found that neutralising antibodies in foals often declined to undetectable levels two to four months after birth, which leads to a greater susceptibility during this period. This problem is further complicated by the breeding season in southern Africa. Foals are born from September to December and AHS outbreaks reach peak levels during autumn from February to the beginning of May. This situation makes foals most vulnerable during the window period when no neutralising antibodies are detected and vaccine administration does not provide protection (Alexander and Mason, 1941; Blackburn and Swanepoel, 1988a, 1988b). Although a recombinant vaccine against AHSV does not have to replicate to confer protection, it is important that the adjuvant-antigen combination is formulated in such a way that protection is provided for at least eight months.

The goals of this part of the investigation were twofold: the first was to determine whether two immunisations with Saponin Q and 10 μg soluble baculovirus-expressed AHSV-5 VP2 will be sufficient to protect horses against challenge two to three months after the booster. The second goal was to determine whether immunity would last for eight months after the booster.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of soluble AHSV-5 VP2

Soluble baculovirus-expressed AHSV-5 VP2 was prepared as described under section 2.2.5.

4.2.2 Preparation of adjuvant

Saponin Q: Was prepared as described under section 3.2.2

4.2.3 Challenge virus

The challenge virus was prepared as described under section 3.2.3.

4.2.4 Immunisation and challenge of horses

Young (one to five years old), mainly female (see Tables 4.1 and 4.2), AHS susceptible horses were used. Animals were stabled as described under section 3.3.3. Challenged horses were monitored as described in 3.2.3 and NAb titres and viraemia were determined as described under sections 2.2.8 and 3.2.5.

Animals were divided into two groups (Groups 6 and 7). Animals in Group 6 were challenged on day 138 and in Group 7 on day 448. Viraemia and temperature monitoring were done for 18 dpc for Group 6 and 16 dpc for Group 7.

4.3 RESULTS

To determine the duration of immunity and level of protection afforded, a group of 10 horses was immunised as described in 3.2.2 on day 0 and day 42 with a 3.0 ml inoculum of 0.6 mg Saponin Q and 10

g AHSV-5 rVP2 prepared in PBS. The animals were then divided in two groups, Groups 6 and 7 (Tables 4.1 and 4.2), with the aim to challenge Group 6 three months and Group 7 eight months later. Due to the limited availability of AHSV susceptible horses in South Africa, it was decided to use the same challenge virus for both groups and to include only one control (C1) for both experiments. Control 1 was injected on day 0 and day 42 with a 3.0 ml inoculum of PBS mixed with 0.6 mg Saponin Q. This was done to determine whether the antigen enhances the inflammatory effect of the adjuvant. However, due to the low (NAb) titres displayed by the immunised animals, the immunisation strategies for both groups were changed (explained later).

4.3.1 Challenge experiment 3 (Group 6): *Determination of inflammatory response and protection observed in horses immunised twice with 0.6 mg Saponin Q plus 10 μg soluble baculovirus-expressed AHSV-5 VP2 and challenged on day 138*

Animals only developed detectable NAbs after the second immunisation on day 42 (Table 4.1). This was in contrast to our previous experiments (Chapter 3), where animals injected with 3.0 mg Quil A (Groups 3, 4 and 5), already displayed NAb titres after the first immunisation. The highest titre of 320 was reached by horse 20 and had dropped to 160 on day 138, the day of challenge. Horses 19 and 22 both had NAb titres of 40 on day 56. Due to their low NAb titres, these two horses received a second booster with the same amount of adjuvant and antigen on day 70. Their titres then increased to 160 on day 84, 14 days after the second booster was given. Animals were challenged on day 138, 68 days after the last booster for horses 19 and 22 and 96 days after the last booster for horses 20 and 21. Challenge inoculations were given subcutaneously at the left side of the neck with a 2.0 ml inoculum, containing homogenised spleen supernatant with a 10^6 $TCID_{50}$/ml AHSV-5 titre. On the day of challenge, horses 19 and 21 had the lowest NAb titres of 40 and 20 respectively. Horses 22 and 20 displayed NAb titres of 160. The control horse (C1) did not show any detectable NAb titre prior to challenge. All the immunised animals had a huge anamnestic response after challenge, which quickly decreased after 56 dpc and then remained constant as displayed in Table 4.1. The control also showed an increase in NAb titre to 5120 in response to the challenge virus. This value remained constant for the whole period of 196 dpc that the animals were monitored.

Horses developing a rectal temperature of 39 ^{0}C or higher after challenge were considered febrile (Fig. 4.1). The control horse (C1) did not die, but it did develop typical clinical signs of AHS, including petechia on the tongue and eyelids, supraorbital oedema, laboured respiration and fever from 5 to 13 dpc (Fig. 4.1). Viraemia was determined by doing infectivity titrations of collected horse blood on Vero cells.

However, inconclusive results were obtained. It was then decided to repeat the viraemia titrations in two-day-old suckling mice using heparinised blood collected on 4, 6, 8, 10, 12 and 14 dpc. Seven mice were allocated per family and two families were used for each horse for each indicated day. The mice were inoculated intracranially with 30 µl heparinised horse blood and were monitored for neurodegenerative symptoms for 14 days after inoculation. Brains of mice showing such symptoms or that died were collected for confirmation of virus replication in Vero cells. Blood samples from the control taken on 6, 8 and 10 dpc displayed viraemia (Table 4.3) in mice. Horse 21 developed fever (Fig. 4.1) and some clinical AHS signs, including increased respiratory rate and supra-orbital oedema from 6 to 10 dpc. Viraemia could be detected in mice from blood samples taken on 6 and 8 dpc (Table 4.3). Horse 19 displayed a slight febrile temperature of 39.3 ^{0}C on 6 and 8 dpc (Fig. 4.1), but no clinical signs or detectable viraemia (Table 4.3). Due to the absence of viraemia, this horse was considered completely protected against disease. Horses 20 and 22 developed no fever (Fig. 4.1), clinical AHS signs or detectable viraemia after challenge and were considered completely protected. Thus, both the horses (19 and 22) that were immunised thrice were completely protected against disease.

Most horses in Group 6 did not show any significant inflammatory response after immunisation with 0.6 mg Saponin Q, even after three immunisations. Some horses developed a very slight swelling at the injection site, which completely subsided after 48 h. The inflammatory response in the control did not differ from those observed in the immunised animals.

Thus, in this group of four horses that were challenged 68 or 96 days after the last booster was given, three (75 %) were completely protected against disease. Although horse 21 was not completely protected, the duration of disease and rectal temperatures of this horse, were not as long and high as for the control. Both horses (horse nos. 19 and 22) that were immunised thrice were completely protected whereas one (horse no 21) of the horses immunised twice was only partially protected. Immunisation with three doses of 0.6 mg Saponin Q and 10 µg rVP2 did not improve NAb titres significantly as compared to two doses.

