T cell and B cell Responses in a Rat Model of Rheumatic Heart Disease

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DECLARATION OF ETHICS

The research presented in this thesis was conducted within the guidelines of the James Cook University Statement and Guidelines on Research Practices which is based on the NHMRC Australian Code for the Responsible Conduct of Research (2007). The proposed research methodology received approval from the James Cook University Animal Ethics Committee (A958 and A1326).

DE Gorton
October 2009

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ABSTRACT

Rheumatic fever (RF) is an inflammatory disease following group A streptococcal (GAS) infection which affects the joints, skin, central nervous system and heart in susceptible individuals. Rheumatic heart disease (RHD) accounts for the majority of acquired heart disease in children and young adults in many parts of the world and is the most serious manifestation of RF. Rarely seen now in most developed nations, RF/RHD remains an important medical and economic public health problem in many developing countries and also in certain indigenous populations within developed nations.

It is known that permanent cardiac damage can manifest after prolonged or repeated episodes of RF or during a severe acute episode. Although all layers of the heart, that is the pericardium, myocardium and endocardium, can be affected, it is the loss of function of the heart valves that is associated with rheumatic carditis. The aetiology of RF/RHD is believed to be autoimmune, whereby an aberrant immune response against GAS cross-reacts with host tissue proteins through molecular mimicry. Studies have implicated mimicry between the GAS M protein, an alpha helical coiled-coil extending from the surface of the bacterium and host tissue proteins such as cardiac myosin. However, despite decades of intensive investigation, the exact pathogenic mechanism(s) involved in initiating disease remains elusive.

In this thesis the cellular and antibody responses elicited in the rat autoimmune valvulitis (RAV) model, were investigated. The RAV animal model may, in part, represent rheumatic carditis in humans. Rats immunised with recombinant streptococcal M5 protein (rM5) developed valvular lesions, distinguished by infiltration of $CD3^+$, $CD4^+$ and $CD68^+$ cells into valve tissue but few $CD8^+$ cells, consistent with human studies that suggest RF/RHD are mediated by inflammatory $CD4^+$ T cells and macrophages.

It will be shown herein that rM5 elicits strong T cell and opsonic IgG antibody immune responses in rats. Immunisation experiments using peptides from the B-repeat and C-repeat regions of streptococcal M5 identified one B-repeat peptide (M5-B.6, amino acids TVKDKIAKEQENKETIGTIK) that contains a strong T cell

epitope and a probable cardiac myosin cross-reactive epitope with the capacity to induce valvular lesions in rats.

Additional experiments were carried out to investigate the immune responses in rats as a result of repetitive immunisation with rM5 protein. Higher T cell and B cell responses with exacerbation of inflammatory changes in heart tissue and prolongation of P-R interval on ECG, in addition to increased IgG cardiac myosin cross-recognition, was demonstrated in rats which received additional booster immunisations with rM5 protein.

Alternative adjuvants and immunisation strategies were also investigated in the current study. A replacement for Freund's complete adjuvant (FCA) was not found in the alternative formulations tested in this study, however in the RAV model, hock immunisation was demonstrated to be superior to the footpad route of immunisation when using FCA. Hock immunisation is shown to elicit less inflammation and pain in the animals whilst maintaining the desired immune responses.

Thus, the data demonstrate that the RAV model displays many of the characteristics of human rheumatic heart disease and thus, is an invaluable tool for studying the immunopathogenic mechanisms involved in RF/RHD.

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COMMONLY USED ABBREVIATIONS

AA	amino acids
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ANOVA	analysis of variance
APC	antigen presenting cell
APL	altered peptide ligand
APSGN	autoimmune post-streptococcal glomerulonephritis
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CD	cluster of differentiation
CM	cardiac myosin
Con A	concanavalin A
cpm	counts per minute
DAB	3, 3'-diaminobenzidine tetrahydrachloride
ddH_2O	deionised water
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease
dNTP	deoxynucleotide phosphate
DTT	dithiothreitol
EAM	experimental autoimmune myocarditis
ECG	electrocardiograph
EDTA	Ethylenediaminetetra acetic acid
EGTA	ethylene glycol tetra acetic acid
ELISA	enzyme linked immunosorbent assay
FBS	foetal bovine serum
FCA	Freund's complete adjuvant
FFPE	formalin fixed paraffin embedded
FIA	Freund's incomplete adjuvant
g	gravity
GAS	group A streptococcus
H&E	haematoxylin and eosin
HBA	horse blood agar

HITC	heart-infiltrating T cells
i.p.	intraperitoneal
IBT	indirect bactericidal assay
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
IU	international units
kDa	kilodaltons
kV	kilovolts
LB	Luria Bertani
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MNC	mononuclear cells
MWCO	molecular weight cut-off
NBF	neutral buffered saline
NHFA	National Heart Foundation of Australia
NHMRC	National Health and Medical Research Council
OD	optical density
p.i.	post immunisation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenyl methanesulfonyl fluoride
PPD	purified protein derivative
RAV	rat autoimmune valvulitis
RF/RHD	Rheumatic fever/rheumatic heart disease
RNA	ribonucleic acid
RNase A	ribonuclease
s.c.	subcutaneous
SAAP	streptavidin conjugated alkaline phosphatase
SBA	sheep blood agar
SD	standard deviation
SDS-PAGE	sodium dodecylsulphate -polyacrylamide gel electrophoresis
SEM	standard error of the mean
SI	stimulation index

TBS	tris buffered saline
TCR	T cell receptor
THYB	Todd Hewitt yeast broth
TLCK	N-α-tosyl-L-lysine chloromethyl ketone·
TNF-α	tumour necrosis factor-alpha
V	volts
WHO	World Health Organisation

CHAPTER 1
GENERAL INTRODUCTION

1.1 BACKGROUND

Rheumatic fever (RF) is an inflammatory disease affecting the joints, heart and central nervous system in susceptible individuals following untreated group A streptococcal (GAS) infections (Cunningham, 2000). Carditis, which affects from 30% to 50% of RF patients, is the most serious consequence of the disease (Binotto *et al.*, 2002). Immediate damage to the heart muscle (acute carditis) can be lethal, manifesting as congestive heart failure. Permanent scarring of the heart valves can cause an insidious, long-term condition, chronic rheumatic heart disease (RHD) which can often be fatal without surgical repair or valvular replacement. Damaged valves either restrict the flow of blood (stenosis) or create severe leaks (regurgitation) that cause heart failure (WHO, 2004).

The heart is comprised of three main structural components: 1) the left and right atria, 2) the left and right ventricles and 3) a fibrous skeleton, called the annulus fibrosis, which provides strength and separates the atria from the ventricles electrically. Externally, the muscle (myocardium) is covered with serous epicardium (pericardium) and is lined internally with endothelium (endocardium). Heart valves are attached around the border of each of four orifices in the annulus fibrosis, the left and right atrioventricular orifices, the pulmonary and the aortic orifices. Pulmonary and aortic valves are comprised of three semi-lunar leaflets, while the atrioventricular valves are comprised of three (right, tricuspid valve) or two (left,mitral valve) leaflets. Six to twelve chordae tendinae attach the valve cusps to papillary muscles projecting from the ventricular walls (Snell, 2007) (Figure 1.1). Previously, heart valves have been thought of as simple structures, functioning in response to pressure gradients to prevent backflow during contraction, thus ensuring a uni-directional flow of blood (Cooper *et al.*, 1966). However, it is now known that valves are much more complex (as reviewed by Flanagan and Pandit, 2003). Histologically, valves are avascular and made up of proteoglycans and a dense collagen network covered by endocardium, with elastic fibrous tissue positioned sub-

endorcardially (Flanagan and Pandit, 2003). Damaged valves either restrict the flow of blood (stenosis) or create severe leaks (regurgitation) that cause heart failure (Saffitz, 2008).

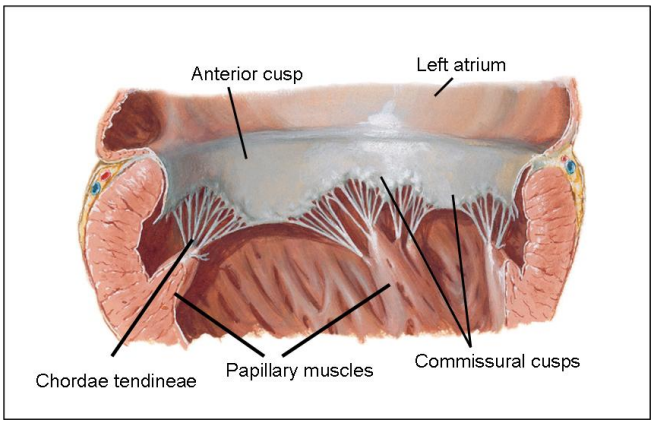

Figure 1.1 Diagram of the normal mitral valve and associated structures
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At the close of the 19th Century and in the first few decades of the 20th Century, RF/RHD contributed greatly to worldwide morbidity and mortality statistics, with numerous hospitals dedicated solely to caring for RF/RHD patients. In the 1920s, RF/RHD accounted for the majority of deaths in young people between five and 20 years and the second major cause of death in 20 to 30 year olds. The delayed appearance of RF/RHD was linked to an infectious organism prior to 1900 (Dalton, 1890). A trend to a more severe condition following prolonged or recurrent episodes of RF was also noted (Poynton, 1905). F.J. Poynton, a paediatrician at the Hospital for Sick Children in London, believed that risk factors associated with RF/RHD included 'climatic and local surroundings, sanitation, and conditions of housing' and declared that 'it is to *prevention*, then, that we look for some advance from this grievous state of affairs' (Campbell, 1944). It was not until the 1930s, after documented cases confirmed a correlation between α-haemolytic streptococcal throat infection two to five weeks prior to the appearance or recurrence of RF, that the GAS bacteria were firmly established as the infectious agent responsible for the development of RF (Coburn, 1931; Swift, 1931; Coburn and Pauli, 1932b; Coburn and Pauli, 1932a; Coburn and Pauli, 1932c).

Since the end of World War II, RF/RHD has declined dramatically in most developed countries; however, this largely preventable disease remains a major cause of acquired heart disease and accounts for a substantial proportion of the health budgets in many less-developed nations and in many indigenous populations within developed countries such as Australia and New Zealand (Stollerman, 1997).

Earliest treatment strategies for RF patients included general blood-letting, subcutaneous injection of carbolic acid or 'morphia'. However, these 'remedies' were replaced by bed rest and salicylates or sometimes, steroid treatment (Chambers, 1863; Young, 1880). It was not until shortly before World War II that it was demonstrated that recurrences of RF could be prevented by the continuous use of sulfonamides to prevent streptococcal infection. For those with severe acute heart damage during an initial bout of RF or with established RHD the prognosis was however, bleak. Later, advances in valve repair surgical techniques and development of biological or mechanical replacement valves improved survival rates in patients, with 10 year survival rates reported in one Toronto study as 88%, 70% and 73% for valve repair, bioprostheses and mechanical replacement respectively (Yau *et al.*, 2000). However, increased post-surgical complication rates and reduced survival have been recorded in underdeveloped countries including Aboriginal and Torres Strait Islander populations in Australia (Carapetis *et al.*, 1999; Walsh *et al.*, 2008). The current gold standard for prevention of RF is secondary penicillin treatment to stop recurrences of RF rather than mass primary prophylaxis to prevent GAS infection, due to high cost and fears of penicillin hypersensitivity and resistance (WHO, 2004). However, secondary treatment programs are not without problems including deficiencies in diagnosis, delivery and compliance issues (WHO, 2005c). The ideal solution is a safe, efficacious vaccine against GAS that not only prevents RF/RHD, but also other serious complications of GAS infection including post-streptococcal glomerulonephritis, necrotising fasciitis and streptococcal toxic shock syndrome (WHO, 2005b).

Vaccine development is based on the principle of acquired immunity to pathogens and has progressed, following Pasteur's theory of 'isolate, inactivate and inject', from whole killed or attenuated pathogens, to sub-unit and conjugate vaccines containing pathogen-derived immunogenic antigens which induce T cell-mediated as

well as antibody immunity. Rebecca Lancefield carried out extensive work over many years to characterise GAS M protein, establishing its importance as a key virulence determinant by endowing the bacterium with anti-phagocytic properties to circumvent host immunity. Lancefield also demonstrated protective antibodies, directed towards the M protein, in the serum of human patients following pharyngitis (Lancefield, 1959; Lancefield, 1962). The M protein is therefore a logical target for a vaccine candidate. Currently however, no vaccine preparation against GAS is licensed for use in the general population.

While M protein-based vaccines have demonstrated some promise in both human and animal trials over the last four or five decades (Beachey and Stollerman, 1972; Fox *et al.*, 1973; Waldman *et al.*, 1975; D'Alessandri *et al.*, 1978; Beachey *et al.*, 1979; Bessen and Fischetti, 1990) progress has been slow, in part due to the necessity of inducing protective immunity against multiple GAS serotypes circulating at different times in different populations. Additionally, there are concerns that the immune response directed against the M protein itself could lead to RF/RHD (Massell *et al.*, 1969). More recently, research has focussed on developing sub-unit vaccines containing M protein epitopes that elicit protective antibody and T cell responses against multiple serotypes while avoiding potentially harmful immune responses.

An intrinsic requirement for designing and testing these vaccine candidates is an understanding of the pathogenic mechanisms involved in the development of RF/RHD following GAS infection. While there is a wealth of evidence to indicate that autoimmunity as a result of molecular mimicry between streptococcal and host tissue proteins play a central role (Cunningham, 2004; Guilherme and Cunningham, 2006), the precise mechanisms are not fully understood. Considerable progress has been made by analysing cellular and humoral responses in peripheral blood and in cells isolated from heart lesions of RF/RHD patients (Guilherme *et al.*, 1995; Guilherme *et al.*, 2000; Guilherme *et al.*, 2001b; Ellis *et al.*, 2005). Cytokine studies using sera and lymphocytes from heart tissue of RF/RHD patients support the inflammatory model of these diseases. High expression of pro-inflammatory cytokines IFN-γ and TNF-α in the myocardium and valves has been demonstrated. However, while high expression of regulatory cytokines IL-10 and IL-4 was shown

in cells infiltrating myocardial tissue, low IL-10 and very low IL-4 expression was found in cells infiltrating valvular tissue. This disparate production of regulatory cytokines may account for the worsening or progressive damage seen in heart valves in contrast to resolution of lesions in the myocardium (Guilherme *et al.*, 2004). However, further studies of T cells and associated cytokines involved in the early stages of the autoimmune process and in the progression to valvular damage in RHD patients are required. Limited access to tissues from RF/RHD patients is a major obstacle to these studies and an acceptable model would facilitate further research. An animal model, in which the immunopathological mechanisms or outcome of disease resembles those that occur in humans, is a logical adjunct to human studies.

A number of animal models including primates, rabbits, mice and rats have been used in the past and have contributed, in part, to our understanding of RF/RHD. However, the pathological changes displayed in these proposed models were not analogous to those seen in human patients (Kishna and Iyer, 1999). The rat autoimmune valvulitis (RAV) model, developed by Cunningham and colleagues (Quinn *et al.*, 2001), has shown promise as a suitable animal model of rheumatic carditis and has been used previously in our laboratory to induce valvulitis/carditis in rats using C-terminal M-protein peptides (Lymbury *et al.*, 2003).

1.1.1 Purpose of the study

Several key questions regarding RF/RHD have not yet been fully answered. What risk factors dictate the susceptibility to RF/RHD in some individuals but not others? What drives the generation of autoreactive T cells following infection by rheumatogenic bacteria? The RAV model may provide answers to some of these important questions and could be a useful tool in the development of treatment strategies. However, an important aspect of any model is to validate its relevance to the human condition. Therefore, the present study was undertaken to further investigate T cell and B cell responses in the RAV model and to compare these immune responses with those reported in RF/RHD patients. A second key objective of this study was to refine our methods of immunisation to minimise pain and stress in the animals under experimentation.

To examine the role of GAS M protein in the development of experimental carditis in Lewis rats, the following hypotheses were tested:

1) Group A streptococcal M protein specific T cells play a significant role in the development of autoimmune valvulitis
2) Valvular and myocardial lesions in rats immunised with GAS M-protein are comprised of the same cell types seen in heart lesions of RF/RHD patients
3) Immunisation methods can be improved to reduce pain and stress in experimental animals whilst retaining the ability to induce autoimmune valvulitis
4) Selected peptides from Group A streptococcal M protein afford protection against infection without generating autoreactive T cells
5) Repetitive exposure of rats to GAS M protein increase the severity of heart damage.
6) M protein-specific T cell lines derived from rats with heart lesions can transfer heart damage to healthy, naïve rats on adoptive transfer.

Animal experiments testing the first two hypotheses are outlined in Chapter 4. The studies carried out to address the third hypothesis using alternative adjuvants and sites of inoculation are outlined in Chapter 5. Chapters 6 to 8 describe the work carried out to test hypotheses four to six respectively. A final chapter (9) will discuss the general findings of this study, including its successful outcomes and its limitations. Future directions for using this RAV model which may supplement this body of work and provide further insights into the mechanisms that initiate development of RF/RHD are also put forward.

CHAPTER 2
A REVIEW OF BACKGROUND LITERATURE

2.1 INTRODUCTION

Rheumatic fever (RF) is an inflammatory disease directed against the joints, heart and central nervous system in susceptible individuals following untreated group A streptococcal (GAS) infections (Cunningham, 2000). Carditis, which affects from 30% to 45% of RF patients, is the most serious manifestation of the disease and can lead to permanent damage of the heart valves, causing chronic rheumatic heart disease (RHD).

While it is unequivocally accepted that GAS is the aetiological agent of RF, the pathogenesis of RF/RHD is still not completely understood. The current understanding is that these diseases are autoimmune in nature and are triggered by an aberrant immune response, both humoral and cellular, to particular streptococcal antigens that cross-react with host tissues through molecular mimicry (Cunningham, 2000).

Current strategies to control RF include the elimination of GAS infection by antibiotic therapy (primary prevention) and treatment of symptoms. After an initial episode of RF, long-term prophylactic treatment (secondary prevention) to prevent subsequent episodes of GAS infection can effectively prevent RF recurrences and reduce severity of heart disease. The World Health Organization (WHO) first addressed the problem of RF/RHD in 1954 by setting up an Expert Committee to study the prevention and control of group A streptococcal infections and RF/RHD. A global prevention strategy initiated in 1984 resulted in a marked decrease in these diseases in some countries. However, in many developing countries and in some populations within developed countries, RF/RHD remain a significant health problem accounting for up to 60% of cardiovascular disease in children and adolescents (WHO, 2004).

The failure of control programs in these regions is multi-factorial and clearly demonstrates the need for a more cost-effective method of prevention. An effective

vaccine would not only prevent RF/RHD, but would protect against a number of other GAS diseases ranging from uncomplicated pharyngitis and pyoderma to severe invasive conditions of necrotizing fasciitis and streptococcal toxic shock syndrome or another post-infectious sequelae, glomerulonephritis. The most studied vaccine candidates to date have been based on the GAS M-protein, but have proven to be problematic due to potential tissue cross-reactivity and the multiplicity of M-serotypes (Dale and Beachey, 1985a; Dale and Beachey, 1985b; Poirier *et al.*, 1985; Bronze *et al.*, 1988a; Good and Brandt, 1997; Guilherme *et al.*, 2001c; Batzloff *et al.*, 2004). Recent progress in the development of multivalent and synthetic peptide-based vaccines has shown promising results (Dale, 1999; Olive *et al.*, 2002; Olive *et al.*, 2003; Batzloff *et al.*, 2006).

This review of literature will discuss the current knowledge of the relationship between group A streptococci and its sequelae of RF/RHD. Recent advances in the understanding of the roles of host susceptibility and humoral and cellular immunopathogenesis will also be reviewed.

2.2 PATHOLOGY & CLINICAL SIGNIFICANCE

2.2.1 Group A streptococcus

It was established long ago that infection by certain strains of *Streptococcus pyogenes*, often referred to as GAS, is a prerequisite to development of RF/RHD. Although the live organism has not been demonstrated in RF-affected tissues, a large body of clinical, epidemiological, immunological and prophylactic evidence strongly supports the view that GAS plays a central role in initiating the disease process.

Morphologically, GAS are non-motile, non-spore forming Gram positive cocci. Individual cells are round to oval in shape forming chains of varying length in broth and clinical specimens. The organism is a catalase-negative, aerotolerant anaerobe requiring enriched medium for growth. On blood agar (BA), colonies are greyish-white in colour and 1 mm or less in diameter, exhibiting a distinct zone of beta-haemolysis (Figure 2.1). Strains that produce a capsule composed of hyaluronic acid are mucoid in appearance (Kilian, 1998).

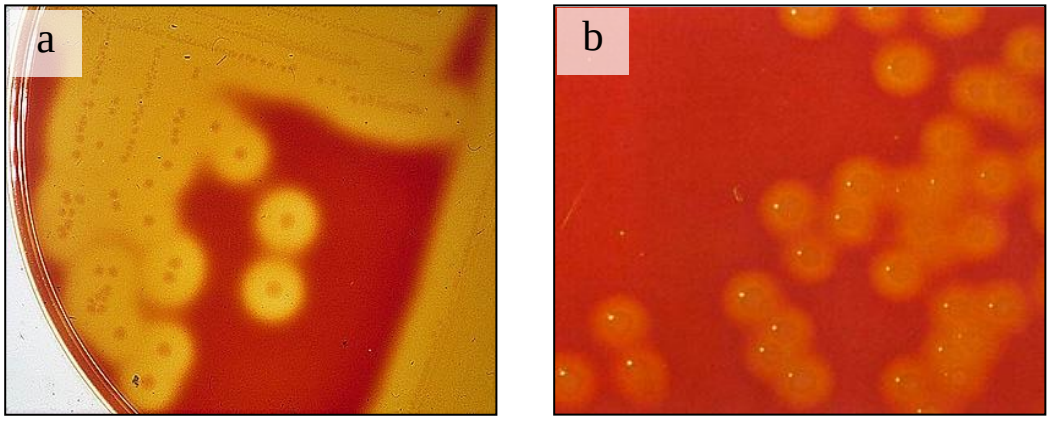


Figure 2.1 Group A streptococcal colonies on 5% sheep blood agar
a) beta-haemolysis and b) mucoid appearance characteristic of RF-associated strains

With no other natural known hosts, GAS are considered exclusively human pathogens. Transmission of GAS is principally caused by the person-to-person spread of respiratory droplets or saliva from patients with an acute infection. Asymptomatic carriers are considered to be less-frequent sources of infection. Crowded conditions such as those that occur in schools, military institutions and nursing homes provide an ideal environment for the spread of the pathogen (Parker, 1978). However, in temperate climates when winter weather sends people indoors, outbreaks occur in all age groups accounting for the seasonal nature of GAS pharyngitis and the associated RF/RHD in these regions.

2.2.2 Classification of group A streptococci

The Lancefield system of separating streptococci into groups is based on serological typing of cell wall carbohydrates. As reviewed by Kilian (1998) and Stollerman (1998), groups A to W are currently recognised. Conventionally, group A streptococci are further subdivided according to antigenic specificity of the surface-expressed M protein (Figure 2.2), based on a precipitation assay as described by Lancefield (1962). The decline in severity of GAS diseases prior to the 1980s, led to a similar decline in the preparation of M typing sera by many reference laboratories. Lack of available typing reagents has meant that many isolates, particularly those collected from outside of Europe and North America, are

serologically non-typeable (Facklam *et al.*, 1999). Attention has therefore focussed on a molecular approach for typing GAS. This method is based on sequencing the heterogeneous 5' end of the *emm* gene which encodes the M-protein (Beall *et al.*, 1996). Two isolates are considered to share to same sequence type if they are >95% homologous over the first 160 bases of the gene. To date, the Centres of Disease Control and Prevention (CDC) in Atlanta, Georgia, USA have validated over 120 different M-types (Facklam, 2002).

The characterization of GAS into so-called 'rheumatogenic' or 'nephritogenic' strains results from numerous studies that have found some strains are more likely to be isolated from RF patients, while other strains are associated with acute post-streptococcal glomerulonephritis (APSGN). RF strains were only recovered from the throat whereas APSGN strains came from either the throat (M types 1, 4 and 12) or skin (Bisno *et al.*, 1970). In general, M serotypes 1, 3, 5, 6, 18, 19 and 24 are associated with throat infection and RF (Kaplan *et al.*, 1989). These rheumatogenic strains are distinguished by their mucoid colonies rich in M-protein and the absence of serum opacity factor protein on their cell surface (Olivier, 2000). The distinction between 'rheumatogenic' and 'nephritogenic' strains is less clear in some tropical regions, where low rates of GAS pharyngitis or throat carriage and isolation of few classic 'rheumatogenic' strains have been demonstrated despite high rates of RF/RHD. Isolation of group C or G streptococci from the throat and high rates of GAS skin infection in these communities have led to speculation, as yet unproven, that pyoderma may be associated with RF in tropical regions (McDonald *et al.*, 2006; Steer *et al.*, 2009a).

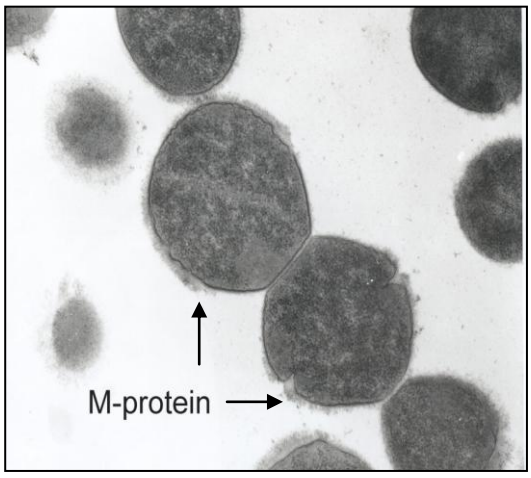


Figure 2.2

Micrograph of GAS bacteria (x30,000)

(Reproduced with permission, N Ketheesan, James Cook University)

2.2.3 Virulence Factors

GAS have developed a wide variety of virulence factors which enable the organism to colonise, multiply and spread in its human host whilst effectively circumventing the immune system (summarised in Table 2.1).

Table 2.1 Established and putative virulence factors of group A streptococci (Salyers and Whitt, 2002)

Virulence Factor	Role in Host
Anti-phagocytic factors	
M protein	Binds fibrinogen and Fc portion of IgG Binds complement factor H
Hyaluronic acid capsule	Inhibits C3b binding; disguises bacteria as 'self'
C5a peptidase	Reduces concentration of C5a; reduced chemotactic signaling for neutrophils
SIC	Interferes in formation of MAC of complement cascade
Extracellular enzymes	
Streptolysins (SLO, SLS)	Lysis of red blood cells, leukocytes
Streptokinase	Activates plasminogen to plasmin; dissolves fibrin clots
SpeB	Cysteine protease; may be a spreading factor
Superantigens	
SpeA, SpeC to SpeJ	Non-specific stimulation of T-cells, release of pro-inflammatory cytokines
Adhesins	
M-protein and M-like proteins	Binds extracellular matrix proteins (fibrinogen, plasminogen, collagen)
Fn-binding proteins	Bind fibronectin

2.2.3.1 Streptococcal M-protein

The M-protein is the best-defined virulence factor of group A streptococci (Figure 2.3). Consisting of four repeat regions (A-D), based on a common seven-residue or heptad periodicity (Manjula and Fischetti, 1980; Fischetti, 1991), the M protein extends from the streptococcal cell as an alpha-helical coiled coil dimer, forming

fibrils on the cell surface (Philips *et al.*, 1981). The helical structure extends throughout the length of the protein with the exception of the unrepeated amino- or N -terminal sequence of 10-20 residues, and the carboxy- or C-terminal proline-rich cell wall-spanning region. The hypervariable N-terminal region is distal to the cell surface and confers serotype specificity. The C-terminal region is highly conserved between serotypes. An LPSTGE motif anchors the protein in the cell wall (Fischetti, 1991).

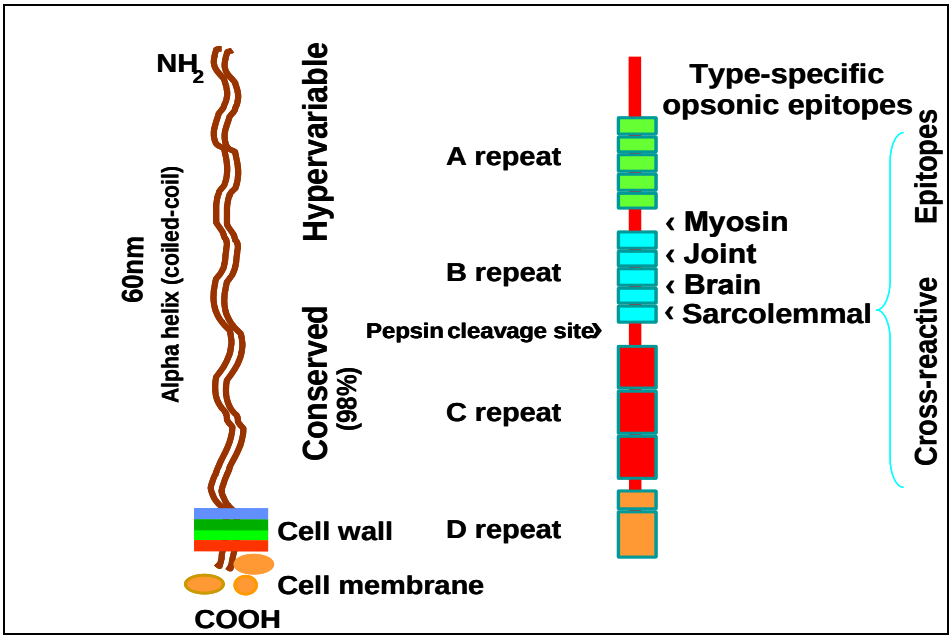


Figure 2.3 Diagrammatic representation of the GAS M protein molecule

Sequencing studies have revealed that despite extensive amino acid substitutions throughout the sequence, the α-helical coiled-coil structure is maintained. Therefore, different M protein types have highly variable primary sequences but are similar in structure and function (Kehoe, 1991). The hypervariable N-terminal half of the protein is released from the cell surface by enzymatic cleavage with pepsin (Beachey *et al.*, 1974). This digest product, termed pepM, is used extensively in GAS studies.

It was established long ago that in human blood deficient in specific antibodies directed against the M protein, GAS strains are resistant to phagocytosis by polymorphonuclear (PMN) leukocytes (Lancefield, 1962). Bisno (1979)

demonstrated that strains deficient in M-protein were cleared through the alternative complement pathway. It was proposed that the antiphagocytic property of M protein results from its ability to bind fibrinogen (Horstmann *et al.*, 1992), thus inhibiting the deposition of complement on the GAS surface. Binding of factor H to M protein also acts to inhibit opsonisation and subsequent phagocytosis of GAS. Factor H is a molecule capable of regulating complement activity by inactivating C3 convertase, an enzyme required for deposition of C3b on the bacterial surface (Horstmann *et al.*, 1992; Perez-Caballero *et al.*, 2000). However, a recent study demonstrated that all strains of GAS tested, including a wild-type AP1 strain and a mutant strain deficient in M-protein, were efficiently phagocytised by neutrophils in non-immune human blood. While the mutant strain was rapidly killed intracellularly, the wild-type was able to survive inside the neutrophils. This led to the postulation that the M-proteins and/or M-like proteins contribute to GAS virulence by enabling the bacteria to evade host defences not only by conferring antiphagocytic properties, but through intracellular survival (Staali *et al.*, 2003).

M protein shares structural homology with cardiac myosin and other mammalian alpha-helical coiled coil molecules such as tropomyosin, laminin and keratin (Manjula *et al.*, 1985; Fischetti, 1991). The homology between M protein and tissue proteins suggests a structural basis for the induction of cross-reactive antibodies occurring in GAS autoimmune sequelae. However, previous studies showing the induction of myocarditis in the Lewis rat by both rat and human cardiac myosin which share a high degree of amino acid sequence similarity, suggests that sequence homology is more important for the induction of this autoimmune myocarditis than structural similarity (Cunningham, 2001). As discussed later, there is much evidence implicating the role of molecular mimicry in the development of autoimmunity and autoimmune diseases.

2.2.4 Diagnosis

RF is a clinical condition that has no single pathognomonic marker or definitive diagnostic test. Symptoms and signs of RF usually occur one to five weeks after onset of GAS infection (which may be apparent in only 60% of cases), and can vary depending on host factors, the organs involved and the severity of the disease.

General manifestations including fever, malaise and loss of appetite may be absent (Hilário and Terreri, 2002). Diagnosis is therefore based on a combination of clinical and laboratory findings. The Jones Criteria, proposed in 1944 by Dr T Duckett Jones, is a set of guidelines to aid in the diagnosis of initial (but not recurrent episodes of) RF (Guzman-Cottrill *et al.*, 2004). There have been several revisions of these criteria, including a requirement for evidence of a prior group A streptococcal infection. In 2004, the WHO published a guide to the diagnosis of RF which was formulated to account for limitations in the revised Jones Criteria. Diagnosis of RF is established on presentation of at least two major criteria, or one major and two minor criteria, in conjunction with evidence of prior GAS infection (Table 2.2).

2.2.4.1 Jones criteria: major manifestations

Polyarthritis is the least specific clinical manifestation of ARF and the most common, occurring in 60-80% of patients. The arthritis, often migratory and painful in nature, is usually transient and self-limited and involves the larger joints including knees, ankles, wrists and elbows.

Sydenham's chorea is a neurological disorder characterised by random, involuntary movement, in-coordination and facial grimacing. It may occur from one to six months after GAS infection in the absence of any other manifestation associated with ARF, and after puberty, is observed almost entirely in females (Stollerman, 2001). Rheumatic chorea has also been associated with post-infectious autoimmune neurological diseases (PANDAS) including tics, Tourette's syndrome and obsessive compulsive behaviour (Swedo, 1994; Swedo *et al.*, 2004).

Erythema marginatum (serpiginous macular lesions) is a characteristic rash which can occur at any time during the course of RF (Guzman-Cottrill *et al.*, 2004). Subcutaneous rheumatic nodules are firm, painless nodules which usually resolve within a month without long-term sequelae. Like erythema marginatum, this manifestation is usually rare and tends to occur more often in patients with RHD.

Table 2.2 2002-2003 WHO criteria for the diagnosis of rheumatic fever (WHO, 2004)

Diagnostic Categories	Criteria
Primary episode of RF [a]	Two major* or one major & two minor** manifestations plus evidence of a preceding group A streptococcal infection***
Recurrent attack of RF in a patient without established rheumatic heart disease [b]	Two major or one major and two minor manifestations plus evidence of a preceding group A streptococcal infection
Recurrent attack of RF in a patient with established rheumatic heart disease	Two minor manifestations plus evidence of a preceding group A streptococcal infection[c]
Rheumatic chorea. Insidious onset rheumatic carditis[b]	Other major manifestations or evidence of group A streptococcal infection not required
Chronic valve lesions of RHD (patients presenting for the first time with pure mitral stenosis or mixed mitral valve disease and/or aortic valve disease)[d]	Do not require any other criteria to be diagnosed as having rheumatic heart disease
* Major manifestations	- carditis - polyarthritis - chorea - erythema marginatum - subcutaneous nodules
** Minor manifestations	- *clinical*: fever, polyarthralgia - *laboratory*: elevated acute phase reactants (erythrocyte sedimentation rate or leukocyte count) - *electrocardiogram*: prolonged P-R interval
*** Supporting evidence of a preceding streptococcal infection within the last 45 days	elevated or rising antistreptolysin-O or other streptococcal antibody, or a positive throat culture, or rapid antigen test for group A streptococci, or recent scarlet fever

[a] Patients may present with polyarthritis (or with only polyarthralgia or monoarthritis) and with several (3 or more) other minor manifestations, together with evidence of recent group A streptococcal infection. Some of these cases may later turn out to be rheumatic fever.

[b] Infective endocarditis should be excluded.

[c] Some patients with recurrent attacks may not fulfil these criteria.

[d] Congenital heart disease should be excluded.

Rheumatic carditis is a pancarditis, affecting the pericardium, myocardium and endocardium. Occurring in approximately 40% - 60% of patients, it is the most serious clinical manifestation of RF and can cause permanent scarring of the mitral and/or aortic valves (rarely the tricuspid or pulmonary valves), presenting as valve stenosis or regurgitation. Recurrent episodes of RF compound the valvular damage produced by an initial episode and increases RHD severity, often leading to development of chronic RHD, cardiac failure or death.

Fraser *et al.,* (1997) described the pathological features of rheumatic carditis as Aschoff nodules in heart valves, comprising a central area of fibrinoid necrosis surrounded by Aschoff giant cells (specialised cardiac histiocytes) and Anitschkow (caterpillar) cells admixed with lymphocytes (Figure 2.4). Immunohistochemical studies of heart valve tissue from patients with active RF indicated that these valvular lesions develop in three stages. The earliest stage contained histiocytes with or without central fibrinoid necrosis, and macrophages secreting inflammatory cytokines interleukin 1 (IL-1) and tumour necrosis factor-α (TNF-α) but with no admixed lymphocytes. IL-2 secreting T-lymphocytes, predominately of CD4+ lineage, infiltrated the lesions during stage 2 followed by B-lymphocytes and occasionally plasma cells in stage 3 (Fraser *et al.*, 1997).

2.2.4.2 Jones criteria: minor manifestations

Minor manifestations include arthralgia, usually involving the large joints without obvious signs of arthritis. Fever is often present at the onset of ARF and generally resolves within several weeks. Elevated levels of acute phase reactants, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) at the onset of disease are characteristic of inflammation, and provide evidence of antecedent streptococcal infection. Prolongation of the P-R interval on electrocardiogram (ECG) is present in 30-35% of ARF patients (Homer and Shulman, 1991).

Up to one-third of RF patients have no overt symptoms of GAS infection. Therefore, laboratory evidence of a recent infection by culture, positive GAS antigen test from throat swab or serology is required for diagnosis of RF. Positive results for

culture or rapid streptococcal antigen tests are uncommon due to the three week latency period between infection and clinical signs of RF. Elevated anti-streptolysin-O (ASO) or anti-deoxyribonuclease-B (anti-DNAse B) titre provides strong serological evidence of prior GAS infection (Cunningham, 2000).

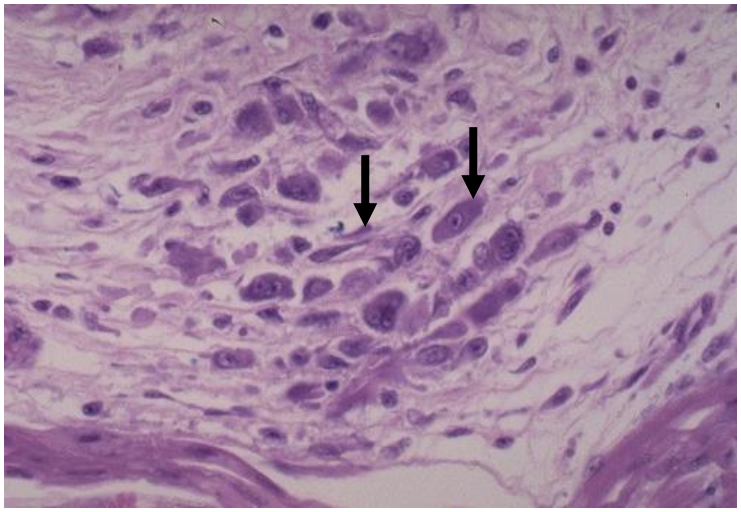


Figure 2.4 Aschoff giant cells and anitschkow cells in RF-affected valve tissue. Anitskchow cells (black arrows) have a round or ovoid nucleus with chromatin condensed at the nuclear periphery and along the centre, which results in the classic owl-eyed and caterpillar appearance. Aschoff giant cells are multinucleate cells formed by the fusion of Anitschkow histiocytes (taken from Fraser *et al.*, 1997).

2.2.5 Prevention and Treatment

Despite over sixty years of use, penicillin remains the treatment of choice for GAS infections. Early administration of penicillin to eradicate the bacteria from the nasopharynx has been shown to prevent an autoimmune response and therefore, development of RF. The primary prevention of RF has been defined as 'adequate antibiotic therapy of GAS upper respiratory tract infections to prevent acute rheumatic fever' (WHO, 2004). The recommended treatment is a single intramuscular (i.m.) injection of 1,200,000 IU of benzathine benzylpenicillin, or 250-500 mg phenoxymethyl penicillin (penicillin V) orally two to three times a day for 10 days.

Fortunately, GAS have not developed resistance to penicillin as have many other pathogens, including other streptococcal groups (Woodford, 2005). Treatment failure

rates of up to 30% have been reported, but are thought to be caused by the co-existence of β-lactamase producing flora residing in the pharynx. Also, GAS which have the ability to internalise into host cells are protected from the effects of penicillin and are able to evade host phagocytic and adaptive immune defence mechanisms and thus, the host immune response (Sela *et al.*, 2000).

It is crucial that patients who have had a previous episode of RF or have documented RHD receive secondary prophylactic treatment to prevent recurrences of GAS infection. Secondary treatment involves continuous administration of antibiotics to prevent colonization and infection by GAS. The recommended regime consists of i.m. injection of benzathine benzylpenicillin (1,200,000 IU) every three to four weeks. Alternatively, oral penicillin can be used for secondary prophylaxis but concerns over consistency of serum antibiotic levels and lack of long-term compliance makes this method less effective. Suppression of the inflammatory response in active RF can be achieved through the use of aspirin while patients with pericarditis respond well to corticosteroids. The use of steroids has been shown to be of benefit during active carditis, but is ineffective against valvular disease (WHO, 2004).

Vaccines against GAS infection would not only prevent infection but also prevent development of RF and RHD. Vaccines candidates which have been considered include GAS M protein based vaccines (see Section *2.7*) and those which target other GAS proteins including C5a-peptidase (Shet *et al.*, 2003) and SPE-B (Kapur *et al.*, 1994).

Other treatment strategies to prevent colonisation or infection by GAS currently under investigation include T-cell receptor peptide immunotherapy (Gold *et al.*, 1997) and bacteriophage lytic enzyme therapy (Nelson *et al.*, 2001). Murein hydrolase (lysin) from the streptococcal bacteriophage C(1) has demonstrated bactericidal properties against GAS *in vitro*. Furthermore, when Nelson *et al.* (2001) administered lysin to mice via the oral cavity, GAS were rapidly killed while other non-pathogenic oral flora were unaffected.

2.3 EPIDEMIOLOGY

Until the middle of the twentieth century, RF/RHD were significant causes of morbidity and mortality worldwide. RF was reported as the leading cause of death in school-age children, and the major cause of heart disease in those under 40 in the United States from 1925-1950 (Massell *et al.*, 1988). It was not uncommon for families to lose several children to this disease despite a substantial decrease in incidence rates which began around the turn of the century. U.S. mortality reports document a fall in incidence from 100 per 100 000 in the 1950s to less than 1 per 100,000 by the mid-1970s (Massell *et al.*, 1988; Kaplan, 1996). Evidence suggests that during this time, the incidence of GAS infection remained unchanged (Stollerman, 2001). The downward trend in RF was also observed in other developed countries, and is associated with improvements in living conditions, health care and changes in the characteristics of GAS strains (Kumar *et al.*, 1999). The availability of sulphonamides in the 1930s which prevented recurrences of RF (secondary attacks) and the introduction of penicillin in the 1940s to prevent initial (primary) attacks, contributed to the rapid decline of RF (Krause, 2002; Stollerman, 2002). By the early 1980s, RF had become almost a rarity in the industrialised western world (Veasy *et al.*, 2004).

In contrast, RF/RHD remain important medical and public health issues in developing nations and certain geographic regions within the developed world (WHO, 2005a). In these RF/RHD-endemic regions, RF is the primary cause of acquired childhood heart disease and chronic RHD continues to be a contributory factor in congestive heart failure (CHF) (Olivier, 2000). It was estimated in 1994 that 12 million cases of RF/RHD occurred worldwide. Of these, three million had CHF that required hospitalisation, a significant proportion requiring cardiac valve surgery within 5-10 years (WHO, 2004). Epidemiological data on the prevalence of RHD in developing countries is limited. However, by systematically searching the MEDLINE database for published articles of RF/RHD surveys, Steer *et al.* (2002) concluded that the highest prevalence of RHD occurred the Pacific region and within certain indigenous communities in developed countries. Disparities between population groups are demonstrated by the disproportionately high rates of RF/RHD between Maoris (6.5 per 1000) and non-Maoris (0.9 per 1000) in New Zealand and

between Aboriginals and non-Aboriginals in northern Australia (Steer *et al.*, 2002). A 2005 WHO report estimated that the median prevalence rates of RHD varies from 0.3 per 1000 in regions with established market economies to more than 7.6 per 1000 in Pacific and indigenous Australian and New Zealand populations, although prevalences may reach more than 20 per 1000 in particular age groups (WHO, 2005a).

In countries such as Cuba, Egypt and Sudan, where WHO strategies for preventing RF/RHD have been implemented, a reduction in both new cases and recurrences of RF has been observed. A ten-year programme (1982-1992) undertaken in the French Caribbean Islands of Martinique and Guadeloupe demonstrated the cost-effectiveness of prevention programmes, with a decrease in RF incidence (74%-78%) and severe carditis (86% - 97%), and a concomitant reduction in medical costs for RF (Bach *et al.*, 1996). Unfortunately, many poorer developing countries lack the medical and financial resources necessary to implement effective secondary prevention programs.

Numerous studies have confirmed rates of RF/RHD in indigenous communities living in Australia's tropical north are among the highest in the world (Neilson *et al.*, 1993; Carapetis *et al.*, 2000; Carapetis *et al.*, 2007). The annual incidence of RF in aboriginal children 5-14 years has been estimated to range from 250 to 350 per 100,000 (Carapetis and Currie, 1997). Similar figures have been reported for Central Australia (Brown *et al.*, 2003) and northern Queensland (Hanna and Heazlewood, 2005). As at the end of 2006, the crude prevalence rate of RHD in Aboriginal and Torres Strait Islanders in Australia's Northern Territory was reported to be 24.8 per 1,000 and 18.1 per 1,000 for the Central Australian Aboriginal population compared to less than 2 per 1,000 in non-indigenous Australians in the same region (AIHW: Penm E, 2008). Although indigenous Australians represent less than 2% of the population, they account for 14% of hospitalisations for RF/RHD and are 20 times more likely to die from RHD than non-indigenous Australians. Females are more likely to be hospitalised for RHD than males, while for RF there is little difference between males and females. Hospital use for RHD increases with age up to age 80, while hospitalisation for RF is more common among the younger age groups (AIHW, 2004).

Recent studies in these Aboriginal groups challenge the long-held view (Unny and Middlebrooks, 1983; Stollerman, 2001) that RF results solely from upper respiratory tract infection by 'rheumatogenic strains' of group A streptococci (Brown *et al.*, 2003). In these Aboriginal communities, throat carriage rates are very low and symptomatic pharyngitis is rare (Carapetis and Currie, 1998). However, streptococcal pyoderma secondary to scabies infestation is endemic (Gardiner and Sriprakash, 1996; Carapetis *et al.*, 1999; Currie and Carapetis, 2000). Among Aboriginal children, the prevalence of pyoderma may be as high as 70%, with multiple serotypes isolated from any one individual or indeed from within a single skin lesion (Carapetis *et al.*, 1995). Gardiner and Sriprakash (1996) detected up to 14 genetically different GAS strains in circulation within individual communities at any one time. In one study of rural Australian Aborigines, a large percentage of GAS types isolated in skin sores were identified by molecular typing as those usually associated with throat infection (Bessen *et al.*, 2000). The proposal that GAS strains originating in the skin are either directly or indirectly involved in the pathogenesis of RF is currently under investigation (McDonald *et al.*, 2004). It has also been hypothesised that group C or G beta-haemolytic streptococcal infections play a role in the development of ARF (McDonald, 2003). These reports have serious implications for control programs that target only pharyngeal GAS infections for the prevention of RF/RHD.

2.4 HOST IMMUNE RESPONSES TO GAS INFECTION

The normal host responses to streptococcal infection comprise innate, non-specific responses and acquired or adaptive responses mediated by antigen-specific T cells and B cells. Both systems act in concert to prevent initial colonisation or, for bacteria which have entered host tissue, to inhibit multiplication or spread of the bacteria through mechanisms including opsonisation, phagocytosis and ultimately, elimination.

2.4.1 Innate Immune Response

Host resistance against GAS infection is mediated primarily by innate, non-specific mechanisms that prevent bacteria adhering to and colonizing host epithelium. These include the protective skin barrier and mucociliary movement of the upper respiratory tract. Immunity to extracellular GAS is mediated by the innate mechanisms of complement activation, phagocytosis and the inflammatory response (Abbas and Lichtman, 2003). Pro-inflammatory and effector cytokines, secreted by activated phagocytes, play an important role in regulating the innate response and for initiating the adaptive immune response to infection (Medzhitov and Janeway, 1998).

Interleukin-1, IL-6 and TNF-α control the recruitment of leukocytes to infection sites and stimulate hepatocytes to produce acute-phase proteins, further enhancing opsonisation and activation of the classical pathway of complement (Kotb *et al.*, 1993; Murphy *et al.*, 2008). Mannose-binding lectin (MBL), an acute phase protein produced by the liver and a soluble pathogen recognition receptor, binds strongly to N-acetyl-glucosamine on the streptococcal surface. MBL plays a major role in the innate response, activating the complement cascade via the lectin pathway and facilitating pathogen opsonisation and enhanced phagocytosis (Jack *et al.*, 2001). High levels of MBL associated with particular genotypes have been reported in chronic RHD patients (Ramasawmy *et al.*, 2008).

2.4.2 Adaptive Immune Response

The humoral arm of the adaptive immune system provides a second line of defence against GAS through opsonisation of streptococci with antibody, leading to phagocytosis and subsequent killing of the bacteria by host polymorphonuclear leukocytes or neutrophils. Anti-streptococcal antibodies in serum activate the classical complement pathway, while bacterial surface molecules activate the alternate pathway (Cunningham, 2000). Antibodies to an abundance of streptococcal cellular and extracellular components including mucopeptide, *N*-acetyl-glucosamine, streptolysin-O, DNase B, NADase and streptokinase can be detected in patient serum following infection by GAS (Cunningham, 2000). B cell proliferation and antibody

production in response to GAS antigens may be either dependent (Malkiel *et al.*, 2000) or independent (Medina *et al.*, 2001) of help from T cells.

M type-specific immunoglobulin G (IgG) antibodies recognising the hypervariable amino-terminus of the M protein persist for up to 30 years following natural infection (Lancefield, 1959), and have been shown to provide opsonic protective immunity (Lancefield, 1962). Secretory IgA (isotype A immunoglobulins, sIgA) antibodies against epitopes of M-protein provide protective immunity by blocking adherence of bacteria to mucosa (Fluckiger *et al.*, 1998). Brandt *et al*. (1996) demonstrated that IgA antibodies in serum specific for the conserved region of peptide p145 derived from M-protein, could opsonise M5 GAS in the presence of complement and polymorphonuclear cells (PMN) *in vitro.* Others have reported the protective role of sIgA against intra-nasal challenge by GAS in animal models. However, while affording protective immunity by preventing colonisation, the opsonic properties of sIgA have yet to be demonstrated *in vivo* (Bronze *et al.*, 1992).

Besides M-protein, other GAS components reported to induce protective or neutralising antibodies include the streptococcal pyrogenic exotoxins A, B and C (Norrby-Teglund *et al.*, 1998), C5a peptidase (Ji *et al.*, 1997), cysteine protease (Kapur *et al.*, 1994) and fibronectin-binding proteins (Courtney *et al.*, 1996; Guzman *et al.*, 1999; Gillen *et al.*, 2002).

While protection against GAS M protein is predominantly through a robust antibody response, T-cell function is also critical. Helper (CD4+) T cells recognise antigens associated with major histocompatibility complex class II molecules (MHC II), and are generally involved in the induction and regulation of immune responses, and are further subdivided into two functional classes. Helper type 1 T cells (T_H1) activate macrophages to kill intravesicular pathogens whereas T_H2 cells activate B-cells to proliferate and differentiate into antibody secreting cells. Many organ-specific autoimmune diseases, including RHD, are thought to be the result of an excessive T_H1, inflammatory response (Guilherme *et al.*, 2001b). A second population of T lymphocytes is the CD8+ cytotoxic subset, responsible for direct killing of infected cells which harbour pathogens in the cytosol. These cytotoxic T cells (CTL) respond to antigenic peptide fragments associated with MHC class I molecules.

A purported third subpopulation of T lymphocytes is that of the regulatory/ suppressor T cells. However, this term is currently applied to CD8+ cells (Zimring and Kapp, 2004), as well as to a subset of CD4+ T cells. CD4+ regulatory T cells comprise around ten percent of the peripheral T cell population and, as discussed later (Section *2.5.1*), are identified by the co-expression of IL-2 receptor α-chain CD25 and Foxp3, (Shevach, 2001; Sakaguchi *et al.*, 2006).

