CHAPTER 4

CONCLUDING REMARKS

A common feature of the smallest dsRNA genome segment of all known orbiviruses is that they encode two related proteins, NS3 and NS3A, from two in-frame overlapping ORFs. Why this feature has been conserved is not yet clear and one of the objectives of this investigation was to analyse and compare the NS3 and NS3A proteins of AHSV. This study forms part of a larger project to understand the role of NS3/A in the life cycle, virulence and pathogenesis of AHSV.

The AHSV segment 10 products may be related to a family of viral proteins that cause membrane permeabilisation, termed viroporins. This is based on the fact that NS3 and NS3A cause membrane damage and eventual cell death when expressed as baculovirus recombinants in insect cells (Van Staden *et al.,* 1995; Van Niekerk *et al.,* 2001a). The NS3 and NS3A proteins also display many of the characteristic structural features common to viroporins. The cytotoxicity of these proteins has been shown to be dependant on their membrane association (Van Niekerk *et al.,* 2001a), but little else is known about the cytotoxic properties of NS3 and NS3A. Several aspects of the functioning of these proteins, therefore, remain to be investigated including whether the proteins cause membrane permeabilisation in mammalian cells and if they are able to exert their effect extracellularly. The effect of NS3 on mammalian cells following extracellular addition of the protein was investigated here.

As the NS3 protein has not yet been purified in a biologically active form, it was decided to use crude extracts from Bac-NS3-infected *Sf9* cells for initial experiments in determining the effect of exogenous NS3 on the membranes of Vero cells. Extracellular NS3 was found to cause an increase in the uptake of the membrane impermeable translation inhibitor, Hygromycin B in Vero cells. Thus the cytotoxic properties of NS3 appear not to be limited to causing membrane damage in insect cells alone. This property of NS3 needs to be investigated as this furthers the idea that NS3 is a viroporin and is involved in the pathogenesis of AHSV. Future

research could investigate the molecular mechanism of membrane damage caused by NS3 and also determine the correlation, if any, between the virulence of a strain of AHSV and the membrane damaging properties of the NS3 protein of that strain. The extracellular cytotoxic effect of NS3 should, furthermore, be compared to that of NS3A.

Three possible molecular mechanisms of toxin-membrane interaction and subsequent increased membrane permeability can be postulated. These are: receptor-mediated, transmembrane ionic channel and/or pore formation and nonspecific membrane pertubation.

The enterotoxic behaviour of rotavirus NSP4, for example, is mediated by its interaction with a putative plasma membrane receptor triggering a phospholipase C-mediated increase in intracellular Ca^{2+} (Dong *et al.,* 1997; Morris *et al.,* 1999). HIV-1 gp120 increases the permeability of rat brain endothelium by a receptor-mediated mechanism involving substance P (Annunziata *et al.,* 1998).

HIV-1 Vpr added extracellularly to intact cells forms ion channels that cause a large inward cation current, depolarization of the plasmalemma and eventual cell death (Piller *et al.,* 1998). This extracellular toxic effect of Vpr is caused by a region of the protein containing the sequence HFRIGCRHSRIG (Macreadie *et al.,* 1996). An amphipathic region (designated lentivirus lytic peptide, LLP-1) of HIV-1 transmembrane protein was also found to be toxic to both prokaryotic and eukaryotic cells when added exogenously. The LLP-1 peptide causes membrane pertubation by forming pores of defined size in cytoplasmic membranes (Miller *et al.*1993). Other examples of pore forming cytolytic proteins include M2 of influenza A, NB of influenza B, Vpu of HIV-1 (Sansome *et al.,* 1998), the synthetic amphipathic peptide GALA (Parente 1990), polypeptide venoms such as melittin, and polypeptide antibiotics such as magainins (Segrest *et al.,* 1990). A common structural feature in many pore forming cytotoxic proteins is the presence of an amphipathic helix motif.

Membrane pertubation may also be nonspecific, occurring via a detergent-like action such as was found with the cytolytic endotoxin Cyt A (Butko *et al.*1996).