4.3.2 *Challenge experiment 4 (Group 7): Determination of duration of protection observed in horses immunised twice with 0.6 mg Saponin Q plus 10 µg soluble baculovirus-expressed AHSV-5 VP2*

As was already explained, the initial plan was to immunise animals in this group twice with 0.6 mg Saponin Q and 10 µg rVP2 and to challenge eight months later with the same virus that was used for Group 6. However, the control horse in Group 6 did not die from the challenge virus strain, although the

challenge virus in previous experiments had the same titre (3.2.3) and was prepared from the same material. A new challenge virus was therefore prepared, as described previously (3.2.3), containing 10^7 $TCID_{50}$/ml AHSV-5. Horses in Group 7 were challenged with a 1.0 ml inoculum of this virus. A second control (C2) had to be included to test the lethality of the challenge strain. This horse did not receive any adjuvant prior to challenge.

As with Group 6, immunised animals only developed neutralising antibodies after the first booster on day 42. NAb values ranged between 40 and 160 (Table 4.2). Since these values had already dropped to below 20 on day 126 (84 days later), all the animals were boosted on day 196 with 0.1 mg Quil A plus 10 µg rVP2. It was decided to use Quil A, due to the results achieved in previous challenge experiments (Chapter 3) where Quil A at a 3.0 mg dose was shown to render complete protection against disease. An amount of 0.1 mg was chosen, since previous sensitivity studies (Chapter 3) indicated that 0.3 mg Quil A generated moderate swellings in horses (Table 3.5). Therefore, it was decided to use an even lower dose. A 1.0 mg/ml Quil A stock solution was prepared as described under section 3.2.2, diluted to 0.1mg/ml in water and filter sterilised again. Each horse was immunised with a 3.0 ml inoculum containing rVP2 in PBS (2.0 ml) emulsified with 0.1 mg Quil A (1.0 ml of the 0.1 mg/ml Quil A solution).

After this second booster, NAb titres ranged between 80 and 640 on day 210, which was on average higher than the values obtained with the Saponin Q. However these values dropped again to between 20 and 40 100 days later (day 292) (Table 4.2). Animals were challenged on day 434 (238 days after the last booster). On the challenge day, NAb titres had dropped to 20 for horses 23, 25 and 26 and less than 20 for horses 24, 27 and 28. The control horse (C2) did not show any detectable NAb titre prior to challenge. All the animals that survived the challenge displayed anamnestic responses (Table 4.2), although not as large as in Group 6.

The control horse (C2) developed fever from 4 to 7 dpc (Fig. 4.2). It displayed clinical AHS symptoms of supraorbital oedema, petechia on the eyelids and increased respiratory rate on 7 dpc and died during the night 7 dpc. Blood samples taken on 4 and 6 dpc tested positive for viraemia in mice (Table 4.4). The death of the control confirmed lethality of the challenge virus. Horse 26 developed fever (Fig. 4.2) and clinical AHS signs from 4 to 10 dpc and died during the night of 10 dpc. Blood taken from this horse on 4, 6 and 8 dpc showed viraemia in mice (Table 4.4). Horses 27 and 28 developed fever from 4.0 to 9.0 dpc and tested positive for viraemia in mice only on 8.0 dpc (Table 4.4). None of these two horses displayed any clinical AHS signs after challenge. Horse 23 also developed fever from 4.0 to 9.0 dpc (Fig. 4.2) and only blood taken on 6.0 dpc showed viraemia in mice (Table 4.4). This horse also did not show

clinical AHS signs after challenge. Horse 24 only had a slightly elevated temperature of 39.0 and 39.1 ^{0}C on 6.0 and 7.0 dpc (Fig. 2) and tested positive for viraemia on 6.0 dpc. This horse also did not develop clinical AHS signs. Horse 25 did not develop any fever (Fig. 4.2), viraemia or clinical signs during challenge and was considered completely protected.

Inflammatory responses after immunisation with 0.6 mg Saponin Q were the same as reported for Group 6. When the animals were injected on day 196 with 0.1 mg Quil A, all six displayed swellings 6.0 h later with an average diameter of 9.0 x 5.5 x 0.7 cm. After 24 h, the swellings had completely subsided in three of the six animals. The remaining animals still displayed swellings, but that had subsided 50%. After 48 h the swellings had completely subsided in all the animals.

Thus, in this group of six immunised horses that were challenged 252 days (8.4 months) after the last booster was given, one horse (horse 25) was completely protected against disease. Four horses (23, 24, 25 & 28) were partially protected by developing fever and a very short viraemia, that could only be detected for one day in mice, but no clinical AHS signs. Only one (horse 26) died after challenge.

Table 4.1 **Neutralising antibody titres of horses in Group 6 immunised twice or thrice with 0.6 mg Saponin Q plus 10 μg soluble baculovirus-expressed AHSV-5 VP2. The control horse was only injected with adjuvant. All animals were challenged 138 days after the first immunisation**

			DAYS *POST* FIRST IMMUNISATION AND CHALLENGE													
Horse	**Adjuvant**	VP2 (g)	d0 [1]**I**	d42 [2]**B**	d56	d70 [3]**B**	d84	d138 [4]**C** **0 dpc**	d152 **14 dpc**	d180 **28 dpc**	d208 **56 dpc**	d236 **84 dpc**	d264 **112 dpc**	d292 **140 dpc**	d320 **168 dpc**	d348 **196 dpc**
19 (F)	3 x 0.6 mg Sap Q	3 x 10	<20	<20	40	[3]40	160	40	>40960	>40960	2560	2560	2560	2560	2560	2560
20 (F)	2 x 0.6 mg Sap Q	2 x 10	<20	<20	320	160	160	160	>40960	>40960	5120	1280	1280	1280	1280	1280
21 (F)	2 x 0.6 mg Sap Q	2 x 10	<20	<20	80	80	80	20	>40960	>40960	40960	5120	2560	2560	2560	2560
22 (F)	3 x 0.6 mg Sap Q	3 x 10	<20	<20	40	[3]40	160	160	>40960	>40960	1280	640	640	640	640	640
C1 (M)	None	None	<20	<20	<20	<20	<20	<20	-	5120	5120	5120	5120	5120	5120	5120

C1: Control 1
[1]First immunisation
[2]First booster
[3]Second booster. Only horses 19 and 22 received two boosters
[4]Challenge

Table 4.2 **Neutralising antibody titres of horses in Group 7 immunised twice with 0.6 mg Saponin Q plus 10 μg soluble baculovirus-expressed AHSV-5 VP2 and once with 0.1 mg Quil A plus 10 μg soluble baculovirus expressed AHSV-5 VP2. The control horse was not immunised. Horses were challenged 434 days after the first immunisation**