Adaptive cellular immunity to many pathogens including streptococci relies in part on the random generation of T cell receptor (TCR) surface molecules providing a means to interact with a wide variety of foreign antigens. The TCR is composed of two disulphide-linked polypeptide chains, alpha (α) and beta (β), each with separate constant and variable domains. Like the B-cell immunoglobulin genes, TCR genes undergo rearrangements during T cell maturation resulting in expression of a diverse repertoire of T cells capable of interacting with a vast array of antigenic moieties. Variability is due to random combinations of multiple germline segments of V, (β chain) D and J coding sequences in addition to junctional diversity, the addition and removal of random nucleotides during recombination events (Abbas and Lichtman, 2003). The V-D-J junction encodes the hypervariable region of the TCR known as the complementarity-determining region (CDR-3). A helper T cell will become activated when the CDR-3 interacts directly with its cognate peptide/MHC class II complex located on the surface of antigen presenting cells (APC).

Early immunological studies led to the belief that the T cell repertoire was restricted, with an enormous but finite number of TCR capable of responding to antigenic peptides. This conclusion was based on observations that only a few regions of an intact protein resulted in the induction of an immune response. Furthermore, textbooks expounded the ‘one T cell – one epitope’ theory and that all self-reactive T cells were eliminated by central and peripheral tolerance. However, new studies have revealed that not only is the T cell repertoire more diverse than originally thought, but T cell recognition is degenerate, with a single T cell able to respond to related, but heterologous ligands (Maverakis *et al.*, 2001). These studies have

provided a greater understanding of the roles of antigen processing, T cell activation, and peptide cross-reactivity in autoimmunity and autoimmune diseases.

Sercarz and colleagues (1997) propose that the manner in which self-proteins are processed and presented to maturing T cells during thymic development determines which ones are recognised as autoreactive and subsequently deleted (dominant epitopes), or which potentially autoreactive T cells escape negative selection (subdominant or cryptic epitopes) and which, under certain conditions, can be later activated in the periphery (breaking of tolerance) (Gammon and Sercarz, 1989; Schneider and Sercarz, 1997; Sercarz and Maverakis, 2003). The process by which antigen presenting cells (APC) degrade native proteins into peptide fragments is subject to variation. Different lineages of APC (dendritic cell, macrophage or B cell) possess different antigen processing machinery, including route of uptake (phagocytosis, receptor-mediated endocytosis or macropinocytosis), proteolytic enzyme repertoire, MHC loading and sensitivity to environmental cytokines. Any of these parameters could determine which peptide fragments are loaded into the peptide groove of MHC molecules, and how avidly the antigenic-peptide/MHC II complex interacts with specific T cells in the thymus. Peptides that are less likely to be loaded into the peptide groove either through differential cleavage by proteolytic enzymes or competition by flanking determinants are considered to be subdominant or cryptic determinants (Maverakis *et al.*, 2001).

Using T-cell clones generated from mice immunised with purified, recombinant serotype 5 M protein (rM5), Robinson *et al.*(1991) identified at least 13 distinct T-cell recognition sites located in both the highly variable N-terminal and in the highly conserved C-terminal regions of the M5 protein. Further studies provided evidence that presentation of two of the immunodominant M5 protein peptides by MHC class II molecules was modified by i) different uptake pathways by macrophages, ii) proteolytic enzymes present on the surface of APC or iii) inflammatory cytokines (von Delwig *et al.*, 2002; von Delwig *et al.*, 2003a; von Delwig *et al.*, 2003b).

Contemporary thinking espouses the affinity model of TCR selection, whereby TCR affinity/avidity for MHC molecules or TCR affinity for self-peptides decides the fate of the T cell in the thymus (Chao *et al.*, 2005).

2.5 AUTOIMMUNE RESPONSES FOLLOWING GAS INFECTION

It is thought that the inflammatory nature of RF is due to a hyperimmune reaction caused by either an allergy to GAS or autoimmunity. Experimental evidence has been presented to support the hyperimmune theory. It includes the detection of immune complexes during the early phase of rheumatic activity in the sera of patients with RF, carditis or chorea, and the heightened cellular reactivity to streptococcal antigens of RF patients compared to non-rheumatic controls indicative of a delayed hypersensitivity reaction (as reviewed by Unny and Middlebrooks, 1983). However, a more popular view is that autoimmunity plays a central role in the pathogenesis of RF and RHD.

2.5.1 Autoimmunity and autoimmune disease

Autoimmunity has been described as an adaptive immune response directed at specific self-antigens following a breakdown in self-tolerance (Abbas and Lichtman, 2003). However, while the majority of self-reactive lymphocytes are deleted by negative selection during development, self-reactive B-cells (and autoantibodies) and self-reactive T-cells are frequently detected in the blood of healthy individuals. Indeed, physiological autoimmunity is now recognised as instrumental in maintaining homeostasis and is an important feature of normal physiology. Germ-line encoded autoreactive antibodies against many diverse self-antigens are present in every compartment of the circulation (Poletaev and Osipenko, 2003), interacting with other immune network components such as cytokines, receptors and B or T cells in response to inflammatory perturbations in ‘self’ (Maslloréns, 2000). Many instances of the protective or homeostatic effects of physiological autoimmunity have been described including a) the elimination of aged red blood cells (Lutz, 2007) b) immunomodulation of serum low density lipoprotein (LDL) cholesterol levels (Alving and Wassef, 1999) and tumour surveillance or clearance (Pardoll, 1999; Toubi and Shoenfeld, 2007).

Autoreactive T-cells have also been shown to function in a protective as well as a damaging manner. For example in a murine model, a single population of autoimmune effector T-cells has demonstrated a dual role in the central nervous

system (CNS), mediating damage in experimental autoimmune encephalomyelitis (EAE), while also providing protection from secondary degeneration following spinal cord injury (Yang *et al.*, 1977; Cohen and Schwartz, 1999; Moalem *et al.*, 1999).

Self-reactive T and B cells are generally unresponsive to self-antigens in the absence of a critical second, or co-stimulatory signal or to low levels of self-antigen (Rose, 1998). A critical step in the pathogenesis of autoimmune disease is the activation and clonal expansion of autoreactive lymphocytes (Wucherpfennig, 2001). The progression from benign, physiological autoimmunity to pathogenic autoimmune disease, often referred to as a 'break in tolerance', may be caused by a disruption to the finely-tuned regulatory immune network as a result of both host genetics and environmental factors. The role of infectious agents including viruses, bacteria and parasites in the development of several autoimmune diseases in humans has been long established. An association between the measles virus and encephalomyelitis (Griffen *et al.*, 1994), Coxsackie B3 virus and autoimmune myocarditis (Cunningham *et al.*, 1992; Rose, 1998), the *Trypanosoma cruzi* parasite and Chagas' disease (Kalil and Cunha-Neto, 1996) as well as *Streptococcus pyogenes* and RF/RHD has been documented. Several mechanisms by which infectious agents induce autoimmunity have been put forward (Table 2.3).

Table 2.3 Proposed mechanisms for activation of autoreactive lymphocytes by infectious agents (Wucherpfennig, 2001)

• Polyclonal activation of B or T lymphocytes
• Viral and bacterial superantigens activating autoreactive T cells that express certain Vβ segments
• Enhanced processing and presentation of autoantigens/ increased expression of MHC (class I or II) molecules
• Release of cytokines that result in activation of immune cells
• Alteration of lymphocyte/macrophage function
• Exposure of modified, cryptic or new antigenic determinants
• Molecular mimicry

The existence of several functionally distinct populations of regulatory T cells dedicated to maintaining the balance between immunity and tolerance is now firmly established. These regulatory T cells (Tregs) have been described according to their origin, expression of cell markers (CD4, CD25 and the specific marker forkhead/winged-helix transcription factor box P3, Foxp3, considered by many to be the master switch for Treg development) and by their mechanism of action. Naturally occurring regulatory T cells (nTregs) are generated in the thymus through MHC II-dependent TCR interactions resulting in high-avidity selection and appear to be involved in prevention of autoimmunity (Curotto de Lafaille and Lafaille, 2009). On the other hand, peripheral naïve $CD4^+$ T cells can be induced to express Foxp3 to form adaptive or antigen-induced Tregs (iTregs) under conditions of sub-immunogenic antigen presentation or chronic inflammation. Cytokines IL-2 and TGF-β are essential for induction of iTregs (Corthay, 2009) and these cells are reported to be involved in mucosal tolerance and control of chronic allergic inflammation (Josefowicz and Rudensky, 2009).

Shevach (2001) proposed that in the normal immune response, $CD4^+CD25^+$ Treg cells are recruited to sites of autoimmune damage where they recognise and are activated by organ-specific antigens. Activation of Tregs via their TCR, independent of co-stimulation, generates effector cells with the ability to non-specifically suppress the activation of other $CD4^+$ or $CD8^+$ cells. Impaired Treg cell function has been shown to contribute to autoimmunity leading to human autoimmune liver disease (Longhi *et al.*, 2004), experimental autoimmune encephalomyelitis (EAE) in rats and autoimmune gastritis in mice (Powrie *et al.*, 1993). Aberrations in regulatory T cell function have also been reported in RF/RHD patients (Bhatia *et al.*, 1989) and more recently, $CD4^+CD25^+$ Treg cell numbers were found to be significantly lower in patients with rheumatic mitral stenosis (Yildiz *et al.*, 2007).

Other populations of T cells integral to maintaining the balance between activation and inhibition of the immune response include $foxp3^-$ TR1 and Th3 and type 1 NKT cells (Jordan and Baxter, 2008; Wilczynski *et al.*, 2008) and 'suppressor' $CD8^+$ T cells (Smith and Kumar, 2008; Suzuki *et al.*, 2008). Ongoing studies to further characterise the diverse phenotypes and functions of the immunomodulatory T cell

repertoire will no doubt provide further insights into the significance of Tregs in the development of autoimmune diseases.

2.5.2 The role of molecular mimicry in autoimmunity

Molecular mimicry originally described the phenomena whereby parasites could evade immune recognition by mimicking the host it infected; some GAS strains disguise as 'self' by being encapsulated by hyaluronic acid, a common component of mammalian tissue (Froude *et al.*, 1989). Applied to autoimmune reactivity, molecular mimicry refers to the close resemblance between epitopes on the 'foreign' infectious agent and epitopes found in the host (self) (Oldstone, 1987). Evidence of molecular mimicry has been demonstrated both at the B-cell and T-cell level.

Several recent studies support the idea that induction of autoimmune diseases following infection by pathogens is caused by the activation and clonal expansion of autoreactive T cells in the periphery through molecular mimicry. It is thought that dominant determinants on the foreign pathogen are able to activate autoreactive T cells that are capable of cross-reacting with subdominant/cryptic determinants in host proteins. Several levels of molecular mimicry have been suggested. The first describes cross-reactivity based on sequence homology and was illustrated by early experiments in which EAE was induced in an animal model by a peptide from hepatitis B virus polymerase which has six consecutive amino acid residues in common with an encephalitogenic determinant of myelin basic protein (MBP) (Fujinami and Oldstone, 1985). A second level of molecular mimicry is that of minimal residue similarity (Gautam *et al.*, 1992) which states that the position of only a few amino acid residues within the 11-20 peptide fragment bound in the MHC groove is critical for TCR engagement and subsequent recognition.

The degeneracy of antigen recognition by T cells was demonstrated by Hemmer *et al.*, (Hemmer *et al.*, 2000) who use synthetic peptide combinatorial libraries to examine the response of myelin basic protein specific human leucocyte antigen (HLA) class II restricted $CD4^+$ T cell clones to truncated ligands. Many cross-reactive ligands were defined, some of which were more potent than the autoantigens originally used to select the T cell clones. Penta-, tetra- and tri-peptides were

recognised and even at low concentrations, could enhance T cell clone survival. T cell degeneracy may drive the autoimmune process in sites of local inflammation where high amounts of activated proteases and peptidases could result in increased degradation of self-proteins released from damaged tissue (Hemmer *et al.*, 2000).

2.5.3 Autoreactive B cells in RF/RHD

Immunological studies over more than half a century have established that high-titre antibodies against streptococcal antigens are present in the sera of RF/RHD patients. Antibody and complement deposits can be detected in cardiac tissues of rheumatic carditis patients, suggesting autoimmune deposition of antibody in or near Aschoff lesions (Carreno-Manjarrez *et al.*, 2000). Most heart-reactive antibody titres decline over time. After three to five years, these titres are often undetectable in patients following a single RF occurrence. Zabriskie and Freimer (1966) proposed an association between GAS and mammalian tissue in the 1960's when it was found that antibodies in rabbit antisera to GAS could cross-react with sarcolemma of cardiac and other striated muscle, as well as endocardium and vessel wall smooth muscle. Numerous other studies in the 1960's and 1970's reported autoantibody responses in RF/RHD patients (Cunningham, 2000). However, it was not until the advent of monoclonal antibodies (mAbs) against GAS and heart tissue and advances in immunofluorescent staining techniques in the 1980's that the heart auto-antigen was identified as myosin.

Using enzyme-linked immunosorbent assays (ELISA), Dale and Beachey (1985b) showed that purified pepsin-extracted M5 protein (pepM5) induced antibodies cross-reactive with cardiac myosin. The anti-pepM5 antibodies reacted with intact myosin but not with myosin light chains, suggesting that the cross-reactive epitope resided on the myosin heavy chain. In the same series of experiments, the myosin-cross-reactive antibodies were totally inhibited by pepM5 and partially inhibited by pepM6 and pepM19, but not by pepM24. The authors also postulated that the structural similarities between M protein and the rod region of the myosin heavy chain might be the reason for immunological cross-reactivity (Dale and Beachey, 1985a).

Cunningham *et al.*, (1988) compared levels of anti-myosin antibodies in sera of patients with uncomplicated GAS infections and RF/RHD patients, to levels in sera of normal, healthy individuals. An exaggerated response to myosin was demonstrated in ARF/RHD patients and fifty percent of those with uncomplicated GAS infection. Anti-myosin antibodies purified from patient sera on myosin affinity columns cross-reacted with GAS or M5 protein. The cross-reactive antibodies were also present in normal sera, although five times as much sera was needed for detection of reactivity. The authors concluded that lymphocyte clones with the ability to produce these antibodies are present in normal individuals and that prior infection with GAS resulted in elevated levels of these antibodies (Cunningham *et al.*, 1988).

Human anti-myosin anti-streptococcal mAbs 1.C8, 1.H9, 5.G3 and 3.B6 produced from peripheral blood lymphocytes of RHD patients and mAb 10.2.5 from tonsillar tissue of a normal patient were also shown to cross-react strongly with the group A carbohydrate *N*-acetyl-glucosamine (GlcNAc) (Adderson *et al.*, 1998). While all mAbs demonstrated cross-reactivity with the light meromyosin (LMM) region of human cardiac myosin, mAb 10.2.5 from a healthy control individual reacted to a distinctly different part of the cardiac myosin LMM. Anti-myosin anti-GlcNAc cross-reactivity was also demonstrated in later studies when, anti-myosin mAbs derived from BALB/c mice were shown to cross-react with GlcNAc (Malkiel *et al.*, 2000). These researchers suggested that myosin and GlcNAc have significant antigenic homology and that anti-myosin reactivity observed in RHD patients is the result of a switch from a T cell-independent anti-GlcNAc response to a T cell-dependent anti-myosin response.

In another study, antibodies against human cardiac tropomyosin, another α-helical coiled-coil protein resembling myosin or M protein, were more commonly found in RF sera than were anti-myosin antibodies (Khanna *et al.*, 1997). This was observed when sera taken from RF patients were tested against crude cardiac extracts, and then against purified heart extracts.

Despite evidence of autoantibodies in myocardial and valvular tissue of RF/RHD patients, no conclusive evidence of a direct role in heart damage has been demonstrated. Autoantibody involvement in heart cell cytotoxicity has been demonstrated only in a limited number of studies. Suggestions that these autoantibodies are in fact generated in response to already damaged tissue have therefore focused RF/RHD research in the direction of cell-mediated responses being the initiators of the disease process.

2.5.4 Autoreactive T cells in RF/RHD

While the role of the humoral immune response in the pathogenesis of RF/RHD has been researched in depth, providing evidence of autoantibodies cross-reactive with streptococcal antigens, there are far fewer published studies addressing the role of the cellular response. High titres of anti-heart antibodies are present in the circulation of acute RF patients but in RHD patients, there is often no evidence of these antibodies in the circulation or at the site of cardiac damage. Moreover, Yang *et al.*, (1977) noted that the absence of antibodies and complement deposits in Aschoff bodies and the granulomatous nature of the Aschoff nodule, a hallmark of rheumatic myocarditis, was indicative of a focal inflammatory reaction. These observations led researchers to focus on the cellular responses in RF/RHD. Current evidence suggests that the cell-mediated response plays a pivotal role in the development of these diseases.

Read *et al.* (1974) reported exaggerated cellular reactivity of peripheral blood lymphocytes to GAS cell membrane antigens in RF patients when compared to patients with uncomplicated streptococcal infections and normal controls. Later, Yang *et al.*, (1977) demonstrated that T lymphocytes from the spleens of adult guinea pigs sensitised to GAS protoplast membrane antigens were cytotoxic to cultured foetal guinea pig heart cells but not to cultured skeletal muscle, liver or skin cells. Immunofluorescence studies confirmed that antigenic determinants present on the membranes of cultured myofibres were cross-reactive with GAS cellular antigens. Interestingly, cytotoxicity was not observed by Yang and colleagues when cultured newborn rat myocardial cells and spleen or lymph node lymphocytes isolated from GAS sensitised adult rats were used during the same series of

experiments (Yang *et al.*, 1977). These early studies established a significant role for the cellular response in the pathogenesis of RF/RHD.

Cytotoxic immune cross-reactivity between heart tissue and GAS antigens was further demonstrated by Dale and Beachey (1987). In these experiments, peripheral blood lymphocytes from normal volunteers which were stimulated with purified peptic-extracted GAS M protein (pepM) from serotypes M5, 6 and 19, were shown to be cytotoxic against cultured human fibroblasts, heart and liver cells, but not against several other animal cell types. Anti-pepM5 antibodies raised in rabbits partially inhibited cytotoxic activity induced by pepM5 in heart cells but not other target cells, indicating that a subset of the lymphocytes were specific for myocardial cells (Dale and Beachey, 1987). Cytotoxic activity in human heart cells by peripheral blood $CD8^+$ T cells stimulated with GAS M protein were demonstrated by other researchers (Kotb *et al.*, 1989).

A prospective study undertaken by Morris *et al.* (1993) further defined the distribution of T-cell subsets in acute RF and RHD patients. In acute RF and acute RHD patients, absolute numbers of T-cells were normal, although an overall increase in the percentage of IL-$2R^+$ lymphocytes and a decrease in percentage of $CD3^+$ cells indicated that a large portion of these cells were of the $CD4^+$ helper type in an activated state. This activation was sustained for up to 48 weeks. These investigators also reported decreased $CD8^+$ T-cell activity in acute RF and acute RHD patients for up to 1 year. Patients with chronic RHD (CRHD), uncomplicated streptococcal pharyngitis or controls showed normal distributions of $CD3^+$ cells. These results were interpreted as evidence that a continued CD4+ helper cell-dependent response is present in active but not quiescent RHD patients. Whether the distribution of lymphocytes in peripheral blood mirrored that of heart infiltrating cells was not investigated in this study (Morris *et al.*, 1993).

In a study using valvular specimens from South African children with both acute and chronic RHD, Kemeny *et al.* (1989) reported that the cellular infiltrates were primarily comprised of T cells and macrophages. In acute valvulitis, 50% of infiltrating cells were HLA-DR positive macrophages and T cells were predominantly of the $CD4^+$ helper type, indicative of an active immunological

process akin to a delayed-type hypersensitivity response. Specimens from patients with chronic valvulitis on the other hand, were more heterogeneous. In five of 8 specimens from chronic valvulitis patients, the number of helper T-cells was up to 2.6 times that of the cytotoxic T cell subset. In the remaining three specimens, the helper T-cells and cytotoxic T cells were present in equal numbers. These findings differed to some extent from those of Raizada *et al*. (1983) who had studied valves removed from older RHD patients, 10–20 years after the initial episode of RF. Raizada also demonstrated the presence of T cell subsets in these valves but reported a more homogeneous result with 70-100% of cells being T-helper type.

To compare reactivity of peripheral blood T-cells (PBMC) and heart infiltrating T cells (HITC) to GAS antigens, Yoshinaga *et al.,* (1995) established T-cell lines from both peripheral blood mononuclear cells and from cells isolated from valvular lesions of seven patients who had documented evidence of RF/RHD. T-cell lines generated by GAS serotype M11 cell wall or cell membrane antigens, or by PHA stimulation were of the $CD4^+$ helper type as established by fluorescence-activated cell sorting (FACS) analysis. Both valvular and peripheral T-cell lines showed reactivity to cell wall and cell membrane antigens from rheumatogenic GAS strains but not to nephritogenic GAS strains. The strongest response was seen in T-cell lines challenged with cell membranes suggesting that reactivity was directed against GAS membrane antigens (Yoshinaga *et al.*, 1995).

T-cells clones derived from myocardium and valvular tissue obtained from four RHD patients were used by Guilherme *et al.* (1995) to investigate the role of HITC in the pathogenesis of RHD. Clones derived from non-rheumatic heart disease patients were used as negative controls. T-cell clones derived from the RHD patients were tested for proliferation against nine peptides derived from the rheumatogenic M5 type GAS strain, protein fractions derived from normal myocardium and normal aortic valves. None of the 42 non-rheumatic T-cell clones recognised the M5 peptides. However, eight RHD T-cell clones demonstrated cross- reactivity, recognising both M5 peptides and protein fractions. Cross-reactive recognition occurred against three peptides (p1-p25, p81-p103, and p163-p170) from the A and B repeat regions of the M5 protein molecule. Peptides from the C-repeat region were not tested in this study. Of interest, cross reactivity at the antibody level had

previously been shown for two of these regions. p81–p103 cross-reactivity with cardiac myosin has been reported in mice, rabbits and humans, and p163–p170 with sarcolemmel antigens in rabbits (Cunningham *et al.*, 1997).

Studies by El-Demellaway *et al*., (1997) confirmed that patients with RHD possess a distinct subset of autoreactive T-cells and that T-cell recognition was enhanced by prior exposure to GAS. PBMC from both RHD patients and normal controls recognised myocardial proteins fractionated by SDS-page into 20 specific bands. One particular fraction (protein band 8), with apparent molecular mass of 50-54 kDa, was recognised by T-cells from 40% of RHD patients, but not by T-cells from controls (El-Demellawy *et al.*, 1997).

To simulate the natural *in vivo* interaction between streptococci and PBMC, streptococci isolated from RF patients (serotypes M1, M6, M24) were cultured in corresponding rabbit polyclonal antiserum to produce opsonised bacteria. Priming of the PBMC with these opsonised streptococci increased the response to protein band 8 by T cells from RHD patients to over 85% but showed no effect on control T cells, with no recognition reported. These results further supported the link between exposure to GAS antigens and specific heart tissue protein cross-reactivity (El-Demellawy *et al.*, 1997).

In a novel approach to define cross-reactive epitopes of M protein, Cunningham *et al*., (1997) used a previously described single immunisation protocol (Gammon *et al.*, 1990) rather than one of hyper-immunisation. Cardiac myosin-immunised BALB/c mice were tested for T cell cross-reactivity against overlapping synthetic peptides that spanned the A, B and C regions of the M5 protein. This allowed researchers to identify the immunodominant regions of the M protein most likely to produce cross-reactive T cell responses following a single exposure to myosin. Interestingly, there was no correlation between peptides that produced strong proliferative responses and those that produced heart lesions. The authors concluded that epitopes of cardiac myosin that are associated with heart damage are cryptic epitopes that do not show dominance when M5 peptides are tested in proliferation assays. Cryptic or sub-dominant epitopes may ‘break tolerance’ under conditions of

hyper-immunisation or chronic exposure. Indeed, recurrent episodes of GAS infection frequently occur prior to development of RF.

2.5.4.1 T cell receptor restriction in GAS infection

It has been hypothesised that M protein acts as a superantigen, capable of non-specifically activating up to 20% of T cells and therefore precipitating the autoimmune state. Superantigens can stimulate T cells by bridging MHC class II molecules, independent of antigen, to regions of the TCR Vβ-chain. GAS possess other powerful superantigens and mitogenic factors including the streptococcal pyrogenic erythrogenic (SPE) toxins responsible for the manifestations of scarlet fever and streptococcal toxic shock syndrome (Cunningham, 2000). Studies in the early 1990's reported that pepM5 preferentially stimulated T cells expressing TCR Vβ chains 2, 4, and 8 (Tomai *et al.*, 1991; Tomai *et al.*, 1992). PepM proteins from other serotypes were found to have their own distinct characteristic set of Vβ specificity (Watanabe-Onishi *et al.*, 1994). These superantigenic properties were later shown to be caused not by pepM, but by contaminating trace amounts of other GAS pyrogenic exotoxins that could be removed by purification (Fleischer *et al.*, 1992; Degnan *et al.*, 1997). Nevertheless, GAS superantigens may play a significant role in the autoimmune process through up-regulation of inflammatory cytokines.

Stimulation of T cells with mitogens, superantigens or nominal antigens results in distinct patterns of TCR β-chain diversity (Plasilova *et al.*, 2003). Investigations into the *in vitro* response to SPE-A by T cells from acute RF and chronic RHD patients have revealed that $CD8^+$ cells undergo anergy in response to GAS superantigens (Bhatnagar *et al.*, 1999). Therefore, analysis of the hypervariable CDR3 region of the TCR β-chain is a useful tool for discriminating between polyclonal T cell expansion due to superantigens and antigen-driven oligoclonal expansion.

The relative frequencies of TCR Vβ families in cell lines derived from PBMC and HITC from six patients with severe RHD were examined by Guilherme *et al.*, (2000) using semi-quantitative PCR analysis. No changes in the TCR Vβ repertoire were detected in either HITC or PBMC cell lines. The T cell Vβ patterns were found to

be consistent with antigen-driven oligoclonal expansion of Vβ families and not a superantigen effect. Furthermore, different dominant T cell clones were found in valve and myocardial tissue, indicating HITC responded to different antigens in these two sites. Cross-reactivity of HITC and PBMC from the same patients to M5 N-terminal peptides was also demonstrated, confirming earlier results by the authors. The same researchers extended their studies by analysing the M5 peptide and heart-specific responses, cytokine profile and TCR Vβ usage of HITC and PBMC from patients with different clinical forms of severe RHD (Guilherme *et al.*, 2001a). HITC clones derived from myocardium and valves from patients produce significant levels of inflammatory cytokines IFN-γ and TNF-α, consistent with a T_H1 type response. TCR Vβ analysis on cell lines indicated that HITC from RF patients were more polyclonal, were present in higher numbers and less inflammatory in nature than were HITC from chronic RHD patients, which were oligoclonal, in lower frequency and had a strong inflammatory nature. Interestingly, PBMC from the RF patient showed no reactivity to M5 peptides or aortic-valve derived proteins, but mitral-valve derived HITC clones displayed 67% reactivity to both antigens (Guilherme *et al.*, 2001a). Other studies involving a much larger patient group (41 with severe RHD and 33 mild RHD) showed that the frequency of PBMC reactivity to heart proteins increased with the severity of RHD (Guilherme *et al.*, 2001c). Together, these results led the authors to propose that either i) T cells that are sensitised to streptococcal M-protein in the periphery during infection migrate to the heart where they cause heart tissue damage following cross-reactivity with heart proteins, or ii) heart-reactive T cells detected in the periphery are the result of a 'spill-over' from the heart.

A recent study by Fae *et al.*, (2004) investigated how degenerate T cell activity might lead to pathological autoimmunity in susceptible individuals. The T cell immune response pattern of a six-year-old severe RHD patient was determined by comparing patterns of antigen recognition between mitral valve and heart papillary muscle T cell lines. They found that four mitral valve clones and one papillary muscle-derived T cell clone representing the same Vβ13 BJ2S7 TCR region recognised different heart tissue or M5 peptide antigens, including the immunodominant M5 peptide (p81-p103) described in earlier work. The papillary

muscle derived T cell clones expressed an alpha chain with different AV AJ segments to those of the mitral valve clones. The degenerate antigen recognition by HITC demonstrated in these experiments led the researchers to speculate that epitope spreading could trigger an amplification of the autoimmune response in RHD (Fae *et al.*, 2004).

Figueroa *et al*., (2002) reported that TCR Vβ usage studies by Guilherme and colleagues may have been flawed because T cell clones generated by the limited dilution method were used, which could have introduced selection bias into results. This assumption was incorrect because Guilherme *et al.*, (2000) looked for oligoclonality within T cell lines only not clones. On the other hand, Figueroa *et al.*, (2002) investigated Vβ usage by analysing T cells directly from damaged tissue. CDR3 region size patterns indicated that a restricted T cell repertoire was present in the hearts of 11 out of 15 patients with longstanding quiescent RHD. In contrast, one control patient with myxomatous heart disease showed widespread expression of TCR Vβ genes, while TCR Vβ were not detected in endocarditic or congenital heart disease controls. Eight out of nine of the highly expressed Vβ regions have been previously associated with superantigens (Watanabe-Onishi *et al.*, 1994). However, since these highly expressed Vβ chains were not sequenced, the question as to whether the observed expansions results from a superantigen-mediated or antigen-specific response could not be resolved (Figueroa *et al.*, 2002). Further studies by the same group in which the Vβ repertoire on CD3, CD4 and CD8 peripheral blood cells from four RF patients, 10 RHD patients and nine healthy controls were analysed by four-colour flow cytometry, showed a skewed TCR Vβ repertoire. A selective depletion of Vβ2 in the CD8+ T cell subset of chronic RHD patients was demonstrated, consistent with an ongoing immune response in these patients (Carrion *et al.*, 2003).

2.6 HOST SUSCEPTIBILITY

While there is a high prevalence of GAS-associated pharyngitis worldwide, only a small proportion of individuals go on to develop RF. One of the most significant factors for developing RF appears to be recurring GAS infection as a consequence of

repeated exposure to GAS. Environmental factors such as crowded living conditions and low socioeconomic status, and pathogen attributes such as changes in virulence characteristics have both been shown to play a crucial role in the disease process. However, studies have indicated that, like many other autoimmune diseases, there is a possible genetic predisposition to RF and RHD in different populations and countries.

High familial incidence of RF noted in early epidemiological studies suggested that autosomal recessive heredity factors might determine susceptibility to disease (Kemeny *et al.*, 1994). As discussed previously, HLA-DR and -DQ are the major histocompatibility complex (MHC) class II molecules encoded by genes located on human chromosome six and different alleles can determine which peptides are presented to T cells. An acknowledged association between the major histocompatibility complex genes and expression of immunomodulatory cell surface antigens turned the attention of researchers to a possible link between the HLA antigens and RF/RHD (Ayoub *et al.*, 1986; Weidebach *et al.*, 1994; Hernandez-Pacheco *et al.*, 2003a; Simonini *et al.*, 2004; Haydardedeoglu *et al.*, 2006).

As reviewed by Guilherme and Kalil (2010), several HLA class II alleles have been found to be associated with RF/RHD. HLA-DR7, now defined as DRB1*07:01 (Marsh, 2010), appears to be the allele most frequently associated with RF/RHD in Brazilian, Turkish, Latvian and Egyptian populations (Guilherme *et al.*, 1991; Ozkan *et al.*, 1993; Guedez *et al.*, 1999; Stanevicha *et al.*, 2003) while HLA-DR4 has been associated with disease in white American (Anastasiou-Nana *et al.*, 1986; Ayoub *et al.*, 1986) and Saudi Arabian populations. A predisposition to more severe forms of heart disease has also been linked to a combination of HLA-DR7 and some DQ alleles (Stanevicha *et al.*, 2003). However, several studies in different ethnic populations have produced some conflicting results (Ayoub *et al.*, 1986; Maharaj *et al.*, 1987; Carlquist *et al.*, 1995; Koyanagi *et al.*, 1996) while some alleles have been implicated in disease protection (Ozkan *et al.*, 1993; Kudat *et al.*, 2006). An association between RF/RHD and polymorphisms in other genes located near the HLA loci on chromosome 6 have also been investigated recently. Several studies have found an association between RF/RHD and single nucleotide polymorphisms

(SNP) in TNF-α alleles (-308A and -238A) (Sallakci *et al.*, 2005; Ramasawmy *et al.*, 2007) or mutations in MBL (Ramasawmy *et al.*, 2008).

Khanna *et al*., (1989) prepared a non-HLA B-cell marker, mAb D8/17, by immunising mice with B cells from a (North American) RF patient. An increased proportion of B cells (10-30%) from RF/RHD patients of diverse ethnic origin expressed a cell surface antigen that reacted with D8/17. Less than 14% of non-rheumatic controls tested positive for D8/17. The use of D8/17 a disease-specific marker has been supported by subsequent studies in Israeli (Harel *et al.*, 2002) and Australian Aborigine populations (Harrington *et al.*, 2006). D8/17 familial distribution studies (Kemeny *et al.*, 1994) supported the hypothesis that this marker occurs in an autosomal recessive manner and is not linked to other MHC markers. However, mAbs of North American origin were found to have less discriminatory ability in some other populations, identifying only 60% and 70% of RF patients in India and Egypt respectively. On the other hand, mAbs produced from RF patients in a homologous population increased the detection of RF/RHD patients. The mAb PGI/MN SII derived from a North Indian patient not only detected a higher percentage of rheumatic patients in the same population, but also detected the marker in over 60% of siblings of RF/RHD patients, suggesting that this mAb could be used to screen for RF susceptibility (Kaur *et al.*, 1998). These results indicate a need to develop mAbs to B cell alloantigenic markers for different ethnic populations.

It is clear from these studies that genetic susceptibility to RF/RHD is not due to a single defined factor but may involve interaction between several genes in a complex immune network. The ability to identify RF-susceptible individuals at birth could, however, have major public health implications including early and effective prophylaxis for susceptible individuals in GAS endemic regions or during GAS outbreaks in other regions.

2.7 VACCINES TO PROTECT AGAINST RF/RHD

The history of prophylactic immunisation against infectious diseases can be dated back to Edward Jenner's successful vaccination against smallpox in 1796 although

there are earlier reports of the practice of smallpox vaccination in China, India and Turkey. The success of vaccination programs are highlighted by the fact that devastating infectious diseases including smallpox, polio, diphtheria and measles have been almost completely controlled in many developed countries.

However, despite over eight decades of research to produce a vaccine to protect humans from GAS infection, no safe and effective vaccine is currently available. Initial attempts to vaccinate against GAS using large amounts of crude streptococcal toxins or whole killed organisms did not appear to prevent primary or recurrent attacks of RF. Early work by Lancefield's team (Lancefield, 1957) and subsequent research over the next 50 years has established that the M protein has the ability to elicit highly protective opsonic antibodies. Therefore, most vaccine research has focused on this protein. However, preliminary M protein vaccine trials faced a number of challenges. Firstly, vaccines using impure M protein preparations displayed toxicity and many failed to elicit an acceptable level of primary response in human subjects at the doses tested (Fox *et al.*, 1973). Secondly, protective immunity based on the hypervariable amino-terminus of M protein is serotype specific and over 100 distinct serotypes are currently known. Thirdly, human trials of an M protein vaccine in 1969 resulted in controversy as several subjects developed RF. Although it was not proven that the RF was vaccine-induced, concerns were raised by some that the vaccine preparation itself might have triggered the development of RF/RHD (Massell *et al.*, 1969). This theory gained support when anti-M protein antibodies demonstrated cross-reactivity with heart tissue (Cunningham *et al.*, 1989). Finally, the dramatic decline in rates of severe GAS infections and RF/RHD during the early to mid-1900s in developed countries was paralleled by a similar decline in interest for developing an anti-GAS vaccine, despite unchanged disease rates in developing countries.

Interest in a vaccine against GAS was renewed following an apparent increase in invasive GAS diseases and in RF/RHD in regions of the United States and other countries during the 1980s. The observation that this resurgence occurred in middle-class areas not previously associated with environmental risk factors such as overcrowding or poor health care, suggested the appearance of highly virulent GAS strains (Kehoe, 1991). Whilst GAS is still extremely sensitive to penicillin,

treatment failure occurs in up to 30% of cases and there is no guarantee that the bacterium will never acquire penicillin resistance. Resistance to other antibiotics including tetracycline, chloramphenicol and erythromycin has already been reported in GAS (Horn *et al.*, 1998). The carriage of penicillin-resistant strains of GAS would present a major problem in health care not only in developing countries but also in developed countries in the future. An effective vaccine against GAS is therefore both desirable and necessary.

Serotype-specific antibodies directed against the N-terminus of the M protein mediate immunity to GAS. Epidemiological studies have established that rheumatogenic serotypes of GAS show global temporal and geographical distribution patterns. Thus, for any vaccine based on N-terminal determinants, multivalent constructs combined either genetically or chemically to protect against local serotypes are required. Dale and Chiang (1995) demonstrated that a hybrid protein, constructed by fusing a 15 amino-acid fragment from M5 protein N-terminus onto the entire B subunit of *Escherichia coli* labile toxin (LT-B), could successfully evoke serum opsonic antibodies in mice. These experiments established the basis for multivalent, type-specific vaccines consisting of synthetic M protein peptides incorporated into hybrid proteins or as individual peptides linked in tandem to unrelated carrier proteins. Tetravalent and octavalent vaccines were later tested in rabbits and shown to induce antibodies to each of the M proteins. However, while none of the antisera raised against the octavalent protein were cross-reactive with heart tissue, only six were opsonic (Dale *et al.*, 1996). This suggests that the size and orientation of each peptide in the hybrid may be very important (Good and Brandt, 1997). More recently, a 26-valent vaccine covering up to 90% of serotypes circulating in North America has been successful in eliciting antibodies, none of which exhibited cross-reactivity to human tissue (Hu *et al.*, 2002). This vaccine candidate and other multivalent vaccines are currently undergoing human clinical trials (Batzloff *et al.*, 2004; Kotloff *et al.*, 2004; McNeil *et al.*, 2005).

The conserved C-region has been studied extensively in an effort to design a vaccine applicable to the populations where GAS is endemic such as the indigenous communities in northern and central Australia. All possible epitopes from the C-repeat region were screened as possible vaccine candidates by preparing fifteen

synthetic peptides, each 20 amino acids in length and overlapping by 10 amino acids. One of these peptides, with the sequence LRRDLDASREAKKQVEKALE (designated p145) was recognised by over 90% of sera from adult Aborigines when tested by ELISA. Antibodies to this peptide were present in only 10% of sera from Caucasians living in regions where GAS infection was much lower (Pruksakorn, 1994). Furthermore, antibodies directed towards p145 was present in about 40% of Aboriginal children and increased with age, paralleling the acquisition of streptococcal immunity due to increased streptococcal exposure with age (Brandt *et al.*, 1996). Affinity purified human antibodies specific for p145 could opsonise M5 GAS *in vitro*. However, mice immunised with p145 developed specific T cells which were shown to react with keratin, and two human T cell lines that reacted with p145 also showed reactivity against human heart proteins (Pruksakorn *et al.*, 1994a). Small linear peptides 8-12 amino acids in length spanning the p145 region were then used to identify the minimal T cell epitope suspected of stimulating harmful T cells.

Minimal B cell epitopes could not be identified using linear peptides due to the loss of conformational structure recognised by B cells. It was found that the α-helical structure of the p145 could be re-formed by flanking the peptides from p145 with regions from the helical DNA-binding protein of yeast, GCN4, to form chimeric proteins (J1 – J8), (Relf *et al.*, 1996). Chimeric proteins J1, J2, J7 and J8 were identified as minimal antibody epitopes by endemic human sera. Murine p145 anti-sera recognised J7, J8 and J9 in an ELISA, but when mice were immunised with one of these three peptides, only J8 stimulated the production of antibodies that recognised the parent p145 peptide or could opsonise M5 GAS. T cells isolated from mice primed with J8 and J14, another chimeric peptide similar to J8, did not proliferate in the presence of p145, keratin, heart extract, myosin, tropomyosin or myosin homologs. Mice immunised with J8 conjugated to a diphtheria toxoid carrier protein (J8-DT) combined with adjuvants alum or SBAS2 demonstrated enhanced survival compared to control groups when challenged with M6 or 88/30 GAS serotypes. J8-DT antisera opsonised multiple GAS serotypes *in vitro*, thus indicating the suitability of J8 as a vaccine candidate (Batzloff *et al.*, 2003).

Recently a heteropolymer, a novel multivalent construct in which J14 and seven N-terminal peptides were assembled into polymers as side chains from an alkane backbone, was shown to induce a strong opsonic response to each of the M types represented by the construct. The heteropolymer delivered with complete Freund's adjuvant (CFA) also protected outbred mice against challenge with two other GAS strains (Brandt *et al.*, 2000a).

A novel self-adjuvanting vaccine delivery system using lipid polylysine core peptide (LCP) compounds has recently been investigated as a safe and effective method for delivering GAS vaccines in humans (Olive *et al.*, 2003). The LCP system incorporates lipoamine acids coupled to a polylysine core containing up to two different antigen targets, and is designed to incorporate antigen, carrier and adjuvant in a single molecule.

2.8 ANIMAL MODELS OF RF/RHD

Group A streptococci are strictly human pathogens, with no other known natural host or environmental reservoir. Other than ethical considerations, the main problems associated with studying human hearts or human heart tissue are those of heterogeneity, obtaining sufficient numbers at predictable times and minimising degradation of tissue, thus ensuring that specimens are suitably fresh for study. Therefore, an animal model in which the pathogenic mechanisms responsible for RF/RHD are accurately reproduced is crucial for furthering our understanding of the disease process and ultimately for designing vaccines against the disease (Hearse and Sutherland, 2000). Developments in molecular biology, genomics, transgenesis, and cloning techniques have enabled researchers to study animals and humans in greater depth. By identifying and studying homologous genes across species, investigators can translate experimental data from animals to humans. Thus, comparative and functional genomic and proteomic studies have been used to identify genes and protein functions responsible for numerous disease processes and disease phenotypes. Over 80% of animals used in biomedical research are comprised of rodent species such as mice, rats and hamsters. Rodents are ideal animal models because they are small, easy to handle, reproduce prolifically and are relatively

inexpensive to feed and maintain. There are several limitations in using rat models for heart studies including differences in contractile function, calcium removal processes and differences in the ratios of α- and β-myosin heavy-chain isoforms (Hasenfuss, 1998). Nevertheless, rat models of several human heart diseases including cardiac hypertrophy, heart failure and ischaemic heart disease have been developed (McGregor and Dunn, 2003).

A major obstacle in the field of RF/RHD research has been the lack of a suitable model. In general, animals are not easily infected by GAS and if an infection is initiated, it is usually not sustained for any length of time. Early experiments to produce animal models of RF were designed on the hypotheses that RF pathology was caused either by i) persistent sub-clinical infection by GAS or ii) direct injury to heart tissue by streptococcal toxins. Therefore, most studies involved the introduction of whole bacteria or crude streptococcal preparations into various animals including rabbits, mice, rats, guinea pigs or non-human primates. While numerous examples of myocardial necrosis, myocarditis and endocarditis following immunisation with GAS were reported, none of the lesions were considered representative of the hallmark of RF, the Aschoff nodule (Unny and Middlebrooks, 1983).

It was not until a shift in thinking from antibody-mediated autoimmunity as a cause of RHD to T cell mediated autoimmunity, that the search for a suitable animal model made any progress. Huber and Cunningham (1996) produced myocarditis in $MRL^{+/+}$ mice immunised with N-terminal peptides from M5 GAS. This animal model was one of the first to indicate that mimicry of a cryptic epitope by a pathogen-associated antigen could break tolerance and trigger autoimmunity in a susceptible host. Studies in BALB/c mice further support the immunological cross-reactivity paradigm. However, the most appropriate animal model for RF and RHD is that of the Lewis rat (Quinn *et al.*, 2001; Galvin *et al.*, 2002), which exhibit myocardial lesions similar to those seen in RF following immunisation with GAS M protein. More importantly, this animal model is the first in which valvular disease has been demonstrated. M protein specific T cells lines generated from M protein immunised Lewis rats has been shown to passively transfer valvulitis into naïve rats, and both

human and rat cardiac myosin (Galvin *et al.*, 2002) produced severe myocarditis or valvulitis in rats, further validating the Lewis rat as a suitable animal model for studying human RHD.

2.9 FUTURE DIRECTIONS

RF/RHD occurs following untreated GAS infections. Primary and secondary prevention programs have been proven to be safe and effective methods of preventing RF/RHD both in developing and developed countries. However, these programs are not effective in some regions, placing a substantial burden on patients, their families and on the economies of these countries. The ineffective control of RF/RHD is associated with poor socioeconomic status resulting in poor nutrition, overcrowding and limited access to effective healthcare. In particular, for those living in remote areas or who cannot afford the costs of antibiotics, the long-term repeated treatments required for secondary prevention of RF are often impossible.

A safe, effective vaccine to prevent infection by group A streptococci would be an ideal method for preventing RF/RHD. As discussed earlier, there is currently no vaccine approved for widespread use in humans, although several promising candidates are undergoing clinical trials. Researchers must be sure that any vaccine released must not only stimulate the production of protective antibodies against the bacteria, but must not elicit host cross-reactive T cell or antibody responses. In order to achieve this, a greater understanding of the mechanisms involved in the development of these diseases is required. Current research strategies are focused on the T cell responses involved in RF/RHD, and while much has been achieved in recent years, the role of cross-reactive T cells and regulatory T cells in the pathogenesis of RF/RHD has not yet been fully elucidated. The development of the Lewis rat as a suitable model for RHD will make it possible to study the immune mechanisms involved in damage to myocardial and valvular tissue.

CHAPTER 3
GENERAL MATERIALS AND METHODS

In this chapter, the general materials and methods used throughout this project are described. Materials or methods which are specific to a particular chapter, or which have been modified significantly from those outlined below, have been included in the relevant chapter. Recipes for buffers and solutions are included in Appendix I. Details for suppliers and distributors are included in Appendix 2.

3.1 MATERIALS

3.1.1 Chemicals and consumables

Unless otherwise stated, all chemicals were purchased from *Sigma, Castle Hill, Australia* and were of the highest grade available. Bacteriological media was purchased from *Oxoid*. Plasticware including microfuge tubes, petri dishes and tubes were from *Sarstedt.*

3.1.1.1 Protein reagents

PD10 desalting/buffer exchange columns, Superflow columns and Ni-NTA resin were purchased from *QIAGEN*. BCA Protein Assay kit was purchased from *Pierce Biotechnology.*

3.1.1.2 Cloning reagents

DNA grade agarose, 0.2 ml thin-walled Eppendorf tubes and *Fermentas* restriction endonucleases *BamHI* and *SalI* were purchased from *Progen.* 1 Kb Plus DNA Ladder^{TM} was purchased from *Life Technologies.* RedHot^{R} DNA polymerase, RedHot^{R} DNA polymerase 10X reaction buffer and $MgCl_2$ were from *Integrated Sciences.* dNTP mixture consisting of dATP, dTTP, dGTP and dCTP was from *Bioline.* Sephadex^{R} G-50 fine DNA grade and DYEnamic^{ET} DNA sequencing kit were from *Amersham BioSciences.* T4 DNA ligase and 10X buffer were from *Promega.* Ultrapure molecular biology grade water and molecular biology grade

ethanol were from *Sigma*. DNA marker 1 Kb Plus DNA Ladder™ was from *Life Technologies*.

3.1.1.3 Histology and immunohistology reagents and monoclonal antibodies

Monoclonal antibodies raised in mouse against rat T helper cells (anti-CD4, clone W3/25), T cytotoxic cells (anti-CD8, clone OX8) and macrophage/neutrophil integrin molecule (anti-CD11b/c, clone OX42) were from *Cedarlane Laboratories.* Pan T cell marker (anti-CD3, clone G4.18) was from *eBiosciences* and anti-rat macrophage (anti-CD68, clone ED1) was from *Chemicon.* Biotinylated secondary antibody (horse anti-mouse IgG), whole horse blocking serum and HRP-ABC VectaStain visualisation kit were all from *Vector Laboratories.* Streptavidin alkaline phosphatase (SAAP) was purchased from *Jackson ImmunoResearch*. Fast Red staining kit was from *BioGenex.* Chromagen 3, 3' diaminobenzidine tetrahydrachloride (DAB) was from *Sigma-Aldrich*.

3.1.1.4 Cell culture reagents

RPMI-1640 medium, HEPES buffer and penicillin/streptomycin antibiotics were from *Gibco-BRL* and purchased from *Life Technologies*. Outbred rat serum was purchased from *Animal Resource Centre* and autologous rat serum was sourced from Lewis rats at the Small Animal Facility at James Cook University. Gibco® Foetal bovine serum (FBS) was purchased from *Invitrogen*. Sera were heat-inactivated by incubating for 30 min in a water bath set at 56 °C. Ficoll-Paque® Plus density gradient solution was purchased from *GE Healthcare.*

3.1.2 Animal Care

3.1.2.1 Rat strains

Lewis rats (LEW/ SsN; Albino: a,h,c: RT^1) were purchased from the Animal Resources Centre (Canning Vale, Western Australia) and bred by sibling mating in the Small Animal Breeding Facility at James Cook University (Townsville, Australia). As higher incidence and prevalence rates RF/RHD and more severe forms of RHD are reported in females (AIHW, 2004), only female rats were used in this study. Rats were maintained in standard plastic containers with wire lids and wood

shavings and/or shredded paper for bedding in 12 hr light/dark cycles. Pelleted protein-rich commercial diet and tap water were provided *ad libitum.* Animals under experimentation were observed daily including weekends and a log was maintained to monitor their well-being. Experimental procedures were conducted in accordance to institutional guidelines under James Cook University Animal Ethic Committee (Approval number A958 and A1326) and in accordance with the National Health and Medical Research Council's (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.1.2.2 Animal anaesthesia

Immediately prior to immunisation, rats were weighed to determine the optimal dose of anaesthetic for each animal. Animals were injected intraperitoneally (i.p.) with Ketamine (*Parnell Laboratories Pty Ltd*) a dissociative anaesthetic, at 10 mg per 250 g body weight and Xylazine (*Troy Laboratories Pty Ltd*), a muscle relaxant and analgesic, at 0.2 mg per 250 g body weight. At this dose, the animals were fully sedated within 5 to 10 mins, and fully recovered within 45 to 60 mins. Anaesthetic was administered using 1 ml syringes (*Terumo, USA*) fitted with 27 gauge needles (*Terumo, USA*).

3.1.2.3 Animal euthanasia

Rats were killed humanely in accordance with Animal Care and Ethics Committee guidelines at the end of experimentation for the purposes of tissue collection and analysis. No animal required euthanasia due to ill health, suffering or abnormal behaviour, however, two rats did not recover from anaesthesia during this project. Rats were killed by CO_2 asphyxiation in a lethal chamber, followed by cervical dislocation to ensure death. If blood samples (or maximum blood collection) were required, cardiac puncture was performed following CO_2 asphyxiation.

3.1.3 Bacterial Strains

3.1.3.1 Streptococcus pyogenes type M5

The *Streptococcus pyogenes* (GAS) M type 5 strain (T 5/B/PS) used in this and previous RF studies (Pruksakorn *et al.*, 1992; Martin *et al.*, 1994; Schmidt *et al.*,

1995) and originally from the Public Health Laboratory Service (PHLS) in London, was provided by Queensland Health Pathology Services at The Townsville Hospital, Townsville, Australia. Frozen stock (-80 ^{o}C in glycerol) was plated out on Horse Blood agar (*bioMérieux*) using a sterile loop and incubated overnight at 37 ^{o}C. GAS were passaged through human blood to increase M protein yield. In aseptic conditions, a single colony of M5 GAS was picked from HBA and suspended in 1 ml of sterile saline (0.9%) before inoculating into 10 ml of heparinised human blood. After 40 h incubation at 37 ^{o}C in an orbital shaker (115 rpm), 0.5 ml of culture was removed and subcultured on HBA overnight at 37 ^{o}C. A single mucoid colony was used to inoculate 10 ml of Todd Hewitt broth supplemented with 0.2% yeast (THYB) and cultured for 5 h at 37 ^{o}C. The GAS were pelleted by centrifugation at 5000 rpm for 10 mins, washed in sterile PBS and centrifuged again prior to re-suspension in 2 ml of THYB. An aliquot (0.5 ml) of cell suspension was added to 0.5 ml (30%) glycerol in sterile cryovials, frozen rapidly in liquid nitrogen and stored at –80 ^{o}C for future use.

3.1.3.2 Bordetella pertussis

Bordetella pertussis cells, commonly used to promote a Th1 response and facilitate the development of autoimmunity in animal models (Silver *et al.*, 1999), were used as a co-adjuvant when immunising rats. *B. pertussis* strain T1F1 was obtained from reference stocks (frozen on beads) at the School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia. Bacteria were grown by rolling a bead over charcoal agar plates containing 40 μg/ml cephalexin (Appendix 1) and incubating in a 37 ^{o}C humidified atmosphere for 4-5 days.

