THE DETECTION OF ANTIBODIES TO GROUP A STREPTOCOCCAL M PROTEIN IN RHEUMATIC FEVER

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Contents

Table of Abbreviations

ADB	AntiDNase B
AHT	Antihyaluronidase titre
ASOT	Antistreptolysin O titre
C-terminal	Carboxyl-terminal
EIA	Enzyme immunoassay
kDa	kilodalton
MAP I / II	M-associated protein I and II
N-terminal	Amino-terminal
OF	Opacity factor
PBS	Phosphate buffered saline
pep M	pepsin extract of M-protein
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TTBS	Tween 20 / Tris buffered saline

Declaration

I declare that the work described herein contains no material that has been accepted for the award of any degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Summary

Rheumatic fever as a sequela of group A streptococcal pharyngitis continues to be an important cause of cardiovascular morbidity not only in the developing world but also in aboriginal communities within Australia. The clinical diagnosis of rheumatic fever or rheumatic heart disease can be difficult, particularly, in remote aboriginal communities. Current serological methods to support the diagnosis are not specific for rheumatic fever. The group A streptococcus (*Streptococcus pyogenes*) has on its outer surface an antiphagocytic protein, M-protein. Antibodies to M-protein have been shown to persist in rheumatic fever. There are however, few studies looking at the use of this as a diagnostic test.

In the first part of this study, pepsin extracts of *Streptococcus pyogenes* M24 and M18 were used in a Western blot against sera from 31 subjects, (both Aboriginal and non-Aboriginal). Eighteen had proven rheumatic heart disease or rheumatic fever. Of these, 16 had definitive bands at regions corresponding to specific M24 and/or M18 proteins. There was no difference between Aboriginal and non-Aboriginal subjects with a history of rheumatic fever or rheumatic heart disease. Of the remaining 13 subjects with no history of rheumatic fever, 12 were non-reactive. This would suggest that the detection of antibodies to M-protein, may have a place in the serodiagnosis of rheumatic fever.

In the second part of this study, linear epitopes of the M24 protein were mapped, using overlapping synthetic biotinylated 16-mer peptides in a streptavidin based enzyme immunoassay system, against sera from eighty two subjects, Aboriginal and non-Aboriginal with either acute rheumatic fever, previous rheumatic heart disease or with no history of disease. Of

the eighty two peptides tested, five commonly reactive peptides were identified. These five peptides (Peptides 89, 95, 102, 103 and 105), were all from the carboxy-terminal end of M24 protein and had significant amino-acid sequence homology with each other. Parameters for the enzyme immunoassay were determined and included using 0.01 μg/well streptavidin, 0.28 μg/well biotinylated peptide and sera at a 1 in 500 dilution.

Sera from Aboriginal subjects with acute rheumatic fever, previous rheumatic fever or rheumatic heart disease, were significantly more reactive with peptides 89 and 95, than sera from matched control subjects,

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There was no significant difference in reactivity between sera from non-Aboriginals with previous rheumatic fever and matched controls.

Using peptides in combination, or using related 20-mer peptides with the same panel of sera, did not reliably differentiate between subjects with rheumatic fever and those without.

It is concluded that related peptides at the C-terminal end of M24 protein represent linear epitopes recognised by sera from Aboriginal subjects with acute rheumatic fever and previous rheumatic fever.

It is proposed that these peptides could be used as antigens in the serodiagnosis of acute rheumatic fever.

Chapter 1

Rheumatic Fever and Streptococcal M protein - A review of the literature

1.1 Introduction

Rheumatic fever is one of the non-suppurative complications of Group A streptococcal (*Streptococcus pyogenes*) pharyngitis. Acute post-streptococcal glomerulonephritis and Sydenham's chorea are two other recognised non-suppurative sequelae with quite different epidemiological features.

Valvular heart disease, as a sequela of acute rheumatic fever, continues to be a significant cause of morbidity and mortality not only in the developing world but also among Aboriginal communities in Australia. The problem is multifactorial but difficulties and delays in the early diagnosis of rheumatic fever play a significant part. The frequent occurrence of group A streptococcal skin and throat infections make the interpretation of standard streptococcal serology (Anti-streptolysin O and anti-deoxyribonuclease B tests) difficult. Often, a retrospective diagnosis of rheumatic fever is made following the chance finding of a cardiac murmur. It is now clear, that following an initial episode of rheumatic fever, subsequent exposure to group A streptococci will lead to an increased risk of carditis. This would have been preventable with prophylactic penicillin had the diagnosis of rheumatic fever been made previously. A serological test with greater specificity for rheumatic fever, would help in making an earlier diagnosis.

1.2 Historical background

Hippocrates, writing in the 4th century, mentions in his aphorisms that "Prolonged fevers are attended either by swellings or pains in the joints" (*In* Hippocratic Writings). The association between pharyngitis and acute rheumatic fever however, was first made by J.K. Fowler in 1880 when he described the occurrence of acute rheumatism, subcutaneous nodules, valvular heart disease together with "rheumatism" of the throat in children. It was not until the introduction of Rebecca Lancefield's grouping system for beta-haemolytic streptococci and her identification of streptococcal M-protein that the epidemiology of this condition was clarified (Lancefield 1928). The diagnosis of acute rheumatic fever is currently based upon the revised (1984) Jones criteria of the American Heart Association (Ad Hoc committee of the council on rheumatic fever 1984).

1.3 Epidemiology

Over the last century there has been a consistent decline in the reported incidence of rheumatic fever in North America and Europe (Mayer et al 1963). A number of explanations have been suggested for this phenomenon and include less crowded living conditions, improved hygiene and widespread use of antibiotics in children with upper respiratory infections. A number of epidemiologic studies have shown a possible correlation between the prevalence of rheumatic heart disease and the degree of crowding within homes (Perry et al 1937; Maddox 1937). This has been supported by the observation that the incidence of rheumatic fever has remained relatively constant in developing parts of the world.
Rheumatic fever continues to be a significant cause of morbidity and mortality not only in the developing world but also amongst Aboriginal

communities in Australia where the reported incidence is as high as 800 per 100,000 (Pruksakorn et al 1994). In Africa, as many as 470 cases of rheumatic fever per 100,000 population have been reported (WHO Programme for the prevention of rheumatic fever), whereas in the United States, the figure is markedly lower at 0.63 per 100,000 population (Veasy et al 1987). In Maori and Pacific Islanders the incidence is reported to be between 50 - 75 per 100,000 poulation (Lennon 1992).

A resurgence of rheumatic fever in the United States during the 1980s has renewed interest in the role of group A streptococci in the pathogenesis of this disease (Veasy et al 1987; Kaplan et al 1989). One unusual feature of these outbreaks was that they occurred predominantly among white middle-class children living in the suburbs. There were also outbreaks of acute rheumatic fever in military training bases (MMWR 1988), a phenomenon that had not been observed for decades.

