



UNIVERSITY OF CAPE TOWN

**EVALUATION OF THE *IN VIVO* ROLE OF VACCINIA VIRUS
COMPLEMENT CONTROL PROTEIN (VCP) FOLLOWING RENAL
ISCHEMIA/REPERFUSION INJURY**

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“I can do all things through Christ who empowers me.” Philippians 4:13

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LIST OF ABBREVIATIONS

A β	amyloid beta
AD	Alzheimer's disease
ADH	antidiuretic hormone
ANOVA	analysis of variance
A $^{\circ}$	Ampere
AOX-1/2	alcohol oxidase 1/ 2
AP	alternative pathway
APES	aminopropyltriethoxysilane
APS	anti-phospholipid syndrome
ARDS	acute respiratory distress syndrome
ARF	acute renal failure
BMGY	buffered glycerol complex medium
BMMY	buffered methanol complex medium
bp	base pair
BSA	bovine serum albumin
BUN	blood urea nitrogen
C3/4/5/6	complement component 3/4/5/6
C4b-BP	complement-4b-binding protein
CCPs	complement control proteins
CD55/59	cluster differentiation 55/59
CIA	collagen induced arthritis
cm 2	centimeter squared
COX	cyclooxygenase
CP	classical pathway
CPV	cowpox virus
CR1/2	complement receptor type1/ 2
CRPs	complement regulatory proteins
Crry	complement receptor type 1-related gene y
CV	column volume

CVF	cobra venom factor
DAB	3,3'-diaminobenzidine
DAF	decay-accelerating factor
dl	deciliter
DNA	deoxyribonucleic acid
dO ₂	dissolved oxygen
DRC	Democratic Republic of the Congo
DS	discovery studio
E	glutamic acid
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
g	centrifugal force
GFR	glomerular filtration rate
GLDH	glutamate dehydrogenase
GSH	Groote Schuur Hospital
GVB	Gelatin veronal-buffer
H	histidine
H&E	hematoxylin and eosin
HAIGG	heat aggregated IgG
HAE	hereditary angioedema
HIV	human immunodeficiency virus
HPLC	high-pressure liquid chromatography
hr(s)	hour(s)
HRP	horseradish peroxidase
hrVCP	humanized recombinant VCP
HSP	heparan sulphate proteoglycan
HUS	hemolytic uremic syndrome
I/R	ischemia/reperfusion
ICAM	intercellular adhesion molecule
IC ₅₀	inhibitory concentration at 50%
IgG/M/A	immunoglobulin G/M/A
IL	interleukin

im	intramuscular
IMP	inflammation modulatory protein
IP	intraperitoneal
IV	intravenous
K	lysine
kDa	kilo dalton
kg	kilogram
K-X-K	lysine-any amino acid-lysine
LAL	Limulus Amebocyte Lysate
LN	lupus nephritis
LP	lectin pathway
LPS	lipopolysaccharide
MAC	membrane attack complex
MBL	mannose binding lectin
MCP	membrane cofactor protein
MD	macular degeneration
MEM	minimal essential medium
mg	milligram
min(s)	minute(s)
MMH	minimal methanol histidine medium
MOI	multiplicity of infection
MOPICE	monkeypox inhibitor of complement enzymes
MPa	mega Pascal's
MPGN	membranoproliferative glomerulonephritis
MPV	monkeypox virus
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NHLS	National Health Laboratory Service
NSAIDs	nonsteroidal antiinflammatory drugs
N-X-S	Asparagine-any amino acid-serine
OD	optical density

p	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PVDF	polyvinylidene difluoride
RA	rheumatoid arthritis
RCA	regulators of complement activation
RDB	regeneration dextrose histidine medium
rev	revolution
RK	rabbit kidney cells
rpm	revolution per minute
SC	subcutaneous
SCR	short consensus repeat
sCR1	soluble complement receptor type 1
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SPICE	smallpox inhibitor of complement enzymes
SPR	surface plasmon resonance
ssRBCs	sensitized sheep red blood cells
STP	standard temperature and pressure
TAE	tris-acetate EDTA
TBM	tubular basement membrane
TBS	tris buffer saline
TNF	tumor necrosis factor
UCT	University of Cape Town
μl	microliter
μm	micrometer
UV	ultraviolet
V	volts
VCP	vaccinia virus complement control protein
VV	vaccinia virus

WHO World Health Organization
WR Western Reserve
YPD yeast extract peptone dextrose medium

DECLARATION

I declare that the site-directed mutagenesis and cloning of the mutant proteins (^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K) characterized in this study together with other uncharacterized mutants were generated by Dr. Odutayo O. Odunuga as part of his postdoctoral research fellowship at the University of Cape Town in the academic years' 2003-2005. I also declare that the rest of the work included in the thesis is my own original research and any significant contribution made by others is acknowledged. Therefore, except the University of Cape Town, which by default holds free license to publish it in part or in whole ("**GP8**"), any part of the thesis shall not be reproduced in any format without the written approval of the candidate.

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Signed by candidate

August 2006

THESIS TITLE: EVALUATION OF THE *IN VIVO* ROLE OF VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN (VCP) FOLLOWING RENAL ISCHEMIA/REPERFUSION INJURY

ABSTRACT: In transplantation, vascularized organs often suffer the consequences of ischemic damage as well as reperfusion injury following the reestablishment of blood flow. The induced ischemia/reperfusion (I/R) damage is usually associated with the accumulation of injurious complement components. The vaccinia virus complement control protein (VCP) has the ability to simultaneously inhibit the classical and the alternative complement pathways by binding to the early components C3b and C4b. The complement component C3 is known to be the central route to all of the known complement activation pathways. As a result, it is involved in a number of complement-mediated ailments including renal ischemia/reperfusion injury.

The objectives of this study were to initially evaluate the *in vitro* roles of the natural VCP and the humanized recombinant VCP (hrVCP), and then to establish their *in vivo* roles in a renal I/R injury model.

The recombinant VCP was produced both in flasks and the fermentor, while the natural VCP was produced in tissue culture. The *in vitro* activities of the proteins were tested for their ability to inhibit the classical and the alternative complement pathways using a hemolysis assay, and the production of pathway-specific fragment (Bb) respectively. The proteins were found to be biologically active in inhibiting complement activation at various concentrations.

The *in vivo* role of VCP/hrVCP was investigated by subjecting Long Evans rats to bilateral I/R injury by clamping both the renal arteries for 60 minutes followed by 24 hours of reperfusion. The animals were randomly allocated to receive VCP/hrVCP, phosphate buffered saline (PBS) or sham groups. Blood samples were collected and the kidneys removed for histopathological and immunohistochemical studies. The biochemical studies showed that the PBS group (n = 4) displayed 2.7-fold and 10-fold increases in the serum urea and creatinine concentrations respectively compared to the VCP/hrVCP groups. Moreover, the histopathological study revealed severe tubular damage in the PBS group compared to the focal tubular injury in the VCP (n = 3) and hrVCP (n = 3) treated animals. The immunohistochemical study displayed marked deposition of C3 in the renal tubules of the PBS group compared to the focal C3 staining in the VCP/hrVCP groups suggesting the ability of VCP/hrVCP to ameliorate renal I/R injury by inhibiting the local biosynthesis of C3.

LITERATURE REVIEW:

CHAPTER 1: COMPLEMENT INHIBITORY PROTEINS

1.1 Poxviruses

The genus *Orthopoxvirus*, is one of the eight genera, which constitute vertebrate poxviruses, and belongs to the family of *Poxviridae*, *Chordopoxvirinae* subfamily. The genus includes a number of poxviruses, which are large cytoplasmic DNA viruses with an average genome size of 200kbp (above 10-fold larger than HIV genome). Some of the better known members of this genus are the camelpox, cowpox, vaccinia, monkeypox and variola (commonly known as smallpox virus) viruses (Smith and Kotwal, 2002). The latter two members have been shown to establish serious infection in humans. The variola virus is the only member known to only infect humans and has claimed thousands of lives before its successful eradication following an efficient vaccination program launched by the World Health Organization (WHO) (Henderson¹, 1999; Henderson², 1999; Henderson et al, 1999; Henderson and Fenner, 2001; Wittek, 2006)

1.2 Complement Inhibitors Encoded by Poxviruses

Besides the molecules for viral replication, poxviruses also encode a stock of proteins that nullify the host immune defense mechanism. Some of the defense molecules engaged in clearing viral infection include cytokines, chemokines and complement (Alcamí et al, 2000; Kotwal, 2000; Smith and Kotwal, 2002). Poxviruses possess more than one defense mechanism for the same antiviral molecule encoded by the host. For example, the viruses encode proteins (known as “*immunomodulatory proteins*”) that block the synthesis of cytokines as well as proteins that bind, and therefore inhibit, already synthesized cytokine molecules (Kotwal, 2000). These lines of defense encoded by poxviruses not only enable them to successfully subvert destruction but also suggest that the viruses have salvage mechanisms to challenge the host defense. Members of poxviruses which encode complement inhibitory proteins (known as “*virokines*” [Kotwal, 2000; Smith and Kotwal, 2001]) include variola, vaccinia, cowpox and monkeypox viruses. The encoded proteins enable the viruses to persistently infect target cells despite the immune challenges from the host. Although this appears to be a common *in vivo* role played by the viral complement inhibitors, there is significant difference in their ability and preference to inhibit host complement. Some of the well-studied complement inhibitors are described below.

1.2.1 Smallpox Inhibitor of Complement Enzymes (SPICE)

The variola (smallpox) virus is known for its virulence. Despite its eradication, the genome of the virus, its mechanisms of disease causation and host discrimination are still being kept under close scrutiny (Dunlop et al, 2003). Understanding the variola genome and its subsequent proteins not only help researchers to prepare for any eventuality of its reemergence (De Clercq, 2001), but also provide the opportunity to use some of its proteins, such as those engaged in inhibiting human complement during infection, for therapeutic purposes in patients suffering from uncontrolled complement disorders such as autoimmune diseases. One of such potentially therapeutic proteins encoded by smallpox virus is SPICE.

SPICE, a potent complement inhibitor encoded by variola virus in the left terminal region of its genome, is a 28.158 kDa (Sfyrera et al, 2005) protein composed of four short consensus repeats (SCRs; usually composed of 60-70 amino acids) of 61 amino acids each. The SCRs are interlinked by disulphide bonds (Zhang and Morikis, 2006).

Although its crystal structure has not yet been determined, both its remarkable amino acid homology (95% identity) and modeling studies suggest that SPICE is structurally similar to the crystallized (Murthy et al, 2001) vaccinia virus encoded complement inhibitor, the vaccinia virus complement control protein (VCP) (Rosengard et al, 2002; Dunlop et al, 2003). Functionally, in addition to its similarity to poxviral proteins such as VCP, the cowpox encoded inflammation modulatory protein (IMP) and the recently characterized monkeypox encoded inhibitor of complement enzymes termed by the authors as MOPICE (Liszewski et al, 2006), SPICE is similar to the cellular complement control proteins (CCPs) (Dunlop et al, 2003) such as decay-accelerating factor (DAF; CD55), Factor H, membrane cofactor protein (MCP; CD46), complement-4b-binding protein (C4b-BP) and soluble complement receptor type 1 (sCR1), which are collectively known as the regulators of complement activation (Liszewski et al, 2000; Smith et al, 2000). *In vitro* studies have demonstrated that SPICE inhibits both the classical and the alternative pathways of human complement more efficiently than its viral homologs, VCP (Rosengard et al, 2002; Sfyrera et al, 2005) and MOPICE (Liszewski et al, 2006).

In vivo, following entry to its host via the respiratory route (Smith and Kotwal, 2002), the variola virus strives to replicate to higher titers in infected cells in order to establish infection. However, it is a prerequisite for the virus to overwhelm the host complement and protect the infected cells by secreting complement inhibitors and shifting the balance to its advantage. Therefore, SPICE, an efficient complement inhibitor, is thought to be the ultimate mechanism by which the virus attenuates its host (Lachmann, 2002; Dunlop et al, 2003). The role of SPICE in smallpox infection appears to be critical in leading to the speculation that immunizing humans with SPICE might be protective against possibly emerging smallpox infection (Lachmann, 2002). Meanwhile, the anti-complement therapeutic potential of SPICE might be further elucidated by developing animal models of different ailments that mimic the clinical scenario, such as head injury, spinal cord injury, xenorejection, sepsis, ischemia/reperfusion injury and arthritis where complement has been shown to play an important role.

1.2.2 Inflammation Modulatory Protein (IMP)

IMP, a homolog of SPICE, MOPICE and VCP, is an anti-inflammatory protein encoded by cowpox virus (CPV), a virus used by Edward Jenner to successfully vaccinate people against smallpox (Moss, 1996; Kotwal, 2000; Viera and Chies, 2005). CPV, in contrast to variola virus which only infects humans, is able to infect wild (rodents, lions, elephants), domestic animals (cattle, cats), and humans (Smith and Kotwal, 2002). Wild type CPV infection manifests less pathologic changes than infection with the mutant CPV lacking the gene that encodes the inflammation modulatory protein (CPV-IMP). For example, Miller et al (1997) demonstrated that both wild-type and C5-deficient BALB/c mice inoculated with the mutant virus displayed severe footpad swelling and aggregation of inflammatory cells compared to the wild-type CPV infected mice suggesting the beneficial role of IMP in the containment of inflammation (Howard et al, 1998).

In vitro studies have shown that IMP possesses four SCRs and retains the ability to inhibit the classical pathway of complement activation. It also binds to heparin (Smith et al, 2000). Moreover, comparative phylogenetic analysis has indicated that IMP together with SPICE and VCP might have emerged from a common ancestral complement inhibitor (Ciulla et al, 2005). However, there are very limited *in vitro* studies on IMP, and the crystal structure and the pharmacological value of purified IMP in curbing complement-mediated diseases remain to be determined.

1.2.3 Monkeypox Inhibitor of Complement Enzymes (MOPICE)

After the successful eradication of smallpox, monkeypox infection has emerged as the most devastating and perhaps life-threatening orthopoxviral disease, with symptoms resembling that of smallpox (Douglass and Dumbell, 1992). Humans (mostly hunters) are primarily infected through contact with infected monkeys (zoonosis) and can secondarily infect other individuals. For example, the two independent outbursts of monkeypox infection in the Democratic Republic of the Congo (DRC) in 1970, and more than three decades later in the United States suggest that monkeypox infection is a relapsing biological threat (Liszewski et al, 2006). The presence of MOPICE in the genome of the DRC-strain appears to have enhanced the virulence of the virus compared to the milder form of monkeypox infection which occurred in the United States (Liszewski et al, 2006).

MOPICE, a recently characterized potent complement inhibitor, is structurally and functionally similar to SPICE, IMP and VCP, with the exception of possessing a truncated 4th SCR as a result of premature stop codon in the carboxy terminal of the protein (Uvarova and Shchelkunov, 2001; Liszewski et al, 2006). Despite the fact that the truncation has reduced the number of putative heparin binding sites from four (SPICE, IMP and VCP) to three (Smith et al, 2000), MOPICE binds heparin as strongly as VCP and SPICE (Liszewski et al, 2006), suggesting that the overall positive charge might be more influential in determining the affinity to the negatively charged heparin column. Interestingly, MOPICE has been shown to bind C3b, a critical complement component (refer "*CHAPTER 4*" of the thesis), more efficiently than VCP, suggesting its preferential application in treating complement-mediated diseases where C3 plays a damaging role, such as renal ischemia/reperfusion injury (Zhou et al, 2000; "*CHAPTER 4*" of the thesis), traumatic brain injury (Keeling et al, 2000) and guinea pig-to-rat heart transplantation (Anderson et al, 2003).

1.2.4 Vaccinia Virus Complement Control Protein (VCP)

Historically, VCP was the first viral complement inhibitor to be identified from vaccinia virus. It was identified during sequencing of the terminally located "*nonessential*" genes (Kotwal and Moss, 1988). The vaccinia virus, known for representing candidate vaccine strains (Smith and Kotwal, 2002), encodes VCP in the left terminus of its genome in order to evade the host immune defense mechanisms. VCP migrates as a ~28 kDa (in a 12% SDS-PAGE gel) (Smith et al, 2000) and ~35kDa protein (in a 10% SDS-PAGE gel) (Kotwal and Moss, 1988; Kotwal et al, 1990), and is composed of four SCRs, sharing significant structural homology to the host

complement-4b-binding protein (C4b-BP). Functionally, VCP blocks both the classical and the alternative pathways of complement activation (Kotwal et al, 1990; Isaacs et al, 2003) and subsequently protects the vaccinia virus from neutralization, and prolongs the survival of infected cells in order to generate more viruses before their death (Isaacs et al, 1992; Kotwal, 2000; Dunlop et al, 2003; Kotwal and Abrahams, 2004). In order to further elucidate the potential benefits of complement inhibition, the VCP gene was amplified from the vaccinia virus Western Reserve (WR) strain genomic DNA and cloned into yeast cells for large-scale production and ease of characterization (Smith et al, 2000). This full length recombinant VCP (rVCP) is probably the most studied recombinant viral complement inhibitor, extending from its ligand binding ability (Smith et al, 2000; Ganesh et al, 2004; Ganesh et al, 2005), crystallization (Murthy et al, 2001), and physicochemical property (Smith et al, 2002), to its multitude of therapeutic potentials (Daly and Kotwal, 1998; Anderson¹ et al, 2002; Anderson² et al, 2002; Hicks et al, 2002; Anderson et al, 2003; Jha and Kotwal, 2003; Scott et al, 2003; Kahn et al, 2003; Reynolds et al, 2004; Jha et al, 2005; Thorbjornsdottir¹ et al, 2005; Ghebremariam et al, 2006).

The recombinant VCP is predominantly a monomer which exists as a 28kDa protein that can tolerate extremes of temperature, pH, and storage (Smith et al, 2002; Liszewski et al, 2006). However, recent studies have shown that the natural VCP secreted from infected mammalian cells has a different oligomerization pattern, which partially accounts for its enhanced functional activity (Liszewski et al, 2006). We have shown that the VCP secreted from vaccinia virus Lister strain is the only glycosylated (N-X-S form) poxviral complement inhibitor and that this glycosylation appears to have affected its functional properties (Odunuga et al, 2005).

Functionally, VCP and rVCP have been shown to modulate a number of complement-mediated ailments. For example, the loop of complement activation by the amyloid-beta (A β) peptide, a reactive molecule known to be progressively deposited in Alzheimer's disease, has been completely abolished using VCP (Daly and Kotwal, 1998). Moreover, intracranial administration of VCP has been shown to improve memory following fluid percussion injury in rats (Hicks et al, 2002). Furthermore, Scott et al (2003) demonstrated that the intravenous administration of rVCP in mice increased the ability of neutrophils to reduce bacterial load following intestinal infection. Moreover, we, in collaboration with Dr. Tom E. Mollnes's laboratory in Norway, have recently shown (Thorgersen et al, manuscript in preparation) that

VCP is a potent inhibitor of pig complement following selective activation of the alternative pathway with zymosan, heat aggregated IgG (HAIGG) and *E. coli* in swine sera and whole blood, as evaluated by measuring tumor necrosis factor (TNF- α), interleukin (IL-1 β) and IL-8. This work suggests a therapeutic potential for VCP in conditions predominantly driven by the alternative pathway of complement activation such as renal ischemia/reperfusion injury.

Other studies have investigated the therapeutic values of VCP/rVCP in transplantation. Al-Mohanna et al (2001) have demonstrated the ability of VCP to competitively block the binding of the preformed anti-galactosyl- α 1,3 galactosyl transferase xenoantibodies to their natural receptors on the surface of non-primate endothelial cells, as well as its additional ability to inhibit the destruction of pig endothelial cells by human inflammatory cells, suggesting its pharmacologic potential in xenotransplantation research. This *in vitro* evidence was supported by the ability of rVCP to significantly prolong the survival of xenografted organs following mouse-to-sensitized rat (Anderson¹ et al, 2002) and guinea pig-to-rat (Anderson et al, 2003) heart xenotransplantations. Moreover, Kahn et al (2003) have demonstrated the ability of rVCP to increasingly downmodulate complement activation following its administration in primates, supporting its therapeutic potential in xenotransplantation. Recently, we reviewed the ethical, psychosocial, biosafety and molecular barriers confounding xenotransplantation research, and discussed various therapeutic approaches towards clinical xenotransplantation (Ghebremariam¹ et al, 2005; Ghebremariam² et al, 2005).

Based on the aforementioned therapeutic promise of the recombinant VCP, its remarkable homology to SPICE (95-95.4% identity depending on the strains; Rosengard et al, 2002; Lachmann, 2002; Dunlop et al, 2003), the extreme potency of SPICE extending from 100 (Rosengard et al, 2002) to 1000-fold (Sfyroera et al, 2005) than rVCP, computer modeling predictions on surface exposure and ligand binding efficiency, and its homology to host complement control proteins, specific amino acids were substituted in rVCP in the hope of enhancing its activity (Ghebremariam³ et al, 2005; Sfyroera et al, 2005).

The novel work by Sfyroera et al (2005) demonstrated the generation of 12 independent rVCP mutants with single, double or multiple amino acid substitution(s) towards their SPICE analog/homologs. The surface plasmon resonance (SPR)-based binding assay and ELISA-based functional assays revealed that all the generated mutant proteins acquired greater ability in regulating complement activation (Sfyroera et al, 2005).

For example, the single amino acid substitution of glutamic acid (E) at position 120 to its SPICE analog (E120K) has immensely upregulated the alternative pathway activity of the protein by about 87-fold (Sfyoera et al, 2005). Furthermore, the authors have demonstrated that not all the amino acid differences between VCP and SPICE (Figure 1) are equally critical, and only few specific amino acid changes are required in order to make rVCP as potent as SPICE.

In the authors *own* words, the combined two amino acid substitutions in rVCP at positions 108 and 120 of the second SCR (SCR-2) to their SPICE analogs (E108K, E120K) is described to be “...THE MINIMAL AMINO ACID SUBSTITUTIONS REQUIRED ON VCP TO BRING ITS ACTIVITY CLOSE TO THE ACTIVITY OF SPICE.” (Sfyoera et al, 2005). Our findings (*In vitro*, “CHAPTER 3” and *in vivo* “CHAPTER 4” of the thesis) supported the work by Sfyoera et al (2005), and demonstrated that SCR-2, a module that displays above 40% of the amino acid differences, is a critical domain that significantly interacts with both the classical and the alternative pathways of complement activation (Ghebremariam³ et al, 2005). Most researchers appear to have been exclusively engaged in identifying critical amino acid(s) in rVCP, and the interaction of these modified protein(s) with complement components, heparin and other ligands (Smith et al, 2000; Murthy et al, 2001; Rosengard et al, 2002; Smith et al, 2003; Ganesh et al, 2004; Ganesh et al, 2005; Ghebremariam³ et al, 2005; Sfyoera et al, 2005). Very little (if any) attention has been given to basic and essential immunogenicity studies of rVCP, SPICE and any of the derivative poxviral complement inhibitors, which we regard as potential anti-complement therapies (Mollnes and Kirschfink, 2006).

1.3 Natural Human Complement Regulators

The human complement regulatory proteins (CRPs) are a subset of proteins that exist in either a soluble form or bound to cell membrane (Lachmann, 2002; Liszewski et al, 2006). They have significant amino acid identity with the poxviral complement inhibitors described above. The physiological role of the human complement regulators is to protect endogenous cells from excessive complement driven self-attack (Mollnes et al, 2002; Dunlop et al, 2003). These proteins are encoded by genes located on the long arm of chromosome 1 (Lachmann, 2002; Liszewski et al, 2006), and belong to the regulators of complement activation (RCA) family (Liszewski et al, 2000; Smith et al, 2000; Ganesh et al, 2004).

The family includes complement-4b-binding protein (C4b-BP), complement receptor type 1 (CR1), membrane cofactor protein (MCP), decay-accelerating factor (DAF), CD59 and Factor H (Cozzi and White, 1995; Smith et al, 2000). A brief description of each of these members of the RCA family is described below.

1.3.1 Complement-4b-Binding Protein (C4b-BP)

As its name implies, this protein binds to the complement component C4b (Kotwal et al, 1990) and inhibits the classical pathway C3 (C4b2a) and C5 (C3bC4b2a) convertases (Kotwal, 1997; Lachmann, 2002; Mollnes and Kirschfink, 2006). C4b-BP exists naturally as a soluble plasma glycoprotein bound to protein S (Wiesel et al, 1990; Lachmann, 2002; Ganesh et al, 2004; Liszewski et al, 2006). A chimeric membrane bound form of C4b-BP has been engineered on the surface of pig endothelial cells, and used in pig-to-human xenotransplantation research (Mikata¹ et al, 1998; Mikata² et al, 1998). C4b-BP is the largest of all the members of the RCA family containing 56 SCRs and has the highest structural homology to VCP with an average amino acid identity of 32% (extending from 28% to 38% within the first 4 SCRs) (Kotwal and Moss, 1988; Kotwal, 2000; Dunlop et al, 2003). C4b-BP (550kDa) is a very potent complement regulatory glycoprotein with both cofactor (for Factor I to degrade C4b) and decay-accelerating (accelerates the decay of the classical pathway C3 and C5 convertases) activities (Kotwal et al, 1990), making it a good candidate as anti-complement therapy. However, the exploitation of C4b-BP's protective role by some microbes in order to escape immune surveillance (Lachmann, 2002) may affect its chances of proceeding to the clinic.

1.3.2 Complement Receptor Type 1 (CR1)

CR is a membrane-anchored protein that exists in two types namely CR1 (CD35) and CR2 (CD21) (Lachmann, 2002). Of the two, CR1 is by far the most studied protein in complement research, not only because CR2 does not possess any significant complement regulatory activity (Mollnes and Kirschfink, 2006) but also because it serves as a receptor for Epstein-Barr virus (Kotwal, 1997; Dunlop et al, 2003), a herpesvirus known to cause Burkitt's lymphoma and infectious mononucleosis in children. CR1, on the other hand, possesses complement regulatory activity similar to that of VCP (Kotwal, 2000).

CR1, composed of 30 SCRs, is the most efficient complement regulator in the RCA family, with cofactor and decay-accelerating activities in the classical and alternative pathways of complement activation (Rosengard et al, 2002; Mollnes and Kirschfink, 2006) making it a candidate protein for therapeutic purposes. Weisman et al (1990) have engineered a soluble form of CR1 (sCR1), and showed its ability to reduce myocardial infarction in rats. Soluble CR1 (sCR1) has a unique ability to inhibit all the three known routes of complement activation namely the classical, alternative and the lectin pathway (Sacks et al, 2003). Subsequently, sCR1 (120kDa) has proven its strong potential in higher-order animal xenotransplantation (Pruitt et al, 1994) and has reached different stages of clinical trials with some promises in vascular surgery (Sacks et al, 2003; Mollnes and Kirschfink, 2006). Mollnes and Kirschfink (2006) have reviewed more than a decade work on sCR1.

1.3.3 Membrane Cofactor Protein (MCP)

MCP, also known as CD46, is expressed on the surface of B and T lymphocytes (Sacks et al, 2003) and is one of the two smallest (4 SCRs) members of the RCA family (Dunlop et al, 2003). It shares significant amino acid homology (35%) and functional identity with VCP (Smith et al, 2000; Sfyroera et al, 2005). As its name indicates, MCP serves as a cofactor in assisting Factor I in order to degrade activated C3 (C3b) and C4 (C4b) complement components (Liszewski et al, 2000). The molecular significance of the different SCRs in MCP have been meticulously elucidated (Liszewski et al, 2000) and shed light on other complement inhibitors (Rosengard et al, 2002; Ghebremariam³ et al, 2005). Despite its small size and modest complement inhibitory activity, its chances of progressing to the clinic may be hampered by the fact that it is used by many bacteria and viruses as a convenient route of entry into cells (lymphocytes, monocytes and keratinocytes) which express MCP (Kotwal, 1997; Liszewski et al, 2000), which undoubtedly increases the chances of subsequent infection(s) if used clinically.

1.3.4 Decay-accelerating Factor (DAF)

DAF (CD55) is a four-SCR protein expressed on the surface of erythrocytes, lymphocytes and antigen-presenting cells (Mollnes et al, 2002; Sacks et al, 2003). It shares 31% amino acid homology to VCP (Sfyroera et al, 2005) and has efficient decay-accelerating activity (Dunlop et al, 2003).

In vivo studies have shown that the deletion of DAF gene in mice increases the susceptibility to ischemia (Yamada et al, 2004) and immune-complex disorders (Mollnes et al, 2002), suggesting its indispensable role. Numerous studies involving the expression of DAF on the endothelial surface of pig organs (Langford et al, 1994; Cozzi and White, 1995; Rosengard et al, 1995) have reported its potential in abolishing hyperacute xenorejection (McCurry et al, 1995; Schmoeckel et al, 1997; Ramirez et al, 2002; Brandl et al, 2003).

However, other researchers are sceptical about the therapeutic promise of DAF, not only because it fails to effectively inhibit complement (Koike et al, 1996; Kotwal, 1997; Van Denderen et al, 1997) but also because it is used as a receptor by enteroviruses and as a shield by HIV in order to evade immune surveillance (Kotwal, 1997). Therefore, its chances of advancing to the clinic might be extremely minimal.

1.3.5 CD59

It is not clear whether CD59 belongs to the RCA family (Cozzi and White, 1995) as the literature has exclusively ignored it (Murthy et al, 2001; Lachmann, 2002; Sfyroera et al, 2005; Mollnes and Kirschfink, 2006). CD59 is expressed on the surface of many cells and is used to inhibit the alternative complement pathway by preventing the formation of membrane attack complex (MAC; C5b-9) (Anderson et al, 2003; Yamada et al, 2004). Failure of CD59 to inhibit the detrimental MAC formation has been described as a primary cause of hyperacute rejection in guinea pig-to-rat heart transplantation (Anderson et al, 2003). Moreover, Mollnes et al (2002) have documented the fragile nature of erythrocytes isolated from CD59-deficient mice indicating the importance of its natural expression. Moreover, other studies have demonstrated the cooperative role of CD59 with DAF in renal ischemia/reperfusion injury (Yamada et al, 2004) and experimental xenotransplantation (McCurry et al, 1995). However, its failure to regulate the classical complement pathway and other biosafety concerns (Kotwal, 1997) may diminish its chances of proceeding to the clinic.

1.3.6 Factor H

Factor H is a soluble protein composed of 20 SCRs (Dunlop et al, 2003) and has the ability to regulate the alternative pathway C3 (C3bBbP) and C5 (C3b2BbP) convertases (Kotwal, 1997; Mollnes and Kirschfink, 2006).

Interestingly, Factor H is the only heparin binding protein among the members of the RCA family. This heparin binding ability allows Factor H to possess multifunctional activities, such as the inhibition of antibodies and inflammatory cells from binding to the surface of endothelial cells, thus, preventing cytotoxicity (Kotwal, 2000).

Deficiency of Factor H is associated with a detrimental genetic disorder, hemolytic uremic syndrome, indicating its critical role in the pathophysiology of complement regulation (Ganesh et al, 2004; Zipfel et al, 2006). Despite its essential role in complement regulation and cytoprotection, its chances of progressing towards clinical trials may be impeded due to biosafety concerns (Kotwal, 1997; Lachmann, 2002).

To efficiently and safely regulate excessive complement activation, all the potential complement inhibitory proteins need to be well characterized and any putative microbial binding site(s) need to be modified or else eliminated. One such key approach in characterizing and refining potentially therapeutic proteins is their molecular recombination in various expression systems (He et al, 1997; Smith et al, 2000; Rosengard et al, 2002; Sfyroera et al, 2005; Liszewski et al, 2006).

1.4 Expression of Recombinant Proteins

Protein biotechnology is being enjoying the extraordinary advances in molecular biology. The engineering of foreign proteins in multiple expression systems has become increasingly simplified. Although *Escherichia coli* was among one of the protein expression systems, its inability to secrete the proteins of interest (Files et al, 2001; Liu et al, 2001), and the folding problems often encountered (Lange et al, 2001; Ouyang et al, 2003), have resulted in the search for other expression systems. The expression of recombinant proteins in eukaryotic systems, such as the yeast and mammalian cells have made major differences in the folding and other posttranslational properties of the expressed proteins (Chen et al, 1997; Lange et al, 2001; Ouyang et al, 2003). However, because of the need for expensive growth and maintenance media, as well as the risk of infectious contaminants, the mammalian cell-based tissue culture expression system is used sparingly (Sudbery, 1996; Chen et al, 1997).

The transformation of yeast cells for the expression of foreign genes has become a widely accepted approach for over two decades (Sudbery, 1996; Chen et al, 1997; Sreekrishna et al, 1997; Cereghino et al, 2002).

A number of yeast strains have been used as vehicles for protein production. *Saccharomyces cerevisiae*, *Pichia pastoris* (*P. pastoris*) and *Hansenula polymorpha* have been successfully used to express one or more potentially therapeutic proteins (Sudbery, 1996). However, the methylotrophic yeast *P. pastoris* has been found to be the most affordable and optimal expression system for diverse proteins (Zhu et al, 1995; Sudbery, 1996; Chen et al, 1997; He et al, 1997; Kobayashi et al, 2000; Files et al, 2001; Lange et al, 2001; Liu et al, 2001; Cereghino et al, 2002; Koganesawa et al, 2002; Ouyang et al, 2003). For example, different modules of the VCP gene were expressed in *P. pastoris* (Smith et al, 2000) and the recombinant proteins have been successfully produced ever since (Smith et al, 2000; Hicks et al, 2002; Anderson¹ et al, 2002; Anderson et al, 2003; Reynolds et al, 2004; Ghebremariam³ et al, 2005; Jha et al, 2005; Ghebremariam et al, 2006).

Similarly, researchers have reported over 40% increase in the production of the free radical scavenger, thioredoxin, by simply switching the expression system to *P. pastoris* (Anna et al, 2003). Other researchers have demonstrated satisfactory yield of their respective recombinant proteins ranging from 300 to 900mg/l when expressed in *P. pastoris* (Chen et al, 1997; Kobayashi et al, 2000; Liu et al, 2001; Lee et al, 2003; Ouyang et al, 2003).

The cloning of foreign genes into shuttle vectors (such as pPIC9) downstream to the well-characterized and engineered alcohol oxidase 1 (AOX1) promoter of *P. pastoris*, allows the constitutively inducible promoter to be upregulated upon the addition of methanol during induction phase of the protein production (Chen et al, 1997). The pseudo regulation of the AOX1 gene, in order to facilitate the interaction between oxygen and methanol, is the basis for the molecular exploitation of the cloning site (Lee et al, 2003). In addition to the optimal yield, *P. pastoris* has the added advantages of minimal glycosylation and endotoxin related problems which often hamper the clinical applications of recombinant proteins (Sudbery, 1996; Chen et al, 1997; Cereghino et al, 2002).

The success of laboratory scale production of proteins using *P. pastoris*, has encouraged scientists to expand the amount of protein production to an industrial scale. The use of fermentors for the production of recombinant proteins has significantly increased the amount of protein produced compared to the flask-based small-scale production (Koganesawa et al, 2002).

A fermentor is a bioreactor-like device which incorporates a vessel, an agitator, a condenser, heat blanket, pH and oxygen probes, a thermometer and a primary control unit which can be connected to an external computer to monitor the progress of the different parameters. It is usually connected to an external pump for delivering acid/base, oxygen and sources of antifoam and other solutions necessary during the process of fermentation.

The tight regulation of temperature, agitation, pH and dissolved oxygen levels in fermentors creates a suitable atmosphere for the yeast cells to grow to high density (Chen et al, 1997; Files et al, 2001; Cereghino et al, 2002; Thiry and Cingolani, 2002; Lee et al, 2003). For example, Zhu et al (1995) demonstrated remarkable differences in the enzymatic activity of the recombinant α -galactosidase following subtle changes in pH, suggesting the crucial role of the acidity/alkalinity of the media used for protein production. The activity of proteases appear to be pH dependent and their proteolytic activity can generally be decreased by lowering the pH of the media (Sreekrishna et al, 1997; Koganesawa et al, 2002; Thiry and Cingolani, 2002); although it seems to be the converse for other proteases (Kobayashi et al, 2000).

Furthermore, the supply of additional source of oxygen (medical or industrial grade) to the recombinant cells appears to positively influence the amount of protein secreted (Chen et al, 1997; Lee et al, 2003).

The *in vitro* and *in vivo* authenticity of the secreted proteins needs to be monitored, as incorrectly folded or hyperglycosylated proteins may be immunogenic and may induce complex reactions when administered clinically for the treatment of various diseases (Sudbery, 1996).

CHAPTER 2: ISCHEMIA/REPERFUSION INJURY

2.1 The Renal System

The renal system includes the kidneys, collecting ducts, urinary bladder, ureters and the urethra. Each of the components performs an important function in order to clear metabolic wastes from circulation. A brief description of the anatomy including blood supply and innervation, histology (microscopic and macroscopic structures), physiology (function), and clinical investigations of the renal system follow.

2.1.1 Anatomy

The kidneys are located in the abdomen, in the retroperitoneum on either side of the vertebral column (Bulgar and Hebert, 1979; Ross, 1972). This anatomical location appears to be uniform across species from man to small laboratory animals including rats (Ross, 1972). The blood vessels (renal artery and vein), the ureter and nerve supply enter the kidney in the hilum. The blood vessels ensure the supply of oxygenated (arterial) blood and the exit of deoxygenated (venous) blood. The ureter drains the urine into the urinary bladder and the sympathetic nervous system innervates the renal artery (Ross, 1972).

2.1.2 Histology

Microscopically, the renal tubules are composed of the functional unit (nephron) and parts of the collecting ducts (Bulger and Hebert, 1979). Moreover, the nephron is further composed of various segmented structures that ultimately contribute to the histologic appearance of the kidney. In summary, the various structures that are distinctively visible under the microscope include (Bulger and Hebert, 1979): the nephron, made up of the following structures: the glomeruli, which is made of Bowman's capsule, the glomerular capillaries and the specialized phagocytic cells known as mesangial cells; the proximal tubule (convoluted and straight portions); loop of Henle (descending and ascending parts); distal tubule (includes the cortex and the medulla-containing straight portion and the convoluted region); and the collecting duct which comprise the cortical, outer medullary and the inner medullary collecting ducts.

2.1.3 Physiology

The kidneys are involved in the excretion of metabolic wastes such as urea and creatinine, acid-base balance as a compensatory mechanism following respiratory acidosis/alkalosis, activation of vitamin D, and synthesis of hormones such as erythropoietin, a hormone which is necessary for red blood cell maturation (Zilva et al, 1988; Ghebremariam¹ et al, 2005; Ghebremariam² et al, 2005), and renin, a proteolytic enzyme involved in maintaining blood pressure through the renin-angiotensin system (Zilva et al, 1988).

Between 180 to 200 liters (125 to 139 ml/min) of fluid gets filtered by the glomeruli per day and about 99% of the filtrate gets reabsorbed through the proximal convoluted tubule (Bulger and Hebert, 1979; Zilva et al, 1988). The loop of Henle is involved in concentrating the 40 to 60 liters of water received, by 20 to 30-fold. The distal convoluted tubule and the collecting duct are involved either in urine dilution, in the absence of the antidiuretic hormone (ADH), or in the concentration of urine in the presence of ADH by reabsorbing water into the ascending vasa recta (Zilva et al, 1988). Finally, the ureters are used as passage routes for the urine to be temporarily stored in the urinary bladder before excretion through the urethra.

2.1.4 Laboratory Investigation of Renal Diseases

The following tests are used to assess renal function:

2.1.4.1 Urea and Creatinine

Urea and creatinine are the two major nitrogenous metabolic waste products. These markers can be clinically used to assess renal function, with the upper limits of 42 mg/dl (urea) and 1.2mg/dl (creatinine). However, each of them is susceptible to exogenous influences such as diet, extrarenal injury (for example, injury to the muscle) and biochemical reagents, when assessed using manual or semi-automated techniques. It is therefore advisable to request the two tests simultaneously to assess renal function. Alternatively, the rate of creatinine clearance can be measured by quantifying the level of creatinine in urine and its concentration in plasma (Zilva et al, 1988). In general, increases in urea and creatinine are suggestive of renal disease(s) that compromise the integrity of the nephrons.