The mechanism by which extracellular NS3 causes membrane permeabilisation could be investigated by determining whether the protein is toxic to both prokaryotic and eukaryotic cells. Membrane damage is unlikely to be via a receptor-mediated mechanism in prokaryotic cell membranes. Alternatively, it may be that the effects of the protein are not limited simply to direct membrane damage alone but also occur through interactions with membrane proteins and receptors. Although extracellular rotavirus NSP4 causes an increase in Ca^{2+} via a receptor-mediated mechanism (Tian *et al.,* 1994), the protein has also been shown to have direct membrane destabilising activity that does not require receptors (Tian *et al.,* 1996). The requirement for receptors could also be investigated by treating eukaryotic cells with protease prior to exogenous addition of the NS3 protein.

To determine which regions of the protein are involved and important in membrane destabilisation a variety of truncated mutants of the NS3 protein could be made and investigated. Synthetic peptides could then be produced, representing this region(/s), and the dynamics and conditions of cell lysis determined. The change in permeability of cell membranes to a variety of compounds of various molecular weights should also be investigated. If a transmembrane pore is formed by NS3 then there is expected to be a size or molecular weight dependance on the leakage or uptake of compounds, while size should not be a factor if the protein acts in a detergent-like fashion.

The role that this aspect of NS3 plays in viral virulence and pathogenicity of AHSV also needs to investigated. Studies of AHSV virulence characteristics have been carried out using a mouse model system and genome segment reassortments between virulent and avirulent strains. Exchange of genome segment 10, and therefore variations in the protein sequence of NS3, were shown to influence the virulence characteristics of the progeny virus strain (O'Hara *et al.,* 1998). This furthermore implicates NS3 in the pathogenicity and virulence of AHSV (O'Hara *et al.,* 1998). The toxic effect of the NS3 protein of virulent and avirulent strains of AHSV should be compared to one another and related to the virulence of the strain.

The specificity of the NS3 extracellular cytotoxic effect can be investigated by using a variety of eukaryotic cells such as equine lung cells and endothelial cells in

membrane permeability assays. Laegried and co-workers (1992) suggest that difference in ability of virulence variants of AHSV to cause acute disease may be related to their ability to infect and damage endothelial cells.

The toxicity of NS3 to mammalian cells exogenously should also be compared to the effect of the protein when expressed endogenously. For this reason the NS3, NS3A and NS3-mutant proteins should be expressed as recombinants in a mammalian expression system. Browne *et al.,* (2000) postulate that the membrane-destabilising and enterotoxic properties of NSP4 may be mediated by different regions of the protein.

Macreadie *et al.,* (1996) propose that the cytotoxic HIV-1 Vpr could be responsible for the death of uninfected bystander cells surrounding HIV-1-infected cells. Cell death in uninfected bystander cells has also been observed during AHSV infection, the role that NS3 may play in this should be investigated. It would also be interesting to determine whether AHSV-infected cells secrete NS3 or a functional peptide thereof. Rotavirus-infected cells have been shown to secrete a functional NSP4 enterotoxin peptide (Zhang *et al.,* 2000).

Another aspect of the functioning of NS3 and NS3A that was addressed in this study was the effect of co-expression of NS3 and NS3A on the cytotoxicity of the proteins to insect cells. This was necessary as AHSV infection of Vero cells results in the synthesis of NS3 and NS3A in equimolar amounts (Van Staden *et al.,* 1995), while expression of S10 in insect cells results in the synthesis of NS3 alone. NS3 and NS3A were therefore co-expressed here in insect cells to mimic the expression patterns seen in AHSV infected cells. This was achieved through the co-infection of insect cells with recombinant baculoviruses expressing NS3 and NS3A. The cytotoxic effect of co-expression of the NS3 and NS3A proteins was similar to the effect of individual expression of either protein. The NS3 protein is therefore representative of NS3 and NS3A together in terms of its cytotoxic effect and no interactions between these proteins occurs that affects this aspect of their functioning.