To prepare stocks of whole-killed *B. pertussis*, colonies were scraped off the agar surface using a sterile loop and suspended in 5 ml of sterile PBS. The bacterial suspension was spread over a further 50 charcoal agar plates using a sterile glass rod. After 4-5 days incubation, these colonies were scraped off the agar and suspended in 10-15 ml sterile PBS. Serial dilutions (10^{-1} to 10^{-20}) were plated out and incubated as above for colony counts. Immediately after preparing these plates, the bacteria in the remaining stock suspension were killed by adding formalin at 1% (v/v) and incubating at 4 ^{o}C. After 24 h, a sample was plated out as above to ensure sterility.

To calculate the concentration of bacteria (counted as colony-forming units, cfu) in the original stock, the number of colonies growing on plates which contained between 30 and 300 colonies were counted after 5-7 days. The concentration was calculated as follows:

cfu / ml = no. colonies counted x dilution factor x volume plated / 1 ml.

Prior to use, the bacterial suspension was centrifuged at 7000 x *g* for 10 mins and the supernatant containing formalin was removed. The cell pellet was washed in PBS and resuspended in an appropriate volume of sterile PBS to achieve 1 x 10^{11} cfu/ml.

3.1.3.3 Escherichia coli

The *E. coli* host strain BL21[pREP4] (*Qiagen*) was a gift from Dr Michael Batzloff at the Queensland Institute of Medical Research (QIMR, Brisbane, Australia). BL21 is commonly used with various vector systems for the production of recombinant proteins. Regulation of recombinant protein expression is controlled by the low copy plasmid pREP4 (*Qiagen*). *E. coli* DH5-α cells were from in-house stock stored at -70 °C at the School of Veterinary and Biomedical Sciences, James Cook University. All *E. coli* strains were grown in Luria-Bertani (LB) broth at 30-37 °C supplemented with, where appropriate, ampicillin alone (100 μg/ml) or ampicillin and kanamycin (25 μg/ml), and stored in 15% (v/v) glycerol at -70 °C.

3.1.4 Plasmids

3.1.4.1 pQE-30

Expression vector *pQE-30 (Qiagen)* (Figure 3.1) was maintained in *E. coli* DH5-α cells at -70 ^{o}C until required. In this plasmid, expression of an inserted gene is under the control of the T5 promoter which is recognised by the *E. coli* polymerase. Two *lac* operator sequences increase *lac* repressor binding and thus ensure more efficient transcription regulation. Other elements of the vector include a 6x*His*-tag sequence on the 5' end of the cloning site to facilitate purification of expressed protein, a ColE1 origin of replication and the β-lactamase (*bla)* gene which confers resistance to ampicillin.

3.1.4.2 pREP4

pREP4 (*QIAGEN*) is a 3.74 kb plasmid used to regulate the extreme transcription rate of expression vector pQE-30 by constitutively expressing the *lac* repressor protein encoded by the *lac 1* gene.

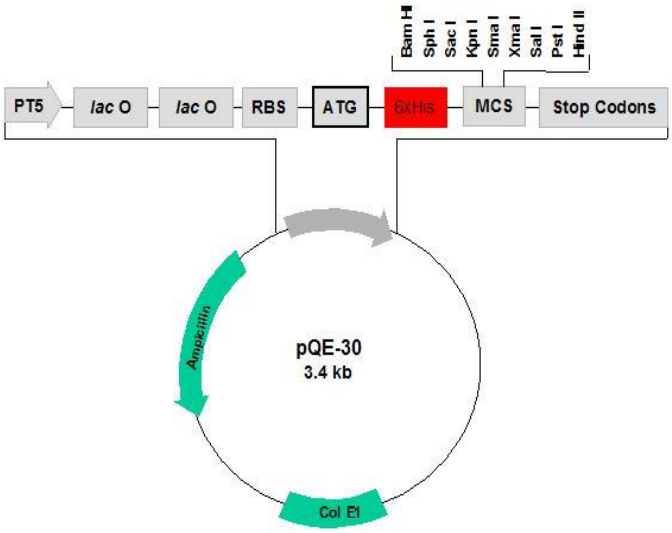


Figure 3.1. Schematic representation of expression vector pQE-30.
PT5: T5 promoter, *lac* O: *lac* operator, RBS: ribosome binding site, ATG start codon, 6xHis: *his*-tag sequence, MCS: multiple cloning site, Ampicillin: Ampicillin resistance gene, Col E1: Col E1 origin of replication

3.2 METHODS

3.2.1 Molecular Biology

The method used to extract nucleic acid was determined by the concentration, purity and quantity of DNA required.

3.2.1.1 DNA preparation by crude alkali lysis

A single colony of bacteria was suspended in 30 µl of 50 mM NaOH and mixed thoroughly. The suspension was incubated in a 95 ^{o}C water bath for 5 mins and then cooled to 4 ^{o}C, before neutralising the mixture by addition of 5 µl of 1 M Tris-Cl, pH 8.0. The suspension was centrifuged at 16,000 x *g* for 1 min to pellet cell debris and the supernatant containing the DNA removed for immediate use.

3.2.1.2 High quality plasmid DNA

High quality plasmid DNA was prepared using a *Sigma* GenElute Plasmid DNA kit according to the manufacturer's protocol. Plasmid DNA was extracted using a *Macherey-Nagel* Nucleospin® Plasmid Quickpure kit (*Integrated Sciences*). The concentration and purity of the eluted plasmid DNA was determined by spectrophotometric analysis (Section *3.2.1.6*). If required, DNA was concentrated using a *Qiagen* MinElute kit as per manufacturer's instructions.

3.2.1.3 High quality genomic DNA

Genomic DNA was isolated from M5 GAS using the *Sigma* GenElute^{TM} Bacterial Genomic DNA kit. The concentration and purity of the eluted genomic DNA was determined by spectrophotometric analysis.

3.2.1.4 DNA restriction

Approximately 1 µg of DNA was digested with 10 units of restriction enzyme in buffer and temperature conditions according to the manufacturer's recommendations for the relevant restriction enzyme. At the end of the digestion period, the enzymes

were either heat-inactivated or inactivated using a *QIAGEN* MinElute Reaction Cleanup kit, eluting in 10-20 µl of water.

3.2.1.5 Separation of nucleic acid fragments by agarose gel electrophoresis

Agarose gel (0.8% - 2.0% w/v) was prepared by melting an appropriate amount of DNA grade agarose in 1x TAE buffer (Appendix 1.). The solution was cooled to 55 ^{o}C before adding ethidium bromide to a concentration of 0.5 µg/ml and poured into an electrophoresis casting tank to set. 5-15 µl of DNA sample was mixed with 6x loading buffer (Appendix 1) and loaded into wells. To allow estimation of DNA fragment size, 1 µl of 1 Kb Plus DNA Ladder™ was included on each gel. Electrophoresis was carried out according to the type of casting tank used. For Hoefer HE 33 tanks (*Hoefer Inc.*), products were electrophoresed for 80 mins at 90V or until the dye front had progressed ¾ of the way down the tank. Liberty tanks (*Biokeystone*) were operated in accordance with the manufacturer's instructions. Products were visualised on a transilluminator under UV light. Digital images were captured by GelDoc 1000 imaging system (*BioRad*), and analysed using GeneSnap V4.00.00 (*Syngene*) software.

3.2.1.6 Determination of nucleic acid concentration

The concentration of DNA and RNA was estimated by measuring the absorption at 260 and 280 nm on a Biophotometer 6131 spectrophotometer (*Eppendorf*). An optical density (OD) of 1.0 260 nm corresponds to 50 µg/ml of DNA and 40 µg/ml of RNA. Purity of samples was determined by 260/280 ratio, with pure DNA samples between 1.8 and 1.95 and pure RNA samples between 1.9 and 2.0.

3.2.2 Polymerase chain reaction

3.2.2.1 Primers

In some experiments, oligonucleotide primer sequences were obtained from previously published studies as indicated. Where required, Oligo6 for Windows software (*Molecular Biology Insights*) was used to design suitable primers based on a number of criteria including specificity for the target DNA, melting temperature (Tm) of each forward and reverse primer pair no more than 5 °C apart, 18-25 bp in

length and GC content of no more than 50% (Eeles and Stamps, 1993). Primers were manufactured by *Sigma Genosys* in lyophilised form and were reconstituted to a stock concentration of 100 µM in Ultrapure molecular biology grade water or molecular grade TE buffer, pH 8.0 (*Sigma*). Stocks were stored at -20 °C until use.

3.2.2.2 Polymerase chain reaction DNA amplification

Polymerase chain reaction (PCR) was used to amplify DNA sequences of interest. A typical 25 µl reaction consisted of:

2.5 µl PCR buffer (10X)
2.5 µM $MgCl_2$
0.2 µM each dNTP
0.1 µM forward (5') primer
0.1 µM reverse (3') primer
0.75 units DNA polymerase
molecular grade DNase-free H_2O to 25 µl.

Immediately prior to thermal cycling, 1- 2 µl of DNA template containing 20-100 ng of DNA was added to reaction tubes.

PCR was carried out in a Mastercycler or Mastercycler Gradient thermocycler (*Eppendorf*) using the following standard conditions unless otherwise stated:

Initial denaturation	94 °C	9 mins
Followed by 30 cycles of:		
Denaturation	94 °C	1 min
Annealing	55-65 °C	45 sec
Elongation	72 °C	1 min 30 sec
Final elongation	72 °C	10 min
Cooled and held	15 °C	

On completion, products were stored at 4 °C until required, then electrophoresed on agarose gel (Section 3.2.1.5) to determine efficiency of amplification.

3.2.3 DNA sequencing

3.2.3.1 Sequencing reactions and thermocycling

Forward and reverse strands of DNA were sequenced using an *Amersham* DYEnamic™ ET dye terminator sequencing kit. Reactions were carried out in triplicate in 20 µl volumes as per manufacturer's instructions. A typical reaction consisted of:

DYEnamic Pre-mix	8 µl
Primer (5 mM)	1 µl
Plasmid DNA	100-400 ng
molecular biology grade H_2O	to 20 µl.

PCR cycling consisted of 35 cycles of the following conditions:

Denaturation	95 ^oC	20 secs
Annealing	50 ^oC	15 secs
Elongation	60 ^oC	1 min 20 secs
Cooling and hold	10 ^oC.	

3.2.3.2 Purification of sequencing PCR product

Prior to sequencing, PCR products were run through Sephadex^R G50 columns to remove unincorporated dye-labelled terminators and primers. Briefly, 1 g Sephadex G50 was mixed with 15 ml deionised water (ddH_2O) and allowed to re-hydrate for at least 3 h. Sephadex suspension (600 µl) was then loaded into *Qiagen* spin columns. The columns were placed into microfuge tubes and centrifuged at 2000 x *g* for 1 min to remove excess water. Columns were transferred to clean microfuge tubes and loaded with individual PCR samples. Purified PCR product was obtained by centrifugation at 2000 x *g* for 1 min and was stored at 4 ^oC short-term prior to DNA sequencing.

3.2.3.3 DNA sequencing

Sequencing was carried out on a MegaBACE™ 1000 DNA Analysis System (*Amersham Biosciences*) by the staff at the Genetic Analysis Facility at James Cook

University, Townsville, Australia. DNA sequence data were analysed using Sequencher^{TM} 4.0 for Windows software.

3.2.4 Molecular cloning techniques

3.2.4.1 Electrocompetent E. coli cells

An aliquot (1 ml) from overnight cultures of *E. coli* was used to inoculate 200 ml of LB medium with antibiotics as required. The culture was grown at 37 °C with vigorous shaking until the OD_{600} reached 0.5-1.0, and then cooled rapidly by swirling the flask in ice/water slurry. The cells were pelleted by centrifugation (4000 x *g*, 15 mins, 4 °C) and the supernatant decanted. A low ionic strength cell suspension was obtained by extensive washing as follows; the cells were resuspended in 200 ml of cold ddH_2O and centrifuged as above, resuspended in 150 ml cold ddH_2O and centrifuged as above, resuspended in 20 ml cold (10%) glycerol and centrifuged as above and finally, resuspended in 600 μl cold (10%) glycerol. Aliquots (50 μl) were frozen immediately in liquid nitrogen and stored at -80 °C.

3.2.4.2 Transformation by electroporation

Electrocompetent bacterial cells (50 μl) were thawed on ice and 2 μl of ligation reaction was added and stirred gently. The mixture was transferred to a pre-cooled (0 °C) 0.2 ml electrocuvette and pulsed at 2.5 kV in an Electroporator 2510 (*Eppendorf*). The pulsed cells were immediately added to warm (37 °C) SOC medium (Appendix 1), mixed gently, then incubated for 1 h at 37 °C with shaking (150 rpm). Aliquots (10, 20, 50, 100 & 200 μl) were plated onto pre-warmed LB agar with 100 μg/ml ampicillin and 25 μg/ml kanamycin and incubated overnight at 37 °C to allow colonies to grow.

3.2.5 Protein Methods

3.2.5.1 Estimation of protein concentration

Protein concentration in samples was measured using a BCA Protein Assay kit (*Pierce Biotechnology*). Bovine serum albumin (BSA) standards and protein samples

were prepared according to instructions. Working reagent was prepared by mixing 50 volumes Part A (containing bicinchoninic acid) and one volume Part B (4% cupric sulphate). A 25 µl aliquot of standard or sample, and 200 µl of working reagent were pipetted into microtitre wells in triplicate and mixed thoroughly. The microtitre plate was incubated at 37 °C for 30 mins, cooled to room temperature and absorbance was measured at 540 nm. A BSA standard curve was prepared and protein concentration of the samples was determined from the standard curve.

3.2.5.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean III system (*BioRad*). Buffer recipes are included in Appendix 1. To prepare samples for electrophoresis under reducing conditions, samples were incubated with SDS-PAGE buffer with β-mercaptoethanol to reduce disulphide bonds as previously described (Laemmli, 1970) for 5 mins at 100 ^{o}C, then loaded onto 0.75 mm 8% or 12% (w/v) polyacrylamide gels. Samples were electrophoresed with running buffer through the stacking gel at 150 V and 200 V through the resolving gel until the dye front reached the end of the gel. Gels were stained with Coomassie Blue stain for 3 h or overnight and destained with destaining solution. The molecular weight of proteins was estimated by comparing bands with *Fermentas* Protein Molecular Weight Marker SM0431. In some experiments, protein was resolved on 1.0 mm 12% LongLife™ Tris-HEPES precast gels (*Gradipore*) with Tris-HEPES-SDS buffer (*Gradipore*) as per manufacturer's instructions.

3.2.6 Immunological methods

3.2.6.1 Preparation of antigens for immunisation

A volume of 200 µl was required to inject 500 µg of antigen per animal. The stock antigens were dissolved in sterile PBS, pH 7.4, to 5 mg/ml and were combined thoroughly with appropriate adjuvant. For Complete Freund's Adjuvant (FCA) and Incomplete Freund's Adjuvant (FIA) (*Sigma*), the antigen was slowly introduced into an equal volume of adjuvant and the mixture was forced through a 24G

connector fixed between two glass mixing syringes until a stable, uniform water-in-oil emulsion was formed.

For experiments using Emulsigen® adjuvant *(MVP Laboratories, Omaha, USA),* antigen was diluted to 3.33 mg/ml and mixed with 25% (v/v) Emulsigen® by vigorous vortexing.

For experiments using Montanide ISA 50V *(SEPPIC, France),* antigen was mixed with an equal volume of Montanide by vigorous vortexing according to manufacturer's instructions.

3.2.6.2 Immunisation regime

The general immunisation schedule used in this study is outlined in Table 3.1. Injections were administered using a 1 ml syringe (*Terumo*) fitted with a 25 or 27 gauge needle. Initial (Day 0) and booster (Day 7) immunisations were administered subcutaneously (s.c.) and were given under general anaesthetic (as per Section *3.1.2.1*). Intraperitoneal (i.p.) injections, including the additional *B. pertussis* adjuvant injections (Days 1 and 3) were given without anaesthetic.

In early experiments, the primary immunisation injection was given in the foot (i.e. subplantar). Later, as discussed in Chapter 5, the hock was found to be a more appropriate injection site. For subplantar immunisation, the entire rear foot was swabbed with iodine, and the emulsion slowly injected s.c. into the bottom of the foot, followed by application of Emla® local anaesthetic cream (lignocaine 25 mg/g, prilocaine 25 mg/g) to the injection site. For hock immunisation, the ankle was first sprayed with 70% ethanol and the emulsion delivered s.c. bevel upwards into the lateral side of the foot directly above the heel and into the space between the fibularis longus and soleus muscles. The rats were monitored in heated cages until fully recovered, then transferred to cages containing shredded paper towel. Booster immunisations were administered s.c. in the flank following application of iodine to the injection site. Control groups were immunised with sterile PBS, pH 7.4, mixed with adjuvant.

Table 3.1 Standard immunisation schedule

Day	Treatment	Injection volume	Dose	Administration route
0	Rats weighed and anaesthetised	various	(10 mg ketamine + 0.2 mg xylazine) per 250 g body wt	intraperitoneal
	Initial immunisation	200 µl	500 µg antigen in adjuvant	subcutaneous (rear foot or hock)
1	Additional adjuvant	100 µl	$1x10^{10}$ whole-killed *B. pertussis* cells	intraperitoneal
3	Additional adjuvant	100 µl	$1x10^{10}$ whole-killed *B. pertussis* cells	intraperitoneal
7	Rats weighed and anaesthetised	various	(10 mg ketamine + 0.2 mg xylazine) per 250 g body wt	intraperitoneal
14	Booster immunisation	200 µl	500 µg antigen in adjuvant	subcutaneous (flank)

3.2.6.3 Isolation of mononuclear cells

Rat spleens and lymph nodes (popliteal and inguinal) were removed aseptically and cells extracted by pushing the organs through a fine mesh metal screen into RPMI medium supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. After debris was allowed to settle mononuclear cells (MNC) were isolated by the density gradient method. The cell suspension was layered onto Ficoll-Paque and centrifuged at 500 x g for 20 mins. The buffy coat at the interface of the Ficoll-Paque and medium was collected and the cells washed three times in fresh medium. Viability was assessed using trypan blue staining and cell concentration adjusted as required.

3.2.6.4 Trypan blue viability assay

Cell preparations were diluted 1:1 in trypan blue solution (0.4% in PBS, pH 7.4) and inspected microscopically in an improved Neubauer haemocytometer. As trypan blue only crosses the cell membrane of dead or damaged cells, total cell numbers as well as the proportion of unstained, viable cells can be assessed simultaneously.

3.2.6.5 Lymphocyte proliferation assay

The proliferative response of lymphocytes was measured in a tritiated [^{3}H] thymidine incorporation assay. Initial experiments were carried out to determine optimal concentration for each stimulating antigen. Typically, MNC extracted from blood or lymphoid tissue (Section *3.2.6.3*) were plated in triplicate wells in U96-well culture plates (*Nunc*) in the presence of 6 to 10 μg of antigen and incubated in a humidified atmosphere for up to 5 days at 3 °C in 5% CO_2. Cells stimulated with 1 μg/well concanavalin A (Con A) were used as positive controls and triplicate wells of unstimulated cells served as negative controls. Autologous culture medium consisted of RPMI medium (*Invitrogen*) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 10 mM HEPES buffer and 2.5% heat-inactivated autologous rat serum in a total volume of 200 μl per well. At 24 h intervals between days 3 and 5 of culture (72 h and 120 h), cells were pulsed with 1.25 μCi/ml [6-^{3}H]-thymidine (*GE Healthcare*) for 4 h prior to harvesting onto glass fibre mats using a Micromate cell harvester (*PerkinElmer*). When dry, scintillation fluid was applied to the mats and counts per minute (cpm) were measured in a Wallac MicroBeta® Trilux scintillation counter. Proliferative response of cells was reported as stimulation index (SI), calculated as a ratio between mean cpm in stimulated cells and cpm in unstimulated cells (cpm of test wells / cpm of control wells). Maximum stimulation indices of three or greater were considered significant.

3.2.6.6 Enzyme linked immunosorbent assays (ELISA)

The antibody responses of rats from each group were evaluated for the presence of immunoglobulin isotype G (IgG) using an indirect ELISA. Antigen was diluted in bicarbonate coating buffer, pH 9.6 and the solution was used to coat Maxisorp F96 plates at 100 μl/well. The plates were incubated at 4 °C overnight, washed five times

with wash buffer (Phosphate-buffered saline [PBS] at pH 7.2, 0.05% Tween 20), and blocked with 200 µl Post-coating Buffer (*TropBio*) for 1 h at 37 °C. The plates were then inverted and blotted on paper to remove residual blocking solution, and either used directly or stored at 4 °C in a sealed plastic bag for future use. Rat sera samples were added to duplicate or triplicate wells at 100 µl/well and the plates were incubated for 1 h at 37 °C. A positive control serum sample, a normal serum sample from unimmunised rats and a diluent only sample were tested in the same manner. The plates were washed five times with wash buffer and horseradish peroxidase (HRP) conjugated goat anti-rat, or for human sera samples (Chapter 6), goat anti-human antibody (*Jackson ImmunoResearch*) was added at 100 µl/well at a 1:5000 dilution. After 1 h incubation at 37 °C, the plates were washed five times and 100 µl of ABTS substrate solution (*TropBio*) was added to each well. After 20 min incubation at room temperature, the absorbance was read at an appropriate wavelength. Titres were calculated as the lowest dilution that gave an absorbance of more than three standard deviations above the mean absorbance of control wells (containing normal rat serum).

3.2.7 Histological techniques

3.2.7.1 Preparation of tissue for histology

As soon as possible after death, organs were aseptically removed and stored in cold transport medium (Appendix 1) until processed. Prior to fixation, the organs were dissected into smaller pieces and trimmed.

For paraffin-embedded sections, tissue was processed routinely by overnight fixation in 10% neutral buffered formalin (NBF), dehydrated through graded alcohol and embedding in paraffin. Sections were cut at 5-7 µm on a microtome, placed on silinised or charged glass slides and dried for 60 min or overnight at 60 °C.

Alternatively, tissue was frozen after fixing in 4% paraformaldehyde for four hours, washing three times in PBS and soaking in 20% sucrose solution for 24-48 h prior to embedding in Tissue-Tek OCT mounting media (*ProSciTech*) and freezing rapidly in liquid nitrogen. Frozen tissue blocks were stored at -70 °C until required. Sections

(5-10 μm) were cut on a cryostat and placed on Superfrost®Plus slides (Menzel-Glaser). After air-drying overnight, the sections were fixed in acetone at 4 °C for 10 min, air-dried and stored at -70 °C, wrapped in aluminium foil.

3.2.7.2 Histology

Using readily available standard protocols, paraffin-embedded sections were stained with haematoxylin and eosin (H&E) or Gomori's Trichrome stain for morphological examination by light microscopy.

3.2.7.3 Immunohistochemistry

All procedures were carried out at room temperature unless otherwise stated. Phosphate buffered saline, PBS (Appendix 1), or Tris buffered saline, TBS, (Appendix 1) pH 7.2 were used for rinsing throughout the staining process.

Formalin-fixed paraffin-embedded (FFPE) sections (4-6 μm) were deparaffinised in xylene and rehydrated with graded ethanol through to water. For antibodies requiring microwave antigen retrieval, sections were treated with citric acid at pH 6.0 for 3 x 5 min then rinsed in PBS.

While several other groups have reported successful staining of $CD4^+$ cells in FFPE rat tissue (Whiteland *et al.*, 1995; Ward *et al.*, 2006), all attempts in this study were unsuccessful despite trialling a number of enzymatic and heat-induced antigen retrieval protocols. Therefore, frozen sections were used when staining for $CD4^+$ cells. Hearts were fixed in 4% paraformaldehyde for 4 h, rinsed in PBS then soaked 24-48 h in 30% sucrose prior to embedding in Tissue-Tek OCT medium and freezing in liquid nitrogen. Cryostat sections (5-7 μm) were mounted on Superfrost®Plus slides (Menzel-Glaser), air-dried overnight and acetone-fixed at 4 °C for 10 min, air-dried and stored at -70 °C. Prior to use, frozen sections were warmed to room temperature and rinsed to remove OCT. When frozen sections were not possible, $CD4^+$ T cells were identified in FFPE sections by staining for $CD3^+$ T cells and for $CD8^+$ T cells in consecutive sections, with the assumption that those T cells which did not stain for $CD8^+$ were of the $CD4^+$ type.

Endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 mins. Sections were rinsed (3 x 5 mins) in PBS and incubated with 1.5% normal horse serum in PBS for 30 mins. Excess serum was removed and sections were incubated overnight at 4 °C with primary antibody against rat CD3, CD4 or CD8 and CD68. After rinsing, biotinylated secondary antibody at 1:50 or 1:100 dilution was applied for 30 min. Following a further 3 x 5 min rinse, sections were incubated for 30 mins with VectaStain ABC reagent and rinsed again. Chromagen solution DAB or nickel-enhanced DAB (*Sigma*) was applied to sections and allowed to develop for 5-10 mins before rinsing in tap water. Slides were counterstained in Mayer's haematoxylin, dehydrated in a series of graded ethanols, cleared in xylene and coverslipped using DPX mounting medium. Positive staining of labelled cells was demonstrated by brown precipitate in tissues. For Fast Red substrate staining, streptavidin alkaline phosphatase (SAAP, *Jackson ImmunoResearch*) at 2 μg/ml was applied in place of ABC reagent, then Fast Red chromagen (*BioGenex*) containing 0.02% Levamisole (*Vector Laboratories*) before counterstaining in haematoxylin and mounting with aqueous Crystal Mount.

Sections were examined using an Olympus BH2 light microscope and scored blindly for evidence of pathology according to Table 3.2. For each animal, a valvular or myocardial score ≥ 2 was classed as positive.

Table 3.2 Scoring system used for histology sections

Tissue	Score	Description
Valves	0	No staining
	1	< 5 infiltrating cells
	2	> 5 cells but no foci present
	3	1- 2 focal lesions with necrosis
	4	> 2 lesions
Myocardium	0	Isolated cells throughout tissue
	1	1-2 small foci
	2	> 2 small foci
	3	≥1 large focal lesion with necrosis
	4	Aschoff-type lesion

3.2.8 Statistical Analyses

Statistical analysis of data was carried out using SPSS 12.0.1 (SPSS Australasia Pty Ltd) or GraphPad Prism 5.00 (GraphPad Software Inc.) software. Data that was normally distributed was analysed using either the least significant difference one-way analysis of variance (ANOVA) or Student's unpaired, two-tailed *t*-test with a p-value of ≤ 0.05 considered significant. If required, data was transformed to obtain normal distribution before analysis.

CHAPTER 4
THE RAT AUTOIMMUNE VALVULITIS MODEL

4.1 INTRODUCTION

Humans are the only natural known hosts of GAS. Consequently, RF/RHD resulting from GAS infection is considered an exclusively human condition. Basic research using human patients is not always practical or ethical and much of our knowledge about the immunopathogenesis of human diseases has been acquired through the use of animal models.

Animal models are important biomedical research tools; their use is recorded as far back as the 2nd century AD when the Greek physician Galen used pigs and dogs as models for human anatomical research. Later, the 19th century French physiologist Claude Bernard introduced the 'induced animal model' where the condition to be investigated is induced experimentally in healthy animals. Induced disease models have been used extensively to further our knowledge of human physiology and in the development of antibiotics and vaccines against infectious diseases such as influenza and polio, as well as drug treatments for a range of disorders, from multiple sclerosis to tuberculosis (Koike, 1993; Gerdts *et al.*, 2007; Basaraba, 2008; Mix *et al.*, 2008).

For many years, attempts to establish a suitable animal model for RF/RHD were unsuccessful, with none of the proposed models displaying the same pathological changes as those seen in human patients (Kishna and Iyer, 1999). More recently, a rat autoimmune valvulitis (RAV) model was developed by Cunningham and associates whereby Lewis rats immunised with recombinant streptococcal M protein developed hallmark RHD lesions in both the myocardium and heart valves (Quinn *et al.*, 2001). Lesions were identified in H&E stained sections by the presence of mononuclear cells and/or neutrophils infiltrates in heart tissue. However, the infiltrating cells were not phenotyped to determine whether the cell populations were comparable to those found in lesions of RHD patients (Kemeny *et al.*, 1989). In a very recent study, both myocarditis and valvulitis were induced in Lewis rats immunised with formalin-killed GAS, demonstrated by H&E staining of heart tissue

(Huang *et al.*, 2009). Antibody responses were reported but T cell responses were not characterised in that study.

RAV studies also supported a role for molecular mimicry involvement in RF/RHD. Peripheral T cells from M protein-immunised rats proliferated in response to cardiac myosin (Quinn *et al.*, 2001) and T cells from heart lesions of cardiac myosin-immunised rats also responded to peptides from the B region of M protein (Galvin *et al.*, 2002). This model has also been used in our laboratory to induce valvulitis by immunising with C-terminal M protein peptides (Lymbury *et al.*, 2003).

4.1.1 Aims

The work described in this chapter was conducted to validate the RAV model for studying RF/RHD in the first instance, and more specifically, to determine the role of GAS M protein specific T cells in the development of autoimmune valvulitis.

The aims of the work described in this chapter were to:-

1) Induce valvulitis in Lewis rats by immunising with M protein from serotype M5 GAS
2) Examine heart valves and myocardium from immunised rats for lesions consistent with rheumatic carditis
3) Determine the specificity and reactivity of lymphocytes from immunised rats to streptococcal protein and their cross-reactivity to other tissue proteins such as myosin, tropomyosin or collagen.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Lewis/SsN A rats used in immunisation experiments were obtained from the Small Animal Breeding Facility at James Cook University and are described in Section *3.1.2*.

4.2.2 Bacterial strains and plasmids

Preparation and storage of *Streptococcus pyogenes* and *Bordetella pertussis* are described in Sections *3.1.3.1* and *3.1.3.2*. The *Escherichia coli* (*E. coli*) host strain BL21 [pREP4] used to express rM5 is described in Section *3.1.3.3*.

4.2.3 Antigens

Porcine cardiac myosin, collagen IV from human placenta and tropomyosin were purchased from *Sigma, Australia*. The methods used to prepare rat cardiac myosin, pepM5 and GAS serotype 5 recombinant M protein (rM5), used in the experiments described in this chapter and throughout the project, are described below.

4.2.3.1 Rat cardiac myosin

Rat cardiac myosin was extracted from the hearts of normal healthy rats using the method described by Tobacman and Adelstein (1984) and modified by Quinn *et al.*, (2001). All procedures were carried out at 4 °C or on ice to reduce enzymatic degradation of protein during the extraction process. Finely chopped heart tissue was homogenised using a Biosonik III sonicator (*Bronwill*) for 15 sec on ice in a low-salt buffer (40 mM KCl; 20 mM imidazole, pH 7.0; 5 mM EGTA; 5 mM dithiothreitol (DTT); 0.5 mM phenylmethanesulfonyl fluoride (PMSF); 1 µg/ml leupeptin, pH 8.0). Myofibrils were collected by centrifugation at 15,000 x *g* for 15 min at 4 °C. The pellet was resuspended in high-salt buffer (0.3 M KCl; 0.15 M K_2HPO_4; 1 mM EGTA; 5 mM DTT; 0.5 mM PMSF; 1 µg/ml leupeptin, pH 6.5) and homogenised on ice for three 30 sec bursts with 30 sec cooling between bursts, followed by a further incubation with gentle stirring at 4 °C for 30 min. After centrifuging as above, the supernatant was transferred to a large beaker and ten volumes of ice-cold distilled water was added, followed by pH adjustment to 6.5 with HCl. DTT was added to 5 mM and the precipitation allowed to continue for 1 h on ice. The actin-myosin was pelleted by centrifugation, 16,000 x *g* for 15 min at 4 °C, the supernatant was removed and the pellet solubilised in high salt buffer to which KCl was added to 0.5 M. To dissociate the actin filaments from the myosin, ATP and $MgCl_2$ were added to 10 mM and 5 mM respectively and the actin was then precipitated with 33% ammonium sulphate. The solution was centrifuged at

20,000 x *g* for 30 min at 4 °C. After determining protein concentration by BCA assay (Section *3.2.5.1*), the supernatant was stored at 4 °C in the presence of protease inhibitors leupeptin and TLCK (N-α-*p*-tosyl-L-lysine chloromethyl ketone).

4.2.3.2 Recombinant M5 (rM5) protein construction

The techniques used to prepare the expression construct have been described (Sambrook *et al.*, 1989) and were based on the general cloning strategy shown in Figure 4.1. rM5 was prepared as follows: the M5 coding region (*smp5*, Genbank accession number M20374) was amplified by PCR using genomic DNA extracted from M serotype 5 GAS as template. Primers were designed using criteria described in Section *3.2.2.1* and are shown in Table 4.1.

Table 4.1 Primer sequences used for the amplification of GAS M5[a]

Primer	Length (bp)	Sequence (5' – 3')	Target[b]
M5-5-169	28	gcgcGGATCCGCCGTGACTAGGGGTACA	169-186
M5-3-1330	31	gcgcGTCGACTTGACCTTTACCTGGAACAGC	1350-1330

[a]Primers contain the restriction sites *BamHI* and *SalI* (underlined) present in the multiple cloning site (MCS) of the expression vector pQE-30 to facilitate cloning. Additional (gcgc) bases were included at the 5' end of the primers ensure optimal binding of restriction enzymes during digestion. [b]Genbank accession number M20374 (Miller *et al.*, 1988).

These primers were designed to amplify a 1162 bp DNA fragment (pos 169-1330 of the published M5 sequence) which is immediately upstream of the N-terminal leader sequence and part way through the Gly/Pro-rich, cell wall spanning region. The final expected PCR product was 1182 bp in length. The published DNA sequence encoding the M5 protein and showing the primer binding sites is included in Appendix 3 of this thesis. Following preliminary PCR optimisation using standard methods (Eeles and Stamps, 1993), PCR amplification of M5 was performed as

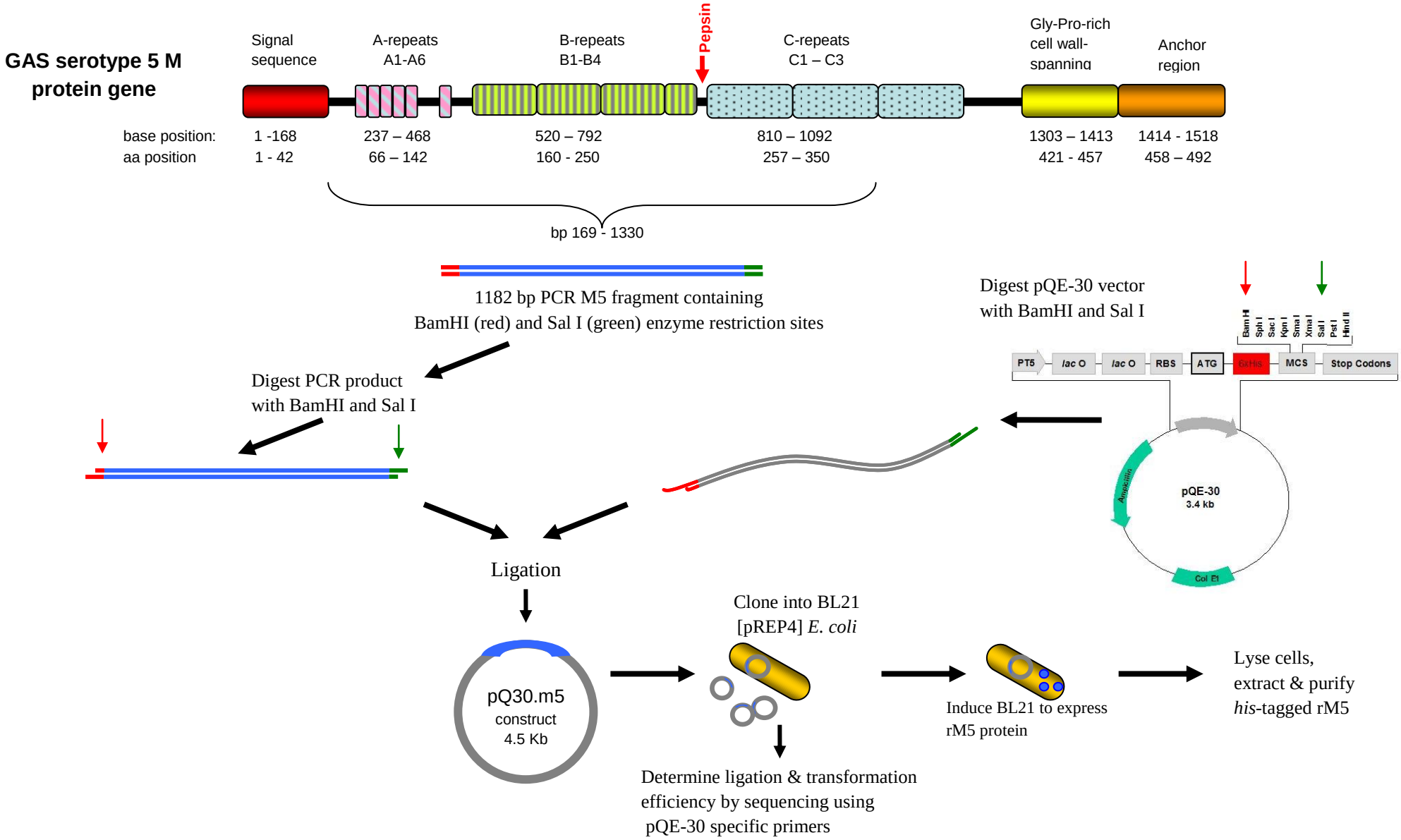


Figure 4.1 Strategy for preparation of recombinant M5 protein

described in Sections *3.2.2.2* and *3.2.2.3* using an optimal annealing temperature of 64 °C. PCR product was purified using a *Qiagen* MinElute kit according to instructions, visualised on agarose gel (Section *3.2.1.5*), and DNA content was determined spectrophotometrically.

Expression vector pQE-30 was extracted from DH5α cells (Section *3.2.1.2*). M5 insert and pQE-30 vector were digested with *BamHI* and *SalI* restriction enzymes (Section *3.2.1.4*) to create compatible 'sticky ends' and allow directional cloning. Insert and vector were ligated at a molar ratio of 5:1 using 1-3 Weiss units of T4 DNA ligase and supplied buffer (*Promega*) in a 10 μl reaction for 2.5 h at room temperature. The reaction was stopped by inactivating the ligase at 70 °C for 10 mins. A sample of ligation reaction was run on 1% agarose gel to confirm the presence of a 1182 bp insert in the plasmid construct (which was designated pQE-30.m5).

Electrocompetent *E. coli* expression strain BL21/pREP4 was transformed with the ligation product pQE-30.m5 by electroporation, a method first described by Neumann *et al.,* (1982). Cells were also transformed with unligated pQE-30 as a positive control and with TE buffer (Appendix 1) alone as a negative control.

E. coli BL21/pREP4/pQE-30.m5 were streaked onto LB/kan/amp selection plates and allowed to incubate overnight at 37 °C. Bacterial colonies which grew overnight were screened for potential clones containing the pQE-30.m5 construct. Screening by the restriction digest of extracted plasmid DNA was not successful, as fragment bands were not visible on agarose gels; therefore, colony PCR screening was used. Frozen glycerol cell stocks were prepared by subculturing one half of each colony into LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin. Next, the remaining half of the each colony was subjected to crude alkaline lysis (Section *3.2.1.1*). One microlitre of the lysate supernatant was added to a PCR reaction which contained the primers M5-5-169 and M5-3-1330 (as above). Positive clones were identified as those which produced a PCR fragment of expected length, ~1182 bp, on agarose gel.

Recombinant plasmid pQE-30.m5, extracted from positive clones was sequenced to confirm that a) the M5 insert was present and ligated in the correct reading frame at the 5' end of the open reading frame and b) the T5 promoter region, TATA-box and ribosomal binding site (RBS) were intact. Three DNA sequencing reactions were prepared for each primer using the methods outlined in Section *3.2.3*. The pQE sequencing primers (*Qiagen*) are shown in Table 4.2.

Table 4.2 pQE sequencing primers

Primer	Length	Sequence (5' – 3')
pQE-forward	18 bp	CCCGAAAAGTGCCACCTG
pQE-reverse	19 bp	GTTCTGAGGTCATTACTGG

Sequencing was carried out on a MegaBACE^{TM} 1000 DNA Analysis System (*Amersham Biosciences*) by staff at the Genetic Analysis Facility at James Cook University, Townsville, Australia. Sequence data was analysed using Sequencher^{TM} 4.0 for Windows software (*Gene Codes Corporation*). After removing vector coding from sequences, the rM5 consensus sequence (Appendix 3) was used to probe the NCBI database using the blastn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to confirm gene identity (Altschul *et al.*, 1990). The deduced amino acid sequence was determined using the translate tool found on the ExPASy protein website and multiple protein alignments were performed using the ClustalW method (http://au.expasy.org/tools/dna.html).

4.2.3.3 Recombinant M5 (rM5) protein expression

A rapid screening method using a small expression culture (as described in the QIAexpressionist™ handbook) was initially performed to determine if the 6x*histidine*-tagged rM5 protein was indeed expressed. This procedure included a time course whereby samples were taken at four one-hourly intervals during incubation to determine the level of expression during induction.

BL21[pREP4] containing construct pQE30.m5 were grown overnight in 10 ml LB medium with 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37 °C with shaking (200 rpm). The following day, cells were pelleted, washed in PBS and resuspended in 10 ml LB, then subcultured (1:50) into fresh medium. Cultures were grown at 37 °C with shaking until an OD_{600} of between 0.4 and 0.6 was reached. A 1 ml aliquot was then removed for SDS-PAGE analysis (Section *3.2.5.2*). The cells pelleted and labelled as the uninduced control (UC) and stored at -20 °C.

Expression of the 6x*histidine*-tagged rM5 protein was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and growth allowed to continue for a further 4 h at 37 °C with shaking. A post-induction sample (induced control, IC) was removed for analysis as above. The remaining induced culture was centrifuged at 4,000 x *g* for 20 mins, the cell pellet weighed and frozen at -20 °C until required.

Large scale expression was carried out in one litre culture volumes using the method described above. Extraction under denaturing conditions produced equal yields of 6x*histidine*-tagged rM5 protein as extraction under native (non-denaturing) conditions as per QIAexpressionist handbook (*Qiagen),* therefore, the latter method was used in this study.

4.2.3.4 Recombinant M5 (rM5) protein purification

Frozen cell pellets were thawed on ice for 15 min and resuspended in Lysis Buffer (Appendix 1) at 2-5 ml per gram wet cell weight. Lysozyme to 1 mg/ml was added and the suspension incubated on ice for a further 30 min, then sonicated on ice for six x 10 sec bursts at 300 W with 20 sec cooling intervals. RNase A to 10 μg/ml and DNase1 to 5 U/ml was added to the lysate to reduce viscosity by nucleic acid degradation. After 15 min incubation on ice, the lysate was centrifuge at 10,000 x *g* for 30 mins at 4 °C to pellet cell debris. A 5 μl sample of supernatant (cleared lysate, CL) was removed for SDS-PAGE analysis and stored at -20 °C. The remaining supernatant was filtered (0.45 μm) and mixed with a Ni-NTA resin slurry (*Qiagen)* in a 4:1 ratio, then mixed gently for 60 min at 4 °C. The mixture was loaded into clean Superflow columns (*Qiagen*) and the flow-through collected by gravity flow.

The resin was washed three times with 10 ml Wash buffer (Appendix 1) before the *his*-tagged protein was eluted four times with 0.5 ml Elution buffer (Appendix 1). Elution fractions containing high concentrations of protein, determined spectrophotometrically, were pooled and buffer was changed to PBS on desalting/buffer exchange PD10 columns (*Amersham*). Protein concentration was measured using the BCA method (Section *3.2.5.2)* and if required, concentrated on Amicon Ultra-4 Centrifugal filter devices (*Millipore*, 10,000 MWCO) or diluted with sterile PBS. The rM5 solution was filter-sterilised through a 0.22 μm filter (*Millipore*) and potential endotoxin contamination was tested by the *Limulus* Amoebocyte Lysate (LAL) method using an E-Toxate kit (*Sigma*) before storage at -20 °C.

4.2.3.5 PepM5

PepM5 was extracted from a 'rheumatogenic' GAS serotype M5 by limited pepsin digestion following the method of Beachey *et al.,* (1977) with some modifications. Pepsin digestion of whole group A streptococci releases a fragment of the native M protein (known as PepM) from the streptococcal cell wall. At a suboptimal pH of 5.8, pepsin specifically cleaves the M protein at a hinge region between the B and C repeat regions (see Figure 4.1). PepM, containing the highly variable N-terminal (A repeat) region of the native M protein and the variable B repeat region, has been shown to elicit serotype-specific opsonic antibodies (Cunningham and Beachey, 1974). PepM from several GAS serotypes have also been shown to elicit host cross-reactive antibodies (Dale and Beachey, 1986; Cunningham *et al.*, 1989). PepM is therefore immunologically active.

Frozen GAS M5 stock was plated out on Horse Blood agar (HBA) using a sterile loop and incubated overnight at 37 °C. A single colony was suspended in sterile saline (0.85%) to 0.5 McFarland units or 1 x 10^8 CFU/ml. Bacterial suspension (1 ml) was inoculated into glass flasks (2.0 L) containing 500 ml of THY broth (Appendix 1) and cultured for 18 h at 37 °C without shaking. The cells were harvested by centrifugation at 7000 x *g* for 20 mins at 4 °C and washed twice in cold (4 °C) sterile PBS, pH 7.4. The cell pellet was weighed after the final centrifugation: the yield of GAS M5 bacteria varied from 2.0 to 4.0 g (wet weight) per litre of

culture. Bacteria were resuspended in warm (37 °C) PBS, pH 5.8 containing 50 μg/ml pepsin in a ratio of 1 g cells to 2 ml of buffer. Digestion was carried out at 37 °C on an orbital shaker at 200 rpm for 45 mins. To stop digestion, the suspension was immersed in ice and sufficient sodium bicarbonate (7.5% w/v) was added to raise the pH to 7.4.

The bacteria were pelleted and the supernatant applied to a PD10 Sephadex G-25 column. The protein was eluted with sterile PBS, concentrated and purity was assessed by SDS-PAGE as described for rM5 protein. The PepM5 solution was then filter-sterilised and stored at -20 °C.

4.2.4 Immunisation of rats

For the induction of experimental autoimmune valvulitis, groups of three to five female Lewis rats (8-12 weeks old), 175 to 230 g in weight, were immunised as described in Section *3.2.6* using the method of Galvin *et al.* (2002) with modifications. For acute RHD, rats were euthanased on day 21 post immunisation (p.i.); for chronic RHD, euthanasia was carried out on day 46 p.i. Organs including hearts, spleens and lymph nodes were aseptically removed and stored short term in cold transport medium (Appendix 1) until required.

4.2.5 Tissue histology and immunohistochemistry

4.2.5.1 Histology

Longitudinal sections (5 μm) of rat hearts were stained with either H&E, Gomori's trichrome or toluidine blue using standard procedures and examined by light microscopy. Pathological changes indicative of valvulitis included infiltration of mononuclear and polymorphonuclear cells into valve tissue, fibrosis (scarring), oedema, neovascularisation of valve tissue and shortening or thickening of chordae tendineae. Myocardial tissue was examined for the presence of focal lesions comprised of infiltrating mononuclear and polymorphonuclear cells, as well as multinuclear giant cells or Anitschkow ('caterpillar')-like cells. Myocyte hypertrophy, necrosis or increased interstitial fibrosis was also indicative of myocardial changes associated with carditis.

4.2.5.2 Immunohistochemistry

Cryostat sections or formalin-fixed, paraffin-embedded sections of rat hearts were immunostained using reagents and methods described in Chapter 3. Antibodies against cell surface molecules CD4, CD8 and T cell receptor molecule CD3 were used to detect T cells and an anti-CD68 (lysosomal glycoprotein) antibody was used to detect macrophages in heart valves and myocardium. Sections were examined microscopically and scored for evidence of pathology as outlined in Chapter 3 Materials and Methods, Section *3.2.7.3.*

4.2.6 Lymphocyte proliferation assays

The specificity and reactivity of lymphocytes from immunised rats was determined by lymphocyte proliferation assay as described in Section *3.2.6.5*. Cells were harvested onto glass fibre mats at 24 h intervals on days 4-7 of culture after pulsing for 4 h with 1.25 μCi/ml ^{3}H-thymidine (*GE Healthcare*). When dry, scintillation fluid was applied to the mats and counts per minute (cpm) were measured in a MicroBeta scintillation counter (*ElmerPackard*). Proliferative response of cells is reported as stimulation index (SI), calculated as the ratio of cell proliferation (cpm) in the presence or absence of stimulating antigen. SI values of ≥ 3 were considered positive.

4.2.7 Statistical analysis

For lymphocyte proliferation assays, results are reported as stimulation index ± standard error of the mean (SEM). To test significance, Student's unpaired two-tailed *t*-test was performed on normally distributed data with significance set at $p \leq 0.05$.

4.3 RESULTS

4.3.1 Rat cardiac myosin

Cardiac myosin was extracted from healthy, non-immunised Lewis rats and resolved on an 8% polyacrylamide gel stained with Coomassie Blue to visualise protein bands. The extract contained a strong protein band with the expected molecular

weight of around 220 kDa, but three lighter bands around 45, 35 and 20 kDa respectively, possibly cardiac myosin light chains as a result of protein break down or actin contamination were also present. However, this rat cardiac myosin was deemed to be sufficiently pure for use when compared with the electrophoretic profile of commercially-sourced porcine myosin (Figure 4.2).

4.3.2 Recombinant GAS M5 (rM5) protein

4.3.2.1 Recombinant M5 protein construction

Oligonucleotide primers were designed to amplify a fragment of the M protein gene from chromosomal DNA of *S. pyogenes* serotype 5 bacteria, including sequences that encode the A-, B- and C- repeat regions, but not the leader sequence, ATG start codon or the cell wall anchoring region. To facilitate directional cloning of the M5 DNA insert in-frame into the pQE-30 expression vector, restriction sites were incorporated into the primers. Preliminary optimisation experiments determined that PCR reactions containing 2.5 μM $MgCl_2$ and 0.1 μM primer and an annealing temperature of 64 °C produced the best results (Figure 4.3).

After digestion with *BamHI* and *SalI* restriction enzymes, the PCR product was ligated into a pQE-30 expression vector to create the pQE30.m5 construct, which was subsequently transformed by electroporation into *E. coli* strain BL21[pREP4] bacteria. As this expression system does not support blue-white screening, all colonies appeared white on LB agar plates containing ampicillin and kanamycin. Growth on this selective agar indicated that colonies were comprised of BL21 with pREP4, thus conferring kanamycin resistance, and transformed with pQE-30 carrying ampicillin resistance. Negative controls (electroporated, no plasmid) produced no growth; positive controls (bacteria transformed with unligated pQE-30) produced a confluent lawn of bacteria.

Plasmid DNA with confirmed inserts, determined by PCR colony screening, was extracted from clones, sequenced and analysed using Sequencher™ software. A GenBank BLASTn search returned seven hits with more than 98% nucleotide identity and zero (0.0) E-values (Appendix A3.3). The rM5 consensus sequence and deduced amino acid sequence, generated using ExPASy are included in Appendix

A.3.4. ProtParam analysis of the rM5 sequence with incorporated *histidine* tag predicted a 395-residue protein with a molecular weight of 45.03 kDa.

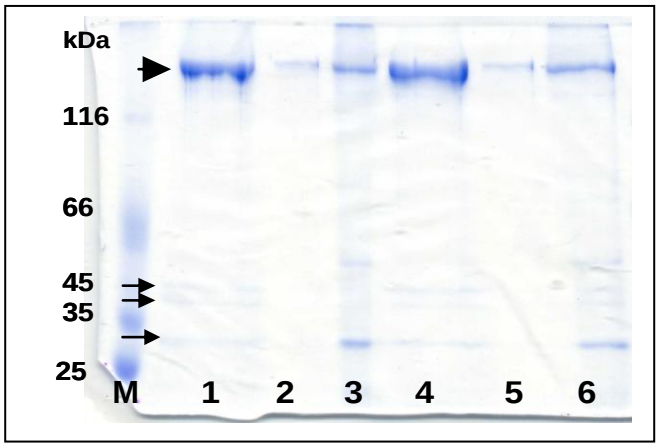


Figure 4.2 Electrophoretic analysis of cardiac myosin extracted from normal rat heart tissue and porcine cardiac myosin.
Lanes 1 and 2: rat cardiac myosin (this study), 20 µg and 5 µg per lane respectively; Lane 3: porcine cardiac myosin (*Sigma***)** 10 µg; Lanes 4-6: same proteins as lanes 1-3, run under reducing conditions (as described in *Section* 3.2.5.2). Arrows indicate expected band at 220 kDa and three smaller bands ~43, 30 and 20 kDa respectively. M= marker (SM0431, *Fermentas*).