2.1.4.2 Electrolytes

It is clinically advisable to measure the plasma or serum levels of some electrolytes when tubular dysfunction is suspected. For example, the concentration of bicarbonate [HCO_3^-] and potassium [K^+] usually decrease as a result of proximal or distal tubule dysfunction as they are involved in reabsorption and electrolyte balance respectively. In addition to the estimation of these electrolytes, the concentrations of phosphate [PO_4^{3-}], magnesium [Mg^{2+}], and uric acid need to be included as they, together with the urinary presence of amino acids, phosphate and glucose, are indicative of injury to the proximal tubule and therefore may help rule out failure in the distal convoluted tubule (Zilva et al, 1988).

2.1.4.3 pH

Urine pH (the level of hydrogen ion concentration [H^+]) can be semiquantified using dipsticks or precisely quantified using pH meter. The hydrogen ion [H^+] is mainly excreted by conjugating with ammonia [NH_3^+] in the form of ammonium [NH_4^+]. The physiological urine pH is 7. A pH of less than 7 is indicative of acidic urine and alkaline urine has pH above 7. Measurement of urine pH is additional yet important clinical information in the accurate diagnosis and nature of kidney stone (calculi). For example, urine pH displays strong acidity (very low pH) in kidney stone patients where the calculi are mainly composed of uric acid. Meanwhile, alkaline urine pH is displayed in patients where the calculi are predominantly composed of calcium and also in patients suffering from infection stones (Fairley, 1979). Furthermore, urine pH can be monitored regularly in such patients following treatment to reverse the acidosis or alkalosis.

2.1.4.4 Protein

Under physiological conditions, every filtered protein must be reabsorbed. However, the presence of protein in urine is not necessarily an indication of renal disease as temporary circumstances such as strenuous exercise and emotional stress (Kassirer and Harrington, 1979) may lead to proteinuria. Yet, the persistent presence of protein (mainly albumin) in urine is a pathologic feature usually suggesting renal dysfunction and therefore should be integrated with other tests suggestive of renal pathology.

2.1.4.5 Renal biopsy (Histology)

Despite its invasiveness, renal biopsy is the definitive diagnostic procedure to diagnose diseases involving different compartments of the kidney. The renal biopsy can be stained with hematoxylin and eosin (H&E) stain and the integrity of the glomeruli, Bowman's capsule, tubules and blood vessels can be assessed (Tisher and Croker, 1979).

Histologic evaluation of kidney biopsy is a reflection of the architecture of the various components of the kidney and unlike most other laboratory tests that use blood, urine, serum or plasma as specimens, it is less susceptible (if at all) to exogenous factors. Moreover, it is particularly useful in patients with sub-clinical changes.

2.1.4.6 Immunohistochemistry

Immunostaining of frozen or paraffin-embedded kidney biopsy is essential in a number of immune complex diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in order to evaluate the effect of auto-antibodies (Tisher and Croker, 1979; Wen et al, 2004). It is also useful in non-autoimmune diseases to look for possible mechanisms of tissue injury. For example, the immunostaining for complement component C3 in renal ischemia/reperfusion injury is crucially important in understanding the mechanism of tissue damage following I/R injury in rats. In addition to the semiquantitative immunohistochemical complement scoring, the degree of complement activation in biological fluids can be accurately and specifically quantified using pathway specific (classical, alternative or lectin pathway) diagnostic kits (Wen et al, 2004).

The Immune System

The immune system is broadly categorized into innate and adaptive (acquired) immunity. The innate immunity is the rapid and non-specific defense mechanism against microbial infections. The skin (as a mechanical barrier), the respiratory system (through the expression and secretion of cilia, mucus and surfactants) and the gastrointestinal system (due to gastric acid secretion) are the main physical defense barriers (Nairn and Helbert, 2002). The innate immunity also possesses extracellular molecules in order to rapidly clear microbial infections. These molecules include interferons, C reactive protein (CRP) and complement.

Interferons, type I α and β , inhibit the replication of double-stranded RNA viruses (by directly interfering the process of transcription), activate natural killer cells and amplify the killing action of CD8⁺ cytotoxic T cells. CRP is among the many molecules that respond during early (acute phase) infection by binding to the bacterial cell wall to induce phagocytosis. Other molecules that are released during the “*acute phase response*” are interleukins (IL-1 & 6) and tumor necrosis factor (TNF) (Nairn and Helbert, 2002). Complement is another, yet important, component of the innate immunity that is involved in speeding up the clearance of microbial infections in various orchestrated ways as described below.

The innate immunity is short lasting, and if the infection is not cleared soon enough, it alerts the adaptive immunity to deal with the established infection (Nairn and Helbert, 2002). The adaptive immunity, though slow to kick in, is highly specific and relatively long lasting (Nairn and Helbert, 2002).

2.2 The Complement System

Although the history of complement dates back to the times of Robert Koch and Louis Pasteur in the 1880s, the term “*complement*” was coined by Ehrlich and colleagues in 1899 (Lachmann, 2006). Following this description, four complement components have been identified between 1907 (C1) and 1926 (C4) (Lachmann, 2006). Between the two arms of the immune system, acquired and innate, the complement cascade is an integral component of the innate immunity and through its remarkable ability to distinguish self from non-self (Mollnes and Fiane, 2003), it serves as an essential defense tool against invading microorganisms (Girardi et al, 2006). However, failure of the system to differentiate self from non-self could well trigger and/or complicate a number of autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Mollnes and Kirschfink, 2006).

Despite the pioneering advances in immunobiology research, the complement system is still not fully understood. So far, about 40 different molecules have been identified as complement components. These molecules exist in different forms and have immensely unique roles in enabling the complement system as a defense mechanism. The system can be activated and regulated by any of the three independent yet interlinked pathways (Zipfel et al, 2006) namely the classical, alternative or lectin pathway of complement activation (Sacks et al, 2003).

Tight regulation of complement is critically important, a lack of complement regulation can result in deleterious deficiency conditions such as the hemolytic uremic syndrome (HUS), and macular degeneration (MD) (Ganesh et al, 2004; Zipfel et al, 2006), and excessive complement activation results in inflammatory conditions such as spinal cord injury (Reynolds et al, 2004), traumatic brain injury (Hicks et al, 2002), xenorejection (Anderson et al, 2003; Ghebremariam¹ et al, 2005; Ghebremariam² et al, 2005), ischemia reperfusion injury (Zhou et al, 2000; Thurman et al, 2003; Thurman et al, 2005; Ghebremariam et al, 2006), arteriosclerosis (Thorbjornsdottir¹ et al, 2006), recurrent miscarriage (Girardi et al, 2006), Alzheimer's disease (Daly and Kotwal, 1998), collagen-induced arthritis (Jha et al, 2005), acute respiratory distress syndrome (ARDS), acid-aspiration to the lungs, sepsis, and systemic inflammatory response syndrome (Mollnes and Kirschfink, 2006).

Although, complement in general plays a damaging role in all these ailments, any given condition is primarily driven by either of the pathways of complement activation. A brief description of each of the three pathways and the mechanisms of tissue injury follow.

2.2.1 The Classical Pathway and Disorders Associated

The classical pathway (CP) was named to reflect its first and classic discovery following the differential understanding of the complement system (Nairn and Helbert, 2002). The CP is composed of five pathway-specific complement components namely the C1q, C1r, C1s, C4 and C2, and the non-specific critical component, C3. In this chain reaction, C1 interacts with IgG (G1, G2 or G3 subunits) or IgM antibodies resulting in direct activation of C2 and C4 components and this results in the formation of classical pathway C3 convertase (C4b2a) (Figure 1). The C3 convertase then catalyses the cleavage of C3 to its active components C3a and C3b (Tosi, 2005).

Being a component of the innate immunity, the CP is physiologically involved in the clearance of invading microorganisms (opsonization) and stimulation of the B cells for antibody production (Whaley, 1985; Nairn and Helbert, 2002). The CP is activated by antigen-antibody interaction, which involves the recognition of an antigen by the Fc receptor(s) of an immunoglobulin and a further complexed interaction with the different components (Whaley, 1985). However, when the CP is defective, it results in a number of genetic diseases including systemic lupus erythematosus (SLE) and hereditary angioedema (HAE), as well as infectious diseases such as streptococcal infection (Rynes and Pickering, 1985). In contrast, excessive activation of the CP results in ischemia/reperfusion (I/R) injury of the skeletal muscle, heart, lung and intestine (Sacks et al, 2003). Brief descriptions of these ailments follow.

2.2.1.1 Systemic Lupus Erythematosus (SLE)

SLE is an autoimmune disease that occurs commonly in patients with complement deficiency. Depending on which classical complement component is defective, the probability of developing SLE varies from about 15% (C2 deficiency) to 90% (C1q deficiency) (Wen et al, 2004). The hallmarks of SLE include juvenile onset, increased susceptibility to light, low white blood cell (WBC) and platelet counts, rash, arthritis and cardiac complications (Rynes and Pickering, 1985; Wen et al, 2004; Abou-Raya and Abou-Raya, 2006).

2.2.1.2 Hereditary Angioedema (HAE)

HAE is non sex-linked (autosomal) disorder that manifests in a heterozygous state as a result of mutation(s) in the C1-Inh gene (Nairn and Helbert, 2002). C1-Inh is a CP regulatory protein for C1r and its natural substrate (C1s) as well as the coagulation cascade molecules such as factor XI, the fibrinogen activator (plasmin) and kallikrein (Wen et al, 2004).

Thus, deficiency of C1-Inh affects three distinct pathways, namely the coagulation, complement and the kinin-kallikrein pathways.

Biochemically, HAE exists in two forms. Type I HAE is characterized by low levels of C1-Inh due to decreased production of antigenic levels and manifests (penetrance rate) in 85% of children with C1-Inh mutation (Wen et al, 2004). Type II HAE is distinguished from type I by its normal or high antigenic levels of C1-Inh (Wen et al, 2004). However, the protein produced is not biologically active probably due to mutation (s) that affect the folding of the protein or other posttranslational properties.

In general, advanced HAE symptoms include edematous swelling in the face and extremities, increased capillary permeability, upper respiratory tract obstruction, and vomiting as a result of bowel wall edema (Nairn and Helbert, 2002; Wen et al, 2004; Bork¹ et al, 2006; Bork² et al, 2006). The possible treatment for type I HAE is the administration of purified C1-Inh to normalize the antigenic levels (Asghar, 1996; Nairn and Helbert, 2002).

The other, probably rare, form of angioedema is the non-hereditary one, known as acquired angioedema (AAE). Here, the antigenic level of C1-Inh produced is normal but the protein is cleared prior to the fulfillment of its physiological duty (Wen et al, 2004).

2.2.1.3 Microbial Infection

Until the development of a fully functional adaptive immunity, the innate immunity is responsible for the defense against microbial infections. Because the adaptive immunity takes over during adolescence, the inability to clear microbial infection as a result of classical complement component deficiency is only noticed during childhood. For example, the lack of functional C2 increases the susceptibility to encapsulated bacterial infection in children (Wen et al, 2004; Sjöholm et al, 2006). One of the infections that occur as a result of a defective classical pathway is pneumonia caused by the gram-positive cocci, *Streptococcus pneumoniae* (Mold et al, 2002; Sjöholm et al, 2006).

2.2.2 The Lectin Pathway (LP)

The lectin pathway (LP) is the most recently identified pathway of complement activation (Tosi, 2005). The LP possesses significant resemblance to the classical pathway (CP). However, unlike the CP, there is no participation of antibodies. This component of the innate immunity contributes to defense against microbial infection by the interaction of its mannose binding lectin (MBL) to saccharide molecules on bacterial surface (Trouw et al, 2005). This interaction activates serine protease enzymes that are homologous to C1r and C1s components of the classical pathway as described earlier. The activated molecules further activate C2 and C4 in order to form the classical pathway C3 convertase (C4b2a) (Figure 1). This results in direct activation of C3, a complement component where all the currently known complement activation pathways converge (Tosi, 2005).

Any aberration or mutation in the coding or promotor region of the MBL gene could deviate the physiology of the aforementioned process of LP activation and may result in decreased levels of MBL in serum that subsequently results in a number of diseases including recurrent bacterial infections, cystic fibrosis, sepsis, spontaneous abortion and SLE (Wen et al, 2004; Trouw et al, 2005). On the other end, an excessive level of MBL is associated with various kidney diseases such as IgA nephropathy and lupus nephritis (Trouw et al, 2005) as well as cardiac ischemia/reperfusion injury (Sacks et al, 2003).

2.2.3 The Alternative Pathway (AP) and Disorders Associated

The AP, a component of the innate immunity, is one of the earliest pathways to be identified. The name was probably coined to indicate the separate (alternative) way of complement activation independent of antibodies (classical pathway) as demonstrated by Ritz in 1912 (Lachmann, 2006). Peter Lachmann, in his recently published extensive review article on the genesis of complement, described H. Ritz as the first researcher to have activated a complement component independent of antibodies by the use of cobra venom. Two years later, Ritz's work was supported by Coca who activated the now called alternative pathway (AP) using the yeast cell wall, zymosan (Lachmann, 2006). More than four decades later, in 1954, Pillimer identified a factor necessary for the alternative pathway activation; he named this factor Properdin (Lachmann, 2006). Sixteen years after revealing Properdin, in 1970, one of the key components of the AP was identified by Boenisch and Alper, and was named as Factor B by Goodkofsky and Lepow in 1972 (Lachmann, 2006). In the same year, Hans Muller-Eberhard and Gotze nomenclatured the complement component C3 and finally, to complete the picture, the last component of the AP was identified and named as Factor D (Lachmann, 2006).

Under physiological conditions, the AP is initiated by the cleavage of C3 into its active form C3b, which then binds to a cleavage product of Factor B, Bb. The zymogen factor B (93kDa) is cleaved by factor D into Ba (33kDa) and the proteolytic enzyme Bb (60kDa) (Quidel corporation, 2004; Ghebremariam³ et al, 2005). Bb interacts with C3b and forms the complex C3bBb (Quidel corporation, 2004), which is also known as the alternative pathway C3 convertase. The Bb fragment further cleaves C3 to the anaphylatoxin peptide C3a and the C3b fragments. The cleaved C3, C3b, interacts with the complex C3bBb to form C3bBbC3b complex also known as the alternative pathway C5 convertase. This proteolytic enzyme then cleaves C5 to the anaphylatoxin peptide C5a and the C5b fragments (Quidel corporation, 2004).

If the alternative pathway is defective as a result of mutation or autoantibodies, or excessively activated as a result of injury, it can lead to a myriad of pathogenic infections and debilitating conditions such as rheumatoid arthritis (RA), hemolytic uremic syndrome (HUS), lupus nephritis, membranoproliferative glomerulonephritis (MPGN), anti-phospholipid syndrome (APS), asthma, neurological ailments such as multiple sclerosis, age-related macular degeneration (ARMD), and renal ischemia/reperfusion injury (Holers et al, 2004; Zipfel et al, 2006). A brief description of these conditions and an overview of the mechanisms of disease causation follow.

2.2.3.1 Hemolytic Uremic Syndrome (HUS)

HUS is a syndrome of diseases broadly classified into:

1) Typical HUS is a non-genetic form caused by members of the gram-negative bacteria capable of releasing shiga toxin to the blood stream (toxemia). For example, members of the *Enterobacteriaceae* family such as *Shigella dysentery* subtype 1 and *Escherichia coli* subtypes O-26, O-111 and O-157 (Zipfel et al, 2006). Typical HUS occurs almost exclusively in children and is characterized by diarrhea (Ganesh et al, 2004), self-limiting and non-recurrent infection that can be cured in majority of the patients (Zipfel et al, 2006).

2) Atypical or adult form HUS is a genetic disorder caused by point mutations or deletions in the genes that code for alternative pathway C3 convertase regulatory proteins.

Most of these mutations were detected in Factor H and some in membrane cofactor protein (MCP, CD46) and Factor I (Ganesh et al, 2004; Zipfel et al, 2006). These mutations result in low levels of the mutated complement control protein such as Factor H and therefore unable to effectively bind to C3b and hence fail to regulate complement activation (Ganesh et al, 2004; Zipfel et al, 2006).

Hyperactivation of the C3 convertase in this circumstance leads to the depletion of the basal circulating complement as manifested by low levels of the classical and the alternative complement activity *in vitro* (Zipfel et al, 2006). This form of HUS is characterized by hemolytic anemia, low platelet count and seeding of platelet thrombi into the kidneys usually causing renal failure. Consequently, patients with atypical HUS usually have poor prognosis (Zipfel et al, 2006).

2.2.3.2 Rheumatoid Arthritis (RA)

RA is an autoimmune disease characterized by joint inflammation, caused by hyperactivation of the alternative pathway. The hallmarks of the disease include decreased AP activity, accumulation of AP breakdown products in the joint fluid, and increased complement activity in the peripheral blood (Holers et al, 2004). The predominant role of AP in RA was demonstrated by the induced resistance of Factor B deficient DBA1/j mice to anti-glucose-6-phosphate isomerase (GPI) antibody-induced arthritis and to collagen induced arthritis (CIA) (Holers et al, 2004). Meanwhile, the beneficial role of anti-complement proteins such VCP further confirmed the role of complement in RA using the CIA mice model (Jha et al, 2005).

2.2.3.3 Lupus nephritis (LN)

LN is an autoimmune disease associated with SLE, which primarily affects the glomerulus causing excretion of protein in urine, deposition of IgG antibodies, and other renal abnormalities (Holers et al, 2004; Li et al, 2006). The role of complement in LN has been implicated through the inhibition of disease progression by the use of soluble and membrane-bound complement inhibitors such as Crry and anti-C5 monoclonal antibody. The predominant role of the alternative complement pathway was demonstrated from the observed protection of Factor B deficient mice and through inhibition of the complement component C5, a building block of MAC (Holers et al, 2004). The anti-inflammatory drug prednisone and the immunosuppressive regimens of cyclophosphamide and mycophenolate mofetil are clinically accepted therapeutic options for LN (Flores-Suarez, 2006).

2.2.3.4 Membranoproliferative Glomerulonephritis (MPGN)

Histologically, there are two types of MPGN, namely MPGN I and II. The deposition of complement in the subendothelial layer distinguishes type I MPGN from type II, where complement is densely deposited in the basement membrane of the glomerular capillary wall (Pickering et al, 2006; Zipfel et al, 2006). Common molecular defects in both forms of MPGN are the significant reduction or absence of the complement regulatory protein Factor H and low levels of circulating complement due to their local consumption usually caused by hyperactivation of the alternative pathway C3 convertase (Dragon-Durey et al, 2004; Zipfel et al, 2006). The severity of the disease may vary based on the number, site and type of mutation (heterozygous, compound heterozygous, homozygous) in Factor H and the presence of autoantibodies in the basement membrane of the glomerulus (Zipfel et al, 2006).

Under physiological conditions Factor H regulates complement activation and contributes to the maintenance of glomerular function. However, due to a single, double or multiple mutation (s) in the active site of the Factor H gene, the encoded protein becomes defective or even absent and hence unavailable to regulate complement activation in the glomerulus (Dragon-Durey et al, 2004; Zipfel et al, 2006). Interestingly, in some MPGN patients, the secretion of Factor H is intact. However, the production of autoantibodies that block the secreted Factor H encourage deregulation of complement activation (Zipfel et al, 2006).

2.2.3.5 Anti-phospholipid Syndrome (APS)

APS is a complication triggered by antiphospholipid (aPL) autoantibodies against placenta during gestation. APS is associated with spontaneous abortion, fetal growth limitation and coagulation cascade disruption (Holers et al, 2004; Velayuthaprabhu and Archunan, 2005; Girardi et al, 2006).

There is direct and indirect evidence that suggests the damaging role of complement in APS, and thus complications during pregnancy. For example, the inhibition of complement in pregnant mice using the complement inhibitor, complement receptor type 1 (CR-1)-related gene γ (Crry), ameliorated spontaneous abortion (Holers et al, 2004; Girardi et al, 2006). Moreover, the absence of Crry endogenous expression induced fetal loss, suggesting the significant role of complement (Holers et al, 2004). Furthermore, applying the principles of knockout technology, several studies (Holers et al, 2002; Girardi et al, 2003; Pierangeli et al, 2005) have demonstrated the crucial roles of Factor B, C3, C4 and C5 complement components in APS, supporting the contributions of the lectin, classical and the alternative pathways of complement activation.

Moreover, by the use of neutrophil depleting monoclonal antibodies, the role of these inflammatory cells (Girardi et al, 2003) and the suggestive role of lethal inflammatory chemicals such as free radicals, cytokines and chemokines in aggravating APS was proposed and the study speculated effective intervention in APS-related fetal loss and growth defects in the clinic (Girardi et al, 2006).

2.2.3.6 Asthma

Asthma is a hypersensitivity reaction induced by allergens resulting in airway inflammation and obstruction (Nairn and Helbert, 2002). The allergens trigger cell-mediated immune responses that involve CD4⁺ T-helper memory cells and the subsequent production of cytokine molecules to participate in airway inflammation (Holt et al, 1999). Although the role of cell mediated response in this pulmonary disease is relatively well realized, the understanding of the involvement of complement system in asthma is still in its infancy. However, preliminary studies have demonstrated the elevation of the inflammatory complement components C3a and C5a in bronchial washings from asthmatic patients (Krug et al, 2001; Holers et al, 2004). Moreover, the lack of the anaphylatoxin peptide C3a receptor in experimental animals had been shown to correlate with reduction in disease progression. Furthermore, inhibition of complement using Crry-Ig or anti-factor B monoclonal antibody in sensitized mice as well as the use of Factor B deficient mice supported the role of complement in experimental asthma and suggested the potential benefits of complement inhibition in clinical asthma (Holers et al, 2004; Taube et al, 2006).

2.2.3.7 Neurological Ailments

There are several reasons to believe that inflammatory chemicals and complement play a role in many diseases of the nervous system. Examples include Alzheimer's disease, Parkinson's disease, head injury, traumatic brain injury, multiple sclerosis and age-related macular degeneration. For example, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce the risk of developing Alzheimer's and Parkinson's diseases, multiple sclerosis, and age-related macular degeneration suggesting the damaging role of inflammation in the development of these neurological ailments (McGeer et al, 2005). A key component that gets excessively activated in the process of neuroinflammation is the complement cascade (Kulkarni et al, 2004). Once complement is activated, it in turn amplifies inflammation through the secretion of the inflammatory C3a, C4a and C5a peptides (Kulkarni et al, 2004; McGeer et al, 2005).

For example, in Alzheimer's disease (AD), complement is activated by the direct interaction of the β -amyloid plaques to the classical pathway component C1q (Daly and Kotwal, 1997; McGeer et al, 2005) suggesting the role of complement in AD. Moreover, complement inhibition using Crry-Ig has been shown to be neuroprotective in head injury mice model (Rancan et al, 2003; Leinhase, 2006).

Therefore, in addition to anti-inflammatory drugs, the use of effective complement inhibitors may also provide promising insights towards neuroprotection (Kulkarni et al, 2004).

Multiple sclerosis is another neurodegenerative disorder where complement in general and the alternative complement pathway in particular are believed to be involved in the destruction of neurons. Indirect evidence of this hypothesis is the significant reduction in disease progression in Factor B deficient mice (Nataf et al, 2000; Holers et al, 2004).

In age-related macular degeneration (ARMD), a progressive degeneration of the macular densa of the eye which results in blindness, the alternative pathway of complement activation regulator, Factor H, has been strongly implicated (Tuo et al, 2006). The point mutation in the short consensus repeat 7 (SCR 7) substitutes histidine for tyrosine (H402Y) rendering Factor H unable to effectively regulate the alternative pathway (Zipfel et al, 2006). The importance of the site is probably due to its location in the heparin and C-reactive protein (CRP) binding site of Factor H. The sequencing of the SCR 7 domain in ARMD patients' has revealed more than 2-fold higher frequency of the mutation than in the normal control groups (Zipfel et al, 2006).

2.3 Role of Complement in Ischemia/Reperfusion (I/R) Injury

Ischemia/reperfusion (I/R) injury is a complication induced and/or amplified by several factors and is known to significantly affect the physiological recovery of the organ being exposed both experimentally and clinically (Inal et al, 2003). Vascularized organs such as the heart, kidney and intestine are mainly susceptible to I/R injury (Daemen et al, 1999).

I/R injury triggered by revascularization surgery and organ transplantation may account for delayed graft recovery, and associated morbidity and mortality (Hughes et al, 1996; Zhou et al, 2000). The multiple factors involved in I/R injury include inflammatory molecules, free radicals, and hyperactivation of the complement system (Zhou et al, 2000; De Vries¹ et al, 2003; Sacks et al, 2003; Mollnes and Fiane, 2003; Mollnes and Kirschfink, 2006). There is strong correlation between deregulation of complement and the severity of ischemia independent of the organ source (Mollnes and Fiane, 2003). However, the driving force, within the three major pathways of complement activation, seems to vary depending on the type of organ being exposed to the injury. According to Sacks et al (2003), the CP drives I/R injury in the heart, lungs, skeletal muscle and intestine.

For example, it has been reported that mice defective in IgG and IgM production as a consequence of deficiency of the complement receptors (CRs) 1 (CR1 = CD35) and 2 (CR2 = CD21), are protected from intestinal I/R injury.

Furthermore, treatment of rabbit and pig cardiac I/R injury models with C1-Inh ameliorated myocardial I/R injury. In addition, C1-Inh has been shown to reduce I/R injury in sheep after lung transplantation suggesting the crucial role of the classical complement pathway in these various injury models (Sacks et al, 2003).

2.3.1 Role of Complement in Renal Ischemia/Reperfusion Injury

The kidneys are among the most susceptible organs to ischemia/reperfusion (I/R)-induced injury. Specifically, the tubular cells are highly susceptible to I/R injury. Due to the lack of oxygen supply during the ischemia time, the injured tubular epithelial cells are able to locally activate the complement pathway through a poorly understood mechanism (Inal et al, 2003). However, the alternative pathway of complement activation has been reported to be predominantly responsible for the I/R injury in the kidneys (Sacks et al, 2003; Stahl et al, 2003). The continuous local production and activation of complement and the lethal MAC generation in the renal tubules overwhelm the natural complement inhibitors and ultimately destroy the renal tubules irreversibly.

The tubular collapse is amplified due to the fact that the tubular epithelial cells are able to provide all the necessary complement activation ingredients when injured (Inal et al, 2003). The main factors involved in renal ischemia/reperfusion injury are the complement (mainly through its alternative cascade), inflammatory chemicals (predominantly derived from activated complement components cleavage) and the highly reactive free radicals mainly generated during reperfusion time. Supportive evidence to the involvement of these variables comes from a number of studies that have shown the effects of the direct inhibition of complement using anti-complement proteins (Wen et al, 2004; Yamada et al, 2004), monoclonal antibodies (Williams et al, 1997; De Vries¹ et al, 2003; Quigg, 2003; DiLillo et al, 2006; Mollnes and Kirschfink, 2006), complement-inhibitor expressing transgenic animals (Schiller et al, 2001), and certain complement component knock out animals (Zhou et al, 2000; Lien et al, 2003; Stahl et al, 2003; Thurman et al, 2003; Yamada et al, 2004; Thurman et al, 2006). All these studies illustrated the essential role of complement inhibition and its possible benefits in the clinic.

Furthermore, the role of free radicals in renal I/R injury has been demonstrated in various studies and is exclusively described in the literature (Rhoden et al, 2002; Fujii et al, 2003; Kielar et al, 2003; Singh and Chopra, 2004; Rodriguez-Reynoso et al, 2004). A brief overview of the different inhibitors and their mechanism of action follow.

2.3.1.1 Therapeutic Role of anti-complement Molecules in Renal I/R Injury

Although the kidney is considered to be resistant to anti-complement therapy following ischemia/reperfusion (I/R) injury (Park et al, 2001; Inal et al, 2003), inhibition of the alternative complement pathway at different stages of complement synthesis has been shown to curb the persistence of I/R-induced renal injury. For example, De Vries² et al (2003) demonstrated the prevention of renal I/R injury using lysophosphatidic acid (LPA). LPA is a multifunctional molecule with anti-apoptotic, anti-inflammatory and anti-complement properties. The anti-apoptotic activity is due to its ability to inhibit activation of caspase-7, an apoptosis pathway molecule, following renal I/R injury. Its anti-inflammatory activity is the result of the inhibition of tumor necrosis factor- α (TNF- α) and inhibition of neutrophils. LPA has the ability to dose dependently inhibit the complement components C3, C6 and C9 (De Vries² et al, 2003). LPA has been shown to immensely ameliorate I/R-induced renal injury in mice (De Vries² et al, 2003). In another study, the same authors demonstrated significant protection from renal I/R injury by inhibition of the complement component C5 using anti-C5 monoclonal antibody termed as BB5.1. Besides its effective complement inhibitory activity, BB5.1 has also been shown to inhibit the aggregation of inflammatory cells (neutrophils) and the MAC-induced late apoptosis in murine model (De Vries² et al, 2003).

Cobra venom factor (CVF), a controversial complement regulator (Park et al, 2001; Yamada et al, 2004 Vs Mollnes and Kirschfink, 2006; Lachmann, 2006) has also been shown to improve I/R-induced renal injury in CD55/CD59 double knock out mice but failed to provide any therapeutic value in wild type mice (Yamada et al, 2004). Meanwhile, the soluble form of Crry was reported to play negligible therapeutic role in renal I/R injury (Park et al, 2001). However, the membrane bound form of the protein was shown to abrogate I/R-induced renal injury (Schiller et al, 2001) supporting the role of complement in renal I/R injury.

Other soluble or membrane bound forms of complement inhibitors with beneficial role in attenuating renal I/R injury might emerge in the future. For example, the newly identified complement inhibitory activity of the herbal formulation Curcumin (Kulkarni et al, 2005),

together with its anti-inflammatory property, may in the future play a role in the prevention of I/R-induced renal injury.

2.3.1.2 Genetic Engineering Tools to Inhibit Complement

Major advances in molecular biology and genetic engineering have allowed researchers to further understand the mechanisms of different diseases. For example, the use of transgenic animals expressing critical molecules, and the use of gene-knock out animal models, have contributed vast knowledge to the field of science in general, and transplantation medicine and immunobiology in particular. Brief overviews of these approaches follow.

2.3.1.2.1 Complement Inhibition using Transgenic Animals

The local expression of Crry, an active murine C3 convertase inhibitor, in the glomerular and tubular membranes of the kidney has been shown to completely abrogate I/R-induced renal injury (Schiller et al, 2001) compared to the markedly elevated tubular damage in the wild type mice, suggesting the injurious role of complement and the therapeutic value of complement inhibition in I/R-induced renal injury.

2.3.1.2.2 Complement Inhibition using Knock Out Animals

A number of studies have supported the damaging role of complement in I/R-induced renal injury. These studies have developed novel techniques to understand the contribution of each complement component using target specific knock out technology. For example, the novel and classically cited (above 90 citations) work by Zhou et al (2000) have meticulously studied the effect of C3, C4, C5 or C6 deficiency in the genesis of renal I/R injury. In their study, the deficiency of C3, C5 or C6 was protective from I/R-induced renal injury in mice, suggesting the crucial role of the alternative complement pathway in general and the membrane attack complex (MAC) in particular. However, the lack of C4 did not provide any degree of protection suggesting the insignificant role of the classical complement pathway in renal I/R injury (Zhou et al, 2000). Moreover, the replacement with human C6 in the C6-deficient mice rendered the susceptibility to a degree of the wild type mice suggesting further the independent role of C6 in renal I/R injury (Zhou et al, 2000). Furthermore, Thurman and his colleagues (2003) also demonstrated the critical role of the alternative complement pathway by using Factor B deficient mice. In the absence of Factor B gene, the animals were partially protected from I/R-induced renal damage compared to the increased tubular injury in the Factor B sufficient mice (Thurman et al, 2003).

Recently, Thurman et al (2006) have further demonstrated the involvement of the alternative pathway by showing the increased susceptibility of Crry heterozygous knockout mice to renal I/R injury.

In addition to the critical role of the alternative complement pathway in renal I/R injury, the contribution of the lectin pathway of complement activation has also been demonstrated by the direct relationship of the mannan-binding lectin (MBL) gene knockout mice, the consequent diminishing of C3 deposition, and the subsequent protection from I/R-induced renal injury (Moller-Kristensen et al, 2005) indicating the role of complement inhibition in conferring resistance to renal I/R injury. Similarly, Yamada et al (2004) evaluated the roles of the natural complement regulators, CD55 and CD59 in renal I/R injury. Although the deficiency of CD55 exposed the animals to a greater degree of I/R injury, the lack of CD59 did not affect the degree of injury. Interestingly, the simultaneous lack of both genes rendered the animals highly susceptible to the injury, suggesting their additive role in providing protection against renal I/R injury (Yamada et al, 2004).

Lien et al (2003) have extensively reviewed the effect of 16 independent knock out genes in the genesis of I/R-induced renal injury. For example, the lack of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) gene (NCX1) provided protection by regulating the accumulation of intracellular Ca^{2+} , known to be involved in hypoxia-induced renal injury (Matsumura et al, 2002; Yamashita et al, 2003). The deficiency of caspase-1, a member of the cysteine proteases involved in activating lethal cytokines, offered significant functional and structural protection from tubular damage as well as reduced the influx of neutrophils by inhibiting the activation of the proinflammatory molecule interleukin-18 (IL-18) (Melnikov et al, 2001). Furthermore, the lack of the growth factor midikine has been reported to protect renal I/R injury by inhibiting the activity of lethal chemokines (Lien et al, 2003). Intercellular adhesion molecule-1 (ICAM-1) deficiency also reduced renal I/R injury and improved survival in mice by preventing the adhesion and aggregation of neutrophils to the renal tubular epithelial cells (Kelly et al, 1996).

In conclusion, there is overwhelming evidence that the complement cascade plays a damaging role in ischemia/reperfusion injuries. For example, different pharmacological agents that directly or indirectly regulate the complement cascade have been shown to attenuate I/R injury in dogs, mice and rats (Stahl et al, 2003; Kitada et al, 2002; Yagmurduur et al, 2003).

In addition, transgenic animals expressing complement control proteins (Schiller et al, 2001; Knight et al, 2005) or critical complement component knock out animals (Zhou et al, 2000; Stahl et al, 2003; Thurman et al, 2003; Thurman et al, 2006) have strongly suggested the role of the complement cascade in I/R injury and provided further insights to the design of target-specific therapeutics.

THEREFORE IT WAS HYPOTHESIZED THAT INHIBITION OF EXCESSIVE COMPLEMENT SYNTHESIS IN THE RENAL SYSTEM FOLLOWING ISCHEMIA/REPERFUSION (I/R) INJURY MIGHT BE A FEASIBLE APPROACH TO AMELIORATE ISCHEMIC DAMAGE. BASED ON THIS HYPOTHESIS, THE THERAPEUTIC ROLE OF A COMPLEMENT INHIBITOR, THE VACCINIA VIRUS COMPLEMENT CONTROL PROTEIN (VCP), WAS ELUCIDATED FOLLOWING ISCHEMIA/REPERFUSION-INDUCED RENAL INJURY IN RATS.

The vaccinia virus complement control protein (VCP) was isolated from vaccinia virus infected cells (Kotwal and Moss, 1988). It has structural and/or functional homology to members of the regulators of complement activation (RCA) family (Smith et al, 2000). VCP regulates both the classical and the alternative pathways of complement activation through its ability to block the activated C3 and C4 early complement components from stimulating further complement components including the formation of the membrane attack complex (MAC, C5b-9) (Kotwal, 1997). This terminal complement component and the C3 are believed to be injurious in renal I/R injury (Zhou et al, 2000).

2.3.1.3 Anti-inflammatory Agents in Renal I/R Injury

Although inflammation may be a direct result of complement activation and cleavage, which generates powerful proinflammatory peptides such as C3a, C4a and C5a, or may be it is initiated through the accumulation of free radicals following reperfusion injury, it is still considered as an important independent factor in I/R-induced renal injury. Its direct inhibition has been accounted to reduced tissue injury. For example, the anti-inflammatory drug, FR167653, has been shown to ameliorate renal I/R injury in dogs. FR167653 works by inhibiting the proinflammatory chemicals IL-1 β and TNF- α (Kitada et al, 2002). Furthermore, the anti-inflammatory property of heparin, apart from its anticoagulant activity, has been shown to reduce the deposition of IgM and C3 as evaluated by immunostaining (Yagmurduur et al, 2003). The heparin treated rats displayed lower lipid peroxidation and serum creatinine than the untreated I/R injury group. The electron microscopic study also revealed an increased proximal and distal tubule injury in the histology of the untreated group suggesting further the anti-inflammatory action of heparin and its potential benefits in limiting I/R-induced renal

injury (Yagmurdur et al, 2003). Moreover, the inhibition of the inflammatory cyclooxygenase (COX) enzymes 1 and 2 using indomethacin or rofecoxib drugs provided significant improvement in renal structure and function following I/R injury (Feitoza et al, 2005).

2.3.1.4 Free Radical Scavengers

The role of free radicals in the pathogenesis of renal I/R injury has been extensively documented. The use of natural and synthetic free radical scavengers in renal I/R injury animal models have identified a number of promising inhibitors that can limit the progression of renal I/R injury. For example, treatment with L-carnosine, an antioxidant and free radical scavenging peptide, prior to ischemic injury has been shown to dose dependently reduce the levels of plasma creatinine and BUN compared to the untreated control group (Fujii et al, 2003). Moreover, the histopathologic findings revealed a decreased medullary congestion and necrosis of the tubular epithelial cells in the rats which received treatment. Meanwhile, pretreatment of rats with the grapefruit derived bioflavonoid herb, naringin, protected the animals from renal I/R injury as evaluated by serum creatinine, BUN and creatinine clearance measurements (Singh and Chopra, 2004). The histopathologic results also supported the protective role of naringin as revealed by decreased tubular cell swelling, tubular dilation, tubular epithelial cell necrosis, interstitial edema and deposition of hyaline casts (markers of renal failure) in the treated animals. The protective effect of naringin was thought to be due to its antioxidant and free radical scavenging properties (Singh and Chopra, 2004). In addition to the aforementioned studies, the therapeutic role of melatonin, a multifunctional hormone secreted by the pineal gland in the brain, was also evaluated in renal I/R injury. This free radical scavenging molecule improved both renal structure (by limiting the degree of tubular damage by about 50%), and function. Moreover, treatment with melatonin has improved the survival rate by 30% compared to the untreated group (Rodriguez-Reynoso et al, 2004).

In conclusion, a vast number of studies have demonstrated the therapeutic potential of blocking complement (mainly the alternative pathway), inflammation and/or free radicals as promising approach in preventing the ischemia/reperfusion-induced renal injury. *Figure-1 (shown above) is reproduced from Mollnes et al (2002) to summarize the initiation, convergence and divergence of the 3 known complement activation pathways and the possible therapeutic intervention spots from complement mediated tissue injury (Mollnes et al, 2002).*

CHAPTER 3

CHARACTERIZATION OF HUMANIZED RECOMBINANT VACCINIA VIRUS COMPLEMENT CONTROL PROTEINS (hrVCPs): IMPLICATIONS IN BIOLOGICAL APPLICATION

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3.4 DISCUSSION

3.1 INTRODUCTION

Vaccinia virus complement control protein (VCP) inhibits both the classical and the alternative pathways of complement activation and enables the vaccinia virus to evade host immune defense. Proficient downmodulation of complement activation decisively depends on the competence of the inhibitory protein to efficiently bind the activated third (C3b) and fourth (C4b) host complement components. Incorporating the resourceful information obtained from sequence alignment with homologous *orthopoxviral* proteins such as the highly efficient human complement inhibitor, the smallpox inhibitor of complement enzymes (SPICE), and host complement inhibitors such as membrane cofactor protein (MCP) and soluble complement receptor type-1 (sCR1), crystallographic structure of the recombinant VCP (rVCP) (Murthy et al, 2001) and molecular modeling exercises (Liszewski et al, 2000; Murthy et al, 2001; Ganesh et al, 2004), putative sites have been identified on the yeast cell expressed rVCP (Smith et al, 2000) as contact sites for C3b/C4b. Site-specific mutagenesis studies substituted amino acid (s) in the postulated sites.

The aim of this study was to characterize the complement inhibitory activities of the generated mutant proteins in comparison to rVCP and the natural VCP isolated from vaccinia virus infected cells. The activities were compared for both the classical and the alternative pathways by employing antibody mediated and zymosan-induced *in vitro* assays respectively.