One of the objectives of this study was to purify large quantities of NS3 and NS3A, expressed in an eukaryotic system for comparative and functional studies.

The NS3 and NS3A proteins were expressed as histidine tag fusion products in insect cells using the baculovirus expression system. The HTc-NS3 protein was found, however, to be only partly soluble and did not bind to charged Nickel resin. This may be due to the folding of the tag in such a way that it is not exposed to the environment. HTc-NS3 and wild type NS3 were furthermore found to differ significantly in solubility, this insolubility of HTc-NS3 may be due to incorrect folding due to the presence of the histidine tag.

The N-terminal NS3 sequences of AHSV, Palyam virus, BRD virus, BTV and EHDV were compared and found to differ significantly in both length and amino acid identity. The presence of the second methionine, representing the N-terminal amino acid of NS3A, was however conserved throughout, indicating a selective conservation of the second start codon in the S10 gene of orbiviruses. Sequence and pattern searches of this region in NS3 of AHSV-3, using protein profile databases, did not reveal potential motifs or domains that may represent a functional domain. The N-terminal region of NS3 of the different serotypes of AHSV furthermore displays little conservation. The presence of a functional domain in this region appears to be unlikely.

The final aim of this study was to produce polyclonal antibodies that would specifically recognise the short N-terminal region of NS3 that is absent in the NS3A protein, for use in distinguishing between these proteins in AHSV infected cells. The AHSV core protein VP7 was used as a vector for immunological display of this small peptide. This would allow for both the production of serum against the N-terminal of NS3 and for an investigation into the suitability of the VP7 protein as an antigen display vector. The chimera, VP7-NS3, was found to still be able to assemble as highly ordered crystal structures and was used for polyclonal antiserum production. The resulting serum contained antibodies directed against VP7, but not NS3, epitopes.

Future research may target different sites on VP7 for the display of such a small peptide or tandem repeats of the peptide could be displayed. Once available this serum could be used in localisation studies in AHSV infected cells and may aid in determining whether both proteins are necessary and involved in virus

morphogenesis. Antiserum directed against the N-terminal amino acids of NS3 may also be used to experimentally determine the orientation of the NS3 protein in the cell membrane.

The orientation of the influenza virus M2 protein was determined in this way (Lamb *et al.*, 1985). Oligopeptides representing the N-terminal residues 2–10 and C-terminal residues 69-79 of M2 were synthesised and coupled to keyhole limpet hemocyanin (Lamb *et al.*, 1985). Rabbit antisera were then prepared against these peptides and used for immunofluorescent-staining of cells infected with the Influenza virus. Only the antibodies directed against the N-terminus were shown to bind to the M2 protein on the cell surface indicating that this region is located in the extracellular space whereas the carboxy terminus is cytoplasmic. M2 is, thus, a type I integral membrane protein (Lamb *et al.*, 1985). The membrane topology of AHSV NS3 has not yet been experimentally elucidated.

Other aspects of NS3 that remain to be investigated include the oligomerisation state of the protein. As discussed in chapter 1, the presence of different polypeptide monomers, like NS3 and NS3A could increase the range of potential complexes formed. As NS3 and NS3A are involved in virus morphogenesis the interaction between these proteins and the viral capsid proteins needs to be examined. Rotavirus NSP4 has been shown to bind VP6 and VP4 on SSP and facilitate translocation of viral particles across the ER membrane (Au *et al.*, 1989; Meyer *et al.*, 1989; Mattion *et al.*, 1994).

The recent development of a reovirus reverse genetics system may be applicable to all dsRNA viruses, including orbiviruses (Roner & Joklik 2001). This system allows for the introduction of mutations into the dsRNA genome and may in the future be a powerful system for studies on the role of S10 in AHSV virulence.

An understanding of the molecular mechanisms of the functioning of NS3 and the role that the membrane damaging properties of this protein plays in the pathogenesis of AHSV, may potentially lead to the development of new therapies for this devastating disease.