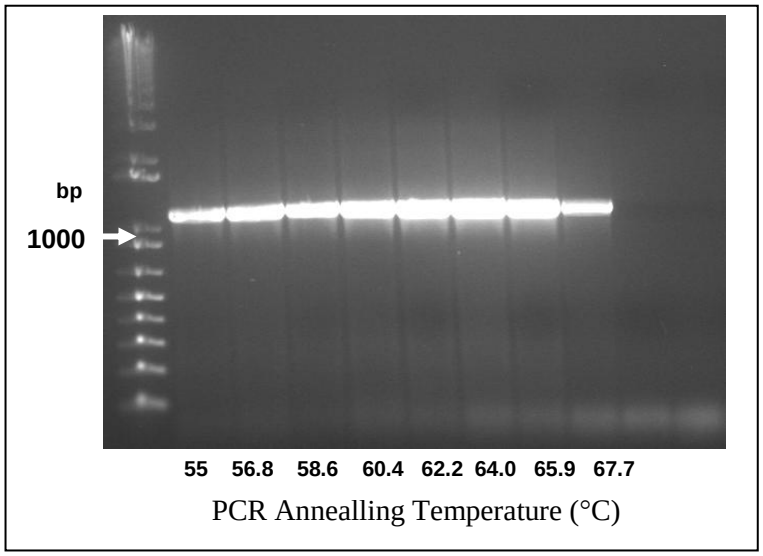


Figure 4.3 Temperature gradient for optimising binding of primers.
Standard PCR conditions using 50 ng GAS DNA template were followed as described in Sections *3.2.2.2* and *3.2.2.3* except that annealing temperature was varied as indicated. Binding of oligonucleotide primers was specific at all temperatures as indicated by single bands. DNA size markers (1 Kb plus DNA Ladder™) are shown on the left and the negative template control is in the far right hand lane.

Plasmid DNA with confirmed inserts, determined by PCR colony screening, was extracted from clones, sequenced and analysed using Sequencher™ software. A GenBank BLASTn search returned seven hits with more than 98% nucleotide identity and zero (0.0) E-values (Appendix A3.3). The rM5 consensus sequence and deduced amino acid sequence, generated using ExPASy are included in Appendix A.3.4. ProtParam analysis of the rM5 sequence with incorporated *histidine* tag predicted a 395-residue protein with a molecular weight of 45.03 kDa.

A BLASTp search of the SWISSProt/TrEMBL database found potentially matching sequences, three of which had very low E-values (Appendix A.3.5). These were accession nos. Q54510 (E-value = -161), A2RGM0 (-146) and P02977 (-145). Multiple sequence alignment analysis (Clustal format for T-Coffee V5.05, [http://www.tcoffee.org]) of rM5 and these three sequences revealed that rM5 shares most identity (99%) with the M5 protein sequence STRPY M5.8193 (Q54510) published by Whatmore and Kehoe (1994) (Table 4.3). Differences between rM5 and Q54510 include a phenylalanine (F) in rM5 pos 347, reported as leucine (L) in other sequences inspected and an alanine in rM5 pos 1, reported as tyrosine (T) in Q54510. When compared to the other two *S. pyogenes* serotype 5 M protein sequences (accession nos. A2RGM0 and P02977), both rM5 and STRPY M5.8193 contain a 14 residue insertion (SNLERKTAELTSEK) between the A5 and A6 region and a 25-residue B2 region repeat sequence deletion (ETIGTLKKILDETVKDKIAKEQENK) (Figure 4.4).

Table 4.3 Results of ClustalW2 Scores Analysis

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	tr|Q54510|Q54510_STRPY	457	2	tr|A2RGM0|A2RGM0_STRPG	492	96
1	tr|Q54510|Q54510_STRPY	457	3	sp|P02977|M5_STRP5	492	96
1	tr|Q54510|Q54510_STRPY	457	4	rM5seq	383	99
2	tr|A2RGM0|A2RGM0_STRPG	492	3	sp|P02977|M5_STRP5	492	99
2	tr|A2RGM0|A2RGM0_STRPG	492	4	rM5seq	383	95
3	sp|P02977|M5_STRP5	492	4	rM5seq	383	95

```
CLUSTAL 2.0.10 multiple sequence alignment

tr|A2RGM0|A2RGM0_STRPG        MARENTNKHYSLRKLKKGTASVAVALSVLGAGLVVNTNEVSAAVTRGTIN 50
sp|P02977|M5_STRP5            MARENTNKHYWLRKLKKGTASVAVALSVLGAGLVVNTNEVSAAVTRGTIN 50
rM5seq                        ----------------------------------------AVTRGTIN 8
tr|Q54510|Q54510_STRPY        ------------------------ALSVLGAGLVVNTNEVSATVTRGTIN 26
                                                                       :*******

tr|A2RGM0|A2RGM0_STRPG        DPQRAKEALDKYELENHDLKTKNEGLKTENEGLKTENEGLKTENEGLKTE 100
sp|P02977|M5_STRP5            DPQRAKEALDKYELENHDLKTKNEGLKTENEGLKTENEGLKTENEGLKTE 100
rM5seq                        DPQRAKEALDKYELENHDLKTKNEGLKTENEGLKTENEGLKTENERLKTE 58
tr|Q54510|Q54510_STRPY        DPQRAKEALDKYELENHDLKTKNEGLKTENEGLKTENEGLKTENERLKTE 76
                              ********************************************* ****

tr|A2RGM0|A2RGM0_STRPG        K--------------KEHEAENDKLKQQRDTLSTQKETLEREVQNTQYNN 136
sp|P02977|M5_STRP5            K--------------KEHEAENDKLKQQRDTLSTQKETLEREVQNTQYNN 136
rM5seq                        KSNLERKTAELTSEKKEHEAENDKLKQQRDTLSTQKETLEREVQNTQYNN 108
tr|Q54510|Q54510_STRPY        KSNLERKTAELTSEKKEHEAENDKLKQQRDTLSTQKETLEREVQNTQYNN 126
                              *              ***********************************

tr|A2RGM0|A2RGM0_STRPG        ETLKIKNGDLTKELNKTRQELANKQQESKENEKALNELLEKTVKDKIAKE 186
sp|P02977|M5_STRP5            ETLKIKNGDLTKELNKTRQELANKQQESKENEKALNELLEKTVKDKIAKE 186
rM5seq                        ETLKIKNGDLTKELNKTRQELANKQQESKENEKALNELLEKTVKDKIAKE 158
tr|Q54510|Q54510_STRPY        ETLKIKNGDLTKELNKTRQELANKQQESKENEKALNELLEKTVKDKIAKE 176
                              **************************************************

tr|A2RGM0|A2RGM0_STRPG        QENKETIGTLKKILDETVKDKIAKEQENKETIGTLKKILDETVKDKLAKE 236
sp|P02977|M5_STRP5            QENKETIGTLKKILDETVKDKIAKEQENKETIGTLKKILDETVKDKLAKE 236
rM5seq                        QENK------------------------ETIGTLKKILDETVKDKLAKE 183
tr|Q54510|Q54510_STRPY        QENK------------------------ETIGTLKKILDETVKDKLAKE 201
                              ****                         ********************

tr|A2RGM0|A2RGM0_STRPG        QKSKQNIGALKQELAKKDEANKISDASRKGLRRDLDASREAKKQLEAEHQ 286
sp|P02977|M5_STRP5            QKSKQNIGALKQELAKKDEANKISDASRKGLRRDLDASREAKKQLEAEHQ 286
rM5seq                        QKSKQNIGALKQELAKKDEANKISDASRKGLRRDLDASREAKKQLEAEHQ 233
tr|Q54510|Q54510_STRPY        QKSKQNIGALKQELAKKDEANKISDASRKGLRRDLDASREAKKQLEAEHQ 251
                              **************************************************

tr|A2RGM0|A2RGM0_STRPG        KLEEQNKISEASRKGLRRDLDASREAKKQLEAEHQKLEEQNKISEASRKG 336
sp|P02977|M5_STRP5            KLEEQNKISEASRKGLRRDLDASREAKKQLEAEQQKLEEQNKISEASRKG 336
rM5seq                        KLEEQNKISEASRKGLRRDLDASREAKKQLEAEHQKLEEQNKISEASRKG 283
tr|Q54510|Q54510_STRPY        KLEEQNKISEASRKGLRRDLDASREAKKQLEAEHQKLEEQNKISEASRKG 301
                              **********************************:***************

tr|A2RGM0|A2RGM0_STRPG        LRRDLDASREAKKQVEKALEEANSKLAALEKLNKELEESKKLTEKEKAEL 386
sp|P02977|M5_STRP5            LRRDLDASREAKKQVEKALEEANSKLAALEKLNKELEESKKLTEKEKAEL 386
rM5seq                        LRRDLDASREAKKQVEKALEEANSKLAALEKLNKELEESKKLTEKEKAEL 333
tr|Q54510|Q54510_STRPY        LRRDLDASREAKKQVEKALEEANSKLAALEKLNKELEESKKLTEKEKAEL 351
                              **************************************************

tr|A2RGM0|A2RGM0_STRPG        QAKLEAEAKALKEQLAKQAEELAKLRAGKASDSQTPDTKPGNKAVPGKGQ 436
sp|P02977|M5_STRP5            QAKLEAEAKALKEQLAKQAEELAKLRAGKASDSQTPDTKPGNKAVPGKGQ 436
rM5seq                        QAKLEAEAKARKEQLAKQAEELAKLRAGKASDSQTPDTKPGNKAVPGKGQ 383
tr|Q54510|Q54510_STRPY        QAKLEAEAKALKEQLAKQAEELAKLRAGKASDSQTPDTKPGNKAVPGKGQ 401
                              **********:***************************************

tr|A2RGM0|A2RGM0_STRPG        APQAGTKPNQNKAPMKETKRQLPSTGETANPFFTAAALTVMATAGVAAVV 486
```

Figure 4.4 ClustalW multiple sequence alignment results.
Red shading indicates discrepancy between rM5 sequence and sequence published by Whatmore and Kehoe (1994), * indicates 100% identity between all sequences, ':' indicates mismatch between one or more sequences.

4.3.2.2 Expression and purification of rM5 protein

Following over-expression in *E. coli* BL21 cultures, rM5 protein was successfully purified by affinity chromatography. SDS-PAGE analysis confirmed that protein with a calculated molecular weight of approximately 47 kDa was expressed in induced samples but not in uninduced samples (Figure 4.5). Endotoxin contamination of rM5, measured by Limulus Amoebacyte Lysate (LAL) assay (E-toxate kit, Sigma), ranged from below 0.05 EU/ml (assay detection limit) to 6 EU/ml. Stocks which were less than 0.10 EU/ml were used in experiments.

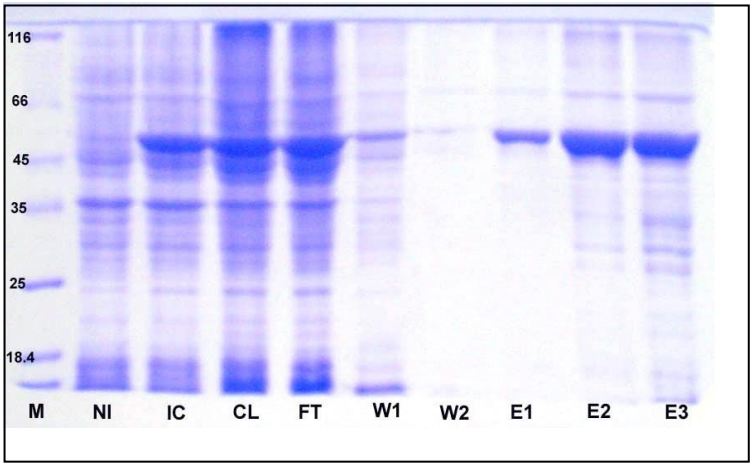


Figure 4.5 Expression profile of rM5 protein.
rM5 purified from *E. coli* lysate under native conditions as described in the QiaExpressionist procedure guide (Qiagen) were stained with Coomassie and resolved on a 12% SDS-PAGE gel. M=molecular weight marker, NI=non-induced, IC=induced control, CL=cleared lysate, FT=flow-through, W1-2=wash fractions, E1-E3=elution fractions.

4.3.3 GAS rM5-induced inflammation in Lewis rat hearts

We sought to determine whether rats immunised with GAS M protein in Freund's adjuvant developed signs of valvulitis or myocarditis which are similar to the lesions described in RF/RHD patients (Saffitz, 2008). Apart from an initial weight loss in some animals in the first seven days after initial immunisation, all animals had a steady weight gain throughout the second and third week and in all cases, rats exhibited no overt signs of disease prior to sacrifice. Hearts from rM5-immunised rats and control rats were examined microscopically for evidence of cardiac lesions and given a score based on the number of infiltrating MNC cells or cardiac lesions

present as per Table 3.2. The extent of pathology in rM5-immunised rats varied between individual animals (Table 4.4).

Table 4.4 Proportion of Lewis rats with myocarditis and valvulitis as determined by immunohistochemical analysis of heart tissue 21 days after primary immunisation

	No. rats with lesions/no. rats immunised	
Antigen	Myocardium	Valves
PBS	1/5	0/5
rM5	4/7	4/7
Cardiac myosin	4/5	4/5
pepM5 [a]	2/2	1/2

[a] 2 of 4 pepM5-immunised rats died during booster immunisation procedure

In H&E sections of valves from control rats, no evidence of valvulitis was detected in any of the rats immunised with PBS and adjuvant only (Figure 4.6 a,b). Two of four rats immunised with pepM5 did not recover from anaesthesia following booster injections. At necropsy, the hearts of these rats were unremarkable and the cause of death was not determined. Of the two remaining rats, one had evidence of valvulitis, including MNC infiltration and fibrinoid necrosis (Figure 4.6 c,d). Moderate to severe valvulitis characterised by MNC infiltration, necrosis and oedema was observed in four of the five rats immunised with positive control antigen porcine cardiac myosin (Figure 4.6 e,f). However, valve thickening and calcification was not observed in any of the valves examined, regardless of immunising antigen.

Four of seven animals immunised with rM5 had histological evidence of either valvular or myocardial lesions. Three rats had both valvular and myocardial infiltrates. Four animals had mild inflammatory infiltration of the valves (Figure 4.7) with both diffuse and focal infiltration of mononuclear cells and/or neutrophils. In valve tissue of one rM5-immunised rat, neovascularisation was also present.

In H&E sections of myocardium from immunised rats four of five rM5-immunised rats had signs of mild myocarditis (Figure 4.8a, b). Lesions were identified by interstitial inflammatory cell infiltrates, predominantly mononuclear, with focal

myocyte necrosis and interstitial oedema. Lesions were observed 1) in the sub-pericardial space, 2) in papillary muscles, 3) in the interstitial space between muscle bundles or 4) around myocardial vessels. However, no Aschoff-like bodies were observed in either the valves or myocardium of rM5-immunised rats.

No evidence of myocarditis was observed in H&E sections of the PBS control rats (Figure 4.8c, d). Both surviving pepM5-immunised rats developed myocarditis, demonstrated by the infiltration of mononuclear cells and/or neutrophils, necrosis and interstitial oedema and intense eosinophilic staining of myocytes with loss of striations (Figure 4.8e, f). The four cardiac myosin-immunised rats with valvulitis had also developed moderate to severe myocarditis, with intense cellular infiltration, myocyte degeneration and necrosis and interstitial oedema affecting more than 40% of the myocardial tissue, particularly in the base of the heart (Figure 4.8g, h). Cellular structures akin to Aschoff bodies were also present in the myocardium of these cardiac myosin-immunised rats.

Collagen is a major component of the heart valve leaflets and chordae tendinae and in the fibrous skeleton which anchors and supports the valves. In normal myocardium, collagen is found under the endothelial layer, around blood vessels, in the basement membrane and also forming narrow strata between muscle fibres, thus providing strength to the heart against mechanical stress (Iyer *et al.*, 2007). Cardiac fibrosis is associated with damage to and subsequent healing (or scarring) of the heart and is characterised by proliferation of fibroblasts and excessive deposition of extracellular matrix proteins such as collagen (Towbin, 2007). In this study, we used Gomori's trichrome, which stains collagen bright blue, to demonstrate fibrosis in the myocardial tissue of rats. We found minimal fibrosis in rM5-immunised animals, with several small focal lesions in the myocardium (Figure 4.9 a,b). In contrast, rats immunised with cardiac myosin developed extensive fibrosis (Figure 4.9 c,d) associated with the areas of inflammatory cell infiltration seen in H&E sections. The majority of PBS-control rats appeared normal, with collagen localised to blood vessels and the endothelium (Figure 4.9 e). Unexpectedly however, one PBS-immunised rat also had evidence of moderate myocardial fibrosis (Figure 4.9 f).

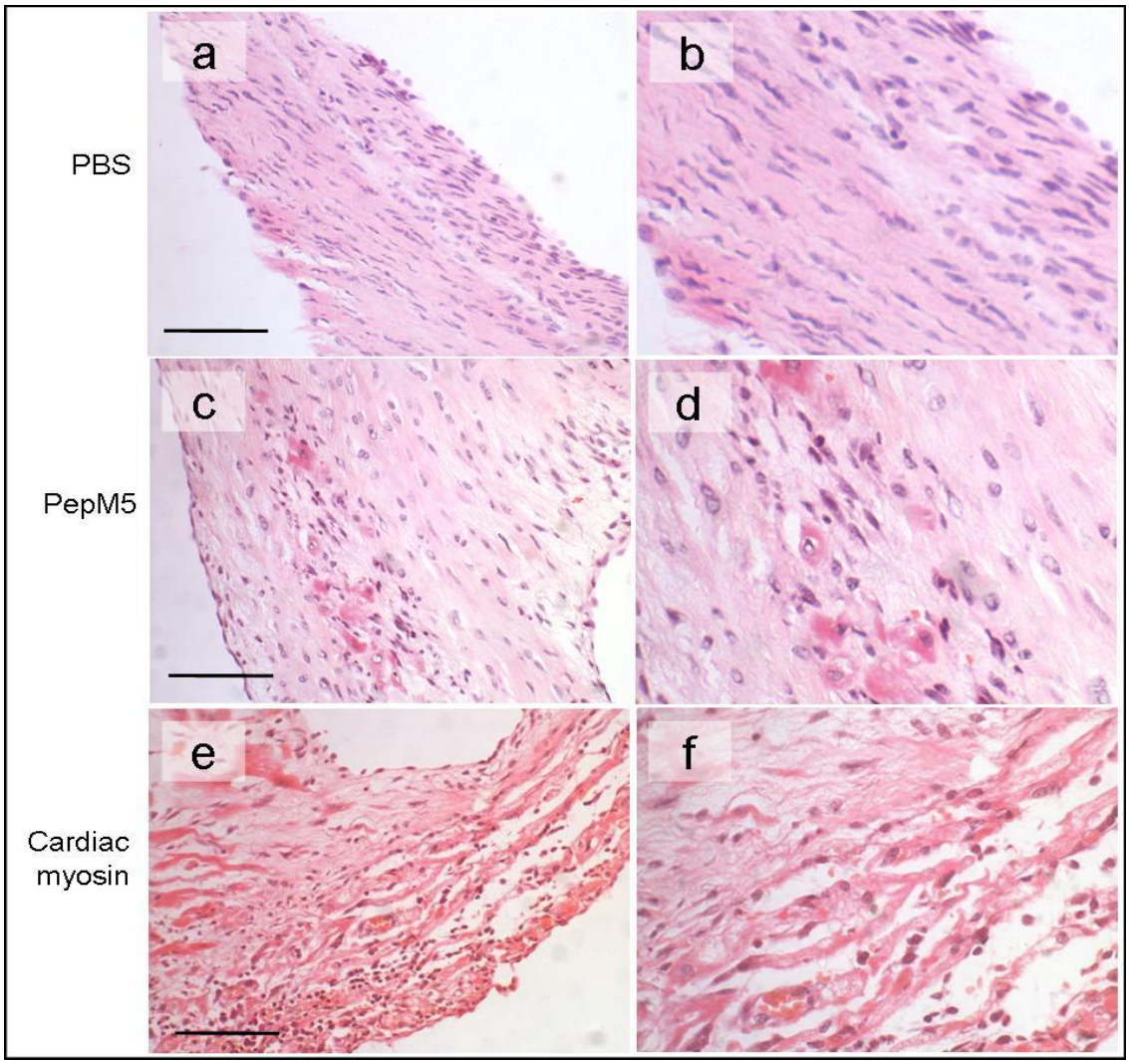


Figure 4.6 H&E stained sections of heart valves from rats.
Heart valves from negative control rats immunised with PBS and adjuvant only (*a*,*b*) showed no evidence of pathology. In comparison, immunisation of positive control animals with pepM5 (*c*,*d*) and cardiac myosin (*e*,*f*) induced lesions characterised by infiltration of the valves with mononuclear cells and neutrophils, necrosis and oedema. Representative photomicrographs on the left were taken using a 20x objective lens; higher magnification (40x) are shown on the right. *Bars = 100 μm.*

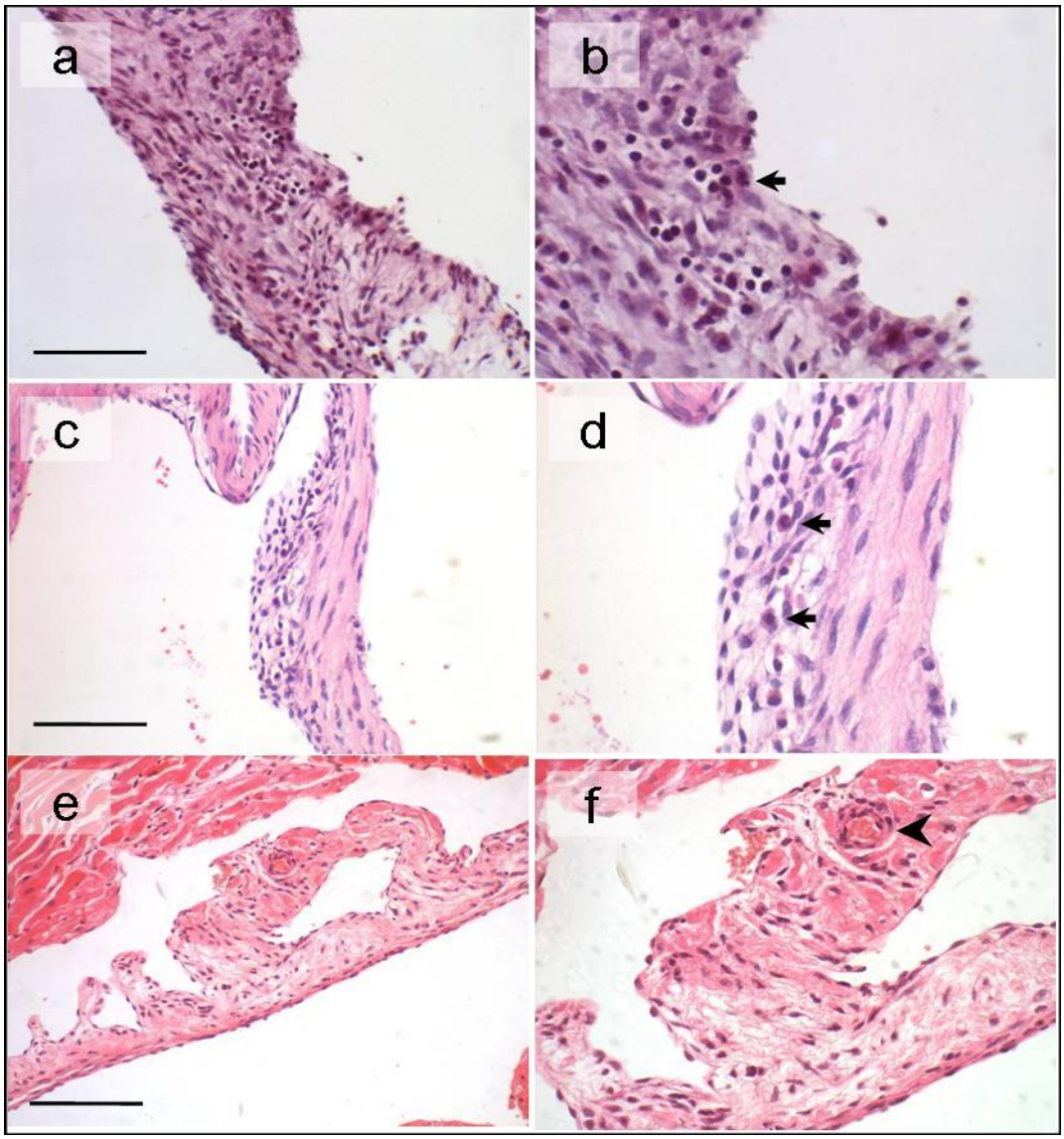


Figure 4.7 Histological features of heart valves from rM5-immunised rats. Lewis rats immunised with rM5 had evidence of valvulitis, characterised by infiltration of mononuclear cells (arrows) into mitral valves (*a*, *b*) and aortic valves (*c*,*d*). Formation of blood vessels was also noted (*e*,*f*) in the normally avascular valve tissue (arrowhead). Representative photomicrographs on the left were taken using a 20x objective lens; higher magnification (40x) are shown on the right. H&E stain. *Bars = 100 μm.*

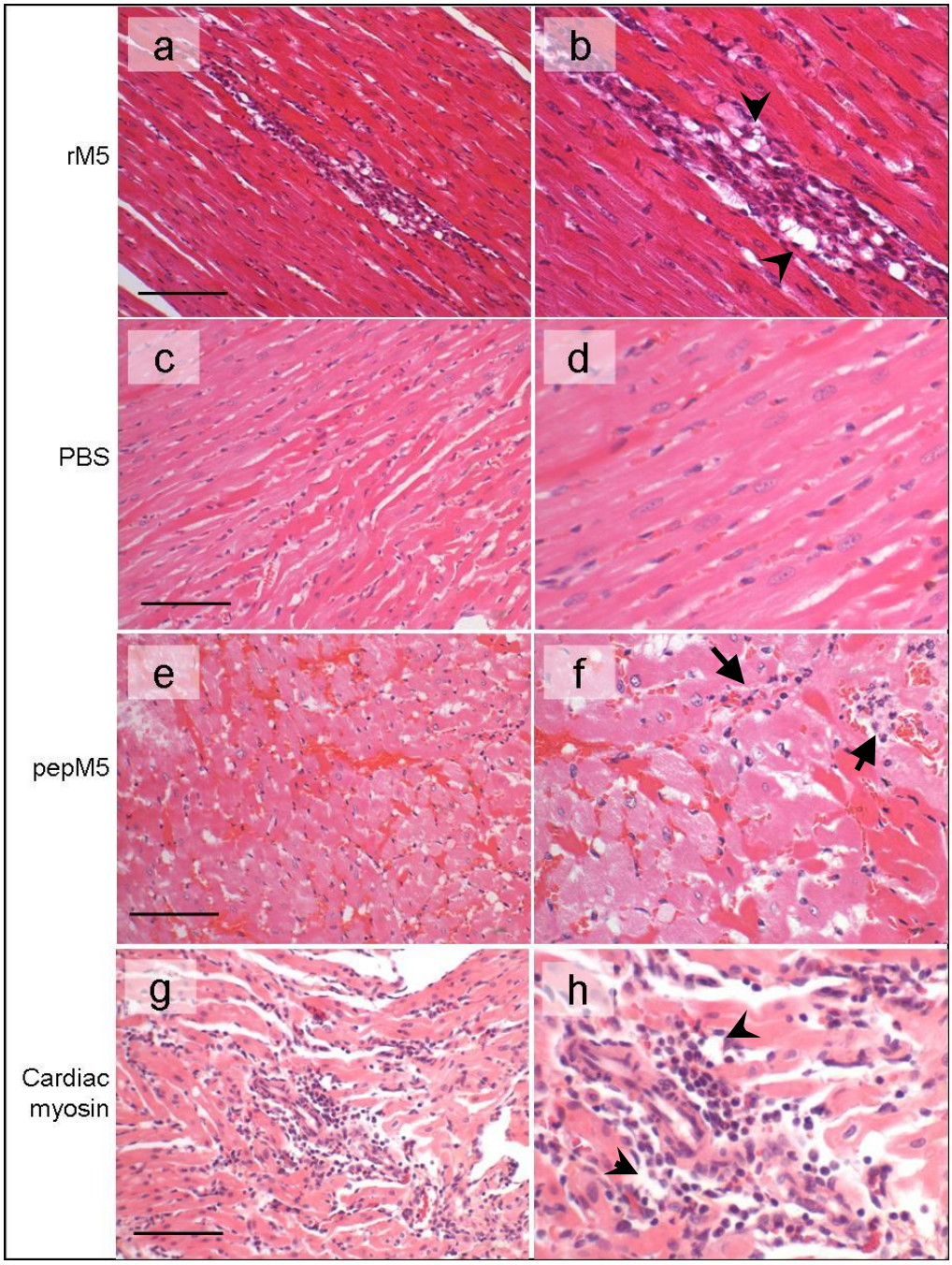


Figure 4.8 Histological features of myocardium from immunised rats.
Rats immunised with (a,b) rM5 had interstitial focal lesions comprised of infiltrating inflammatory cells and myocyte necrosis (arrowheads). (c,d) normal myocardium from PBS control rat. (e,f) foci of mononuclear infiltrates in pepM5-immunised rats (arrows). (g,h) Cardiac myosin-immunised rats developed severe myocarditis with extensive mononuclear infiltrates and necrosis (arrowheads). Images on the left were taken using a 20x objective lens; higher magnification (40x) are shown on the right. H&E stain. *Bars = 100 μm.*

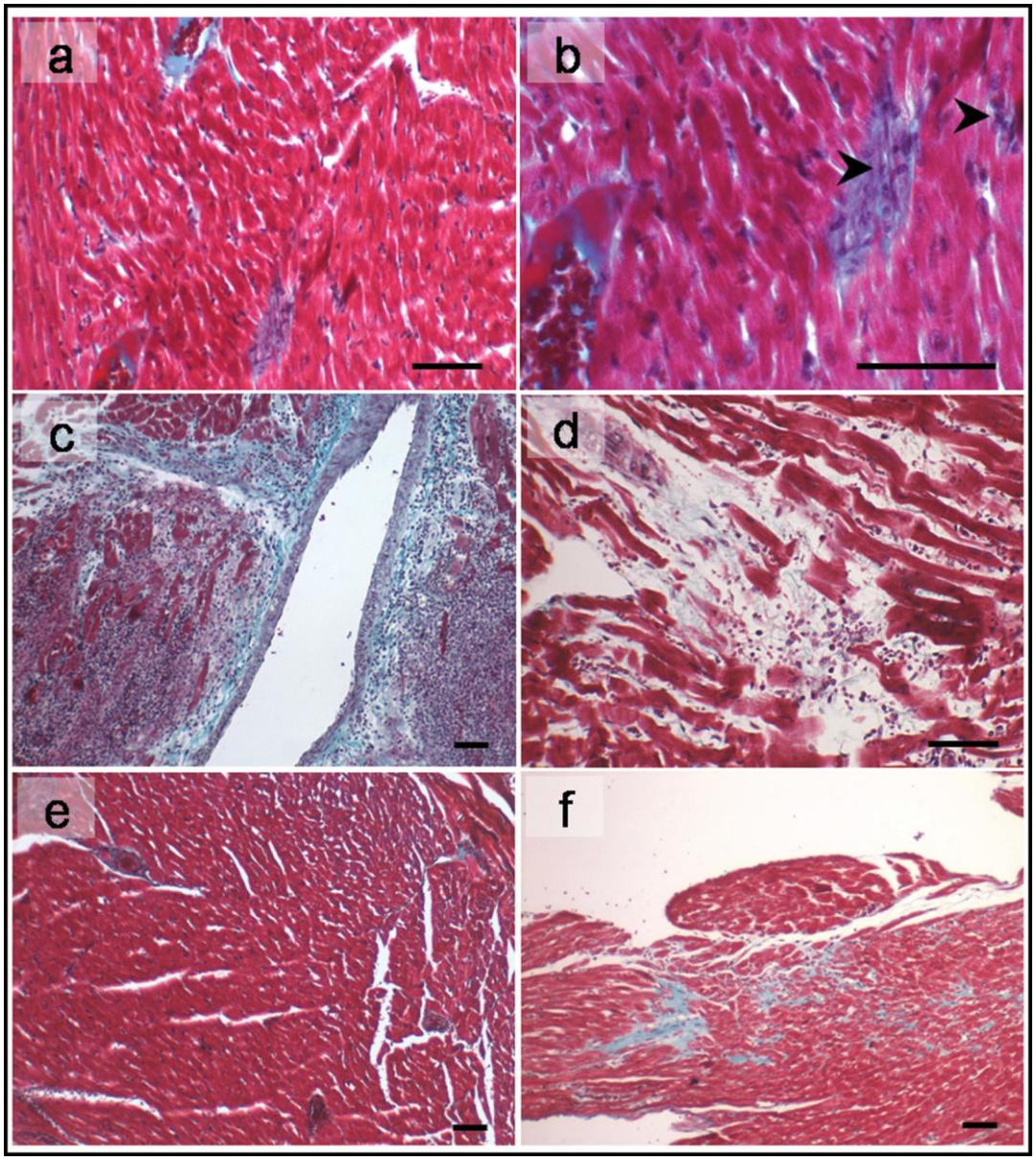


Figure 4.9 Fibrosis in rat myocardium demonstrated by collagen deposition. a) Isolated fibrotic lesion in myocardium of rM5-immunised rat (20x) b) higher magnification of (a) showing Anitschkow-like cells (arrowheads) (40x); c) extensive fibrosis in cardiac myosin-immunised rats (10x); d) myocardial lesion in a cardiac-myosin-immunised rat showing MNC infiltration and necrosis (20x); e) in the myocardium of PBS control rats, collagen was mainly located adjacent to blood vessels; f) one PBS rat had evidence of myocardial fibrosis (10x). Gomori's trichrome stain (blue = collagen). *Bars = 100 μm*

4.3.4 T cells and macrophages infiltrate heart tissue in rM5-immunised rats

Immunohistochemical staining of paraffin sections of hearts from rM5-immunised rats revealed that mononuclear cells in valvular lesions consisted mainly of $CD3^+$ (Figure 4.10A) and $CD68^+$ cells (Figure 4.10E) with few $CD8^+$ cells present (Figure 4.10C). Foci of infiltrating mononuclear cells in the myocardium of these rats stained positive for CD3, CD8 and CD68 (Figures 4.10 B, D and F respectively). Staining of frozen sections showed that $CD4^+$ cells were also present in valves and myocardium (not shown). In comparison, no signs of pathology were observed in control rats immunised with PBS and adjuvant which did not stain or stained weakly for CD3, CD4 and CD8 cells (Figure 4.11). It should be noted that $CD68^+$ cells were also detected in control rat valves, however, these were in very low numbers compared to rM5-immunised rats, and staining was generally confined to the endothelial layer with few cells actually infiltrating the valve tissue. Although isolated CD3, CD4, CD8 and CD68 cells were present in the myocardium of control rats, commonly in perivascular regions, no foci were evident. Numerous intact and de-granulated mast cells, identified by toluidine blue staining of granules, were located adjacent to blood vessels and scattered throughout cardiac tissue.

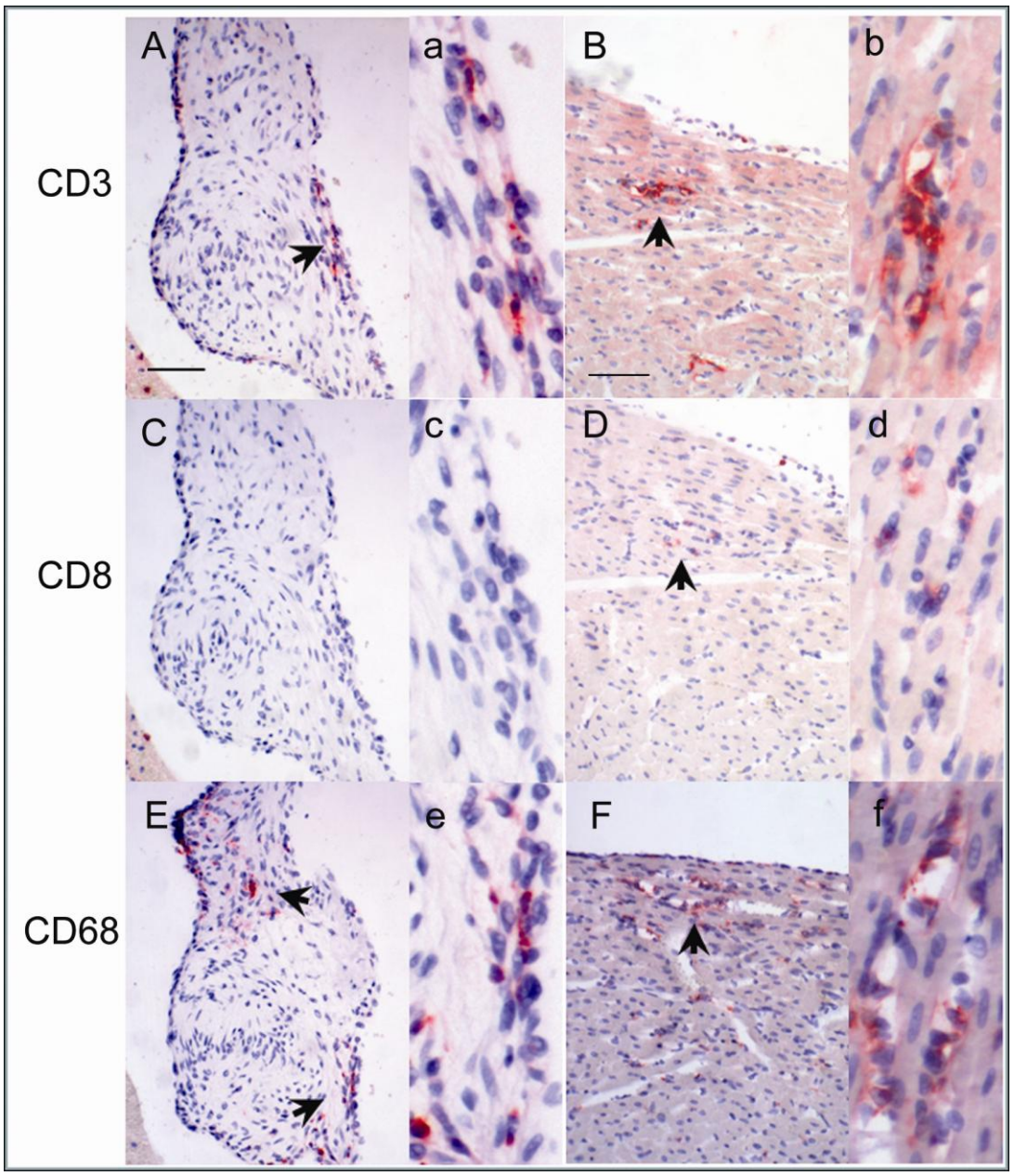


Figure 4.10 Immunohistochemical staining of hearts from rM5-immunised rats. Infiltrating MNC in heart valves of rM5-immunised rats stained for (*A* and at higher magnification, *a*) $CD3^+$ T cells and (*E, e*) $CD68^+$ macrophages but (*C, c*) not $CD8^+$ T cells. Myocardial lesions in rM5-immunised rats were characterised by (*B, b*) $CD3^+$, (*D, d*) $CD8^+$ and (*F, f*) $ED1^+$ infiltrating cells. *Bars = 50 μm*

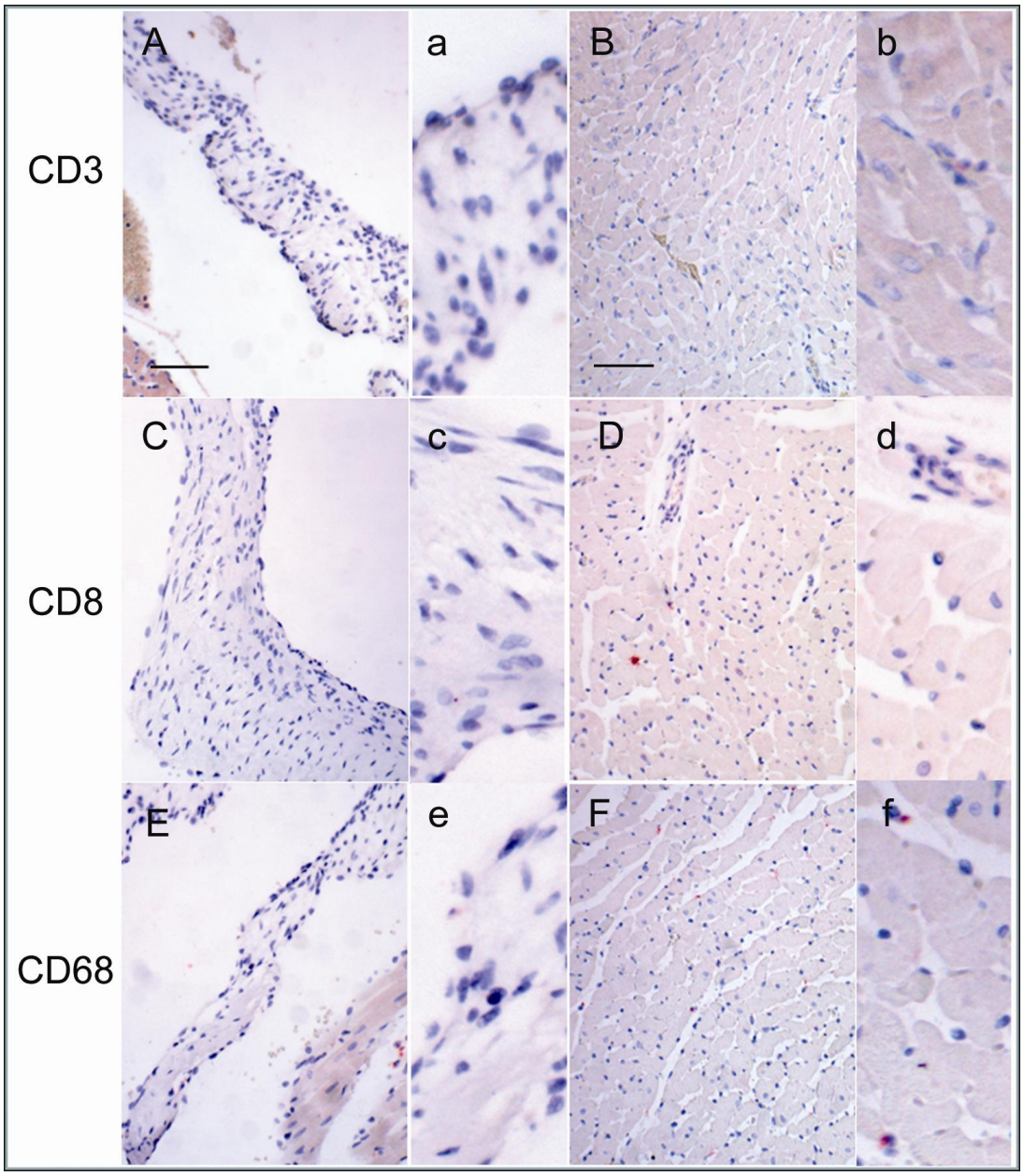


Figure 4.11 Immunohistochemical staining of hearts from PBS-immunised rats. Heart valves from control rats immunised with PBS and adjuvant only did not stain for (*A* and at higher magnification, *a*) $CD3^+$ T cells or (*C*, *c*) $CD8^+$ T cells and (*E*, *e*) minimally for $ED1^+$ macrophages. Staining for (*B*, *b*) $CD3^+$, (*D*, *d*) $CD8^+$ and (*F*, *f*) $ED1^+$ cells in the myocardium of control rats was restricted to isolated cells dispersed throughout the tissue, with no foci observed. *Bars = 50 μm*

4.3.5 T cell responses in rM5-immunised rats

The specificity and cross-reactivity of T cells derived from spleens of immunised rats were determined by measuring lymphocyte proliferation in response to antigenic stimulation in a standard tritiated [^{3}H] thymidine incorporation assay. In this assay the amount of radio-labelled thymidine incorporated into newly synthesised DNA of proliferating cells is measured in a liquid scintillation counter as counts per minute (cpm) and reported as a stimulation index (SI = cpm stimulated cells/cpm unstimulated cells).

Preliminary experiments revealed that rats immunised with rM5 responded vigorously *in vitro* to rM5 with a maximum SI of 106 ± 21, whereas control rats immunised with PBS did not respond to rM5 (maximum SI = 1.1 ± 0.3) (Figure 4.12). In the same assay, both rM5-immunised and control rats responded to T cell mitogen Con A with maximum SI ± SEM values of 152 ± 25 and 67 ± 35 respectively (not shown), indicating that these Lewis rats have a competent immune system capable of responding to the intact rM5 protein.

In the same experiments, the ability of rM5-immunised rats to cross-recognise host proteins cardiac myosin and tropomyosin was also investigated. Splenocytes from rM5-immunised rats were cultured for 72 h to 120 h in the presence of cardiac myosin or tropomyosin and pulsed with [^{3}H] thymidine as described above. In Figure 4.13 it is shown that T cells from rM5-immunised rats had a more than five-fold higher response towards cardiac myosin than control rats (SI= 5.3 ± 2.8 *vs* 0.9 ± 0.2) although this was not found to be statistically significant (Student's t-test, two-tailed). Similarly, rM5-immunised rats also had a higher response towards tropomyosin (SI= 2.9 ± 1.0 *vs* 0.6 ± 0.1) which was not statistically significant.

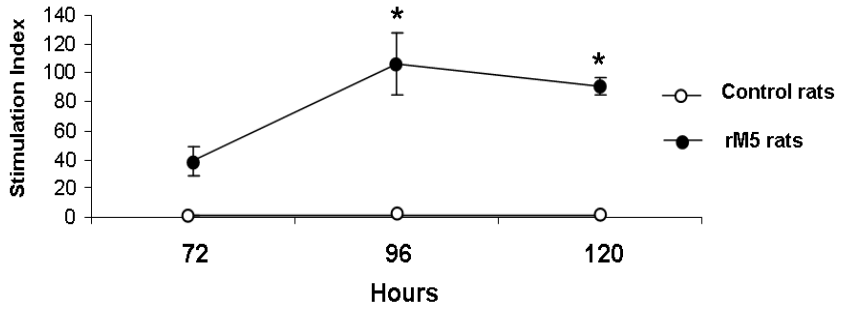


Figure 4.12 Proliferative response of rat T cells to *in vitro* stimulation with rM5. MNC from spleens of Lewis rats immunised with rM5 (n=4) or PBS (n=3) were cultured for 72-120 h in autologous culture medium in 50 μg/ml of rM5. Proliferation was measured using ^{3}H-thymidine incorporation and results are expressed as mean SI. Error bars represent SEM. * $p < 0.05$ *vs* control rats. One representative of three independent experiments is shown.

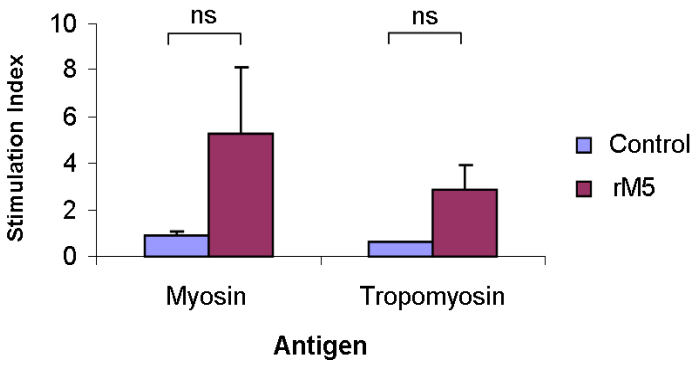


Figure 4.13 T cells from rM5-immunised rats cross-recognise host proteins. MNC from spleens of Lewis rats immunised with rM5 (n=4) or control rats immunised with PBS (n=3) were stimulated with cardiac myosin or tropomyosin for 72 h in autologous culture medium. Cells in negative control wells were left unstimulated. Proliferative response is expressed as mean SI. Error bars represent SEM.

4.4 DISCUSSION

One of the first objectives of the work described in this thesis was to validate the rat autoimmune valvulitis (RAV) model of RF/RHD. The results presented here are in agreement with the findings of Quinn *et al,* (2001) and support the hypothesis that a cell-mediated immune response to GAS M protein can result in damage to the heart valves and myocardium in the RAV animal model.

Rodent models have been used for over 25 years to study immune mediators of inflammatory heart disease caused by infectious agents including viruses, protozoa, fungi and bacteria. A case for autoimmunity as the pathologic mechanism responsible for disease initiation and progression has been well-established in experimental autoimmune myocarditis animal models using both mice and rats (Neu *et al.*, 1987; Smith and Allen, 1991; Huber, 1997a; Afanasyeva *et al.*, 2004b; Daniels *et al.*, 2008). Experimental autoimmune myocarditis (EAM) is histologically similar to human myocarditis with mononuclear cell infiltration consisting of around 80% monocytes/macrophages and 16% T cells and neutrophil infiltration, in addition to myocyte hypertrophy, necrosis and fibrosis (Afanasyeva *et al.*, 2004a; Afanasyeva *et al.*, 2004b). $CD4^+$ T cells were found to predominate over $CD8^+$ T cells in both the acute and chronic forms of the disease in contrast to Coxsackievirus B3-induced myocarditis which is characterised by a $CD8^+$ T cell predominance (Huber, 1997b). Similarly, acute and severe myocarditis can be elicited in Lewis rats by immunising with cardiac myosin in Freund's complete adjuvant (Kodama *et al.*, 1990; Galvin *et al.*, 2002; Li *et al.*, 2004). In our study, cardiac myosin immunisation also produced severe myocarditis in rats. However, the EAM model is primarily used to investigate the role of cardiac myosin in myocardial injury, with less attention on valvular damage. For this reason, the EAM model is not an appropriate model for RF/RHD studies.

In 2001, Quinn *et al.* reported that immunisation of Lewis rats with streptococcal M6 protein, induced valvulitis and myocarditis which was more analogous to the pathology seen in RF/RHD patients than cardiac myosin-induced pathology. This rat

autoimmune valvulitis (RAV) model has also been used previously in our laboratory (Lymbury *et al*, 2003).

In this present study, initial experiments were designed to induce valvulitis in the RAV model. To achieve this, Lewis rats were immunised with pepM5 or purified rM5 protein, representing a rheumatogenic strain of GAS (M5) which shares 54% and >80% homology with GAS M6 in the B- and C-repeat regions respectively (Fischetti, 1989). PepM5 was not used after two out of four animals given pepM5 failed to recover from anaesthesia in initial experiments. PepM reactogenicity due to mitogenic impurities in the preparation have been previously reported (Fleischer *et al.*, 1992) and may account for the death of the animals.

It is shown here that immunisation with rM5 protein does indeed elicit inflammatory changes in the valves and myocardium that can be detected 21 days post primary immunisation. However, the observed changes were not as marked as those reported by others in myocardial or valvular tissue obtained from RHD patients (Saffitz, 2008). Macroscopically, the hearts from rM5-immunised rats appeared normal at necropsy and Aschoff-like cellular structures, often purported to be pathognomonic for RHD, were not observed in histology sections. The absence of Aschoff-like cellular structures in our rats may be a question of timing and 21 days may not be sufficient for pathognomonic valvular or myocardial lesions to develop completely. Therefore, it may be necessary to extend the time between immunisation and examination of cardiac tissue. Furthermore, we hypothesised that since carditis in patients often manifests following recurrent or prolonged GAS infection, additional immunisations in the form of extra booster injections may be needed to drive the inflammatory autoimmune process. This hypothesis will be tested in the work described in Chapter 8.

Another possible explanation for the relatively milder pathology observed in our rats could be that in patients with established carditis, tissue samples are often only taken for analysis during surgery to repair or replace heart valves or by endomyocardial biopsy (EMB) from patients with suspected carditis. In one human study, all patients demonstrating Aschoff nodules had overt clinical congestive heart failure, however, the sensitivity of EMB to detect Aschoff nodules and histiocytic aggregates in

patients with active rheumatic carditis was reported to be only 27% (Narula *et al.*, 1993). This suggests that EMB sensitivity may be dependent on whether lesions happen to be present in the sampling site or that typical Aschoff nodules are more likely to be present only in severe forms of carditis. Similar biopsy results were reported in 2007 in a Brazilian study following 25 young patients with severe RHD (Sampaio *et al.*, 2007). In that study, Aschoff nodules were detected in only 21% of acute RHD patients. All patients had mitral valve disease, with 32%, 4% and 28% also involving the aortic, tricuspid or all three valves respectively. Around 50% of patients had either valvular neovascularisation or fibrosis, ranging from mild to severe. Patients undergoing valve surgery due to an acute attack of RF usually also presented with myocarditis, although inflammatory reactions in the myocardial fragments analysed were considered as mild in intensity. Notably, despite the presence of interstitial inflammatory cells in the myocardium, myocardial cell damage was not marked (Sampaio *et al.*, 2007).