The peak age of incidence of rheumatic fever is between 5 and 15 years. It is noticeably uncommon in children under 4 years of age. Between 1 and 5% of individuals who have group A streptococcal pharyngitis may develop acute rheumatic fever if untreated (Fischetti 1989). This, however, varies with the epidemiologic circumstances. The frequency is higher during epidemics of streptococcal pharyngitis in enclosed populations such as military recruits. Endemic streptococcal infection among populations of children is less easy to define. Rates of acquisition of group A streptococci are highest among school children and their families (Meyers et al 1962). Rapid human passage provides the conditions under which rheumatic fever can occur with greatest frequency (Stollerman et al 1965). There is epidemiologic evidence to suggest that repeated, often subclinical attacks of rheumatic fever may result in progressive worsening of rheumatic heart

disease (Williams 1994). This is particularly true of rural Aboriginal populations in Australia. A combination of problems including regional isolation, difficulties with clinical diagnosis and a reluctance to seek medical attention all serve to make rheumatic fever and rheumatic heart disease an ongoing problem in these communities.

Jose et al (1970) reported that organic heart murmurs, indicating valvular damage secondary to rheumatic fever, were ten times more common in Aboriginal children than in Sydney school children. Group A streptococcal strains most strongly epidemiologically associated with recent outbreaks of acute rheumatic fever, belong to a subset of the >80 serotypes that have been defined. Some of these rheumatogenic serotypes are the M types 1, 3, 5, 6, 14, 18, 19, 24, 27 and 29. In addition, a high proportion of these strains had mucoid colonial appearances and were generally also rich in M protein (Bisno 1991). It has, however, also been demonstrated that virulent strains of other M types can initiate acute rheumatic fever under appropiate epidemiologic conditions (Rammelkamp et al *In* Rheumatic Fever 1952; Martin et al 1994). Strains of group A streptococci have now been shown to be genetically polymorphic within the same M type (Single et al 1992). For any particular strain of group A streptococci, its phase of virulence may be the critical determinant of rheumatogenicity. Whether or not unique rheumatogenic antigens are expressed preferentially by such strains remains unknown.

1.4 The pathogenesis of rheumatic fever

The pathogenesis of both acute rheumatic fever and acute post-streptococcal glomerulonephritis remains unclear. There are distinct structural differences between the M proteins of streptococci associated with acute rheumatic fever and those known to cause acute

glomerulonephritis (Bisno 1991). The causal relationship between group A streptococcal pharyngitis and subsequent acute rheumatic fever was first clearly documented over forty years ago (Rammelkamp et al 1952). While group A streptococcal pyoderma has been implicated in post-streptococcal glomerulonephritis, a definitive association with acute rheumatic fever remains unproven. It is however possible that transfer of organisms from skin lesions to the throat can occur with the subsequent development of rheumatic fever. This may be the mechanism in Australian Aboriginal communities, where streptococcal impetigo is common.

Rheumatic fever is a good example of molecular mimicry between a foreign agent and autologous host tissue. A number of components of the group A streptococcus have been shown to cross react with various human host tissues. These include cardiac myosin, heart sarcolemmal membrane and heart valves (Kaplan et al 1962; Kaplan 1963; van de Rijn et al 1977). Two mechanisms suggested whereby molecular mimicry could induce rheumatic fever are : Firstly, group A streptococci infect the pharynx and sensitise both B cells primed for a humoral response and a cell mediated response against T cell crossreactive epitopes within host heart tissue. A second mechanism is the sharing of antigens between the streptococcus and components of heart tissue which may allow a state of partial tolerance to exist. This reduces the normal immune eliminative mechanisms of the host. Repeated streptococcal infections then result in continuing cell mediated damage to tissues with cross-reactive antigens (Read et al 1974).

Another hypothesis which may explain the pathology of this condition suggests that streptococcal components are released by bacteria growing at a localized site of infection and are carried in the bloodstream, possibly linked to antigen presenting cells, to target organs. Formation of *in situ*

immune complexes leads to local inflammation, unmasking of tissue components such as laminin, type IV collagen, myosin and heparan proteoglycans, which in turn stimulates the formation of specific autologous antibodies that exacerbate the lesions (Michael *In* Streptococcal diseases and the immune response; Makino et al 1986; DeScheerder et al 1984; Fillet et al 1985; Kefalides et al 1986). No specific streptococcal component has been conclusively implicated in this role. More recently, a 9 kDa glycosaminoglycan binding protein was isolated from a number of different M types of group A streptococci and this was subsequently found to bind selectively to the basal laminae of human cardiac muscle (Winters et al 1993).

Cross-reactivity between components of streptococcal M protein and mammalian proteins will be discussed in section 1.13.

Antibodies against α -helical coiled structures such as streptococcal M protein have also been found to be cytotoxic to heart and fibroblast cell lines. They also reacted with the viral capsid antigen of Coxsackieviruses B3 and B4, which can be aetiological agents of myocarditis. The significance of this is that in susceptible individuals exposed to infectious agents, a hyperresponsive production of crossreactive immunity may lead to the development of autoimmunity and damage to host tissues as seen in acute rheumatic fever and myocarditis (Cunningham et al 1992). The question of what drives the continuing chronic inflammatory response once the organism has been eradicated and the signs of acute inflammation have died down, remains unclear. One possible explanation is persistence of antigen within target tissues or as messenger RNA within antigen presenting cells. These would then be capable of re-presenting antigen to specifically reactive B and T cells.

The role of genetic susceptibility as a possible predisposing factor for rheumatic fever has been considered. This is in relation to Major Histocompatibility Class II (MHC II) restricted antigenic processing. No clear HLA association has been identified. One study showed an increase of HLA-DR4 in white subjects, whereas HLA-DR2 was increased among black patients (Ayoub et al 1986). Markers on B cells have been defined which appear to be related to susceptibility to rheumatic fever. Their exact nature remains to be determined but there does not appear to be any direct link with known histocompatibility antigens (Khanna et al 1989).

A mouse model has been used for the study of experimental induction of rheumatic fever-like lesions. This showed that macrophages played a central role by selecting from the antigenic determinants of streptococci which showed cardiac cross reactivity (Dos Reis et al 1980).

1.5 The clinical diagnosis of rheumatic fever

Rheumatic fever is essentially a multi-system disorder. The clinical presentation may therefore be quite varied, involving the joints, heart, skin and central nervous system. Possible presentations include fever, polyarthritis, lethargy, new cardiac murmurs, pericarditis, cardiac failure, subcutaneous nodules, erythema marginatum and chorea.