	DAYS *POST* FIRST IMMUNISATION AND CHALLENGE																	
Horse	d0 [1]**I**	d42 [2]**B**	d56	d70	d98	d126	d196 [3]**B**	d210	d264	d292	d350	d434 [4]**C**	d448 **14 dpc**	d476 **56 dpc**	d504 **84 dpc**	d532 **112 dpc**	d560 **149 dpc**	d588 **210 dpc**	d616 **238 dpc**
23(F)	<20	<20	40	20	20	<20	<20	320	80	40	40	20	10240	5120	2560	2560	2560	1280	640
24 (F)	<20	<20	40	40	40	<20	<20	320	40	20	<20	<20	10240	10240	2560	2560	2560	1280	1280
25 (F)	<20	<20	40	40	40	<20	<20	160	20	20	20	20	10240	1280	640	640	640	320	320
26 (M)	<20	<20	160	160	80	<20	<20	640	40	40	40	20	-	-	-	-	-	-	-
27 (F)	<20	<20	80	40	<20	<20	<20	80	20	<20	<20	<20	20480	5120	5120	2560	2560	1280	1280
28 (F)	<20	<20	80	40	<20	<20	<20	160	20	20	<20	<20	40960	40960	10240	10240	10240	2560	2560
C2 (M)	-	-	-	-	-	-	-	-	-	-	-	<20	-	-	-	-	-	-	-

C2: Control 2
[1]First immunisation
[2]First booster
[3]Second booster
[4]Challenge

Table 4.3 **Viraemia analysis of horses in Group 6 immunised twice or thrice with 0.6 mg Saponin Q plus 10 μg soluble baculovirus-expressed AHSV-5 VP2. The control horse (C1) was only injected with adjuvant. All animals were challenged 138 days after the first immunisation**

			Viraemia detection in mice Days *post* infection					
Horse	**Adjuvant**	**rVP2**	**4**	**6**	**8**	**10**	**12**	**14**
19	3 x 0.6 mg Saponin Q	3 x 10 μg	[1]-	-	-	-	-	-
20	2 x 0.6 mg Saponin Q	2 x 10 μg	-	-	-	-	-	-
21	2 x 0.6 mg Saponin Q	2 x 10 μg	-	[2]+	+	-	-	-
22	3 x 0.6 mg Saponin Q	3 x 10 μg	-	-	-	-	-	-
C1	2 x 0.6 mg Saponin Q	None	-	+	+	+	-	-

[1] indicates that no signs of viraemia could be detected in two-day-old suckling mice injected with horse blood from horses challenged with AHSV-5.
[2] indicates that two-day-old suckling mice injected with blood from horses challenged with AHSV-5 displayed clinical neurological signs of AHSV.

Table 4.4 **Viraemia analysis of horses in Group 7 immunised twice with 0.6 mg Saponin Q plus 10 g solublebaculovirus-expressed AHSV-5 VP2 and once with 0.1 mg Quil A plus 10 μg soluble baculovirus-expressed AHSV-5 VP2. The control horse (C2) did not receive any adjuvant or antigen prior to challenge. Horses were challenged 434 days after the first immunisation**

			Viraemia detection in mice Days *post* infection						Death
Horse	**Adjuvant**	**rVP2**	**4**	**6**	**8**	**10**	**12**	**14**	
23	2 x 0.6 mg Saponin Q 1 x 0.1 mg Quil A	3 x 10 μg	[1]-	[2]+	-	-	-	-	No
24			-	+	-	-	-	-	No
25			-	-	-	-	-	-	No
26			+	+	+	-			Yes
27			-	-	+	-	-	-	No
28			-	-	+	-	-	-	No
C2	None	None	+	+					Yes

[1] indicates that no signs of viraemia could be detected in two-day-old suckling mice injected with horse blood from horses challenged with AHSV-5.
[2] indicates that two-day-old suckling mice injected with blood from horses challenged with AHSV-5 displayed clinical neurological signs of AHSV.

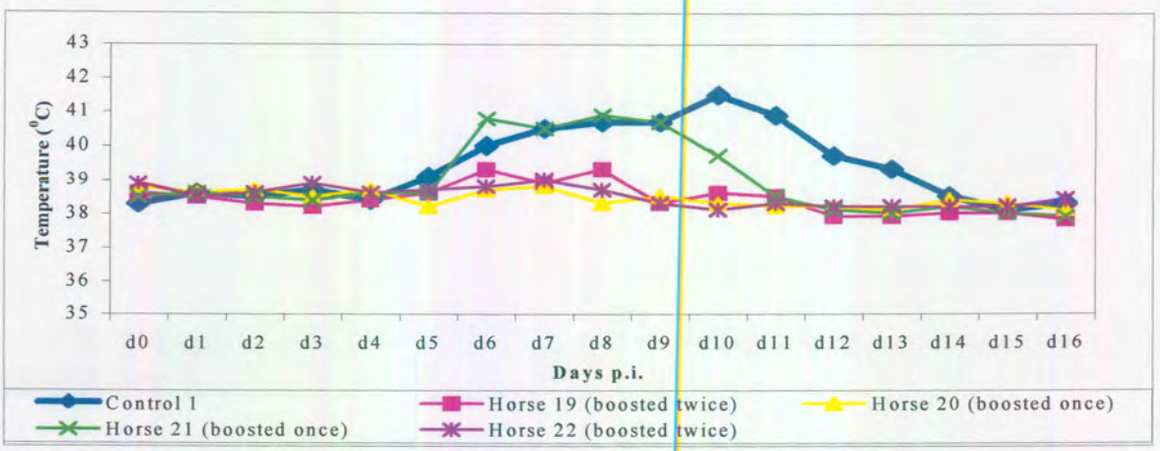

Fig. 4.1 Temperature profiles of horses in Group 6 after challenge. Horses 20 and 21 were immunised twice with 0.6 mg Saponin Q plus 10 μg soluble baculovirus-expressed AHSV-5 VP2. Horses 19 and 22 received three doses of the same vaccine. The control was injected with only adjuvant. Animals were challenged subcutaneously on the left side of the neck on day 138. Each horse received a 2.0 ml inoculum of homogenised spleen supernatant containing 10^6 $TCID_{50}$/ml AHSV-5.