Whether myocarditis and subsequent heart failure are a direct result of valve damage or if myocarditis itself contributes to RHD has been controversial for some years (Essop *et al.*, 1993; Veasy and Tani, 2005; Mishra *et al.*, 2007). Indeed, myocarditis or pericarditis in the absence of detectable valve involvement is considered not diagnostic for rheumatic carditis (Stollerman, 1997). While some suggest that myocarditis precedes valvulitis, with Kumar *et al.* (1999) stating that rheumatic 'myocarditis can be so severe that resulting cardiac dilation causes functional mitral insufficiency and even congestive heart failure', others believe that mechanical factors such as acute and chronic volume and pressure loading as a result of valvular changes are the major contributors to myocardial damage. In support of this theory, Gentles *et al.* (2001) found that during and after acute RF, development of ventricular contractile dysfunction is closely related to the degree and type of valve regurgitation. Results from Doppler echocardiography on rheumatic carditis patients in conjunction with the absence of elevated cardiac troponin I (cTnI), a sensitive and specific marker of myocardial injury, led Kamblock *et al.* (2003) to concluded that congestive heart failure in RF patients results from mitral regurgitation rather than myocardial involvement. Recovery of acute rheumatic patients with intractable heart failure after valve repair or replacement provides additional support to this proposal (Lewis *et al.*, 1979; Pomerantzeff *et al.*, 2000).

In this thesis, we considered RHD to be primarily a disease affecting the heart valves and therefore deemed inflammatory changes in the valve leaflets, tendinous chords and associated endocardium to be indicative of disease. However, the presence of foci of inflammatory infiltrates in myocardial tissue in rats immunised with rM5 but not in PBS-immunised control rats were also considered as evidence of disease.

Immunostaining of heart valves showed that the majority of infiltrating mononuclear cells was comprised of macrophages expressing the $CD68^+$ lysosomal protein, intermixed with $CD3^+$ T cells. Since the valves did not stain for $CD8^+$ T cells, this would suggest that the $CD3^+$ cells belong to the $CD4^+$ T helper type. Indeed, positive $CD4^+$ staining in frozen sections confirmed that $CD4^+$ T cells did comprise part of the inflammatory infiltrate present in valves of immunised rats. These findings are in agreement with human studies which have shown that RHD is mediated by $CD4^+$ T helper cells and macrophages (Kemeny *et al.*, 1989; Fae *et al.*, 2005). On the other hand, flow cytometry studies have shown that only around 50% of heart-infiltrating $CD3^+$ T cells in a murine model of cardiac myosin-induced myocarditis were of the CD4+ or CD8+ sub-type (Afanasyeva *et al.*, 2004a). This led to speculation that the population of $CD3^+CD4^-CD8^-$ cells may have been a subset of double-negative regulatory T cells, natural killer T cells, or possibly γδ T cells, a subset of T cells shown previously to accumulate in the myocardium of patients with fulminant myocarditis and in coxsackievirus B3-induced myocarditis in mice (Eck *et al.*, 1997). This was not the case in a different study, where all T cell lines generated from heart tissue fragments from RHD patients were αβ TCR^+ and $CD3^+$ and most were of the $CD4^+$ type (Guilherme *et al.*, 2000). We did not investigate whether $CD3^+CD4^-CD8^-$ T cells were present in the hearts of rats used in this study. Due to inferior tissue morphology in frozen tissue, the majority of histological and immunohistochemical analysis was performed using formalin-fixed paraffin-embedded sections which were not conducive to $CD4^+$ staining.

One interesting question raised in the course of this investigation was whether cardiac mast cells play a role in either initiating or driving inflammation in RF/RHD. Upon staining heart sections with toluidine blue, we observed an abundance of intact and degranulated mast cells in the myocardium, perivascularly and associated with

valvular tissue in rat hearts, as well as increased numbers of mast cells in the draining lymph nodes of immunised rats. Mast cells are a source of a number of pre-formed inflammatory molecules including histamine, heparin, proteases and cytokines such as TNF-α and IL-4 as well as inducible cytokines, chemokines and growth or angiogenesis factors (Galli *et al.*, 2005). Cardiac mast cell numbers are known to increase in response to mitral regurgitation in the dog (Stewart *et al.*, 2003) and increased mast cell numbers during an acute response to myocardial volume overload have been shown to be due to maturation and differentiation of a resident population of cardiac mast cells (Forman *et al.*, 2006). Selvaraj *et al.*,(2005) also found a correlation between myocardial fibrosis and mast cell density in a Lewis rat model of myocarditis and a role for mast cells in autoimmunity was the focus of several recent studies (Christy and Brown, 2007). Furthermore, recent studies have revealed an important role for activated mast cells in the maturation and migration of dendritic cells to lymph nodes (Suto *et al.*, 2006) and in enhanced T cell activation (Nakae *et al.*, 2006). To this author's knowledge, a role for mast cells in the pathogenesis of RF/RHD has not been examined. While further investigation into the significance of mast cells in the RAV model of RF/RHD was beyond the scope of this study, this aspect of the inflammatory response presents a possible direction for future studies.

One disappointing outcome of this current study was our failure to generate T cell lines from heart infiltrating T cells (HITC). As fewer cellular infiltrates are present in the hearts of RAV rats compared to the number found in cardiac myosin-induced myocarditis, we found that traditional enzymatic methods were inefficient at extracting enough viable cells to generate T cell lines. Therefore, this particular section of work has not been included in this thesis. However, improved extraction methods, as described by Afanasyeva *et al.*, (2004a), may permit further characterisation of the T cell subsets present in the RAV model by flow cytometry in future studies.

Having established that inflammatory cells are involved in RAV pathology, we next set out to determine T cell specificity in immunised rats. We found that peripheral T cells isolated from spleens of rM5-immunised rats responded strongly to *in vitro* stimulation with rM5 protein but not T cells from control rats. Therefore, we can

conclude that the inflammatory response in the rM5-immunised rats is not a direct effect of the adjuvant. Furthermore, when compared to the T cell response in control rats towards heart proteins cardiac myosin and tropomyosin, the higher response observed in rM5-primed rats lends credit to the notion that molecular mimicry is involved in the autoimmune pathology observed in the RAV model. Other studies have provided compelling evidence that RF/RHD is a T cell-mediated autoimmune condition triggered by molecular mimicry between cardiac proteins and streptococcal M protein (Guilherme *et al.*, 2001c; Fae *et al.*, 2005; Fae *et al.*, 2006). Moreover, although anti-streptococcal antibodies have been shown to be cross-reactive with heart proteins, transfer of immune serum does not result in disease, suggesting that T cells are important in the disease process. Data from the study by Sampaio *et al.*, (2007) supports the findings of others by demonstrating that during the acute phase of RHD there is expansion of predominantly $CD4^+$ anti-streptococcal auto-reactive T cells in the heart, followed by a decline in numbers during the chronic phase. Additionally, this group found that intralesional T cell clones derived from RHD patients recognised the same heart tissue proteins and streptococcal M5 peptides as peripheral T cells, supporting the idea that heart lesions in RHD patients are initiated by streptococcal-primed T cells in the periphery that migrate to the heart and cause damage.

CHAPTER 5
T CELL RESPONSES TO GROUP A STREPTOCOCCAL M5 PEPTIDES

5.1 INTRODUCTION

Experiments described in Chapter 4 of this thesis showed that Lewis rats mounted a cellular immune response following immunisation with recombinant streptococcal M5 (rM5) protein. This result was predicted based on reports by others who have previously demonstrated a T cell response in Lewis rats immunised with streptococcal rM6 protein (Quinn *et al.*, 2001; Lymbury *et al.*, 2003). In a recent study, immunisation with formalin-killed group A streptococci (GAS) in complete Freund's adjuvant induced inflammatory changes in Lewis rats, demonstrated by clinical signs of arthritis, histological evidence of carditis and increased anti-myocardial IgG antibodies. However, the strain and M protein status of the GAS used in the study was not reported and T cell responses were not examined (Huang *et al.*, 2009).

It is now generally accepted that many chronic autoimmune conditions involve autoreactive T cells which, having escaped negative selection in the thymus, could induce pathology. A role for autoreactive T cell-mediated responses in the development of RF/RHD has already been well-established in several previous studies (Raizada *et al.*, 1983; Dale and Beachey, 1987; Pruksakorn *et al.*, 1994b; Guilherme *et al.*, 1995; Guilherme *et al.*, 2001c). Previous studies have also defined cross-reactive T cell epitopes in GAS M protein (Table 5.1) which may be responsible for triggering the deleterious response against myosin in cardiac tissue.

Some GAS strains have a predilection for colonising the mucous membranes of the respiratory tract and others for the skin. On encounter with GAS, the innate immune system mobilises to abolish or contain the infection and also to activate and direct an adaptive immune response against the bacteria. An important feature of this adaptive response is that of immunological memory whereby lymphocytes previously exposed to a specific pathogen persist long term in an individual. Following

re-exposure to the same pathogen, these lymphocytes are able to rapidly proliferate and mount an enhanced response to efficiently clear the pathogen. It is this principle that underlies most current vaccine strategies, including those being developed against GAS infection based on the M protein.

Table 5.1 Summary of streptococcal M5 peptides containing T cell epitopes cross-reactive with myosin and heart tissue

Peptide (amino acids)	Sequence	Reference
B1B2 (137-154)	VKDKIAKEQENKETIGTL [a]	Cunningham *et al*., 1997
B2 (150-167)	TIGTLKKILDETVKDKIA [a]	Cunningham *et al*., 1997
B3A (176-193)	IGTLKKILDETVKDKLAK [a]	Cunningham *et al*., 1997
C2A (254-271)	EASRKGLRRDLDASREAK [a]	Cunningham *et al*., 1997
C3 (293-308)	KGLRRDLDASREAKKQ [a]	Cunningham *et al*., 1997
p145 (337-357)	LRRDLDASREAKKQVEKALE [a]	Pruksakorn *et al*., 1994
M5 (163-177)	ETIGTLKKILDETVK [b]	Fae *et al.*, 2006

Amino acid positions and sequences taken from the M5 protein sequences reported by [a] Miller *et al.*, (1988) and [b] Manjula *et al.*, (1985). Amino acid sequences that are identical are highlighted in either blue or red.

The work described in this section was designed to investigate the immunogenicity of peptides from M5 protein. The particular peptides chosen are located in the B-repeat region and C-repeat region of the protein and several have been implicated in the development of carditis by other studies (Manjula *et al.*, 1985; Cunningham and Quinn, 1997; Fae *et al.*, 2006). The purpose of these experiments was to determine whether peptides from the variable B-region or conserved C-region of M5 protein contain immunodominant or sub-dominant T cell epitopes and more importantly, whether these peptides cross-react with myocardial epitopes in the RAV model.

5.1.1 Aims

The aim of the work described herein was conducted to further define the role of GAS M protein in development of carditis in the RAV model by identifying epitopes that induce a T cell response and/or cause myocardial lesions.

The specific aims were to:-

1) Induce valvulitis in Lewis rats by immunising with peptides derived from the B and C regions of GAS M protein
2) Examine heart valves and myocardial tissue from immunised rats for signs of pathology
3) Determine the specificity and reactivity of lymphocytes from heart lesions and the periphery of immunised rats to streptococcal M5 protein-derived peptides and their cross-reactivity to other proteins found in the heart.

5.2 MATERIALS AND METHODS

5.2.1 rM5 protein

The method of preparing Group A streptococcal recombinant M5 protein (rM5) used in this section of work is described in Chapter 4 of this thesis.

5.2.2 M5 peptides

Overlapping 20-mer peptides spanning parts of the B and C repeat regions of the M5 protein were synthesised at the Queensland Institute of Medical Research (QIMR), Brisbane, Australia by solid-phase peptide synthesis using Boc (*tert*-butoxycarbonyl) chemistry as previously described (Houghten, 1985), and contained a free amine on the N-terminus and a free amide on the C-terminus. The location and sequences of the peptides, designated M5-B.1 to M5-B.6 and M5-C.7 to M5-C.10 are shown in Table 5.2 and Figure 5.1 respectively. Peptides were assessed for purity by high performance liquid chromatography (HPLC), lyophilised and stored at -20 °C. Prior

to use, the peptides were reconstituted in sterile ddH_2O to 5 mg/ml. While the heptad periodicity of hydrophobic residues in M protein confers a tendency to form α-helical coiled-coils and a conformational epitope has been reported within peptide 'LRRDLDASREAKKQVEKALE' (M5-C.8, aa 337-356) (Relf *et al.*, 1996), secondary structures were not determined for the peptides used in this study.

Table 5.2 M5 peptides and sequences used in this study[a]

Peptide	Amino acid sequence	Position	Region	Repeat [b]
M5-B.1	TRQELANKQQESKENEKALN	153-172	B	B1A
M5-B.2	ESKENEKALNELLEKTVKDK	163-182	B	B1B
M5-B.3	ELLEKTVKDKIAKEQENKET	173-192	B	B1-B2
M5-B.4	IAKEQENKETIGTLKKILDE	183-202	B	B2
M5-B.5	IGTLKKILDETVKDKIAKEQ	193-212	B	B2-B3
M5-B.6	TVKDKIAKEQENKETIGTIK	203-222	B	B3
M5-C.7	NKISEASRKGLRRDLDASRE	327-346	C	C3A
M5-C.8	LRRDLDASREAKKQVEKALE	337-356	C	C3B
M5-C.9	AKKQVEKALEEANSLKAALE	347-366	C	C3C
M5-C.10	EANSKLAALEKLNKELEESK	357-376	C+	C3+

[a] Sequence and amino acid position taken from the M5 sequence reported by Whatmore and Kehoe (1994). [b] Repeat designation as per Miller *et al.*, (1988)

5.2.3 M5 peptide-induced valvulitis

Immunisation of female Lewis rats (n=3 or 4 per group) was carried out under anaesthesia using the method described in Chapter 3. Individual peptides, pools of peptides or rM5 protein were suspended in sterile PBS to a volume of 100 µl and emulsified 1:1 with either Freund's complete adjuvant (FCA) (Day 0, priming dose, in rear foot) or Freund's incomplete adjuvant (FIA) (Day 7, booster, in flank) and administered subcutaneously as per Table 5.3. Control rats were injected with PBS and adjuvant only. On Days 1 and Day 3, $1x10^{10}$ whole formalin-killed *B. pertussis* cells were injected intraperitoneally as an additional adjuvant to enhance the immune response. On Day 21 following primary immunisation (21 p.i.), the animals were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Under aseptic conditions, hearts, spleens and blood serum was harvested for analyses.

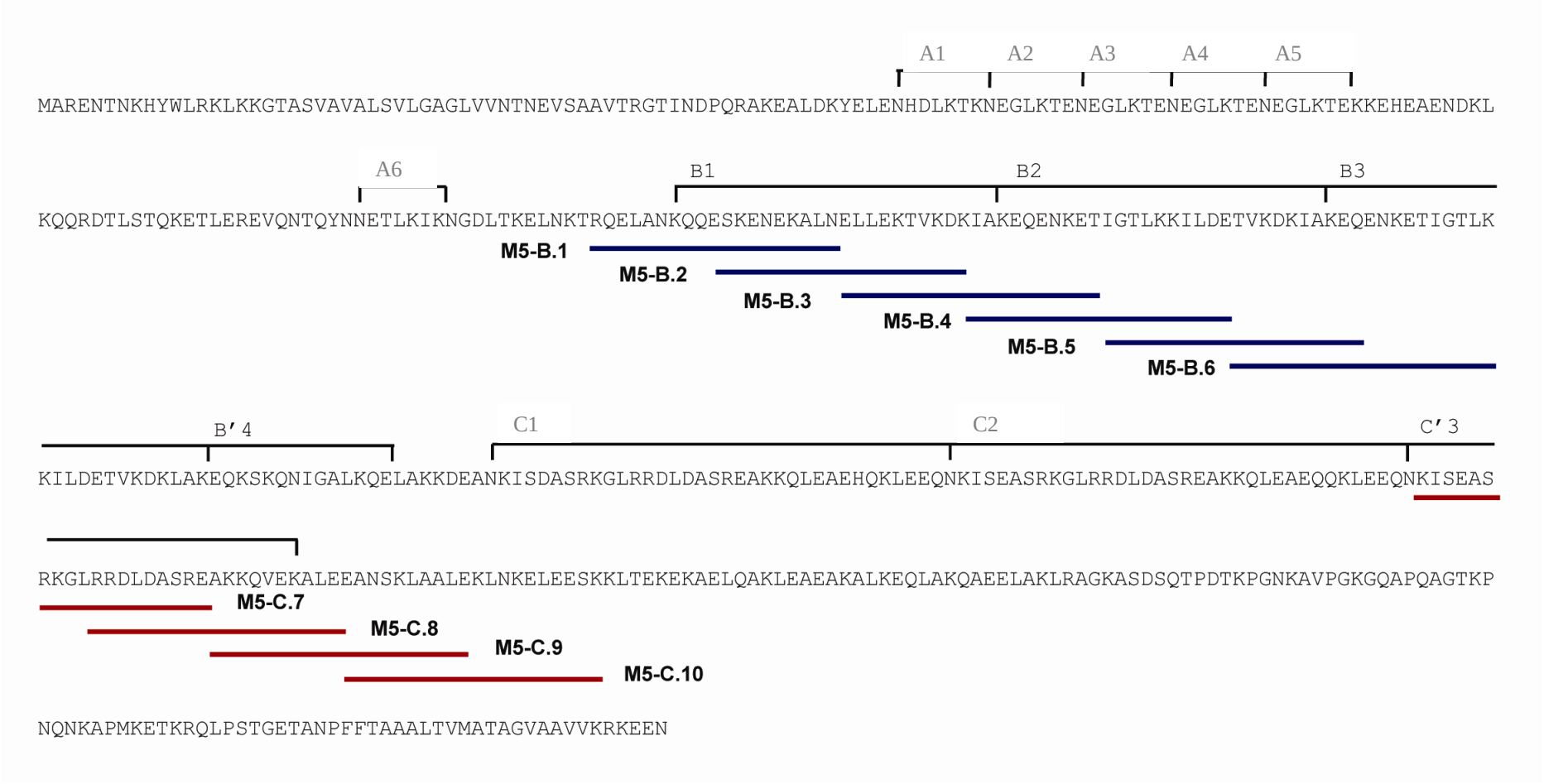


Figure 5.1 Locations of the overlapping 20-mer GAS M5 protein peptides used in this study. M5 sequence taken from Miller *et al.*,(1988).

Table 5.3 Pooled peptide antigens and control antigens used to immunise rats

Antigen	Antigen per rat (µg)	Antigen volume per rat (µl)	Adjuvant volume (µl)	Total injection volume (µl)
Single peptide	500 µg peptide	100	100	200
Pooled peptides (M5-B.1-M5-B.3)	150 µg per peptide	100	100	200
Pooled peptides (M5-B.4-M5-B.6)	150 µg per peptide	100	100	200
Pooled peptides (M5-C.7-M5-C.10)	150 µg per peptide	100	100	200
rM5	500 µg	100	100	200
PBS only	-	100	100	200

5.2.4 Detection of carditis in M5-peptide immunised rats

Sections (4-7 µm) of formalin fixed paraffin embedded hearts (FFPE) from normal, non-immunised and rats immunised with rM5, M5 protein-derived peptides or PBS were stained with H&E and examined by light microscopy. Evidence of myocarditis and valvulitis included infiltration of mononuclear cells and polymorphonuclear cells, fibrosis (scarring) and neovascularisation of valve tissue. Immunohistochemistry, using antibodies as described in Chapter 3 against T cell markers CD3 and CD8 and against macrophage lysosomal membrane molecule CD68, was performed on FFPE sections of hearts to phenotype cellular infiltrates. Anti-CD4 antibody was used on frozen sections of rat hearts to determine the presence of $CD4^+$ T helper cells. Immunohistochemical methods have been described previously in Chapter 4.

5.2.5 T cell responses in peptide-immunised rats

The antigen-specific responses of rats immunised with M5 peptides, PBS control rats or normal, non-immunised rats were measured by tritiated thymidine incorporation in a lymphocyte proliferation assay as described in Chapter 3 General Materials and Methods (Section *3.2.6.5*). In brief, spleen MNC were plated in U96-well culture plates in triplicate at 10^5 cells/well in a total volume of 200 µl of culture medium (Appendix 1) and stimulated with 10 µl of various antigens. The different antigens and previously optimised concentrations used to stimulate the cells are outlined in Table 5.4. Cells in positive control wells were stimulated with the T cell mitogen Con A and cells in negative control wells were left unstimulated. The cells were cultured for 3 to 5 days and pulsed with 1.25 µCi/ml of ^{3}H-thymidine for the final 4 h of culture. Proliferative response to antigenic stimulation was determined as the ratio of counts per minute (cpm) of stimulated cells to cpm of unstimulated cells, reported as stimulation index (SI). Although SI values as low as 2.0 have been reported as positive for peptide re-stimulation (Iwai *et al.*, 2007), in this study we chose an SI $\geq$ 3.0 as representing a positive response.

Table 5.4 Antigens used to stimulate lymphocytes derived from spleens of peptide-immunised rats

Antigen	Concentration
Individual peptides (M5-B.1 to M5-C.10)	10 µg/ml
rM5	50 µg/ml
Cardiac myosin	30 µg/ml
Collagen IV	30 µg/ml
PPD	15 µg/ml
Con A	5 µg/ml

5.3 RESULTS

5.3.1 rM5-sensitised T cells recognise a B-region epitope in rM5

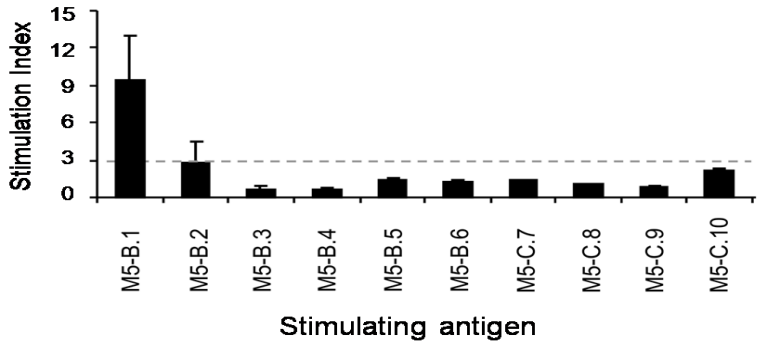
As demonstrated in Chapter 4, a recombinant M protein derived from a rheumatogenic strain of GAS (rM5) induces autoimmune valvulitis in Lewis rats, confirming previous findings by Quinn *et al.* (2001). In order to identify T cell epitopes in rM5, we examined the proliferative response of splenic T cells from rM5-immunsed rats to a panel of peptides that span sections of the B-repeat and C-repeat regions of M5. These regions were selected because they have previously been implicated in the development of carditis in humans and murine models of RF/RHD (Robinson *et al.*, 1991; Guilherme *et al.*, 1995; Cunningham *et al.*, 1997).

In Figure 5.2a it is shown that only one B-region peptide (M5-B.1, TRQELANKQQESKENEKALN) induced significant proliferation (SI = 9.5 ± 3.5) in rM5-primed cells. A B-region peptide which overlaps M5-B.1 by ten amino acids (M5-B.2, ESKENEKALNELLEKTVKDK) (overlapping sequence is underlined), resulted in a minor but non-significant response (SI = 2.8 ± 1.8).

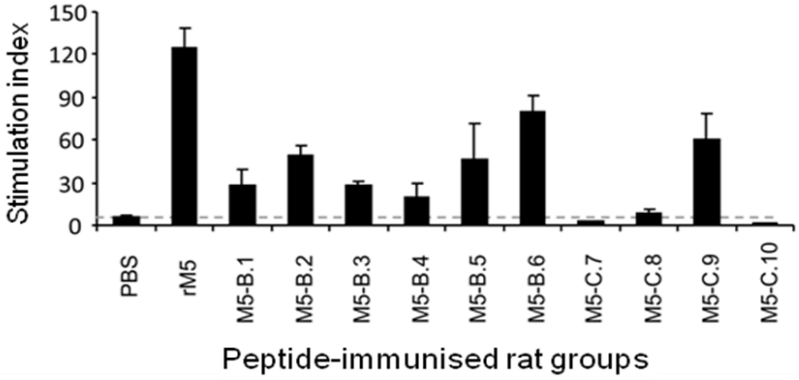
5.3.2 Proliferative response of lymphocytes from peptide-immunised rats

In another set of experiments to further define T cell epitopes involved in carditis in the RAV model, rats were immunised with either pooled peptides or individual peptides (Table 5.3) as described in Materials and Methods (Section *5.2.3*).

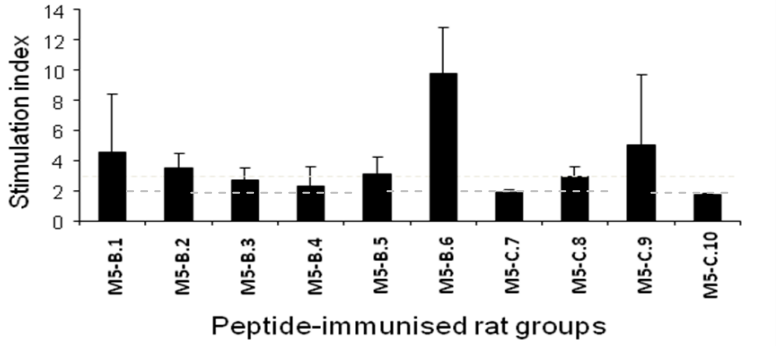
Out of the rats (n=3-4 per group) immunised with individual peptides (n=10), lymphocytes from of all but two groups (M5-C.7 and M5-C.10) recognised epitopes in full-length rM5 protein, producing a strong proliferative response (SI >15) (Figure 5.2b). A lower but nonetheless significant response against rM5 was also observed in rats immunised with C-region peptide M5-C.8 (SI = 8.4 ± 2.5). When stimulated with the original immunising peptide (Figure 5.2c), five of the 10 peptide groups (M5-B.1, M5-B.2, M5-B.5, M5-B.6 and M5-C.9) had a significant recall response (SI ≥ 3).



a. Responses of rM5-immunised rats to individual peptides



b. Responses of peptide-immunised rats to rM5



c. Responses of peptide-immunised rats to original peptide

Figure 5.2 T cell proliferative responses in Lewis rats (n=4 per group). Proliferative response is reported as stimulation index ± SEM, determined as cpm of stimulated cells/cpm unstimulated cells in triplicate wells. Dashed line indicates significant proliferative response (SI=3).

To test the hypothesis that the carditis observed in the RAV model is associated with a self-reactive autoimmune response towards cardiac tissue proteins, lymphocytes derived from the spleens of rM5-immunised and peptide-immunised rats were stimulated *in vitro* with collagen IV and cardiac myosin, two proteins found in heart tissue and previously implicated in RF/RHD (Dinkla *et al.*, 2003; Cunningham, 2004; Fae *et al.*, 2006; Guilherme and Cunningham, 2006).

Figure 5.3 shows that lymphocytes from rM5-immunised rats responded strongly to collagen IV (SI=10.4 ± 3.8). Four rat groups immunised with B-region peptides and one group immunised with C-region peptides responded to collagen IV. These were M5-B.2 (SI ± S.E.M. = 4.5), M5-B.3 (3.5 ± 0.9), M5-B.5 (4.0 ± 1.2), M5-B.6 (3.5 ± 2.4) and M5-C.8 (3.2 ± 1.1).

Only one group (M5-B.6-immunised) responded significantly to cardiac myosin. Control rats immunised with PBS in adjuvant and normal, non-immunised control rats did not respond to either collagen or cardiac myosin.

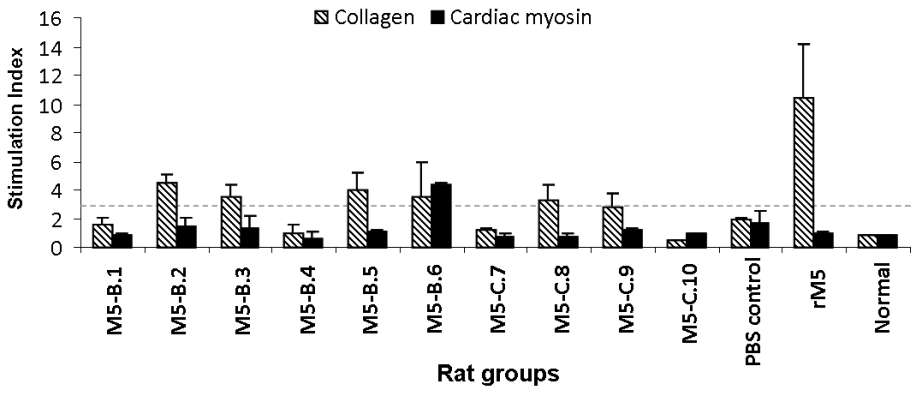


Figure 5.3 Proliferative responses of lymphocytes from rats immunised with M5-peptide, PBS or rM5 or normal, non-immunised rats (n=3-4 per group) to stimulation with collagen or cardiac myosin.
Dashed line indicates significant proliferative response (SI ≥ 3).

5.3.3 Peptide sequence M5-B.6 contains a strong T cell and pathogenic epitope

Lymphocytes derived from the spleens of rats immunised with peptide M5-B.6 (TVKDKIAKEQENKETIGTIK), representing amino acid residues 161-180 of M5 protein B-repeat region, also recognised tissue proteins collagen type IV (SI= 3.5 ± 2.4) and cardiac myosin (SI = 4.4 ± 0.1) (Figure 5.4). In contrast, no positive reactivity with cardiac myosin was demonstrated by any of the other peptide groups, with SI values of 2.1 or less (data not shown).

Furthermore, cardiac tissue from one of three rats immunised with peptide M5-B.6 also had evidence of pathology. Frozen sections of heart tissue from this rat stained positive for CD4 and CD68 (Figure 5.5), indicating that inflammatory T cells and macrophages infiltrated the heart valves and myocardium.

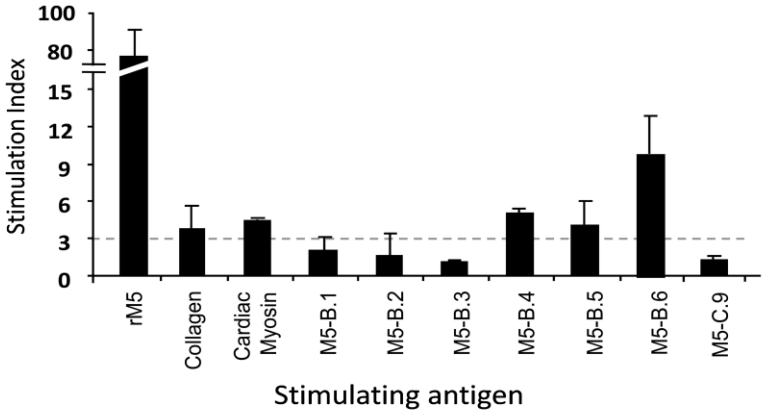


Figure 5.4 Responses of T lymphocytes from M5-B.6-immunised rats.
T cells from M5-B.6-immunised rats (n=3) proliferated in response to M5-B.6 and other peptides and selected host proteins. Only one C-region peptide (M5-C.9) was available for this assay. Proliferative response is reported as stimulation index. Error bars represent SEM. Dashed line indicates significant proliferative response (SI ≥ 3).

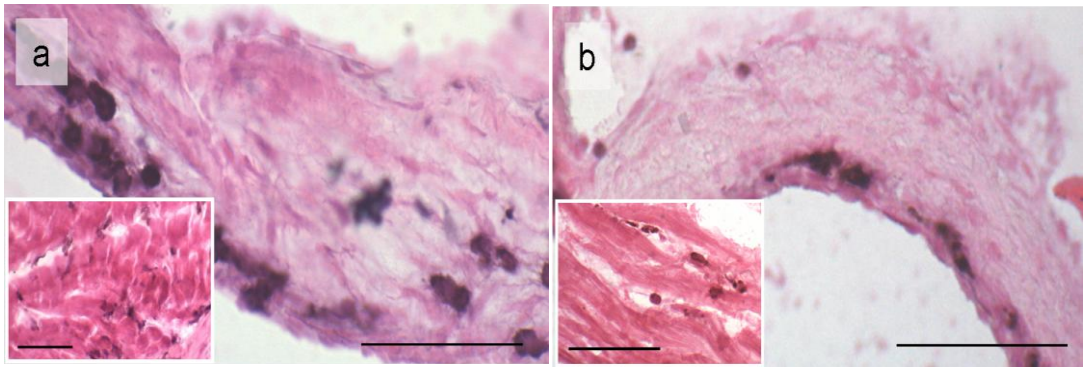


Figure 5.5 Inflammatory cells in heart tissue from an M5-B.6-immunised rat. a) $CD4^+$ T cells and b) $CD68^+$ macrophages in the valves and myocardium (inset) in a rat immunised with streptococcal M protein peptide M5-B.6. Chromagen is nickel-enhanced DAB. *Bars = 50 μm*

5.4 DISCUSSION

The ability to mount specific responses against 'foreign' pathogens while ignoring 'self' is a defining characteristic of the adaptive immune response. However, this system is not perfect and the vast field of research dedicated to trying to unravel the mechanisms involved in autoimmune diseases attests to its complexity. Active immunisation against bacteria such as GAS can be elicited by vaccination with antigens from the bacterium. M protein from GAS is highly immunogenic and has been targeted by many research groups as a likely vaccine candidate.

Development of a vaccine against GAS has been slow, however, due in part to a lack of a clear understanding of why infection with this bacterium can lead to RF/RHD in some individuals. Concerns that anti-GAS vaccines may have the potential to trigger autoimmune disease in vaccinees have not yet been fully addressed. A vaccine should not only induce a protective cellular or antibody response, it should not itself cause disease. For this reason, the selection of which antigens to incorporate into a vaccine formulation is critical to its efficacy. Ideally, any anti-GAS vaccine should contain not only a B cell epitope to induce production of protective antibodies, but

also epitopes that activate $CD4^+$ T helper type 2 (Th2) cells to facilitate the long term B cell response in the form of antibody producing plasma cells.

The requirement for a broad-based vaccine that could provide protection against a large range of GAS serotypes makes the M protein conserved region an attractive choice for sub-unit vaccine research. Studies have demonstrated C-region specific opsonic antibodies in populations in GAS endemic regions (Brandt *et al.*, 1999) and in mice immunised with C-region peptides (Pruksakorn *et al.*, 1992). However, studies in mice (Pruksakorn *et al.*, 1994) and the RAV model (Lymbury *et al.*, 2003) suggest that the C-region may also contain T cell epitopes that may contribute to the pathogenesis of RF/RHD.

In Chapter 4 of this thesis, it was established that GAS rM5 protein not only induces strong T cell responses in the RAV model but more importantly is carditogenic, producing changes in myocardium and heart valves reflective of RF/RHD. However, evidence from human studies implicate distinct T cell epitopes within GAS M protein with the pathogenesis of rheumatic carditis (Pruksakorn *et al.*, 1994; Cunningham *et al.*, 1997; Fae *et al.*, 2006). To further validate that the RAV model is appropriate for studying RF/RHD immunopathogenesis, it was therefore necessary to demonstrate M protein peptide-induced valvulitis in Lewis rats.

We therefore set out to determine whether selected peptides from the variable B-repeat region and conserved C-repeat region of M5 protein, some of which are either currently the focus of anti-GAS vaccine research (Guilherme *et al.*, 2006; Pandey *et al.*, 2009) or which have previously been shown to be associated with development of RF/RHD, were also capable of eliciting an immune response and/or carditis in this rat model.

When we examined the ability of peptides to stimulate proliferation of lymphocytes from peptide-immunised rats, peptide M5-B.6 (TVKDKIAKEQENKETIGTIK) was found to contain a strong T cell epitope as well as a myosin-cross-reactive epitope. Since M5-B.6 was not recognised by rM5-primed lymphocytes cells, it is therefore deemed to contain a subdominant or cryptic epitope (Sercarz *et al.*, 1993). We consider the cross-recognition seen in M5-B.6-sensitised T cells towards cardiac

myosin as significant. While recognition of 'foreign' antigen by peripheral T cells is readily observed *ex vivo*, cross-recognition of 'self' antigen is more difficult to demonstrate. T cells with high affinity for self antigens are usually removed from the repertoire during intrathymic deletion; those that escape to the periphery most likely have low to intermediate affinity for self antigens (Barnaba and Sinigaglia, 1997). Auto-reactive T and B cells can be isolated from the periphery of healthy individuals who present no signs of autoimmunity (Diamond, 2005; Moudgil and Sercarz, 2005; Anderton, 2006). This includes auto-antibodies and auto-reactive T cells with the ability to recognise cardiac myosin (Pruksakorn *et al.*, 1994b). Cryptic epitopes in human cardiac myosin have been shown to induce T cell-mediated myocarditis in Lewis rats (Li *et al.*, 2004) and may also be involved in coxsackievirus B3-induced myocarditis (Huber, 2006).

Under normal conditions, these autoreactive lymphocytes are kept in check by peripheral tolerance, including mechanisms which render the cells anergic towards self-proteins or by regulatory T cells which act to suppress response to self (Walker and Abbas, 2002). Certain events or conditions such as inflammation may 'break tolerance' leading to immune cell recognition of self tissue and resulting in systemic or organ-specific autoimmune disease.

Ellis *et al* (2005) demonstrated significant cardiac myosin cross-reactivity in T cell clones derived from RHD patients. These clones had a 100-fold higher sensitivity to streptococcal M protein than to cardiac myosin when measured in dose response studies. The amino acid sequence for M5-B.6, identified in this current study as containing a potential cardiac myosin cross-reactive epitope, lies within the region reported by others to contain a strong myosin cross-reactive epitope recognised by T cells from ARF patients (Guilherme *et al.*, 1995) and BALB/c mice (Cunningham and Quinn, 1997). The demonstration that M5-B.6 causes cardiac lesions in the RAV model provides further evidence that this peptide may contain a T cell epitope that triggers the myocardial damage observed in RHD.

Only one peptide (M5-B.1) out of the 10 examined in this study elicited a significant T cell response in rM5-primed rat spleen cells. Sercarz (1993) defines an immunodominant epitope as one that induces a 'strong T cell response upon

immunisation of a native antigen in adjuvant'; therefore, a dominant T cell epitope is present in peptide M5-B.1 (TRQELANKQQESKENEKALN). The absence of cardiac lesions in M5-B.1-immunised rats suggests however, that this epitope may not be adequate to cause cardiac damage, although an almost identical M5 peptide, B1A (TRQELANKQQESKENEKAL), was shown to induce mild cardiac lesions in BALB/c mice (Cunningham *et al.*, 1997). Cunningham *et al*., (1997) also noted that immunodominant M5 peptides that consistently produced strong proliferative responses in T cells from BALB/c mice were different from those peptides that induced heart lesions. It is often found that T cells taken from animals that have been immunised with whole protein show minimal proliferative responses when stimulated with individual peptides spanning the whole protein (Gammon *et al.*, 1990; Miyakoshi *et al.*, 2003). This may be attributed to the low frequencies of T cells in the periphery that respond to each individual peptide, whereas multiple dominant epitopes present in M5 protein induce a high proliferative response to the whole protein.

The disparity between immunodominance and carditogenic potential as observed with peptide M5-B.1 in this study is consistent with the concept, described above, that cross-reactive T cell epitopes are more likely to be subdominant or cryptic in nature. Further studies such as cytokine analysis of M5-B.1-primed T cells may be able to establish if the immunodominant epitope in this peptide is associated with a polarised Th1/Th2 response. Inflammatory Th1 responses, characterised by cytokines interferon-γ (IFN-γ) tumour-necrosis factor-α (TNF-α) and interleukins 2 and 6 (IL-2, IL-6) have been implicated in RF/RHD in human studies (Fraser *et al.*, 1997; Hernandez-Pacheco *et al.*, 2003b; Guilherme *et al.*, 2004).

Of the rat groups immunised with individual peptides, all B-region peptide-immunised rat groups but only one C-region-immunised rat group (M5-C.9) responded very strongly to the entire rM5 protein (Figure 5.2b). These results contrast with those obtained by one study carried out in B10.A mice to define T cell determinants in hen egg lysozyme (HEL) (Gammon *et al.*, 1990). In that study, lower responses to whole HEL than to recall peptide were observed in T cells from peptide-immunised mice. It was proposed that inefficient *in vitro* processing of the HEL protein into smaller fragments for antigen presentation was responsible for that result. Gammon *et al.* (1990) also reported marked variability between syngeneic

animals in T cell 'fine specificity patterns' examined in primary cell cultures and in T cell lines derived from HEL-primed mice.

In our experiments, the very high stimulation indices obtained in peptide-primed rat cells following rM5 stimulation may be due to a high frequency of peptide-specific T cells induced in these rats. It is also feasible that a loss of viability in unstimulated control cells during the culture period resulted in artificially inflated SI values. On the other hand, lymphocytes from the same peptide-immunised rats displayed lower proliferative responses to recall peptide with SI values < 10 (Figure 5.2c), suggesting that the population of T cells capable of responding to rM5 was indeed higher in rats immunised with M5 peptides. While it is plausible that the rM5 antigen prepared for this study was contaminated with superantigens or mitogenic factors such as LPS, this was considered unlikely. No (or minimal) LPS was detected in rM5 by *Limulus* Amoebocyte Lysate (LAL) assay (Section *4.2.3.2*) and more compelling, no response to rM5 was observed in lymphocytes from normal, non-immunised rats.

It is a widely held view that molecular mimicry, also known as epitope mimicry (Davies, 1997), whereby antigenic determinants on infectious micro-organisms resemble antigenic determinants on host tissue, is a key factor in the induction of many autoimmune diseases (Oldstone, 1998). Molecular mimicry may occur between epitopes of similar conformation (Oldstone, 1998; Lovitch and Unanue, 2005), linear epitopes with primary sequence homologies (Guilherme and Cunningham, 2006) or epitopes with limited sequence similarities (Evavold *et al.*, 1995), precipitating cross-reactivity at the B cell or T cell level. Substantial support for the role of molecular mimicry is provided by a strong correlation between microbial infections and autoimmune diseases including *Trypanazoma cruzi* infection and Chagas disease (Cunha-Neto *et al.*, 2006), *Borrelia burgdorferi* and Lyme disease (Sigal, 1997), herpes simplex virus and myasthenia gravis (Schwimmbeck *et al.*, 1989) and coxsackievirus and myocarditis (Gauntt *et al.*, 1995), as well as *Streptococcus pyogenes* and RF/RHD.

In addition, degenerate TCR reactivity, where a single T cell can recognise more than one peptide sequence presented by APC, has been demonstrated in several autoimmune diseases (Gran *et al.*, 1999; Martin *et al.*, 2001; Fae *et al.*, 2005; Cunha-

Neto *et al.*, 2006) and provides further insights into the role of molecular mimicry in autoimmunity. For instance, altered peptide ligands (APLs), in which one or two TCR contact sites have been altered can act to either suppress (Ruiz *et al.*, 1999) or enhance (Tang *et al.*, 2007) immunogenicity of a determinant. These APLs have also been shown to induce partial T cell activation, mediating some T cell functionality such as cytokine secretion but without inducing proliferation (Sloan-Lancaster and Allen, 1995; Cao and Braciale, 1996). Moreover, peptide fragments processed from infectious agents may be presented on MHC molecules in a conformation that resembles self proteins (Lovitch and Unanue, 2005). This molecular mimicry and T cell degeneracy may account for the cross-recognition observed between GAS M protein and some host proteins such as cardiac myosin, tropomyosin and laminin, which have similar α-helical coiled-coil structures or similar sequence homology. In this body of work, M5-specific T cells also recognised collagen type IV. However, whether this response is significant in the context of RF/RHD requires further investigation. Collagen IV α-1 and α-5 chains are expressed in high levels in basal laminae in the heart and are associated with laminin (Miner and Sanes, 1994), another α-helical coiled-coil protein that may be involved in recruiting auto-reactive T cells to valvular endothelium in RF/RHD (Cunningham *et al.*, 1993; Antone *et al.*, 1997) .

The work described in this chapter demonstrates that peptides from GAS M protein have the capacity to induce a peptide-specific T cell response in the RAV model. In addition, peptide M5-B.6 from the variable B region of M protein (amino acid sequence TVKDKIAKEQENKETIGTIK) most likely contains a subdominant or cryptic epitope that cross-reacts with cardiac myosin, a potent auto-antigen implicated in the pathogenesis of carditis in RF/RHD patients.

An important caveat in using an animal model to identify cross-reactive GAS epitopes with carditogenic potential, is that the immunological distance between rats or other rodents and humans prevents a direct translation of results between the two species. That is, epitopes that cause carditis in the rat model may not necessarily be carditogenic in the human context. Moreover, since presentation of immunodominant and cryptic epitopes to T cells is influenced by a number of factors

including MHC haplotype, site of immunisation and adjuvant used and thus APC processing, the cross-reactive peptide identified in this study may be relevant only in the RAV model. Notwithstanding this observation, the work described in this chapter provides further evidence that the RAV model displays appropriate immunological responses which parallel those seen in RF/RHD patients.

It is however, important that T cell cross-reactivity is more fully characterised in this model. One possible direction for future work would be to expand the range of M5 peptides used in order to map T cell epitopes in more detail. In addition, T cell lines and T cell clones derived from peptide-immunised rats need to be established before fine T cell specificity, phenotyping or cytokine analysis can be examined. In our current work, all attempts to establish T cell lines were unsuccessful, most likely due to sub-optimal culture conditions. One observation made during this study was that rat lymphocytes appear to be less 'robust' than human or murine lymphocytes; they are more fastidious in their culture requirements and more susceptible to loss of viability. Therefore, to address these limitations in our current data, T cell lines/clones should be investigated in future work.

CHAPTER 6
B CELL RESPONSES IN THE RAT AUTOIMMUNE VALVULITIS MODEL

6.1 INTRODUCTION

Antibodies provide protection from extracellular pathogens or microbial toxins by several effector mechanisms. The three most important functions of antibody are:- 1) neutralisation of pathogens or their toxins by preventing their adherence to, or entry into host cells, 2) antibody-dependent cellular cytotoxicity and 3) enhancing destruction of pathogens by phagocytes which recognise the F_C portion of antibodies coating the pathogen or by activating deposition of complement onto pathogens, a process known as opsonisation (Murphy *et al.*, 2008).

The humoral arm of the adaptive immune system produces antibodies against a range of antigenic GAS components including streptolysin-O (SLO), DNase B, streptokinase, C5a peptidase and M protein which can be readily detected in patient serum following GAS infection (Cunningham, 2000). Elevated SLO and DNase B titres are still the standard tests for serodiagnosis of preceding GAS infection. While early infection is dominated by an IgM antibody response, proliferation and differentiation of antigen-specific B cells in conjunction with somatic hypermutation and antibody isotope class switching leads to production of high affinity IgG and IgA antibodies which characterise the adaptive response.

The specific immune response to different serotypes of GAS is directed against the hypervariable N-terminal region of M protein. Therefore, exposure to one serotype does not lead to broad protection from infection by the other 100 or so other serotypes. The M protein has been shown to confer protection against bacterial challenge in murine (Batzloff *et al.*, 2003; Bruner *et al.*, 2003) and human (D'Alessandri *et al.*, 1978; Hu *et al.*, 2002) immunisation trials. Brandt *et al.*, (2000a) have shown induction of opsonising antibodies in mice following immunisation with N-terminal M protein peptides conjugated to tetanus toxoid. Bruner *et al.* (2003) have also shown that synthetic, unconjugated N-terminal M protein peptides can elicit a functional antibody response in mice and significantly

reduced nasopharyngeal colonisation following subcutaneous immunisation. The latter study also demonstrated limited cross-opsonisation and cross-protection between heterologous M serotypes using N-terminal peptides, possibly due to some degree of sequence or structural similarity between peptides.

Recent studies have also identified conserved regions in the C-repeat region of M protein that elicit protective antibodies in RF patients (Bessen *et al.*, 1995; Brandt *et al.*, 1996; Brandt *et al.*, 1999; Vohra *et al.*, 2005) and in mice (Bessen and Fischetti, 1990; Batzloff *et al.*, 2004).

In the series of experiments described in this chapter, sera from rM5-immunised rats were first tested for functional activity against GAS M5 bacteria using a standard bactericidal assay. The immunoreactivity of recombinant streptococcal M5 (rM5) protein and synthetic peptides derived from the B-repeat region and C-repeat region was determined by enzyme-linked immunabsorbant assay (ELISA).

The work described herein was carried out in order to study the humoral response in the RAV model following immunisation with recombinant M5 protein or the selected M5 protein peptides described in previous chapters.

6.1.1 Aims

The aims of the work described here were to:-

1. Determine the immunogenicity of rM5 protein by measuring total IgG antibody titres in sera from rM5-immunised and control rats using an ELISA
2. Determine the immunogenicity of selected M5 peptides by measuring total IgG antibody titres in sera from rM5-immunised and control rats
3. Demonstrate opsonic antibodies against M5 GAS in serum from rats immunised with rM5 in a standard indirect bactericidal test (IBT).

6.2 MATERIAL AND METHODS

6.2.1 Bacteria

Preparation and storage of *S. pyogenes* serotype M5 is described in Section *3.1.3.1*. A single colony picked from SBA was grown overnight in 5% CO_2 at 37 °C in 5 ml of Todd-Hewitt broth supplemented with 0.2% yeast (THYB). Two hundred microlitres of overnight culture was sub-inoculated into 5 ml of fresh, warm THYB and incubated for 2 to 4 h until OD_{600} ~ 0.12 or until logarithmic phase as assessed by kinetic growth pattern. Bacteria were then serially diluted to 10^{-4} in sterile PBS and 50 µl was immediately plated on SBA for overnight incubation to determine colony-forming units (cfu). Diluted bacteria were kept on ice until used.

6.2.2 Antigens and reagents

Recombinant M5 protein and rat cardiac myosin were prepared according to the protocols described in Chapter 4 Materials and Methods. Peptides (20-mer) derived from streptococcal M5 protein from the B-repeat region and C-repeat region of M5 protein are identical to those described in Chapter 5 and are summarised in Table 6.1. Porcine cardiac myosin, collagen type IV and tropomyosin were purchased from *Sigma*. Bicarbonate coating buffer (pH 9.6), Post-coating (blocking) buffer, antibody diluent and ABTS (2,2'-azino-bis(3-ethylbenzthiazioline-6-sulfonate) were all purchased from *TropBio*. Goat anti-human peroxidise-conjugated IgG and goat anti-rat peroxidase-conjugated IgG (*Jackson Immunoresearch*) were a gift from Dr Jan Smith, James Cook University, Townsville.

6.2.3 Source of rat antisera for antibody assays

Six to ten week old Lewis rats were immunised subcutaneously with 500 µg rM5 or M5 peptide emulsified in FCA and boosted seven days later as outlined in Chapter 4. On day 21 post immunisation (p.i.) rats were euthanased by CO_2 asphyxiation. Blood was collected either by terminal cardiac puncture during euthanasia or soon after from the chest cavity after snipping the vena cava. Blood was left to clot before centrifuging at 200 x *g* for 20 min to harvest serum, which was then stored at -20 °C.

Table 6.1 GAS M5 peptides used in this study.
Peptide sequences were taken from the M5 sequence reported by Whatmore and Kehoe (1994) and were synthesised as 20-mers with a 10 amino acid overlap.

M5 Region	Synthetic peptide	Sequence
B repeat	M5-B.1	TRQELANKQQESKENEKALN
	M5-B.2	ESKENEKALNELLEKTVKDK
	M5-B.3	ELLEKTVKDKIAKEQENKET
	M5-B.4	IAKEQENKETIGTLKKILDE
	M5-B.5	IGTLKKILDETVKDKIAKEQ
	M5-B.6	TVKDKIAKEQENKETIGTLK
C repeat	M5-C.7	NKISEASRKGLRRDLDASRE
	M5-C.8	LRRDLDASREAKKQVEKALE
	M5-C.9	AKKQVEKALEEANSLKAALE
	M5-C.10	EANSKLAALEKLNKELEESK

6.2.4 Source of donor blood for bactericidal assays

Initially, sheep's blood was used as a readily available source of neutrophils and complement in bactericidal assays. However, this proved to be unsuitable due to extensive platelet clumping within minutes of adding bacteria/antiserum mixture to blood samples. Furthermore, data indicate that many M proteins bind complement regulatory molecules such as C4b binding protein (C4BP) in human plasma but not in mouse or rat plasma, suggesting that the anti-phagocytic properties of M-protein should be tested in human blood (Accardo *et al.*, 1996; Carlsson *et al.*, 2005). Whole human blood was therefore used in later experiments. Heparinised blood (14 ml per subject) was obtained from human volunteers following informed consent. All blood was stored at 4 °C and used within 4 hrs of collection. Before being used in the bactericidal assay, sera from human donors were pre-screened by ELISA for the presence of anti-M5 antibodies as described below in Section *6.2.8*.

6.2.5 Optimisation of ELISA by chequerboard titration

To optimise ELISA conditions, a chequerboard titration was performed using the principles described online (www.iaea.org/nafa/d3/public/ras-ai-elisaii.pdf, accessed 15 September 2006). The underlying rationale for conducting a chequerboard titration involves the dilution of two reagents against each other to assess the activities present at different combinations of dilutions, with the aim of empirically determining the optimal concentration of reagents which permit signal detection without background signal. The signal-to-noise ratio is the signal generated by a sample with analyte, relative to a sample without analyte. As the signal-to-noise ratio increases, the assay becomes more sensitive at detecting the analyte. In this study, a 1:5,000 dilution of the HRP-conjugated goat anti-rat IgG antibody was used as recommended by *Jackson ImmunoResearch*. Plate coating antigen concentrations and sera dilutions which gave the greatest distinction between the positive signal (absorbance) and background noise were then chosen for subsequent assays.