The latent period between the preceding streptococcal infection and the onset of symptoms suggestive of acute rheumatic fever, ranges between one and five weeks. Acute polyarthritis is the commonest presentation, occurring in 75% of cases, carditis in 40-50%, chorea in 15% and subcutaneous nodules and erythema marginatum in less than 10%. Carditis occurs more frequently in the paediatric age group and is uncommonly seen in adults having a first attack. It remains the single most important manifestation due to its capacity to cause permanent organ damage and

death. While early recognition may be possible in the epidemic situation, it can on occasions be difficult to make a definitive clinical diagnosis of acute rheumatic fever. The importance of accurate clinical diagnosis is highlighted by the fact that long - term penicillin prophylaxis would be instituted where a diagnosis of rheumatic fever is made. The revised Jones criteria (*Ad Hoc* Committee of the Council on Rheumatic fever, 1984), provided clinical guidance in the diagnosis of this condition but stipulated the need for supporting evidence of preceding streptococcal infection. This would be a positive throat culture for the group A streptococcus or serological evidence of streptococcal infection. Apart from the epidemic situation, positive throat cultures are uncommonly obtained. Less than half of the patients with group A streptococcal pharyngitis will have positive throat cultures after two weeks. Reasons for this include normal eradication of the causative organism and concomitant antibiotic therapy. The isolation and M typing of group A streptococci from throat swabs of patients with rheumatic fever is therefore, generally the exception rather than the rule. This is particularly true in rural Aboriginal communities where there may be delays in seeking medical attention. Serology therefore, remains the commonest method of demonstrating previous group A streptococcal infection. There are two types of group A streptococcal antibody tests. The first detects antibodies to extracellular antigens and will be discussed here. The second detects antibodies to cellular antigens such as group - specific polysaccharide, M-associated protein, and M protein. These will be discussed in section 1.17.

1.6 Serological tests used to demonstrate previous streptococcal infection (antibodies to extracellular antigens)

Current serological techniques (anti-streptolysin O test and anti-deoxyribonuclease B test) for the detection of streptococcal antibodies are not considered diagnostic for acute rheumatic fever. Numerous factors affect their interpretation including patient variability in demonstrating rising titres and the effect of other intervening group A streptococcal infections.

(a) Anti-streptolysin O test. (ASOT)

The basis of this test is the neutralisation of the haemolytic activity of streptolysin O toxin by specific antibodies present in the patient's serum. A highly elevated ASO titre (>256) or more significantly, a four-fold rise (or greater) in the titres of paired sera are evidence of a recent streptococcal infection. A significant disadvantage of this test is that the titre falls relatively rapidly and may be low at the time of presentation with rheumatic fever. Up to 15% of patients with acute rheumatic fever do not have an elevated ASO titer (Ayoub et al 1962).

(b) Anti-deoxyribonuclease B test. (ADB)

Group A streptococci produce four DNases, i.e: A,B,C and D. DNase B is found in nearly all strains of group A beta-haemolytic streptococci. The ADB test is a neutralisation test detecting specific antibodies in serum to the nuclease DNase B. Titres of 256 or greater in school-age children are considered elevated although, as with the ASOT, a rise in titre with paired sera would be considered diagnostic. The ADB titre reaches a peak between 4 to 8 weeks after infection and unlike the ASOT, remains elevated for several months. A patient may therefore present with an illness unrelated to streptococcal infection and have an elevated ADB titre (Ferrieri 1986). Caution should therefore be used in linking an elevated

ADB titre to a clinical entity. In practice, both the ASOT and the ADB tests are used to provide evidence of prior streptococcal infection.

(c) Antihyaluronidase test (AHT)

This is another neutralisation assay to detect antibodies against the streptococcal enzyme, hyaluronidase. Its main advantage lies in detecting antibodies after group A streptococcal skin infections which are not normally detected by the ASOT. However, as group A streptococcal skin infections are not linked to the aetiology of rheumatic fever, it has a limited role in the diagnosis of this condition (Ferrieri 1986).

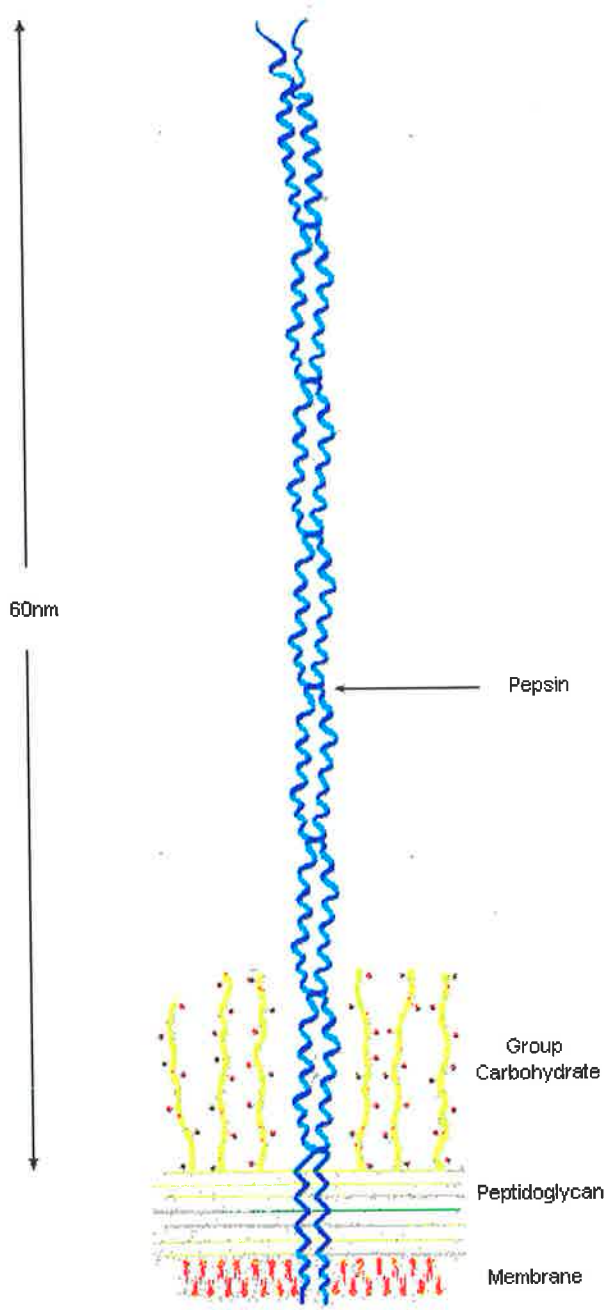
1.7 Streptococcal M protein - background

Streptococcal M protein was identified over 60 years ago by Rebecca Lancefield (Lancefield 1928) who subsequently went on to define this protein as a major virulence factor of the group A streptococcus (Lancefield 1962). It forms the basis of the typing scheme still in use. There are currently over eighty M types of group A streptococci. Not all isolates, however, are typable. Reasons for this include poor M-protein production by the organism, or unavailability of typing sera for the strain to be tested. The M serotype represents at least one marker of potential rheumatogenicity. With some exceptions, the M serotypes known to be strongly associated with acute rheumatic fever are M types 1, 3, 5, 6, 14, 18, 19 and 24 (Bisno 1991).