3.2 MATERIAL AND METHODS

3.2.1 Identification of Critical Amino Acids for VCP Mutagenesis

Based on a number of independent observations, putative amino acids have been identified in the rVCP sequence as critical sites to enhance its complement inhibitory activity. Alignment of amino acid sequence with SPICE, crystallized structure of rVCP and computer based modeling exercises have deduced the C3b/C4b contact points in the rVCP sequence as described below.

3.2.1.1 Sequence Alignment

The conceptually translated amino acid sequence of recombinant VCP (rVCP) was aligned with the amino acid sequence of serine protease inhibitor of complement enzymes (SPICE) searched using the NCBI protein-protein blast (blastp) search tool. The blast alignment revealed 95% identity with the D15L protein (known as SPICE) of the Variola virus Congo-1965 strain (Massung et al, 1996) as shown in Figure-1 below. The two proteins were then aligned using the blast 2 sequences (bl2seq) tool and the differences are displayed as shown in Figure-1. The difference was mainly in SCR-2 (41.7%) and minimal in SCR-1 (0%) suggesting the role of the former SCR in offering SPICE a differential advantage in inactivating human complement about 100-fold more than rVCP (Rosengard et al, 2002). Therefore, SCR-2 was a fine candidate for mutagenesis studies.

rVCP	CCTIPSRPINMKFKNSVETDANANYNIGDTIEYLCCLPGYRKQKMGPIYAKCTGTGWTLFN	60
SPICE	60
rVCP	QCIKRRCPSPRDIDNGQLDIGGVDFGSSITYSCNSGYHLIGESKSYCELGSTGSMVWNPE	120
SPICEH.....Y....Y....K.....K	120
rVCP	APICESVKCQSPPSISNGRHNGYEDFYTDGSVVITYSCNSGYSLIGNSGVLCSSGGEWSDPP	180
SPICEL.....N.....N..	180
rVCP	TCQIVKCPHPTISNGYLSSGFKRSYSYNDNVDFKCKYGYKLXXXXXXXXXXPGNTWKPELP	240
SPICEL.....T.....L.....Q....	240
rVCP	KCVR	244
SPICE	244

Figure 1: Amino acid sequence alignment of rVCP (WR strain) with SPICE (Congo-1965 strain) revealing 12 amino acid differences.

3.2.1.2 Modeling Studies

Computer based modeling studies are the backbone of pharmacognosy and computational chemistry due to their ability to provide essential information regarding the physicochemical properties of molecules with known structures, and rational prediction of unknown structures and hence better insights to the design of target-specific drugs. Here, the Swiss-Model program (www.expasy.org) was used to draw the space filling models of rVCP, ^{rVCP}E108K and ^{rVCP}H98Y, E102K, E120K (abbreviated elsewhere in the thesis as hrVCPs) as displayed in Figure 2 (A-C). *Figure 2D was generously modeled by Dieter Blaas (University of Vienna, Austria) using the "GRASP" software.* The Discovery Studio Viewer Pro (DS Viewer pro; Accelrys Inc., San Diego, USA) software package was used to view and edit the modeled structures. The DS viewer Pro is user-friendly tool to view, understand, sketch and modify molecular structures.

3.2.1.3 Crystallography

Determining the crystal structure of a protein provides original information about the structural appearance (linear, helical or coiled) of the protein, hidden/exposed sites and therefore their importance in mutagenesis and target-oriented drug design studies. The crystal structure of rVCP was determined at 2.1Å⁰ (Murthy et al, 2001). This information was crucial in identifying the critical amino acids in rVCP for site-specific mutagenesis studies.

3.2.2 Cloning of hrVCPs

The cloning of hrVCPs into pPIC9 expression vector and the transformation of GS 115 *Pichia pastoris* yeast cells was recently published (Ghebremariam³ et al, 2005) (*These experiments were performed by Dr. Oduyayo O. Odunuga as part of his postdoctoral research as described in the "Declaration" section of the thesis.*) The yeast cells putative to express the VCP gene were screened by PCR (Figure 3) using the AOX-1 universal primers (Invitrogen Life Technologies) and the actual recombinant clones were used for further expression studies. The conditions of the PCR used in the screening experiments are summarized in Table 1A and B below. The amplified products (15µl each) were resolved in a 1.2% agarose gel in a horizontal gel system using the conducting buffer Tris-Acetate EDTA (TAE) (Appendix A) at 70V for 2 hours. The 1kb⁺ molecular weight marker (Promega Life Science, WI, USA) (Appendix B) was used as a standard size marker.

Table 1A: Components of the AOX-1 PCR performed on putative recombinant yeast cells

Component	Stock concentration	Final concentration
#Primers	50pmol/μl	1pmol
dNTPs	200mM	40mM
MgCl ₂	25mM	1.5mM
Reaction buffer	10X	1X
Taq polymerase	5U/μl	1.25U
DNA	Single colony	-

#Primers used in the 5' → 3' orientation are: the forward (GACTGGTTCCAATTGACAAGC) and the reverse (GCAAATGGCATTCTGACATCC). The reaction mix was in 25μl and the samples were amplified using the PCR Sprint (ThermoHybaid) PCR machine. The conditions of the reaction are shown in Table 1B.

Table 1B: Amplification conditions of the AOX-1 PCR performed on putative recombinant *P. pastoris* colonies

Step	Temperature (°C)	Time (min)	Cycle
Initial denaturation	94	2	1
Denaturation	94	1	30
Primer annealing	55	1	30
Extension	72	1.3	30
Final extension	72	5	1

3.2.3 Production of rVCP/hrVCPs in Flask

The recombinant proteins (^{rVCP}E108K and ^{rVCP}H98Y, E102K, E120K) were produced by inoculating positively identified single colonies from regenerated dextrose base (RDB) plates (Appendix A) into 10ml of buffered minimal glycerol medium (BMGY) (Appendix A) at 30°C at 200rpm for 24 hours in a shaker incubator. This starter culture was further inoculated into 490ml of BMGY until the OD₆₀₀ spectrophotometric reading reached above 2. The growth media was clarified by centrifugation at 4000g for 4 minutes and the harvested cells were induced with 125ml of buffered minimal methanol medium (BMMY) (Appendix A) containing 4% filter sterilized methanol (Merck Chemicals) for a period of 96 hours. The supernatant containing secreted protein(s) was harvested by centrifugation at 13200 rpm for 10 minutes.

Twenty μl of each sample was mixed with 8 μl of loading buffer mix (Appendix A) and 23 μl of the sample mix was SDS-PAGE analysed at 110 volts for 2 hours (Figure 4A). The gel was coomassie blue stained (Appendix A for staining solution) for 30 minutes and destained (Appendix A for destaining solution) for 45 minutes. The samples which showed a band at the size of the standard VCP (rVCP) were filter sterilized using either 0.22 μm syringe filters (Millipore products) or 0.45 μm filter units (Adcock Ingram), diluted in binding buffer (50mM sodium acetate, pH 5.5; sometimes pH 7.0) (Amersham Pharmacia Biotech cue card) and accommodated into a sample loop. The HiTrap heparin columns (3 X 5ml connected in series to make a 15ml column) were equilibrated with 5-column volume (5CV; 1CV = 5ml) of filter sterilized and theoretically degassed distilled water and then with the binding buffer (Appendix A) prior to the injection of the sample accommodated in the sample loop (Amersham Pharmacia Biotech). The sample was then injected at a flow rate of 1ml/min in a low-pressure *AKTA prime* chromatographer (Amersham Pharmacia Biotech) with the aid of the built-in peristaltic pump. The flow-through (unbound) sample was collected through the outlet valve and the bound protein was eluted at a linear NaCl gradient of 0 (binding buffer) to 100% (1M NaCl prepared in binding buffer) and collected in a series of fraction tubes (3ml/tube). The peak fractions were SDS-PAGE analysed and those containing pure ^{hrVCP}E108K or ^{hrVCP}H98Y, E102K, E120K were pooled, concentrated and SDS-PAGE analysed (Figure 4B) prior to biological activity test.

For large scale VCP purification, the high-pressure liquid chromatography (HPLC) technique was applied in a 10S explorer (Amersham Pharmacia Biotech) using the UNICORN 4.10 software package (Amersham Biosciences, Uppsala, Sweden). This explorer purifies large sample volume of up to 500ml and tolerates a flow rate elevation of 10ml/min due to the higher column pressure limit (up to 25MPa) compared to the *AKTA prime* (1MPa). The property of the HiTrap heparin column allows the chromatographic techniques to separate VCP based on the principles of affinity (to heparin) and ionic interaction (cation-exchange) with the negatively charged heparin matrix.

3.2.4 Production of rVCP/hrVCP in Fermentor

Large-scale production of different recombinant proteins applying the process of fermentation is a widely employed procedure in various biotechnology and pharmaceutical laboratories.

The use of fermentors for industrial scale protein production has a clear and unambiguous advantage over the condition- and scale-limited production of foreign proteins in flasks. Fermentors provide a tight regulation of different parameters such as temperature, pH, agitation and the level of dissolved oxygen in the culture. These factors, alone or in combination, have significant effect on the expressed protein and on the expression system. Here, a series of fermentation experiments (over 20 fermentations' with a number of modifications) were undertaken to optimize the production of the recombinant VCP (rVCP) using a 14-liter BioFlo 110 Benchtop Fermentor (New Brunswick Scientific Co, Inc., New Jersey, USA) (Appendix B). Some of the fermentation trials are described below.

3.2.4.1 Fermentation-1

Single colony putative to express the VCP gene was initially inoculated into 1-liter of BMGY and then into 16-liter at 30°C at 200 rpm in a shaker incubator until the OD₆₀₀ spectrophotometric reading reached above 2. Pre-induction sample was taken and the cells were harvested by centrifugation at 10000 rpm for 10 minutes. The cell pellet from the 16-liter culture was inoculated into 4-liter of BMMY containing 0.5% methanol in the fermentor, where as the pellet from the 1-liter was inoculated into 250ml of BMMY (0.5% methanol) in the flask as an external control. The initial fermentation parameters used in this experiment are shown in Table 2A below.

Table 2A: Parameters used in Fermentation-1

Parameter	Value	Setpoint (Low – High)	Control
Temperature (°C)	30	30 (29.5 – 30.5)	Auto
Agitation (rpm)	200	200 (150 – 250)	Auto
pH	5.77	0	Off
Dissolved oxygen (%)	0	0	Off

After 24 hours of induction, a final concentration of 2% methanol was added to the fermentor and to the control flask, and a further 1% after 48 hours of induction. Samples were taken every 24 hours (for 72 hours) from the flask and only at 72 hours from the fermentor (due to the difficulty in sampling at this stage). The samples were SDS-PAGE analysed (at 70V for 2 hours) in a 4-12% pre-cast gel as shown in Figure-5.

3.2.4.2 Fermentation-2

Based on the outcome of the first fermentation trial; that is, growing the cells in flasks and inducing them in the fermentor, the present experiment attempted growing the cells in the fermentor and inducing them in the same fermentor after a short “starvation period” in order to allow the cells to switch from glycerol (in the BMGY) to the methanol (in the BMMY) as a source of carbon. Here, a single colony from YPD plate (Appendix A) was inoculated into 100ml of BMGY at 30°C at 200 rpm in a shaker incubator. After 48 hours, the inoculum was inoculated into 5-liter of BMGY in the fermentor. The 24 hours post-inoculation readout is displayed in Table 2B.

Table 2B: Parameters used in Fermentation-2

Parameter	Value	Setpoint (Low – High)	Control
Temperature (°C)	29.9	30 (29.5 – 30.5)	Auto
*Agitation (rpm)	200	205 (200 – 350)	Dissolved oxygen
Pump C (%)	0	40	#Off/On
pH	6.14	6.10 (5.0 – 6.5)	Auto
Dissolved oxygen (%)	18.6	20 (20 – 30)	Auto

Footnote: *the cascade of the agitation was changed from “Auto” (in Fermentation-1) to “Dissolved oxygen” (dO₂) that means when the level of oxygen in the culture drops, the agitation automatically increases to uniformly distribute the available oxygen throughout the culture.

#switched on when the cells needed additional carbon source as described below.

After ~29 hours of growth, the OD₆₀₀ reading started to drop (2.124 to 1.8) suggesting cell death and therefore 1-liter of 50% glycerol was fed through an external pump (pump C) at a rate of 165ml/hour. Consequently, the cell growth started to improve (OD₆₀₀ rose to 2.024). After ~35 hours of growth, 1-liter of the culture was centrifuged at 3500g for 45 minutes and the cells were resuspended in 250ml of BMMY containing 4% methanol. This induction phase took place in a shaker incubator (control). The remaining cells in the fermentor were starved for 30 minutes in order to allow exhaustion of the glycerol in the culture and then fed with 50ml of filter-sterilized methanol over a period of 24 hours. Following the start of the induction phase, industrial oxygen was pumped from an externally connected cylinder (Afrox, Cape Town, South Africa) in order to increase the supply of oxygen and hence the efficiency of methanol metabolism.

However, as a result of high cell yield, large amount of foam was produced in the system and significantly blocked the exit of air, as a result the fermentor was turned off and the supernatant (~5-liter) was harvested (at 90 hours instead of 96 hours post-induction as planned initially) by centrifugation at 3500g for 45 minutes. Finally, the sample was filter-sterilized and then concentrated using the process of ultrafiltration as described below.

3.2.4.2.1 Concentration by Ultrafiltration

The process of ultrafiltration allows fast and effective concentration and partial purification of large volume samples compared to the small volume centricon, centriprep or amicon filters (Millipore). In this experiment, peristaltic pump assisted pellicon XL filter device (Millipore) with a nominal molecular weight cut-off 10kDa was used to concentrate the 5 -liter supernatant harvested from the fermentor into a volume of 500 ml (10X) within few minutes. Because the cassette has a molecular weight (MW) cut-off 10kDa, any material with MW less than 10kDa is collected through the “permate” tube while the molecules above 10kDa are collected through the “retinate” tube. Therefore, the rVCP (28.8kDa) was collected through the “retinate” partially purified by the size-exclusion nature of the device. Sixteen μ l of the concentrated sample was mixed with 7 μ l of loading buffer and then analysed by SDS-PAGE in a 12% resolving gel.

3.2.4.3 Fermentation-3

In order to avoid the foaming problem encountered in the previous fermentation, antifoam-204 was purchased from Sigma-Aldrich and 1ml was added for every 5-liter culture. In this experiment, 5-liter of BMGY was first poured into the fermentor in order to adjust the temperature of the media to 30°C prior to inoculating the starter culture. High-purity nitrogen was then pumped (~100kPa) into the fermentor in order to calibrate the amount of dissolved oxygen (dO₂) in the media so that the dO₂ displayed in the screen panel reflects valid readings. The starter culture (125ml) was then inoculated into the fermentor. The initial fermentation readout is shown in Table 2C below.

Table 2C: Parameters used in Fermentation-3

Parameter	Value	Setpoint (Low – High)	Control
Temperature (°C)	30.2	30 (29.5 – 30.5)	Auto
Agitation (rpm)	212	500 (300 – 550)	Dissolved oxygen
Pump C (%)	0	100	*Off/On
pH	6.13	5.00 (5.0 – 6.5)	Auto
Dissolved oxygen (%)	46.6	30 (25 – 35)	Auto

*after ~20 hours, pump C was turned on and the cells were fed with 500ml mixture of 50% sterile glycerol, basal salts (Appendix A) and PTM1 trace salts (Appendix A) at a rate of 40ml/hour.

Meanwhile, ~25ml of the culture was pumped out of the fermentor and was incubated in the shaker incubator as a control (for contamination). After 45 hours of growth in the fermentor, high cell mass was formed (OD₆₀₀ was 3.0 compared to 2.4 in the flask) and the cells were harvested by centrifugation at 4000g for 5 minutes. The cells were then induced in ¼ volume of buffered methanol (Appendix A) and incubated in the shaker incubator for 48 hours. Finally, the supernatant was harvested by centrifugation at 4500 rpm for 20 minutes and the sample was SDS-PAGE analysed.

A number of subsequent fermentation trials were attempted to produce significant and relatively pure rVCP. The parameters that were continuously modified in order to optimize the process of fermentation were the agitation, pH, fed-batch (amount and composition), and dissolved oxygen. Moreover, a synthetic media, composed of basal salts and PTM1 trace salts, was used as a growth media in one instance, independent of the complex BMGY. Among the promising trials was the production of rVCP using the parameters described in Fermentation-4 Table 2D below.

3.2.4.4 Fermentation-4

Single colony putative to express the rVCP gene was inoculated into 500ml of BMGY at 30°C at 200 rpm in a shaker incubator until the OD₆₀₀ spectrophotometric reading reached above 2. The starter culture was then inoculated into 8-liter of BMGY in the fermentor. The fermentation parameters used in this experiment are shown in Table 2D below.

Table 2D: Parameters used in Fermentation-4

Parameter	Value	Setpoint (Low – High)	Control
Temperature (°C)	30	30 (29.5 – 30.5)	Auto
*Agitation (rpm)	400	400 (350 – 800)	Dissolved oxygen
Pump C (%)	0	25	#Off/On
**pH	5.9	5.5 (5.0 – 6.5)	Auto
##Dissolved oxygen (%)	19.7	30 (20 – 35)	Auto

Footnote: *as the cells continue to consume the supplied oxygen (when actively dividing), the speed of agitation increases towards the upper limit (800rpm) in response to the ‘scarcity’ of oxygen.

**as the cells metabolise the nutrients, they release respiration by-products such as CO₂, which acidify the media and causes a drop in pH. The drop in pH below 5.5 automatically activates the external peristaltic pump to deliver 25% ammonia solution (Merck Chemicals, Saarchem, Midrand, South Africa) until the pH rises above 5.5.

#after 20 hours of growth, 1-liter of sterile 50% glycerol pumped at a rate of 30ml/hour.

##the source of the dissolved oxygen was mainly the atmospheric air. An oil-free Bambi compressor DT/30 (Norgen, USA) extracts oxygen out of the atmospheric air and feeds the cells. However, when the cells needed more oxygen, industrial oxygen was supplied from an external cylinder (Afrox, Cape Town, South Africa).

The samples were SDS-PAGE analysed and those containing a band at the size of the standard VCP were confirmed by western blotting (Figure 6B) as described below. The rVCP/hrVCP containing samples were purified using the procedure described above. The purified proteins were then dialysed (Spectropore membrane tubing) against phosphate buffered saline (PBS, pH 7.2) and the protein concentration was estimated using the Biorad microplate assay protocol as described below.

3.2.5 Estimation of Protein Concentration

The concentration of the purified proteins was estimated using the bovine serum albumin (BSA) standards (Pierce Laboratories; Rockford, Illinois, USA) and the Bio-Rad DC protein assay reagents in a 96-well microplate assay. This colorimetric assay is based on the principle of the Lowry assay and involves the initial reaction of the protein (standard or sample) with the supplied alkaline copper tartrate solution (reagent A). This reduction reaction (Cu^{2+} to Cu^{+}) further reacts with specific amino acids constituting the protein and upon the addition of Folin reagent (reagent B), blue color, equivalent to the protein concentration, develops and this can be measured spectrophotometrically or using a microplate reader (Caprette, 1997).

In this experiment, 5 μl of the samples or standard was mixed with 25 μl of the supplied reagent A, mixed well and then 200 μl of reagent B was added into each well. The mixture was incubated at room temperature for 5 minutes and the protein concentration was measured at 595nm using microplate reader (anthos 2010, Salzburg, Austria). Finally, the quantified pure recombinant VCP was monitored for bacterial endotoxins using the QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) quantitative assay (BioWhittaker, Walkersville, MD) prior to biological use.

3.2.6 Screening for Endotoxin

Bacterial endotoxins are usually active contaminants of genomic DNA, plasmid and proteins in solutions. These toxins also known as lipopolysaccharides (LPSs) are often derived from gram-negative bacteria (Vélez et al, 2006). Vaccines or any other therapeutic materials need to be monitored for endotoxins prior to their *in vivo* use as they may cause septic shock, hypotension and multiple organ dysfunction syndrome (Perrella et al, 1996).

The Chromogenic Limulus Amebocyte Lysate (LAL) test is a widely used enzymatic assay for the quantification of endotoxins and applies the catalysis of a proenzyme into its active form by bacterial endotoxins resulting to the development of a yellow color proportional to the concentration of endotoxin present in the sample (BioWhittaker Manual). In this experiment, the microplate assay method (BioWhittaker Manual) was used. Initially, a 96-well plate and all the reagents were pre-warmed at 37°C. Fifty µl of blank (endotoxin free water), standard or sample was added into each well and then 50µl of the supplied LAL was added into each well and mixed prior to 10 minutes incubation at room temperature. The supplied substrate solution (100µl) preheated to 37°C was added into each well, mixed and incubated for 6 minutes at 37°C. The reaction was then stopped by adding 100µl of stop reagent (10% SDS) and the endotoxin concentration was measured at 405 nm using a microplate reader.

Production of VCP from natural infection

3.2.7 Tissue Culture Experiments

When the vaccinia virus infects mammalian cells such as the African green monkey kidney (BSC-1) cells, the vaccinia virus complement control protein (VCP) is majorly secreted from the infected cells to the media (Kotwal and Moss, 1988). In this experiment, the natural VCP was produced by infecting BSC-1 cells at a titer of 3×10^6 cells with the laboratory adapted vaccinia virus vGK5 strain (Kotwal and Abrahams, 2004) at a multiplicity of infection (MOI) of 1 in a serum-free minimal essential medium (MEM) Earle's Base (Highveld Biological, Lyndhurst, South Africa) containing 1X triple antibiotics (Penicillin, Streptomycin and Fungizone, Highveld Biological) for 48 hours. The produced VCP was confirmed by western blotting as shown in Figure 6B. The western blot protocol used in this experiment was as described below.

3.2.7.1 Western Blot

The natural VCP and the hrVCP were SDS-PAGE (Appendix A) analysed and confirmed by western blotting (Figure 6B). In this experiment, the resolved samples were transferred (Appendix A) from the gel into an Immobilon-P (PVDF) membrane with a pore size of $0.45 \mu\text{m}$ (Millipore Corporation, Bedford, MA) at a constant current of 0.14A° for 1.3 hours. The membrane was then removed and stained with Ponceau S (Appendix A) stain for 1-2 minutes in order to assess the success of the transfer. The membrane was rinsed with TBS (Appendix A) prior to blocking with freshly prepared 5% blocking solution (Appendix A) at 4°C overnight. The blocking solution was discarded and the membrane was incubated with 1:1000 diluted rabbit anti-VCP primary antibody (10mg/ml) (Vector Laboratories, Burlingame, CA) (Appendix A) for 1 hour at room temperature with continuous shaking at a rate of 100revs/min (Orbital Shaker SO3, Stuart Scientific, United Kingdom). The membrane was washed with 30ml of 0.1% TBS-T (Appendix A) twice for 10 minutes each. The membrane was washed again with 30ml of 0.5% blocking solution twice for 10 minutes each and incubated with 1:12500 diluted anti-mouse/anti-rabbit IgG peroxidase secondary antibody (Appendix A) (Roche Diagnostics, Mannheim, Germany) for 30 minutes with continuous shaking. The membrane was then rinsed and washed with TBS-T (30ml each) four times (15 minutes each). The bands were detected by adding premixed detection reagent (Appendix A) for 60 seconds. Finally, the blot was inserted into a film cassette and exposed for various time points and the X-ray film was processed by transferring it into a Developer (~2 minutes), Stopper (2% acetic acid) (quick dip) and a Fixer (~2 minutes).

Following the western blot confirmation, the supernatant containing VCP was then purified, concentrated, dialysed, and monitored for endotoxins as illustrated above. The production and passaging of BSC-1 cells as well as the titration and infectivity of the vaccinia virus vGK5 strain are described below.

3.2.7.2 Production and Passaging of BSC-1 cells

Pre-produced stock of African green monkey kidney (BSC-1) cell line was generously provided by Prof. Girish J. Kotwal (Division of Medical Virology, UCT) for further cell line production and maintenance experiments as described below.

The cells ($\sim 10^7$) were inoculated into 20ml of MEM containing 10% fetal calf serum (FCS) (Highveld Biological, Lyndhurst, South Africa) and 1X triple antibiotics (as above) in a 75 cm² pyrogen free sterile cell culture flask (Corning Incorporated, NY, USA) and incubated at 37°C in a Thermocyt incubator with 5% CO₂ (ESI-FluFrance, Wissous, France). At day four, the cells were fully confluent as revealed under 40X Olympus CK2 light microscope (Japan). Thereafter, the entire medium was aspirated, leaving the adhered cells behind, and the cells were treated (1 minute) with trypsin/EDTA in order to remove any traces of anti-trypsin prior to partial trypsinization with 2ml of fresh beef pancreas-derived gamma irradiated Trypsin/EDTA (Highveld Biological, Lyndhurst, South Africa) at 37°C with occasional vigorous shaking for 5 minutes. All the cells were detached with the application of gentle mechanical force and in-and-out aspiration as confirmed under a microscope. The cells were resuspended in 10ml of fresh MEM and transferred into 15ml tube for centrifugation at 100g at 20°C for 5 minutes (Heraeus Multifuge 3 L-R, Kendro Laboratory Products, Hanau, Germany). Finally, the supernatant was discarded and the cell pellet was resuspended in 0.5ml of cryoprotective medium (basal eagle's medium containing Hanks' BSS and 15% DMSO) (BioWhittaker, Walkersville, MD). The cell-suspension was aliquoted into 2ml cryovials and stored at -80°C and the cells were thawed and passaged when necessary.

3.2.7.3 Virus Titration

The BSC-1 cells (0.5ml x 6) were plated out in 6-well plates (Corning Incorporated, NY, USA) until confluency (~72 hours) and each well, except the negative control, was infected with the pre-titrated vaccinia virus vGK5 strain (initial stock kindly provided by Walter Rangel De Campos, MSc graduate) at a titer range of $10^6 - 10^{10}$ plaque forming units (pfu) per ml as follows (Kotwal and Abrahams, 2004).

The original stock (2×10^7 pfu/ml) was diluted by mixing $1\mu\text{l}$ of the virus with 1ml of fresh MEM (without FCS and antibiotics). A μl of the mix was added to the well labeled as 10^6 , mixed, and $100\mu\text{l}$ of this mix was transferred into the well labeled as 10^7 and so on up to the 10^{10} labeled well. The cells were infected by incubating them with the different viral titers' at 37°C for 48 hours. The supernatant was removed and the wells were stained with crystal violet stain (Appendix A). Subsequently, the number of plaques was counted (Figure 7A) and the viral titer was determined as described above.

3.2.7.4 Confirmation of Infectivity and Virus Production

To confirm the infectivity of the newly produced vGK5 strain, confluent 6-well plate grown BSC-1 cells were infected with vGK5 virus at a multiplicity of infection (MOI) of 0.1 by adding $1.5\mu\text{l}$ of virus stock (3×10^4 viral particles) into 3×10^6 cells in 1ml. After 24 hours of infection, the entire medium was sucked out and the cells were resuspended in 1ml of serum-free MEM and incubated further at 37°C for 24 hours. After a total of 48 hours of infection, the supernatants were harvested and the wells were stained with crystal violet for 1 hour at room temperature (Figure 7B). Once the infective ability of the virus was confirmed, virus stocks were produced by infecting fully confluent BSC-1 cells with a viral titer of 2×10^8 pfu/ml for 48 hours. The cell monolayer was washed twice with 0.5ml of PBS (pH 7.2) and the cells were detached using a cell scraper. The cell suspension was harvested and freeze-thawed between -80°C and 37°C three times followed by sonication using an ultra sonic cell disrupter (VirTis, NY, USA) for 30 seconds on ice. This step was done in order to destabilize the cell membrane and release the internalized viral particles. The supernatant was clarified by centrifuging at $4000g$ for 5 minutes and the viral particles were aliquated in eppendorf tubes and stored at -80°C for further use.

3.2.8 Inhibition of Complement *In Vitro*

The bioactivity of VCP, rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K was tested for the classical pathway and that of rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K for the alternative pathway of complement activation in microplate based *in vitro* assays. For the classical pathway, the ability of normal human serum to lyse 95% of sensitized sheep red blood cells (ssRBCs) (Diamedix Corporation; Miami, Florida, USA) was first tested in serum assay.

3.2.8.1 Serum Assay

The level and potency of circulating complement in human blood varies from one individual to another. Therefore, before using any given serum as a source of complement in assays measuring classical complement inhibition, its ability to lyse 95% of ssRBCs need to be confirmed by serial dilutions. In this experiment, three sterile eppendorf tubes were labeled as 1:30, 1:60 and 1:90, and 75 μ l of ssRBCs suspension was added into each tube. Ten μ l of the supplied gelatin-veronal-buffer (GVB) was added into each tube and mixed well. Normal human serum was thawed from -80°C and diluted 1:30, 1:60 and 1:90. Fifteen μ l of each dilution was added into the respective tube and the samples were incubated at 37°C for 1 hour prior to centrifugation at 7000 rpm for 30 seconds. Finally, 75 μ l of each sample was transferred into a well in a 96-well plate. The absorbance, proportional to the percentage of lysis, was measured at 405nm measurement filter and the dilution that yielded 95% lysis was used for the subsequent hemolysis assay as illustrated below.

3.2.8.2 Modulation of Classical Complement Pathway

The efficiencies of the natural VCP, rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K to modulate the antigen-antibody triggered classical complement activation was demonstrated by incubating the respective protein with IgG-sensitized sheep RBCs prior to activation of the pathway using human serum at a dilution established by the serum assay. We have recently published the details of the assay (Ghebremariam³ et al, 2005). In this experiment, 75 μ l of sensitized sheep red blood cells (ssRBCs) suspended in GVB were transferred into clean eppendorf tubes and then different amounts of each protein (20 – 100ng) were added to the respectively labeled tubes. Finally, 15 μ l of 1:60 diluted human serum (determined using serum assay as described above) was added into each tube except the negative control. The samples were incubated at 37°C for 1 hour, centrifuged at 7000 rpm for 30 seconds and 75 μ l of the supernatant was transferred into each well of a 96-well plate.

The absorbance, proportional to the percentage of lysis, was measured at 405nm and was subtracted from 100% to calculate the percentage of inhibition. The result was standardized in the background of the negative control in order to account the complement independent spontaneous lysis of the cells. The inhibition of lysis by the various VCPs and comparison of their potency in modulating the classical pathway of complement activation is shown in Figure 8 A-C.

3.2.8.3 Modulation of the Alternative Complement Pathway

The alternative component of the complement pathway is an active defense tool against microbial infections during childhood. However, excessive activation of the alternative pathway can result in a number of disorders as described in the literature review. In this experiment, the alternative pathway was selectively activated in normal human serum using zymosan (Fearon and Austen, 1977; Kazatchkine et al, 1979; Fishelson et al, 1984; Mahesh et al, 1999) and then used the “*Quidel Bb Fragment Enzyme Immunoassay*” kit (Quidel Corporation, San Diego, USA) to quantify the abilities of the VCPs to regulate the activated pathway. This assay, modified for the first time for this purpose, was published recently (Ghebremariam³ et al, 2005). The avidity of the different VCPs in modulating the alternative pathway of complement activation is shown in Figure 9.

Briefly, the alternative complement pathway was selectively activated using zymosan, a component of the yeast cell wall. Each recombinant protein was preincubated with human serum and the mix was then incubated with 12.5µg of double-sonicated zymosan at 37°C for 1 hour. Subsequently, 10µl of each mix was transferred into clean eppendorf tubes and 90µl of the supplied complement specimen diluent (Quidel corporation) (Appendix A) was added in order to make 1:10 dilution. Prior to the addition of samples, each well in the 8-well strip was washed with 200µl of 1X wash buffer (Appendix A) three times (1 minute each). The samples (100µl each) were added into the prewashed wells coated with mouse anti-human Bb antibody and incubated for 30 minutes at room temperature in order to allow the capture of the generated Bb fragments by the monoclonal antibody. The samples were then discarded and the wells were washed 5X using a multi-channel pipetter (1 minute per wash). Horseradish peroxidase (HRP)-coupled goat anti-human Bb (50µl) was added and incubated for 30 minutes to detect the bound Bb. The conjugate was then discarded and each well was washed 5X as described above. The color was developed by adding 100µl freshly prepared substrate solution (Appendix A) and incubated at room temperature for 30 minutes.

Finally, 50 μ l stop solution (contains 250mM oxalic acid as described in the insert provided) was added to stop the reaction and the absorbance of the developed color, equivalent to the concentration of Bb fragment, was measured at 405nm measurement filter. In this experiment, naïve serum and serum sample preincubated with equivalent amount of zymosan (in the absence of the protein) were used as negative and positive controls respectively. The kit is normally used to assess the activation of Factor B in patients' sera. In the present study, we have tuned the protocol in order to evaluate the ability of a complement inhibitor to regulate the alternative complement pathway by selectively activating the alternative pathway in normal human serum.

3.3 RESULTS

3.3.1 Modeling Studies

The computer-based modeling exercises (using the Swiss-Model and “GRASP” programs) revealed the surface potentials of rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K (Figure 2A-D). The study showed that the substitution of the amino acid glutamic acid (E) to lysine (K) at position 108 (E108K) has minimized the acidity of rVCP (Figure 2A and 2D) in the SCR-2 as shown in red and subsequently increased the overall positive charge of the protein as displayed in blue color (Figure 2B and 2D). This single amino acid substitution has minimally reduced the overall hydrophobicity (water solubility) of the protein as glutamic acid has a hydrophobicity of -3.5 while lysine has a slightly reduced water solubility (-3.9).

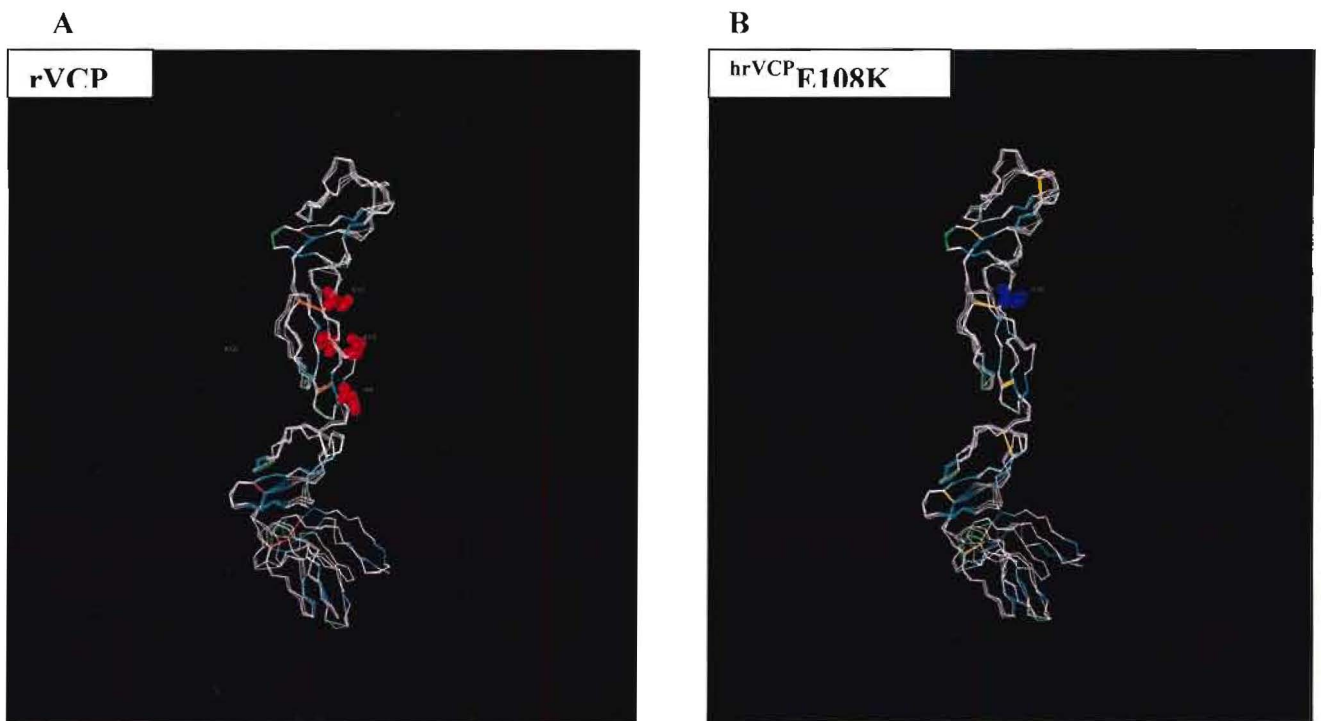


Figure 2A and B: CPK (space filling) display style of rVCP (A) and ^{hrVCP}E108K (B) models showing a single amino acid change (E108K) in the SCR-2. **Note:** the red colors indicate acidic amino acids while the blue color designates basic amino acid (Modeled using the Swiss-Model software, and edited using DS ViewerPro software package).

The substitution of the amino acids histidine (H) to its SPICE and MCP analog tyrosine (Y) at position 98 (H98Y); glutamic acid (E) to lysine (K) at position 102 (E102K) and the substitution of glutamic acid to its SPICE and sCR1 homologs at position 120 (E120K) has immensely increased the basic (positive) amino acid content of the modified protein (^{hrVCP}H98Y, E102K, E120K) as shown in Figure 2C and 2D below.

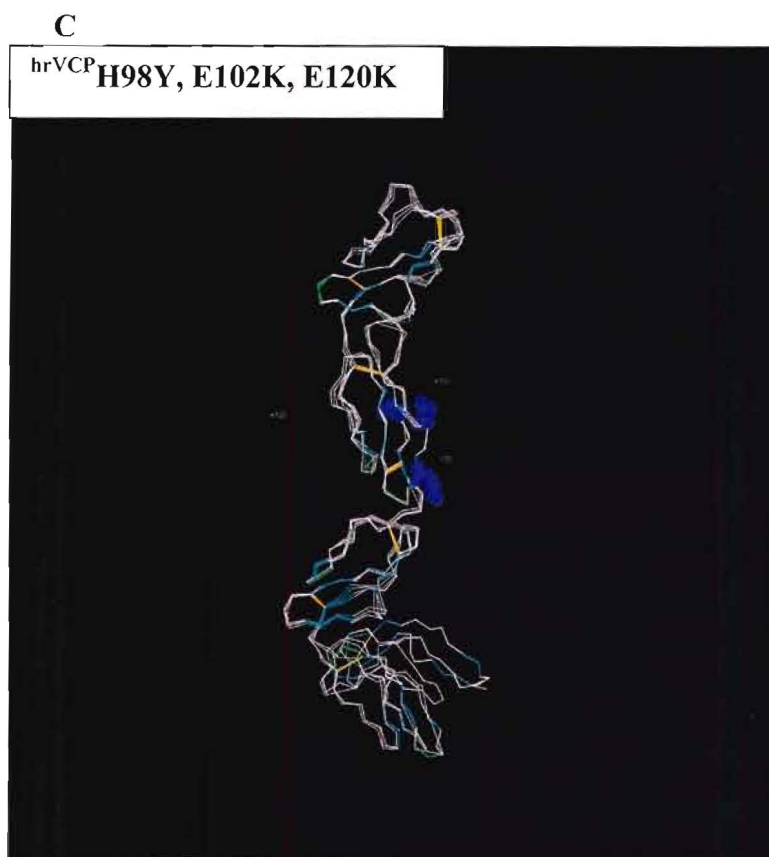


Figure 2C: CPK (space filling) display style of ^{hrVCP}H98Y, E102K, E120K based on the crystal structure of rVCP (protein databank ID 1G40). The blue colors indicate the basic amino acids substituted in the rVCP (Figure 2A) to generate this modified protein (Modeled using the Swiss-Model software and edited using DS ViewerPro software package).

In addition to my modeling studies (Figure 2A – 2C) using the Swiss-Model program, *Figure 2D was kindly drawn by Dieter Blaas of the University of Vienna, Austria using the “GRASP” software package.* This modeling shows the sites and proximity of the modified amino acids in the single (E108K) and triple (H98Y, E102K, E120K) mutant proteins (Figure 2D).