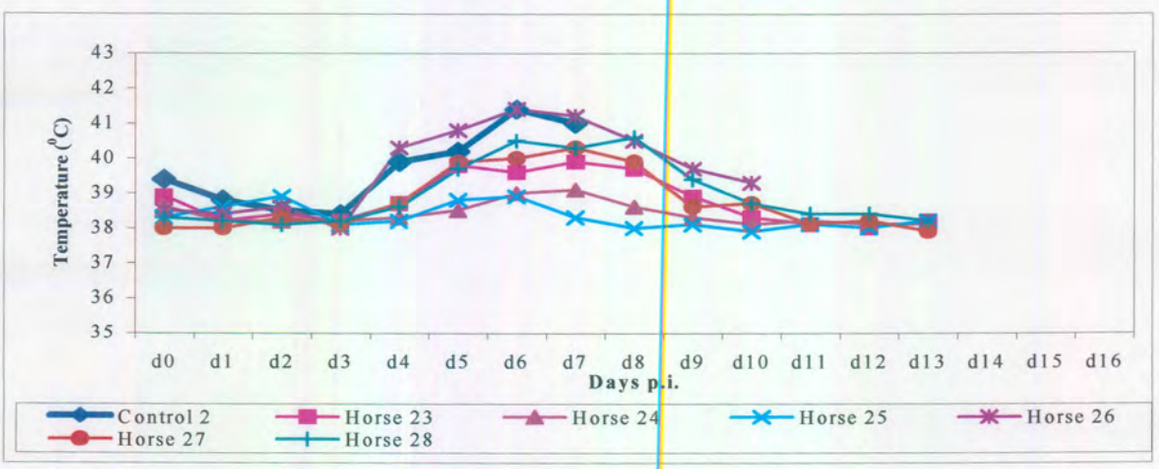

Fig. 4.2 Temperature profiles of horses in Group 7 after challenge. Immunised animals received two doses of 0.6 mg Saponin Q plus 10 μg soluble baculovirus-expressed AHSV-5 VP2 and one dose of 0.1 mg Quil A plus the same amount rVP2. The control did not receive any antigen or adjuvant prior to challenge. Animals were challenged subcutaneously on the left side of the neck on day 434 (8.4 months after the booster). Each horse received a 1.0 ml inoculum of homogenised spleen supernatant containing 10^7 $TCID_{50}$/ml AHSV-5.

4.4 DISCUSSION

The first aim of this investigation was to determine whether a concentration of 0.6 mg Saponin Q is sufficient to protect against challenge two to three months after the last booster was given. This dose of adjuvant was previously shown to initiate only a slight hypersensitivity response in horses (Chapter 3) when administered with 10 µg rVP2. This was the first time horses were only injected with this adjuvant at the given dose. In previous trials, horses were either injected twice with 3.0 mg Quil A or first injected with 3.0 mg Quil A, followed with a second injection of 0.6 mg Saponin Q as adjuvant. Immunisation at these doses was found to render complete protection against disease 84 days after the booster was given (Chapter 3).

It was previously demonstrated that initial immunisation with 3.0 mg Quil A renders NAb titres 28 days later (Chapter 3). However, in this experiment it was found that initial immunisation with 0.6 mg Saponin Q did not induce any detectable NAbs. Booster immunisation with the same adjuvant elicited NAbs, albeit lower than what was observed with the Quil A/ Saponin Q experiment (Chapter 3). Two of the four horses that were used for the first experiment still had low NAb titres of 40 one month after the first booster was given. These two horses then received a second booster with the same amount of adjuvant and antigen. After challenge, both the horses that were immunised thrice were completely protected and one of the horses that were immunised twice became ill. However, the duration of disease in this horse (horse 21) was not as long as for the control. The fact that the control did not die complicated interpretation of the results. However, this was the first challenge experiment where the control was injected with the tested adjuvant prior to challenge. In previous experiments, the control did not receive any adjuvant prior to challenge. It could thus be that this horse's immune system had been primed prior to challenge, but he could also have been one of the "lucky" 5-10 % susceptible horses that survive AHSV challenge.

The second aim was to determine whether horses would still be protected when immunised with above vaccine and challenged 310 days later than in the first experiment. This was the first time where the duration of protection achieved by a recombinant VP2-based vaccine for AHSV was determined. Due to low NAb titres observed 154 days after the first booster, a second booster immunisation was done with 0.1 mg Quil A and 10 µg rVP2. Five of the six immunised horses were protected against death and clinical signs 238 days (eight months) after the second booster was given. These results clearly demonstrate the potential of a recombinant AHSV vaccine to provide long-term protection. As with the first experiment, it was found that two immunisations with 0.6 mg Saponin Q could not increase and

maintain the NAb titres as efficiently as when 3.0 mg Quil A was used as single adjuvant or followed by a booster of 0.6 mg Saponin Q (Chapter 3). Even a booster injection of 0.1 mg Quil A did not maintain NAb titres. This was demonstrated by the fact that the NAb titres had already dropped significantly on day 264, 68 days after the booster with Quil A. Horse 26 displayed the highest NAb titres throughout the period prior to challenge, but still was not protected against death. Horse 25, whose NAb titre prior to challenge did not differ much from the other horses in Group 7, was completely protected against death and disease. These results confirm previous results from this study (Chapter 3) and published results which showed that other immune mechanisms than the humoral response (e.g. cell-mediated immunity) might also be involved in protection against AHSV in horses (Martínez-Torrecuadrada *et al.*, 1996, Stone-Marchat *et al.*, 1996).

The obtained results confirm and extend previous findings (Chapter 3) regarding the protective potential of a VP2-based recombinant AHSV vaccine. However, the formulation of the adjuvant and the antigen (10 μg rVP2), will have to be adapted to provide complete protection against disease in all immunised animals after two immunisations within one season. This is particularly of importance to foals that are at highest risk of contracting the disease during peak season.

It seems that Quil A as adjuvant was even at a concentration of 0.1 mg too toxic for the horses, since they still developed large sensitive swellings at the injection sites. The use of this adjuvant alone in a recombinant AHSV vaccine is thus not recommended. It could, however, be used in an ISCOM (immunostimulating complex) formulation. Since it is known that the adjuvant action of saponin is dependent on its capability to bind to membrane cholesterol, the inflammatory response can be markedly reduced by addition of cholesterol-containing liposomes. Adjuvant activity is not modified with this treatment (Scott *et al*, 1985). This principle has been widely used in the manufacturing of ISCOMS (Morein *et al*, 1984). The terms ISCOM and ISCOMS are commercial trademarks of Isotec AB, Sweden. Two types of ISCOMS have been described. The classic form contains cholesterol, saponin, phospholipid and viral envelope proteins and the basic form, which does not contain viral proteins. The basis of the unique matrix structure of an ISCOM, is the interaction between saponin (usually Quil A) and cholesterol (Barr and Mitchell, 1996). Good antibody responses and protection against equine influenza by making use of ISCOMS, have been reported. Horses showed no significant local reactions even after three immunisations and their performance was not adversely influenced (Sundquist, Lövgren and Morein, 1988; Mumford *et al,* 1994).

To provide effective adjuvant activity with a saponin-based adjuvant in a VP2-based recombinant AHSV vaccine, a balance will have to be found between inflammatory response and protection. Previously (Chapter 3), it was found that when 3.0 mg Quil A is used to immunise twice, or once followed by a 0.6 mg Saponin Q booster, 10 or 50 µg rVP2 did not influence protection determined over a three month period. However, when 10 µg rVP2 was used with only 0.6 mg Saponin Q as adjuvant, one of four horses was not completely protected after three months. In previous hypersensitivity studies (Chapter 3, Table 3.5), it was shown that Saponin Q, even at a concentration of 3.0 mg, did not elicit such severe inflammatory responses as Quil A. Another approach that is therefore recommended, is that a candidate vaccine with an initial dose of 50 g rVP2 and 3.0 mg Saponin Q, followed with a booster of 50 g rV P2 and 0.6 mg Saponin Q should be prepared for field trials in larger groups of horses. The larger dose of VP2 is recommended, since the 10 g dose does not seem to protect completely when Saponin Q is used in all immunisations. The use of Saponin Q in a recombinant subunit AHSV vaccine is still preferred above the use of ISCOMS due to much lower costs involved in the preparation of this adjuvant. To lower production costs further, techniques to improve the solubility of the rVP2 produced by baculovirus expression should also be investigated.