1.8 The nature and structure of M protein

Streptococcal M protein is a filamentous molecule consisting of two protein chains in a coiled coil configuration extending about 60nm above the surface of the organism (Fischetti 1989). This is shown in Figure 1.1. M protein appears to be strongly associated with peptidoglycan in the cell wall. The carboxyl (C) terminus of the molecule is closely associated with the cell surface while the amino (N) terminus protrudes outwards from the cell. A repeating seven-residue periodicity of amino acids has been noted in a number of M proteins including M types 5, 6 and 24. M5 protein has six A-repeat blocks at the N-terminal end, four B-repeat blocks, and three C-repeat blocks at the C-terminal end (Miller et al 1988). These repeating residues are a basic characteristic of α-helical coiled-coil proteins like tropomyosin (Manjula et al 1980a). This structure is also unique amongst bacterial surface molecules (Fischetti 1989). Epitopes in the C-terminal regions are conserved among different M-types, while those at the N-terminal are highly variable. On the intact cell, the conserved C-terminal epitopes are masked by the N terminal epitopes. This C-terminal region in M24 protein is virtually identical to that of M5 (Miller et al 1988). Comparisons of the amino acid sequences for various M proteins are provided in Figure 1.2.

Figure 1.1 A diagrammatic representation of streptococcal M6 protein. Adapted from Fischetti (Streptococcal M protein: molecular design and biological behaviour. Clin Microbiol Rev. 1989;2:285-314)

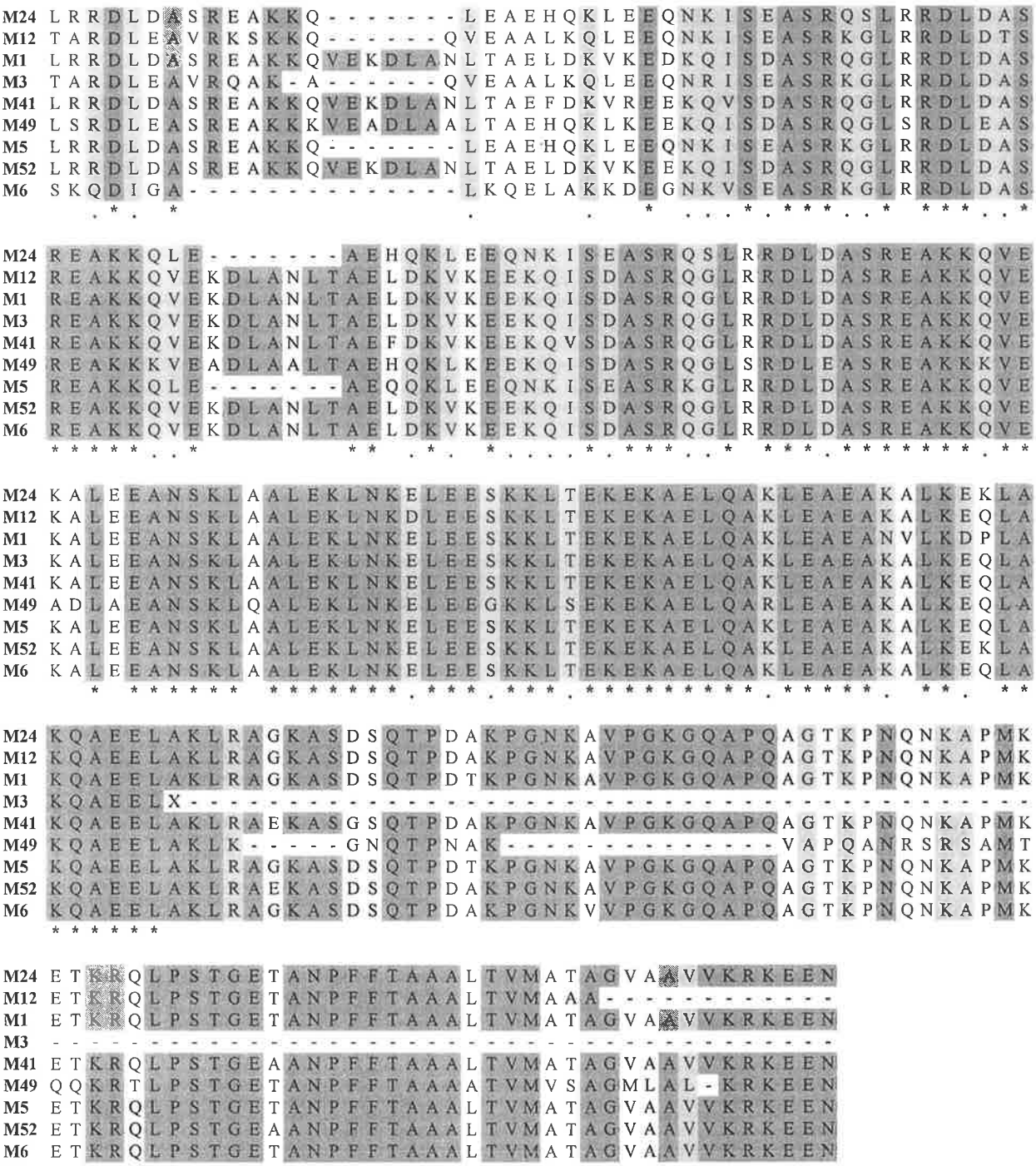


Serologically different M proteins are built around this basic scheme of an extended central coiled-coil rod domain flanked by functional end domains, namely the wall-associated anchor region at the C-terminal end and the non-helical N-terminal region. The amino acid sequence of this region is unique in each M sub-type (Manjula et al 1984). This N-terminal region contains a significantly higher net negative charge than the C-terminal end and plays an important role in the biological activity of M protein. Antibodies generated to this region opsonise the specific M type of streptococci. This hypervariable region is said to be the functional domain of M protein. In support of this, it has been shown that synthetic peptides derived from the N-terminal sequence (residues 1 to 20) of the M5 molecule, stimulated production of type-specific antibodies which did not cross-react with M protein from other serotypes (Dale et al 1983). The ability of this organism to evolve antigenic variants of M protein provides the species with an effective defence to the host immune response, thus allowing group A streptococcal infections to recur. Since a large number of streptococcal strains isolated are nontypeable (Kehoe et al 1985), it is likely that they represent derivatives of typable strains with sequence changes within the non-repetitive N-terminal end. The coiled-coil design therefore has a number of functions. Firstly the central rod region acts as a shaft to position the N-terminal antiphagocytic domain away from the cell surface and secondly the variability of sequences necessary for the coiled-coil structure allows for specific tailoring of the domains.

The complete amino acid sequence of M 24, one of the rheumatogenic strains, has been determined (Mouw et al 1988). It was found to have a molecular weight of 58,804 daltons and consisted of 539 amino acids. As mentioned earlier, it had the characteristic seven residue repeating sequence.

Figure 1.2: A comparison of inferred amino-acid sequences of nine M proteins (M24, M12, M1, M3, M41, M49, M5, M52, M6). Rheumatogenic types are M24, M1, M3, M5 and M6.

Figure 1.2: Continued



1.9 M associated proteins and Class I and II M proteins

M-associated proteins (MAP) have been described. They are surface components of M protein bearing organisms which co-purify with the type specific organism. Unlike M protein, they lack type specificity. Two distinct classes of MAP have been found. They appear to be associated with the production of serum opacity factor (OF), a lipoproteinase which is a virulence factor for the group A streptococcus. Serotypes which produce OF express MAP II antigen. Conversely, serotypes which do not produce OF express MAP I antigen. Patients with rheumatic fever have been shown in one study to have high complement-fixing titres to MAP I antigen, suggesting that this may be a virulence determinant (Widdowson et al 1971).