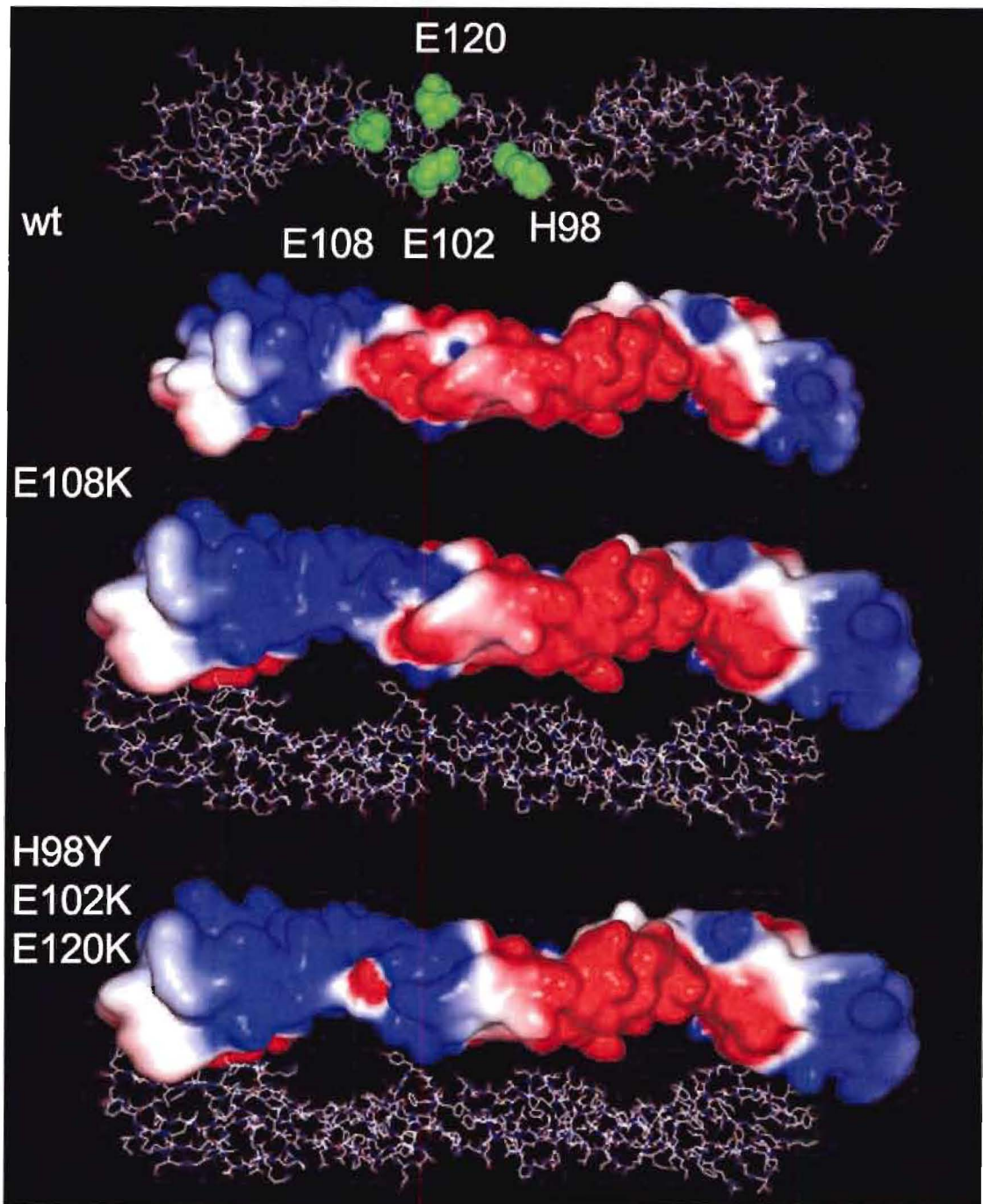


Figure 2D: Surface potential and line drawings of rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K displaying the sites of the mutated amino acids (shown in green clusters) and the effect of the amino acid change(s) in the charge distribution of the protein. Blue color indicates basic amino acids while red color shows acidic amino acids. (This was kindly modeled by Dieter Blaas of the University of Vienna, Austria using the “GRASP” software package).

3.3.2 Screening of rVCP/hrVCP putative clones

The transformed recombinant GS115 yeast clones were PCR-screened for the integration of the $hrVCP^{E108K}$ and $hrVCP^{H98Y, E102K, E120K}$ DNA in their genome. As shown in Figure-3 below, the recombinant clones (Figure-3 lanes 4-7) showed a major band running at the size of the positive control (AOX-1/rVCP; 1222bp) suggesting size of the rVCP gene (732bp) flanked by the alcohol oxidase-1 (AOX-1) gene sequence (490bp), the gene where the rVCP is cloned down-stream to. These actual recombinant clones were used for subsequent protein production experiments.

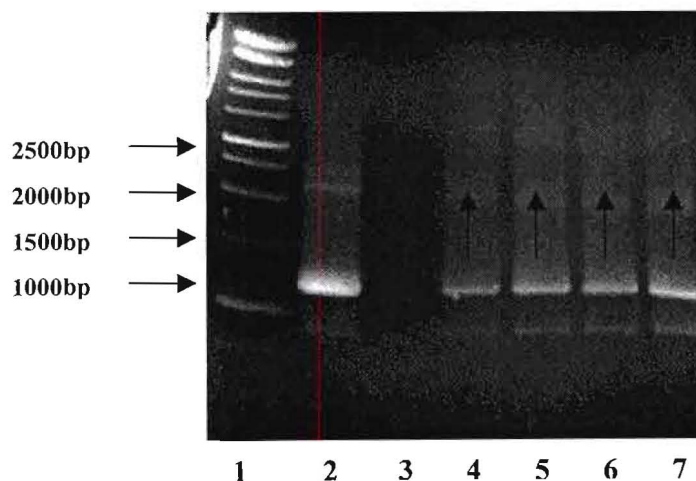


Figure-3: Confirmation of putative rVCP/hrVCPs recombinant yeast clones in a 1.2% agarose gel electrophoresis. Lane-1: 1kb⁺ MW marker; lane-2: rVCP/AOX-1 and 2 control (1222bp and 2200bp respectively); lane-3: negative (H₂O) control; lanes-4 and 5: rVCP genes (1222bp each) representing colonies 1 and 2; lane-6: $hrVCP^{H98Y, E102K, E120K}$ gene; lane-7: $hrVCP^{E108K}$ gene. The up-arrows, inside the gel, show a second band (~2200bp) in each of the lanes suggesting the presence of the yeast's own alcohol oxidase gene (AOX-2; 2200bp).

3.3.3 Production of rVCP/hrVCPs in Flask

The rVCP/hrVCP proteins were produced using the protocol described above. The pre and post induction samples were SDS-PAGE analysed (Figure 4A) and the samples containing a band at the size of standard rVCP were filter sterilized, concentrated and then purified using heparin column chromatography. The purified samples were then SDS-PAGE analysed (Figure 4B) and the pure fractions were concentrated and dialysed prior to their use in the *in vitro* assays described below.

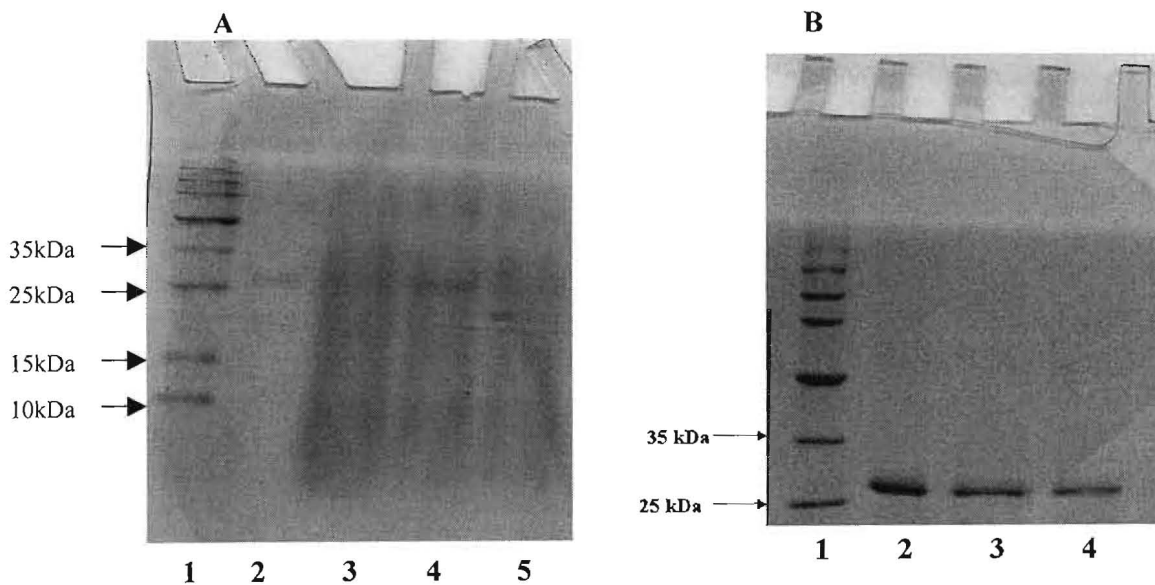


Figure 4: SDS-PAGE (12%) analysis of recombinant VCPs produced in flask. (A): lane-2: standard rVCP; lane-3: unpurified supernatant of rVCP; lane-4: unpurified supernatant of $^{hrVCP}E108K$; lane-5: unpurified supernatant of $^{hrVCP}H98Y, E102K, E120K$. (B): heparin-column purified samples of rVCP (lane-2); $^{hrVCP}E108K$ (lane-3) and $^{hrVCP}H98Y, E102K, E120K$ (lane-4). Lane-1 is a broad range molecular weight marker (Promega). (Figure 4B is published in the *Ann. N.Y. Acad. Sci* 1056: 113-122 (2005); page-116 Ghebremariam³ et al).

3.3.4 Production of rVCP/^{hr}VCP^{H98Y, E102K, E120K} in Fermentor

The production of recombinant VCPs appear to greatly vary from the flask to the fermentor even when using a split of the same colony (Figure 5). This could be due to the differences in the major variables in the flask and the fermentor. For example, temperature, pH, agitation and the level of oxygen available in the system are significant contributors of foreign protein production in *Pichia pastoris* yeast expression system. The appearance of abundantly secreted proteins as shown in Figure-5 (lanes 5-7) may be due to the effect of methanol toxicity causing the release of endogenous yeast proteins.

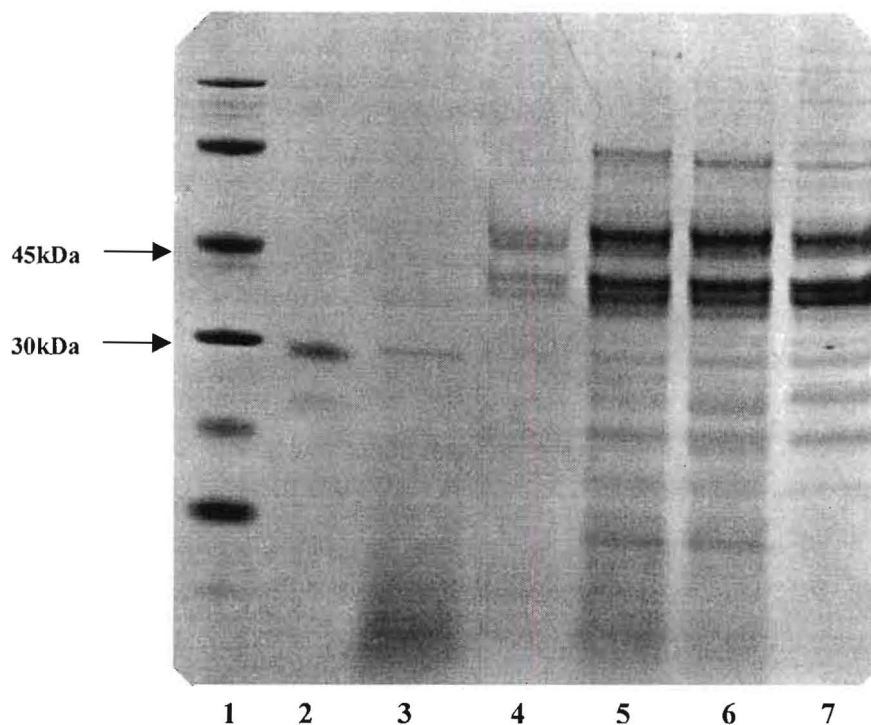


Figure 5: Coomassie-stained SDS-PAGE (4-12% precast gel) analysis of recombinant VCP. Lane-1: molecular weight marker; lane-2: standard recombinant VCP (28.8 kDa); lane-3: preinduction sample; lane-4: 72 hours postinduction (fermentor); lanes 5-7: 24, 48, 72 hours postinduction samples (flask).

However, following the second fermentation approach (growing and inducing the recombinant yeast cells in the same fermentor as described in “SECTION 3.2.4.2”), there was no band at the size of the standard VCP in any of the lanes (data not shown). The absence of VCP corresponding band in the fermentor sample may be due to the inhibition of methanol by glycerol (Invitrogen Life Technology, 2002) as the cells were induced after a short ‘starvation period’ without clarifying the growth media and therefore, this may not be an efficient mechanism to exhaust the glycerol (without killing the cells) supplied with the complex growth

medium. However, following extensive optimization trials in the flask and fermentor, the production of recombinant VCP was significantly improved as shown by SDS-PAGE analysis (Figure 6A) and western blotting (Figure 6B). Moreover, the western blotting (protocol described above) result also confirmed the ability of the anti-rVCP (rabbit anti-VCP) antibody to recognize the triple mutant (^{hrVCP}H98Y, E102K, E120K) (Figure 6B lanes 2-5) and the vaccinia virus-infected mammalian cell secreted natural VCP (lane-6) as well as their equal mobility despite their functional differences (Figure 8 and 9). Furthermore, optimization of the protein production simplifies the challenges of purification.

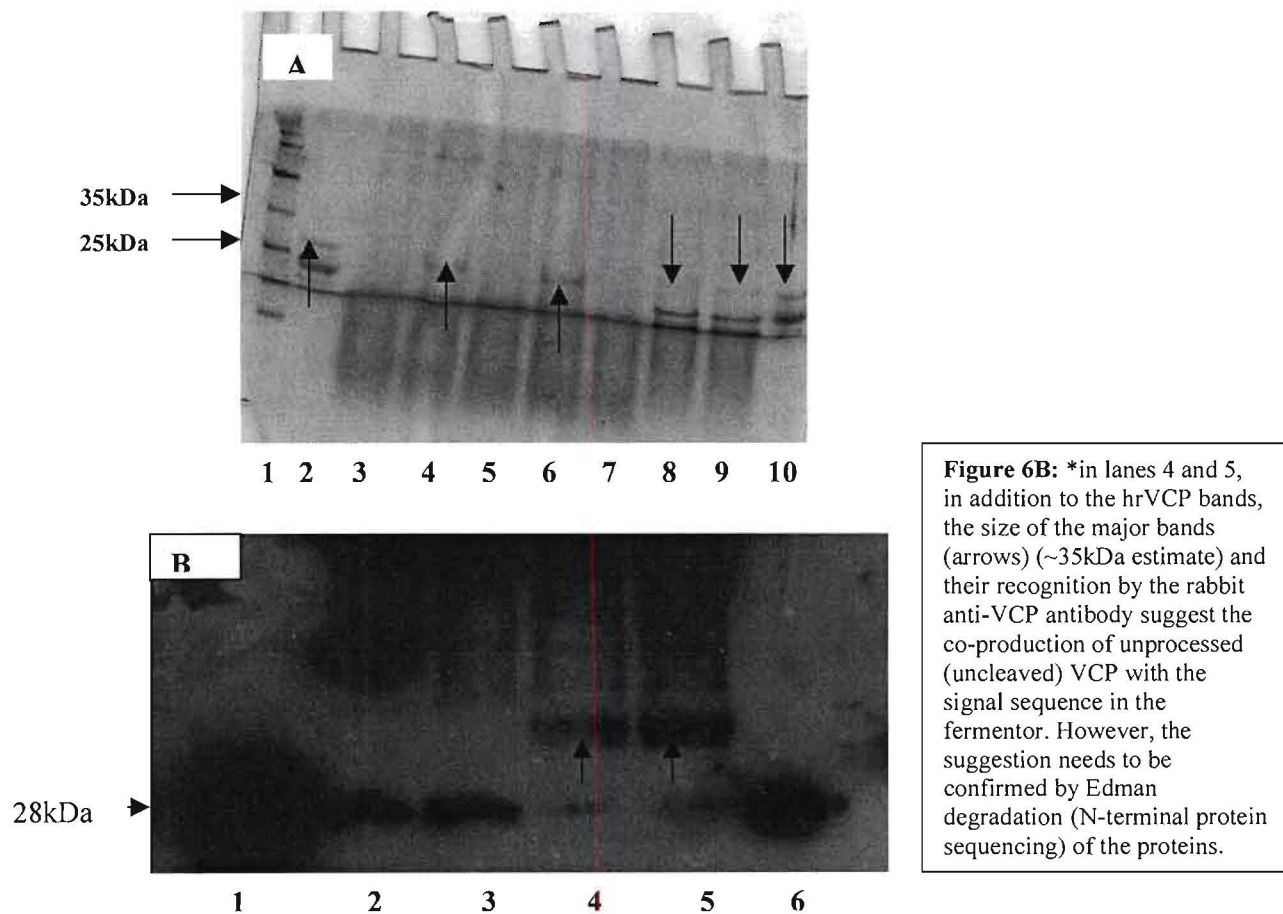


Figure 6B: *in lanes 4 and 5, in addition to the hrVCP bands, the size of the major bands (arrows) (~35kDa estimate) and their recognition by the rabbit anti-VCP antibody suggest the co-production of unprocessed (uncleaved) VCP with the signal sequence in the fermentor. However, the suggestion needs to be confirmed by Edman degradation (N-terminal protein sequencing) of the proteins.

Figure 6: Analysis of recombinant VCPs (A): SDS-PAGE (12%) showing lane-1: broad range molecular weight marker; lanes 2 and 10: standard rVCP (arrows); lane-3: preinduction sample (colony-1, flask); lane-4: 72 hours postinduction (colony-1, flask); lane-5: preinduction sample (colony-2, flask); lane-6: 72 hours postinduction (colony-2, flask); lane-7: preinduction sample (colony-1, fermentor); lane-8: 72 hours postinduction (colony-1, fermentor); lane-9: 96 hours postinduction (colony-1, fermentor). (B): western blotting of lane-1: rVCP standard (28.8 kDa); lanes 2 and 3: ^{hrVCP}H98Y, E102K, E120K 24 and 48 hours postinduction (flask); *lanes 4 and 5: ^{hrVCP}H98Y, E102K, E120K 24 and 48 hours postinduction (fermentor); lane-6: natural VCP produced from infection of mammalian cells.

3.3.5 Production of VCP from natural infection

The tissue culture based production of VCP is usually guaranteed as long as the virus used in the experiment is infective enough (high titers). Our comparative experiments have shown that the VCP produced from vaccinia virus Lister strain (242 amino acids long) infected cells runs at a higher molecular weight than the VCP produced from vaccinia virus vGK5 strain (244 amino acids). Despite its fewer number of amino acids, the lower mobility was found to be due to its two novel glycosylation sites (Odunuga et al, 2005).

The production of VCP from vGK5 strain is confirmed by western blot as shown in Figure-6B above. The virus titration (plaques shown in the wells) and confirmation of infectivity are shown in Figure 7A and 7B respectively.

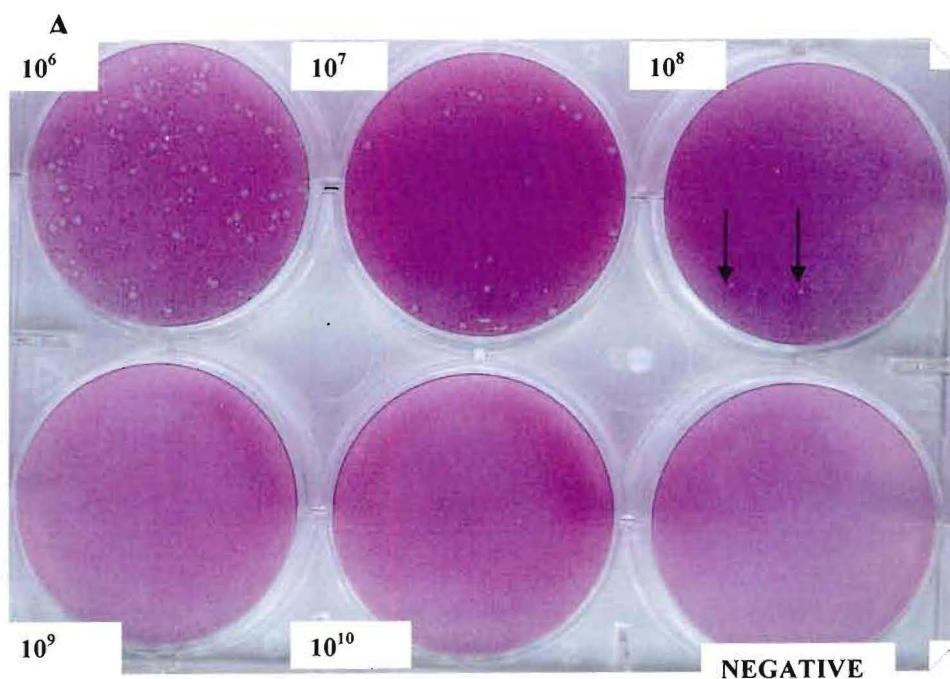


Figure 7A: Six-well based quantitative viral assay showing titration (quantification) of vaccinia virus vGK5 strain (dots showing viral plaques) at the dilutions shown. This titration assay shows a viral titer of 2×10^8 virus particles/ml (arrows). Uninfected BSC-1 cells are shown as “NEGATIVE” control.

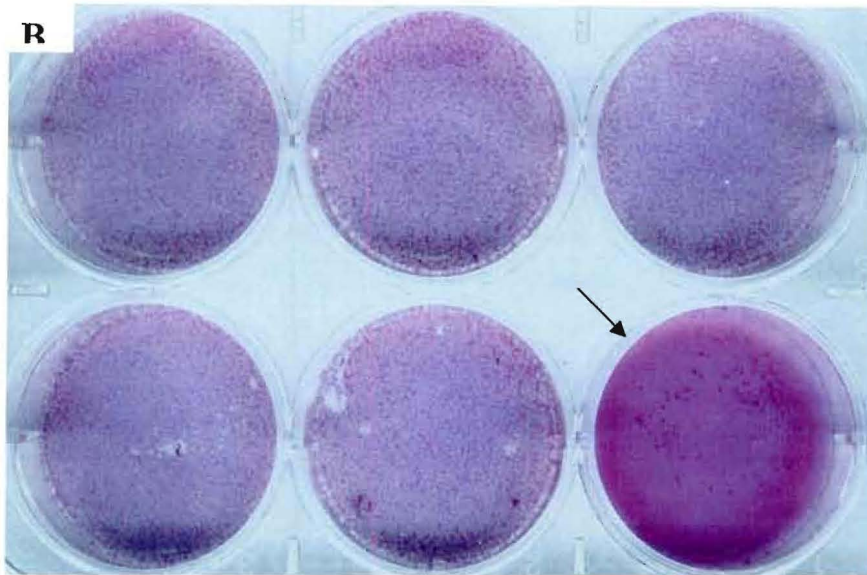


Figure 7B: Six-well based qualitative viral assay displaying the ability of the quantified vGK5 strain to infect mammalian (BSC-1) cells (monolayer peeled-off) at a titer of 3×10^4 . The uninfected (negative) control is shown by an intact monolayer of cells (arrow).

3.3.6 Inhibition of Complement *In Vitro*

The abilities of the recombinant VCPs (rVCP/^{hrVCP}E108K/^{hrVCP}H98Y, E102K, E120K) to inhibit either of the two major pathways of complement activation appear to vary significantly.

3.3.6.1 Classical Complement Pathway Inhibition

Using antibody-sensitized sheep red cells, the efficacies of the rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K were determined. This assay involves the formation of antigen-antibody complex and measures the resultant lysis of the red cells bearing the complex. We have recently published (Ghebremariam³ et al, 2005) the outcomes of the classical complement inhibition by the different VCPs in a series of dilutions. The single mutant ^{hrVCP}E108K has been found to possess approximately 25-fold increased activity (ranging from 19.7 to 33.7-fold) while the triple mutant revealed about 100-fold (91.8 to 116-fold) increased activity than rVCP in inhibiting the classical pathway of complement activation (Figure 8A-C). In a separate experiment, we have also shown the ability of the natural VCP to be about 80-fold more active than the rVCP in inhibiting the classical complement pathway (Odunuga et al, unpublished data).

Inhibition of CP by rVCP/hrVCPs

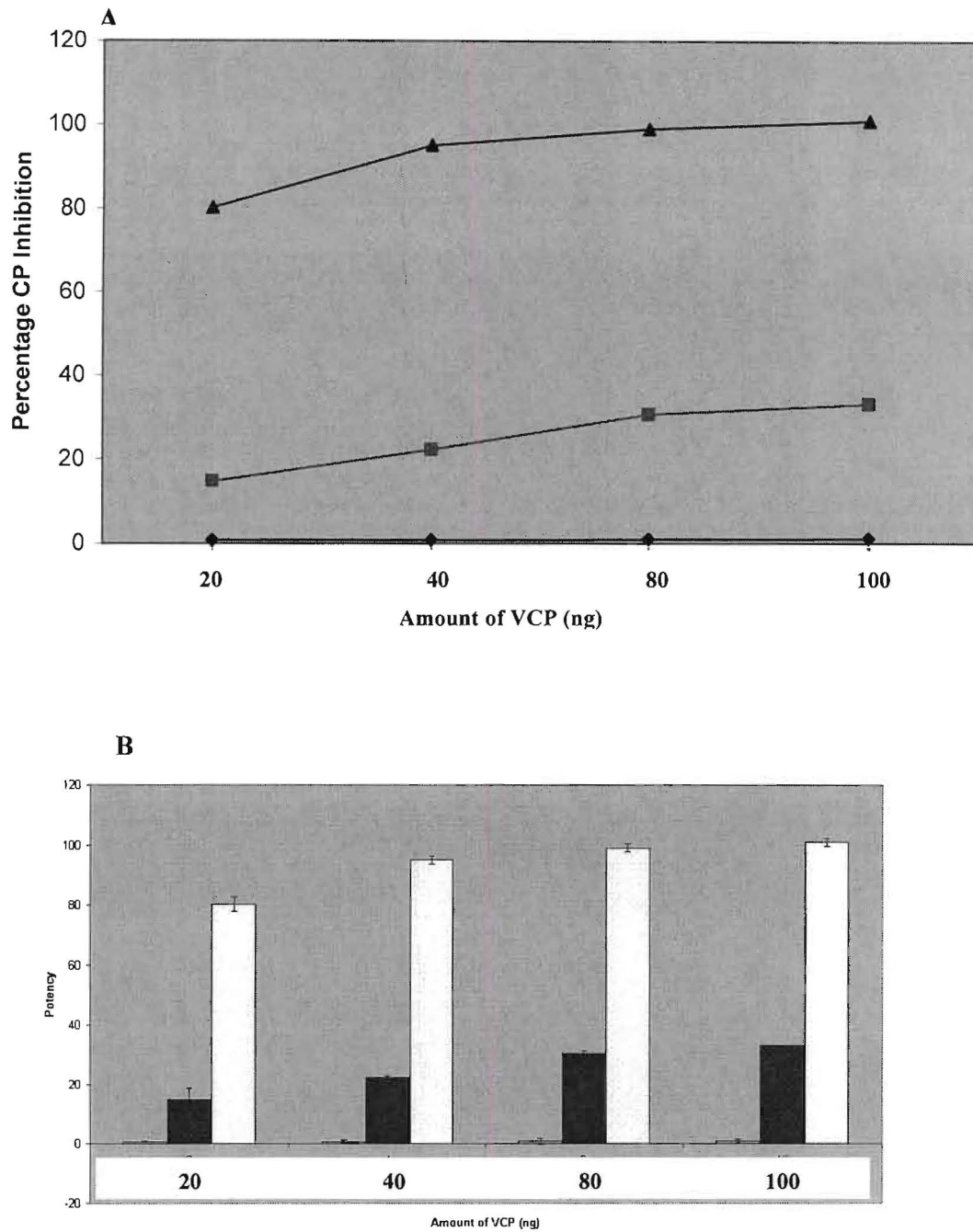


Figure 8: Comparative *in vitro* analysis of classical complement pathway inhibition at various dilutions. (A): percentages of classical pathway inhibition by rVCP (diamonds), ^{hrVCP}E108K (squares) and ^{hrVCP}H98Y, E102K, E120K (triangles) at the indicated amounts of the proteins. (B): comparison of rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K potency at the indicated amounts of the proteins. Experiments were performed in duplicates and the bars indicate standard errors of sample mean. (Figure 8 is published in the *Ann. N.Y. Acad. Sci* 1056: 113-122 (2005); page-117 Ghebremariam³ et al).

3.3.6.2 Alternative Complement Pathway Inhibition

In addition to the comparative analysis of the classical complement pathway, the activities of the different recombinant VCPs (rVCP/^{hrVCP}E108K/^{hrVCP}H98Y, E102K, E120K) were also compared for the alternative pathway of complement activation by measuring an alternative pathway specific breakdown product. We have published this novel assay and its feasibility in measuring the degree of inhibition by complement control proteins (Ghebremariam³ et al, 2005). Our findings have shown that the recombinant VCP with single amino acid substitution (^{hrVCP}E108K) possessed almost 55-fold increased ability, and the triple mutant (^{hrVCP}H98Y, E102K, E120K) revealed almost 100-fold increased activity. The inhibitory concentrations at 50% (IC₅₀) were 32ng and 16ng respectively, compared to the weaker inhibition by rVCP (IC₅₀ value of 1760ng) as shown in Figure-9 below.

Inhibition of AP by rVCP/hrVCPs

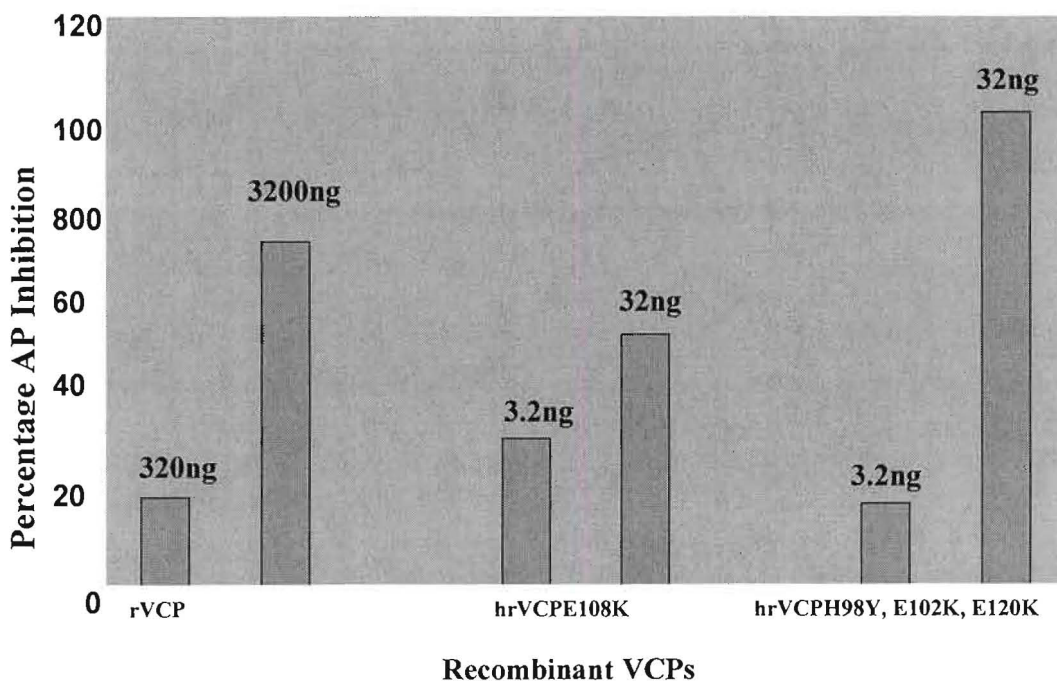


Figure 9: comparison of rVCP, ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K in inhibiting the alternative pathway of complement activation showing ~55-fold and ~100-fold increased activity by ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K respectively. (Figure 9 is published in the *Ann. N.Y. Acad. Sci* 1056: 113-122 (2005); page-118 Ghebremariam³ et al).

The observed increase in the inhibitory activities of both the classical and the alternative pathways of complement activation following one and three amino acid substitution(s) suggest that the putative amino acids genuinely contact with C3b/C4b complement components and allowed the modified proteins to efficiently block complement activation.

Moreover, we have identified a new putative heparin binding site of the K-X-K type (Smith et al, 2000) in the triple mutant ^{hrVCP}H98Y, E102K, E120K created by the substitution of glutamic acid (E) to lysine (K) at position 102 (E102K) (Ghebremariam³ et al, 2005). Therefore, the number of putative heparin binding sites in the recombinant VCP has increased from four (in rVCP) (Smith et al, 2000) to five.

3.4 DISCUSSION

The classic observation that rabbit kidney (RK-13) cells infected with wild-type vaccinia virus Western Reserve (WR) strain majorly secrete a polypeptide of approximately 35kDa; together with the amino-terminal sequencing of the polypeptide led to the discovery of the vaccinia virus complement control protein (VCP) (Kotwal and Moss, 1988). The search for VCP homologs in the protein database has revealed the greatest hit (38%) to human complement-4b binding protein (C4b-BP) (Kotwal and Moss, 1988). It was therefore this observation together with the homology search in the databank that brought the stories of human proteins, complement and the mimicry of poxviral proteins together.

Consequently, the roles of VCP as a complement inhibitory protein and as a virulence factor (in enabling the vaccinia virus to evade host defense) were established (Kotwal et al, 1990; Isaacs et al, 1992). Since then the number of poxviral complement inhibitory proteins has been increasing. The sequencing of variola virus genome (Massung et al, 1996), the *in vivo* cowpox work (Miller et al, 1997) and the monkeypox VCP homolog DNA sequencing (Uvarova and Shchelkunov, 2001) have contributed to the identification of SPICE, IMP and MOPICE respectively. These poxviral proteins, known as complement regulatory proteins (CRPs), share significant structural and/or functional homology with the human regulators of complement activation (RCA) family. These poxviral CRPs, with the exception of IMP, have been expressed as recombinant proteins (Smith et al, 2000; Rosengard et al, 2002; Sfyroera et al, 2005; Liszewski et al, 2006) and demonstrated their sequence identity, mobility, complement inhibitory activity and heparin binding affinity among themselves and in reference to some members of the RCA family (Rosengard et al, 2002; Sfyroera et al, 2005; Liszewski et al, 2006). The deduced amino acid sequence alignment of the recombinant poxviral CRPs revealed above 90% identity (Liszewski et al, 2006) suggesting the paradigm conservation of complement inhibitors during the evolution of poxviruses (Ciulla et al, 2005). Yet, despite the remarkable amino acid identity, their efficiency in inhibiting either pathways of complement activation, their contribution as cofactors and their decay-accelerating efficacy appear to differ substantially (Rosengard et al, 2002; Sfyroera et al, 2005; Liszewski et al, 2006). For example, the few amino acid differences (11-12 amino acids) between VCP and SPICE seem to have endowed SPICE with the ability to inhibit human complement 100 to1000-fold more efficiently than VCP (Rosengard et al, 2002; Sfyroera et al, 2005) suggesting their ultimate role in complement inactivation (Rosengard et al, 2002; Ganesh et al, 2004; Ghebremariam³ et al, 2005; Sfyroera et al, 2005; Liszewski et al, 2006).

However, not all the amino acid differences are equally critical, as some of the residues are located in sites that are inferred not to make significant contact with human complement components C3b/C4b (Murthy et al, 2001; Rosengard et al, 2002; Ganesh et al, 2004). Moreover, point mutation studies in MCP, a member of the RCA family sharing 35% amino acid identity with VCP (Sfyrera et al, 2005), have shown that some residues at specific positions are necessary for cofactor activity while others are important for C3b and/or C4b binding (Liszewski et al, 2000) suggesting the explicit role of individual amino acid residues. Other studies have speculated that the amino acid differences between VCP and SPICE in module 2 (SCR-2) are critical in offering SPICE an increased C3b/C4b and/or cofactor activity (Murthy et al, 2001; Rosengard et al, 2002; Ganesh et al, 2004; Ghebremariam³ et al, 2005). Sfyrera et al (2005) published a study addressing the importance of individual amino acid residue and entire module differences between VCP and SPICE. The study evaluated the significance of the amino acid differences at positions 77, 98, 103, 108 and 120, all located in SCR-2 (a module that composes above 40% of the VCP/SPICE amino acid differences) individually or in various combinations. They also elucidated the effects of double amino acid substitutions in VCP SCR-2 to the SPICE homologs (E108K, E120K), triple amino acid substitutions in VCP SCR-3 or in VCP SCR-4, multiple amino acid substitutions in VCP SCR-2/3, SCR-2/4, SCR-3/4 and finally generated SPICE out of VCP (Sfyrera et al, 2005) [only second to Rosengard et al (2002)]. The computer modeling exercises, surface plasmon resonance (SPR)-based binding assays and functional studies that were employed in the work by Sfyrera et al (2005) addressed a number of essential questions and supported our concentration in the amino acid differences localized in SCR-2 (H98, E108, E120).

The novel work by Sfyrera et al (2005) showed that the generated recombinant SPICE was about 75- and 1000- folds more potent than the recombinant VCP (rVCP) in inhibiting the classical and the alternative pathways of complement activation respectively, when using human sera as a source of complement. Their work supported the previously documented (Rosengard et al, 2002) potency of recombinant SPICE over rVCP with some discrepancies (~10-fold) that may have risen from the differences in the assays used. Interestingly, the single amino acid residue substitutions in VCP to the SPICE analog significantly enhanced the function of the recombinant proteins in inhibiting at least the alternative complement pathway (Sfyrera et al, 2005). For example, the respective amino acid substitutions in rVCP at position 77 (Q77H), 98 (H98Y), 103 (S103Y), 108 (E108K) and 120 (E120K) have upregulated the ability of the individual modified rVCPs by 11-, 28-, 24-, 17- and 87- folds in inhibiting the

alternative complement pathway respectively (Sfyroera et al, 2005). Moreover, the modified proteins generated from amino acid substitutions in SCR-2 and SCR-4 to their SPICE homologs have been found to be increasingly associated with Factor I in degrading C3b to its inactive components (Sfyroera et al, 2005).

Our studies have demonstrated that the single amino acid substitution in rVCP at position 108 from glutamic acid (E) to lysine (K) to match its SPICE analog (E108K) increases the ability of the modified recombinant protein, that we named "*humanized recombinant VCP (hrVCP E108K)*", by about 25- and 55- folds in inhibiting the classical and the alternative pathways of complement activation respectively (Ghebremariam³ et al, 2005), again confirming the crucial role of the residue in human C3b/C4b interaction. Additionally, with the guidance of sequence alignment (Liszewski et al, 2000; Rosengard et al, 2002), crystallography (Murthy et al, 2001) and computer modeling studies (Ganesh et al, 2004), we generated a modified recombinant protein with three amino acid substitutions in the rVCP sequence (H98, E102, E120). The inclusion of tyrosine (Y) at position 98 appears to have contributed to the increase in cofactor activity in MCP, as its substitution by alanine (Y98A) has diminished MCP's C4b cofactor activity to less than 40% (Liszewski et al, 2000). Moreover, its conservation in SPICE and the inference from modeling studies predicting that the residue at this position is surface-exposed (Liszewski et al, 2000; Ganesh et al, 2004) makes it a suitable candidate for mutagenesis studies. In addition, the H98Y targeted mutation in rVCP has been shown (Sfyroera et al, 2005) to enhance the ability of the modified protein to inhibit the alternative complement activation by about 28-fold indicating the residues genuine role. Furthermore, the amino acid residue at position 102 is a critical site for C3b/C4b binding and C3b/C4b cofactor activities as its substitution in MCP affected the complement binding and cofactor activities for both the classical and the alternative complement pathways (Liszewski et al, 2000). Moreover, MCP and VCP modeling studies have proposed that position-102 is surface-exposed and therefore expected to influence complement activity (Liszewski et al, 2000; Murthy et al, 2001; Ganesh et al, 2004) identifying it as one of the critical sites for interaction with complement. The E102K substitution has created an additional putative heparin-binding site of the K-X-K type. Previously, Smith et al (2000) have identified four putative (K/R-X-K/R) heparin-binding sites in the rVCP amino acid sequence.