CHAPTER 5

Concluding discussion

The current South African live AHSV attenuated vaccine has become inadequate, due to the absence of serotypes 5 and 9 and the consequence of vaccination that makes it impossible to distinguish between seropositive, vaccinated and naturally infected horses. This necessitated the development of recombinant subunit vaccines. Work done prior to the commencement of this study established proof of concept for the development of baculovirus-expressed AHSV VP2-based subunit vaccines. However, only partial protection was obtained with the first batches of AHSV-5 rVP2. The main goal of this study was to determine the cause of variation, establish whether conditions exist under which AHSV-5 rVP2 is fully protective and, if so, determine whether baculovirus expression of rVP2s would be a practical option for recombinant subunit vaccine production.

The two most important findings of this study are firstly that only the soluble form of rVP2 is biologically active to induce NAbs and provide complete protection against a lethal challenge of AHSV. Thus, by using the soluble form of AHSV-5 rVP2, it was shown that AHSV-5 rVP2 alone, without the inclusion of other AHSV structural proteins e.g. VP3, VP5 and VP7, can induce complete protection against lethal challenge. Secondly it was shown that the type of adjuvant determines the efficacy and safety of soluble rVP2. Soluble rVP2 can only induce complete protection if it is administered with specific adjuvants. Saponin adjuvants are effective, but induce dose-related side effects. The results reported here for the first time indicated that AHSV VP2 induces protective cell-mediated immunity in addition to humoral immunity. This conclusion was made from the fact that saponin, which rendered the best protection with rVP2, induces a Th1-type CMI. Aluminium phosphate, which is known to induce an allergic Th2 response, only rendered partial protection. Our results, therefore, argue against a Th2 response as the major protective response to AHSV.

Although saponin adjuvants are widely used in veterinary vaccines, one of the major disadvantages is batch variation that may cause differences in adjuvanticity and skin reactions (Kensil, 1996). However, Harrison *et al.*, (1999) found that the use of dialysed saponin reduced the number of site reactions, without losing adjuvanticity, when more than 20 mg saponin was used with a recombinant *Taenia ovis* vaccine in sheep. Dialysis allowed the increase of saponin to 30 mg doses. According to Dalsgaard (1978), dialysis removes lower molecular weight irritant material. Dialysis of Saponin Q for use with a recombinant AHSV vaccine could thus be considered to limit skin reactions even more.

Preliminary studies (see Appendix) indicated that the solubility of the baculovirus-expressed AHSV-5 rVP2 is greatly influenced by the combination of MOI/TOI/TOH at infection, the insect cell line and the incubation medium. Sf9 cells used with Grace's insect medium rendered the best rVP2 yield. The yield of soluble rVP2 was increased from 10 % to 30 % when exponentially growing Sf9 cells were infected at MOI 2.0, TOI 2.5 x 10^6 cells/ml, TOH 48 h and collected at a final cell density of 2.0 x 10^7 cells/ml. Quantification showed that infection of Sf9 cells in shaker cultures at MOI 2.0, TOI 2.0 x 10^6 cells/ml and TOH 48 h yielded approximately 0.25 mg soluble rVP2 per 25 ml infected cells, counting 2.5 x 10^6 cells/ml at TOH and lysed at 2.0 x 10^7 cells/ml. This corresponds to 1900 50 µg rVP2 doses per 5.0 L medium (1.25 x 10^{10} infected cells) when the Sf9 cells are maintained by shaker incubation. However, it is generally known that cell densities between 10^7 to 10^8 cells/ml can be maintained by large scale insect cell culture. In the worst case scenario of 10^7 cells/ml, 7600 doses of 50 µg rVP2 can be obtained from one 5.0 L fermenter run under optimised conditions. However, if incubation conditions can be optimised to reach cell densities of 10^8 cells/ml, 76 000 doses of 50 µg rVP2 can be produced from one 5.0 L fermentation run. Since there are about 200 000 horses in SA, this means that under optimised conditions six 5.0 L fermenter runs should deliver enough 50 µg rVP2 doses per horse for one season in SA. Preliminary results indicate that even higher soluble rVP2 yields can be obtained if infection is done at higher MOIs. Therefore, infections at MOIs 10, 15 and 20 need more investigation. Thus, the production of an economically viable recombinant subunit AHSV vaccine appears to be feasible. Methods to conserve the final vaccine (e.g. freeze drying compared to storage at 4 ^{0}C or –20 ^{0}C) and the influence thereof on AHSV-5 rVP2 solubility should now be investigated in order to define the final product.

Previously different serum-free media namely Sf 900II™ (Invitrogen Corporation), Ex-Cell™ 401 (JRH Biosciences, Lenexa, KS) and HyQ® SFX-Insect medium (HyClone® Laboratories, Inc.) were tested for AHSV-5 rVP2 production in Sf9 cells. No rVP2 expression could be shown in these media (results not shown). It is, therefore, recommended that Grace's insect medium should be used for large-scale production. One of the major disadvantages of this medium for upscaling is, however, that it is supplemented with 10 % FCS. The problem with using FCS is that it causes undesired foaming in fermenters. In addition, FCS suffers from variability between batches and the risk of contamination with pathogens such as prions. However, since it is commonly known that Sf9 cells can grow at lower than 10 % foetal calf serum (FCS) concentrations, rVP2 production in cells adapted to lower serum concentrations may be further investigated. The passage number of the insect cells from initiation number is also an important factor (Maruniak, 1996). This study indicated, that under laboratory conditions cells younger than 15 passages after initiation generally delivered higher rVP2 yields (results not shown).

The recent outbreak and quick spread of bluetongue virus (BTV) in the Mediterranean (Baylis, 2002; Mellor and Wittmann, 2002) emphasised the need for a recombinant subunit vaccine for this disease as well. Although 24 serotypes of BTV are known (Roy, 2001), outbreaks are usually caused by only a few serotypes. The immediate availability of a subunit vaccine for the serotype(s) causing the outbreak, is thus of the utmost importance to hinder further spread. Although the effectiveness of BTV VLPs has been demonstrated in sheep (Roy, French and Erasmus, 1992), the use of a recombinant vaccine consisting of the VP2 proteins of only the implied serotypes should also be considered, since the cloning and expression of only one protein is much more simple than the expression of VLPs. The availability of a cost-effective recombinant subunit vaccine for BTV is of the utmost importance due to the need for large numbers of animals to be immunised. Our results suggest that when rVP2 is injected with the right adjuvant, cost-effective production can be achieved. The use of saponin in a VP2-based recombinant BTV vaccine has never been tested and should also be considered. It is generally known that sheep and cattle can tolerate higher doses of adjuvant than horses. Studies show that sheep and cattle can tolerate up to 1.0 mg Quil-A in different vaccines (Kensil, 1996). Harrison *et al.* (1999), as mentioned above, found that when an unpurified mixture of saponin was used in sheep, larger doses could be tolerated.