Two distinct classes of M protein molecules have also been described. These are based on the presence of an antigenically conserved domain, within the surface exposed portions of M protein, of certain M types. These have been named Class I and Class II M proteins. Class 1 isolates react with M protein specific monoclonal antibodies as do rheumatogenic serotypes and are OF negative. Class 2 isolates lack immunoreactivity to the monoclonal antibodies, are non-rheumatogenic and are OF positive (Bessen et al 1989). Class I M protein molecules share the surface exposed, antigenic domain comprising the C repeat region as defined for M6 protein. Class II M proteins lack these antigenic epitopes. Nearly all streptococcal serotypes associated with outbreaks of acute rheumatic fever express Class I M protein.

Most group A streptococcal serotypes therefore, fall into one of two major classes of M protein which closely parallel the MAP I and II antigenic types (Bessen et al 1989).

1.10 Antiphagocytic activity of M protein.

M protein is a major virulence factor of the group A streptococcus. It exerts its mode of action by inhibiting opsonophagocytosis as a result of reduced complement deposition on organisms. This is achieved by M protein binding factor H in serum. Factor H inhibits the formation of particle bound C3 convertase (C3b,Bb) and serves as a co-factor in the conversion of C3b to iC3b by factor I. This binding reduces the amount of C3b deposited on the organism. Without opsonisation by complement, phagocytosis is ineffective (Fischetti 1989).

1.11 The role of M protein in adherence.

It was first shown by Ellen and Gibbons (1972) that streptococci with M-protein adhere better to epithelial cells in vitro than M-protein deficient organisms. M-protein binds specifically to F actin filaments which is present on the surface of a wide range of cells including fibroblasts and lymphocytes (Chalovich et al 1986; Owen et al 1978; Fischetti 1989). The clinical significance of this is still unclear but it would appear to provide the organism with an attachment factor.

1.12 Other virulence factors of the Group A streptococcus

Other virulence factors include the Ig-Fc-binding M-related proteins (Heath et al 1989), the complement factor inactivating C5a peptidase (Chen et al 1990) and streptococcal inhibitor of complement mediated lysis (SIC).

Surface coating of the organism with serum proteins such as the Ig-Fc-binding M-related proteins, may disguise it from the immune system. Activation of the complement cascade is therefore prevented.
C5a peptidase is a highly specific protease which cleaves C5a. This eliminates the chemotactic gradient and prevents recruitment of phagocytes.
SIC is a recently described protein produced by the organism which binds to the membrane attack complex that is normally formed by the complement cascade system to lyse an invading organism. This binding by SIC inhibits lysis of the organism (Akesson et al 1996).

1.13 Structural similarity and immunological cross-reactivity between components of group A streptococci and mammalian protein

Group A streptococcal M protein shares a number of features with rabbit skeletal muscle tropomyosin. These are thermal stability of the dimers, elongated shape, fibrous appearance on the cell surface and 40% homology of peptide sequences. M24 peptides have up to 40% identity with tropomyosin (Hosein et al 1979). Many of the observed similarities correspond to the core hydrophobic amino acids in the seven residue repeat pattern necessary to maintain the coiled-coil structure of M protein. It has

been suggested that this similarity may be relevant in the pathogenesis of streptococcal diseases (Fischetti 1989; Hosein et al 1979). Myosin and other coiled-coil proteins such as keratin also show significant homology with M proteins.

Immunological cross-reactivity has been shown between M protein and a variety of mammalian proteins. Cross-reactions were initially reported between purified streptococcal membranes and human cardiac muscle sarcolemma (Zabriskie et al 1966). Several M proteins have also been shown to be cross-reactive with heart proteins (Dale et al 1985a). It has been shown that type 5 M protein evokes both protective and heart cross-reactive antibodies and that some of these antibodies also react with types 6 and 19 M proteins (Dale et al 1985b).

Synthetic peptides based on the amino acid sequence of M5 protein have been shown to share antigenic epitopes with a 40 kDa protein in cardiac sarcolemmal membrane (Manjula et al 1984). These peptides were from the carboxy terminal of M5 protein which is highly conserved among different M types of group A streptococci.

Using a pepsin extract of M5 protein, heart tissue cross-reactive antibodies were produced which were specific for sarcolemmal membrane proteins (Sargent et al 1987) and the heavy chain of myosin (Dale et al 1985b, Dale et al 1986a). Using synthetic peptides representing the primary structure of the pepsin extract of M5, both the myosin and sarcolemmal membrane cross-reactive epitopes were subsequently shown not to be situated at the N-terminal end. They were located at peptides 84-116 and 164-197 respectively (Dale et al 1986b, Sargent et al 1987).

Synthetic peptides representing the N-terminal 20 amino acids of type 5 (Dale et al 1983) and type 6 (Beachey et al 1986) M proteins, were shown to evoke opsonic, type specific but not heart reactive antibodies. This suggested that the heart tissue cross-reactive epitopes were not located at the N-terminal end of the M proteins tested. The N-terminal was therefore type specific and opsonic, but not heart cross-reactive.

A study by Cunningham et al (1988) looking at human monoclonal antibodies reactive to a pepsin extract of type 5 M protein and myosin, showed that they reflected the antibodies seen in sera from patients with group A streptococcal disease or rheumatic fever (Cunningham et al 1988). Following this study, it was shown that most mouse and human myosin crossreactive antibodies, recognised an epitope within the 14 residue C terminus of a pepsin extract of M5. This was mapped using synthetic peptides spanning the sequence of this extract. The epitope that was identified involved the amino acid sequence QKSKQ. As this was a pepsin extract, epitopes further towards the C-terminal end of the entire M protein molecule were not tested (Cunningham et al 1989).

In a separate study, a pepsin extract of M19 evoked both opsonic and heart-crossreactive antibodies in rabbits. Overlapping synthetic peptides representing the first 24 amino acid residues of the N-terminal were then synthesised and used as immunogens in rabbits (Bronze et al 1988).
Although the initial goal was to identify an immunogen that stimulated protective and not cross-reactive antibodies, antisera so obtained, cross reacted with sarcolemmal membranes of human myocardium. The antibodies specifically recognised a 60-kDa myocardial protein and the cross reactions were completely inhibited by the pepsin extract of M19. A particular peptide was identified SM19 (11-24)C as being particularly

cross-reactive. The amino acid sequence of this peptide was KLKKIIDDLDAKENC.

This would suggest that, contrary to the findings of Dale, Beachey and others as above, at least part of the amino acid sequence of the pepsin extract of M19 represents the N-terminal of the protein and could be cross-reactive with human myocardium (Bronze et al 1988). The authors suggested that epitopes which stimulate autoimmune responses might be located within the hypervariable, type-specific N-terminal region.