6.2.6 Detection of anti-M5 antibodies in rat serum by ELISA

The antibody responses of rats from each group were evaluated for the presence of immunoglobulin isotype G (IgG) against GAS serotype 5 M protein or peptides M5-B.1 to M5-C.10 using an indirect ELISA. Titration experiments (*6.2.5*) were carried out initially to determine optimal ELISA conditions. Antigen was diluted to 5 μg/ml (rM5) or 10 μg/ml (peptides) in bicarbonate coating buffer, pH 9.6 and the solution was used to coat Maxisorp F96 plates (*Nunc*) at 100 μl/well. The plates were incubated at 4 °C overnight, washed five times with wash buffer (Phosphate-buffered saline [PBS] at pH 7.2, 0.05% Tween 20), and blocked with 200 μl Post-coating Buffer for 1 h at 37 °C. The plates were then inverted and blotted on paper to remove residual blocking solution, and either used directly or stored at 4 °C in a sealed plastic bag for future use. Rat sera, pooled in each immunisation group and at an initial dilution of 1:100 were serially diluted two-fold to a final dilution of 1:51,200. Each sample was added to duplicate or triplicate wells at 100 μl/well and the plates were incubated for 1 h at 37 °C. A positive control serum sample, a normal serum sample from unimmunised rats and a diluent only sample were tested in the same manner. The plates were washed five times with wash buffer and

horseradish peroxidase (HRP) conjugated goat anti-rat antibody was added at 100 µl/well at a 1:5,000 dilution. After 1 h incubation at 37 °C, the plates were washed five times and 100 µl of ABTS substrate solution was added to each well. After 20 min incubation at room temperature, the absorbance was read at 414 nm against a background at 492 nm. For each sample the titre was defined as the lowest dilution that gave an absorbance of more than three standard deviations above the mean absorbance of control wells (containing normal rat serum).

6.2.7 Detection of cross-reactive antibodies in immunised rats

In this section of work, the ability of rM5 or M5 peptides to elicit antibodies that react with proteins found in the heart including cardiac myosin and collagen IV was determined by ELISA using the protocol described above. After initial chequerboard titrations, porcine cardiac myosin, which shares >97% amino acid identity with rat cardiac myosin, was coated onto Maxisorp F96 plates (*Nunc*) as solid phase antigen at 10 µg/ml. Sera were serially diluted two-fold from a 1:100 to 1:25,600 dilution and sera from PBS-immunised rats was used at 1:100 as a negative control. Collagen type IV was coated at 1, 10 and 100 µg/ml and sera was titrated at doubling dilutions starting at 1:10 to 1:320.

In another set of experiments, sera were reacted against denatured forms of porcine cardiac myosin and rat cardiac myosin in ELISA. Myosin was denatured by heating at 95 °C for 15 min, followed by rapid cooling on ice. ELISA was performed as described above with modifications. In these assays, all sera were diluted to 1:4 except for positive control serum (1:500 dilution) from rats immunised with porcine cardiac myosin.

6.2.8 Pre-screening of human donor blood for anti-M5 antibodies

Human donor blood was first screened by an indirect ELISA using rM5 as solid-phase antigen to ensure that it did not contain antibodies against GAS serotype 5 M protein (Lancefield, 1957). After coating wells in a Maxisorp F96 microtitre plate (*Nunc*) with rM5 (1 µg/well in a total volume of 100 µl) overnight at 4 °C, the antigen was removed and wells were blocked with 200 µl Post-coating Buffer for 1

hour at 37 °C. Human donor sera were applied at two-fold dilutions from 1:100 to 1:25,600. In the absence of established human controls, pooled serum from rM5-immunised rats was used at a 1:20,000 dilution as positive control; a negative control consisted of pooled sera from PBS-immunised control rats diluted 1:100. Each sample was added to individual duplicate wells at 100 µl/well and the plates were incubated at 37 °C for 1 h, then washed five times in wash buffer.

Peroxidase-conjugated goat anti-rat or goat anti-human IgG (*Jackson ImmunoResearch*) diluted 1:5,000 was added to appropriate wells at 100 µl/well and incubated for 1 h at 37 °C. After incubation, the plates were washed five times and 100 µl of ABTS was added to each well. After 20 minutes incubation at room temperature, the absorbance at 414 nm was read against a background at 492 nm on a Multiskan microplate reader (*Titertek*). Data was processed using Genesis V3.00 software (*Labsystems*). After correcting for background, a positive cut-off value was set at the mean absorbance of more than three standard deviations above the mean absorbance of control wells (containing control rat serum, 1:100). Additionally, human donor blood which was negative for anti-M5 antibodies by ELISA was considered non-opsonic if it could support the growth of bacteria to at least 32 times the inoculum in a 3 h incubation at 37 °C (Brandt *et al.*, 1996).

6.2.9 Bactericidal assays

The ability of sera from rM5-immunised rats to promote the opsonisation of GAS M5 bacteria was determined in a standard bactericidal assay. A single mucoid colony of M5 was cultured overnight in 5 ml of THYB at 37 °C in a humidified 5% CO_2 incubator, then 200 µl of this culture was sub-inoculated into 5 ml of fresh, warm (37 °C) THYB and cultured as above until early log phase (OD_{600} = 0.15). The bacteria was serially diluted 10-fold in sterile PBS and samples of 10^{-3} to 10^{-6} dilutions were plated on 5% sheep blood agar (SBA) plates for colony counts to determine inoculum.

Fifty microlitres of bacteria was mixed with 50 µl of pooled heat-inactivated (56 °C, 30 min) sera from rM5-immunised rats or control rats and incubated at 37 °C for 10

min. After incubation, 400 μl of non-immune heparinised donor blood was added and the mixtures were rotated end-over-end at 37 °C for 3 h. Fifty microlitres of mixture from each tube was plated out in duplicate using the pour-plate method into 5% SBA, incubated overnight and the number of cfu counted. Additional control tubes containing bacteria and blood with normal, non-immunised rat serum and tubes with bacteria and blood only were also included in each experiment. Bactericidal activity of immune sera was calculated as the percent reduction in cfu in immune sera compared to cfu grown in control sera using a standard formula (Hayman *et al.*, 1997) as follows:-

$$\% \text{ Bactericidal activity} = 1 - \frac{\text{mean cfu in the presence of immune rat sera}}{\text{mean cfu in the presence of control sera}} \times 100$$

6.2.10 Inhibition assays

The ability of rM5 protein to inhibit opsonisation of M5 GAS by antisera raised against rM5 was evaluated in a direct opsonisation inhibition assay. Anti-rM5 antisera (50 μl) from immune and control rats were incubated with 100 μg of purified rM5 protein for 30 min at 37 °C. The mixture was then centrifuged at 10,000 x *g* for 10 min at 4 °C to remove any precipitates (Beachey and Cunningham, 1973) and the supernatant antiserum added to the M5 GAS and blood mixture as described in Section *6.2.9.* Percent blocking was determined by comparison of the number of colonies growing in the presence of rM5 protein to the number of colonies growing in the absence of rM5 protein.

6.3 RESULTS

6.3.1 Reactivity of antibodies in rat antisera

6.3.1.1 rM5 elicits a strong antibody response in Lewis rats against rM5 protein and M5 peptides

The IgG response in rats immunised with rM5 or M5 peptides was tested day 21 p.i. by indirect ELISA. Initially, sera pooled from rM5-immunised rats (n = 5) was evaluated against individual peptides to determine whether the peptides contained epitopes that were recognised by antibodies raised against the full-length rM5 protein. High reactivity (defined as titres of ≥ 6,400) to peptides M5-B.3, M5-B.5 and M5-B.6 from the B-repeat region and M5-C.7 from the C-repeat region and medium reactivity (800 ≥ titre ≤ 3,200) to peptides M5-C.2 and M5-C.8 was observed in rM5 antiserum. As expected, sera from rM5-immunised rats had high reactivity (titre >25,600) to rM5 protein (Figure 6.1). In contrast, sera from PBS-immunised control rats did not react with rM5 or M5 peptides (data not included in Table 6.2).

Table 6.2 IgG antibody reactivity of sera from rM5-immunised rats to full length rM5 and M5 peptides.
Titres are provided for pooled rat sera (n=5) reacted with 10 μg M5 peptide and 1 μg rM5 per ml in an ELISA.

Coating Antigen	Titre
M5-B.1	200
M5-B.2	3,200
M5-B.3	6,400
M5-B.4	400
M5-B.5	6,400
M5-B.6	25,600
M5-C.7	12,800
M5-C.8	1,600
M5-C.9	100
M5-C.10	200
rM5	>25,600

6.3.1.2 Reactivity of anti-peptide sera to full-length rM5 or recall M5 peptide

The reactivity of each of the pooled sera from rats immunised with individual M5 peptides toward the immunising, homologous M5 peptide or the full-length rM5 protein is shown in Table 6.3. Five of 10 groups of sera from peptide-immunised rats reacted (titres > 1,600) with their respective immunising M5 peptides. These were B-region peptides M5-B.2, M5-B.4 and M5-B.5 and C-region peptides M5-C.9, and M5-C.10. Four of these anti-M5 peptide sera (M5-B.2, M5-B.4, M5-B.5, and M5-C.10) also reacted to the full-length rM5 protein. Sera from rats immunised with PBS in adjuvant reacted minimally, but slightly more than (titres $\leq$ 100) sera from normal, unimmunised control rats which did not react to any of the peptides tested or to rM5. Several anti-peptide sera also displayed low reactivity with other overlapping and non-overlapping peptides which shared amino acid sequences in ELISAs (data not shown).

Table 6.3 IgG antibody reactivity of sera from GAS M5 peptide-immunised Lewis rats to homologous peptide or full-length rM5. Titres are provided for pooled rat sera (n=3-5 per group) reacted with 1 μg rM5 and 10 μg M5 peptide per ml in an ELISA.

	Titres	
	ELISA coating antigen	
Sera	rM5	Recall peptide
M5-B.1	<100	<100
M5-B.2	800	3200
M5-B.3	400	200
M5-B.4	3200	>25600
M5-B.5	1600	>25600
M5-B.6	100	1600
M5-C.7	<100	400
M5-C.8	<100	<100
M5-C.9	400	12800
M5-C.10	1600	>12800
rM5	>25600	n/a[a]

[a] not applicable

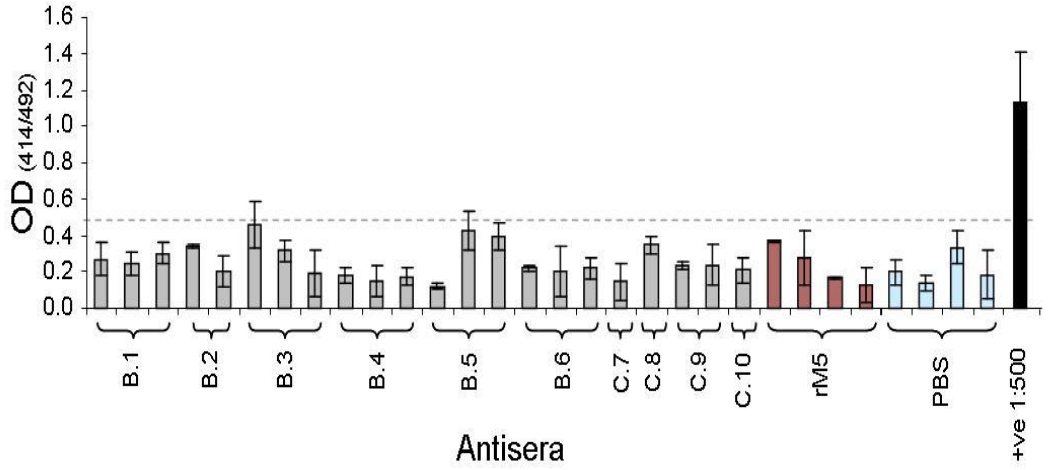
6.3.1.3 Cross-reactivity of rat antisera to cardiac myosin

In human and mice, GAS M protein or M protein peptides induce antibodies that recognise host tissue proteins and could potentially play a role in the induction of autoimmune diseases such as RF/RHD and post-streptococcal glomerulonephritis. To determine whether anti-cardiac myosin cross-reactive antibodies are induced in the RAV model, sera collected day 21 p.i. from full length rM5- and M5 peptide-immunised rats, diluted 1:100 to 1:25,600, were titrated against native porcine cardiac myosin at 10 μg/ml (Table 6.4).

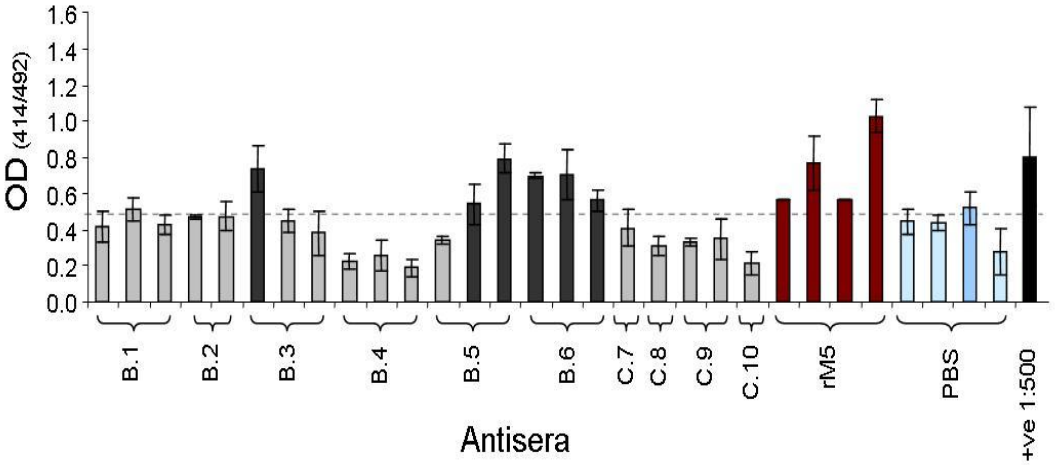
Table 6.4 IgG reactivity of anti-peptide sera with native cardiac myosin. Sera were serially diluted two-fold and reacted with 10 μg of porcine cardiac myosin per ml in an ELISA. Titres were calculated as the mean OD(414-492 nm) + 3SD of normal serum on each ELISA plate.

Antisera	Titre against cardiac myosin
M5-B.1	100
M5-B.2	200
M5-B.3	100
M5-B.4	100
M5-B.5	200
M5-B.6	200
M5-C.7	<100
M5-C.8	<100
M5-C.9	400
M5-C.10	<100
rM5	400

To address whether antibodies against streptococcal rM5 protein which were cross-reactive against host proteins such as cardiac myosin, were present in antisera from rats immunised with GAS M protein or M5 peptides, ELISA plates were coated with heat-denatured porcine or rat cardiac myosin and where possible, serum samples were tested individually at a 1:4 dilution. Higher dilutions were tested initially without success. There was variation between individual animals in the responses to cardiac myosin, as shown in Figure 6.1.



a. Heat denatured porcine cardiac myosin



b. Heat denatured rat cardiac myosin

Figure 6.1 IgG reactivity of rat antisera against denatured cardiac myosin. Antisera at 1:4 dilution raised in rats immunised with M5 peptides (grey bars), rM5 (red bars) or PBS (blue bars) were reacted against heat-denatured a) porcine and b) rat cardiac myosin in ELISA. Positive control serum (black bars) was from cardiac myosin-immunised rats diluted 1:500. Darker bars in each group depict animals that demonstrated positive reactivity. Dashed line represents positive cut-off value set at mean OD + 3SD of sera pooled from normal rats.

While no positive cross-reactivity was observed against denatured porcine cardiac myosin in sera from rats immunised with M5 peptides or rM5 (Figure 6.1.a), reactivity against denatured rat cardiac myosin was observed in rats immunised with peptides M5-B.3 (1/3 rats), M5-B.5 (2/3 rats) and M5-B.6 (3/3 rats) as well as all rM5-immunised rats (4/4) (Figure 6.1b). There were insufficient sera from rats immunised with C-region peptides to test all samples individually at this time, therefore the antibody responses in each of these animals could not be assessed. On the other hand, positive control serum (diluted 1:500) from experimental autoimmune myocarditis (EAM) rats which had developed severe carditis following immunisation with porcine cardiac myosin, reacted strongly with cardiac myosin in ELISA.

To test whether anti-collagen IV antibodies were also elevated in the sera of RAV animals, sera diluted two-fold from 1:10 to 1:320 were titrated against 1, 10 and 100 μg/ml collagen type IV in an ELISA. However, no significant antibody cross-reactivity to collagen IV was detected in these rats (data not shown).

6.3.2 Pre-screening for non-immune human donor blood

Human donor blood was used as a source of complement and polymorphonuclear (PMNs) cells in bactericidal assays. Prior to the assays, pre-screening by ELISA was carried out to select suitable non-opsonic donor blood which did not contain antibodies that recognised rM5 protein and which could support growth of M5 GAS to at least 32 times the inoculum in three hours. In the absence of a recognised human negative control serum sample, we used sera from normal, unimmunised rats as these animals were unlikely to have been previously exposed to GAS. The results from one of two titration experiments which screened six different potential donors is shown in Figure 6.2. Donor A had high levels of antibodies that recognised rM5, possibly due to previous exposure to serotype M5 GAS. Donor C had levels of anti-rM5 antibodies that were comparable to the levels detected in our control serum from normal, unimmunised rats. On the other hand, Donor B had very low levels of anti-rM5 antibodies indicating that this donor had not been previously exposed to M5 GAS and was therefore selected as a source of non-immune blood for bactericidal assays.

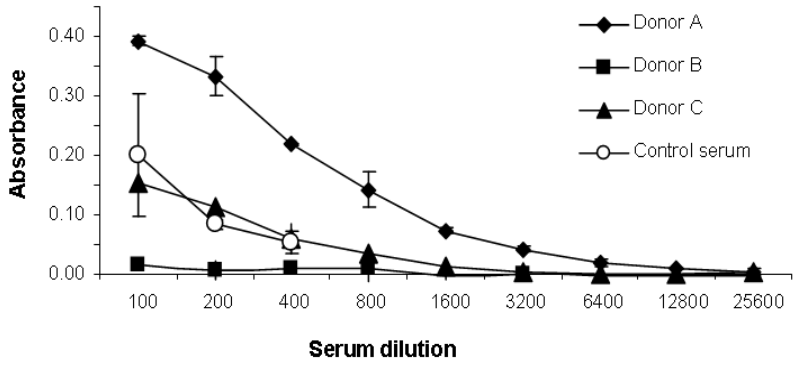


Figure 6.2 Pre-screening for non-immune human donor blood. Sera were titrated against rM5 protein (1µg/ml) in an ELISA to screen potential donors for the absence of anti-M5 antibodies (Lancefield, 1957). Donor B blood was considered non-opsonic as it was negative for anti-M5 antibody and supported bacterial growth to 32 times the inoculum.

6.3.3 Bactericidal activity in anti-rM5 rat sera

Antibodies from immunised rats were tested for their ability to kill M5 GAS in an indirect bactericidal assay. As depicted in Figure 6.3, heat-inactivated sera from rM5-immunised rats had significantly higher bactericidal activity (~98%) than sera from control rats ($p = 0.001$), reducing the number of cfu from an inoculum size of 370 ± 40 (mean ± SEM) to 14 ± 9 after 3 h of incubation in human blood. In the presence of sera from control rats, the number of cfu increased to 1,146 ± 89. Pre-absorption of the rM5 antisera with rM5 protein completely abrogated the bactericidal activity against GAS serotype M5 (1,168 ± 123 cfu).

After 30 min incubation, samples inoculated with M5 GAS in the presence of anti-rM5 antiserum contained streptococci throughout the entire sample (Figure 6.4, upper panel), with numerous chains or single bacterial cells internalised by PMN. After 120 min (Figure 6.4, lower panel), a significant reduction of extracellular bacteria was observed and there was evidence of extensive neutrophil degeneration.

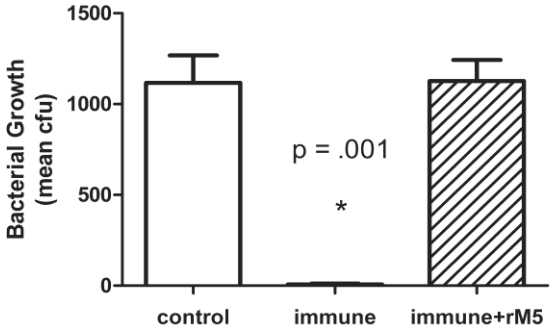


Figure 6.3 Bactericidal activity in rM5 antiserum against M5 GAS. In an indirect bactericidal assay, rM5 antisera produced a 98% reduction of GAS M5 bacteria in non-immune human blood. (* *p* =.001 immune *vs* control serum by unpaired Student's *t*-test, two-tailed). Pre-incubation of sera with rM5 abrogated the bactericidal activity. Results are expressed as mean cfu ± SEM from duplicate assays and two independent experiments.

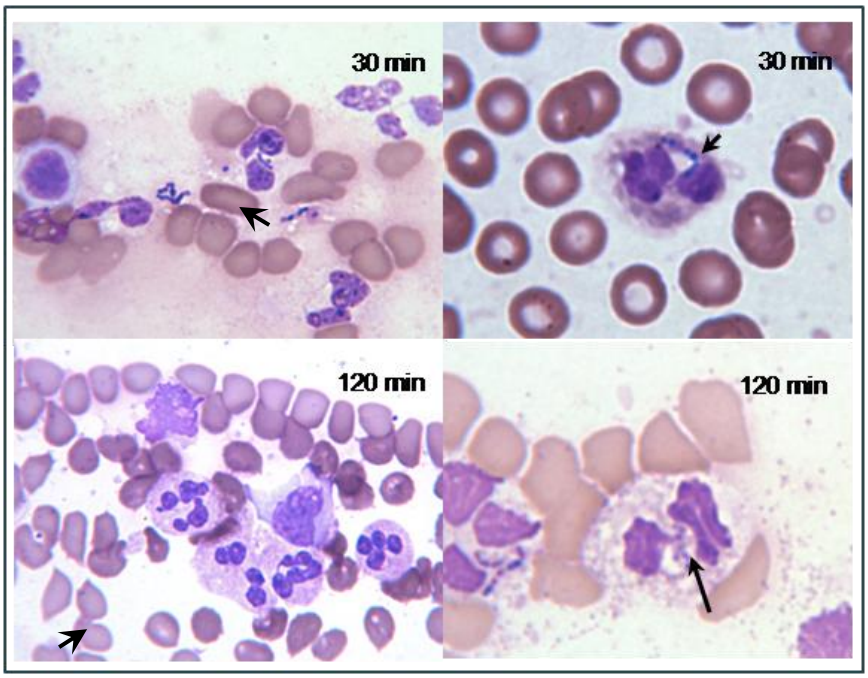


Figure 6.4 Microscopic analysis of bactericidal activity of serum from rM5-immunised rats. Samples taken after 30 min incubation contained abundant extracellular and phagocytosed streptococcal chains (upper panel) compared to samples taken after 120 min (lower panel). Images on the right at higher magnification show phagocytosed bacteria (short arrow) and degranulating neutrophils (long arrow). Blood smears taken from a 1:10 dilution of incubation mixture stained with Diff Quik (original images at 40x and 100x objectives).

6.4 DISCUSSION

Antibody responses towards GAS streptococcal M protein have been studied in depth in both humans and various animal models (Beachey *et al.*, 1988; Bronze *et al.*, 1988b; Cunningham *et al.*, 1989; Quinn *et al.*, 1995; Brandt *et al.*, 2001). The problems associated with designing anti-GAS vaccines based on the M protein have been well documented. Firstly, serotype-specific antibodies do not generally protect against other serotypes (Lancefield, 1962), although several groups have reported cross-reactivity and limited cross-opsonisation in heterologous GAS strains using amino-terminal peptides derived from isolates in an endemic population (Brandt *et al.*, 2000b). Secondly, studies have shown that many M protein epitopes outside of the amino-terminal region elicit host tissue cross-reactive antibodies and T cells that are thought to be involved in the development of post-streptococcal autoimmune diseases such as RF/RHD (Cunningham and Quinn, 1997). In addition, antibodies against most other M protein epitopes outside the N-terminal region were previously thought to have either low or no opsonic capability (Lancefield, 1962; Jones and Fischetti, 1988). The challenge facing vaccine research is to find B cell epitopes on the M protein which result in a protective antibody response against a broad range of serotypes without eliciting cross-reactive autoantibodies or T cells.

M protein confers resistance to phagocytosis in the absence of specific antibody in the host (Horstmann *et al.*, 1992; Fischetti *et al.*, 1995) by inhibiting the activation of complement (Weineisen *et al.*, 2004; Carlsson *et al.*, 2005; Sandin *et al.*, 2006). The M5 protein has been shown to inhibit deposition of complement protein C3b through the binding of fibrinogen at the B region. Nevertheless, this complement inhibition may be overridden by antibodies against the hypervariable N-terminal region of M protein (Sandin *et al.*, 2006).

In this study, the induction of anti-rM5 IgG antibodies in the RAV model was demonstrated by enzyme-linked immunosorbent assay (ELISA). The ELISA is a sensitive analytical tool for detecting and measuring antibodies in serum. The sensitivity and specificity of ELISA is dependent on two important biological properties: 1) the highly discriminatory power of antibodies, based on the ability of the immune system to produce a vast array of antibodies of exquisite specificity and

2) the extremely high catalytic power of enzymes which facilitates the detection of minute amounts of antibody.

In the RAV model, subcutaneous immunisation with the recombinant M5 protein comprising a 6x*histidine*-tagged type-specific N-terminal region, all of the A, B and C repeat regions and a truncated membrane-spanning C-terminal region consistently induced high titres (> 12,800) of anti-rM5 antibodies when measured by ELISA. Our next goal was to investigate whether these antibodies had a functional role in the RAV model, that is, could anti-rM5 antiserum raised in Lewis rats opsonise M5 GAS bacteria?

Several of the peptides (three from the B region and two from the C region) generated antibodies against the original peptide when delivered with an adjuvant. Reactivity to the rM5 protein was also demonstrated to all but one of these five peptides (M5-C.9), whereas no reactivity to either the original peptide nor to rM5 was shown by the remaining five anti-peptide sera. Interestingly, although antisera from rM5-immunised rats recognised peptides M5-B.2, M5-C.7 and M5-C.8, direct immunisation with these peptides did not elicit antibodies against either the original peptides or rM5 protein. This suggests that the immunogenic epitopes in these peptides may be conformational in nature. However, the amino acid sequence of M5-C.8 (LRRDLDASREAKKQVEKALE) is identical to a peptide (p145), shown previously to contain an immunodominant antibody binding epitope recognised by human antisera from a majority of adults in regions of high GAS exposure (Pruksakorn *et al.*, 1994a). In that study, p145 was also found to be cross-protective, inducing opsonisation of different streptococcal serotypes and reducing pharyngeal colonisation in mice.

Further studies by the same group to map the minimal B cell epitope in p145 revealed that the immunodominant epitope was located in amino acids 2-13 (RRDLDASREAK) and was most likely conformational (Relf *et al.*, 1996). The construction of novel, p145-based chimeric peptides with an alpha helical propensity resulted in conformational epitopes which maintained their B cell immunogenicity but without stimulating cross-reactive T cells (Brandt *et al.*, 1996; Brandt *et al.*, 1997; Hayman *et al.*, 1997; Brandt *et al.*, 2000a; Hayman *et al.*, 2002). Peptide p145 and its derivatives J8 and J14 are the basis of intensive past and current vaccine

research and have shown the most potential for a safe and broadly effective vaccine (Hayman *et al.*, 2002; Batzloff *et al.*, 2003; Vohra *et al.*, 2005; Yoonim *et al.*, 2006). Further development of novel adjuvant formulations e.g. lipid-core peptides or diphtheria toxin has resulted in the enhanced immunogenicity of these peptides in mice (Olive *et al.*, 2004; Batzloff *et al.*, 2006; Pandey *et al.*, 2009).

It should be noted however, that the low antibody titres against M5-C.7 and M5-C.8 observed in anti-peptide sera in our study are in agreement with those reported in murine models where multiple boosters (4-5) of peptide p145 in Freund's adjuvant were required to obtain higher antibody titres (Hayman *et al.*, 2002). It is therefore logical to assume that if a similar regime of multiple boosters had been used in the current study, comparable antibody titres in peptide-immunised rats would have been achieved. Unfortunately, due to the high cost of synthetic peptides, only one booster was feasible for these experiments.

In humans and mice, GAS M protein or M protein peptides induce antibodies that recognise host tissue proteins and could potentially play a role in the induction of autoimmune diseases such as RF/RHD (Guilherme *et al.*, 1995; Cunningham, 2000) and post-streptococcal glomerulonephritis (Burova *et al.*, 2003; Zheng *et al.*, 2009).

Studies conducted over 25 years ago first demonstrated anti-streptococcal antibodies in sera from RF patients that cross-reacted with human cardiac myosin, leading to the speculation that molecular mimicry between host proteins and streptococcal antigens, in particular the M protein, may be important factor in the pathogenesis of RF/RHD (Dale and Beachey, 1985a). Several other studies have also indicated a role for cardiac myosin in the pathogenesis of RHD (Krisher and Cunningham, 1985; Cunningham *et al.*, 1993; Cunningham *et al.*, 1999; Cunningham, 2003). When we tested sera from peptide-immunised rats against native cardiac myosin in ELISAs, four B-region peptides and one C-region peptide, as well as rM5, generated antibodies only weakly cross-reactive with cardiac myosin. It was anticipated that higher levels of reactivity against cardiac myosin would be observed, given that cross-reactivity between GAS M protein and cardiac myosin has been repeatedly demonstrated in other studies (Cunningham *et al.*, 1997).

Immunisation with overlapping peptides M5-B.2 (SKENEKALNELLEKTVKDK) and M5-B.3 (ELLEKTVKDKIAKEQENKET) which share some sequence identity with the B-region peptide B1B (ENEKALNELLEKTVDKI), reported to be a high inducer of anti-myosin antibodies (titre=102,400) by Cunningham *et al*. (1997), also induced anti-myosin reactivity in our work, albeit at much lower levels. It was hypothesised that the reason for the low titres of anti-myosin antibodies shown in our experiments may be due to differences in assay parameters. Firstly, we pooled the sera from each peptide-immunised rat group before performing ELISAs. Secondly, the anti-myosin antibodies present in the polyclonal antisera may have been diluted out, reducing the concentration of cross-reactive antibodies to below detection limits and thirdly, conformational changes could have occurred in the native cardiac myosin when it was coated onto microtitre wells. Vashishtha and Fischetti (1993) found that antisera to peptides from GAS M6 protein cross-reacted more to denatured forms of cardiac myosin rather than to native myosin in ELISA. A plausible explanation for the observed antibody reactivities proposed by Vashishtha and Fischetti (1993), is that antibodies reactive to denatured forms of cardiac myosin are induced because epitopes shared between M protein and denatured myosin are not usually seen as part of the B cell self repertoire, whereas native myosin would be. Cross-reactive anti-M protein antibodies could then bind to denatured forms of myosin exposed in cardiac tissue damaged through other mechanisms including inflammation. The cross-reactive antibodies could then contribute to further inflammation and damage.

While the low reactivity towards cardiac myosin demonstrated in our study could be the result of *in vivo* expansion of naturally occurring autoreactive B cells due to a generalised inflammatory response, when compared to serum from adjuvant control rats, the reactivity of four peptides was found to be significant. Another logical explanation for lack of high cross-reactivity to cardiac myosin in these experiments is that little damage has occurred in the myocardium of the rats. Histological examination of heart tissue supports this view; only isolated, mild lesions were observed in rM5-immunised and one peptide-immunised rat whereas positive control rats (immunised with cardiac myosin) had evidence of severe myocarditis and corresponding high titres of anti-myosin antibodies.

Recent studies have found high levels of antibodies against cardiac basement membrane protein collagen IV in sera from acute RF patients, but not in GAS pharyngitis patients or healthy individuals (Dinkla *et al.*, 2003). However, in this work, no cross-reactivity was observed in sera of rM5-immunised rats towards collagen IV when tested both by ELISA or Western blots.

Opsonophagocytosis, mediated by serum opsonins such as antibodies or complement, is a key mechanism for host protective immunity against streptococcal infection. Protection has been correlated with the presence of opsonic antibodies directed against the N-terminal hypervariable region of M protein (Todd and Lancefield, 1928; Lancefield, 1959; Fischetti, 1989; Sandin *et al.*, 2006). In the immune host, M type-specific antibodies bind to the distal portion of the M protein which extends beyond the hyaluronic capsule, rendering the GAS bacterium susceptible to rapid clearance by phagocytic neutrophils (Fischetti, 1991). Further, while a locally protective mucosal response to intranasal immunisation has been demonstrated in mice mediated by non-opsonic secretory IgA antibodies (Bessen and Fischetti, 1988), systemic protection against GAS infection is afforded by antibodies of the IgG isotype (Fischetti, 1991; Cunningham, 2000).

The indirect bactericidal test measures growth or killing of haemolytic streptococci in human blood and has been used extensively to demonstrate opsonic anti-M protein antibodies in sera of mice (Cunningham *et al.*, 1989) and humans (Lancefield, 1959; Cunningham *et al.*, 1989; Brandt *et al.*, 1997; Yoonim *et al.*, 2006).

The data generated from this study show conclusively that a functional IgG antibody response is generated in the RAV model upon exposure to GAS M5 protein. This was demonstrated by more than 98% reduction of bacteria in human blood in the presence of immune rat sera.

The work described here has shown that GAS M protein is antigenic and immunogenic in the RAV model. Moreover, immunisation with rM5 protein induces functional antibodies with bactericidal activity. However, these antibodies are most likely directed towards the hypervariable N-terminal region of rM5. In order to

determine whether peptides located outside of the N-terminal region have the capacity to elicit opsonic antibodies, bactericidal assays using anti-peptide sera are necessary. The next step would then be to challenge peptide-immunised rats with GAS to gauge the protection afforded by individual peptides.

CHAPTER 7
ALTERNATIVE IMMUNISATION STRATEGIES

7.1 INTRODUCTION

A major consideration during this project was the choice of adjuvant to be co-administered with antigen in immunisation injections. Adjuvants (from the Latin, *adjuvare*: to help) are compounds which provide a critical link between the initial innate response and subsequent adaptive response to an antigen. Their inclusion is very important in sub-unit or synthetic peptide vaccine formulations which are themselves poorly immunogenic, due to their ability to 1) enhance antigen uptake and processing by antigen presenting cells (APC), 2) prolong exposure of the immune system to antigen ('depot' effect), 3) up-regulate expression of co-stimulatory molecules on APC and 4) modulate the required Th1/Th2 response to an antigen (Guy, 2007). Greater understanding of the molecular and cellular modes of action of various adjuvants *in vivo*, as reviewed elsewhere (Cox and Coulter, 1997; Schijns, 2000; Stills, 2005; Perrie *et al.*, 2008), have enabled researchers to develop safer, more efficient vaccines which require a smaller antigen dose.

Many protocols currently used for inducing experimental organ-specific autoimmune diseases in animal models such as experimental autoimmune encephalomyelitis (Conant and Swanborg, 2004), myocarditis (Maier *et al.*, 2005) and uveitis (Mor *et al.*, 2003) require the use of Freund's Complete adjuvant (FCA) for the primary immunisation. A potent immunostimulatory formulation, FCA is composed of a mineral oil, a surfactant agent (mannide mono-oleate) and heat-killed mycobacterial cells and cannot be used in humans due to reported severe inflammatory side-effects (Hughes *et al.*, 1970; Billiau and Matthys, 2001). Animal studies carried out to resolve the issue of pain and distress associated with FCA have reported mixed results, with some researchers finding no evidence of physiological or behavioural changes indicative of pain or stress in rabbits (Halliday *et al.*, 2000) and others documenting clinical signs of both pain and distress in mice and rats (Leenaars *et al.*, 1998; Toth, 2000). Stills (2005) suggested that the granulomatous lesions elicited by FCA are in fact, relatively painless. Conversely, FCA is used in the adjuvant-

induced arthritis (AIA) rat model of chronic pain (Naito and Inoue, 2008). In our early experiments, we observed that FCA produced moderate to severe arthritis in the ankle joints and necrotic lesions in the treated paws of our rats, as discussed below. We therefore explored the possibility of improving our method of immunisation with the goal to reduce unwanted side-effects. The use of FCA in animals is currently tightly regulated and/or actively discouraged due to the issues mentioned above. However, it is clear that the extent of inflammation, distress and pain following administration of any adjuvant is dependent on species, age, dose volume, site of injection and immunisation schedule.

7.1.1 Aims

The aim of the work carried out in this chapter was to : -

1) Test alternative adjuvant formulations to find a replacement for FCA which would produce the same Th1 response involved in the development of autoimmune valvulitis in rats observed in previous studies with a concomitant reduction in adverse side-effects
2) Establish a refined immunisation protocol which meets the Australian National Health and Medical Research Council's guidelines to promote the wellbeing of animals used for scientific purposes.

7.2 MATERIALS AND METHODS

7.2.1 Adjuvants

Freund's complete adjuvant (FCA), a non-metabolisable mineral oil supplemented with 5 mg of heat-killed mycobacteria H37RA per ml and Freund's Incomplete Adjuvant (FIA) without mycobacteria were purchased from *Sigma*. Emulsigen® (*MVP Laboratories INC*) was provided by *InterVet, Bendigo East, Australia.* Montanide ISA50V (*SEPPIC*) was provided by the *Tall Bennett Group*, Mona Vale, Australia.

7.2.2 Preparation of antigen/adjuvant injections

Preparation of antigen in Freund's adjuvants is described in Chapter 3. Emulsigen® was added to aqueous antigen or PBS, to a concentration of 25% v/v in a sterile 1.5 ml microfuge tube and emulsified by vigorous vortexing for 3-5 mins. For Montanide ISA50V preparation, antigen or PBS was added to Montanide ISA50 and mixed by vortexing as per manufacturer's directions. Antigen/adjuvant emulsions were loaded into 1 ml sterile disposable syringes (*Terumo*) fitted with 25 gauge needles (*Terumo*).

7.2.3 Immunisation of rats

For comparison of adjuvants, female Lewis rats were immunised according to the protocol outlined in Section *3.2.6.2*. In brief, a primary immunisation consisting of 500 μg antigen or PBS emulsified in adjuvant in a total volume of 200 μl was delivered s.c. subplantar in the right hind foot. Formalin-killed whole *B. pertussis* cells (10^{10}) were given i.p. on days 1 and 3 post initial immunisation (p.i.) as additional adjuvant (Section *3.1.2.5*). Rats were boosted on day 7 p.i. with 500 μg antigen emulsified in FIA delivered subcutaneously (s.c.) in the flank and animals were sacrificed by CO_2 asphyxiation followed by cervical dislocation on day 21 p.i.

7.2.4 Measurement of inflammatory response

Inflammation following injection of FCA and Emulsigen® were compared by measuring the amount of swelling induced in the injected paw by each emulsion. Digital laboratory callipers were used to determine the difference in foot thickness between the injected right hind foot and the non-injected left hind foot at days 7 and 21 p.i. As the non-injected left foot also became swollen in some FCA-immunised rats, indicating adjuvant-induced polyarthritis (Pearson, 1963), the protocol for quantitating inflammation was changed in subsequent experiments.

To give a more accurate comparison between rat groups, the inflammatory effects of FCA and Montanide ISA50V were compared by measuring the increase in paw thickness of the right hind foot on days 7 and 21 p.i. and compared to Day 0 prior to immunisation.

7.2.5 Antibody responses elicited by alternative adjuvants

To compare the antibody responses in rats immunised with rM5 emulsified in either FCA or Montanide ISA50V, serum IgG levels were measured by ELISA using the method described in Section *7.27*.

7.2.6 Immunisation via the hock

Subplantar (footpad) injection of FCA resulted in most animals developing inflammation in the whole foot and ankle and in some cases secondary infection, leading to debilitation and lameness. A novel immunisation method described by Kamala (2007) involves injection into the lateral tarsus region just above the ankle, or hock, and is purported to induce comparable immune responses in mice without the associated impairment of mobility and pain.

Rats were immunised with antigen/FCA as described previously except that the needle was inserted subcutaneously, bevel upwards, into the lateral side of the foot directly above the heel and into the space between the fibularis longus and soleus muscles so that the needle was inserted approximately 3-5 mm under the skin. A maximum 200 µl of mixture was injected by slow but firm pressure on the syringe plunger. A small 'bubble' of mixture could be seen directly underneath the skin (Figure 7.1). Gentle pressure was then applied with sterile cotton wool as the needle was removed to prevent any leakage out of the injection site.

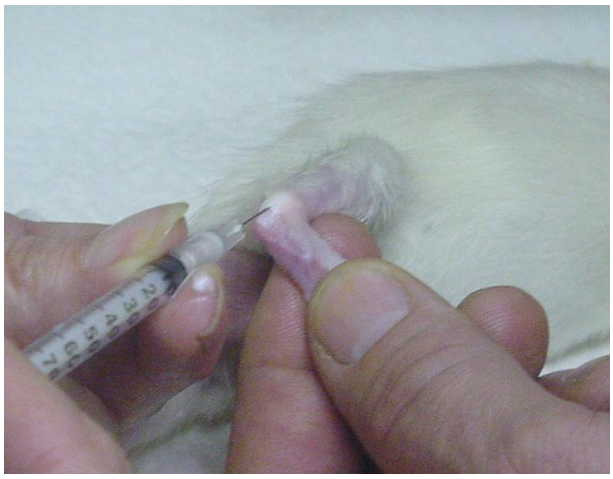


Figure 7.1 Injection of antigen/FCA emulsion into the hock.

7.2.7 Lymphocyte proliferation assays

The effect of adjuvant composition on the cellular immune response in rats was measured in a standard ^{3}H-thymidine incorporation assay (Section *3.2.6.5*). Mononuclear cells derived from spleens of rats immunised with antigen emulsified in either FCA, Emulsigen or Montanide ISA50 were cultured in triplicate in culture medium (Appendix 1) in the presence of antigen. Four hours prior to harvesting at 24 h intervals between 72 and 144 h of culture, cells were pulsed with ^{3}H-thymidine at 1.25 μCi/ml. ^{3}H-thymidine incorporation was measured in a MicroBeta liquid scintillation counter. Proliferative response is reported as mean stimulation index (SI ± SEM), calculated as mean counts per minute (cpm) of stimulated cells ÷ mean cpm of unstimulated cells.

7.2.8 Histology and immunohistochemistry

Formalin-fixed paraffin-embedded sections of hearts from immunised rats were prepared and stained as per Section *4.2.5* and examined for evidence of valvulitis and carditis.

7.2.9 Statistical analysis

Statistical differences between adjuvant groups were assessed by ANOVA and LSD post-hoc tests with statistical significance set at $p \leq 0.05$.

7.3 RESULTS

7.3.1 Footpad immunisation with FCA produces severe inflammation

Initial experiments carried out in the present study found that subplantar injection of antigen/FCA emulsion produced granulomatous lesions with superficial necrosis at the injection site accompanied by oedema and erythema in the entire paw. Daily visual inspection of the rats indicated that inflammation was present on day 1 p.i. with antigen/FCA. By day 7 p.i., oedema and erythema had spread to the ankle joint

in all rats, including vehicle control rats immunised with PBS/FCA. Swelling gradually decreased in the foot over 7-14 days but returned between days 14 and 21 p.i. (Figure 7.2). On several occasions, animals exhibited signs of pain such as limping and vocalisations during handling. In some animals, the emulsion had migrated to the upper surface of the foot or to the ankle resulting in lesions at these sites. Whilst most lesions were sterile, purulent secretions from a lesion on one animal grew microflora on blood agar. However, bacteraemia was not detected in any of the rats and no granulomas were found in other organs such as lymph nodes, kidneys or lungs. Since it was speculated that swollen paws might be susceptible to secondary infection from faecal-contaminated splinters, sawdust in cages was replaced entirely by soft shredded paper. Although the rats appeared to be more comfortable following this change, inflamed paws continued to be a concern and an alternative adjuvant to FCA was investigated.

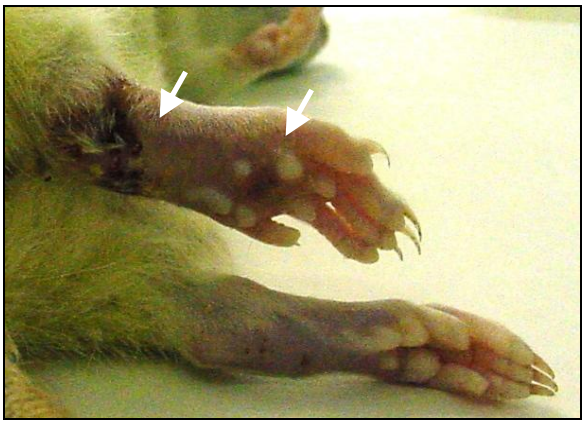


Figure 7.2 Inflammation and lesions (arrows) on Day 21 following s.c. immunisation using FCA as adjuvant.

7.3.2 FCA vs Emulsigen®

7.3.2.1 Inflammatory response after FCA or Emulsigen immunisation

In the first experiment, FCA was compared to Emulsigen®, a water-in-oil emulsion of undisclosed composition. During the first seven days following subplantar injection of antigen/Emulsigen® into the right hind foot, oedema and erythema in the treated feet and ankle joints was comparable to that of rats immunised using FCA. However, after 7 days there was a steady reduction in inflammation concomitant with the disappearance of the Emulsigen® from the injection site and

by 21 days, the mean difference between (right) antigen/Emulsigen®-immunised feet and (left) untreated feet was <1 mm (Figure 7.3). In comparison, FCA-immunised rats still showed signs of significant inflammation after 21 days. In some FCA-immunised animals, swelling of the non-injected left foot and ankle were also observed.

7.3.2.2 Cellular immune response after FCA or Emulsigen immunisation

The proliferative response of MNC derived from rats immunised with either FCA or Emulsigen® is shown in Figure 7.4. MNC from rats immunised with positive control antigen cardiac myosin mixed with Emulsigen® proliferated less to *in vitro* stimulation with myosin (SI=1.6 ± 0.6) than MNC from rats immunised using myosin mixed with FCA (SI=3.2 ± 1.3). MNC from control rats immunised with PBS/FCA responded poorly to cardiac myosin stimulation (SI=0.7 ± 0.1, not shown). The difference in proliferative response between myosin/FCA and myosin/Emulsigen-immunised rats was not found to be statistically different. However, Emulsigen did not elicit a cellular response which was considered biologically significant (SI < 3.0) (Figure 7.4).

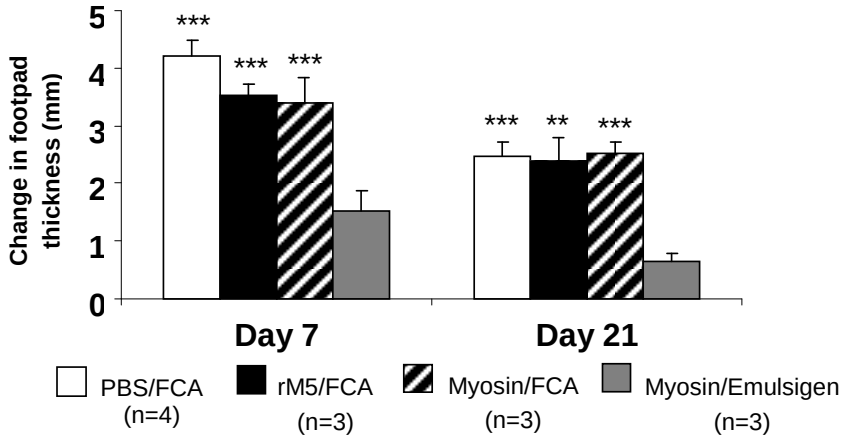


Figure 7.3 Comparison of inflammatory effects of Emulsigen® and FCA. The hind feet of rats that received s.c. injection of antigen emulsified in FCA were more inflamed than the feet of rats that were given antigen mixed with Emulsigen. Results are expressed as the change in foot thickness (mm) between the treated and untreated hind feet, measured on days 7 and 21 p.i. Error bars represent SEM. Asterisks = significant difference between rats immunised using FCA and rats immunised using Emulsigen (ANOVA, F=9.624, ** = p<0.01, *** = p< 0.001 *vs* myosin/Emulsigen).

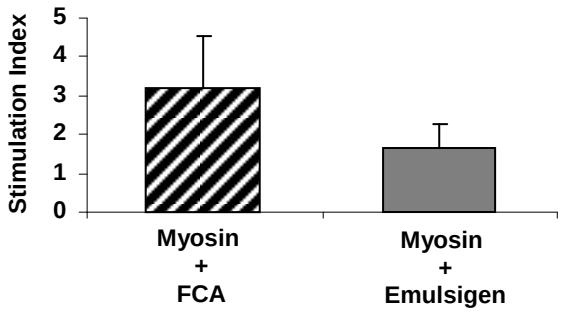


Figure 7.4 Proliferative response of T cells from rats immunised using FCA or Emulsigen.
Pooled (n=3 per group) MNC from rat spleens were stimulated for 96 h with 50 µg/ml cardiac myosin in triplicate wells. Proliferation was measured by ^{3}H-thymidine incorporation in the final 4 h of culture and is expressed as maximum stimulation index (SI) calculated as mean cpm of stimulated cells/mean cpm of unstimulated cells. SI for both immunisation groups was >100 in positive control wells stimulated with Con A. Error bars represent SEM.

7.3.3 FCA vs Montanide ISA50V

7.3.3.1 Inflammatory response after FCA or Montanide ISA50V immunisation

In the second experiment, FCA was compared to Montanide ISA50V a purified, non-metabolisable mineral oil plus emulsifier adjuvant similar to FIA. Montanide ISA50V was found to be equally as inflammatory as FCA, producing oedema and erythema in the treated foot, however, necrotic lesions seen in previous immunisation experiments were absent in these rats. There was no significant difference in foot thickness 7 days after primary immunisation between any of the treatment groups (ANOVA, df=3, F=0.642, P=0.606). By 21 days, inflammation had reduced considerably in all groups, however, again there was no difference in footpad thickness between rats immunised using FCA or Montanide ISA50V (ANOVA, df=3, F=0.275, P=0.842) (Figure 7.5).

7.3.3.2 Cellular immune response after FCA or Montanide ISA50V immunisation

Lymphocytes from rats immunised with rM5 emulsified in Montanide ISA50V did not mount a robust cellular response to *in vitro* stimulation with rM5 (SI= 3.3 ± 0.9) compared to lymphocytes from rats immunised with rM5 in FCA which, as shown in

Figure 7.6, had a significantly higher maximum proliferative response (SI= 26 ± 8.2) (ANOVA, t=3.835, df=3, $p < 0.05$). Control rats immunised with PBS/FCA or PBS/Montanide also responded weakly to stimulation with rM5 antigen with SI values of 5.0 ± 0.5 and 3.3 ±0.9 respectively.

7.3.3.3 Antibody response after FCA or Montanide ISA50V immunisation

To determine the effect of FCA or Montanide on IgG antibody response in immunised rats, sera were tested in ELISA. Low antibody titres were observed in sera from rats immunised with PBS, regardless of whether FCA (titre = 2,000) or Montanide (titre = 4,000) was used as adjuvant. In comparison, rats immunised with rM5 using either FCA or Montanide as adjuvant elicited equivalent high antibody titres (titre = 32,000).

7.3.4 Foot immunisation vs hock immunisation

Finding that Emulsigen® or Montanide ISA50V did not produce the desired immunological responses in the rats, an alternative immunisation protocol was trialled whereby the antigen/FCA emulsion is injected into the hock instead of the sole of the foot.

7.3.4.1 Inflammatory response following hock immunisation

In this experiment, localised inflammation was apparent in the majority of rats one day after injection of antigen/FCA into the hock, observed as swelling and erythema in the ankle which spread to the thigh and foot (Figure 7.7a) over the following days. Swelling gradually decreased in the foot on days 7-14 but did not recur as observed in subplantar-immunised rats. Small cutaneous lesions observed in some animals by Day 6 (Figure 7.7b) progressed to necrosis and sloughing of the skin by Day 21 (Figure 7.7c). Compared to subplantar-injected rats, hock-injected rats showed minimal signs of pain during the entire observation period, with rats suffering no lameness or impairment of weight-bearing ability in the injected limb (Figure 7.7d). Lesions were less extensive than those observed in subplantar-immunised rats and localised to the injection site (Figure 7.7e), with adjacent tissue appearing normal when examined histologically (Figure 7.7f).