During the course of this study, the full set of cloned and sequenced AHSV VP2 genes was cloned from reference AHSV strains (Potgieter *et al.*, 2003). This achievement makes the development of a VP2-based AHSV subunit vaccine, protecting against all nine AHSV serotypes, imminent. The knowledge gained with the study reported here, should now be implemented and tested on the other eight AHSV rVP2s. These proteins have already been expressed in the BEVS and currently soluble rVP2s are being tested in guinea pigs for immunogenicity. Once immunogenicity of each of the newly expressed AHSV rVP2s has been confirmed, combination studies of the different AHSV serotypes should be started in guinea pigs to determine the optimum combination/s to be administered to horses. Potgieter *et al.* (2003) showed that the amino acid sequences of AHSV serotypes that cross-neutralise (serotypes 1 & 2, 3 & 7, 5 & 8 and 6 & 9) share higher identity with each other and group together. Therefore, cross-protection between these serotypes with VP2-based vaccines should be further investigated to minimise the number of different rVP2s included in the final vaccine. This will lead to a reduction of production costs. Final formulation of such vaccines with Saponin Q should be tested in horses by challenge. The effect of the combination of different rVP2s on the solubility and biological activity of these proteins also needs investigation and optimisation.

The availability of the full set of cloned and sequenced AHSV VP2 genes provides molecular tools to attempt to improve rVP2 solubility further. Sequence analysis of these genes revealed that the antigenic

regions are mainly located in the 220-450 amino acid region of the AHSV VP2 proteins (Potgieter *et al.*, 2003). This is in accordance with the findings of Bentley *et al.* (2000) for AHSV-3 (amino acids 324-543); Venter, Napier and Huismans (2000) for AHSV-9 (amino acids 252-486) and Martínez-Torrecuadrada *et al.* (2001) for AHSV-4 (amino acids 321-339 and 377-400). The study of immunogenicity and production yield of truncated baculovirus-expressed VP2 proteins consisting of mainly the identified common antigenic regions may also be rewarding.

It has been shown for AHSV (Wade-Evans *et al.*, 1998) and BTV (Wade-Evans *et al.*, 1996) that VP7 protects against a heterologous serotype virus challenge possibly via a cell-mediated mechanism. The inclusion of purified VP7 protein from one AHSV serotype in a VP2-based subunit vaccine can therefore be considered for enhancement of the immune response induced by such vaccines.

Thus, to summarise: the knowledge generated during this study, combined with the recent cloning of the VP2 genes of all nine AHSV serotypes, provides a route for the development of a complete recombinant vaccine that will offer protection against all nine AHSV serotypes and could well free the restraint on the import and export of horses to and from South Africa.

PUBLICATIONS AND PRESENTATIONS

Parts of the results presented in this thesis have been published:

1. Du Plessis, M., Cloete, M. Aitchison, H. & Van Dijk, A.A. 1998. Protein aggregation complicates the development of baculovirus-expressed African horsesickness virus serotype 5 subunit vaccines. *Onderstepoort Journal of Veterinary Research*, 65: 321-329.

2. Scanlen, M., Paweska, J.T., Verschoor, J.A. & Van Dijk, A.A. 2002. The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine is determined by adjuvant. *Vaccine*, 20: 1079-1088.

Parts of the results presented in this thesis have been presented at scientific meetings:

1. Van Dijk, A.A., Vreede, F.T., Du Plessis, M., Huismans, H., Filter, R. & Aitchison, H. The development of recombinant subunit vaccines for African horsesickness. Biotech SA 97, Grahamstown, 1997.

2. Van Dijk, A.A., Du Plessis, M., Koekemoer, J.J.O., Gehringer, M.M., Louw, I. & Kruger, H. Characteristics of African horsesickness virus outer capsid protein genes and expressed proteins that impact on the development of subunit vaccines and new diagnostic reagents for serotyping. The 6th International Symposium on Double-stranded RNA Viruses, Mexico, 1997.

3. Van Dijk, A.A., du Plessis, M. Cloete, M., Aitchison, H., Venter, D. and Louw, I. Characterisation of outer capsid baculovirus expressed African horsesickness virus VP2 proteins as subunit vaccines. Eighth International Conference on Equine Infectious Diseases, Dubai, UAE, 1998.

4. Van Dijk, A.A., Du Plessis, M., Maree, S., Koekemoer, J.J.O., Potgieter, A.C. & Cloete, M. Importance of protein folding in African horsesickness virus VP2-based subunit vaccine development and demonstration of feasibility of serotype-specific probes. Virology, 98, Cape Town, 1998.

5. Du Plessis, M. and A.A. Van Dijk. Importance of protein folding in African horsesickness virus VP2-based subunit vaccine development. Biochemistry in Africa, Potchefstroom, 1998.

6. Scanlen, M., Paweska, J.T. & Van Dijk, A.A. Protein aggregation complicates the development of a baculovirus-expressed VP2-based recombinant subunit vaccine for African horsesickness. The 16th European Society for Animal Cell Technology Meeting, Switzerland, 1999.

7. Scanlen, M., Paweska, J.T & Van Dijk, A.A. Protein aggregation complicates the development of a baculovirus-expressed VP2-based recombinant subunit vaccine for African horsesickness. The 9th International Symposium of the World Association of Veterinary Diagnosticians, USA, 1999.

8. Van Dijk, A.A., Scanlen, M., Paweska, J.T., Koekemoer, J.J.O. & Potgieter, A.C. African horsesickness virus: Crucial role of protein folding and adjuvant for eliciting protective immunity with recombinant VP2 in horses- and development of serotype-specific probes. The 11th International Congress of Virology, Australia, 1999.

9. Scanlen, M., Paweska, J.T. & Van Dijk, A.A. The importance of vaccine formulation for protection against African horsesickness with baculovirus-expressed VP2. 30th Annual Conference of the Australasian Society of Immunology, Sydney, Australia, 2000.

10. Potgieter, A.C., Scanlen, M., Paweska, J.T. & Van Dijk, A.A. The importance of vaccine formulation for protection of against African horsesickness with baculovirus-expressed VP2. 7th International Symposium on Double-stranded RNA-viruses, Aruba, 2000.

11. Van Dijk, A.A., Potgieter, A.C., Koekemoer, J.J.O., Scanlen, M., Paweska, J.T., Maree, S. Cloning of complete genome sets of six segmented dsRNA viruses (human rotavirus, reovirus, AHSV, BTV, EEV, bacteriophage phi 12) and applications in research, recombinant vaccine development and new diagnostics. 1st Australian Virology Group Meeting, Queensland, Australia, 2001.