Therefore, this new site has increased the number of putative heparin-binding sites from one to two in SCR-2 and to five in the whole protein (Ghebremariam³ et al, 2005). The third amino acid residue that we considered for modification was glutamic acid (E) at position 120 (E120) to its sCR1 and SPICE homolog (E120K). This amino acid substitution has been shown to affect the C3b binding and cofactor activity of secreted CR1 (Krych et al, 1991) and the C4b cofactor activity of MCP (Liszewski et al, 2000) making it an ideal residue for modification. Moreover, the residue at this position is predicted to be surface-exposed in VCP and MCP surface potentials. To confirm this, the site-directed mutagenesis of this amino acid residue to its SPICE analog (E120K) has been shown to be the most critical single amino acid substitution to bridge the gap between SPICE and VCP in inhibiting the alternative complement pathway (Sfyoera et al, 2005). This single amino acid change has bestowed on the modified rVCP an 87-fold increased activity in regulating the alternative pathway (Sfyoera et al, 2005). We demonstrated that the modified VCP with triple amino acid residue substitutions which we named “*humanized recombinant VCP (hrVCP^{H98Y, E102K, E120K})*” due to its closeness to the human-complement specific [SPICE] amino acid sequence, was nearly 100-fold more active than rVCP in regulating both the classical and the alternative pathways of complement activation (Ghebremariam³ et al, 2005) indicating a synergistic/additive effect of the individual amino acid potency. However, these were not the ultimate amino acid residues in SPICE responsible for the 100-1000-fold increased potency compared to rVCP. Our amino acid residue K102 exists in SPICE as E102 making it difficult to appreciate the specific role of this residue. In addition, Sfyoera et al (2005) reported that with only two amino acid residue substitution at positions 108 and 120 in the rVCP sequence to their SPICE homolog (E108K, E120K), a SPICE-like potency had been achieved in inhibiting the alternative pathway of complement activation and in facilitating the Factor I mediated degradation of C3b (C3b cofactor activity).

Our (Ghebremariam³ et al, 2005) and the Sfyoera et al (2005) studies confirm that not all the amino acid differences between SPICE and VCP are equally crucial in interacting complement. The slight differences (~3-fold) in the alternative pathway activity of the single mutant (E108K) may be largely due to the different expression systems used (yeast vs. bacteria) and probably due to the differences in the mechanism of alternative pathway activation (zymosan vs. LPS), measurements of different alternative pathway components (Factor Bb vs. C3b) and the different substrates used in developing the reactions.

In conclusion, we have effectively modified the *in vitro* activity of the recombinant VCP in inhibiting both the classical and the alternative pathways of complement activation (Figures 8 and 9). Our modeling studies (Figure 2A-D) have demonstrated that the substituted amino acids have increased the overall positive charge (basic amino acid content) of the modified proteins compared to rVCP. This is also true in the modeling studies with various amino acid substitution(s) in the rVCP sequence (Sfyroera et al, 2005).

Although, there was no noticeable difference in the heparin-binding abilities of rVCP and ^{hrVCP}H98Y, E102K, E120K, the newly created putative heparin-binding site in the latter protein may have created some *in vivo* retention advantage for the humanized recombinant VCP. It has been previously reported that the heparin binding ability of VCP is responsible for its sustained bioavailability in tissues and for blocking the aggregation of inflammatory cells to the site of injury (Kotwal, 2000; Hicks et al, 2002; Jha et al, 2003). The efficient increase in the *in vitro* complement inhibitory activity of the humanized rVCP (^{hrVCP}H98Y, E102K, E120K) has encouraged us to elucidate its *in vivo* role in modulating ischemia/reperfusion (I/R)-induced renal injury, a complication known to cause excessive complement activation as described in “CHAPTER 4” of the thesis.

CHAPTER 4

**EVALUATION OF THE *IN VIVO* ROLE OF VACCINIA VIRUS
COMPLEMENT CONTROL PROTEIN (VCP) FOLLOWING
RENAL ISCHEMIA/REPERFUSION INJURY**

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4.3.4.2 Renal Histopathology

4.3.4.3 Immunohistochemistry

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4.4 DISCUSSION

4.1 INTRODUCTION

Renal transplantation is performed in most major centres throughout the world. During the transplant procedure there is an ischemia reperfusion (I/R) injury that accounts for a delayed physiological recovery of the graft. This surgically and biologically induced injury results in inflammatory response that involves activation of the complement system. The alternative pathway of complement activation through Factor B, C3 or the membrane attack complex (MAC; C5b-9) components has been shown to be the predominant route of complement activation and one of the major causes of tissue injury in renal I/R injury (Zhou et al, 2000; Sacks et al, 2003; Thurman et al, 2003; Thurman et al, 2006). The vaccinia virus complement control protein (VCP) regulates both the classical and the alternative pathways of complement activation by preventing the formation of C3b, a complement component where both pathways converge.

The aim of the study was to investigate the effect of the VCP produced from vaccinia virus infected cells (VCP), the recombinant VCP (rVCP) and the humanized recombinant VCP (hrVCP) collectively and independently on I/R injury of the kidney. A number of procedures involving various techniques were attempted in order to develop a feasible renal I/R injury model. Two of the five procedures were found to be candid approaches in provoking renal I/R injury in rat models. In the first model (unilateral I/R injury), I/R injury was induced by subjecting Long Evans rats to 60 minutes ischemia of the right kidney followed by 24 hours reperfusion time. The animals were randomly allocated to receive VCP produced from natural infection and the yeast cell expressed hrVCP combination or phosphate buffered saline (PBS) or sham group. In the second model (bilateral I/R injury), the I/R injury was induced by subjecting Long Evans rats to laparotomy, mobilization of both kidneys and clamping of the renal arteries for 60 minutes followed by 24 hours reperfusion. The rats were randomly allocated to receive natural VCP, hrVCP or PBS or sham groups. In both models, blood samples were collected at various intervals for BUN and creatinine studies, and the kidneys harvested for histopathological and immunohistochemical studies.

4.2 MATERIALS and METHODS

4.2.1 Expression of recombinant VCP (^{hrVCP}H98Y, E102K, E120K)

The PCR-based mutagenesis and cloning of the humanized recombinant VCP (hrVCP) in the *Pichia pastoris* yeast expression system was described previously (Ghebremariam³ et al, 2005). The expression of two alcohol oxidase genes (AOX-1 and AOX-2) allows the recombinant yeast clone to metabolize methanol faster (Mut⁺) than the mere AOX-1 expressing clones (Mut^s) upon induction with methanol.

4.2.2 Screening of recombinant clones

Selective plates such as the minimal methanol histidine (MMH) medium (Appendix A) are often used to screen for putative yeast transformants. However, they do not differentiate the actual recombinants from those harboring the Ppic9 vector (Appendix B) without the gene of interest. Therefore, it usually needs to be confirmed by PCR. The putative recombinant clones were screened using the forward (5'- GACTGGTTCCAATTGACAAGC - 3') and the reverse (5' - GCAAATGGCATTCTGACATCC - 3') AOX-1 universal primers (Invitrogen life technologies) and the amplified products were analysed in a 1.2% agarose gel (Figure 1). The ^{hrVCP}H98Y, E102K, E120K gene-expressing recombinant clones were used for subsequent protein production experiments.

The recombinant protein was expressed by inoculating positively identified colonies into 30ml of buffered minimal glycerol complex medium (BMGY) at 30°C at 200 rpm for 48 hours and then further inoculated into 970ml of BMGY until the OD₆₀₀ read above 2. The cells were harvested by centrifugation at 4000g for 4 minutes, rinsed twice with distilled water and resuspended in 250ml of buffered minimal methanol complex medium (BMMY) containing HPLC grade methanol at a final concentration of 4% for 96 hours.

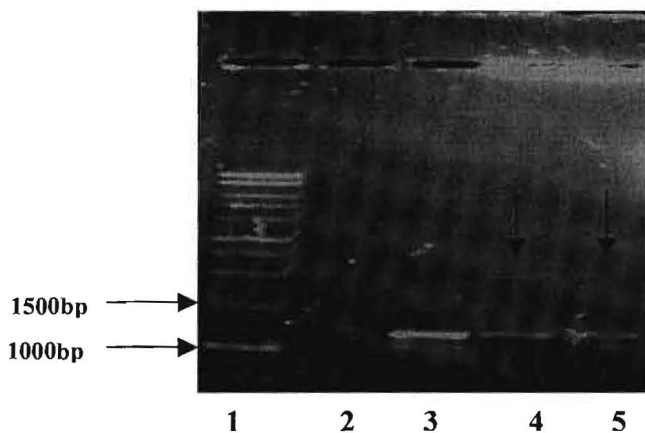


Figure 1: Agarose gel electrophoresis (1.2%) of recombinant *Pichia pastoris* yeast genomic DNA performed using AOX-1 universal primers. Lane-1: 1kb⁺ MW marker; lane-2: negative (H₂O control); lane-3: rVCP/AOX-1 gene control amplified from Ppic9 vector (1222bp); lanes 4 & 5: hrVCP genes amplified from Ppic9 (1222bp each). Note: AOX-2 genes (2200bp) indicated by upper arrows (lanes 4&5).

The samples were SDS-PAGE analysed and those expressing a band at the size of the standard recombinant VCP (rVCP) were purified using a series of HiTrap heparin columns (Ghebremariam³ et al, 2005). The purified fractions were then SDS-PAGE (12%) analysed (Figure 2A) and confirmed by western blotting as described above.

4.2.2.1 Western Blot

The purified natural VCP and the hrVCP were SDS-PAGE (Appendix A) analysed and confirmed by western blotting (Figure 2B). This experiment was performed using the protocol described in the previous chapter.

4.2.3 Tissue Culture Experiments

4.2.3.1 Production of VCP from natural infection

The natural VCP was produced in tissue culture by infecting BSC-1 cells with vaccinia virus vGK5 strain as described in "CHAPTER 3". The post-infection supernatant containing secreted VCP was purified from the medium under similar conditions to the hrVCP^{H98Y}, E102K, E120K containing supernatant (Figure 2A) as described above. The fractions containing pure VCP and hrVCP^{H98Y}, E102K, E120K were confirmed by western blotting (Figure 2B) and then pooled and dialysed against distilled water. The protein concentration was estimated using the BioRad assay as described earlier. The dialysed protein was lyophilized and resuspended in phosphate buffered saline (PBS; pH 7.2). The final protein was then monitored for bacterial endotoxins using the QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) quantitative assay (BioWhittaker, Walkersville, MD) described above.

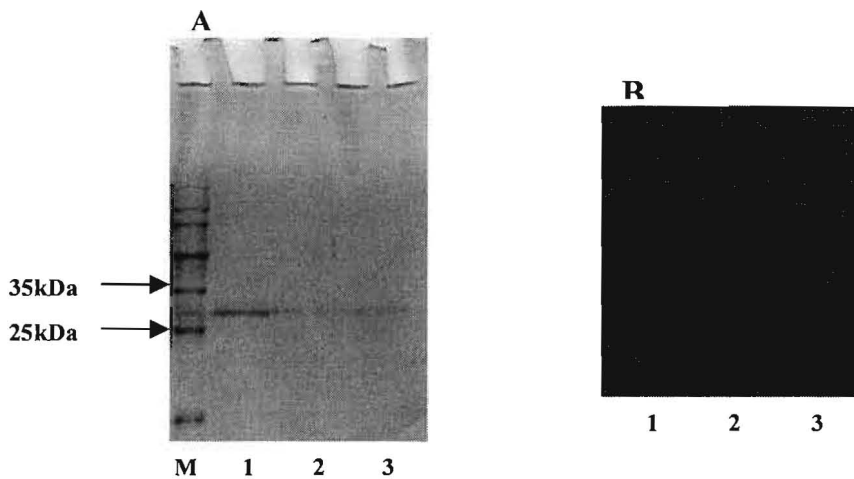


Figure-2: A) SDS-PAGE analysis (12%) & B) Western blotting of rVCP (lane-1); hrVCP^{H98Y}, E102K, E120K (lane-2) and natural VCP (lane-3). Lane-M (Fig. 2A) is molecular weight marker.

4.2.4 *In Vitro* Complement Inhibition

The biological ability of VCP and ^{hrVCP}H98Y, E102K, E120K to inhibit the classical and/or the alternative complement pathway(s) was evaluated in 96-well based *in vitro* assays (Ghebremariam³ et al, 2005).

4.2.4.1 Serum Assay

This assay demonstrates the ability of a given serum to lyse 95% of sensitized sheep red blood cells (ssRBCs). Because not every individual has the same level of circulating complement, the amount of serum responsible to lyse 95% ssRBCs needs to be determined when using sera from different sources and even when using serum from the same source stored for an extended period of time. In this assay, the experiment described in "SECTION 3.2.8.1" was reproduced.

4.2.4.2 Inhibition of the Classical Complement Pathway

The abilities of VCP and ^{hrVCP}H98Y, E102K, E120K to block complement mediated lysis of sheep red cells (Diamedix Corporation, Miami, USA) were tested by preincubating the IgG-sensitized red cells with the respective protein and activating the antigen-antibody dependent classical pathway using human serum as a source of complement as demonstrated earlier (Kotwal et al, 1990, Ghebremariam³ et al, 2005). The experiment was performed using the protocol described above.

4.2.4.3 Inhibition of the Alternative Complement Pathway

The ^{hrVCP}H98Y, E102K, E120K was evaluated for its ability to inhibit the alternative complement pathway by measuring a cleavage product of an alternative pathway specific enzyme, Bb (Quidel Corporation; San Diego, CA, USA) as described previously (Ghebremariam³ et al, 2005). The details of the procedure used in this experiment are described in the previous chapter.

Animal Experiments

4.2.5 Procedure-1

Four male Long Evans rats weighing 147-157g were used in this study. The animals were caged under standard conditions including a regular light and dark cycle with unlimited access to rat chow and water prior to the start of the experiment. The research protocol was approved by the University of Cape Town animal research ethics committee.

4.2.5.1 Ischemia/Reperfusion Injury

The animals were anesthetized with a combination of diethyl ether inhalation and ketamine hydrochloride (100mg/kg, im). The abdomen was cleaned with a cleaning solution and shaved with surgical blade. Laparotomy was performed by midline incision and both kidneys were dissected. The animals were catheterized and baseline blood and urine were collected prior to ischemia. Ischemic damage was induced by clamping both the right and left renal arteries (bilateral clamping) with non-traumatic vascular clamps for 45 minutes. The flow of blood was reestablished by removing the clamps and the abdominal cavity was closed with 4-0 prolene blue monofilament polypropylene suture (Ethicon, PA). In addition to the baseline, blood samples were collected at the start of reperfusion and every 2 hours after the reestablishment of blood flow until sacrifice. Blood and urine samples were collected for urea and creatinine studies (serum) and for SDS-PAGE analysis (urine). Both kidneys were harvested and fixed in 10% formalin for histopathological studies.

4.2.5.2 Administration of rVCP

In the VCP recipient group, the recombinant VCP (rVCP) was administered both intravenously (IV) and intraperitoneally (IP) in one animal. In order to potentially prevent or at least minimize ischemia, an IP injection of 25mg/kg was administered two hours before the laparotomy. Moreover, an IP injection of 30mg/kg was given just after closing the abdominal wall. Furthermore, 15 minutes before clamping, a bolus IV dose of 8mg/kg was administered through the femoral vein. Subsequently, rVCP was administered IV (1.5mg/kg) 15 minutes before releasing the clamps (that is 30 minutes after applying the clamps), just after removing the clamps and every 15 minutes for 2 hours in order to increase the bioavailability of the protein.

In order to comparatively select the most suitable route of administration and possibly cut down the tedious and engaging IV administration, rVCP was administered (n = 1) only IP in the same interval and at the same dosage to the IP route of the first animal. Blood and urine samples were collected at the same intervals. In one animal, phosphate buffered saline (PBS, pH = 7.2) was administered both IV and IP at equivalent intervals to the administration of rVCP in the first animal. In this procedure, one animal was included as a sham control in order to understand the effects of anesthesia and surgical procedure-induced stress in the blood biochemistry. In this animal, the kidneys were similarly isolated, however, no clamps were applied and the animal did not receive any injection.

4.2.5.3 Blood Urea Nitrogen (BUN) Assay

The blood samples were allowed to clot at room temperature overnight. The serum was separated by centrifuging at 3000 rpm for 25 minutes and kept at -20°C until use. The samples were thawed once prior to the biochemical study. Here, the Kinetic UV method was used to measure the serum BUN levels. One reagent tablet was first dissolved in 1ml of supplied buffer (Boehringer, Mannheim). After 15 minutes, $10\mu\text{l}$ of serum or standard (precinorm U at 50mg/dl) was added to every ml of the solution and mixed gently. Finally, two absorbencies were taken at 340nm at 30 seconds (A_1) and 3 minutes (A_2) after the addition of the sample or standard and the concentration of urea was calculated as:

$$C \text{ (mg/dl)} = \frac{A_1 - A_2}{B_1 - B_2} \times 50$$

Where: A_1 & A_2 are sample and B_1 & B_2 are standard absorbance at 30 sec & 3 min. respectively

4.2.5.4 Creatinine Assay

The blood samples were separated as described above in the BUN assay and $50\mu\text{l}$ of each serum was diluted (1:1) with distilled water. Two hundred μl of sodium tungstate was added and mixed well. Thereafter, $200\mu\text{l}$ of sulphuric acid was added, mixed and centrifuged at 3000 rpm for 15 minutes. Following the spinning, $200\mu\text{l}$ of each supernatant was transferred into a clean eppendorf tube and was mixed with $200\mu\text{l}$ of picric acid in order to precipitate down other metabolites and separate them from interfering with the creatinine readout (Sharpe, 1925). Finally, $200\mu\text{l}$ of NaOH (0.75 M) was added and incubated for 20 minutes at room temperature. The absorbance was taken at 520nm .

This experiment was done using the HiTachi U-2000 spectrophotometer and two standards and a positive control with known creatinine concentration were used to justify and calculate the concentration of creatinine in the sample. The creatinine concentration was calculated as follows.

$$C \text{ (mg/dl)} = \frac{\text{Sample absorbance}}{\text{Standard absorbance*}}$$

***N.B:** The “standard absorbance” was the averaged result of two independent absorbencies.

4.2.5.5 Urinalysis

In order to confirm the previously suggested hypothesis (Jha et al, 2003) of the rapid exclusion of VCP from circulation and to clarify the presence of high molecular weight proteins following ischemia/reperfusion injury, urine samples (from baseline and sacrifice) were comparatively elucidated via SDS-PAGE. The samples were kept at 4°C and then 20µl of each sample was transferred into clean eppendorf tubes and centrifuged at 7000 rpm for 5 minutes. The supernatant was separated and 16.5µl was mixed with 6.5µl of loading buffer mix and then analysed by SDS-PAGE (Figure 3).

4.2.6 Procedure-2

Seven male Long Evans rats weighing 240-274g were used in the study. The animals were housed under standard temperature and pressure (STP) including a regular light and dark cycle with free access to rat chow and water 24 hours prior to the procedure and then free access to water for the duration of the experiment.

4.2.6.1 Ischemia/Reperfusion Injury

The animals were initially anesthetized with a combination of diethyl ether inhalation and ketamine hydrochloride (100mg/kg, im) (Centaur Labs). The abdominal region was cleaned with a surgical detergent and laparotomy was performed by midline incision. With the exception of the sham control, acute renal failure was induced by removal of the right kidney (right nephrectomy) followed by 45 minutes occlusion of the left renal pedicle (artery and vein).

In this procedure, the animals were allowed to stabilize following the nephrectomy prior to the contralateral clamping. The perfusion of blood was reestablished by removing the vascular clamps and the abdominal cavity was closed with silk black-braided 5-0 Sharpoint precut suture (Sharpoint, USA). In addition to the baseline, blood samples were collected at the time of reperfusion, and then every 2 hours until sacrifice or the completion of the experiment. The right kidney (trimmed for identification) was pre-fixed in 10% formalin and the left kidney was harvested during sacrifice and fixed likewise.

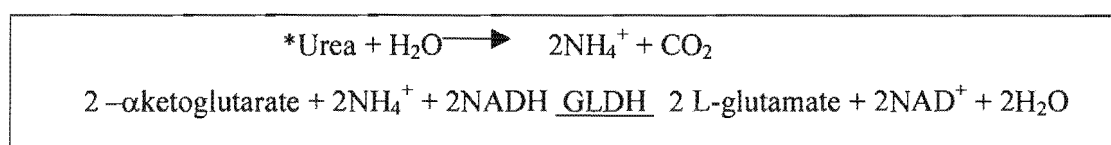
4.2.6.2 Administration of rVCP

The recombinant VCP (rVCP) was administered both intravenously (IV) and intraperitoneally (IP) in two of the rVCP recipients. A bolus dose of 8mg/kg was injected IV (through the renal vein), 60 minutes before applying the clamps and 30 minutes after clamping (although the renal vein was still clamped during this administration, the first blood that flows following reperfusion was expected to immediately transport the protein once the blood flow was re-established). An IP dose of 30mg/kg was administered 2 hours before the start of the experiment, just after the establishment of blood perfusion and then every 4 hours until sacrifice or the completion of the experiment. In two vehicle control animals, normal saline (0.9%) (Adcock Ingram, Johannesburg, South Africa) was administered both IV and IP at equivalent intervals to the administration of rVCP. Moreover, in order to observe the effect of a non-complement inhibitor protein on ischemia/reperfusion injury, one animal was administered (IV & IP) with bovine serum albumin (BSA) at equivalent intervals to the administration of rVCP and normal saline. BSA was chosen due to the easier availability of the protein.

In addition to the aforementioned control animals, one animal was used as a nephrectomy control without contralateral clamping. From this model, it was expected to draw lessons on the ability of a single intact kidney to clear metabolic wastes, the degree of stress and discomfort triggered by nephrectomy, and most importantly to evaluate the effect of proteins; complement inhibitor and non-inhibitor, in renal ischemia/reperfusion injury. A sham control was also included to understand the effect of anesthesia and the possible stress created by lengthy operations. In this animal, a similar surgical procedure was followed to dissect the kidneys. However, neither nephrectomy nor clamping was applied and the animal did not receive any injection during the course of the study. The body temperature of the animals was maintained by covering them with a regulated warming blanket.

4.2.6.3 Blood Urea Nitrogen (BUN) Assay (method adapted from the MPR2 Urea Kinetic UV method supplied insert)

The blood samples were allowed to clot at room temperature for 2 hours and the serum was separated by spinning at 3000 rpm for 25 minutes and stored at -20°C until use. In this biochemical experiment, the Kinetic UV method was used to quantify the concentration of urea in the serum samples. This method applies the breakdown of the urea in the sample into ammonium (NH_4^+) by the help of the catalytic enzyme urease and then the generated NH_4^+ undergoes an oxidation reaction with alpha-ketoglutarate and the coenzyme nicotinamide adenine dinucleotide (NADH) in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and NAD^+ which is proportional to the concentration of urea in the sample. The chemical reaction is displayed as follows:



***N.B:** The chemical representation of the reaction was reproduced from the MPR2 Urea Kinetic UV method supplied insert (Boehringer, Mannheim).

The amount of sample used, time of incubation and absorbance measurement filter was similar to the one explained under “*procedure-1*” above.

4.2.6.4 Creatinine Assay

The same batch of serum (to the ones used for BUN assay), and all other required solutions as well as the amount of sample used, time of incubation, standards and absorbance measurement filter described above for the creatinine assay were used in this set of experiment.

4.2.6.5 Histopathological Study

Both kidneys of each animal were sectioned longitudinally and then fixed, dehydrated, cleared, wax impregnated and embedded in paraffin. The sections were cut in $1\text{-}2\mu\text{m}$ sections and stained with Hematoxylin and Eosin (H & E) stain. A blinded pathologist evaluated the sections.

4.2.7 Procedure-3

Six male Long Evans rats weighing 156-176g were used in this study. The animals were caged under standard conditions including a regular light (12-hour) and dark (12- hour) cycle with free access to rat chow and water prior to the experiment. The animals were diet restricted from the day before the procedure.

4.2.7.1 Ischemia/Reperfusion Injury

The animals were anesthetized with a combination of ether inhalation and ketamine hydrochloride (100mg/kg, im). The abdominal area was sterilized with a cleaning solution and was shaved to expose the abdominal skin. Laparotomy was performed by midline incision and both kidneys were isolated. The animals were randomly allocated to receive rVCP, normal saline or sham controls (n = 2 each). Baseline blood was collected prior to the induction of ischemia, which was provoked by ceasing the flow of blood in both the right and left renal arteries for 45 minutes. The flow of blood was reestablished by removing the clamps and the abdominal cavity (skin and muscle layer) was closed with 4-0 and 6-0 prolene blue monofilament polypropylene sutures. The animals were then stabilized and returned to their cages. In the sham group, both the kidneys were dissected similarly without the application of clamps. Blood samples were collected 8 hourly for 24 hours and the kidneys were harvested for histopathological study.

4.2.7.2 Administration of rVCP

To further elucidate other potential route of rVCP administration, the rVCP was injected subcutaneously (SC) at 30mg/kg 2 hours before the surgical procedure and then every 4 hours for the duration of the study period. Normal saline (0.9%) was administered to the (saline) control animals at equivalent intervals to the administration of rVCP. No injection was given to the sham group during the study period.

4.2.7.3 Blood Urea Nitrogen (BUN) Assay

The BUN assay was performed as demonstrated above.

4.2.7.4 Analysis of serum Complement

In order to evaluate the efficacy of rVCP administration SC and the expected subsequent complement inhibition, the level of circulating (classical) complement was semi-quantified indirectly by hemolysis assay. First, the dilution of serum necessary to lyse 95% of the sensitized sheep red blood cells (ssRBCs) was determined by serum assay as described above.

Once the serum dilution was determined, 75µl of the ssRBCs were mixed with 10µl of the supplied GVB buffer and 15µl of serum from the rVCP, saline or sham control rats collected after 24 hours I/R injury. In the presence of classical complement inhibitors such as VCP the level of complement is expected to be minimal and therefore less of the ssRBCs would be lysed. The reaction mix was incubated at 37°C for 1 hour and then spun at 7000 rpm for 30 seconds. Seventy-five µl of each sample was transferred into a 96-well microplate. The absorbance was measured at 405nm and the percentage of lysis, proportional to the level of complement, was calculated and compared among the groups. Here, 75µl of ssRBCs were incubated without serum as a negative control and human serum at a dilution known to lyse 95% of ssRBCs was used as a positive control.

4.2.7.5 Histopathological Study (protocol adapted from Marilyn Tyler's adapted protocol, Department of Surgery, Groote Schuur Hospital (GSH), UCT)

The kidneys were sectioned longitudinally and then fixed in 10% formalin, dehydrated, cleared, wax impregnated and embedded in paraffin. The sections were cut in 1-2µm sections on a sliding microtome (Leica Microtome sliding 2000R), floated onto microscopic slides and stained with Hematoxylin and Eosin (H & E) stain as briefly described below.

4.2.8 Procedure-4

Eighteen male Long Evans rats weighing 345-418g were used in this study. The animals were housed under standard temperature and pressure (STP) including a regular light and dark cycle with free access to rat chow and water.

4.2.8.1 Ischemia/Reperfusion Injury

The animals were anesthetized with ketamine hydrochloride (100mg/kg, im). The abdominal cavity was cleaned with plasmalyte B cleaning solution and shaved with gamma radiation sterilized surgical blade (Paramount Surgimed Ltd). Laparotomy was performed by midline incision and the right kidney was dissected under surgical microscope. Baseline blood was collected from the inferior vena cava and ischemia was induced by clamping the right renal artery with non-traumatic microvascular clamps for 60 minutes. The perfusion of blood was reestablished by removing the clamp, and the abdominal wall was closed with 4-0 prolene blue monofilament polypropylene continuous suture (Ethicon, PA). The animals were randomly allocated to receive VCP or PBS (n = 6 each). Similarly, in the sham group (n = 6), the right kidney was dissected, however, no clamp was applied. At the end of the 24 hours experiment, the animals were overanesthetized and blood was collected from the aorta for biochemical studies. Moreover, the kidneys (left and right) were harvested for histopathological and immunohistochemical studies.

4.2.8.2 Administration of VCP/^{hrVCP}H98Y, E102K, E120K

In the VCP recipient group, a combination of the natural infection derived VCP and the newly characterized recombinant VCP (^{hrVCP}H98Y, E102K, E120K; abbreviated in the text as hrVCP) was administered both intravenously (IV) and intraperitoneally (IP). Due to its efficient *in vitro* complement inhibitory activity (Ghebremariam³ et al, 2005), lower doses of the hrVCP were administered in this model compared to the amount of rVCP used in the previous procedures. The hrVCP was initially administered IP at 3mg/kg, 2hours before the laparotomy. Subsequently, 5 minutes before clamping, 15 minutes before and 5 minutes after releasing the clamps, an IV dose of 600µg/kg was administered through the dorsal vein. To maintain the bioavailability of the protein in circulation, an amount of 3mg/kg was given IP every 4 hours until the completion of the study. The control group received similar volumes of phosphate buffered saline (PBS) at equivalent intervals to the administration of VCP/^{hrVCP}H98Y, E102K, E120K.

In this model, the normal saline administration was replaced by PBS because it was found that a previous study has shown the perfusion of rat kidney with normal saline causes increased structural and functional damage compared to a similar perfusion with PBS (Hughes et al, 1996). The sham group did not receive any injection during the entire study period.

4.2.8.3 Blood Urea Nitrogen (BUN) Assay

Despite its susceptibility to exogenous factors such as protein-rich diet in one extreme and starvation on the other, the BUN is routinely analysed to assess renal function. In this experiment, the serum levels of urea from the baseline and following the I/R injury were compared. Blood samples were allowed to clot for 45 minutes at room temperature and were centrifuged at 2500 rpm for 30 minutes. The concentration of urea was estimated using the Urease-UV Kinetic enzymatic assay kit (Kat Medical, Gauteng, South Africa) following the procedure inserted (Kat Medical).

4.2.8.4 Creatinine Assay

Serum samples obtained using the procedure described above were analysed for creatinine concentration (Merck Chemicals, Gauteng, South Africa) using the HiTachi U-2000 spectrophotometer (HiTachi, Japan) and the mean concentration was compared among the groups (Figure 4).

4.2.8.5 Histopathological Study (protocol adapted from Marilyn Tyler's adapted protocol, Department of Surgery, Groote Schuur Hospital (GSH), UCT)

The harvested kidneys were sectioned longitudinally before fixing them in 10% formalin (prepared in PBS). The sections were then processed (dehydrated, cleared and wax impregnated; Leica TP 1020 processor, Nussloch, Germany), embedded in paraffin wax, and cut in 1-2 μ m sections on a sliding microtome (Leica Microtome sliding 2000R). The sections were floated onto glass slides and heat fixed at 55^oC prior to Hematoxylin and Eosin (H & E) stain. The tissues were initially cleared in xylol for 3 minutes and then twice for 1 minute each prior to hydration in a series of absolute, 96%, 70% alcohol for 1 minute each. The sections were rinsed with tap water for 1 minute and then hematoxylin stained for 9 minutes. The sections were then rinsed in running tap water for 1 minute and dipped in acid alcohol for 10 seconds prior to rinsing with Scott's blue water for 1 minute and in running tap water for another minute. The sections were counterstained with eosin (1%) for 2 minutes and rinsed before dehydrating them in 70%, 96% and absolute alcohol respectively. The sections were finally cleared with xylol and covered with Canada balsam cover slip prior to examination.

The histology was interpreted by a pathologist blinded to the treatment that the animals received and the degree of injury was scored (Figure 8).

4.2.8.6 Immunohistochemical Study (*The VCP immunostaining was performed by Amod P. Kulkarni using protocols modified from Marilyn Tyler's; Department of Surgery, GSH, UCT and Amod P. Kulkarni's; Division of Medical Virology, UCT, adapted protocols using the proteolytic enzyme antigen retrieval method, and the C3 staining was performed by Heather Iisley, chief medical technologist in the Division of Anatomical Pathology, UCT*)

The paraffin embedded tissue sections (mounted on APES coated slides) were immunostained for VCP and the complement component C3. For the VCP staining (Figure 9), the sections were heat fixed at 60⁰C for about 1 hour prior to dewaxing in xylol for 20 minutes (4 changes, 5 minutes each) and then rehydrated from absolute alcohol to 96% (2 changes, 2 minutes each) and quick rinsed in PBS. Afterwards, the tissue's own (endogenous) peroxidase activity was inhibited using 2% commercially available H₂O₂, diluted in distilled water, for 5 minutes and then quick rinsed in PBS. Thereafter, both the pressure cooker and the proteolytic enzyme antigen retrieval methods were attempted. In the pressure cooker method, the citrate buffer (pH 6.0) was initially boiled and the slides were immersed in the buffer. The cooker was switched on and the boiling pressure was maintained for 2 minutes to 'cook' the tissue before releasing the pressure by the process of conduction. Alternatively, in the proteolytic enzyme antigen retrieval method, the slides were treated with the supplied ready-to-use proteolytic enzyme (DakoCytomation, Denmark) for 8 minutes. The slides were transferred to running tap water for 10 minutes and then rinsed in PBS-T(ween 20) (Appendix A) for 5 minutes. The sections were blocked with 1:20 diluted normal goat serum (DakoCytomation, Denmark) for 10 minutes and then incubated with chicken anti-VCP primary antibody (1:150) at 4⁰C overnight. For the negative controls, no primary antibody was added. The slides were rinsed with PBS-T for 10 minutes prior to 50 minutes incubation with 1:350 diluted goat anti-chicken secondary antibody (Vector Laboratories, Burlingame, CA). The slides were quick rinsed with PBS-T and then incubated for 30 minutes with 1:400 diluted avidin (Dako A/S, Denmark) and rinsed again with PBS-T prior to 5 minutes incubation with the chromogenic substrate 3,3'-diaminobenzidine (DAB) (Novocastra) and then rinsed with PBS and water before enhancing the color with 1% copper sulphate solution for 5 minutes. The sections were counter stained with hematoxylin for 30 seconds and then treated with Scott's blue water for 30 seconds.

Finally, the slides were rinsed with tap water for 5 minutes, dehydrated (in a series of 70%, 96% and absolute alcohol), xylol cleared and then mounted in Entellan (Marienfeld, Germany). The specificity and the degree of antibody staining were interpreted by a pathologist and scored semi-quantitatively.

4.2.8.7 Complement Component C3 staining

For the complement staining, APES coated slides were used. The sections were xylol dewaxed and rehydrated prior to treatment with protease (5mg/ml) at 37⁰C for 30 minutes. The slides were rinsed with PBS for 5 minutes and then blocked for endogenous peroxidase activity using 1% H₂O₂ for 10 minutes and then rinsed in tap water and PBS sequentially. The tissues were blocked with 1:3 diluted normal sheep serum for 20 minutes. Afterwards, the serum was drained off and the slides were incubated with 1:700 diluted rabbit anti-human polyclonal C3 antibody (Dako A/S, Denmark) for 45 minutes and then washed with PBS for 5 minutes prior to incubation with 1:160 diluted swine anti-rabbit peroxidase conjugated secondary antibody. The slides were washed with PBS for 10 minutes and then the color was developed for 2 minutes using DAB. The slides were rinsed and the color was enhanced with copper sulphate solution (1%) for 5 minutes followed by a brief wash with distilled water and counterstaining with Mayer's hematoxylin for 30 seconds. Finally, the sections were treated with Scott's blue water for 30 seconds and then dehydrated, cleared and mounted for immunohistochemical examination. Here, the uninjured (left) kidney of each group mounted on the same slide was used as a background control and the intensity of C3 staining in the right kidneys were compared among the different groups (Figure 10). Rabbit anti-human polyclonal antibody (Dako A/S, Denmark) with good cross-reactivity to rat C3 was used as a primary antibody and the staining was performed in the department of anatomical pathology, UCT.

Statistical Analysis

The creatinine concentration results are demonstrated as mean \pm SEM of n independent experiments. Microsoft Excel was used to plot the creatinine charts.

4.2.9 Procedure-5

Fourteen male Long Evans (LE) rats weighing 422 – 479g were used in this study. The animals were housed under standard conditions (temperature, pressure and humidity) including a regular light (12-hour) and dark (12-hour) cycle with free access to rat chow and distilled water. The modified protocol was amended by the University of Cape Town animal research ethics committee.

4.2.9.1 Ischemia/Reperfusion Injury

Based on the various invasive approaches applied to establish ischemia/reperfusion-induced acute renal failure, the optimization procedure was finally refined in my own hands. In order to stimulate an ischemia model with both structural and functional feasibility, the following procedure was applied in this experiment. The animals were anesthetized with forane (isoflurane) inhalation (Abbott Laboratories S.A. (pty) Ltd) and the abdominal cavity was cleaned with a cleaning solution. The abdominal skin was exposed and laparotomy was performed by midline incision. Both kidneys were carefully dissected under surgical microscope and baseline blood was collected from the inferior vena cava. Acute renal failure was triggered by ceasing the flow of blood (as confirmed by visual inspection of the kidneys) in both right and left arteries for 60 minutes followed by 24 hours reperfusion. The abdominal cavity was closed with 3-0 ethilon blue monofilament polyamide 6 non-boilable continuous suture (Ethicon, Johnson&Johnson, PA). The animals were allowed to recover after the surgical procedure and were administered with 100µl of the analgesic drug temgesic (300µg/ml) (Schering-Plough Ltd, R & C Pharmaceuticals, UK) intramuscularly (IM), to minimize the degree of pain and discomfort provoked by the procedure. The renal failure was induced in the VCP, the ^{hrVCP}H98Y, E102K, E120K recipients (n = 3 each) and the PBS vehicle control groups (n = 4). In the sham group (n = 4), both the kidneys were similarly isolated without the application of vascular clamps. After 24 hours, the animals were overanesthetized and blood was collected from the aorta for BUN and creatinine studies. The kidneys were harvested for histopathological and immunohistochemical studies.

4.2.9.2 Administration of VCP/^{hrVCP}H98Y, E102K, E120K

Based on the promising therapeutic role of these proteins, it was decided to elucidate the therapeutic potential of each protein independently. Three animals received the yeast-cell expressed humanized recombinant VCP, with three critical amino acid differences to the rVCP, (^{hrVCP}H98Y, E102K, E120K) and the other three animals were administered with the mammalian cell derived natural VCP.

On the basis of the previous partial protection outcomes, higher doses of the proteins (to assess whether the amount of protein administered was a limiting factor) was administered both intravenously (IV) and intraperitoneally (IP). Two hours before the laparotomy, an IP dose of 4mg/kg was administered initially. To effectively knock down the level of complement, an IV (dorsal vein) dose of 1mg/kg was then injected 5 minutes before applying the clamps, 45 minutes after clamping and 5 minutes after releasing the clamps. Due to its slow absorption and hence relatively delayed elimination from the body, the IP dose was administered sparingly every 4 hours for the 24 hours study period. The vehicle control group received the same volumes of PBS at equivalent intervals to the administration of VCP. The sham group did not receive any injection during the study period.

4.2.9.3 Blood Urea Nitrogen (BUN) Assay

The serum urea nitrogen concentration was comparatively estimated between the baseline level and following the I/R injury. The blood samples were allowed to clot for 2 hours at room temperature and the sera was separated by centrifugation at 3000g for 30 minutes. This enzymatic assay was performed using the Urease-UV Kinetic kit following the procedure provided as described above.

4.2.9.4 Creatinine Assay

The serum creatinine concentration was estimated using the procedure described above and was repeated in an independent laboratory (The National Health Laboratory Service (NHLS), Clinical Pathology laboratory, GSH, UCT) using an automated technique.