Pruksakorn in a more recent study (1992), looked at specific tissue cross-reactivity between murine antibodies generated to selected, synthetic, overlapping 20-mer peptides. These peptides spanned the conserved C-terminal segment of M5 protein and were labelled peptides 145 through to 162. Antibodies to peptides 146, 150 and 151 reacted to human atrial tissue and porcine heart myosin. Peptides 145 and 149 generated antibodies which reacted weakly with human atrial tissue but strongly to porcine myosin (Pruksakorn et al 1992). This would support the earlier findings, that conserved C-terminal epitopes might be important in the pathogenesis of rheumatic fever.

Epitopes of M5 protein have also been shown to be cross-reactive with antigens of mouse articular cartilage and synovium. The cross-reactive epitopes were localised to peptides representing the C-terminal region of a pepsin extract of M5 protein (Baird et al 1991).

In summary therefore, selected streptococcal M proteins share immunologically cross-reactive epitopes with components of heart tissue, muscle and articular tissue. The location of these epitopes in the M protein

molecule remains unclear although the balance of evidence would suggest they are located at the C-terminal end.

1.14 The extraction of M protein

A number of methods have been used to extract M protein from group A streptococci. The original method was a hot acid extraction as described by Lancefield (1928). The final product obtained by hot acid extraction was thought not to represent the native M protein molecule (Beachey et al 1977) and has subsequently been shown to be less immunogenic than other extracts. Other methods used include the use of pepsin digestion at a suboptimal pH (Beachey et al 1977), nonionic detergent (Fischetti et al 1976) and lysin extraction (Fischetti et al 1971). The extracts obtained from the use of nonionic detergent and lysin were not as homogenous as the pepsin-derived molecule or were associated with cell wall fragments. Pepsin cleavage at a suboptimal pH of 5.8, releases a highly immunogenic, biologically active fragment of M protein termed pepM, from the cell wall. It was also noted to be free of non-type specific immunoreactivity. The mobility of pep M24 upon electrophoresis in 10% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was consistent with an average molecular weight of 33,500 daltons (Beachey et al 1977). M proteins extracted with pepsin are almost half the size of the lysin extract. The point of pepsin cleavage is approximately halfway along the length of the coiled-coil molecule (Figure 1.1). There is however, considerable variation in the molecular weights of M-protein from different M types and strains of the same type extracted by the pepsin method. Pep M5, for example has a molecular weight of 19,000 when estimated by SDS - PAGE (Manjula et al 1980b).

1.15 The *emm* gene coding for M protein and its role in antigenic variation

The gene for M6 protein was first cloned from streptococcal strain D471 (*emm-6.1*) into *Escherichia coli* (Hollingshead et al 1986; Scott et al 1983). M protein genes for a number of other streptococcal serotypes have been cloned and immunologically active M protein has been produced. These include M5, M24 and M12 (Mouw et al 1988; Robbins et al 1987). DNA from 56 different serotypes tested contained homologous *emm* genes. All strains (except one) which did not produce M protein also had identifiable *emm* genes. The reason for this non-expression of the M phenotype is not known (Scott et al 1985).

It has also been shown that the 3' region of the *emm-6.1* gene is conserved while the 5' segment is variable (Scott et al 1983). Antigenic variation among M proteins can be explained as a result of point mutations, intragenic recombination between repeat sequences and intergenic recombination.

One copy of the *emm* gene was detected in an M6 strain (Scott et al 1985) and in nine other serotypes (Scott et al 1986a). This supports the hypothesis that antigenic variation as a result of recombination, is an intragenic event. However, the M5 strain was noted to have two copies of the *emm* gene (Kehoe et al 1985). The significance of this remains unclear. In organisms that undergo a higher frequency of antigenic variation than *S. pyogenes*, multiple copies of homologous sequences have been detected in the genome which can recombine to generate variants (Miller et al 1988).

It has been suggested that the high level of sequence homology in the conserved and signal sequence regions of the M protein genes of M types 5, 6 and 24, reflects the very close evolutionary relationship between these sequences and indicates that the point mutation rate during their divergence has not been high (Miller et al 1988). There is, however, a high level of variation in gene sequences between these two conserved regions. This variation would be due to the presence of repeat sequences within M protein genes. Recombination between imperfect copies of repeated sequences could alter epitopes in regions of M proteins encoded by repeated sequences.

The unique, hypervariable 5' region of M protein genes may also be a result of intergenic recombination leading to the acquisition of unrepeated, 5' sequences that encode opsonogenic type-specific epitopes. This is suggested by the similarity between the C repeat region of the M5 serotype M protein gene (*smp5*) and the M6 serotype M protein gene (*smp6*). This could be facilitated by genetic exchange, mediated by transduction and conjugation (Miller et al 1988).

A positive regulator of *emm* expression has been shown to play a part in high level M protein production in an M6 strain (Scott et al 1986b). This gene has been named *mry* and its role together with environmental factors in the expression of virulence factors requires further study. This is perhaps best illustrated by the decline of M protein production following repeated laboratory subculture and an increase with human blood or animal passage (Becker 1964). The *emm* gene has also been found to be under the positive control of the *vir* R locus in a wide range of different M serotypes. This *vir* gene has 95% homology with the *mry* gene described above (Podbielski 1993).

1.16 B and T-cell epitope mapping of M protein

Conserved T and B cell epitopes have been identified on the C-terminal segment of the M5 protein (Pruksakorn et al 1992). In that study, two conserved peptides LRRDLDASREAKKQVEKALE and KLTEKEKAELQAKLEAEAKA stimulated peptide specific antibodies in mice and proliferation of specific helper T (Th) cells. The T cell epitopes defined were able to help B cells make antibody to the peptide epitope itself. This helper function of T cell epitopes for specific native protein, has not been described for streptococcal M protein but has been described for malaria and hepatitis B (Good et al 1987; Milich et al 1987). It is possible that a similar situation exists with rheumatic fever. It was postulated that these peptides (called peptides p145 and p149) could be the basis of a vaccine against rheumatic fever (Pruksakorn et al 1994).

1.17 The serological detection of antibodies to cellular streptococcal antigens (including M protein)

A number of cellular antigens have been used to demonstrate antibody responses in rheumatic fever. These include M associated protein (Martin et al 1984; Widdowson et al 1971) group A specific carbohydrate (Barrett et al 1983), M protein extracts (Lancefield 1959; Bisno et al 1982) and synthetic peptides representing the B and C repeat regions of M protein (Hartas et al 1995; Bessen et al 1995).

(a) Antibodies to M associated protein (MAP)

MAP I and II have been described in section 1.9. Antibodies to these proteins have been studied in subjects with rheumatic fever (Martin et al 1984; Widdowson et al 1971).

In one study, 67.4% of subjects with rheumatic fever had complement - fixing antibodies to MAP I antigen of >40, when compared with 21.7% of matched controls (Martin et al 1984). These were determined by complement fixation using an acid extract of *Streptococcus pyogenes.* Titres remained elevated for a mean of 10.3 weeks before showing a fourfold decrease. These antibodies however, reflect infection by a serotype of the potentially rheumatogenic MAP I group (or Class I group A streptococcus), rather than being definitive evidence of rheumatic fever.