12. Scanlen, M., Paweska, J.T. & Van Dijk, A.A. The importance of adjuvant in the formulation of an effective recombinant subunit vaccine against African horsesickness. 12th Biennial SASM Congress, Bloemfontein, 2002.

APPENDIX

Pilot scale-up experiments to optimise the yield of soluble baculovirus-expressed AHSV-5 VP2

Commercialisation of the candidate recombinant subunit AHSV-5 vaccine, described in Chapters 2 to 4 is hampered by the finding that only 10 % of the baculovirus-expressed AHSV-5 VP2 is produced in a soluble form that is biologically active. Therefore, the production of a cost effective commercial VP2-based recombinant subunit AHSV vaccine, will benefit by finding ways to increase the yield of soluble rVP2 in the BEVS.

The influence of MOI, TOI, TOH and host insect cell line on the production of soluble AHSV-5 rVP2 has never been investigated before. Thus, the first goal of the study reported here, was to determine the effectiveness of another commonly used cell line, High Five, as a host for baculovirus-expressed AHSV-5 VP2 production. This cell line is known for the production of large quantities recombinant protein (Schlaeger *et al.*, 1995) whereas Sf9 is commonly used for the production of high titre baculovirus stocks. The second goal was to attempt to increase the soluble rVP2 yield by establishing optimum production procedures on laboratory scale. To achieve this, different MOI/TOI/TOH combinations were investigated.

A.1 INFECTION OF HIGH FIVE CELLS WITH rBAC-5

High five insect cells (BTI-Tn-5BI-4) (Invitrogen, Leek, The Netherlands) were maintained in shaker cultures without serum in Excell 405 medium (JRH Biosciences, Lenexa, KS) supplemented with antibiotics as described in section 2.2.1. Infections at different TOIs and MOIs as well as collection of infected cells at different TOHs were done as described in A.2 for Sf9 cells.

A.2 DIFFERENT INFECTION PROCEDURES OF SF9 CELLS WITH rBAC-5

Sf9 cells were grown to a density of 1.0 x 10^6 to 2.5 x 10^6 cells/ml in shaker culture as described in section 2.2.1. The cells were then centrifuged and resuspended in different batches of five ml Grace's without FCS in 100 ml Erlenmeyer flasks to give the required TOIs in a 25 ml volume after infection with

P3 stocks (section 2.2.3) at the required MOI. Infections were done by gentle shaking at room temperature (± 25 °C) for two h whereafter complete medium was added to a final volume of 25 ml. Cells were harvested at different TOHs by collecting 1.5 ml of each culture. One ml of each collection was put on ice for at least one h and the remaining 0.5 ml used to determine cell viability microscopically by Trypan blue exclusion. After one h, the cooled cells were centrifuged at 1000 x g for five min and washed in one ml ice cold PBS. The cells were again centrifuged and washed in 500 µl PBS. After final centrifugation, the cells were resuspended in PBS to yield a final concentration of 2.0 x 10^7 cells/ml. Soluble and aggregated rVP2 were prepared as described in section 2.2.5. Quantity estimations were done as described in section 2.2.6 by using 12 % polyacrylamide gels that were run overnight at 25 mA in a 18 x 16 cm Hoefer® SE 600 (S.A. Scientific Products) electrophoresis unit.

A.3 RESULTS AND DISCUSSION

A.3.1 Determination of the yield of soluble AHSV-5 rVP2 obtained by using High Five cells as the host for rBac-5

To establish whether expression of biological active AHSV-5 VP2 can be improved by using High Five insect cells, two MOI/TOI combinations (Table A.1) were chosen for initial experiments. These combinations were chosen to represent a relatively high as well as a low MOI. Data was analysed on a 12 % polyacrylamide SDS-PAGE gel run in a 7.0 x 10 cm Hoefer® Mighty Small™ (SA Scientific Products) electrophoresis unit.

Table A.1 TOI/MOI/TOH combinations investigated in High Five insect cells to determine soluble AHSV-5 rVP2 production by rBac-5

MOI	TOI	TOH (hours p.i.)
0.02	0.5 x 10^6	24, 48, 72, 96
2.0	1.0 x 10^6	24, 48, 72, 96

No expression of rVP2 was observed at TOH 24 h. Expression was observed at TOH 48 h when the cells were infected at TOI 1.0 x 10^6 cells/ml and MOI 2.0. However, two bands were observed at the expected MW position for rVP2 and most was insoluble (Fig. A.1, lane 5). At TOH 72 and 96 h, expression of

rVP2 could not be demonstrated (not shown). This finding indicates that the High Five insect cell line is not suitable for rVP2 production in the BEVS.

Another insect host cell line that can be used for recombinant protein production with the BEVS is Sf21. We have however, tested this cell line previously (results not shown) and found that the yield of rVP2 is exactly the same than in Sf9 cells. It is, therefore, recommended that Sf9 cells should be used for large scale production of the AHSV-5 VP2.

A.3.2 Determination of the effect of different infection procedures on the yield of soluble AHSV-5 rVP2 produced in Sf9 cells by the baculovirus expression system

Sf9 insect cells were infected with P3 rBac-5 stocks at the MOIs and TOIs indicated (Table A.2). A control infection was included where the Sf9 cells were infected at MOI 2.0 and TOI 2.0 x 10^6 cells/ml as described before in Chapters 2 to 4. After infection the cells were harvested at the indicated TOHs (Table A.2) and resuspended at a final density of 2.0 x 10^7 cells/ml as previously. Quantity estimations with the larger SDS-PAGE gels (18 x 16 cm) were found to be more accurate than the minigel system used in Chapters 2 to 4.

Table A.2 TOI/MOI/TOH combinations investigated in Sf9 insect cells to determine soluble AHSV-5 rVP2 production by rBac-5

MOI	TOI (x 10^6 cells/ml)	TOH (hours p.i.)
0.001	0.2 0.3 0.5	24, 48, 72, 96
0.02	0.2 0.5 1.0	24, 48, 72, 96
0.1	0.5 1.0 2.0	24, 48, 72, 96
2.0	1.0 2.0 2.5	24 & 48

The yield of rVP2 at 24 h p.i. was very low for all the MOIs tested. This was expected since the *polyhedron* promoter, under which the VP2 gene is cloned, is only activated from 18 h p.i. until 72 h p.i. or until the cells lyse (Murphy *et al.*, 1997). Aggregation of rVP2 increased after cell viability dropped below 80 % , typically at about 72 hp.i. for the MOIs smaller than 2.0 (0.001 – 0.1). For MOI 2.0 the 80

% viability point was reached at 48 h p.i. The maximum cell density that could be maintained by shaker incubation in Grace's insect medium was 2.5 x 10^6 cells/ml.