4.2.9.5 Histopathological Study

This experiment was performed using the protocol adapted from Marilyn Tyler's (Department of Surgery, GSH, UCT) adapted protocol. The formalin (10%) fixed kidney tissues were dehydrated by immersing them in an increasing percentage of ethanol, the dehydrated sections were then xylol cleared and wax impregnated; Leica TP 120 processor, Nussloch, Germany). The tissues were then embedded in liquefied paraffin wax (55-60°C), and cut in 1-2µm sections on a sliding microtome (Leica Microtome sliding 2000R). The sections were mounted onto microscopic slides and heat fixed at 55°C incubator. The H & E staining protocol was as demonstrated under procedure-4 above. The histology was examined and interpreted by a pathologist blinded to the treatment that the animals received and the degree of injury was semi-quantitatively scored.

4.2.9.6 Immunohistochemical Study

To assess the density and distribution of the complement component C3, the paraffin embedded tissue sections mounted on APES coated slides were immunostained as described under procedure-4 above. In this experiment, only the proteolytic enzyme antigen retrieval method was used. The specificity, degree and the distribution of antibody staining was interpreted by a blinded pathologist and graded semi-quantitatively.

Statistical Analysis

The BUN and creatinine concentration results are demonstrated as mean \pm SEM of n independent experiments. The values were subjected to one-way analysis of variance (ANOVA). ANOVA, developed by Sir Ronald A. Fisher in the 19th century, is a scientifically valid statistical method of performing multiple comparisons simultaneously and hence has a differential advantage over pairwise comparisons employed by *Student's t-test* when dealing with multiple groups at a time (Durrheim, 2002). In the present experiment, the analysis was performed using both the "STATISTICA" and "R" software packages. "STATISTICA" was used when there was homogeneity of variance in the data, and "R" was used when 'relaxation' of variance was needed. Both the programs were kindly run by *Dawit Y. Ghebrehiwet, Department of Zoology, UCT.*

4.3. RESULTS

4.3.1 Procedure-1

4.3.1.1 Mortality

The small size of the animals, frequent bleeding (bled up to 4 times of 500-700µl each time), unavoidable surgical stress, the possible hypoxia triggered by the anesthesia as well as the induced ischemia, accounted for rapid deterioration of the animals' health. As a result, the experiment was humanely terminated by sacrificing the animals between 5 hours 20 minutes and 8 hours and 30 minutes post-reperfusion.

4.3.1.2 Serum Creatinine and BUN

Due to the fewer number of animals used in this experiment, the short survival of the animals, and the extreme fluctuation in the biochemical data analysed, there was statistically insignificant (extremely high variance) levels of BUN and creatinine within and among the groups. Therefore, the serum urea and creatinine concentration results were rejected based on the null hypothesis (Hoffman, 2002).

4.3.1.3 Urinalysis

The urine samples obtained through catheterization from the baseline and the sacrifice times were SDS-PAGE (12%) analysed to assess the filtration capacity of the renal system for large proteins that could otherwise had been reabsorbed under physiological conditions. Despite the short survival (reperfusion injury) there was significant excretion of bulky proteins following the ischemia/reperfusion injury suggesting early pathological changes in the renal system of the animals exposed to I/R injury (Figure 3). The intensity of the post-reperfusion sample in the PBS vehicle control group appears slightly dense (Figure-3A, lane-6) compared to the VCP treated animals (Figure-4A, lanes 2& 4) suggesting the therapeutic value of VCP. Interestingly, the post-reperfusion urine sample in the sham group also displayed significantly brighter banding patterns (Figure- 3B, lane-3) compared to the baseline protein bands (Figure-3B lane-2) suggesting the possible reperfusion injury due to stress, anesthesia and dehydration-induced hypovolemic shock.

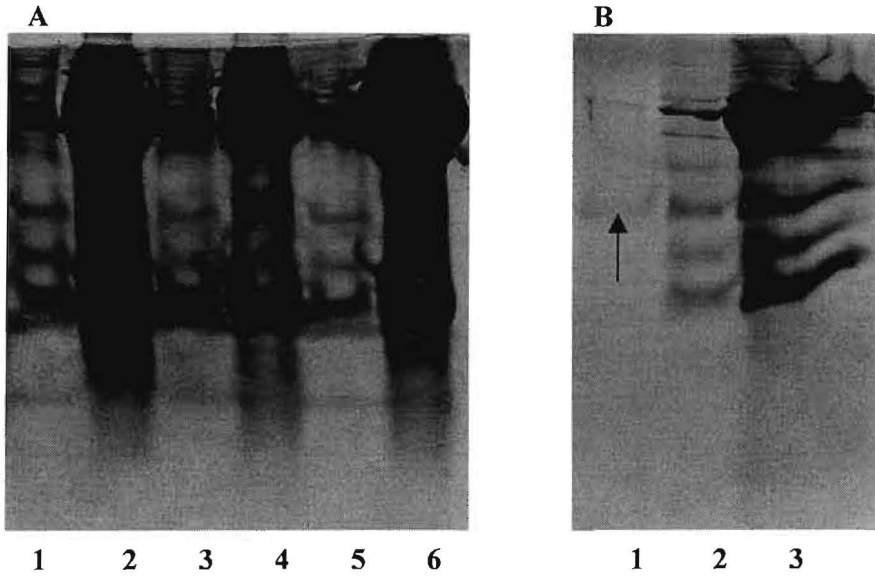


Figure 3 (A&B): SDS-PAGE analysis of urine samples **A)** pre (lanes 1 & 3) and post I/R injury (lanes 2 & 4) of VCP treated rats. Pre (lane-5) and post (lane-6) I/R injury of PBS group. **B)** Standard VCP (28.8 kDa) indicated by arrow (lane-1); baseline control from sham 'injured' (lane-2) and sampled at sacrifice (after ~ 8 hours) (lane-3).

4.3.2 Procedure-2

4.3.2.1 Serum Creatinine and BUN

The animals were kept lying on the operation (OR) table (the animals were needed to be sedated due to the IV catheterization) for the duration of the experiment or until death. This caused significant swelling in the extremities due to insufficient circulation. In addition, the animals were frequently bled for biochemical studies. Consequently, the rVCP treated, one (of two) PBS control, the nephrectomy control and even the sham control animals died between 5 hours 35 minutes and 17 hours 30 minutes. Neither the BUN nor the serum creatinine levels had significantly deviated from the baseline values in these animals.

One of the saline group animals survived for 23 hours post reperfusion and therefore the BSA administered animal was sacrificed for comparison. In these two animals, the BUN levels significantly increased from 21.5mg/dl baseline in the BSA group to 121.4 mg/dl at sacrifice and from 18.68mg/dl baseline in the saline group to 192.40mg/dl at sacrifice suggesting a significant accumulation of the nitrogenous waste following the induced ischemia/reperfusion injury. However, there was no significant difference in the creatinine levels.

4.3.2.2 Renal Histopathology

The animals that died few hours post-reperfusion did not show any significant histologic changes, in the 'injured' left kidneys', beyond mild apoptosis and some condensation of chromatin that are markers of early cell damage. However, the saline control and the BSA 'treated' animals displayed extensive tubular necrosis. Surprisingly, the BSA 'treated' rat showed even more pathologic changes than the saline control vehicle (data not shown) suggesting that administration of bovine serum albumin (BSA) did not only fail to provide any protection but also contributed further and unanticipated damage possibly by increasing the plasma volume and therefore reperfusion injury.

4.3.3 Procedure-3

4.3.3.1 Blood Urea Nitrogen

Surprisingly, the BUN levels post ischemia/reperfusion injury was unequivocally lower than the corresponding baseline samples in 5/6 (83.3%) of the animals' suggestion either decreased urea production by the liver or starvation. To rule out the first possibility, liver tissues from each group were H & E stained and interpreted by an expert. The liver histology revealed normal cytology and tissue architecture (data not shown). It is very likely that the second possibility, starvation, was the reason as the animals were starved from the day before the experiment to avoid exogenous factors such as diet influencing the outcome. Moreover, the pre and post procedure weighing of the animals revealed a significant drop in the body weight (the average weight loss was 18 grams per animal).

4.3.3.2 Complement Analysis

The serum complement level before and following ischemia reperfusion injury was analysed. All the animals showed similar levels of circulating complement (classical component) despite the injury or lack of injury and despite the administration of rVCP suggesting that the injury did not stimulate hyperactivation of the classical component of complement pathway and the administered rVCP did not inhibit the classical pathway of complement activation suggesting that the subcutaneous (SC) route is not an effective route for systemic delivery. This was in agreement to previously observed outcomes (Pers. Comm. Scott A. Smith, University of Louisville, KY, USA). However, because renal ischemia/reperfusion injury predominantly involves the alternative complement pathway, this observation may only have peripheral importance.

4.3.3.3 Renal Histopathology

The 45 minutes bilateral clamping approach did not induce a fine ischemia/reperfusion injury model in my own hands. The histology revealed only focal necrotic changes and was not feasible to compare the effect of rVCP administration.

4.3.4 Procedure-4

4.3.4.1 Serum Creatinine and BUN

As shown in Figure 4 below, there was considerable difference (1.2-fold \pm 0.127) in the serum creatinine levels between the untreated injured (PBS) group and the VCP/hrVCP treatment group, suggesting that the VCP improved renal function and enabled the renal system to excrete the metabolic waste better than the equally injured animals in the untreated group. The sham group displayed the lowest serum creatinine levels as expected. However, there was no statistically significant difference in the serum urea and creatinine levels among any of the groups.

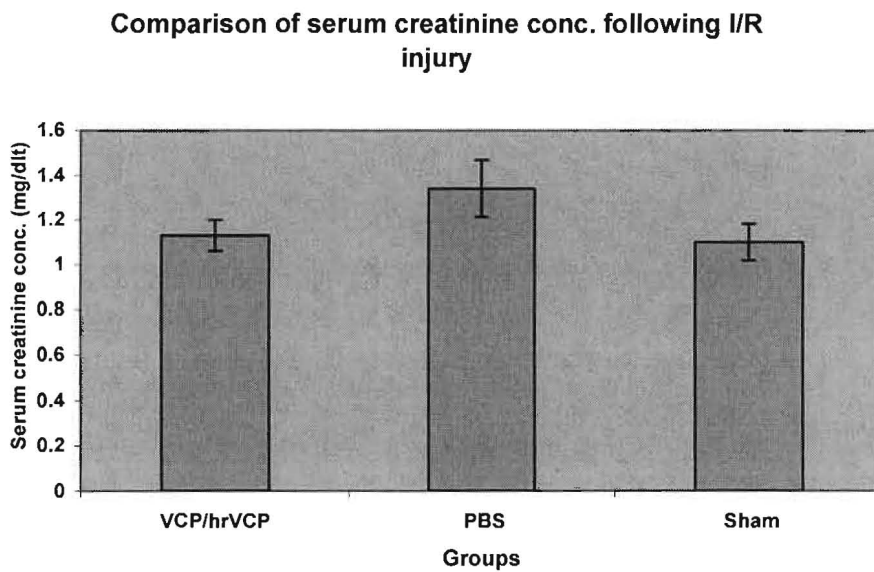


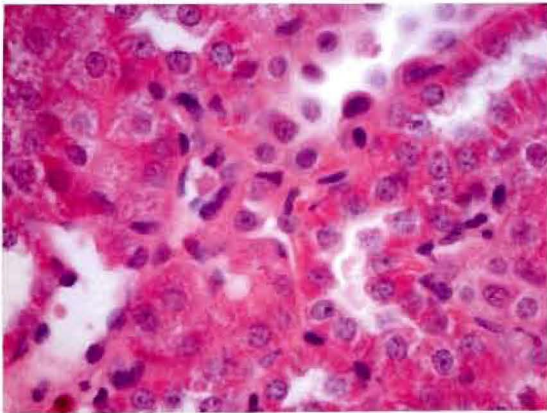
Figure-4: Mean Serum creatinine concentration after 24hrs I/R injury. Bars represent standard errors of sample mean (mean \pm SEM).

Interestingly, one additional, bilaterally clamped, animal showed a 2.9-fold rise in serum creatinine and a 25.3-fold rise in serum BUN levels after 24 hours of I/R injury compared to its baseline concentrations suggesting that acute renal failure (ARF) was induced in this animal.

4.3.4.2 Renal Histopathology

Figure 5, 6 and 7 show the representative renal histologies of the sham, VCP and PBS groups respectively. In the sham group, the tissue displayed normal glomerular (not shown in Figure) and tubular histology (Figure 5A & B) in both kidneys. In the VCP group (Figure 6B), focal necrosis of the tubular epithelial cells was observed in 5/6 (83%) of the animals compared to the markedly elevated and diffuse necrosis of the tubular epithelium in the PBS group (Figure 7B). The uninjured kidneys were stained for intra-group comparison (Figure 6A for the VCP & Figure 7A for the PBS group). The representative right kidneys from each group are displayed for simultaneous comparisons (Figure 8 A-C).

A



B

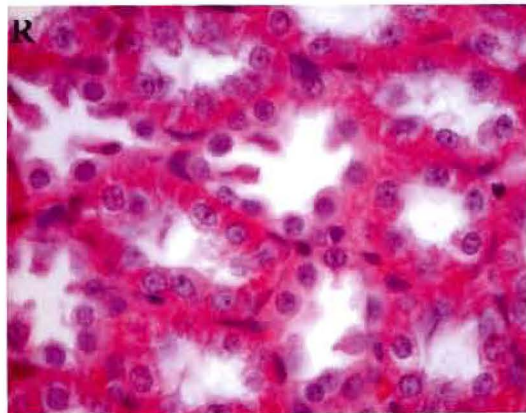
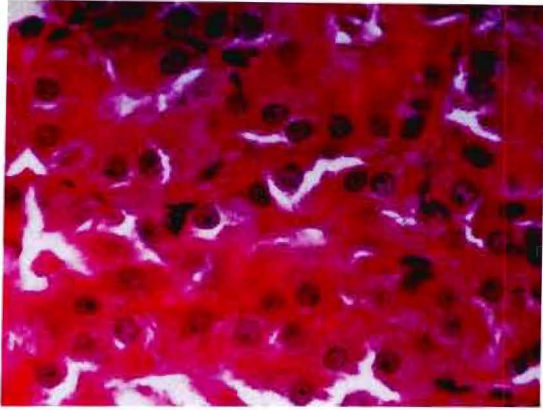


Figure 5: H & E stained left (**A**) and right (**B**) kidney histology displaying normal tubular epithelial cells following sham ‘injury’ (photographs taken at 40X objective magnification at different contrast of visible light).

A



B

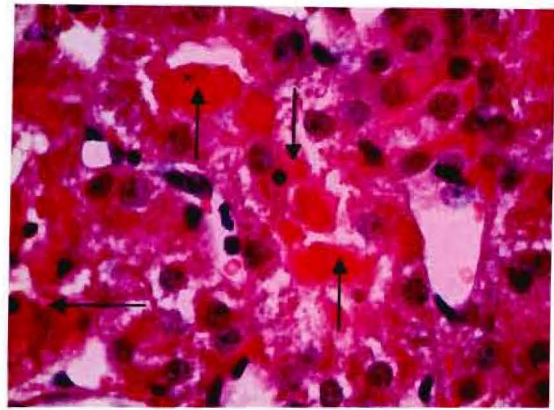


Figure 6: H & E stained kidney histology in the VCP treated group demonstrating normal tubular epithelial cell population in the left kidney (**A**) and focal necrosis (arrows) of tubular epithelial cells (**B**) following 24-hours ischemia/reperfusion injury (40X objective magnification).

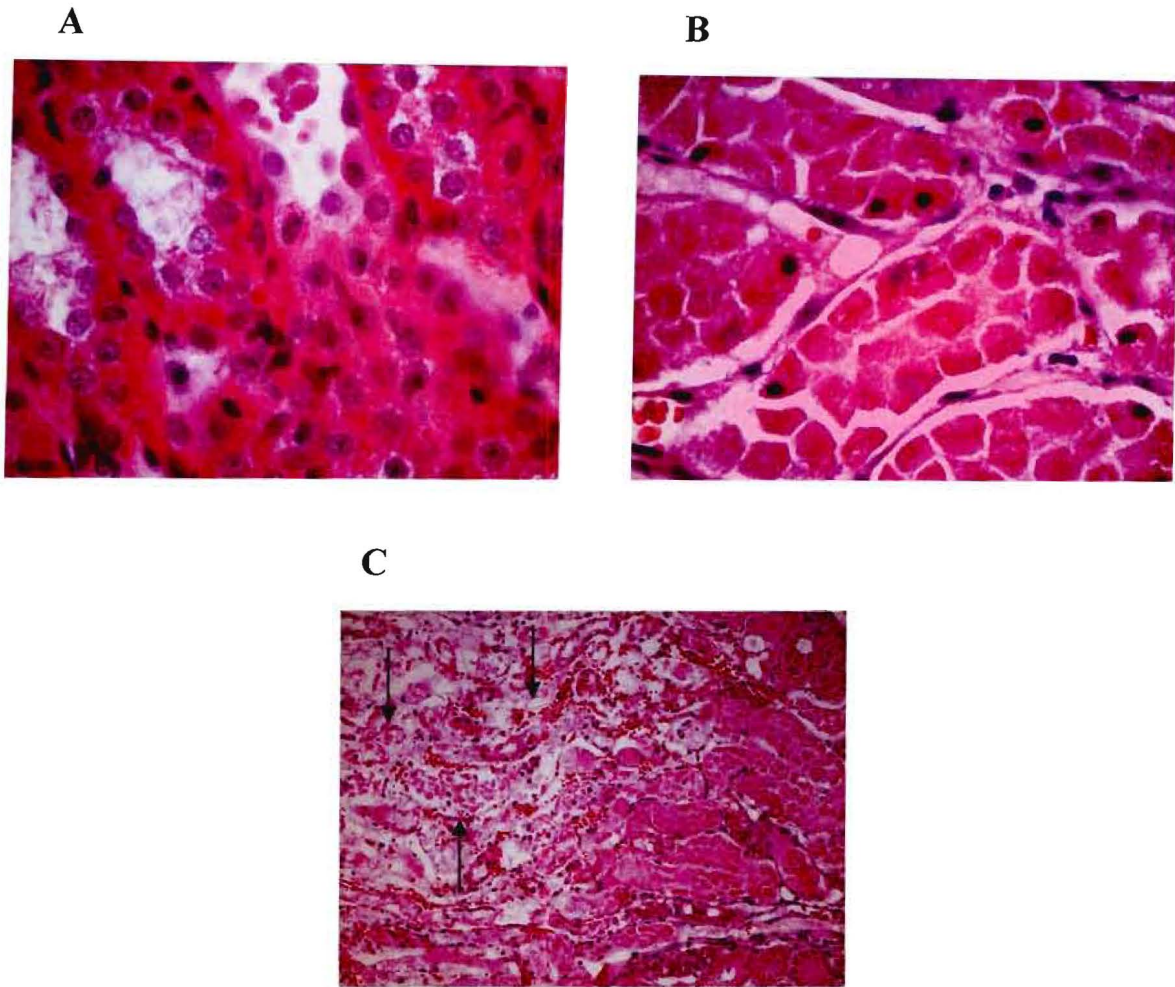
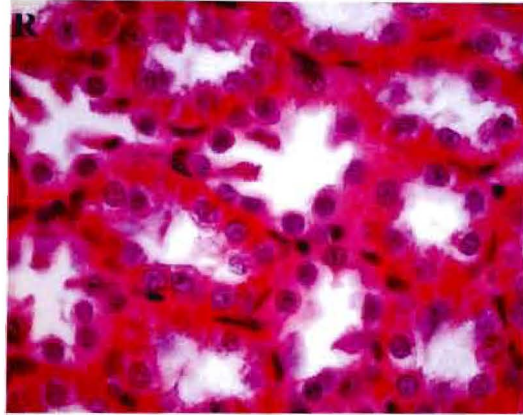
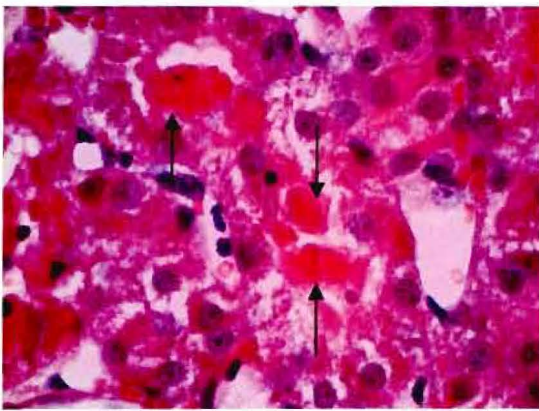


Figure 7: H & E stained kidney histology in the PBS vehicle control group portraying normal tubular epithelial cell population in the left kidney (A) and extensive tubular epithelial cell necrosis in the right renal cortex (B). C) shows cortico-medullary (arrows) necrosis following 24-hours ischemia/reperfusion injury. All, except C) at 10X, were at 40X objective magnification.

A



B



C

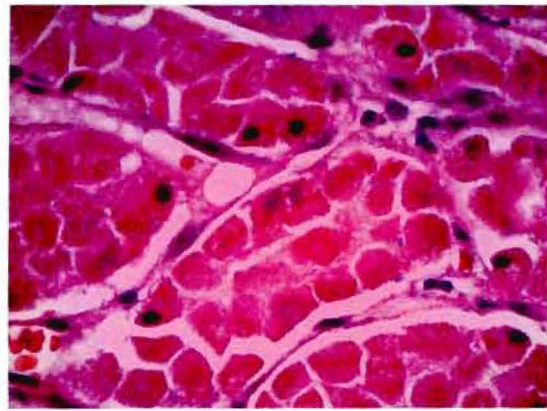


Figure 8: H & E stained right kidney histology: **A)** normal tubular epithelial cells (sham group); **B)** focal necrosis (arrows) of tubular epithelial cells (VCP group) and **C)** extensive tubular epithelial cell necrosis (renal cortex) (PBS group). (40X objective magnification).

4.3.4.3 Immunohistochemistry

The antibody staining for VCP revealed that the protein administered systemically reached the kidneys and was retained significantly in the renal tubules and minimally in the apical membranes of the renal cortical tubular cells as well as the Bowman's capsule epithelium (Figure 9A). This is most likely due to the heparin binding property of VCP (Smith et al, 2000), as the heparin like molecule, heparan sulfate proteoglycan (HSP), is an essential constituent of the tubular basement membrane (Lelongt et al, 1988). Both the kidneys have almost equally retained the administered protein despite the level of injury to the right kidney. Both the PBS and the sham group (Figure 9B) did not show any VCP staining as expected.

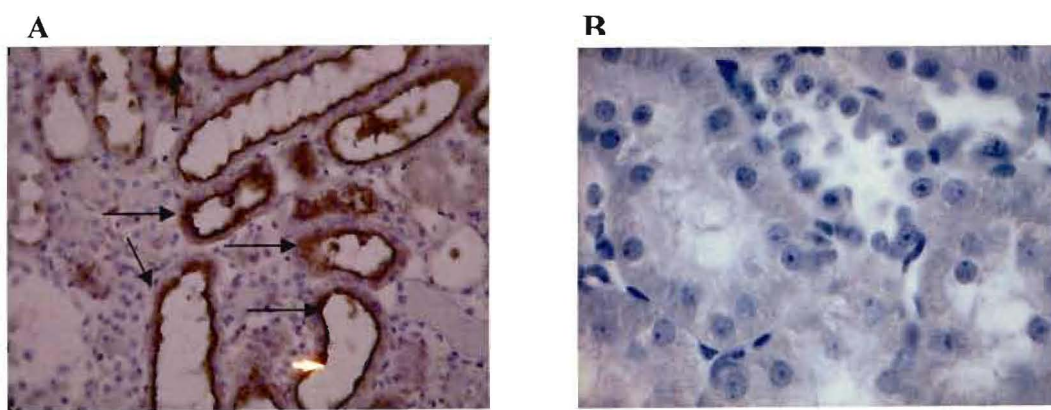


Figure 9: Immunostaining for VCP showing **A)** strong tubular epithelium stain (arrows) and **B)** negatively stained section from untreated group. Magnification at 40X.

The C3 immunostaining for the VCP group showed minimal and extremely focal (<5%) C3 deposition in the epithelium of the injured kidney (Figure 10A). However, the PBS group displayed a strong tubular epithelium stain (25-50%) in the injured kidney (Figure 10B) suggesting high levels of C3 deposition in the renal tubules following I/R injury. The sham group (Figure 10C) and the left (uninjured) kidneys of the VCP and the PBS group showed only basal staining between the epithelia.

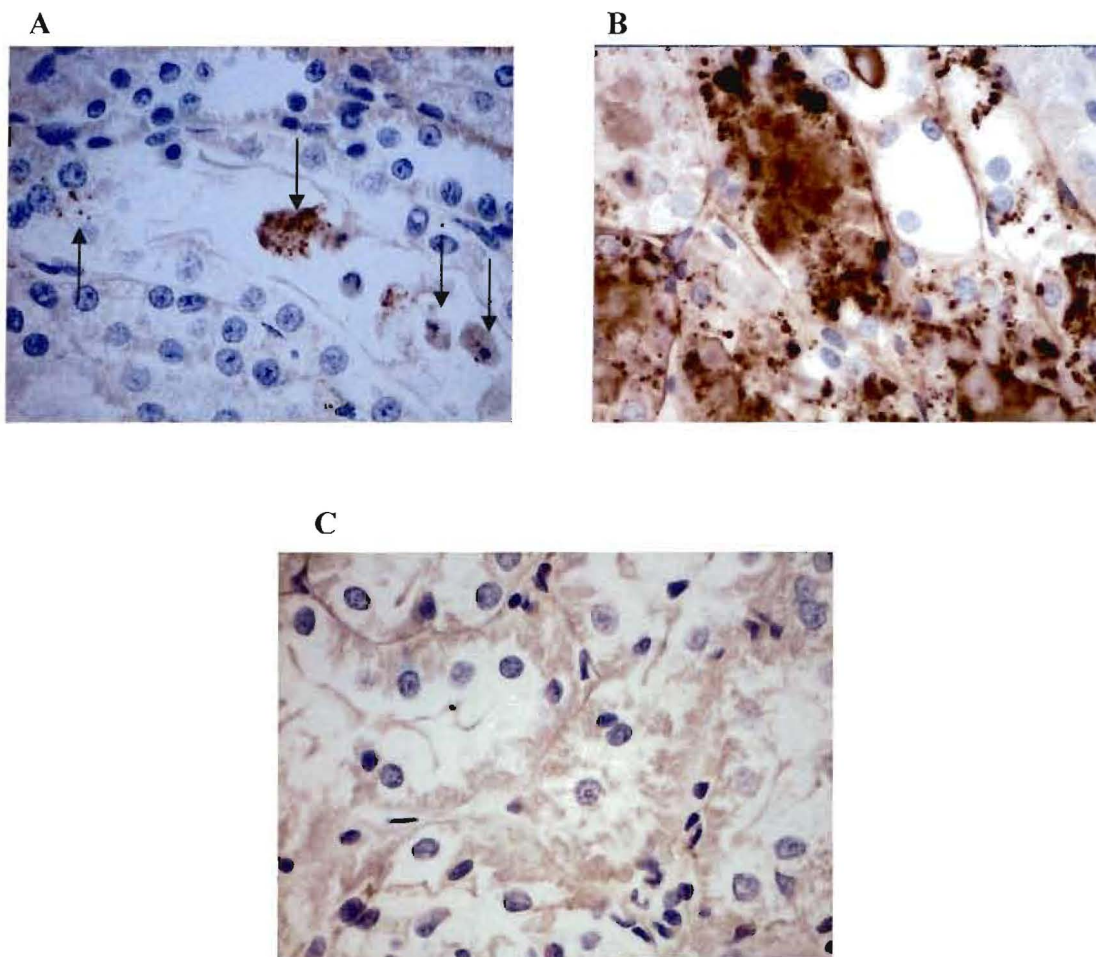


Figure 10: Immunostaining for C3: **A)** focal deposition in the tubular epithelium of the VCP group (arrows) & **B)** markedly elevated deposition in the renal tubules of the PBS group. **C)** shows basal staining between the epithelium in the sham group. Magnification at 40X.

4.3.5 Procedure-5

4.3.5.1 Blood Urea Nitrogen (BUN)

As shown in Figure 11A below, there were substantial differences (3.7-fold) in the serum BUN levels between the natural VCP treatment group and the untreated injured (PBS) group suggesting the effective therapeutic potential of VCP. Moreover, there was about 1.73-fold lower accumulation of urea in the yeast cell derived humanized recombinant VCP (hrVCP) treated animals compared to the PBS group suggesting further the therapeutic value of the modified recombinant protein.

Comparison of serum Urea concentration following I/R injury

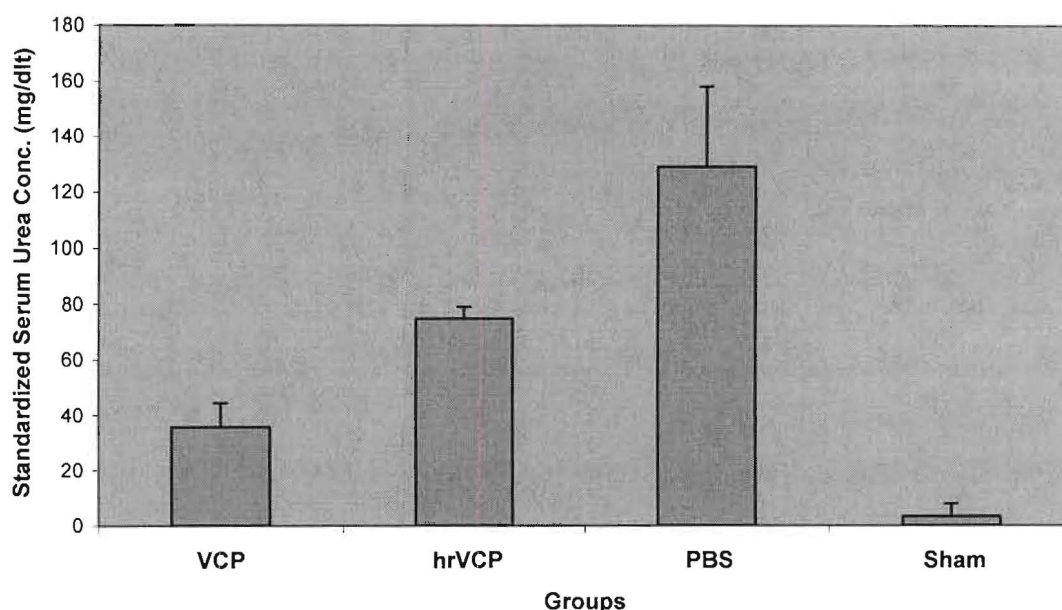


Figure 11A: Mean Serum Urea concentration following 24 hours bilateral I/R injury (Note: a 3.7-fold and 1.73-fold rise in the PBS group compared to the VCP and the hrVCP treatment groups respectively. The Sham group showed almost intact urea concentrations. Bars represent standard errors of sample mean (mean \pm SEM).

Moreover, a powerful statistical method was used to analyse the significance of the differences in serum urea concentration among the different groups. The natural VCP treated animals ($p < 0.05$) and the sham controls ($p < 0.01$) showed statistically significant difference compared to the PBS group (Figure 11B) suggesting the therapeutic potential of the natural VCP. Despite the noticeable differences in the mean values, treatment with hrVCP did not show statistically significant improvement ($p > 0.05$) in clearing urea from the circulation in comparison to the PBS group as shown in Table 1 below.

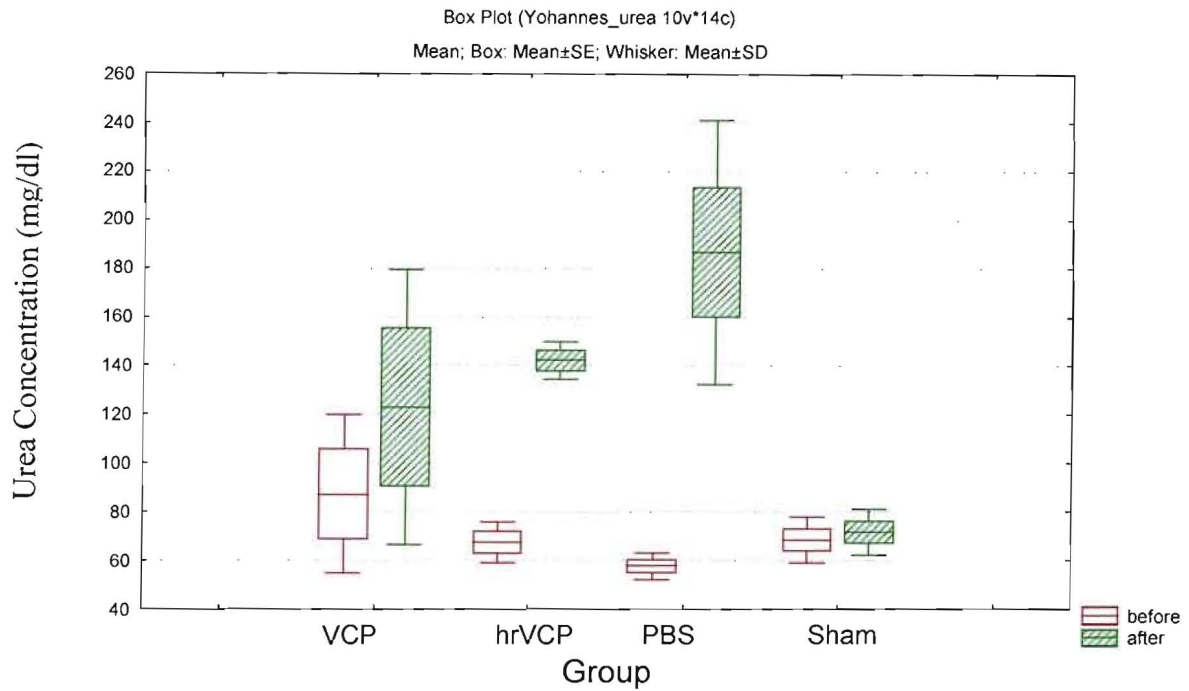


Figure 11B: Comparison of serum urea concentration at baseline (open boxes) and following 24 hours ischemia/reperfusion injury (grid boxes) of the VCP, hrVCP, PBS and the Sham groups. The boxes show the mean value \pm standard error and the whiskers display the mean \pm standard deviation. *The analysis was performed by Dawit Y. Ghebrehiwet, Department of Zoology, UCT using the “R” software package.*

Group	VCP	hrVCP	PBS	Sham
VCP	-	0.555049	0.026913	0.639774
hrVCP	0.555049	-	0.248003	0.096248
PBS	0.026913	0.248003	-	0.002569
Sham	0.639774	0.096248	0.002569	-

Table 1: Analysis of variance followed by the *Tukey HSD test*. The statistically significant differences in urea concentration ($p < 0.05$) are displayed in boldface type.

4.3.5.2 Serum Creatinine Concentration

The creatinine study also showed noticeable rise in the PBS group suggesting the failure of the kidneys to excrete the metabolic waste. However, following treatment with the natural VCP or the humanized recombinant VCP (hrVCP), the levels of serum creatinine dropped by about 8.5-fold and 11.6-fold respectively (Figure 12A). However, the ANOVA did not detect statistical significance at a p value below 0.05 among the treatment (VCP/hrVCP) and the PBS group (Figure 12B).

Comparison of serum Creatinine concentration following I/R injury

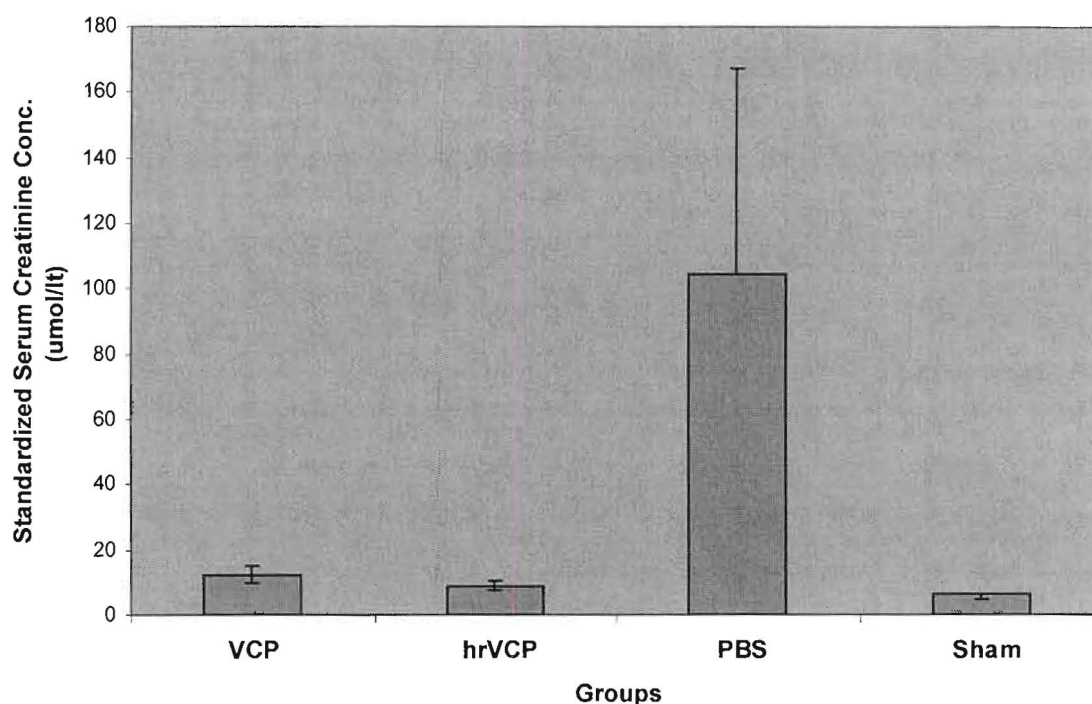


Figure 12A: Mean Serum Creatinine concentration after 24 hours Ischemia/Reperfusion (I/R) injury (Note: ~8.5-fold and 11.6-fold increase in the PBS group compared to the VCP and the hrVCP treatment groups respectively. The sham group showed normal creatinine concentration. Bars represent standard errors of sample mean (mean \pm SEM).

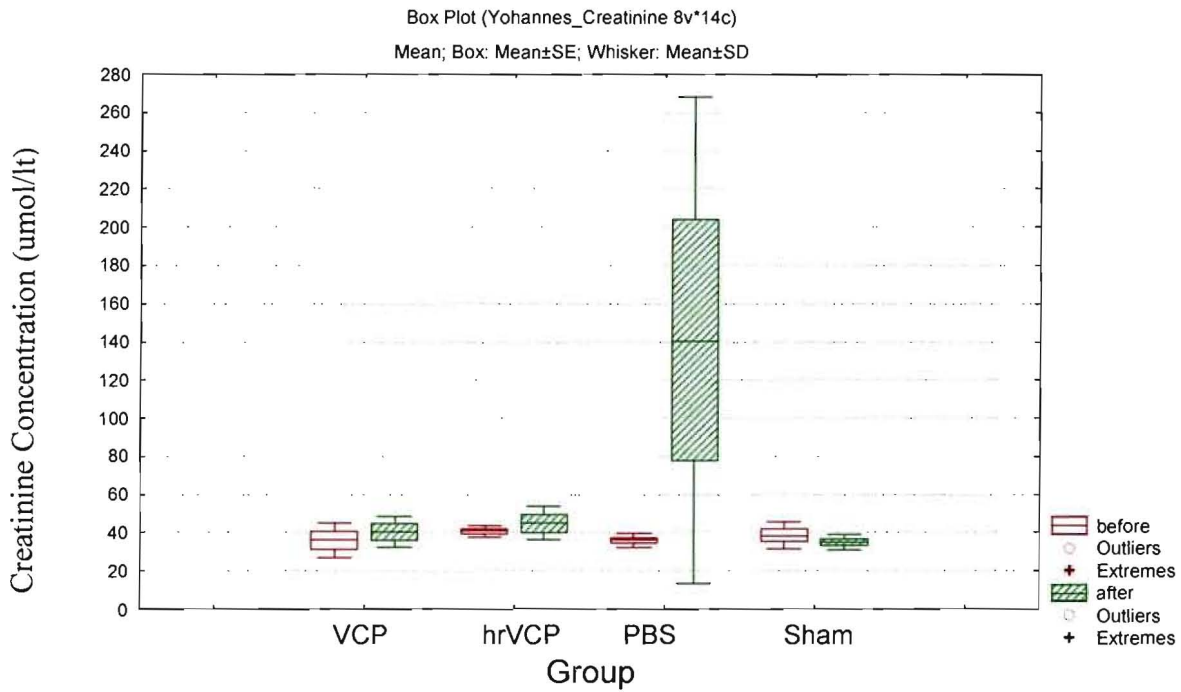


Figure 12B: Comparison of serum creatinine concentration displaying the baseline (open boxes) and after 24 hours ischemia/reperfusion injury (grid boxes) of the VCP, hrVCP, PBS and the Sham groups. The boxes show the mean values \pm standard error and the whiskers show the mean \pm standard deviation. *The analysis was performed by Dawit Y. Ghebrehiwet, Department of Zoology, UCT using the “R” software package.*

4.3.5.3 Renal Histopathology

The interpretation of the renal histologies by a blinded pathologist revealed that all the PBS group experienced severe and diffuse tubular necrosis (3+ to 4+) in the cortex and partly involving the medulla following the ischemia/reperfusion injury (Figure 13D). The severity of injury was equivalent in both kidneys suggesting that acute renal failure was induced. However, following treatment with VCP or hrVCP, the necrosis was correspondingly focal (1+ to 2+) and limited to the cortex (Figure 13B&C) indicating the beneficial role of intervention with VCP/hrVCP. The sham group showed normal renal architecture (0 injury score) in both kidneys (Figure 13A).