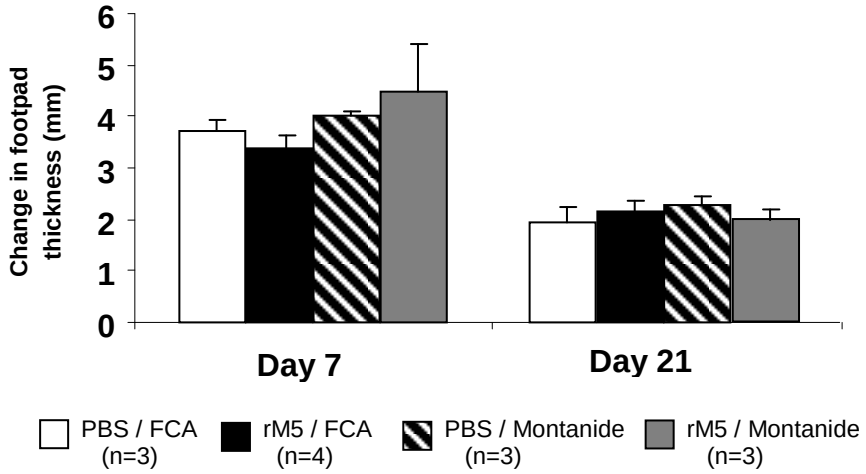


Figure 7.5 Comparison of inflammatory effects of FCA and Montanide ISA50V.
Footpad thickness was measured on days 7 and 21 p.i.and compared to thickness on day 0 prior to immunisation. Results are expressed as mean change in footpad thickness ± SEM. There was no significant difference between immunisation groups.

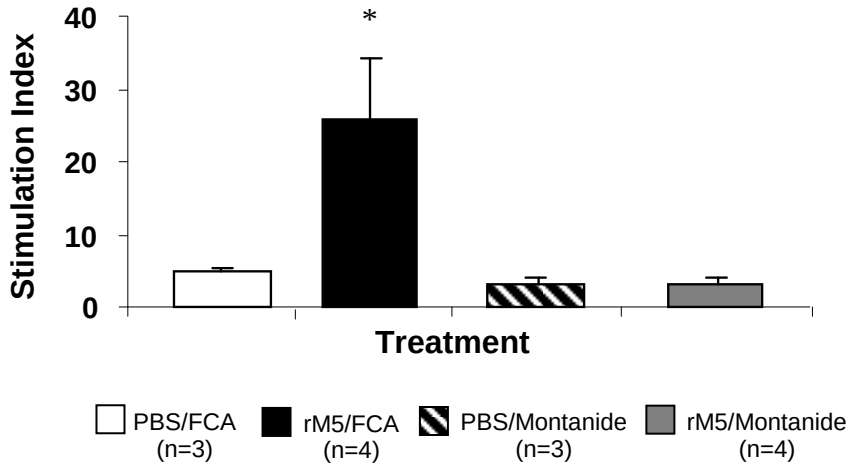


Figure 7.6 Proliferative response of splenic T cells from rats immunised using FCA or Montanide ISA50V.
Lymphocytes from rats immunised with rM5 or PBS emulsified in FCA or Montanide ISA50V were stimulated with 50 ug/ml rM5 in a lymphocyte proliferation assay. Datapoints represent mean maximum SI ± SEM of three replicates. SIs for positive control (Con A-stimulated) wells in each treatment group were between 14.1 ± 5.6 (rM5/Montanide rats) and 55.1 ± 13.1 (rM5/FCA rats). * $p \leq 0.05$ compared to other immunisation groups.

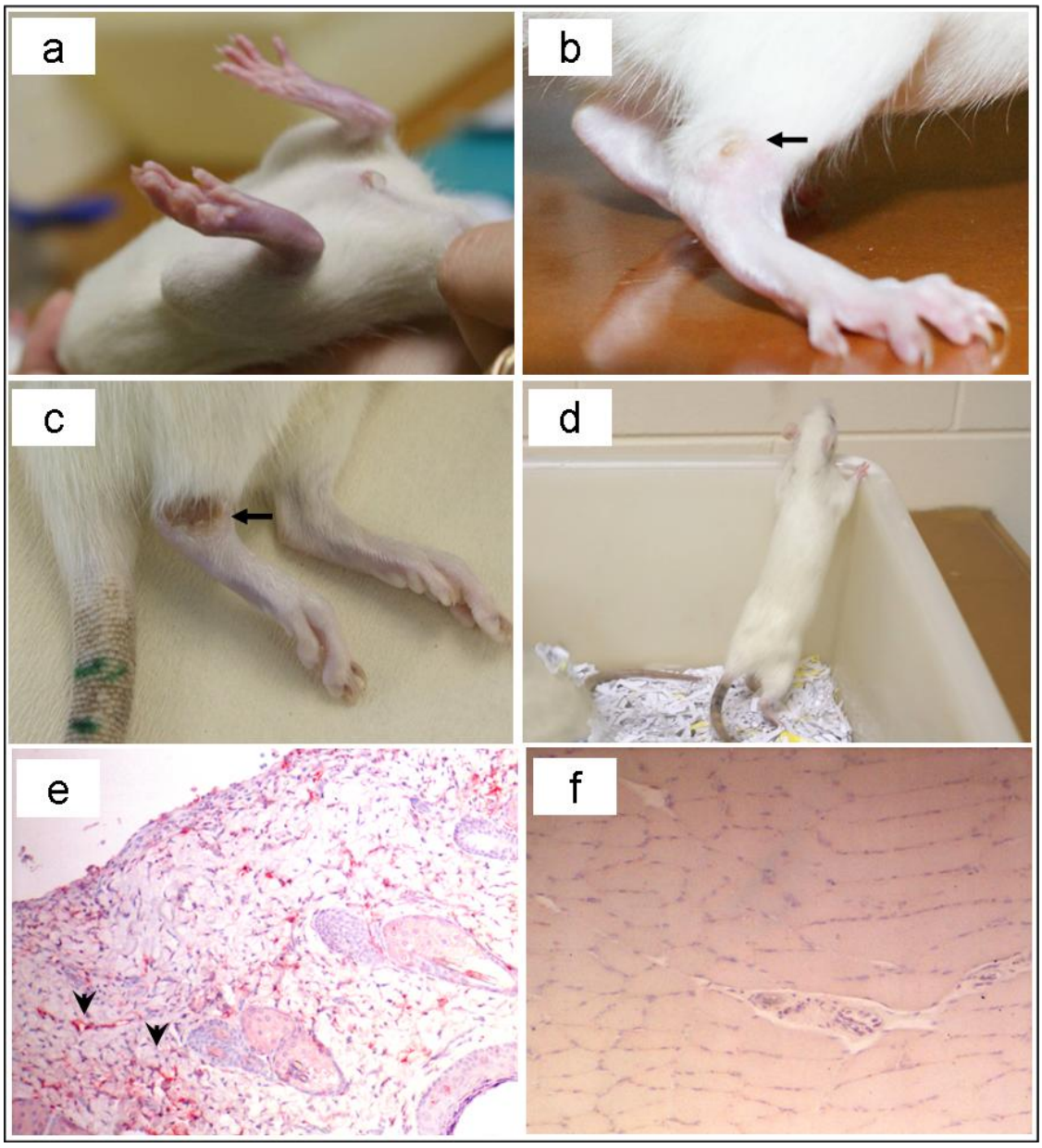


Figure 7.7 Inflammatory effects of hock immunisation.
Oedema and erythema were present in the left hind foot on Day 1 following injection of rM5/FCA into the hock (a) but had reduced considerably by Day 6 (b). FCA-induced necrotic lesions developed over Days 6-21 (b,c arrows) but rats retained full weight-bearing use of the injected limb (d). Lesions at the injection site (e) stained for infiltrating $CD3^+$ T cells (red cells) and macrophages (not shown) while inflammatory cells were absent in adjacent muscle (f).

7.3.4.2 Cellular immune response following hock immunisation

A robust rM5-specific proliferative response was detected in lymphocytes derived from rats immunised in the hock and was comparable to the response in lymphocytes from rats immunised in the foot with rM5, with no significant difference ($p = 0.9967$) in stimulation indices between hock-immunised or foot-immunised rats (Figure 7.8). Weak proliferative responses to rM5 were detected in PBS-control rats regardless of the route of immunisation. These results suggest that in Lewis rats at least, hock immunisation induces T cell responses which are comparable to footpad immunisation.

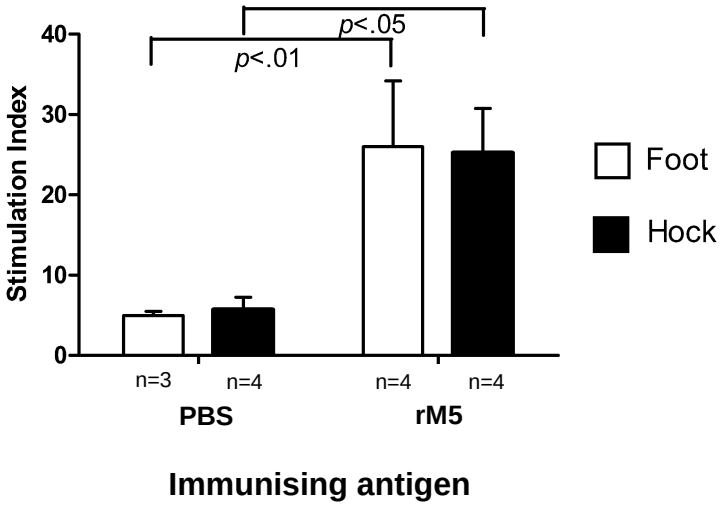


Figure 7.8 Proliferative response of T cells from foot-immunised vs hock-immunised rats.
Shown is the mean maximum SI ± SEM of three replicates. There was no significant difference in proliferative response between rats immunised via the footpad or hock.

7.3.5 Histological evidence of valvulitis following hock immunisation

Histological and immunohistochemical staining of rat hearts revealed that hock immunisation induced valvular (and myocardial) lesions that were identical to those seen in rats immunised in the footpad (Figure 7.9). Out of seven rats immunised in the hock with rM5/CFA, 5 (71%) had either valvulitis or myocarditis or both. Four (57%) had evidence of mild myocarditis and 3 (43%) had valvular lesions.

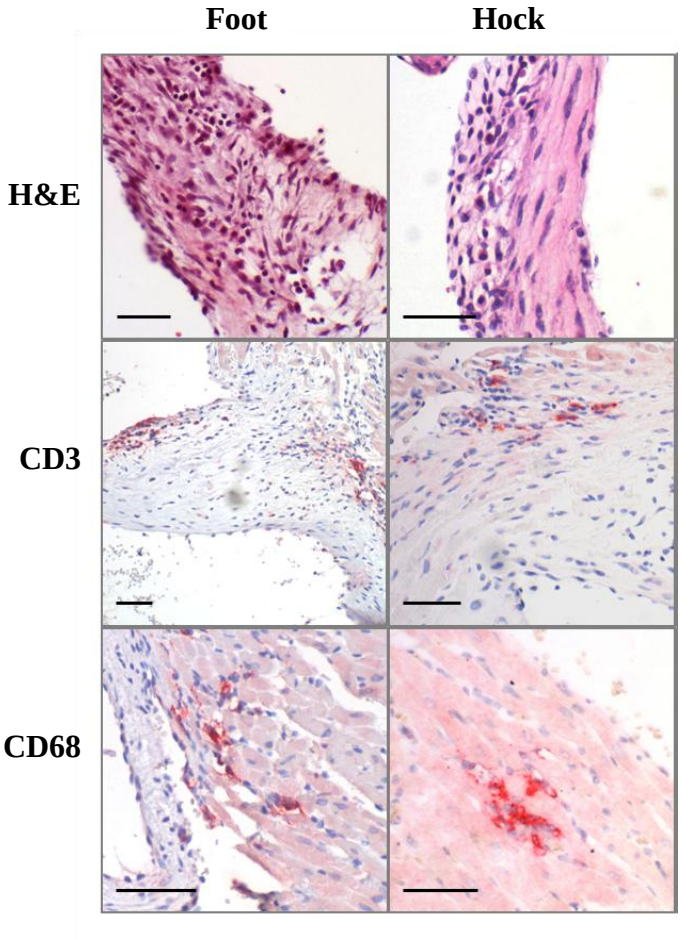


Figure 7.9 Immunohistochemical analysis of rat heart tissue.
Carditis induced in rats immunised in the foot (left panel) or the hock (right panel). Depicted are representative images of rat heart valves stained with H&E (top row) or with Fast Red using anti-T cell CD3 mAb (middle row) and myocardium stained with anti-CD3 (bottom row). 20x original objective. *Bars = 50 μm*.

7.4 DISCUSSION

An initial aim of the present study was to trial alternative adjuvants to FCA for inducing autoimmune valvulitis in the Lewis rat. FCA has been used in research for over five decades to induce experimental autoimmune diseases in animals. When delivered as a water-in oil emulsion, FCA's effectiveness has been attributed to its ability to 1) prolong exposure of autoantigens to the immune system (the 'depot' effect), 2) provide a vehicle for antigen transport through the lymphatic system to

lymph nodes and spleen to effector cells, 3) enhance antigen uptake by antigen presenting cells and 4) stimulate release of cytokines which skew the immune system towards a Th1-type response due to the presence of mycobacteria in the formulation (Cox and Coulter, 1997). Recently however, its use has become increasingly discouraged due to animal welfare concerns.

A number of criteria dictate which adjuvant is most suitable for a particular application, including the nature of the antigen, their ease of use, cost and the type of immune response required e.g. humoral, cellular or both (Cox and Coulter, 1997). Formulations which target a Th2 antibody response (e.g. aluminium salts) or were reported to cause severe reactogenicity (e.g. TiterMax, RIBI) (Leenaars *et al.*, 1998) or low incorporation efficiency (e.g. liposomes) (Cox and Coulter, 1997) were not considered for trialling. Pre-formed immune stimulating complexes (ISCOMS) reported as safe for use in animals (Cox and Coulter, 1997) and for inducing strong Th1 and Th2 responses, good presentation and targeting but require complicated adjuvant preparation (Leenaars *et al.*, 1995), were unavailable for this project and so could not be investigated.

In the current study, both Emulsigen® and Montanide ISA50V adjuvants were found to be unsuitable alternatives to FCA. Emulsigen was well tolerated, producing minor inflammation in the first few days following injection and minimal inflammation or adverse side effects thereafter. However, lack of a T cell response was most likely due to the absence of pro-inflammatory components in the Emulsigen formulation which are necessary for skewing the immune system towards a cellular response. In addition, the apparent disappearance of the emulsion from the injection site within seven days may indicate rapid clearance of the antigen and therefore a deficit in APC function. Recent studies have found that different formulations of Emulsigen elicit high antibody responses in pigs immunised with *Mycoplasma hyosynoviae* but no cellular response, measured by IFN-γ production (Lauritsen *et al.*, 2009).

In agreement with other studies (Raman *et al.*, 2004), Montanide ISA50V produced an inflammatory reaction that was equal to FCA but it also failed to induce the desired T cell-mediated immune response. In contrast, Esteves *et al.*, (2004) reported that Montanide ISA50 induced a T_H1-skewed response in *Ehrlichia ruminantium*-

immunised goats. This is not to say that other Montanide formulations, had they been trialled, would have been equally unsuccessful.

Billiau and Matthys (2001), however, argue that further research into the modes of action of adjuvants, both at the cellular and molecular level, is warranted before comparing the pathogenic mechanisms involved in experimental autoimmune diseases and naturally occurring human autoimmune diseases. It has been questioned whether adjuvants such as FCA have a greater role than merely driving Th1 cell differentiation through mycobacterial innate immunity stimulation. This argument is based on increasing evidence of prolonged myelopoiesis and aspecific activation of mononuclear phagocytes, dendritic cells and T lymphocytes by FCA and FIA in the absence of added antigen in autoimmune diseases such as adjuvant induced arthritis and oil induced arthritis.

Having not found an alternative to FCA in the above adjuvants, attention was turned to investigating a protocol for immunisation using FCA which would result in less pain and stress to the rats, whilst replicating the valvular pathology previously observed in the RAV model.

Kamala (2007) immunised six strains of mice in either the hock or footpad with two different protein antigen/FCA emulsions. After assessment of mobility and comparison of T cell, cytokine and antibody responses in different mouse strains he concluded that hock immunisation targets the same draining lymph nodes as footpad immunisation, inducing comparable local and systemic immune responses but without similar impairment of mobility. In our opinion, hock injection as described by Kamala (2007) in a mouse model and which was used in this study on rats is a superior method. This method of immunisation generates a similar immune response, is easy to perform and is more humane than subplantar (footpad) injection, particularly when combined with FCA.

CHAPTER 8
REPETITIVE EXPOSURE OF RATS TO GROUP A STREPTOCOCCAL M PROTEIN

8.1 INTRODUCTION

A major documented risk factor for developing RF/RHD is repeated GAS infections in susceptible individuals due to increased exposure to the bacteria (WHO, 2005a). GAS epidemiology in many regions where RF/RHD is endemic, including Aboriginal communities of northern Australia, northern Thailand and in Pacific Islander populations, is highly dynamic with rapid turnover of diverse M types contributing to multiple infections and high household acquisition rates (Brandt *et al.*, 2001; McDonald *et al.*, 2008; Steer *et al.*, 2009b). Although an initial attack of RF can result in carditis, the increased incidence and severity RHD is attributed to cumulative damage in the heart as a result of recurrent episodes of RF (Carapetis *et al.*, 2005a). For this reason, the principle strategy for preventing re-occurrence of RF is penicillin prophylaxis to prevent further GAS infections (WHO, 2005c).

In several early experimental models of RF/RHD, attempts to emulate rheumatic carditis by repetitive GAS immunisation failed to produce lesions which were unequivocally rheumatic in nature. In 1949 Murphy and Swift tested the hypothesis that rheumatic-like lesions might be induced in animals by 'successive group A streptococcal infections, each caused by a serological type heterologous to those previously employed' (Murphy and Swift, 1949). New Zealand rabbits inoculated 3 to 10 times intradermally or subcutaneously with live GAS over a period of up to 20 months developed inflammatory cardiac lesions characteristic of acute or chronic carditis. However, only a very small proportion of rabbits exhibited these lesions, and a high number of test animals died from bacteraemia.

Repetitive pharyngeal inoculation (for three or six days) of Swiss albino mice with rheumatogenic GAS serotype M18 resulted in five out of 165 (3.03%) of the animals developing histopathological evidence of cardiac lesions, although these were not characteristic of rheumatic carditis (Stephen *et al.*, 1998). Moreover, intranasal

inoculation with a skin-associated GAS serotype (M49) could elicit mild to chronic endocardial lesions in three of 30 (10%) of these mice (Stephen *et al.*, 1998). In further studies, the development and dynamics of the antibody response against the M protein was also determined in the Swiss albino mouse model. Mice inoculated intranasally with M18 GAS for one, two or three days developed high type-specific IgG responses to M18 protein, peaking between days 42 and 70 p.i. but waning by Day 84. Interestingly, although variations in titres were found between different mice, there was an overall negative correlation between maximum antibody titres and number of doses, leading the authors to suggest that just one episode of pharyngeal GAS infection could elicit high type-specific immunity in the host (Gladstone *et al.*, 2005). However, while these data provide important insights into antibody responses against GAS infection, T cell responses after repetitive exposure were not addressed in this model, nor were the infiltrating inflammatory cell populations present in the lesions identified.

Recently, Huang *et al*. (2009) used a Lewis rat model to test whether immunisation with formalin-inactivated GAS could induce carditis without the need for *B. pertussis* adjuvant. In this study, four of five rats which were boosted three times developed focal myocardial inflammatory cell infiltration and two rats developed valvulitis while of eight rats given one booster only, three developed myocarditis and none had valvulitis. Again, evaluation of the immune responses elicited by repeated exposure to GAS was confined to H&E staining of heart tissue and assessment of antibody levels: T cell responses were not investigated.

Our initial studies provided evidence that immunisation of Lewis rats with GAS M5 protein or peptides from M5 develop carditis which is histologically and immunologically similar to RF/RHD. We then asked whether, in this RAV model, repeated exposure to GAS M protein could emulate the repetitive occurrences of RF seen in human patients and lead to exacerbation of carditis. To address this question, we investigated responses in rats immunised with rM5 protein and boosted one, two or three times. We also recorded electrocardiograms on selected rats to determine whether the RAV model exhibits the same electrophysiological alterations of the heart, namely P-R interval prolongation, as seen in patients with rheumatic carditis (NHFA, 2006). It was hypothesised that the antibody and T cell responses would

increase in a dose-dependent manner. Furthermore, it was anticipated that repetitive immunisation would increase the incidence and/or severity of carditis in rats.

8.1.1 Aims

The work described in this chapter was conducted to: -

1) Compare the T cell proliferative responses and IgG antibody responses in Lewis rats subjected to a primary immunisation with GAS rM5 protein and boosted one, two or three times
2) Employ histological and immunohistochemical methods to compare the incidence and severity of carditis in rats receiving one, two or three boosters of GAS rM5 protein
3) Investigate whether, like patients with RF/RHD, the RAV model exhibits alterations to the functional properties of the heart, detected as a prolongation of the P-R interval by ECG.

8.2 MATERIALS AND METHODS

8.2.1 Antigens and adjuvant

Rat cardiac myosin and recombinant GAS M5 protein (rM5) were prepared as per Sections *4.2.3.1* and *4.2.3.2* respectively. Porcine cardiac myosin was purchased commercially (*Sigma).* Freund's complete adjuvant (FCA), with 5 mg of heat-killed mycobacteria H37RA per ml and Freund's incomplete adjuvant (FIA) without mycobacteria were purchased from *Sigma*.

8.2.2 Experimental animals

Female Lewis rats (LEW/ SsN; RT^1) were purchased from the Animal Resources Centre (Western Australia) and housed in the Small Animal Breeding Facility at James Cook University (Townsville, Australia) as described in Section *3.1.2.1*. The rats were acclimatised for one week prior to experimentation and were used between 6 and 9 weeks of age. Experimental procedures were conducted in accordance to institutional guidelines under James Cook University Animal Ethics Committee (Approval number A1326).

8.2.3 Immunisation of rats

Under general ketamine and xylazine anaesthesia (Section *3.1.2.2*), rats (120 to 171 g in weight) in groups of 3-4 were given an initial immunisation of 500 µg of rM5 antigen emulsified in FCA in a total volume of 200 µl, delivered s.c. in the right hind hock as described in Chapter 5 of this thesis. Control rats received PBS and adjuvant only. All rats were given 10^{10} whole formalin-killed *B. pertussis* cells in 100 µl PBS as additional adjuvant, delivered i.p. 1 and 3 days after primary immunisation (d.p.i.). Booster immunisations comprising the same antigen emulsified in FIA were given s.c. in the flank. Groups of rats (n=3 or 4) were immunised according to the schedule outlined in (Table 8.1). All rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation 14 days after receiving their final booster.

Table 8.1 Rat immunisation groups

Group	Antigen	Treatment
1A	PBS	Primary + 1x booster
1B	rM5	Primary + 1x booster
2A	PBS	Primary + 2x boosters
2B	rM5	Primary + 2x boosters
3A	PBS	Primary + 3x boosters
3B	rM5	Primary + 3x boosters
Normal	None	Non-immunised

Two different immunisation schedules were used in these experiments and an overview of each is shown in Figure 8.1. For the first immunisation schedule used, all six treatment groups (1A and 1B; 2A and 2B; 3A and 3B) received a primary boost on Day 0. Group 1 rats received one booster only at day 7 p.i.; Group 2 rats were boosted at days 7 and 14 days p.i. and Group 3 rats received 3 boosts, given at days 7, 14 and 28 p.i. The rats were then sacrificed on Days 21, 28 and 35 respectively (see Figure 8.1.A).

The schedule was subsequently altered so that all groups were sacrificed on the same day (Figure 8.1.B). Therefore, primary injections were given to groups 3A and 3B, 2A and 2B and 1A and 1B on Days 0, 7 and 14 respectively.

8.2.4 Lymphocyte proliferation response following immunisation with rM5

To determine whether rats had heightened T cell responses following repetitive immunisation with rM5, the proliferative response of T lymphocytes derived from spleens or draining lymph nodes (popliteal and inguinal) was measured in a standard lymphocyte proliferation assay. Single cell suspensions of mononuclear cells were prepared from rat spleens or lymph nodes, counted by trypan blue exclusion and stimulated in culture using the procedures described in Sections *3.2.6.3, 3.2.6.4* and *3.2.6.5* of General Materials and Methods. Cells were cultured in triplicate wells for 72 to 120 h in the presence of rM5 or collagen type IV at 50 μg/ml, 200 μg/ml M5 peptide, porcine cardiac myosin at 12.5, 50 and 150 μg/ml, PPD at 7.5 μg/ml or Con A as positive control at 5 μg/ml. Negative control wells were left unstimulated. Responses are expressed as stimulation index ± standard error (SI ± SEM) unless otherwise stated.

8.2.5 Antibody responses following repetitive immunisation with rM5

Total serum IgG levels in the sera of rats boosted one, two or three times were measured by ELISA. Maxisorp F96 plates were coated with rM5 at 1 μg/ml, porcine cardiac myosin at 10 μg/ml, rat cardiac myosin at 50 μg/ml or tropomyosin at 10 μg/ml as solid phase antigen. Rat serum samples were applied at a 1:4 dilution in duplicate wells. Control wells contained either 1) serum from normal, unimmunised rats, 2) no antiserum or 3) no antigen and no antiserum. All remaining steps in the ELISA procedure were the same as those described in Chapter 6, Methods and Materials, Section *6.26*. For each sample the titre was defined as the lowest dilution that gave an absorbance of more than three standard deviations above the mean absorbance of control wells (containing normal rat serum).

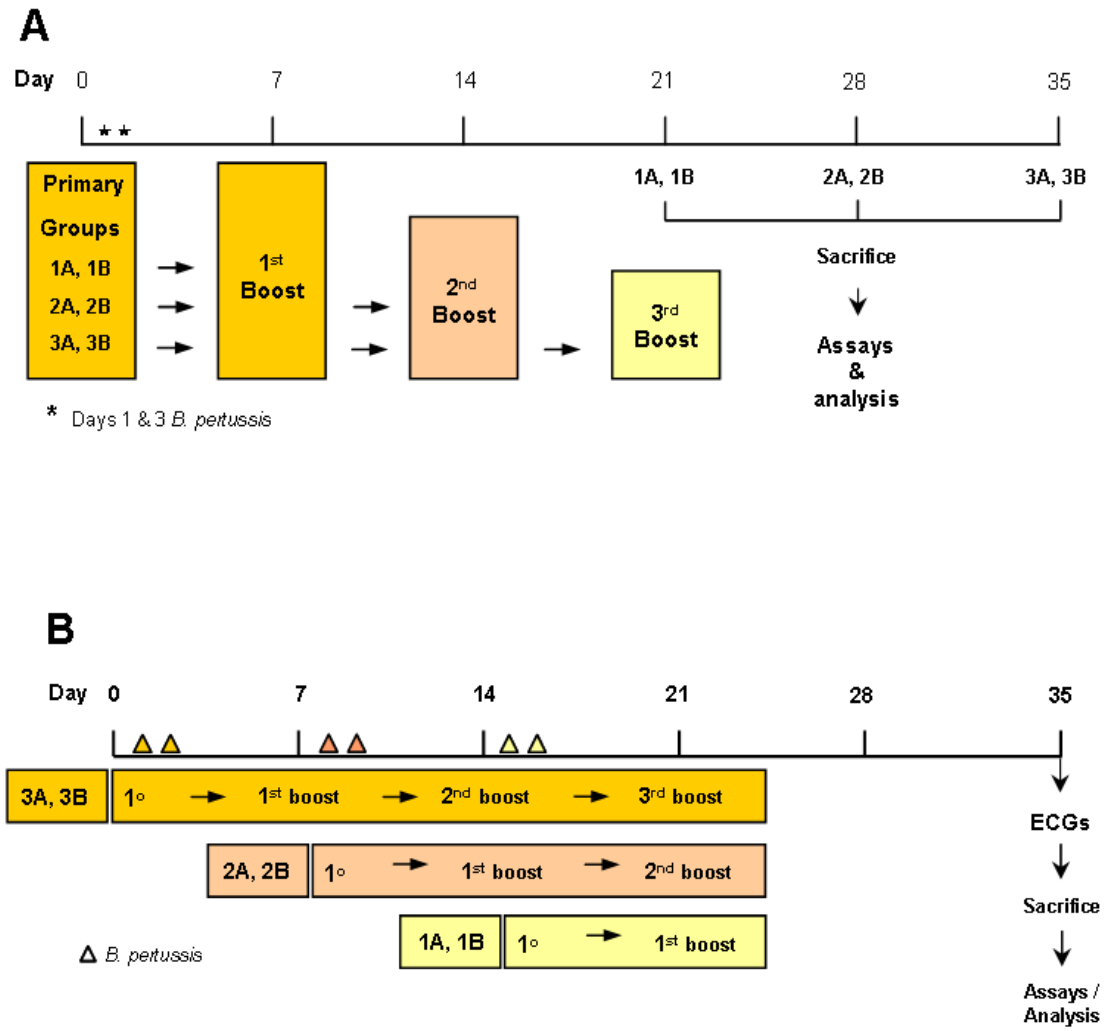


Figure 8.1 Immunisation schedules for rats receiving 1, 2 or 3 boosters. Rats were immunised s.c. with 500 µg rM5 protein in FCA and boosted with 500 µg rM5 in FIA. Animals were euthanased 14 days after final booster. **A**) All rat groups were given the primary immunisation on Day 0 and Groups 1, 2 and 3 were killed on Days 21, 28 and 35 respectively. **B**) Primary immunisation was staggered at 7 day intervals to allow all rats to be euthanased on the same day.

ELISA was also used to examine C-reactive protein (CRP), in sera from rats immunised with 500 µg of rM5 and boosted one or three times to determine whether elevated CRP, a marker of inflammation and tissue injury, could be used as an indicator of cardiac damage in the RAV model. One rat only per treatment group was tested. Control serum from a PBS-immunised rat was used at the same dilution as test sera (1:4,000) for a negative control and serum from a rat immunised with 500 µg cardiac myosin and boosted once was used at a dilution of 1:16,000 as positive control. CRP levels were measured using a Rat C-Reactive Protein ELISA kit (*BD Biosciences*) according to instructions.

8.2.6 Histological examination of rat hearts

Hearts were excised and fixed in neutral buffered formalin (NBF) prior to paraffin embedding and preparation of tissue sections. Heart sections (5 µm) were either stained with H&E or subjected to heat-induced antigen retrieval prior to immunohistochemical staining using antibodies against CD3, CD8 and CD68 to detect infiltrating T cells and macrophages (as outlined in Section *3.2.7.3*). Myocardium and valve tissue was examined microscopically and scored blindly for the presence and severity of carditis using the scoring system described in Chapter 4, Table 3.2.

8.2.7 Electrocardiographs

Additional experiments were carried out to investigate whether electrophysiological abnormalities could also be observed in the RAV model. Electrocardiograph (ECG) traces were recorded 28 days after receiving a third booster (49 days after initial immunisation) for rats immunised with rM5, PBS control rats and normal, non-immunised rats (n=3 per group). Anaesthesia was initially induced in rats by placing the entire rat in a large animal nose cone attached to a vaporisor and exposing the rat to 5% isofluorane in 100% oxygen (Figure 8.2). After sufficient depth of anaesthesia was obtained, determined by cessation palpebral reflex (eye-blinking) and loss of pedal withdrawal reflex, the rats were maintained under anaesthesia using 1.75% isofluorane. Heart rates whilst under anaesthesia were within a normal range of 350 beats per minute (bpm) to 450 bpm. Electrocardiographs were detected with a

PowerLab 8/30 acquisition system fitted with an Animal BioAmp and recorded with Labchart Pro software (ADInstruments). Leads were attached to the animals using the Lead II configuration and after an initial resting period, ECG traces were recorded for 20 min. All animals recovered within 5 min following removal of isofluorane. ECG traces were analysed manually by measuring the P-R intervals (in milleseconds) in four to nine 'clean' ECG complexes for each rat and comparing mean P-R intervals for each immunised rat group to normal, non-immunised control rats.

8.2.8 Statistical analysis

Data were analysed using SPSS S 17.0 software or Graphpad Prism 5.00. Group mean and standard error of the mean (SEM) were calculated using standard formulae. Significance of differences between groups for antibody levels was calculated using two-way ANOVA with Bonferroni post hoc test and a two-tailed, unpaired Student's *t*-test was used to determine significance of differences between groups for P-R interval data. A p-value of < 0.05 considered significant.

Figure 8.2 Anaesthetised Lewis rat undergoing ECG.
The large nose cone (centre of picture) was used for initial anaesthetisation

8.3 RESULTS

To examine the hypothesis that, in the RAV model, repeated exposure to GAS could lead to exacerbation of carditis and that the observed pathological immune responses parallel those seen in RF/RHD patients, Lewis rats were given a primary immunisation with rM5 protein and boosted one, two or three times. The T cell proliferative response following *in vitro* stimulation, the serum IgG antibody response and the changes in cardiac tissue were all evaluated.

8.3.1 Repeated exposure to streptococcal M protein results in increased T cell proliferation in response to stimulation

Mononuclear cells (MNC) derived from the spleens of immunised rats were cultured in the presence of antigens including rM5 and selected 'self' proteins cardiac myosin, tropomyosin or collagen IV. Cell proliferation in response to *in vitro* stimulation was measured using a standard ^{3}H-thymidine incorporation assay as described in Section *3.2.6.5*.

8.3.1.1 T cell responses induced by immunisation using schedule A

Rats subjected to immunisation schedule A (Section *8.2.3*), whereby all rats received a primary immunisation on the same day then boosted on Day 7 only (Groups 1A and 1B) or Days 7 and 14 (Groups 2A and 2B) or Days 7, 14 and 21 (Groups 3A and 3B), were sacrificed at seven day intervals (on Days 21, 28 and 35 post primary immunisation).

The maximal proliferative responses in lymphocytes from rat spleens to *in vitro* stimulation with various antigens are shown in Figure 8.3. rM5-immunised rats which received only one booster responded significantly more vigorously (SI ± S.E.M. = 25.2 ± 5.5) to stimulation with rM5 protein than PBS-immunised control rats boosted once (5.67 ± 1.6), two-tail, t = 3.406, df = 4, $p = 0.03$. Similarly, rM5-immunised rats had significantly higher responses than control rats to rM5 stimulation following two (SI = 24.5 ± 2.9 *vs* SI = 10.2 ± 2.1, two-tail, $t = 4.013$, df = 4, $p = 0.016$) or three boosters (SI = 68.43 ± 9.8 *vs* 6.93 ± 2.8, two-tail, $t = 6.07$,

df = 4, p = 0.004). Con A-stimulated control cells from all rat groups responded with SIs > 30 (not shown).

Significant differences in proliferation were observed between rats boosted three times after a primary immunisation with rM5 and those boosted one (Student's, two-tailed, t = 3.8539, df = 4, p = 0.0182) or two times (t = 4.3138, df = 4, p = 0.0125). In fact, no differences were observed in the proliferation profile for the 1 boost and 2 boost rM5-immunised rats over the 120 h culture period (Figure 8.4).

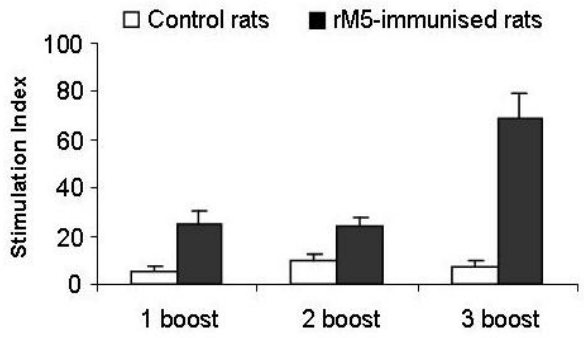


Figure 8.3 T cell responses in rats after repetitive exposure to GAS rM5 using immunisation schedule A.
Lewis rats (n=4 per group) were immunised with rM5 in adjuvant and boosted 1 – 3 times at seven day intervals. The proliferative response in splenic MNC is expressed as maximal SI ± SEM, measured over 3-5 days of culture. Mean counts per minute of spleen cells from control rats in the absence of antigen was 505 ± 68.

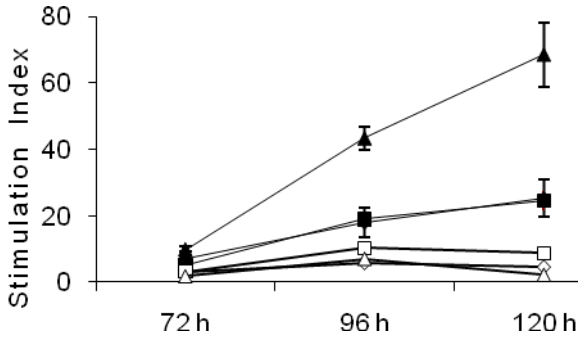


Figure 8.4 Proliferation profile of rM5-sensitised T cells.
Shown is the proliferative response over 120 h culture of T cells derived from Lewis rats immunised with rM5 protein (closed symbols) and control rats (open symbols) boosted (♦) one, (■) two or (▲) three times using schedule A. Vertical bars = SEM.

8.3.1.2 T cell responses induced by immunisation schedule B

Because immunisation Schedule A entailed euthanasing booster groups at seven day intervals, there were concerns that variations in cell processing or culture conditions between batches could affect results. During these experiments cell cultures were subjected to fluctuating incubator temperature (34 ºC – 39 ºC) and CO_2 levels. Consequently, the immunisation protocol was re-designed to reduce potentially confounding conditions. Under immunisation schedule B, all booster groups were killed and processed on the same day so that cell manipulation was more consistent and proliferation assays were run in parallel. In addition, variability in incubator conditions were resolved.

As with schedule A, rM5-immunised rats responded significantly more than control rats to stimulation with rM5 regardless of the number of boosters (Figure 8.5), although stimulation indices were consistently lower than those from schedule A. Con A stimulation (positive controls) induced proliferation of splenic T cells from all groups (SI >3). Unexpectedly, the highest proliferative response was seen in rats given two boosters compared to those given one (two-tailed, unpaired; $t = 5.20$, df = 4, $p = 0.007$) or three boosters ($t = 6.37$, df = 4, $p = 0.003$).

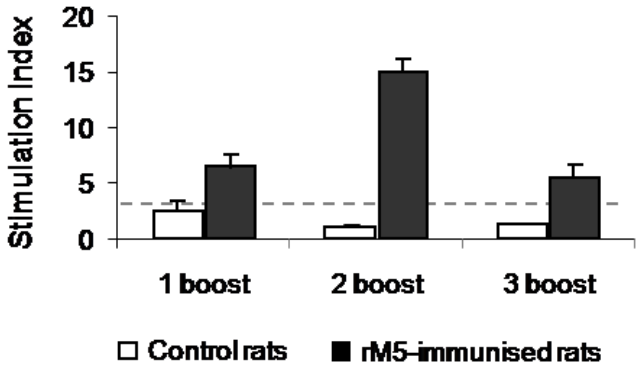


Figure 8.5 T cell responses in rats after repetitive exposure to GAS rM5 protein using immunisation schedule B.
Lewis rats (n = 3-4 per group) immunised with rM5 in adjuvant and boosted 1–3 times at seven day intervals had a higher response than PBS-immunised control rats to stimulation with (50µg/ml) rM5. The proliferative response is expressed as maximum SI ± SEM measured at 24 h intervals over 4 days of culture. Mean counts per minute of spleen cells from control rats in the absence of antigen was 182 ± 38. Dashed line denotes a positive response (SI = 3).

When cultured in the presence of basement membrane protein collagen type IV (CIV), proliferation in T cells from rats immunised with rM5 using Schedule A increased with each booster received. However a similar trend, albeit at lower levels, was also observed in control rats (Figure 8.6). No evidence of T cell cross-recognition of myosin was observed as stimulation with porcine cardiac myosin resulted in any significant proliferation in either treatment group, regardless of the number of boosters. Microscopic examination of plates revealed that in contrast to cells in other wells, which sedimented to form a typical 'button' in the rounded bottom of the well, cells stimulated with collagen and cardiac myosin tended to disperse evenly over the base of the well.

On the other hand, both rM5-immunised and control rats responded (SI >3) to control antigen purified protein derivative (PPD) (data not shown), a constituent of the complete adjuvant used in these studies and to the T cell mitogen Con A, indicating that the animals did have competent immune responses.

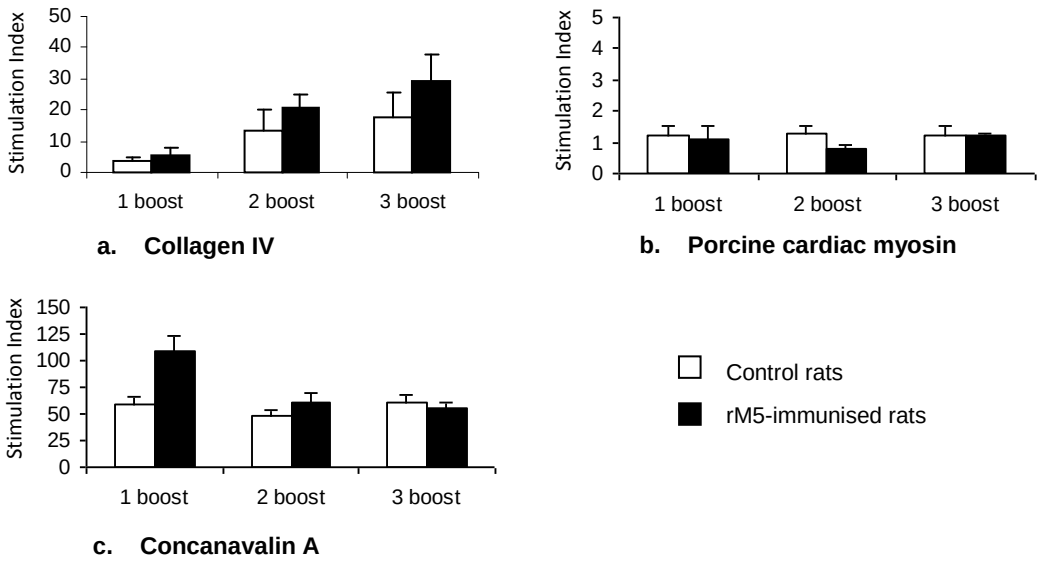


Figure 8.6 Proliferative responses following antigenic stimulation of T cells from rats immunised using schedule A.
MNC derived from the spleens of rats boosted 1, 2 or 3 times were cultured in the presence of a) collagen type IV (50 µg/ml), b) porcine cardiac myosin (50 µg/ml) and c) T cell mitogen concanavalin A (5 µg/ml). Shown is the maximum proliferative response of T cells from control rats (open boxes) or rM5-immunised rats (closed boxes) expressed as stimulation index + SEM (vertical bars).

Consistent with our data from rats immunised using schedule A, no evidence of cross-recognition was observed in splenic T cells stimulated with porcine cardiac myosin at 5 µg/ml or 50 µg/ml, rat cardiac myosin at 50 µg/ml or tropomyosin at 5 µg/ml (SI < 2 for all rat groups), regardless of the number of boosters (Figure 8.7). However, both rM5-immunised rats and controls responded (SI>3) to PPD and Con A (not shown).

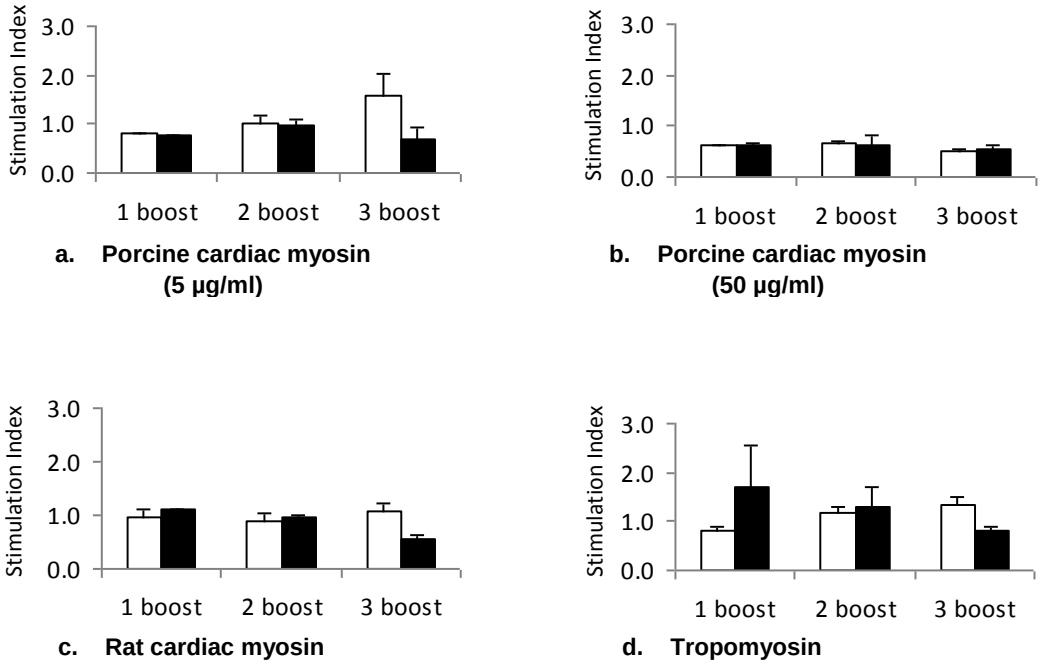


Figure 8.7 Proliferative responses following antigenic stimulation of T cells from rats immunised using schedule B.
MNC derived from the spleens of rats boosted 1, 2 or 3 times were cultured in the presence of **a**) 5 µg/ml porcine cardiac myosin **b**) 50 µg/ml porcine cardiac myosin **c**) 50 µg/ml rat cardiac myosin or **d**) 5 µg/ml tropomyosin. Shown is the maximum proliferative response of control rats (open boxes) and rM5-immunised rats (closed boxes) expressed as maximal SI + SEM.

8.3.2 Repetitive immunisation with GAS M5 protein enhances antibody responses in Lewis rats

8.3.2.1 Antibody responses against GAS rM5 protein

Levels of rM5-specific IgG antibodies were measured in sera obtained from rats immunised with streptococcal rM5 protein and boosted one, two or three times at 7 d intervals. As all sera were collected 14 d after the final booster (at time of death) and frozen until used in ELISAs, rats receiving the same number of boosters administered using either immunisation schedule A or B should have equivalent antibody responses. As shown in Figure 8.8 this is indeed the case, with IgG levels increasing as a function of number of boosts. Compared to control rats which did not react with rM5, a significantly higher ($p < 0.0001$) anti-rM5 antibody response was observed in rats immunised with rM5 protein.

Regardless of the immunisation schedule used, rM5-immunised rats given one booster had a lower response to rM5 protein, with more variability between individual animals than rats that had been boosted two or three times. However, there was no difference ($p > 0.05$) rats boosted two or three times.

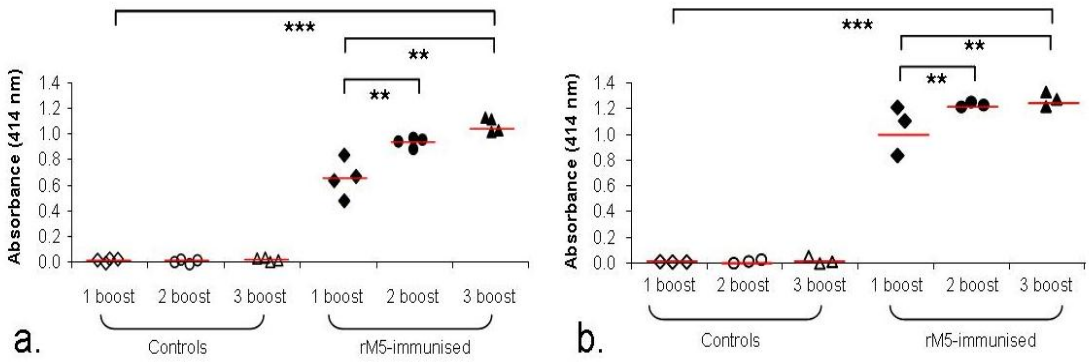


Figure 8.8 Serum IgG responses to rM5 protein after 1, 2 or 3 boosters
Rats subjected to **a)** immunisation schedule A or **b**) schedule B were immunised with rM5 protein (closed symbols) or PBS as control (open symbols) and boosted (♦) once, (●) twice or (▲) three times. *** $p < 0.0001$ rM5-immunised *vs* control rats by two way ANOVA. ** $p < 0.05$ rM5-immunised rats boosted once *vs* rM5-immunised rats boosted two or three times. Horizontal bars = mean absorbance of 3 or 4 rats per group.

8.3.2.2 Antibody cross-reactivity with denatured rat cardiac myosin

When sera from rM5-immunised rats were tested in ELISA, IgG cross-reactivity with rat cardiac myosin varied both within and between booster groups. As shown in Figure 8.9, a higher proportion of sera from rM5-immunised rats that had received two or three boosters recognised heat denatured rat cardiac myosin than rats boosted once only. Three of 4 rats boosted two times and two of 3 rats boosted three times showed positive myosin cross-reactivity, determined as three standard deviations above the mean absorbance of sera from control rats, while antiserum from only one of 4 rats that received one booster reacted with cardiac myosin (Figure 8.9).

8.3.2.3 C-reactive protein as a marker of cardiac damage in immunised rats

The concentration of CRP in sera from rats was quantitated from a standard curve by ELISA. Equivalent CRP levels were detected in rM5-immunised rats boosted once (148 ± 1.1 µg/ml) or three times (136 ± 0.5 µg/ml) and in the normal rat (164 ± 2.6 µg/ml), as shown in Figure 8.10. In contrast, serum from a cardiac myosin-immunised rat with severe myocarditis had a CRP concentration of 408 ± 9.6 µg/ml. It should be noted however, that due to only one rat per treatment being tested, this data was not amenable to statistical analysis.

8.3.3 Repetitive booster immunisation exacerbated histological perturbations in rat heart tissue

Microscopic analysis of hearts from rM5-immunised rats revealed that animals that received two or three booster immunisations had more infiltrating inflammatory $CD3^+$ and $CD68^+$ cells in both the myocardium and valves than animals that had received only one booster (Figure 8.11). $CD8^+$ T cells were not observed in the valves of any of the rM5-immunised or PBS control rats.

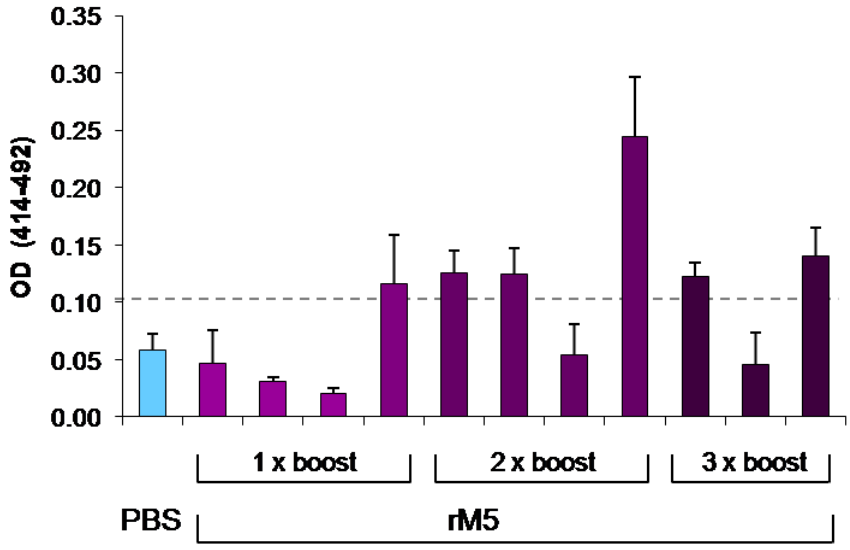


Figure 8.9 Antibody cross-reactivity with denatured rat cardiac myosin in rM5-immunised rats boosted 1, 2 or 3 times.
Antisera from individual rats immunised with rM5 as per schedule A were tested for cross-reactivity with heat denatured cardiac myosin coated at 50 µg/ml in an ELISA. Only three serum samples from 3x boosted rM5-immunised rats was available for testing and results from all PBS control rats (boosted 1-3 times) is represented by a single mean OD value. Dashed line represents mean absorbance of control serum from normal, non-immunised rats ± 3SD.

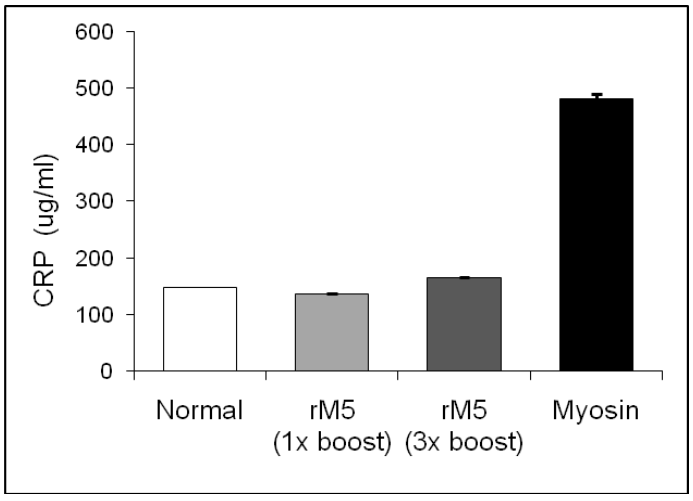


Figure 8.10 Serum C-reactive protein in immunised and control rats.
CRP concentrations in rat sera were measured by ELISA. Equivalent levels of CRP were detected in normal and rM5-immunised rats. In contrast, positive control serum from cardiac myosin-immunised rats had a very high level of CRP.