A collection of some of the rVP2 yields 48 h after infections with the indicated MOI/TOI combinations is shown in Fig. A.2. When the Sf9 cells were infected with the lower MOIs (0.001 – 0.1), the production levels of aggregated and soluble rVP2 were very low (Fig. A.2, lanes 3 – 6) compared to the control (Fig. A.2, lane 7: aggregated; lane 8: soluble), where the cells were infected with MOI 2.0, TOI 2.0 x 10^6 cells/ml and TOH 48 h. However, the percentage of soluble rVP2 seemed to increase to 30 % for the control (Fig A.2 lanes 7 and 8) and not 10 % as before (Chapter 2). This could be due to the fact that the cells were resuspended at a cell density of 2.0 x 10^7 cells/ml and not 1.0 x 10^8 cells/ml as previously. It could thus be that rVP2 tends to aggregate more easily beyond a certain saturation point after cell lysis. This needs further investigation to optimise upscaling.

Thus, to summarise: we have found that soluble rVP2 production in the BEVS is influenced by infection procedures, media and host cell line. The best soluble rVP2 production seemed to be obtained at MOI 2.0, TOI 2.0 x 10^6 cells/ml and TOH 48 h in Sf9 host cells, incubated in Grace's insect cell medium supplemented with 10 % FCS. It is clear from these findings that the cost-effective large-scale production of a baculovirus-expressed VP2-based AHSV-5 vaccine should be practical.

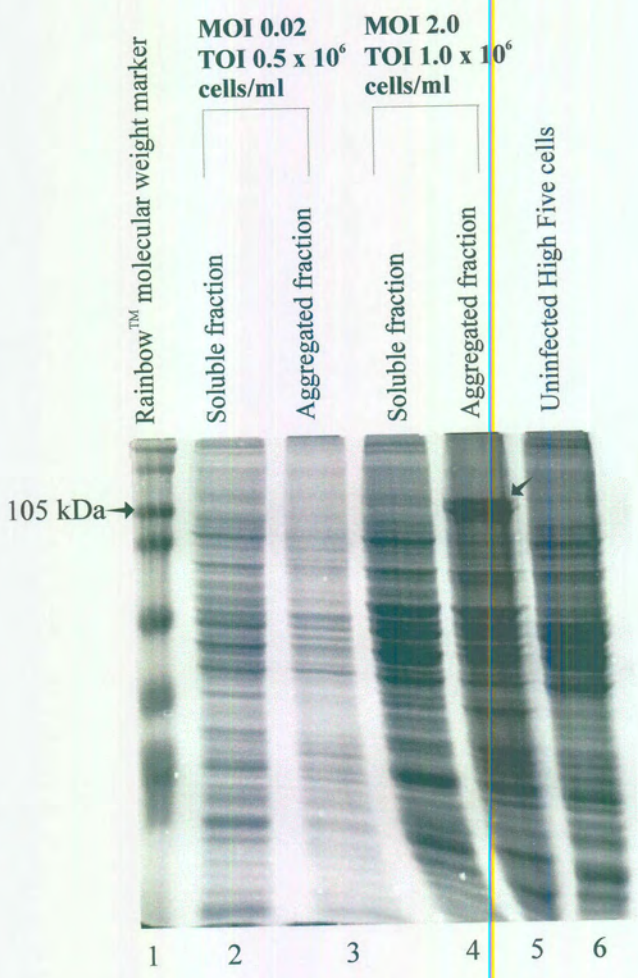

Fig. A.1 Coomassie® brilliant blue stained 12 % SDS-PAGE analysis of crude High Five cell lysates 48 h after infection with rBac-5 at indicated TOIs and MOIs.

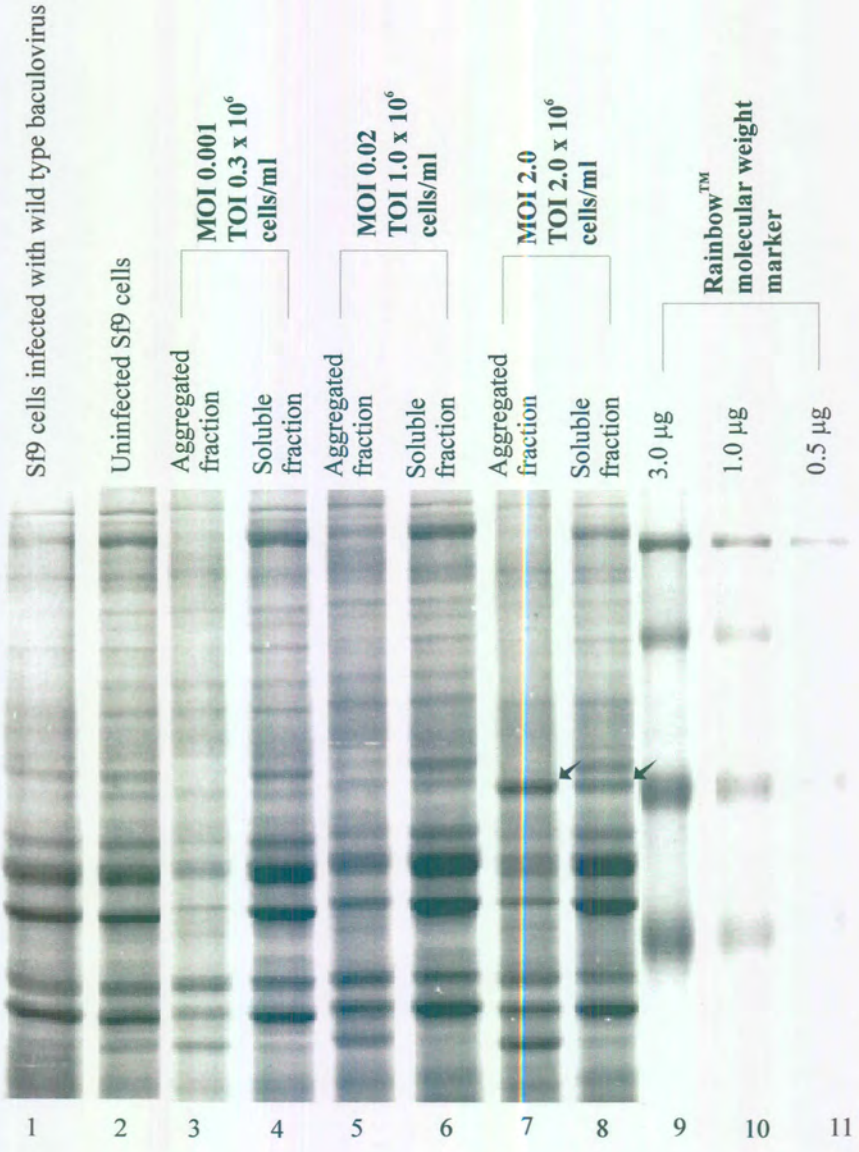

Fig. A.2 Coomassie® brilliant blue stained 12 % SDS-PAGE analysis of crude Sf9 cell lysates 48 h after infection with rBac-5 at indicated MOIs. The positions of the aggregated and soluble fractions of rVP2 obtained after infection at MOI 2.0 and TOI 2.0 x 10^6 cells/ml are indicated by arrows.

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