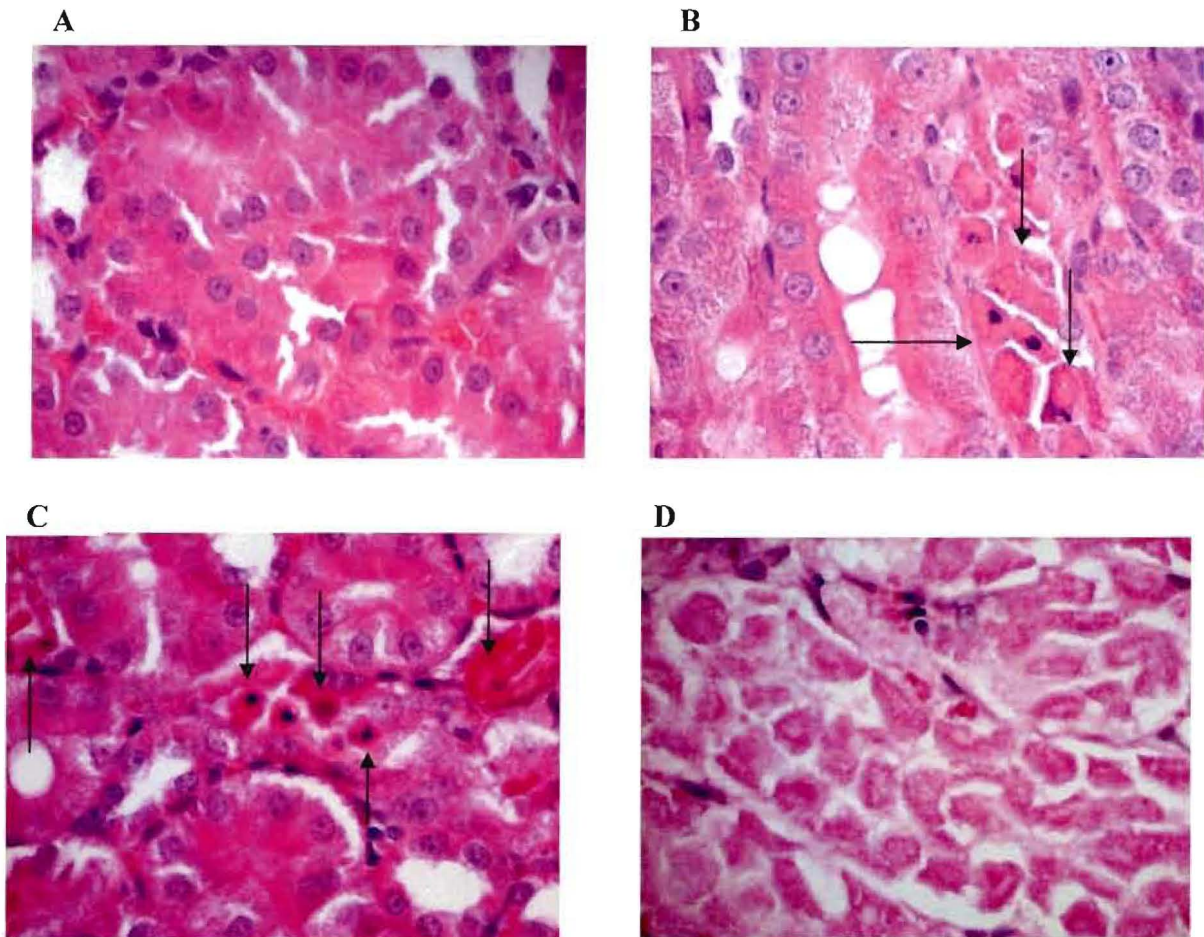


Figure 13: H & E stained renal histology showing **A)** normal tubular epithelial cells following sham 'injury', **B)** focal necrosis (arrows) of tubular epithelial cells following natural VCP treatment, **C)** focal necrosis (arrows) of tubular epithelial cells following recombinant VCP (hrVCP) treatment and **D)** diffuse necrosis of tubular epithelial cells in the PBS group following 24-hours ischemia/reperfusion (I/R) injury. (Representative fields of the Sham, VCP, hrVCP and the PBS groups are shown) (40X objective magnification).

4.3.5.4 Early Migration of Inflammatory Cells

Interestingly, the H&E stained kidney sections of the PBS group showed aggregation of few neutrophils suggesting early migration of inflammatory cells (Figure 14). However, there were no neutrophils in the Sham or the VCP/hrVCP groups indicating that VCP/hrVCP inhibited migration of inflammatory cells to the site of injury following 24 hours ischemia/reperfusion injury.

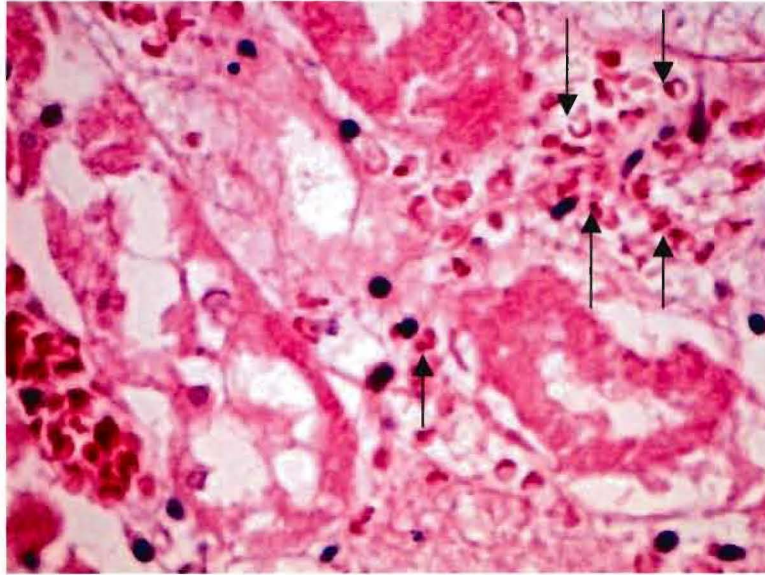


Figure 14: H & E staining showing recruitment of inflammatory cells (neutrophils) following renal I/R injury in the PBS group (arrows) (40X objective magnification).

4.3.5.5 Immunohistochemistry

In accordance with the previous findings (Figure 10) and the literature described above, there was a direct relationship between C3 deposition and tubular injury. The PBS group showed extensive C3 deposition in the renal tubules following 24 hours I/R injury (Figure 15D). However, the VCP/hrVCP treatment has abrogated the local C3 deposition (Figure 15B&C). The sham group displayed only basal levels of C3 between the epithelia (Figure 15A).

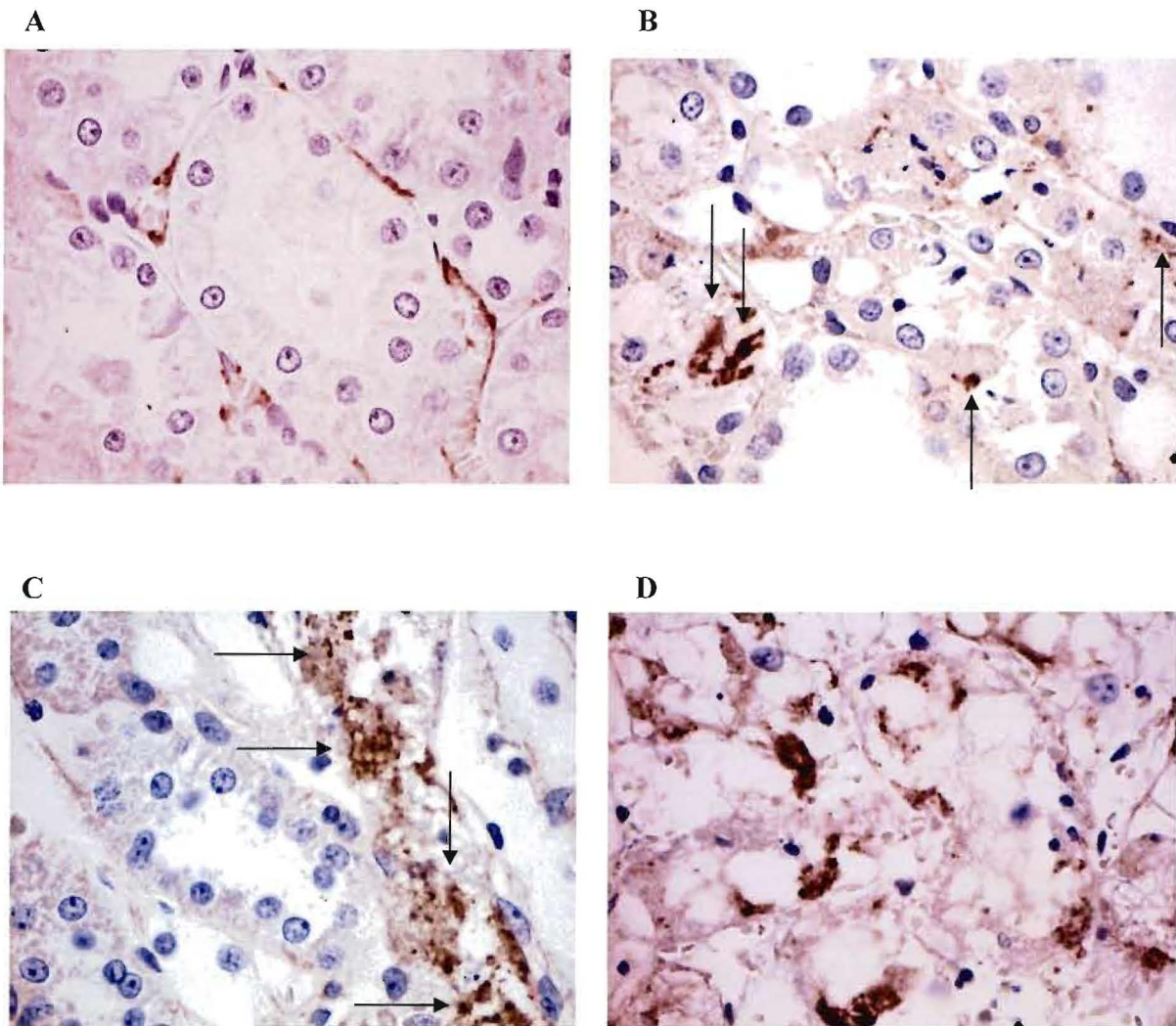


Figure 15: Immunostaining for complement component C3: **A)** basal staining between the epithelia in the sham group; **B)** minimal deposition in the tubules of the natural VCP treatment group (arrows), **C)** focal deposition in the tubule of the recombinant VCP (hrVCP) treatment group (arrows) **D)** markedly elevated C3 deposition in the renal tubules of the PBS group. (40X objective magnification).

Summary of the I/R injury techniques employed in the different procedures described above

Procedure	Ischemia induced by	Duration of Ischemia (min)	Remarks
1	Bilateral clamping (n = 4)	45	Animals too small, multiple bleeding caused dehydration and rapid deterioration of their well-being. Animals did not survive for 24 hrs
2	Right nephrectomy followed by left clamping (n = 7)	45	Animals were under continuous anesthesia and lying on the OR table for prolonged period of time. Animals did not survive for 24 hrs. BSA did not have any protective role.
3	Bilateral clamping (n = 6)	45	rVCP administered subcutaneously (SC) did not inhibit the level of circulating complement as evaluated by hemolysis assay
4	Unilateral clamping (n = 19)	60	The VCP/hrVCP combination delivered both IV and IP has improved kidney structure. No significant differences in the serum BUN and Creatinine probably due to the compensatory effect of the contralateral kidney.
5	Bilateral clamping (n = 14)	60	The VCP/hrVCP treated animals showed significant structural and functional integrity compared to the PBS group

4.4 DISCUSSION

The direct and indirect involvement of the complement system in a number of animal models that mimic human diseases has been strongly implicated. Among the spectrum of diseases, it is worth acknowledging the role of complement in atherosclerosis (Thorbjornsdottir² et al, 2006), head injury (Reynolds et al, 2004), traumatic brain injury (Hicks et al, 2002), xenorejection (Kahn et al, 2003; Ghebremariam¹ et al, 2005; Ghebremariam² et al, 2005), cardiopulmonary bypass (Mollnes and Kirschfink, 2006), the recently established recurrent miscarriage (Girardi et al, 2006) and ischemia/reperfusion (I/R) injury.

The role of the complement cascade in renal I/R injury has been repeatedly demonstrated and inhibition of the complement system at C3 or C5 level or knocking out Factor B, C3 or C6 complement components in mice (Zhou et al, 2000; Thurman et al, 2003; Thurman et al, 2006) have been shown to protect the animals from renal I/R injury suggesting the critical role of the complement cascade in general and the alternative complement pathway in particular in the pathophysiology of I/R injury. In this study, we evaluated the roles of the complement inhibitors, vaccinia virus infected-mammalian cells derived natural VCP and the recently generated humanized recombinant VCP (hrVCP), in modulating renal I/R injury. Previous studies have demonstrated the interrelationship between C3 deposition and tissue injury (Stahl et al, 2003; Anderson et al, 2003). Moreover, 50% reduction in renal I/R injury in C3-deficient mice have been reported (Zhou et al, 2000). It was therefore important to investigate the significance and implication of C3 inhibition by VCP in I/R injury animal model. Recombinant VCP (rVCP) has been previously shown (Anderson et al, 2003) to reduce C3 deposition in guinea pig hearts and its subsequent contribution to prolonged graft survival in a guinea pig-to-rat cardiac xenotransplantation model. In this study, we report the relationship between C3 deposition and renal tubular damage and the significant reduction in C3 deposition and consequent contribution in conferring partial protection of the VCP/hrVCP treated rats from I/R-induced renal injury. There was a significant reduction in the degree of necrosis of tubular epithelial cells following treatment. The inhibition of C3 may either be directly protective or indirectly either by preventing the assembly of the detrimental membrane attack complex (MAC) on tubular cells (Zhou et al, 2000) or by retaining the heparin like molecule, heparan sulfate proteoglycan (HSP), in the tubular basement membrane (TBM). HSP, like heparin, might have complement inhibitory activity and therefore maintenance of HSP in the TBM using heparin-binding proteins such as VCP may prevent local complement activation and hence tubular collapse.

For example, Yagmurdu et al (2003) have demonstrated the therapeutic potential of heparin following renal I/R injury and was accounted to its complement inhibitory activity and not to the anticoagulant property of heparin.

The lack of complete protection may either be due to the retrospectively speculative lower dose of VCP/hrVCP administered or may be due to partial role of complement in renal I/R injury. Therefore, to rule out the first assumption, further studies may be needed to elucidate the role of VCP/hrVCP at higher doses and probably more frequent administration of the proteins.

Even under physiological conditions, VCP has been previously suggested to be speedily eliminated from circulation as a result of its smaller size and charge distribution (Jha et al, 2003). Our immunohistochemical staining results for VCP indicate a strong and specific staining of the renal tubules (Figure 9A), where C3 deposited in the PBS group, and some staining on the apical membranes of the renal cortical tubular cells as well as in the Bowman's capsule epithelium. Based on this results and observations, we also strengthen the previous hypothesis that the fate of the systemically administered soluble VCP may be rapid exclusion from circulation mainly when maiming the renal system itself. Therefore, we suggest coupling of the protein with some molecules that do not regress its function. However, the size of the conjugating molecule needs to be taken into account as a large (160 kDa) complement inhibitor was shown not to improve renal I/R injury (Park et al, 2001) probably because of its size and therefore lack of bioavailability in and around the tubules (Thurman et al, 2003; Thurman et al, 2006). It is also advisable if VCP can be coupled with other compounds such as free radical scavengers that have healing potentials (Fujii et al, 2003; Rodriguez-Reynoso et al, 2004; Singh and Chopra, 2004) for a synergistic effect in the treatment of renal I/R injury while preventing the rapid elimination of the protein from circulation.

Interestingly, Park et al (2001) queried the advantage of inhibiting the complement system in ameliorating renal ischemia/reperfusion injury following the evaluation of the role of soluble Crry in mice prior to I/R-induced renal injury and their article quoted Stein¹ and colleagues (1985) work as they did not achieve any therapeutic value following the abrogation of complement using the glycoprotein cobra venom factor (CVF). However, Riedemann and Ward (2003) referenced the same work by Stein² et al (1985) as a therapeutic gain following the abrogation of C3 using CVF. It appears that there is a controversial report on the same work.

Unfortunately, I was not able to access the original article by Stein et al (1985) and therefore can support neither of the reports. Meanwhile, Yamada et al (2004) evaluated the therapeutic role of CVF in wild type and CD55/CD59 double knock out mice following renal ischemia/reperfusion injury and demonstrated that CVF partially protected the highly susceptible CD55/CD59 deficient mice to a degree of resistance/susceptibility of the wild type mice. However, CVF did not offer significant protection in the wild type animals suggesting the limited potential of the glycoprotein. Conversely, a recent report (Mollnes and Kirschfink, 2006) described CVF as “*potent complement activator*” rather than “*complement inhibitor*” likewise; Lachmann (2006) described CVF as an alternative complement pathway activator. Therefore the inability of the glycoprotein to reduce tubular necrosis following I/R-induced renal injury, at least in wild type rats, may be accounted to its bulky size (149 kDa) and therefore limited bioavailability where required, and probably due to its controversial biological effect.

In the unilateral I/R injury model, we did not perceive any evidence of exogenous cells such as neutrophil recruitment to the site of injury in any of the groups. However, in the bilateral I/R injury model, there was early migration of neutrophils to the site injury in the untreated injured (PBS) group suggesting the severity of the induced injury in the latter model.

In conclusion, the lack of significant elevation in BUN and creatinine levels in the unilateral I/R injury model, relative to the structural damage, may be due to the compensatory ability of the intact contralateral kidney to excrete metabolic wastes. Subclinically, this model implies that in conditions where BUN and creatinine are used as surrogate markers of renal function the patient may be misdiagnosed due to the masking of the injured kidney by the compensatory effect of the contralateral kidney to expel the metabolic wastes from circulation. However, the ability of the kidneys to excrete these metabolic wastes was significantly compromised in the untreated (PBS) group of the bilateral I/R injury model. Therefore, this model may be relevant to a clinical condition reflecting reduced excretion of metabolic wastes as a result of tubular damage. Overall, the natural VCP showed a better preservation of kidney structure and function than the hrVCP following renal I/R injury.

It is also sensible to establish different animal models where the renal I/R injury is induced by various independent causes such as drug, surgical procedure or sepsis in order to verify if the alternative complement pathway remains the driving force despite the etiology of the injury.

CHAPTER 5: PROJECT SUMMARY

The first two chapters of the thesis explored the genesis of poxviruses and their set of proteins known as complement regulatory proteins (CRPs) with emphasis on the vaccinia virus complement control protein (VCP). Historically, it is the significant amino acid homology (38%) of VCP to the human complement-4b binding protein (C4b-BP) that caught the minds of researchers. Subsequently, the mimicry of poxviral proteins to host complement control proteins was elucidated in some other members of the poxviruses.

For about 18 years following its identification, a vast number of researches have been done to understand and exploit the potential benefits of VCP. A cluster of the work cited in this section include identification of the abilities of VCP to inhibit the classical and the alternative pathways of complement activation, its heparin binding property, its cloning and expression in *Pichia pastoris* yeast system, its crystal structure and modeling studies as well as its robustness to a handful of adverse conditions. The present work has characterized the functions of two modified proteins with single and triple amino acid changes in the recombinant VCP (rVCP) sequence (“CHAPTER 3”). The proteins, named ^{hrVCP}E108K and ^{hrVCP}H98Y, E102K, E120K; inhibited both the classical and the alternative complement pathways better than rVCP with the latter protein displaying up to 100-fold increased activity in inhibiting both pathways independently. Moreover, the modeling study revealed significant increase in the net positive charge of the modified proteins. In addition to the enhanced *in vitro* activity, the *in vivo* role of ^{hrVCP}H98Y, E102K, E120K (abbreviated as hrVCP) was evaluated in a rat ischemia/reperfusion (I/R) injury model (“CHAPTER 4”).

Both the unilateral and the bilateral I/R injury models established in this project have indicated the beneficial roles of the natural VCP produced from vaccinia virus-infected cells and the yeast cells expressed hrVCP in conferring partial protection from I/R-induced renal injury. The serum profile of the VCP/hrVCP treated animals showed low levels of the nitrogenous wastes (urea and creatinine) compared to the accumulated levels in the phosphate buffered saline (PBS) administered group. Similarly, the histological study showed partial damage of the tubular epithelial cells compared to the extensive necrosis in the PBS group. Moreover, the immunohistochemical study revealed focal levels of the injurious complement component (C3) in the renal tubules of the VCP/hrVCP groups compared to the diffuse C3 staining in the PBS group.

RECOMMENDATION

This project addressed various research queries from different angles. The first part of the work has tried to optimise the production of recombinant VCP both in the flask and fermentor. The work carried out a battery of fermentation trials with various modifications to the levels of dissolved oxygen (dO_2), pH and agitation. However, this work has only solved the puzzle of optimisation in part and therefore future work needs to continue the process of optimisation for consistent and large-scale production of VCP. The up-coming work needs to give particular emphasis to the pH, agitation and the level of dO_2 supplied, as they appear to be the most critical variables even when changed subtly. Moreover, it might be essential to compare and contrast the production of VCP using both synthetic and complex media in the fermentor. Furthermore, it is advisable to use self-autoclavable fermentors to minimize the risk of contamination and also it is important to incorporate proper cooling system (rather than a chiller) in order to cope with the load of heat generated during the growth phase.

The second part of the work has established two animal models of renal ischemia/reperfusion (I/R) injury that mimic clinical condition. The work has addressed the beneficial role of complement inhibition using VCP/hrVCP in minimizing renal I/R injury. However, the work was done in small animals (rats) and fewer in number. Therefore, future work may need to expand the findings in large number of animals. Moreover, despite their equivalent *in vitro* activity in inhibiting human complement, the natural VCP showed relatively better ability in preserving the rat kidneys than the *humanized* recombinant VCP (hrVCP) suggesting that 'humanizing' VCP may have increased the specie-distance from the rodent model used here and therefore its therapeutic potential may need to be evaluated in higher-order animals preferably non-human primates. The future work may also need to evaluate the role of VCP at higher doses and probably more frequent injections to elucidate if it is possible to achieve dose dependent additional protection. Meanwhile, it may be worthy to couple VCP with a free radical scavenger to investigate if there is a synergistic effect in diminishing I/R-induced renal injury while delaying the rapid elimination of, the otherwise small, VCP from circulation. However, the total size after conjugation has to be taken into account as the protein needs to be available in and around the tubules to prevent necrotic damage.

The last part of my work (*not included in the thesis due to the lack of direct relevance to the scope of my proposed project*) has started the long and obscured immunogenicity study of VCP using sera from smallpox vaccinated and unvaccinated (or unaware of their vaccination) subjects. Although very preliminary, the findings indicated the presence of antibodies in human sera reactive to VCP despite the status of vaccination suggesting that the administration of VCP in humans may cause some immune reaction and therefore should initially be performed under close clinical scrutiny or it might be pertinent to perform simple skin allergy test in order to grasp the immediate effect of VCP administration in humans before proceeding to clinical trials. However, the immunogenic potential of VCP need to be first confirmed by screening large number of vaccinated and unvaccinated individuals (preferably individuals born after the global eradication of smallpox) both by ELISA and western blot.

Moreover, the anti-VCP antibody raised in rabbits (rabbit anti-VCP) had also been shown to significantly react to the vaccinia virus total protein in the absence of VCP and following preadsorption with VCP at various dilutions, suggesting some sort of cross-reactivity. Future work may need to screen different batches of rabbit anti-VCPs and may need to expand the work by immunizing rabbits with VCP, monitoring the generation of antibodies, and then challenging the animals with vaccinia virus to elucidate if there is *in vivo* cross-reactivity and hence cross-protection against poxviral challenge compared to unimmunized controls.

LIST OF PUBLICATIONS

FULL TEXT ARTICLES:

Title: “Human Xenotransplantation: An Immunological and Ethical Challenge.”

Y.T. Ghebremariam, S.A. Smith, J.B. Anderson, D. Kahn & G.J. Kotwal

Published in “*New Research on Immunology*” pp. 127-145.

Nova science Publishers (2005), Inc. Editor: Barbara A. Veskler

Title: “Human Xenotransplantation: An Immunological and Ethical Challenge.”

Y.T. Ghebremariam, S.A. Smith, J.B. Anderson, D. Kahn & G.J. Kotwal

Re-published by the editors in “*Contemporary Ethical Issues*” pp. 63-86.

Nova science Publishers (2005), Inc. Editor: Albert G. Parkis

Title: “Intervention Strategies and Agents Mediating the Prevention of Xenorejection”

Yohannes T. Ghebremariam, Scott A. Smith, J.B. Anderson, D. Kahn &

Girish J. Kotwal

“*Annals of the New York Academy of Sciences*” Volume 1056 (2005): 123-143.

Title: “Humanized recombinant Vaccinia Virus Complement Control Protein (hrVCP)

with 3 amino acid changes, H98Y, E102K, E120K, Creating an Additional Putative Heparin Binding Site, Is 100-fold more active than rVCP in blocking both Classical & Alternative Complement Pathways”

Yohannes T Ghebremariam, Odutayo O. Odunuga, Kristen Janse & Girish J. Kotwal.

Presented at the First International Conference on Natural Products & Molecular

Therapy. “*Annals of the New York Academy of Sciences*” Volume 1056(2005): 113-122.

Title: “Curcumin inhibits the Classical & Alternate Pathways of Complement Activation.”

Amod P. Kulkarni, Yohannes T. Ghebremariam & Girish J. Kotwal

Presented at the First International Conference on Natural Products & Molecular

Therapy. “*Annals of the New York Academy of Sciences*” 1056 (2005): 100-112.

Title: “Anti-HIV, Anti-poxvirus, and anti-SARS Activity of a Non toxic, Acidic Plant Extract from the *Trifolium* Species Secomet-V/anti-Vac Suggests That It Contains a Novel Broad-Spectrum Antiviral.”

Girish J. Kotwal, Jennifer N. Kaczmarek, Stephen Leivers, Yohannes T. Ghebremariam,

Amod P. Kulkarni, Gabriele Bauer, Corena De Beer, Wolfgang Preiser and

Abdu R. Mohamed

Presented at the First International Conference on Natural Products & Molecular

Therapy. “*Annals of the New York Academy of Sciences*” Volume 1056 (2005): 293-302

Title: “Candidate Inhibitors of Porcine Complement”

Ebbe B. Thorgersen, Yohannes T. Ghebremariam, Joshua M. Thurman, Michael Fung, Erik Wagge Nielsen, V. Michael Holers, Girish J. Kotwal and Tom Eirik Mollnes. *Molecular Immunology* (2006) (In Press, Corrected Proof).

ABSTRACTS:

Title: “Lister homologue of vaccinia virus complement control protein is two amino acids shorter, has putative glycosylation sites and other functional and structural differences”
O. O. Odunuga, Y. T. Ghebremariam, and Girish J. Kotwal
Special Symposia FEBS Journal 272(S1) (2005), 15-16.

Title: “Inhibition of Complement by Curcumin”
Amod P. Kulkarni, Yohannes T. Ghebremariam & Girish J. Kotwal
Presented at the 10th Meeting on Complement in Human Disease
Published in *Molecular Immunology* 43(S) (2006) 43, 190.

Title: “Vaccinia Virus Complement Control Protein (VCP) Improves Kidney Structure and Function Following Ischemia/Reperfusion Injury in Rats.”
Yohannes T. Ghebremariam, Del Kahn, Gert Engelbrecht, Marilyn Tyler, Zoë Lotz, Dhiren Govender and Girish J. Kotwal. Presented at the “Experimental Biology 2006” Conference held in San Francisco, CA. Published in *The FASEB Journal* 20(5) 2006: A1072.

APPENDIX A

MEDIA, BUFFERS, AND SOLUTIONS

MEDIA

MMH:

For 1l:

Dissolve 15g agar in 700ml water & autoclave

Cool to ~50°C and add:

100ml 10X YNB (dissolve 13.4g in 100ml water & filter sterilize)

2ml 500X biotin (dissolve 0.02g D-biotin in 100ml water & filter sterilize)

100ml methanol (10%, filter sterilized)

Adjust volume with distilled water

Pour & store plates at 4°C

YPD:

For 500ml:

5g yeast extract

10g peptone

10g agar

Dissolve in ~300ml distilled water & autoclave

Add 100ml filter sterilized glucose [20%(w/v)]

Adjust volume with distilled water

Pour & store plates at 4°C

RDB:

For 250ml:

Composition-1 (prewarmed to 45°C):

Dissolve 5g (w/v) dextrose in 25ml water & filter sterilize

Add:

25ml filter sterilized 10X YNB

0.5ml 500X filter sterilized biotin

2.5ml 100X amino acids (0.5g each of glutamic acid, lysine, leucine, isoleucine & methionine in 100ml of distilled water. Filter sterilize)

20ml autoclaved water

Composition-2:

Dissolve 46.5g of sorbitol & 5g of agar in 175ml water

Autoclave

Cool down & mix to composition-1 (above)

Pour & store plates at 4°C.

BMGY:

For 1l:

Dissolve 10g yeast extract & 20g peptone in ~600ml water

Autoclave, cool to ~45°C

Add:

100ml autoclaved phosphate buffer (1M; pH 6.0) (dissolve ~780ml of 1M KH₂PO₄ and mix with ~120ml 1M K₂HPO₄ adjust pH to 6.0 with KOH & adjust volume with distilled water)

100ml autoclaved glycerol (10%)

100ml filter sterilized YNB (10X)

2ml filter sterilized 500X biotin

Adjust volume with distilled water

BMMY (4%):

For 1l:

Dissolve 10g yeast extract & 20g peptone in ~600ml water

Autoclave, cool to ~45°C

Add:

100ml autoclaved phosphate buffer (1M; pH 6.0) as above

100ml filter sterilized methanol (4%)

100ml filter sterilized YNB (10X)

2ml filter sterilized 500X biotin

Adjust volume with distilled water

Buffered Methanol:

For 1l:

96.5ml phosphate buffer (pH 6.0)

38.5ml filter sterile absolute methanol

Adjust volume with distilled water

Basal Salts:

For 1l:

40g glycerol

18g dipotassium sulphate (K_2SO_4)

15g magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)

4.13g potassium hydroxide (KOH)

27ml Phosphoric acid (85%) H_3PO_4

0.9g Calcium sulphate ($CaSO_4$)

Filter sterilize & store at 4°C

PTM1 Trace Salts:

For 1l:

Add:

6g cupric sulphate ($CuSO_4 \cdot 5H_2O$)

0.08g sodium iodide (NaI)

3g manganous sulphate monohydrate ($MnSO_4 \cdot H_2O$)

0.02g boric acid (H_3BO_3)

24g $Na_2MoO_4 \cdot 2H_2O$

0.5g cobalt chloride ($CoCl_2$)

20g zinc chloride ($ZnCl_2$)

65g ferrous sulphate ($FeSO_2 \cdot H_2O$)

3.4ml 500x biotin

5ml sulphuric acid (H_2SO_4)

Filter sterilize & store at 4°C

BUFFERS & SOLUTIONS**Tris-Acetate EDTA (TAE) Electrophoresis buffer:**

For 1l:

Dissolve 48.4g Tris-base in 11.42ml glacial acetic acid

Add 20ml EDTA (0.5M; pH 8.0)

Adjust volume with distilled water

SDS-PAGE Loading Buffer Mix:

Mix 2 volumes of supplied loading buffer (Invitrogen) with 1 volume of 2-mercaptethanol

Coomassie Staining Solution:

Dissolve 1.25g Coomassie R-250 in 227ml methanol

Add 227ml of distilled water & mix

Add 46ml glacial acetic acid

Filter sterilize before use

Destaining Solution:

For 280ml:

90ml absolute methanol

30ml acetic acid

160ml distilled water

Chromatography Binding Buffer:

50mM sodium acetate

Adjust to pH 5.5

SDS-PAGE Resolving Gel (12%):

2.5ml distilled water

3ml acrylamide 30% (29.2g acrylamide & 0.8g bis-acrylamide mix)

1.9ml 1.5M Tris-base (pH 8.8)

75 μ l SDS (10%)

75 μ l APS (10%)

5 μ l TEMED

SDS-PAGE Stacking Gel (5%):

2ml distilled water

0.5ml acrylamide (as above)

375 μ l 1M Tris-base (pH 6.8)

30 μ l SDS (10%)

30 μ l APS (10%)

5 μ l TEMED

Western Blot Transfer Buffer:

For 1l:

Dissolve 5.8g Tris-base & 2.93g glycine in water

Add 200ml absolute methanol

Adjust volume with distilled water

Store at 4°C

Ponceau S Stain:

For 100ml:

Dissolve 0.1g ponceau S in 1% glacial acetic acid

Cover the bottle with aluminium foil & store at room temperature.

Tris-Buffered Saline (TBS):

For 1l

Dissolve 7.56g Tris-base & 11g NaCl

Adjust volume with distilled water & store at room temperature.

TBS-T (0.1%):

For 500ml:

Mix 449.5ml TBS (from above) with 0.5ml Tween-20

Store at room temperature

Western Blot Blocking Solution (5%):

For 100ml:

Dissolve 5g non-fat milk in TBS (above)

Adjust volume with TBS

Western Blot Blocking Solution (0.5%):

For 100ml:

Dissolve 0.5g non-fat milk in TBS (above)

Adjust volume with TBS

Primary (Rabbit anti-VCP) dilution (1:1000):

Mix 10ml of 0.5% blocking solution (above) with 10µl of rabbit anti-VCP

Secondary (donkey anti-rabbit) dilution (1:12500):

Mix 12.5ml of 0.5% blocking solution (above) with 1 μ l of POD-linked donkey anti-rabbit

Detection Reagent Mix: Mix the supplied solution B to solution A at a ratio of 1:100 in a dark room.

Crystal Violet Stain:

For 100ml:

10ml crystal violet stock solution

10ml formaldehyde stock solution

80ml distilled water

Complement Specimen Diluent:

"Contains PBS, 0.05% Tween-20, 2.5% protein stabilizers, 0.035% Pro Clin 300." (Quidel Corporation Insert)

Quidel Wash Buffer (1X):

"Contains PBS, 0.05% Tween-20, 0.01% thimerosal." (Quidel Corporation Insert)

Quidel Substrate Solution:

Mix 50 μ l of the supplied substrate concentrate with 1ml of substrate diluent (supplied)

ELISA Blocking Solution:

For 100ml:

Mix 0.5g BSA with 0.5ml Tween-20

Adjust volume with PBS (pH 7.2)

ELISA Wash Buffer:

For 100ml:

Mix 100 μ l of Tween-20 with PBS (pH 7.2)

PBS-T (0.1%):

For 100ml:

Mix 100 μ l of Tween-20 with PBS (pH 7.2)

APPENDIX B

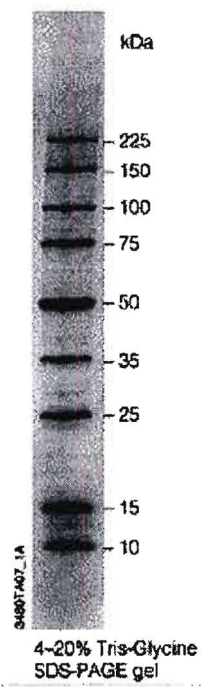


Figure: Protein Molecular Weight Marker (*Reproduced from www.promega.com for the sole purpose of demonstration*).

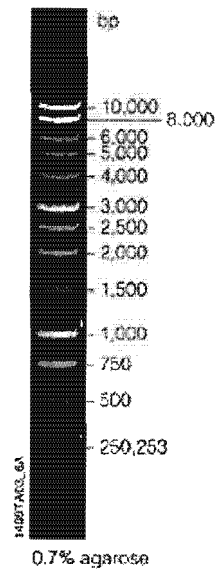


Figure: DNA Molecular Weight Marker (1kb⁺) (*Reproduced from www.promega.com for the sole purpose of demonstration*).

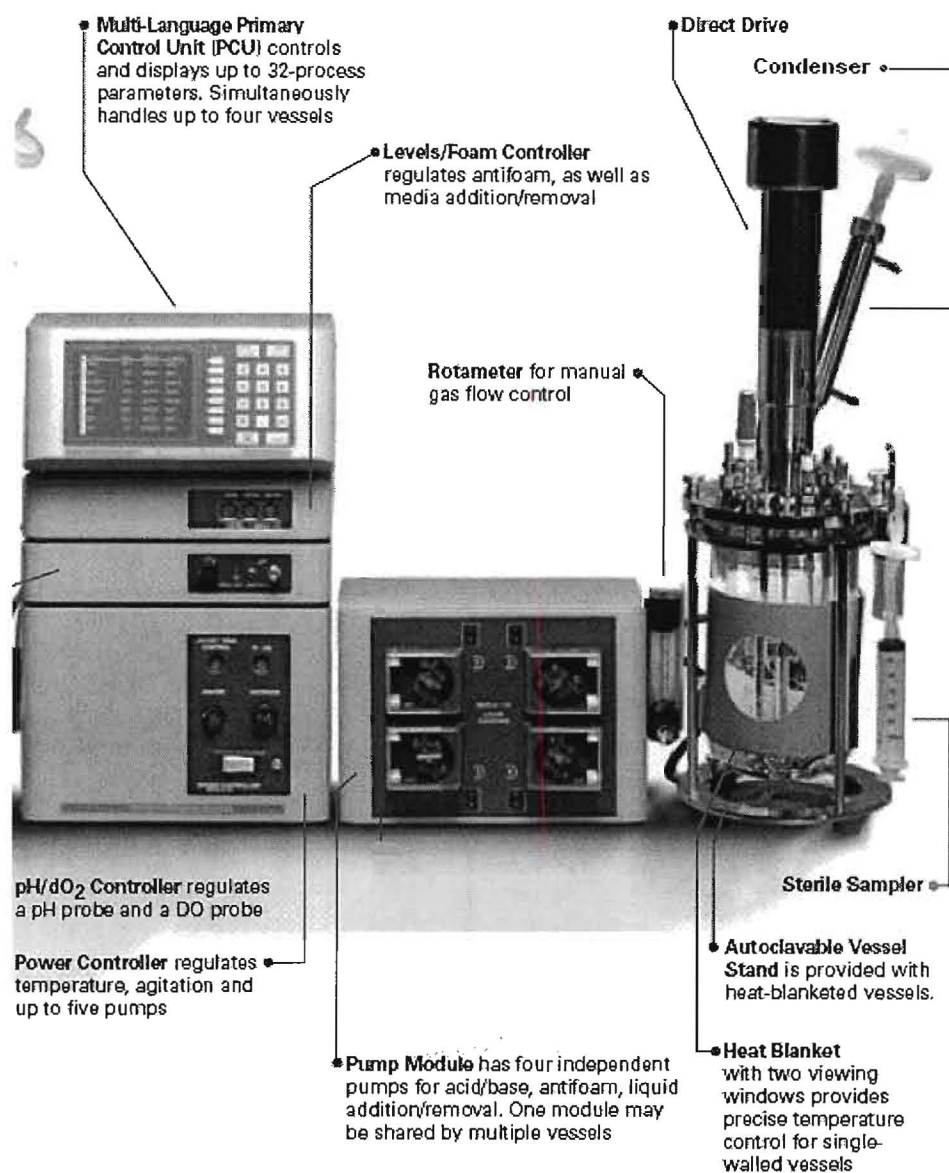


Figure: BioFlo 110 Benchtop Fermentor (*Reproduced from www.enzyme.ucd.ie/Science/Equipment/fermentor for the sole purpose of demonstration*).