(b) Antibodies to group A specific carbohydrate

A correlation between persistence of antibodies to this cell wall polysaccharide and the development of chronic rheumatic mitral valve disease has been shown (Barrett et al 1983). This test has been proposed as a way to distinguish mitral valve disease of rheumatic origin from that due to a non-rheumatic etiology. The antigen used is a hot formamide extract of the cell wall polysaccharide, conjugated to poly-L-lysine. This antibody however, rises after any group A streptococcal infection as do the ASO and ADB titres. It also persists longer after normalisation of the other antibodies. It therefore would have a limited role in the diagnosis of acute rheumatic fever.

(c) Antibodies to M protein

Immunity to group A streptococcal infection is associated with the development of opsonic antibodies to anti-phagocytic epitopes of M-protein. Immunity was originally thought to be type specific and lasting (Lancefield 1959). More recently however, antibody has been found to be strain specific rather than type specific (de Malmanche 1994)

The opsonophagocytic test of Lancefield (bactericidal test), has been used as a standard for M-antibody detection (Lancefield 1957). It is however, cumbersome and not suited to a routine diagnostic laboratory.

The enzyme-linked immunosorbent assay has been used to detect antibodies to M protein (Bisno et al 1982). Antibodies to pepsin extracts of M types 5, 6 and 24 were assayed for in patients with acute rheumatic fever. It was found that type 5 was the predominant rheumatogenic type in the population studied. However, a significant number of controls were noted to have antibodies to type 6. Neither patients nor controls had antibodies to type 24.

(d) Antibodies to peptide fragments representing the B terminal repeat region of M protein

The immunodeterminants of the M6 protein molecule have been mapped (Fischetti et al 1988). In this study, non-overlapping peptides of varying sizes ranging from 20-mer to 10 mer, spanning 82% of the molecule were used in an enzyme immunoassay against rabbit immune sera and four human sera. The rabbit immune sera was prepared by immunising rabbits with whole M6 streptococci or purified M6 protein. The B repeat region was found to be immunodominant with almost a complete lack of immunoreactivity to the C repeat region. Reactivity to one C repeat region was however noted.

Another study however, looking at the antibody reactivity of the B repeat region of the M5 protein, failed to detect any definitive correlation with rheumatic fever in Aboriginals (Hartas et al 1995). In this study, 15 out of 22 sera from subjects with acute rheumatic fever and 9 out of 18 subjects with rheumatic heart disease, reacted with the epitopes KQQESK (B1) and EQKSKQ (B4) used. In a separate patient group of this study, sera from

only two out of thirteen subjects with rheumatic heart disease, reacted with these epitopes. This would suggest that antibodies to this region either do not persist in rheumatic fever or are not specific.

The apparent discrepancy between these studies may be explained by the quite different sized peptides used in each. In Fischetti's study, as overlapping peptides were not used, it is possible that that some determinants in the C-terminal were split and not represented in the relatively large peptides used. More importantly, the hyper-immune rabbit sera used may simply reflect streptococcal infection and not rheumatic fever as sera from subjects with rheumatic fever were not tested with these peptides.

(e) Antibodies to peptide fragments representing the C terminal repeat region of M protein

The detection of antibodies to the conserved C - terminal region has been described (Pruksakorn et al 1994). A 20-mer, peptide based enzyme immunoassay, was used. No difference was found between both Aboriginal and Thai controls and subjects with rheumatic fever in that study. This peptide was similar to that used in the previous study on T and B cell epitope mapping of M protein (Pruksakorn et al 1992).

Another study looking at the C-terminal repeat region of M6 protein was that of Bessen et al (1995). In this study a distinction was made between Class 1 and Class 2 isolates of group A streptococci as previously described in Section 1.9. The aim was to localise class specific epitopes within the C-terminal repeat region.

Peptides representing the Class 1 and Class 2, C-terminal repeat regions were synthesised. These 20-mer peptides corresponded to amino acid

residues 240-260 of M6 protein. The Class 2 peptide was identical to the Class 1 except for amino acid substitutions at residues 243 and 249. Subjects with rheumatic fever showed elevated levels of serum IgG against the Class 1 peptide and lacked immunoreactivity to the Class 2 peptide. This would suggest that sera from patients with rheumatic fever react with C-terminal epitopes of Class I group A streptococci. Controls included subjects with uncomplicated group A pharyngitis and those with no known recent group A streptococcal infection. A highly significant difference in EIA reactivity was noted between this group and those with rheumatic fever, for each peptide ($p<0.001$).

The difference in findings btween these two studies looking at the C-terminal repeat region can only be possibly explained by the use of different peptides. It may be that reactivity in rheumatic fever is restricted to a few discrete C-terminal epitopes. Since neither of these studies used overlapping, smaller peptides, this could not be determined.

1.18 Streptococcal M protein and rheumatic fever - Conclusions

Streptococcal M protein is an important virulence factor of the group A streptococcus. It is implicated in the pathogenesis of rheumatic fever although the precise mechanism of this remains unclear. Current evidence would suggest that antibodies to pepsin extracts of M protein may reflect the antibodies seen in sera from patients with group A streptococcal disease or rheumatic fever. These extracts have also been shown to evoke heart cross-reactive antibodies. The position of these cross-reactive epitopes in the M protein molecule is unclear although the B and C repeat regions of the M protein molecule, have been both implicated and discounted in separate studies.

1.19 Aims of this study

The clinical diagnosis of rheumatic fever can be difficult in remote communities where the access to and provision of medical services may not be optimal. Current serological methods to support a clinical diagnosis of rheumatic fever (ASOT and ADB) are non-specific, particularly in communities with a high incidence of streptococcal infections. This study aims to determine whether antibodies to epitopes of M protein can distinguish between subjects with rheumatic fever and controls. This could then be the basis of an improved serologic test for rheumatic fever.

Aims:

1. To use a mild pepsin extraction method to obtain M protein from M types 18 and 24 group A streptococci.

2. To use the M protein extracts in Western immuno-blot, using sera from Aboriginal and non-Aboriginal subjects with known rheumatic fever or rheumatic heart disease, to determine immune status to M18 and M24.

3. To determine whether the pepsin extracts of streptococcal M protein as used above, can distinguish between subjects with rheumatic fever and matched controls and to determine whether these M-types would universally react with aboriginal sera.

4. To use overlapping peptide banks based upon the known amino-acid sequence of M24 protein to map linear epitopes, universally reactive with sera from subjects with rheumatic fever, using an enzyme immunoassay system.

5. To determine the optimal parameters for an enzyme immunoassay using the universally reactive peptides.

6. To assess the use of universally reactive peptides as antigens in this enzyme immunoassay for the serodiagnosis of rheumatic fever.

Chapter 2

Study Population

Serum from a total of eighty nine individuals, comprising five subject groups, were tested in the two parts of this study. These groups were: Aboriginal subjects with acute rheumatic fever, Aboriginal subjects with previous rheumatic fever, Aboriginal controls, non-Aboriginal subjects with previous rheumatic fever and non-Aboriginal controls. All sera were accessed following Institutional Ethics Committee approval.