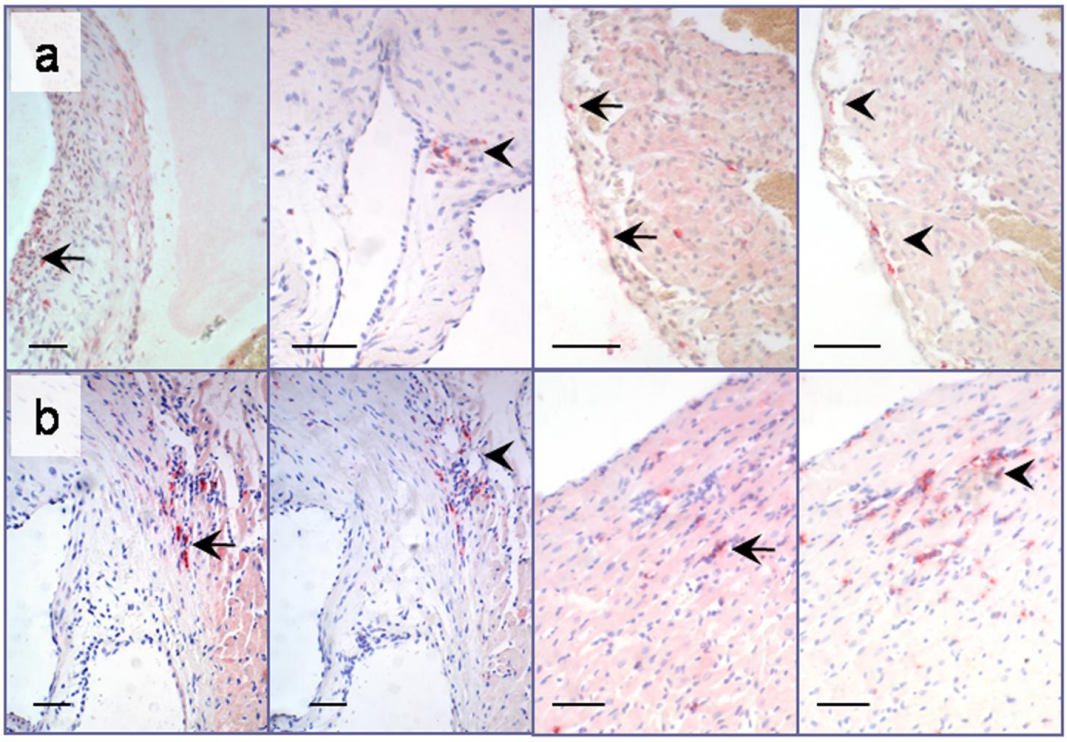


Figure 8.11 Inflammatory infiltrates in heart valves and myocardium in immunised rats following 1 or 3 booster(s).
Hearts from rats immunised with rM5 protein and boosted a) once or b) three times were stained using anti-$CD3^+$ antibody to detect infiltrating T cells (arrows) and anti-$CD68^+$ antibody to detect macrophages (arrowheads).

8.3.4 Electrocardiographic analysis

P-R interval prolongation is a common finding in rheumatic carditis and is one of the minor manifestations included in the Jones Criteria for diagnosis (WHO, 2004). Forty-nine days after primary immunisation, P-R intervals were examined in ECG traces from rats boosted three times immunised with either rM5 or PBS and compared to the P-R intervals in normal healthy, non-immunised rats. A significantly ($p < 0.01$) longer mean P-R interval (70.92 ± 0.8 ms) was observed in rats immunised with rM5 compared to normal, healthy rats (58.93 ± 0.6 ms). PBS-immunised control rats (data not shown) also had a longer, but non-significant mean P-R interval compared to normal rats (Figure 8.12).

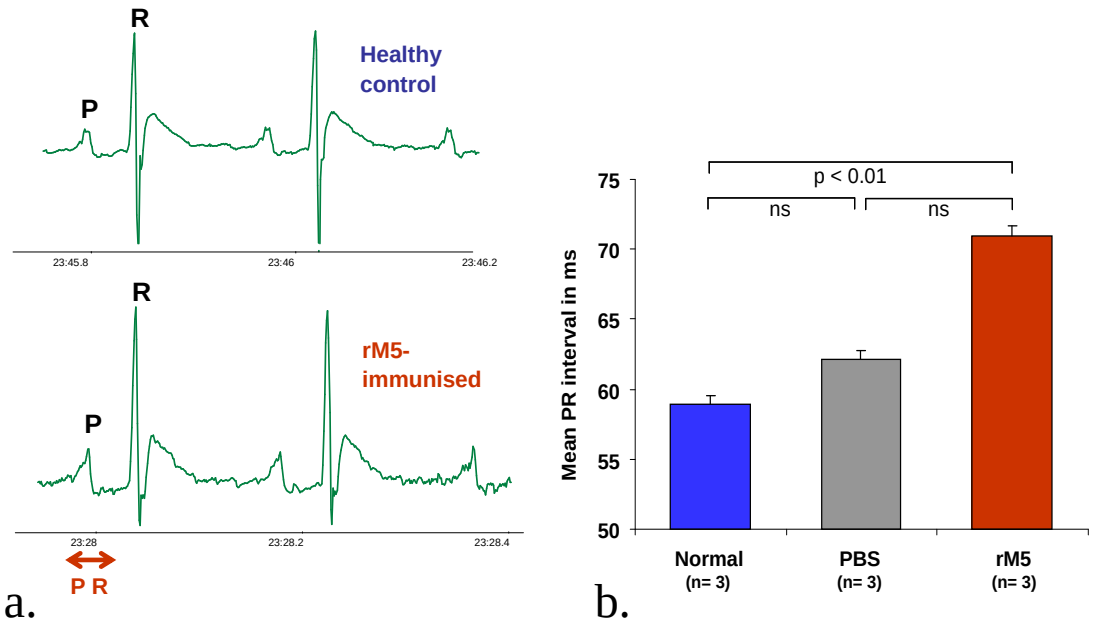


Figure 8.12 P-R interval analysis following ECG in Lewis rats.
a) Representative ECG complexes from a healthy, unimmunised and a rM5-immunised rat recorded under anaesthesia as described in Methods and Materials. b) Compared to age- and sex-matched healthy controls, rM5-immunised rats had a significantly longer (*$p < 0.01$) P-R interval. No significance differences were found between normal and PBS control rats or PBS-control and rM5-immunised rats.

8.4 DISCUSSION

The aim of the work described in this chapter was to investigate whether repeated booster immunisations in the RAV model could induce conditions that emulate recurring RF episodes in human patients and lead to exacerbation of carditis. It is well documented that recurrent RF is a major risk factor for the development of RHD, with progressive damage to heart valves leading to debilitating disease or even death from heart failure (WHO, 2004; Carapetis *et al.*, 2005a). While permanent heart damage can occur during a first episode of RF, in some milder cases pancarditis involving the myocardium, pericardium and endocardium (including valvular endocardium) is transient and resolves on recovery from RF with no long term sequelae (Binotto *et al.*, 2002). RHD may also occur sub-clinically, becoming apparent only after a subsequent episode of RF (Marijon *et al.*, 2007; Rayamajhi *et al.*, 2009). Repeated infection with GAS bacteria is the aetiological cause of recurrent RF in susceptible individuals and may account for the high prevalence of

initial RF episodes in 5-14 year old children as they are exposed to successive strains of GAS in their environment.

We administered additional booster immunisations of rM5 protein to Lewis rats to investigate the immune responses induced by repetitive exposure to GAS. Rats were boosted at seven day intervals with a subcutaneous injection of 500 µg of rM5 in FIA in the flank, with the first booster given seven days after initial immunisation in the hock.

T cells from rats that were immunised using Schedule A and boosted either one or two times demonstrated no difference in proliferative response to *in vitro* rM5 stimulation, whilst rats that had received three boosters had a significantly higher T cell response. In the second experiment (Schedule B) on the other hand, rats boosted twice had the highest T cell response, whilst rats boosted one or three times displayed a similar, but lower T cell response.

In the first experiment, each group of rats was sacrificed 14 days after the final boost and lymphocytes assays were therefore established at seven day intervals, which may have resulted in subtle differences between culture conditions for each group. Therefore, it may be imprudent to conclude, based on these results, that one primary immunisation followed by three boosters induces the highest T cell response in the RAV model. In contrast, by staggering the day of initial immunisation in the second experiment, all rat groups were sacrificed concurrently 14 days after the final boost and proliferation assays for each immunisation group were run in parallel, which should have maintained consistency in assay set-up and culture conditions. The results from this experiment suggest that, in the RAV model, the highest number of activated T cells occurred after an initial immunisation followed by two booster immunisations at seven day intervals.

The disparity in proliferative responses between the schedules A and B were also reflected in positive control wells stimulated with PPD and with the mitogen Con A, suggesting that the proliferation assay itself, not the immunisation protocol, was responsible for the different results. Overall, the proliferative response of cells from rats immunised as per schedule B, measured as mean counts per minute and

consequently stimulation indices, were only 25% of those from rats immunised using schedule A. The consistently reduced T cell responses in the second experiment may have been a matter of timing when setting up proliferation assays. Assays had to be set up for only two treatment groups each week under schedule A whereas under schedule B, cells from all six treatment groups had to be processed in one day. The increased time between initial isolation from the spleen to plating in culture medium may have affected the level of response to stimulation.

Dynamics and kinetics of antigen-specific T cell responses *in vivo* are complex and are dependent on the number of precursor T cells available for activation and MHC affinities for antigen, in addition to antigen type and dose or timing of secondary exposure. In a primary response to infection by a replicating micro-organism such as GAS, a number of naive T cells with both high- and low affinity for antigen/MHC complexes displayed on APC, will be induced to clonally expand and acquire effector functions. Helper $CD4^+$ T cells function to either activate B cells to differentiate into antibody producing plasma cells or to activate cytotoxic $CD8^+$ T cells to expand and eliminate infected cells. The T cell response starts to decline around 15 days later as 90-95% of effector cells rapidly die due to activation-induced cell death (Lanzavecchia and Sallusto, 2005; Williams *et al.*, 2008) although the presence of inflammatory cytokines can extend survival time (Krammer *et al.*, 2007); the remaining 5-10% convert to quiescent long term memory cells. It is this pool of memory cells which expand rapidly into new effector T cells upon later re-exposure to the same antigen during re-infection (or re-immunisation), a process known as a secondary response.

In murine studies using fluorescent multimeric MHC II molecules, Rees *et al.* (Rees *et al.*, 1999) tracked the frequency and relative affinities of peptide/MHC-binding T cells during both primary and secondary immune responses and found that with repeated or prolonged exposure to antigen, T cells with higher affinity receptors were selected for with limiting doses of antigen. In that study, the frequency of antigen specific CD4 T cells, detected eight days after primary immunisation with antigen in FCA was 1% of $CD4^+CD44^{high}$ T cells, an approximate 1000-fold increase compared to mice that did not receive immunisation. An inverse relationship between antigen dose and apparent T cell receptor affinity was indicated by skewing

to brighter-staining responder T cells following 1) exposure to low antigen doses two months after primary immunisation, 2) after secondary immunisation and 3) during *in vitro* expansion, but not during the primary response (Rees *et al.*, 1999). Secondary immunisation with high antigen doses resulted in the recruitment of more low-affinity T cells into the memory pool.

In the current study, the same high antigen dose (500 µg of rM5 protein) was administered for primary and booster immunisations. The literature states that, with antigen/FCA immunisation, the half-life of antigen elimination from the injection site is about 14 days (Hendriksen and Hau, 2003). Therefore, in this model at least, there is a sustained release of antigen over the entire experimental period. Animals that received two or three boosters were subjected to T cell stimulation for a longer period (35 days compared to 21 days for animals that were boosted once only) and therefore might have possessed a higher frequency of rM5-specific memory T cells.

Not unexpectedly, rats that were boosted once only had lower serum IgG levels than rats that had been boosted two or three times. However as discussed above for the T cell response, the higher antibody levels observed in the latter two groups of rats may have been due to the different time periods between initial immunisation and sera sampling in the different immunisation rat groups. In both immunisation schedules used in this study, sera were taken at 21, 28 and 35 days after initial immunisation for the 1x boost, 2x boost and 3x boost rat groups respectively. In mice at least, antibody titres plateau or start to decline around three weeks after immunising with aqueous antigen but this extends to four weeks when a depot-forming adjuvant such as Freund's is used (Hendriksen and Hau, 2003). Therefore, it is conceivable that the anti-rM5 antibody levels had not reached their peak in the 1x boost rats by 21 days.

Interestingly, although cross-recognition by rM5-specific T cells or antibody cross-reactivity towards cardiac myosin in its native form was not observed, when we used heat denatured cardiac myosin in ELISAs, a higher proportion of rats boosted two or three times exhibited cardiac myosin IgG cross-reactivity than did rats which received only one booster. Control rats immunised with PBS and adjuvant only and normal, non-immunised rats did not react with cardiac myosin. It may be

that prolonged or repetitive exposure of B and T cells to GAS rM5 protein allows for the activation of these lymphocytes by subdominant epitopes that are cross-reactive with cardiac myosin molecules undergoing degradation, as would occur during myocyte turnover or myocardial injury. The question as to whether these cardiac myosin cross-reactive antibodies are involved in initiating the pathogenic process or are the result of myocarditic events were not addressed in this work.

In patients, the acute phase of valvulitis is characterised by oedematous valves and chordae tendinae with some infiltration of inflammatory cells. Verracue (vegetations), comprised predominantly of platelets or fibrin (Sampaio *et al.*, 2007), may be present along the cusps with fibrinoid necrosis and fibroblast proliferation beneath. Mitral and/or aortic regurgitation is often diagnosed on auscultation or echocardiography (Saffitz, 2008). Due to the inflammatory nature of RF, a minor criterion for the diagnosis of RF is elevated levels of serum C-reactive protein (CRP), a non-specific marker for inflammation. Ongoing inflammation, driven by repetitive bouts of RF leads to fibrosis and calcification of the mitral, aortic and also the tricuspid valves resulting in commissural fusion, shortening and thickening of the chordate tendinae and ultimately, valve stenosis and/or regurgitation (Binotto *et al.*, 2002).

As stated previously, CRP is an acute phase reactant produced by hepatocytes in response to macrophage-derived interleukin-6 (IL-6) during inflammation and tissue trauma and is reported to facilitate macrophage phagocytic activity. Basal concentration of human CRP is reported to be around 0.1 µg/ml, rising more than 300-fold in RF/RHD patients to $\geq$ 30 µg/ml (NHFA, 2006). To assess whether CRP is a suitable marker for inflammation or cardiac damage in the RAV model, we measured CRP concentrations in sera from rM5-immunised rats and compared them to sera from a normal, non-immunised rat and a positive control rat immunised with cardiac myosin. There was no difference in serum CRP concentration between rM5-immunised rats and the normal control which did not receive immunisation. CRP was higher in rats immunised with cardiac myosin: this is consistent with the severe inflammatory lesions and myocyte necrosis observed in this experimental autoimmune model of myocarditis (EAM). Major differences exist in human and rat CRP including structural properties, isoelectric point and importantly, concentrations

in the normal physiological and inflammatory states. Literature on rat CRP levels is sparse, however, a Medline search (http://www.ncbi.nlm.nih.gov/sites/entrez) found variation in the reported CRP levels for rats in the absence and presence of inflammation. De Beer *et al.*, (1982) reported serum CRP concentrations ranging from 100 to 600 µg/ml in normal rats, rising less than two-fold during acute inflammation. In agreement with our findings, others have reported minor increases in CRP, peaking at 100-150 µg/ml during inflammation in rats (Giffen *et al.*, 2003; Cai *et al.*, 2006). In contrast, Banerjee *et al.*, (2003) found that CRP concentration increased two-fold from only 0.12 ± 0.0 ng/ml in a rat model of adjuvant-induced arthritis. An increase from only 1.16 ±0.35 µg/ml to 6.28 ± 1.61 µg/ml has been reported in a rat model of atherosclerosis (Ramesh *et al.*, 2010). There is therefore, disagreement in the literature as to the normal CRP concentration in rats. Since we found little difference in CRP levels between normal, non-immunised and rM5-immunised rats, it was concluded, in agreement with other studies (Giffen *et al.*, 2003) that, unlike in the human RF/RHD condition, CRP concentration was not a suitable marker of inflammation in the RAV model.

Microscopic examination of hearts from rM5-immunised rats revealed that increased numbers of boosters were associated with increased inflammatory changes in the valves and myocardium. Higher numbers of $CD3^+$ T cells and $CD68^+$ macrophages were evident in the heart valves of rats that were given two or three boosters compared to rats that were boosted once only. We did not observe $CD8^+$ T cells in valvular tissue, indicating that the T cells were of the $CD4^+$ T helper type. The presence of both $CD4^+$ and macrophages in the valves suggests that repetitive immunisation with rM5 enhanced the inflammatory process. However, as discussed previously, the longer time period since primary immunisation would have permitted an ongoing inflammatory process and might account for this observation. Evidence of verrucae was present in only two rats, one that was boosted two times and one boosted three times. Moreover, no Aschoff-like cell structures were apparent in valvular tissue. In regards to the effect of repeated immunisations on rat myocardium, we also observed an increase in foci of inflammatory infiltrates, particularly in the atria, papillary muscles and perivascularly, with cellular changes less marked in the ventricular and interventricular walls.

In order to draw further parallels between RF/RHD and the RAV model, we conducted ECGs on rM5-immunised rats and compared them to PBS-immunised control rats and also to healthy, non-immunised rats. Specifically, the goal was to determine whether there was a difference in P-R interval between treatment groups. We indeed found that in comparison to non-immunised rats, those boosted three times with rM5 had a significantly longer P-R interval, while PBS control rats had slight, but not significant longer P-R intervals than non-immunised rats. Prolonged P-R interval is included in the revised Jones Criteria for diagnosis of RF and along with dysrrythmia, is indicative of electrophysiological cardiac disturbances associated with RHD (Seferovic *et al.*, 2006). This finding is important, not only because it strengthens the similarities between this animal model and RF/RHD, but because it affords further avenues of research into RF/RHD pathogenesis.

CHAPTER 9
GENERAL DISCUSSION AND CONCLUSIONS

The focus of the current project was to investigate the B- and T-cell responses in an animal model of RF/RHD. The studies described herein provide valuable scientific information regarding the RAV model which was first established in the laboratory of Professor Madeleine Cunningham at the Oklahoma University Health Sciences Center (OUHSC) in the USA. This model has shown potential for being the most ideal for studying the pathologic mechanisms involved in the initiation of the disease process in RF/RHD (Quinn *et al.*, 2001).

RF/RHD is the leading cause of acquired heart disease in children and young adults in the developing world, responsible for a large number of premature deaths in these patients (WHO, 2005a). Although RF/RHD has all but disappeared in developed countries, disparate rates of disease still exist in many indigenous populations with the developed world. This includes the Aboriginal and Torres Strait Islander communities in Australia and in many South Pacific nations (Carapetis *et al.*, 2005b). While these diseases are theoretically totally preventable, the high rates worldwide and the high economic and social burden caused by RF/RHD remain a concern.

An understanding of the mechanisms underlying the pathogenesis of RF/RHD can provide further information that would assist in treatment strategies and ultimately, in development of a vaccine to prevent GAS infection. An efficacious and safe anti-GAS vaccine could result in RF/RHD and severe GAS-causing diseases such as necrotising fasciitis or streptococcal toxic shock-like syndrome being all but eradicated.

The first task undertaken in this thesis was to construct a recombinant form of the M protein, a recognised GAS virulence factor implicated in the pathogenesis of RF/RHD. M protein from serotype M5 GAS, a strain closely linked with outbreaks of RF was selected for this study. Recombinant M5 (rM5) protein was prepared using standard cloning strategies and sequencing studies determined that this rM5 was most similar in amino acid sequence to that reported by Whatmore and Kehoe in

1994. After expressing the protein in *E. coli* bacteria, rM5 was used to immunise Lewis rats.

We report here that rM5 induces valvulitis in the RAV model, illustrated by the infiltration of $CD4^+$ T cells and macrophages into valvular tissue, as evidenced by histological and immunohistological analysis of heart tissue. However, despite using syngeneic animals, not all of those immunised with rM5 developed valvulitis or myocarditis, demonstrating the variability of disease development between individuals. Lymphocytes from rM5-immunised rats responded to rM5 *in vitro* by proliferating, however, no cross-recognition with host proteins cardiac myosin or tropomyosin was observed, possibly as a result of assay methodology.

Chapter 5 describes the work carried out to identify T cell epitopes in selected M5 peptides from the variable B-repeat region and conserved C-repeat region of GAS M5. Proliferation assays in conjunction with immunohistochemical analysis of rat hearts revealed that for the RAV model, an immunodominant epitope is present in a peptide from the B-region (M5-B.1) but this epitope is insufficient to cause carditis in Lewis rats. T cells specific for B-region peptide M5-B.6 on the other hand also recognised cardiac myosin. Furthermore, immunisation with this peptide was sufficient to induce inflammatory changes in rat heart valves and myocardium. This particular body of work was limited by the number of peptides that were available for T cell epitope mapping. Ideally, peptides from the hypervariable N-terminal region of M5 protein, including peptides identified in human and murine studies as containing carditogenic T cell epitopes studies would have augmented these studies by providing further comparisons of T cell responses in human, mouse and rat.

One initial ambition of this project which was not successful, was to adoptively transfer carditis to naive rats using labelled heart infiltrating M5-specific T cells in order to determine whether these cells migrate directly to the heart and cause damage. An intrinsic requirement for this was to extract infiltrating T cells from heart tissue to establish T cell lines. It was found that insufficient valvular material, and therefore insufficient T cells were present for extraction. Furthermore, all attempts to establish T cell lines derived from rat spleens were equally unsuccessful

using standard protocols. In this investigator's hands, rat T cells lost viability during the second cycle of stimulation. Refinement of cell culture conditions are therefore warranted in future work using rat T cell lines.

B cell immune responses in the RAV model were assessed by ELISA to determine serum IgG antibody reactivity and by opsonophagocytic assays to determine bactericidal activity in sera from immunised rats. Several M5 peptides from both the B-region and C-region were found to contain immunodominant and sub-dominant B cell epitopes. However, not all M5 peptides that have been reported in other studies using mice to contain B cell epitopes induced a B cell response in the RAV model. One explanation for the disparate results between studies may be that different MHC II haplotypes within species or between species may process and therefore present peptides differently to T helper cells that are important for activating B cells and macrophages to respond (Sinha *et al.*, 2007).

In this study, IgG antibody cross-reactivity was observed against rat but not porcine cardiac myosin. Why this is so is an intriguing question, given that porcine, rat and human cardiac myosin shared over 97 % amino acid similarity and porcine cardiac myosin is routinely used in these types of studies. It is may be that the rat cardiac myosin prepared in our laboratory contained contaminants which were sufficient to cause cross-reactivity in our ELISAs but were not apparent on SDS-PAGE analysis. We also provide conclusive evidence that rM5 elicits a functional IgG response in this rat model, with sera from rM5-immunised rats exhibiting over 98% bactericidal activity against GAS M5 bacteria. Future work, whereby bactericidal activity is assessed in peptide-immunised Lewis rats would present a logical extension to this study.

The studies described in Chapter 7 of this thesis were underpinned by an obligation on the part of the researchers to ensure that the welfare of the animals under experimentation was not only maintained but improved during the investigation. One particular goal was to trial alternative adjuvants to FCA. Failure of the two potential replacements to reduce the adverse inflammatory effects of FCA whilst maintaining the required T cell response, prompted us to investigate alternative injection sites to footpad immunisation.

Delivery of antigen in FCA into the hock proved to elicit T cell and B cell responses that were comparable to those elicited by footpad immunisation. This method of immunisation was easier to administer and crucially, caused less pain and stress in experimental animals. By changing the site of immunisation from the footpad to the ankle, the animals suffered less inflammation and subcutaneous lesions at the injection site. This was most likely due to no longer having to bear direct weight on the treated foot, in conjunction with the site not being subject to contamination from the cage bedding. As a result of these findings, hock immunisation is the preferred immunisation site for future work, in particular when working with FCA.

The work described in Chapter 8 of this thesis was carried out in order to assess whether additional booster immunisations in Lewis rats exacerbated the carditis observed in rats immunised with rM5 protein and boosted once. We show here that repetitive immunisation in the RAV model not only results in increased inflammatory changes in rat hearts, but both T- and B-cell immune responses are heightened. Another finding from this study is that repetitive boosting appears to increase the frequency of cardiac myosin cross-reactive B cells but not T cells. However, it would be necessary to carry out similar repetitive boosts using individual peptides in order to map T- and B-cell cross-reactive epitopes with the potential to cause disease. This strategy was, unfortunately, not possible in the time frame available.

Results obtained from the studies described in Chapter 8 led to the conclusion that further insights into the pathogenic mechanisms involved in RF/RHD may be achieved by refinement of the RAV model. To more accurately determine the frequency of antigen-specific T cells (or B cells) following repeated boosts, it may be more appropriate to extend the time period between each booster injection. In this way, only cells in the memory pool would be assessed as it has been shown that during a secondary immune response, effector cells are dominated by cells from the memory pool rather than from differentiation and expansion of newly-activated naive cells (Chao *et al.*, 2004).

Availability of a characterised animal model of RF/RHD such as the Lewis rat model described herein provides an additional avenue for studying the immune mechanisms involved in the development of RF/RHD in man. This model has the capacity to address immunomechanistic and pathophysiologic hypotheses regarding the initiation of disease in RF/RHD patients following GAS infection.

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APPENDIX 1
MEDIA AND REAGENTS

Unless otherwise stated, all reagents and chemicals were sourced from the following companies: -

BDH Chemicals (AnalaR), Kilsyth, VIC, Australia
Ajax Finechem Pty Ltd (Univar), Taren Point, N.S.W. Australia
Sigma-Aldrich, Castle Hill, N.S.W. Australia.
Microbiological culture media was sourced from Oxoid, Thebarton, South Australia.

A.1.1 Bacterial Broths and Agars

A.1.1.1 Todd Hewitt Broth with 0.2% Yeast (THYB)

Todd Hewitt Base (Oxoid, CM0189)	36.4 g
yeast extract (Oxoid, LP0021)	2 g
dH_2O	to 1000 ml

Combine ingredients, stirring until dissolved. Autoclave at 121 °C for 15 min. Store at 4 °C.

A.1.1.2 Todd Hewitt Yeast Agar

THYB	500 ml
Agar Technical No.3 (Oxoid, LP0013)	15 g

Combine ingredients and boil for 15 min until agar has completely dissolved. Autoclave at 121 °C for 15 min, cool to 50 °C and pour into petri plates.

A.1.1.3 Charcoal Agar

Charcoal agar base (Oxoid, CM0119)	25.5 g
Defibrinated sheep blood	50 ml
40 mg/ml cephalexin (*1.3.3*)	0.5 ml
dH_2O	to 500 ml

Thoroughly dissolve charcoal agar base in water by boiling for 15-30 mins. Autoclave at 121 °C for 15 min, cool to 50 °C and aseptically add sheep blood and cephalexin. Mix by gently swirling and pour into petri plates.

A.1.1.4 Luria-Bertani (LB) Medium

tryptone (Oxoid, LP0042)	10 g
yeast extract (Oxoid, LP0021)	5 g
NaCl	10 g
dH_2O	to 1000 ml

Combine ingredients, stirring until dissolved. Autoclave at 121 °C for 15 min. Store at 4 °C.

A.1.1.5 Luria-Bertani Agar

LB medium	500 ml
Agar Technical No.3 (Oxoid, LP0013)	15 g
antibiotics as required	

Dissolve agar base in LB medium by boiling for 15 min. Autoclave at 121 °C for 15 min, cool to 50 °C and aseptically add antibiotics before pouring into petri plates.

A.1.1.6 5% Sheep Blood Agar

Blood Agar Base No.2 (Oxoid, CM0271)	40 g
sheep blood, sterile defibrinated	50 ml
dH_2O	to 1000 ml

Dissolve base in water and boil for 15 min until agar has completely dissolved. Autoclave at 121 °C for 15 min, cool to 50 °C and aseptically add the blood. Mix by gently swirling and pour into petri plates.

A.1.1.7 SOC Medium

tryptone (Oxoid, LP0042)	2 g
yeast extract (Oxoid, LP0021)	0.5 g
NaCl (1M)	1 ml
KCl (1M)	0.25 ml
Mg^{2+} (2M) stock (*1.2.5*)	1 ml
glucose (2M)	1 ml
dH_2O	to 100 ml

Dissolve tryptone, yeast extract, NaCl and KCl in 97 ml distilled water. Autoclave at 121 °C for 15 min and cool to room temperature. Add Mg^{2+} stock and glucose, each to a final concentration of 20mM. Add sterile, distilled water to 100 ml. Final pH should be 7.0.

A.1.2 General Buffers and Solutions

A.1.2.1 0.5M Ethylenediamine tetra-acetate (EDTA)

Na_2-EDTA	186.1 g
ddH_2O	to 1000 ml

Dissolve EDTA in 800 ml water with vigorous stirring. Adjust pH to 8.0 with concentrated NaOH to completely dissolve EDTA. Make up to 1000 ml with double distilled water. Autoclave at 121 °C for 15 min.

A.1.2.2 2M Glucose

Glucose	36 g
ddH_2O	to 100 ml

Dissolve glucose completely in 80 ml water. Make up to 100 ml with double distilled water. Filter sterilise through a disposable 0.22 micron filter.

A.1.2.3 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG)

IPTG	2.383 g
ddH_2O	10 ml

Dissolve IPTG thoroughly in water. Filter sterilise (22 µm) and store in 1 ml aliquots at -20 °C.

A.1.2.4 Lysozyme (100 mg/ml)

Lysozyme	1.0 g
ddH_2O	10 ml

Dissolve lysozyme thoroughly in water. Filter sterilise (0.22 µm) and store in 1 ml aliquots at -20 °C.

A.1.2.5 2M Magnesium stock (Mg^{2+})

$MgCl_2.6H_2O$	20.33 g
$MgSO_4.7H_2O$	24.65 g
ddH_2O	to 100 ml

Combine ingredients until completely dissolved. Filter sterilise through a disposable 0.22 micron filter.

A.1.2.6 Phosphate buffered saline (PBS) (10x stock)

NaCl	80 g
KH_2PO_4	2 g
Na_2HPO_4	11.5 g
ddH_2O	to 1000 ml

Combine ingredients with stirring until completely dissolved and adjust pH to 7.2 or 7.4 as required. Autoclave at 121 °C for 15 min. For working solution, add 100 ml of 10X stock to 900 ml double distilled water.

A.1.2.7 Tris-acetate buffer (TAE) (50x stock)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA, pH 8.0	100 ml
ddH_2O	to 1000 ml

Combine all ingredients thoroughly. Autoclave at 121 °C for 15 min. For working solution, dilute 1:50 with double distilled water.

A.1.2.8 Tris buffered saline (TBS) (10x stock)

NaCl	80 g
KCl	2 g
Tris base	30 g
ddH_2O	to 1000 ml

Dissolve ingredients in 800 ml water and adjust to required pH using HCl. Autoclave at 121 °C for 15 min. For working solution, add 100 ml of 10X stock to 900 ml double distilled water.

A.1.2.9 1 M Tris-Cl, pH 8.0

Tris base	12.14 g
ddH_2O	to 100 ml

Dissolve tris in 80 ml water and adjust pH to 8.0 using HCl. Add double distilled water to 100 ml. Autoclave at 121 °C for 15 min.

A.1.3 Buffers for protein purification

A.1.3.1 Lysis buffer

		[Final]
NaH_2PO_4	6.9 g	50 mM
NaCl	17.54 g	300 mM
Imidazole	0.68 g	10 mM
ddH_2O	to 1000 ml	

Combine ingredients with stirring until completely dissolved and adjust pH to 8.0 using NaOH. Autoclave at 121 °C for 15 min.

A.1.3.2 Wash buffer

NaH_2PO_4 6.9 g	50 mM	
NaCl	17.54 g	300 mM
Imidazole	1.36 g	20 mM
ddH_2O	to 1000 ml	

Combine ingredients with stirring until completely dissolved and adjust pH to 8.0 using NaOH. Autoclave at 121 °C for 15 min.

A.1.3.3 Elution buffer (100 ml)

NaH_2PO_4	0.69 g	50 mM
NaCl	1.754 g	300 mM
Imidazole	0.136 g	250 mM
ddH_2O	to 100 ml	

Combine ingredients with stirring until completely dissolved and adjust pH to 8.0 using NaOH. Autoclave at 121 °C for 15 min.

A.1.3 Antibiotic solutions

A.1.3.1 Ampicillin (100 mg/ml)

Ampicillin	10 g
ddH_2O	100 ml

Dissolve ampicillin in the water. Filter sterilise (0.22 μm) and store at -20 °C in 1 ml aliquots.

A.1.3.2 Kanamycin (25 mg/ml)

Kanamycin sulphate	2.5 g
ddH_2O	100 ml

Dissolve kanamycin in the water. Filter sterilise (0.22 μm) and store at -20 °C in 1 ml aliquots.

A.1.3.4 Cephalexin (10 mg/ml)

Cephalexin hydrate	100 mg
ddH_2O	10 ml

Dissolve cephalexin in water. Filter sterilise (0.22 μm) and store at 2 °C to 8 °C.

A.1.4 Reagents for Electrophoresis

A.1.4.1 10% sodium dodecyl sulphate (SDS)

Lauryl sulphate	10 g
dH_2O	to 100 ml

Combine ingredients. Do not autoclave.

A.1.4.2 10 % ammonium sulphate (APS) solution

Ammonium persulphate (Bio-Rad)	100 mg
dH_2O	to 100 ml

Combine ingredients. Use within 24 h.

A.1.4.3 12% Separating gel (1 gel)

30% acrylamide/bis (Bio-Rad, 161-0158)	2 ml
1.5M Tris-Cl buffer, pH 8.8	1.3 ml
10% SDS solution (see 1.4.1)	50 µl
10% APS solution (see 1.4.2)	50 µl
ddH_2O	1.6 ml
TEMED (Bio-Rad, 161-0800)	2 µl

Combine first four ingredients thoroughly. Add TEMED immediately prior to pouring the gel.

A.1.4.4 Stacking gel (1 gel)

30% acrylamide/bis (Bio-Rad, 161-0158)	0.17 ml
1M Tris-Cl buffer, pH 6.8	0.13 ml
10% SDS solution (see 1.4.1)	10 µl
10% APS solution (see 1.4.2)	10 µl
ddH_2O	0.68 ml
TEMED (Bio-Rad, 161-0800)	1 µl

Combine first four ingredients thoroughly. Add TEMED immediately prior to pouring the gel.

A.1.4.5 Electrode buffer (5x)

Tris base	15 g
Glycine (Gibco BRL 15527-013)	72 g
Lauryl sulphate	5 g
ddH_2O	1000 ml

Dissolve ingredients thoroughly in water and store at 4 °C. Prior to use, dilute 1:5 with distilled water.

A.1.4.6 SDS-PAGE reducing sample buffer (2x)

Tris-HCl, pH 6.8	0.218 g
Glycerol	4 ml
Lauryl sulphate	0.4 g
Bromophenol blue	4 mg
ddH_2O	16 ml

Dissolve ingredients thoroughly in water and store at 4 °C. Prior to use, aliquot volume required and add 2-β-mercaptoethanol (2 v/v).

A.1.4.7 Coomassie Brilliant Blue staining solution

Coomassie Brilliant Blue R-250	2 g
Methanol	500 ml
Glacial acetic acid	100 ml
dH_2O	400 ml

Combine ingredients in fume hood. Store at room temperature away from direct light.

A.1.4.8 Coomassie Destaining solution

Methanol	100 ml
Glacial acetic acid	70 ml
dH_2O	830 ml

Combine ingredients in fume hood. Store at room temperature away from direct light.

A.1.5 Cell culture media and reagents

(all media preparation was carried out in a biological laminar flow hood using aseptic technique)

A.1.5.1 Transport medium

RPMI 1640 (Gibco BRL)	
Penicillin (Gibco BRL)	100 IU/ml
Streptomycin (Gibco BRL)	100 µg/ml

Mix antibiotics thoroughly into RPMI and store at 4 °C.

A.1.5.2 Rat cell culture medium (2X strength)

RPMI 1640 (Gibco BRL)	
Penicillin	200 IU/ml
Streptomycin	200 µg/ml
Rat serum, autologous, heat inactivated	5%
L-glutamine	4 mM
HEPES (Gibco BRL)	20 mM

Mix all ingredients thoroughly and store at 4 °C until required. Use within 24 h.

A.1.5.3 L-glutamine (15 mg/ml)

L-glutamine (Sigma, G8540)	150 mg
double distilled water	10 ml

Warm (37 °C) L-glutamine in water until completely dissolved. Store at -20 °C in 3 ml aliquots until required.

APPENDIX 2
MANUFACTURER/SUPPLIER ADDRESSES

Company	Address
Amersham Biosciences	Buckinghamshire, UK
Bayer	Leverkusen, Germany
BD Biosciences	San Diego, CA, USA
BioGenex	San Ramon, CA, USA
Biokeystone	El Monte, CA
Biomeda Corporation	Foster City, CA, USA
bioMérieux Australia	Baulkham Hills, NSW, Australia
Bio-Rad Laboratories	Regents Park, NSW, Australia
Bronwill Scientific	Rochester, NY, USA
Cedarlane Laboratories	Hornby, Ontario, Canada
Chemicon Australia Pty Ltd	Boronia, VIC, Australia
Crown Scientific Pty Ltd	Minto, NSW, Australia
Eppendorf	Hamburg, Germany
Fermentas Inc	Glen Burnie, MA, USA
GE Healthcare	Baulkham Hills, NSW, Australia
Genes Codes Corp	Ann Arbor, MI, USA
GibcoBRL	Grand Island, NY, USA
Gradipore Ltd	Frenchs Forest, NSW, Australia
Graphpad Software Inc	La Jolla, CA, USA
Hoefer Inc	Holliston, MA, USA
In Vitro Technologies	Noble Park, VIC, Australia
Integrated Sciences	Chatswood, NSW, Australia
Invitrogen Australia Pty Ltd	Mt Waverley, VIC, Australia
Jackson Immunoresearch	West Grove, PA, USA
Macherey-Nagel	Düren, Germany
Menzel-Gläser	Braunschweig, Germany
Millipore Australia	North Ryde, NSW, Australia
Molecular Biology Insights	Cascade, CO, USA
MVP Laboratories	Omaha, NE, USA
Nunc	Roskilde, Denmark

Company	Address
Parnell Laboratories Pty Ltd	Alexandria, NSW, Australia
PerkinElmer	Glen Waverley, VIC, Australia
Pierce Biotechnology	Rockford, IL, USA
Progen	Darra, QLD, Australia
Promega	Alexandra, NSW, Australia
ProSciTech	Kirwan, QLD, Australia
Quantum Scientific	Murarrie, QLD, Australia
QIAGEN	Doncaster, VIC, Australia
Sarstedt	Ingle Farm, SA, Australia
Seppic S.A.	Puteaux, France
Sigma-Genosys	Castle Hill, NSW, Australia
SPSS Inc	Chicago, IL, USA
Syngene	Frederick, MD, USA.
Terumo	Somerset, NJ, USA
Titertek Instruments Inc	Huntsville, AL, USA
TropBio	Townsville, QLD, Australia
Troy Laboratories Pty Ltd	Smithfield, NSW, Australia
Vector Laboratories	Burlingame, CA, USA

APPENDIX 3
rM5 SEQUENCING AND PROTEIN ANALYSIS

A.3.1 M5 protein DNA sequence as per Genbank accession number M20374 (Miller *et al.*, 1988). Sequences for forward and reverse PCR primers used to amplify the *S. pyogenes* M5 gene fragment (in blue) are depicted by arrows. The forward primer incorporates a BamH1 restriction site and the reverse primer incorporates a Sal1 site. Bases 1-42 = signal sequence; 1263-1361=Gly/Pro-rich, cell wall spanning region; 1362-1386=sorting signal LPXTG; box = start codon

A.3.2 rM5 consensus sequence. Sequence was deduced from forward (n=3) and reverse (n=3) sequencing reactions of pQE-30.m5 construct using Sequencher™ 4.0 software

gccgtgactaggggtacaataaatgacccgcaaagagcaaaagaagctcttgacaagtat
gagctagaaaaccatgacttaaaaactaagaatgaagggttaaaaactgagaatgaaggg
ttaaaaactgagaatgaagggttaaaaactgagaatgaaaggttaaaaactgagaagagt
aacttagaacgtaagactgctgagttaacaagtgagaagaaagaacatgaagcagaaaac
gataagttaaaacaacagagggatacgttatctactcagaaagaaactcttgaaagagaa
gtacagaacacgcaatacaataatgaaacgttaaagattaagaatggtgacttaactaaa
gagttgaataaaactcgacaagaattagcaaataaacagcaagagagtaaagaaaatgaa
aaggcccttaatgaactcttggaaaagacagtaaaagataaaattgctaaggagcaagaa
aataaagaaaccattggtacccttaaaaaaatcttggatgagacagtaaaagataaactt
gcgaaagagcaaaaaagtaaacaaaacattggtgcccttaaacaagaattagctaaaaaa
gatgaggcaaacaaaatttcagacgcaagccgtaagggtcttcgtcgtgacttagacgca
tcgcgtgaagctaagaagcaattagaagctgaacaccaaaaacttgaagaacaaaacaag
atttcagaagcaagtcgcaaaggccttcgccgtgacttagacgcatcgcgtgaagctaag
aagcaattagaagctgaacaccaaaaacttgaagaacaaaacaagatttcagaagcaagt
cgcaagggccttcgccgtgatttagacgcatcacgtgaagctaagaaacaagttgaaaaa
gctttagaagaagcaaacagcaaattagctgctcttgaaaaacttaacaaagagcttgaa
gaaagcaagaaattaacagaaaaagaaaaagctgagctacaagcaaaacttgaagcagaa
gcaaaagcattcaaagaacaattagcaAaacaagctgaagaacttgcaaaactaagagct
ggaaaagcatcagactcacaaacccctgatacaaaaccaggaaacaaagctgttccaggt
aaaggtcaa

A.3.3 Results of GenBlastn analysis of rM5_consensus sequence

Blastn results Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY394553.1	Streptococcus pyogenes strain NS39 M protein (emm) gene, emm5.7 type, partial cds	1249	1537	65%	0.0	99%
U02480.1	Streptococcus pyogenes NCTC8193 antiphagocytic M5.8193 protein (emm5.8193) gene, partial cds, and IgG-binding protein Enn5.8193 gene, complete cds	2109	3181	99%	0.0	99%
AY394551.1	Streptococcus pyogenes strain NS362 M protein (emm) gene, emm5.7 type, partial cds	902	1094	43%	0.0	99%
AJ890457.1	Streptococcus pyogenes partial emm gene for M5 protein, from Addis Ababa	726	919	34%	0.0	99%
AM295007.1	Streptococcus pyogenes Manfredo complete genome	1290	2670	100%	0.0	98%
M20374.1	S.pyogenes smp5 gene encoding serotype 5 M protein, complete cds	1284	2659	100%	0.0	98%
AF502098.1	Streptococcus pyogenes strain JS114 M5 (emm) gene, emm5-5 type, partial cds	869	1061	47%	0.0	98%
U40231.1	Streptococcus pyogenes M3 protein (emm3.1) gene, complete cds	593	593	30%	5e-166	97%
U65900.1	Streptococcus pyogenes M protein gene, partial cds	604	763	46%	2e-169	97%
DQ114470.1	Streptococcus pyogenes subtype emm 5.50 M protein (emm) gene, partial cds	1170	1350	72%	0.0	96%
AY346405.1	Streptococcus pyogenes strain JS23 M protein (emm) gene, emm5-7 type, partial cds	881	1217	54%	0.0	96%
EF460480.1	Streptococcus pyogenes M protein (emm) gene, emm74.0 type, partial cds	911	1623	57%	0.0	95%
EU660380.1	Streptococcus pyogenes strain Sp32 M protein (emm) gene, emm-74 allele, partial cds	776	1488	50%	0.0	95%
AY139417.1	Streptococcus pyogenes isolate NS50 M protein (emm) gene, st854 type, partial cds	959	1472	60%	0.0	95%

A.3.4 **Deduced amino acid sequence and characterisation of rM5 following ExPASy analysis.** Coding in blue depicts vector pQE-30 sequence

```
atgagaggatcgcatcaccatcaccatcacggatccgccgtgactaggggtacaataaat
 M  R  G  S  H  H  H  H  H  H  G  S  A  V  T  R  G  T  I  N
gacccgcaaagagcaaaagaagctcttgacaagtatgagctagaaaaccatgacttaaaa
 D  P  Q  R  A  K  E  A  L  D  K  Y  E  L  E  N  H  D  L  K
actaagaatgaagggttaaaaactgagaatgaagggttaaaaactgagaatgaagggtta
 T  K  N  E  G  L  K  T  E  N  E  G  L  K  T  E  N  E  G  L
aaaactgagaatgaaaggttaaaaactgagaagagtaacttagaacgtaagactgctgag
 K  T  E  N  E  R  L  K  T  E  K  S  N  L  E  R  K  T  A  E
ttaacaagtgagaagaaagaacatgaagcagaaaacgataagttaaaacaacagagggat
 L  T  S  E  K  K  E  H  E  A  E  N  D  K  L  K  Q  Q  R  D
acgttatctactcagaaagaaactcttgaaagagaagtacagaacacgcaatacaataat
 T  L  S  T  Q  K  E  T  L  E  R  E  V  Q  N  T  Q  Y  N  N
gaaacgttaaagattaagaatggtgacttaactaaagagttgaataaaactcgacaagaa
 E  T  L  K  I  K  N  G  D  L  T  K  E  L  N  K  T  R  Q  E
ttagcaaataaacagcaagagagtaaagaaaatgaaaaggcccttaatgaactcttggaa
 L  A  N  K  Q  Q  E  S  K  E  N  E  K  A  L  N  E  L  L  E
aagacagtaaaagataaaattgctaaggagcaagaaaatgaaaccattggtacccttaaa
 K  T  V  K  D  K  I  A  K  E  Q  E  N  E  T  I  G  T  L  K
aaaatcttggatgagacagtaaaagataaaattgctaaggagcaagaaaataaaaaagaa
 K  I  L  D  E  T  V  K  D  K  I  A  K  E  Q  E  N  K  K  E
accattggtacccttaaaaaaatcttggatgagacagtaaaagataaacttgcgaaagag
 T  I  G  T  L  K  K  I  L  D  E  T  V  K  D  K  L  A  K  E
caaaaaagtaaacaaaacattggtgcccttaaacaagaattagctaaaaaagatgaggca
 Q  K  S  K  Q  N  I  G  A  L  K  Q  E  L  A  K  K  D  E  A
aacaaaatttcagacgcaagccgtaagggtcttcgtcgtgacttagacgcatcgcgtgaa
 N  K  I  S  D  A  S  R  K  G  L  R  R  D  L  D  A  S  R  E
gctaagaagcaattagaagctgaacaccaaaaacttgaagaacaaaacaagatttcagaa
 A  K  K  Q  L  E  A  E  H  Q  K  L  E  E  Q  N  K  I  S  E
gcaagtcgcaaaggccttcgccgtgacttagacgcatcgcgtgaagctaagaagcaatta
 A  S  R  K  G  L  R  R  D  L  D  A  S  R  E  A  K  K  Q  L
gaagctgaacaccaaaaacttgaagaacaaaacaagatttcagaagcaagtcgcaagggc
 E  A  E  H  Q  K  L  E  E  Q  N  K  I  S  E  A  S  R  K  G
cttcgccgtgatttagacgcatcacgtgaagctaagaaacaagttgaaaaagctttagaa
 L  R  R  D  L  D  A  S  R  E  A  K  K  Q  V  E  K  A  L  E
gaagcaaacagcaaattagctgctcttgaaaaacttaacaaagagcttgaagaaagcaag
 E  A  N  S  K  L  A  A  L  E  K  L  N  K  E  L  E  E  S  K
aaattaacagaaaaagaaaaagctgagctacaagcaaaacttgaagcagaagcaaaagca
 K  L  T  E  K  E  K  A  E  L  Q  A  K  L  E  A  E  A  K  A
ttcaaagaacaattagcaaaacaagctgaagaacttgcaaaactaagagctggaaaagca
 F  K  E  Q  L  A  K  Q  A  E  E  L  A  K  L  R  A  G  K  A
tcagactcacaaacccctgatacaaaaccaggaaacaaagctgttccaggtaaaggtcaa
 S  D  S  Q  T  P  D  T  K  P  G  N  K  A  V  P  G  K  G  Q
gtcgacctgcagccaagcttaattagctga
 V  D  L  Q  P  S  L  I  S  -
```

rM5

```
AVTRGTINDP QRAKEALDKY ELENHDLKTK NEGLKTENEG LKTENEGLKT ENERLKTEKS
NLERKTAELT SEKKEHEAEN DKLKQQRDTL STQKETLERE VQNTQYNNET LKIKNGDLTK
ELNKTRQELA NKQQESKENE KALNELLEKT VKDKIAKEQE NKETIGTLKK ILDETVKDKL
AKEQKSKQNI GALKQELAKK DEANKISDAS RKGLRRDLDA SREAKKQLEA EHQKLEEQNK
ISEASRKGLR RDLDASREAK KQLEAEHQKL EEQNKISEAS RKGLRRDLDA SREAKKQVEK
ALEEANSKLA ALEKLNKELE ESKKLTEKEK AELQAKLEAE AKAFKEQLAK QAEELAKLRA
GKASDSQTPD TKPGNKAVPG KGQ
```

Theoretical pI/Mw 8.89 / 43628.83 Da (http://au.expasy.org/tools/pi_tool.html)

A.3.5 ExPASy analysis of rM5 deduced amino acid sequence

```
Program: NCBI BLASTP 2.2.17 [Aug-26-2007]
Database: UniProtKB  Bacteria+Archaea
          4,044,863 sequences; 1,243,345,219 total letters
UniProt Knowledgebase Release 14.3 consists of:
UniProtKB/Swiss-Prot  Release 56.3 of 14-Oct-2008:  399749 entries
UniProtKB/TrEMBL      Release 39.3 of 14-Oct-2008: 6678831 entries
```

List of potentially matching sequences

Db	AC	Description	Score	E-value
tr	Q54510	_STRPY M5.8193 protein (Fragment) [emm5.8193] [Streptoc...	572	e-161
tr	A2RGM0	_STRPG M protein, serotype 5 precursor [emm5] [Streptoc...	521	e-146
sp	P02977	_M5_STRP5 M protein, serotype 5 precursor [emm5] [Strep...	518	e-145
tr	Q45UE1	_STRPY M protein (Fragment) [emm] [Streptococcus pyogenes]	402	e-110
tr	Q6TLQ0	_STRPY M protein (Fragment) [emm] [Streptococcus pyogenes]	399	e-109
tr	Q93SL9	_STRPY Emm type protein precursor [Streptococcus pyogenes]	354	4e-96
tr	Q54718	_STRPY M protein [emm19.1] [Streptococcus pyogenes]	332	2e-89
tr	Q8GL90	_STRPY M protein (Fragment) [emm] [Streptococcus pyogenes]	329	1e-88
tr	A4UNS7	_STRPY M protein (Fragment) [emm] [Streptococcus pyogenes]	318	3e-85
tr	Q6V9N4	_STRPY M protein (Fragment) [emm] [Streptococcus pyogenes]	314	4e-84
tr	Q6TLQ2	_STRPY M protein (Fragment) [emm] [Streptococcus pyogenes]	313	6e-84
tr	Q9AMC4	_STRPY M18 protein [emm18.2] [Streptococcus pyogenes]	307	5e-82

PUBLICATIONS ARISING FROM THIS THESIS:

Gorton, D., Govan, B., Olive, C. and Ketheesan, N. (2006) A role for an animal model in determining the immune mechanisms in the pathogenesis of rheumatic heart disease. *In*: Streptococci – New Insights into an Old Enemy. *Proceedings of the XVIth Lancefield International Symposium on Streptococci and Streptococcal Diseases,* Palm Cove, Australia. ICS 1289, pp289-292.

Gorton, D., Govan, B., Olive, C. and Ketheesan, N. (2009) B- and T-cell responses in group A streptococcus M protein- or peptide-induced experimental carditis. *Infection and Immunity*, **77**(5): 2177-2183. (Spotlight article).

Gorton, D., Blyth, S., Gorton, J.G., Govan, B. and Ketheesan, N. (2010) An alternative technique for the induction of autoimmune valvulitis in a rat model of rheumatic heart disease. *Journal of Immunological Methods,* (*In press*) doi: 10.1016/j.jim.2010.02.013

Gorton, D., Bryant, S., Chilton, L. and Ketheesan, N. Repetitive exposure to group A streptococcal M protein exacerbates carditis in a rat model of rheumatic heart disease. *(In preparation).*