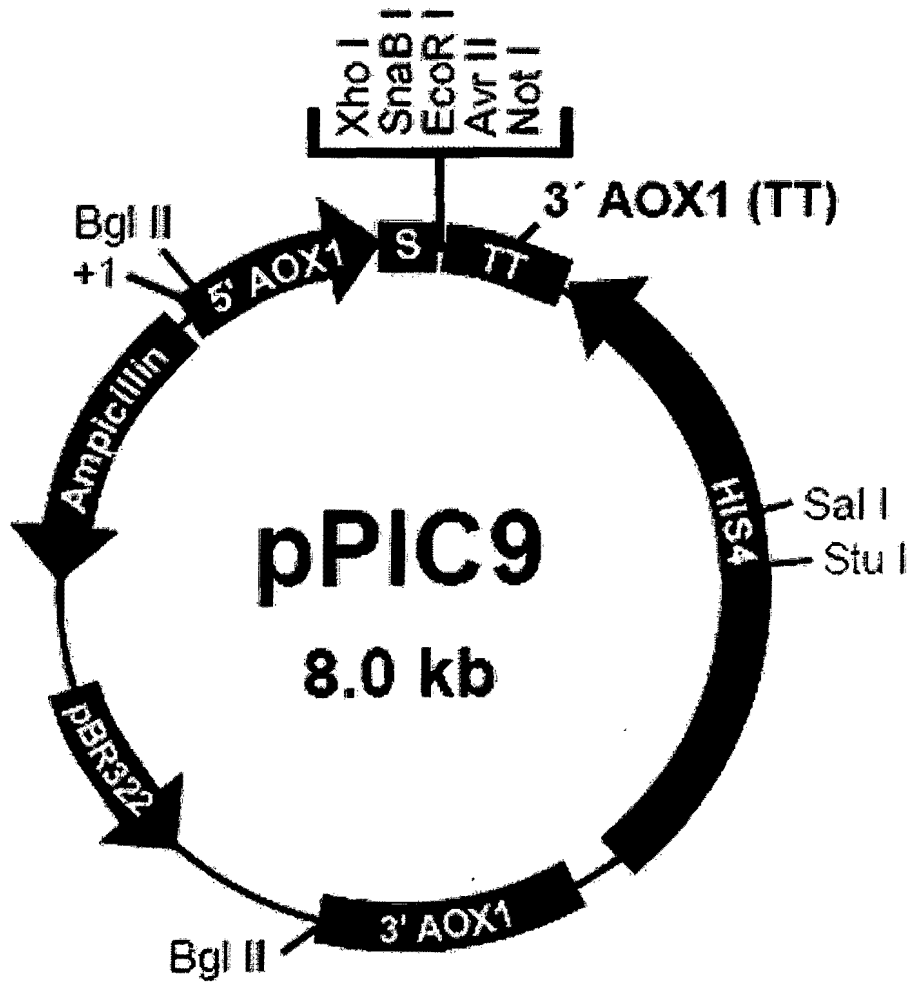


Figure: pPIC9 vector Map (Reproduced from www.invitrogen.com for the sole purpose of demonstration).

REFERENCES

- Abou-Raya A, Abou-Raya S. Inflammation: A pivotal link between autoimmune diseases and atherosclerosis. *Autoimmunity Reviews* 2006; 5(5): 331-337.
- Alcamí A, Symons JA, Smith GL. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. *Journal of Virology* 2000; 74: 11230-11239.
- Al-Mohanna F, Parhar R, Kotwal GJ. Vaccinia virus complement control protein is capable of protecting xenoendothelial cells from antibody binding and killing by human complement and cytotoxic cells. *Transplantation* 2001; 71(6): 796-801.
- Anderson¹ JB, Smith SA, van Wijk R et al. Vaccinia Virus Complement Control Protein Ameliorates Hyperacute Xenorejection by Inhibiting Xenoantibody Binding. *Transplantation Proceedings* 2002; 34: 3277-3281.
- Anderson² JB, Smith SA, Kotwal GJ. Vaccinia Virus Complement Control Protein Inhibits Hyperacute Xenorejection. *Transplantation Proceedings* 2002; 34: 1083-1085.
- Anderson JB, Smith SA, van Wijk R, Chien S, Kotwal GJ. Vaccinia virus complement control protein inhibits hyperacute xenorejection in a guinea pig-to-rat heterotopic cervical cardiac xenograft model by blocking both xenoantibody binding and complement pathway activation. *Transplant Immunology* 2003; 11(2): 129-135.
- Anna DF, Rosa M, Emilia P et al. High-level expression of *Alicyclobacillus acidocaldarius* thioredoxin in *Pichia pastoris* and *Bacillus subtilis*. *Protein Expression and Purification* 2003; 30(2): 179-184.
- Asghar SS. Pharmacological manipulation of the complement system in human diseases. *Frontiers in Bioscience* 1996; 1(1): e15-25.
- BioWhittaker. Limulus Amebocyte Lysate. QCL-1000. Quantitative Chromogenic LAL. BioWhittaker, Inc. A Cambrex Company, Walkersville, MD.
- Bork¹ K, Staubach P, Eckardt AJ, Hardt J. Symptoms, course, and complications of abdominal attacks in hereditary angioedema due to C1 inhibitor deficiency. *American Journal of Gastroenterology* 2006; 101(3): 619-627.
- Bork² K, Meng G, Staubach P, Hardt J. Hereditary angioedema: new findings concerning symptoms, affected organs, and course. *American Journal of Medicine* 2006; 119(3): 267-274.
- Brandl U, Michel S, Erhardt M et al. 25 days of baboons after orthotopic xenotransplantation of hDAF transgenic pig hearts using a moderate immunosuppression. *Journal of Heart Lung Transplantation (Abstract)* 2003; 22:S100-S101.

Bulger RE, Hebert SC. Structural-Function correlations along the renal tubule. In *Diseases of the Kidney* 4th Edition, Volume I. Eds Schrier RE, Gottschalk CW. Little, Brown and Company Inc. 1988; pp. 3-64.

Caprette DR. Hartree-Lowry and Modified Lowry Assays. Rice University, Houston, Texas. <http://dwb.unl.edu/Teacher/NSF/C10/C10Links/www.ruf.rice.edu/~bioslabs/methods/protein/lowry.html>

Cereghino GPL, Cereghino JL, Ilgen C et al. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Current Opinion in Biotechnology* 2002; 13: 329-332.

Chen Y, Cino J, Hart G et al. High protein expression in fermentation of recombinant *Pichia pastoris* by a fed-batch process. *Process Biochemistry* 1997; 32(2): 107-111.

Ciulla E, Emery A, Konz D, Krushkal J. Evolutionary history of orthopoxvirus proteins similar to human complement regulators. *Gene* 2005; 355: 40-47.

Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. *Nature Medicine* 1995; 1(9): 964-966.

Daemen M, Veer C, Denecker G et al. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *The Journal of Clinical Investigation* 1999; 104(5): 541-549.

Daly J, Kotwal GJ. Neurodegeneration in Alzheimer's Disease. Pivotal Role of the Inflammatory Response. *The Immunologist* 1997; 5(5): 157-165.

Daly J, Kotwal GJ. Pro-inflammatory complement activation by the A beta peptide of Alzheimer's disease is biologically significant and can be blocked by vaccinia virus complement control protein. *Neurobiology of Aging* 1998; 19: 619-627.

De Clercq E. Vaccinia Virus Inhibitors as a Paradigm for the Chemotherapy of Poxvirus Infections. *Clinical Microbiology Reviews* 2001; 14(2): 382-397.

De Vries¹ B, Matthijssen RA, Wolfs T et al. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: Inhibition of late apoptosis and inflammation. *Transplantation* 2003; 75(3): 375-382.

De Vries² B, Matthijssen RA, Van Bijnen AAJHM et al. Lysophosphatidic Acid Prevents Renal Ischemia-Reperfusion Injury by Inhibition of Apoptosis and Complement Activation. *American Journal of Pathology* 2003; 163(1): 47-56.

DiLillo DJ, Pawluczkoysz AW, Peng W et al. Selective and efficient inhibition of the alternative pathway of complement by a mAb that recognizes C3b/iC3b. *Molecular Immunology* 2006; 43: 1010-1019.

Douglass N, Dumbell K. Independent Evolution of Monkeypox and Variola Viruses. *Journal of Virology* 1992; 66(12): 7565-7567.

- Dragon-Durey MA, Fremeaux-Bacchiv V, Loirat C et al. heterozygous and homozygous factor h deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. *Journal of American Society of Nephrology* 2004; 15(3): 787-795.
- Dunlop LR, Oehlberg KA, Reid JJ, Avci D, Rosengard AM. Variola virus immune evasion proteins. *Microbes and Infection* 2003; 5: 1049-1056.
- Durrheim K. The logic of analysis of variance In *STATISTICS (Numbers, Hypotheses & Conclusions: A course in statistics for the social sciences)* Edited by Colin Tredoux & Kevin Durrheim. Published by Lansdowne, South Africa: UCT Press, 2002.
- Fairley KF. Clinical Evaluation of the Kidney. In *Diseases of the Kidney* 4th Edition, Volume I. Eds Schrier RE, Gottschalk CW. Little, Brown and Company Inc. 1988; pp. 359-391.
- Fearon DT, Austen KF. Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *Prot. Natl. Acad. Sci. USA* 1977; 74(4): 1683-1687.
- Feitoza CQ, Camara NOS, Pinheiro HS et al. Cyclooxygenase 1 and/or 2 blockage ameliorates the renal tissue damage triggered by ischemia and reperfusion injury. *International Immunopharmacology* 2005; 5: 79-84.
- Files D, Ogawa M, Scaman CH, Baldwin SA. A *Pichia pastoris* fermentation process for producing high-levels of recombinant human cystatin-C. *Enzyme and Microbial Technology* 2001; 29: 335-340.
- Fishelson Z, Pangburn MK, Müller-Eberhard HJ. Characterization of the initial C3 convertase of the alternative pathway of human complement. *The Journal of Immunology* 1984; 132(3): 1430-1434.
- Flores-Suarez. Remission of severe relapsing or persistent lupus nephritis using mycophenolate mofetil. *Archives of Medical Research* 2006; 37(1): 68-73.
- Fujii T, Takaoka M, Muraoka T et al. Preventive effect of L-carnosine on ischemia/reperfusion-induced acute renal failure in rats. *European Journal of Pharmacology* 2003; 474: 261-267.
- Ganesh VK, Smith SA, Kotwal GJ, Murthy KHM. Structure of vaccinia complement protein in complex with heparin and potential implications for complement regulation. *Proc. Natl. Acad. Sci. USA* 2004; 101(24): 8924-8929.
- Ganesh VK, Muthuvel SK, Smith SA et al. Structural Basis for Antagonism by Suramin of Heparin Binding to Vaccinia Complement Protein. *Biochemistry* 2005; 44: 10757-10765.

Ghebremariam YT, Kahn D, Engelbrecht G et al. Vaccinia Virus Complement Control Protein (VCP) Improves Kidney Structure & Function Following Ischemia/Reperfusion Injury in Rats. The FASEB Journal 2006; 20(5): A1072 (Presented at the “*Experimental Biology 2006*” conference held in San Francisco, CA).

Ghebremariam¹ YT, Smith SA, Anderson JB, Kahn D, Kotwal GJ. Human Xenotransplantation: An Immunological and Ethical Challenge. In *New Research on Immunology* Nova Science Publishers, Inc. 2005; pp. 127-145.

Ghebremariam² YT, Smith SA, Anderson JB, Kahn D, Kotwal GJ. Intervention Strategies and Agents Mediating the Prevention of Xenorejection. Annals of the New York Academy of Science 2005; 1056: 123-143.

Ghebremariam³ YT, Odunuga OO, Janse K, Kotwal GJ. Humanized recombinant Vaccinia Virus Complement Control Protein (hrVCP) with 3 amino acid changes, H98Y, E102K, and E120K, Creating an Additional Putative Heparin Binding Site, Is 100-fold more active than rVCP in blocking both Classical & Alternative Complement Pathways. Annals of the New York Academy of Science 2005; 1056: 113-122.

Girardi G, Berman J, Redecha P et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. Journal of Clinical Investigation 2003; 112(11): 1644-1654.

Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. Molecular Immunology 2006; 43: 68-77.

He C, Alexander JJ, Lim A, Quigg RJ. Production of the Rat Complement Regulator, Crry, as an Active Soluble Protein in *Pichia pastoris*. Archives of Biochemistry and Biophysics 1997; 341(2): 347-352.

Henderson¹ DA. Smallpox: Clinical and Epidemiological Features. Emerging Infectious Diseases 1999; 5(4): 537-539.

Henderson² DA. The Looming Threat of Bioterrorism. Science 1999; 283: 1279-1282.

Henderson DA, Fenner F. Recent Events and Observations Pertaining to Smallpox Virus Destruction. Clinical Infectious Diseases 2001; 33: 1057-1059.

Henderson DA, Inglesby TV, Bartlett JG et al. Smallpox as a Biological Weapon. JAMA 1999; 281(22): 2127-2137.

Hicks RA, Keeling KL, Yang M-Y et al. Vaccinia Virus Complement Control Protein Enhances Functional Recovery after Traumatic Brain Injury. Journal of Neurotrauma 2002; 19(6): 705-714.

Hoffman HS (2002) Statistics Explained: Internet Glossary of statistical terms. Null Hypothesis. <http://www.animatedsoftware.com/statglos/sgnullhy.htm>

Holers VM, Girardi G, Mo L et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *Journal of Experimental Medicine* 2002; 195(2): 211-220.

Holers VM, Thurman JM. The alternative pathway of complement in disease: opportunities for therapeutic targeting. *Molecular Immunology* 2004; 41: 147-152.

Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. *Nature* 1999; 402: B12- B17.

Howard J, Justus DE, Totmenin AV et al. Molecular mimicry of the inflammation modulatory proteins (IMPs) of poxviruses: evasion of the inflammatory response to preserve viral habitat. *Journal of Leukocyte Biology* 1998; 64: 68-71.

Hughes JD, Mattar SG, Chen C et al. Renal Artery Perfusion Modifies Ischemia/Reperfusion Injury. *Journal of Surgical Research* 1996; 60: 321-326.

Inal JM, Pascual M, Lesavre P, Schifferli J-A. Complement Inhibition in renal diseases. *Nephrology Dialysis Transplantation* 2003; 18: 237-240.

Invitrogen Life Technology (2002) (*Pichia pastoris* catalog).

Isaacs SN, Argyropoulos E, Sfyroera G et al. Restoration of Complement-Enhanced Neutralization of Vaccinia Virus Virions by Novel Monoclonal Antibodies Raised against the Vaccinia Virus Complement Control Protein. *Journal of Virology* 2003; 77(15): 8256-8262.

Isaacs SN, Kotwal GJ, Moss B. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. *Prot. Natl. Acad. Sci. USA* 1992; 89: 628-632.

Jha P, Kotwal GJ. Vaccinia complement control protein: Multi-functional protein and a potential wonder drug. *Journal of Bioscience* 2003; 28(3): 265-271.

Jha P, Smith SA, Justus DE, Kotwal GJ. Prolonged Retention of Vaccinia Virus Complement Control Protein Following IP injection: Implications in Blocking Xenorejection. *Transplantation Proceedings* 2003; 35: 3160-3162.

Jha P, Smith SA, Justus DE, Kotwal GJ. Vaccinia virus complement control protein ameliorates collagen-induced arthritic mice. *Annals of the New York Academy of Science* 2005; 1056: 55-68.

Kahn D, Smith SA, Kotwal GJ. Dose-Dependent Inhibition of Complement in Baboons by Vaccinia Virus Complement Control Protein: Implications in Xenotransplantation. *Transplantation Proceedings* 2003; 35: 1606-1608.

Kassirer JP, Harrington JT. Laboratory Evaluation of Renal Function. In *Diseases of the Kidney* 4th Edition, Volume I. Eds Schrier RE, Gottschalk CW. Little, Brown and Company Inc. 1988; pp. 393-441.

Kat Medical (Pty) Ltd. KATTM UREA Urease-UV Kinetic Method. Kit Insert

Kazatchkine MD, Fearon DT, Silbert JE, Austen KF. Surface-associated heparin inhibits zymosan-induced activation of the human alternative complement pathway by augmenting the regulatory action of the control proteins on particle-bound C3b. *Journal of Experimental Medicine* 1979; 150: 1202-1215.

Keeling KL, Hicks RA, Mahesh J et al. Local neutrophil influx following lateral fluid-percussion brain injury in rats is associated with accumulation of complement activation fragments of the third component (C3) of the complement system. *Journal of Neuroimmunology* 2000; 105: 20-30.

Kelly KJ, Williams WW Jr., Colvin RB et al. Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *Journal of Clinical Investigation* 1996; 97(4): 1056-1063.

Kielar ML, Jeyarajah DR, Zhou XJ, Lu CY. Docosahexaenoic Acid Ameliorates Murine Ischemic Acute Renal Failure and Prevents Increases in mRNA Abundance for both TNF- α and Inducible Nitric Oxide Synthase. *Journal of American Society of Nephrology* 2003; 14: 389-396.

Kitada H, Sugitani A, Yamamoto H et al. Attenuation of renal ischemia-reperfusion injury by FR167653 in dogs. *Surgery* 2002; 131: 654-662.

Knight KR, Shinkel TA, Cowan PJ et al. Transgenic expression of human complement regulators reduces skeletal muscle ischemia/reperfusion injury in mice. *Clinical Science* 2005; 108(1): 47-53.

Kobayashi K, Kuwae S, Ohya T et al. High-Level Expression of Recombinant Human Serum Albumin from the Methylotrophic Yeast *Pichia pastoris* with Minimal Protease Production and Activation. *Journal of Bioscience and Bioengineering* 2000; 89(1): 55-61.

Koganesawa N, Aizawa T, Shimojo H et al. Expression and purification of a small cytokine growth-blocking peptide from armyworm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification* 2002; 25: 416-425.

Koike C, Isobe K, Nakashima I et al. Establishment of a human DAF/HRF 20 double transgenic mouse line is not sufficient to suppress hyperacute rejection. *Surgery Today* 1996; 26(12): 993-998.

Kotwal GJ. Microorganisms and their interaction with the immune system. *Journal of Leukocyte Biology* 1997; 62: 415-429.

Kotwal GJ. Poxviral mimicry of complement and chemokine system components: what is the end game? *Immunology Today* 2000; 21: 242-248.

Kotwal GJ, Abrahams MR. Growing Poxviruses and Determining Virus Titer In *Methods in Molecular Biology: Vaccinia Virus and Poxvirology: Methods and Protocols*. Humana Press Inc. 2004; 269: pp. 101-112.

Kotwal GJ, Isaacs SN, Mckenzie R, Frank MM, Moss B. Inhibition of the Complement Cascade by the Major Secretory Protein of Vaccinia Virus. *Science* 1990; 250: 827-829.

Kotwal GJ, Moss B. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* 1988; 335: 176-178.

Krug N, Tschernig T, Erpenbeck VJ et al. Complement Factors C3a and C5a Are Increased in Bronchoalveolar Lavage Fluid After Segmental Allergen Provocation in Subjects with Asthma. *American Journal of Respiratory and Critical Care Medicine* 2001; 164(10): 1841-1843.

Krych M, Hourcade D, Atkinson JP. Sites within the complement C3b/C4b receptor important for the specificity of ligand binding. *Prot. Natl. Acad. Sci. USA* 1991; 88(10): 4353-4357.

Kulkarni AP, Ghebremariam YT, Kotwal GJ. Curcumin Inhibits the Classical and the Alternate Pathways of Complement Activation. *Ann. NY Acad. Sci.* 2005; 1056: 100-112.

Kulkarni AP, Kellaway LA, Lahiri DK, Kotwal GJ. Neuroprotection from complement-mediated inflammatory damage. *Ann. NY Acad. Sci.* 2004; 1035: 147-164.

Lachmann P. Complement before molecular biology. *Molecular Immunology* 2006; 43: 496-508.

Lachmann PJ. Microbial subversion of the immune response. *Prot. Natl. Acad. Sci. USA* 2002; 99(13): 8461-8462.

Lange S, Schmitt J, Schmid RD. High-Yield expression of the recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast *Pichia pastoris*. *Journal of Immunological Methods* 2001; 255: 103-114.

Langford GA, Yannoutsos N, Cozzi E et al. Production of pigs transgenic for human decay accelerating factor. *Transplantation Proceedings* 1994; 26(3): 1400-1401.

Lee CY, Lee SJ, Jung KH et al. High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. *Process Biochemistry* 2003; 38: 1147-1154.

Leinhase I, Schmidt OI, Thurman JM et al. Pharmacological complement inhibition at the C3 convertase level promotes neuronal survival, neuroprotective intracerebral gene expression,

and neurological outcome after traumatic brain injury. *Experimental Neurology* 2006; 199(2): 454-464.

Lelongt B, Carone FA, Kanwar YS. Decreased *de novo* synthesis of proteoglycans in drug-induced renal cystic disease. *Proc. Natl. Acad. Sci. USA* 1988; 85: 9047-9051.

Li Y, Tucci M, Narain S et al. Urinary biomarkers in lupus nephritis. *Autoimmunity Reviews* 2006: In press

Lien Y-H H, Lai L-W, Silva AL. Pathogenesis of renal ischemia/reperfusion injury: lessons from knockout mice. *Life Sciences* 2003; 74: 543-552.

Liszewski MK, Leung M, Cui W et al. Dissecting Sites important for complement regulatory activity in membrane cofactor protein (MCP; CD46). *Journal of Biological Chemistry* 2000; 275(48): 37692-37701.

Liszewski MK, Leung MK, Hauhart R et al. Structure and Regulatory Profile of the Monkeypox Inhibitor of Complement: Comparison to Homologs in Vaccinia and Variola and Evidence for Dimer Formation. *The Journal of Immunology* 2006; 176: 3725-3734.

Liu PT, Ta TV, Villarete LH. High-Yield Expression and Purification of Human Interferon α -1 in *Pichia pastoris*. *Protein Expression and Purification* 2001; 22: 381-387.

Mahesh J, Daly J, Cheadle WG, Kotwal GJ. Elucidation of the early events contributing to zymosan-induced multiple organ dysfunction syndrome using MIP-1 α , C3 knockout, and C5-deficient mice. *Shock* 1999; 12(5): 340-349.

Massung RF, Lopaaarev VN, Knight JC et al. Terminal region sequence variations in variola virus DNA. *Virology* 1996; 221(2): 291-300.

Matsumura Y, Yamashita J, Kita S et al. Pathophysiological roles of Ca(2+) overload via the Na(+)/Ca(2+) exchanger and endothelin-1 overproduction in ischemia/reperfusion-induced acute renal failure. *Clinical Science (London)* 2002; 48: 389S-392S.

McCurry KR, Kooyman DL, Alvarado CG et al. Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. *Nature Medicine* 1995; 1(5): 423-427.

McGeer EG, Klegeris A, McGeer PL. Inflammation, the complement system and the diseases of aging. *Neurobiology of Aging* 2005; 26S: S94-S97.

Melnikov VY, Ecdler T, Fantuzzi G et al. Impaired IL-18 processing protects caspase-1-deficient mice from ischemic acute renal failure. *Journal of Clinical Investigation* 2001; 107(9): 1145-1152.

- Mikata¹ S, Miyagawa S, Kamiike W et al. New Approach to Prevent Complement Activation on Xenografts: Effects of a Surface-Bound Form of Human C4b Binding Protein. *Transplantation Proceedings* 1998; 30: 65-66.
- Mikata² S, Miyagawa S, Iwata K et al. Regulation of complement-mediated swine endothelial cell lysis by a surface-bound form of human C4b binding protein. *Transplantation* 1998; 65(3): 363-368.
- Miller CG, Shchelkunov SN, Kotwal GJ. The Cowpox Virus-Encoded Homolog of the Vaccinia Virus Complement Control Protein Is an Inflammation Modulatory Protein. *Virology* 1997; 229: 126-133.
- Mold C, Rodic-Polic B, Du Clos TW. Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc gamma receptors. *Journal of Immunology* 2002; 168(12): 6375-6381.
- Moller-Kristensen M, Wang W, Ruseva M et al. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injuries. *Scandinavian Journal of Immunology* 2005; 61(5): 426-434.
- Mollnes TE, Fiane AE. Perspective on complement in xenotransplantation. *Molecular Immunology* 2003; 40: 135-143.
- Mollnes TE, Kirschfink M. Strategies of therapeutic complement inhibition. *Molecular Immunology* 2006; 43: 107-121.
- Mollnes TE, Song W-C, Lambris JD. Complement in inflammatory tissue damage and disease. *TRENDS in Immunology* 2002; 23(2): 61-64 (*Figure-1 reproduced from page-62*).
- Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc. Natl. Acad. Sci. USA* 1996; 93: 11341-11348.
- Murthy KHM, Smith SA, Ganesh VK et al. Crystal structure of a complement control protein that regulates both pathways of complement activation and binds heparan sulfate proteoglycans. *Cell* 2001; 104: 301-311.
- Nairn R, Helbert M. 2002. Innate Immunity in *Immunology for Medical Students*. Louise Crowe Ed. pp. 157-165. Mosby International Ltd. Printed in China.
- Nataf S, Carroll SS, Wetsel RA et al. Attenuation of experimental autoimmune demyelination in complement-deficient mice. *Journal of Immunology* 2000; 165(10): 5867-5873.
- Ogunuga OO, Ghebremariam YT, Kotwal GJ. Lister homologue of vaccinia virus complement control protein is two amino acids shorter, has putative glycosylation sites and other functional and structural differences. *Special Symposia FEBS Journal* 2005; 272(S1): 15-16.

- Ouyang J, Wang J, Deng R et al. High-level expression, purification, and characterization of porcine somatotropin in *Pichia pastoris*. *Protein Expression and Purification* 2003; 32: 28-34.
- Park P, Haas M, Cunningham PN et al. Inhibiting the Complement System Does Not Reduce Injury in Renal Ischemia Reperfusion. *Journal of the American Society of Nephrology* 2001; 12: 1383-1390.
- Perrella MA, Hsieh C-M, Lee W-S et al. Arrest of endotoxin-induced hypotension by transforming growth factor β 1. *Proc. Natl. Acad. Sci. USA* 1996; 93: 2054-2059.
- Pickering MC, Warren J, Rose KL et al. Prevention of C5 activation ameliorates spontaneous and experimental glomerulonephritis in factor-H deficient mice. *Proc. Natl. Acad. Sci. USA* 2006; 103(25): 9649-9654.
- Pierangeli SS, Vega-Ostertag M, Liu X, Girardi G. Complement activation: a novel pathogenic mechanism in the antiphospholipid syndrome. *Annals of the New York Academy of Science* 2005; 1051: 413-420.
- Pruitt SK, Kirk AD, Bollinger RR et al. The effect of soluble complement receptor type 1 on hyperacute rejection of porcine xenografts. *Transplantation* 1994; 57(3): 363-370.
- Quidel Corporation. An enzymatic immunoassay for the quantitation of Bb fragment of Factor B of the alternative complement pathway. Kit insert 2004. San Diego, CA.
- Quigg RJ. Complement and the kidney. *The Journal of Immunology* 2003; 171(7): 3319-3324.
- Ramirez P, Chavez R, Majado M et al. Transgenic pig-to-baboon liver xenotransplantation: clinical, biochemical, and immunologic pattern of delayed acute vascular rejection. *Transplantation Proceedings* 2002; 34: 319-320.
- Rancan M, Morganti-Kossmann MC, Barnum SR. Central Nervous System – Targeted Complement Inhibition Mediates Neuroprotection After Closed Head Injury in Transgenic Mice. *Journal of Cerebral Blood Flow & Metabolism* 2003; 23(9): 1070(Abstract).
- Reynolds DN, Smith SA, Zhang YP et al. Vaccinia virus complement control protein reduces inflammation and improves spinal cord integrity following spinal cord injury. *Annals of the New York Academy of Science* 2004; 1035: 165-178.
- Rhoden EL, Rhoden CR, Lucas ML et al. The role of nitric oxide pathway in the renal ischemia-reperfusion injury in rats. *Transplant Immunology* 2002; 10: 277-284.
- Riedemann NC, Ward PA. Complement in Ischemia Reperfusion Injury. *American Journal of Pathology (Commentary)* 2003; 162(2): 363-367.
- Rodriguez-Reynoso S, Leal C, Buen EP, Castillo JC, Ramos-Solano F. Melatonin Ameliorates Renal Ischemia/Reperfusion Injury. *Journal of Surgical Research* 2004; 116: 242-247.

- Rosengard AM, Carry NRB, Langford GA et al. Tissue expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs. *Transplantation* 1995; 59(9): 1325-1333.
- Rosengard AM, Liu Y, Nie Z, Jimenez R. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. *Prot. Natl. Acad. Sci. USA* 2002; 99: 8808-8813.
- Ross BD. *Perfusion Techniques in Biochemistry. A Laboratory Manual in the use of isolated perfused organs in Biochemical experimentation.* Clarendon press Oxford Inc. 1972; pp. 221-245.
- Rynes R, Pickering RF. Inherited complement component deficiencies in man in *Methods in Complement for Clinical Immunologists*. Keith Whaley Ed. 1985; pp. 292-321. Longman Group Limited. Printed in Great Britain.
- Sacks SH, Chowdhury P, Zhou W. Role of the complement system in rejection. *Current Opinion in Immunology* 2003; 15: 487-492.
- Schiller B, Cunningham PN, Alexander JJ et al. Expression of a Soluble Complement Inhibitor Protects Transgenic Mice from Antibody-Induced Acute Renal Failure. *Journal of the American Society of Nephrology* 2001; 12: 71-79.
- Schmoeckel M, Nollert G, Shahmohammadi M et al. Transgenic human decay accelerating factor makes normal pigs function as a concordant species. *Journal of Heart Lung Transplantation* 1997; 16(7): 758-764.
- Scott MJ, Burch PT, Jha P et al. Vaccinia Virus Complement Control Protein Increases Early Bacterial Clearance during Experimental Peritonitis. *Surgical Infections* 2003; 4(4): 317-326.
- Sfyroera G, Katragadda M, Morikis D, Isaacs SN, Lambris JD. Electrostatic Modeling Predicts the Activities of Orthopoxvirus Complement Control Proteins. *The Journal of Immunology* 2005; 174: 2143-2151.
- Sharpe JS. The Determination of Guanidines in Urine as Picrates. *Biochemical Journal* 1925; 19(2): 168-171.
- Singh D, Chopra K. The effect of naringin, a bioflavonoid on ischemia-reperfusion induced renal injury in rats. *Pharmacological Research* 2004; 50: 187-193.
- Sjöholm AG, Jönsson G, Braconier JH et al. Complement deficiency and disease: An update. *Molecular Immunology* 2006; 43: 78-85.
- Smith SA, Kotwal GJ. Virokines: novel immunomodulatory agents. *Expert Opin Biol Ther.* 2001; 1(3): 343-357.

Smith SA, Kotwal GJ. Immune response to poxvirus infections in various animals. *Critical Reviews Microbiology* 2002; 28(3): 149-185.

Smith SA, Krishnasamy G, Murthy KHM et al. Vaccinia virus complement control protein is monomeric, and retains structural and functional integrity after exposure to adverse conditions. *Biochimica et Biophysica Acta* 2002; 1598: 55-64.

Smith SA, Mullin NP, Parkinson J et al. Conserved Surface-Exposed K/R-X-K/R Motifs and Net Positive Charge on Poxvirus Complement Control Proteins Serve as Putative Heparin Binding Sites and Contribute to Inhibition of Molecular Interactions with Human Endothelial Cells: a Novel Mechanism for Evasion of Host Defense. *Journal of Virology* 2000; 74(12): 5659-5666.

Smith SA, Sreenivasan R, Krishnasamy G et al. Mapping of regions within the vaccinia virus complement control protein involved in dose-dependent binding to key complement components and heparin using surface plasmon resonance. *Biochimica et Biophysica Acta* 2003; 1650: 30-39.

Sreekrishna K, Brankamp RG, Kropp KE et al. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* 1997; 190: 55-62.

Stahl GL, Xu Y, Hao L et al. Role for the Alternative Complement Pathway in Ischemia/Reperfusion Injury. *American Journal of Pathology* 2003; 162(2): 449-455.

Stein¹ JH, Osgood RW, Barnes JL et al. The role of complement in the pathogenesis of postischemic acute renal failure. *Miner Electrolyte Metabolism* 1985; 11(4): 256-261. *Quoted by* Park P, Haas M, Cunningham PN et al. Inhibiting the Complement System Does Not Reduce Injury in Renal Ischemia Reperfusion. *Journal of the American Society of Nephrology* 2001; 12: 1383-1390.

Stein² JH, Osgood RW, Barnes JL et al. The role of complement in the pathogenesis of postischemic acute renal failure. *Miner Electrolyte Metabolism* 1985; 11(4): 256-261. *Quoted by* Riedemann NC, Ward PA. Complement in Ischemia Reperfusion Injury. *American Journal of Pathology (Commentary)* 2003; 162(2): 363-367.

Sudbery PE. The expression of recombinant proteins in yeasts. *Current Opinion in Biotechnology* 1996; 7: 517-524.

Taube C, Thurman JM, Takeda K et al. Factor B of the alternative complement pathway regulates development of airway hyperresponsiveness and inflammation. *Proc. Natl. Acad. Sci.* 2006; 103(21): 8084-8089.

Thiry M, Cingolani D. Optimization scale-up fermentation processes. *TRENDS in Biotechnology* 2002; 3(20): 103-105.

Thorbjornsdottir¹ P, Kolka R, Gunnarsson E et al. Vaccinia Virus Complement Control Protein Diminishes Formation of Atherosclerotic Lesions: Complement Is Centrally Involved in Atherosclerotic Disease. *Annals of the New York Academy of Science* 2005; 1056: 1-15.

Thorbjornsdottir² P, Kolka R, Gunnarsson E et al. Complement is a rate-limiting step in the development of atherosclerotic lesions. *Molecular Immunology (Abstract)* 2006; 43: 130-131.

Thurman JM, Ljubanovic D, Edelstein CL, Gilkeson GS, Holers VM. Lack of a Functional Alternative Complement Pathway Ameliorates Ischemic Acute Renal Failure in Mice. *The Journal of Immunology* 2003; 170(3): 1517-1523.

Thurman JM, Ljubanovic D, Royer PA et al. Altered renal tubular expression of the complement inhibitor Crry permits complement activation after ischemia/reperfusion. *Journal of Clinical Investigation* 2006; 116(2): 357-368.

Thurman JM, Lucia MS, Ljubanovic D, Holers VM. Acute tubular necrosis is characterized by activation of the alternative pathway of complement. *Kidney International* 2005; 67: 524-530.

Tischer CC, Croker BP. Indications for and Interpretation of the Renal Biopsy: Evaluation by Light, Electron, and Immunofluorescence Microscopy. In *Diseases of the Kidney* 4th Edition, Volume I. Eds Schrier RE, Gottschalk CW. Little, Brown and Company Inc. 1988; pp. 527-556.

Tosi MF. Innate immune responses to infection. *Current reviews of allergy and clinical immunology* 2005; 116: 241-249.

Trouw LA, Seelen MA, Duijs JMGJ et al. Activation of the lectin pathway in murine lupus nephritis. *Molecular Immunology* 2005; 42: 731-740.

Tuo J, Ning B, Bojanowski CM et al. Synergic effect of polymorphisms in ERCC6 5' flanking region and complement factor H on age-related macular degeneration predisposition. *Proc. Natl. Acad. Sci. USA* 2006; 103(24): 9256-9261.

Uvarova EA, Shchelkunov SN. Species-specific differences in the structure of orthopoxvirus complement-binding protein. *Virus Research* 2001; 81: 39-45.

Van Denderen BJ, Salvaris E, Romanella M et al. Combination of decay-accelerating factor expression and alpha 1,3-galactosyltransferase knockout affords added protection from human complement-mediated injury. *Transplantation* 1997; 64(6): 882-888.

Velayuthaprabhu S, Archunan G. Evaluation of anticardiolipin antibodies and antiphosphatidylserine antibodies in women with recurrent abortion. *Indian Journal of Medical Sciences* 2005; 59(8): 347-352.

Vélez ML, Costamagna E, Kimura ET et al. Bacterial Lipopolysaccharide Stimulates the TSH-dependent Thyroglobulin Gene Expression at Transcriptional Level by Involving the

Transcription Factors TTF-1 and Pax8. *Endocrinology* 2006; April 20 doi: 10.1210/en.2005-0789.

Vieira GF, Chies JAB. Immunodominant viral peptides as determinants of cross-reactivity in the immune system – Can we develop wide spectrum viral vaccines? *Medical Hypothesis* 2005; 65: 873-879.

Weisman HF, Bartow T, Leppo Mk et al. Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* 1990; 249(4965): 146-151.

Wen L, Atkinson JP, Giclas PC. Clinical and laboratory evaluation of complement deficiency. *Current reviews of allergy and clinical immunology* 2004; 113: 585-593.

Whaley K. An Introduction to the Complement System in *Methods in Complement for Clinical Immunologists*. Keith Whaley Ed. 1985; pp. 1-20. Longman Group Limited. Printed in Great Britain.

Wiesel M-L, Charmantier J-L, Freyssinet J-M et al. Screening of protein S deficiency using a functional assay in patients with venous and arterial thrombosis. *Thrombosis Research* 1990; 58(5): 461-468.

Williams P, Lopez H, Britt D et al. Characterization of Renal Ischemia-Reperfusion Injury in Rats. *Journal of Pharmacological and Toxicological Methods* 1997; 37: 1-7.

Wittek R. Vaccinia immune globulin: current policies, preparedness, and product safety and efficacy. *International Journal of Infectious Diseases* 2006; 10: 193-201.

Yagmurdur MC, Colak T, Emiroglu R et al. Anti-inflammatory Action of Heparin via the Complement System in Renal Ischemia-Reperfusion. *Transplantation Proceedings* 2003; 35: 2566-2570.

Yamada K, Miwa T, Liu J, Nangaku M, Song W-C. Critical Protection from Renal Ischemia Reperfusion Injury by CD55 and CD59. *The Journal of Immunology* 2004; 172: 3869-3875.

Yamashita J, Kita S, Iwamoto T et al. Attenuation of ischemia/reperfusion-induced renal injury in mice deficient in Na⁺/Ca²⁺ exchanger. *Journal of Pharmacology and Experimental Therapeutics* 2003; 304(1): 284-293.

Zhang L, Morikis D. Immunophysical properties and prediction of activities for vaccinia virus complement control protein and smallpox inhibitor of complement enzymes using molecular dynamics and electrostatics. *Biophysical Journal* 2006; 90(9): Abstract.

Zhou W, Farrar CA, Abe K et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *The Journal of Clinical Investigation* 2000; 105(10): 1363-1371.

Zhu A, Monahan C, Zhang Z et al. High-Level Expression and Purification of Coffee Bean α -Galactosidase Produced in the Yeast *Pichia pastoris*. *Archives of Biochemistry and Biophysics* 1995; 324(1): 65-70.

Zilva JF, Pannall PR, Mayne PD. The kidneys: renal calculi In *Clinical Chemistry in Diagnosis and Treatment* 5th ed. A Lloyd-Luke publication. 1988; pp. 1-24.

Zipfel PF, Heinen S, Jozsi M, Skerka C. Complement and diseases: Defective alternative pathway control results in kidney and eye diseases. *Molecular Immunology* 2006; 43: 97-106.