2.1 Subjects with acute rheumatic fever

Four sera were from Aboriginal subjects (mean age 21 years) with acute rheumatic fever. Three of these were reviewed by cardiologists and the author. The fourth, from who serum 33 was obtained, was reviewed by a paediatrician. There were no non Aboriginals with acute rheumatic fever.

Serum 31

From a 25 year old Aboriginal male, who presented with fever, polyarthritis, cardiomegaly and a mitral systolic murmur. There was no definitive history of previous rheumatic fever although it is unlikely that this was his first episode.

Jones criteria (revised): polyarthritis, carditis, elevated erythrocyte sedimentation rate (ESR), fever.

ASOT - 256, ADB - 512

An echocardiogram confirmed cardiomegaly which was predominantly left ventricular. Moderate mitral regurgitation was noted.

Serum 32

From a 24 year old Aboriginal female who presented with fever, worsening cardiac failure, arthralgia and a mitral stenotic murmur. She had a past history of rheumatic fever although the status of her penicillin prophylaxis was unclear.

Jones criteria (revised): carditis, arthralgia, past history of rheumatic fever, fever, elevated ESR.

ASOT - 192, ADB - 1024

An echocardiogram confirmed moderately severe mitral stenosis.

Serum 33

From a 13 year old Aboriginal female who presented with fever, polyarthritis, an elevated ESR and C-reactive protein. There was no clinical evidence of carditis or of previous rheumatic fever. An echocardiogram was not performed.

Jones criteria (revised): polyarthritis, fever, elevated ESR and C-reactive protein.

ASOT - 128, ADB - >3072

Serum 57

From a 25 year old Aboriginal male who presented with cardiac failure, mitral regurgitant and stenotic murmurs and a past history of rheumatic fever. It was unclear as to the frequency of penicillin prophylaxis used.

An echocardiogram confirmed the presence of moderately severe mitral valve disease with left ventricular dysfuction.

Jones criteria (revised): carditis, previous history of rheumatic heart disease, elevated ESR.

ASOT - 1536, ADB - >3584

2.2 Aboriginal subjects with previous rheumatic fever

Fifteen sera were from Aboriginal subjects (mean age 33 years) with previous rheumatic fever or rheumatic heart disease. All subjects in this group had been reviewed by a number of different physicians and the initial diagnosis of rheumatic fever had been made some time prior. The precise time between the last episode of acute rheumatic fever and serum sampling for this study, was unknown in all cases. Most had been admitted to hospital for assessment of cardiac valvular function prior to consideration of valve replacement. Clinical details were obtained from case notes and discussion with clinicians involved in the management of individual cases.

Serum 6

From a 33 year old Aboriginal woman with previous rheumatic fever, who had a mitral valve replacement for rheumatic mitral valve disease. This was confirmed on histology.

ASOT and ADB were not performed because of insufficient sera being available.

Serum 22

From a 53 year old Aboriginal man with a past history of rheumatic fever who presented in cardiac failure. This responded to standard therapy and valve replacement was not performed. Due to insufficient specimen, only a Western Blot was performed on this serum.

Serum 24

From a 24 year old Aboriginal woman with previous rheumatic fever, who had a mitral valve replacement for rheumatic mitral valve disease. This was confirmed on histology.

ASOT - 256, ADB - 1024

Serum 25

From a 16 year old Aboriginal male with deteriorating cardiac function secondary to rheumatic valvular heart disease. A mitral valve replacement was done and histology supported a rheumatic origin for the valvular disease.

ASOT - 512, ADB - 128.

It is possible that this subject had an acute episode of rheumatic fever. There were no other criteria however, to support this.

Serum 34

From a 24 year old Aboriginal female with a past history of rheumatic fever was assessed for rheumatic heart disease. Apart from cardiac symptoms, she was relatively asymptomatic.

ASOT - 192, ADB - 256

Serum 35

From a 30 year old asymptomatic Aboriginal female with a past history of rheumatic fever. Reviewed for a routine follow up assessment.

ASOT - 256, ADB - 512

Serum 42

From a 56 year old Aboriginal male with a history of rheumatic valvular heart disease for at least 10 years. A mitral valve replacement was done and histology of excised valve confirmed a rheumatic aetiology.

ASOT - 256, ADB - 384

Serum 45

From a 46 year old Aboriginal man with no documented past history of rheumatic fever, presented with a movement disorder which was diagnosed by the attending physician as Sydenham's (rheumatic) chorea. There were no cardiac signs. The author did not have the opportunity to review this patient. The diagnosis could be in doubt, as rheumatic chorea is uncommon in this age group and gender. It is possible that the high ADB titre may have influenced the diagnosis.

ASOT - 256, ADB - 3584

Serum 46

From a 39 year old Aboriginal male with a past history of rheumatic fever, reviewed to assess cardiac function.

ASOT - 192, ADB - 256

Serum 52

From a 23 year old Aboriginal female who had a first documented episode of rheumatic fever at the age of 14 years. At review she was asymptomatic.

ASOT - 64, ADB - 128

Serum 64

From a 24 year old Aboriginal male admitted for mitral valve replacement. Histology of excised valve tissue confirmed a rheumatic aetiology.

ASOT - 64, ADB - 512

Serum 65

From a 22 year old Aboriginal female with previous rheumatic mitral valve disease who had a mitral valve replacement. Histology of excised valve tissue confirmed a rheumatic aetiology.

ASOT - 512, ADB - 768

Serum 71

From a 17 year old Aboriginal male who had a mitral valve replacement for rheumatic heart disease. This was confirmed on histology.

ASOT - 256, ADB - 768

Serum 76

From a 49 year old Aboriginal woman with a past history of rheumatic fever with mitral valve involvement.

ASOT - 256, ADB - 256

Serum 85

From a 57 year old Aboriginal female who had a mitral valve replacement for progressively worsening cardiac function secondary to rheumatic heart disease.

ASOT - 128, ADB - 192

2.3 Control sera from Aboriginals with unrelated, non-rheumatic disorders

Thirty six sera from Aboriginals (mean age 37 years) who had no record of rheumatic fever were obtained. All individuals were reviewed by the author to exclude rheumatic fever or rheumatic heart disease, based on a history and clinical examination. Details are tabulated in Table 4.3

This control group consisted of the following :

One subject with non-rheumatic valvular heart disease.

Two subjects with coronary artery disease.

One subject with Group A streptococcal sepsis of undetermined M type.

Two subjects with post-streptococcal glomerulonephritis.

One subject with group G streptococcal sepsis.

Ten subjects with sepsis relating to a broad range of causative agents. These included *Staphylococcus spp*, microaerophilic streptococci, *Nocardia spp*, *Camplobacter spp*, *Mycobacterium tuberculosis* and streptococci of the viridans group.

Nine subjects with trauma.

Ten subjects with miscellaneous conditions. These included tumour, syphilis, chronic liver disease, subarachnoid haemorrhage and spinal injury.

2.4 Non-Aboriginals with rheumatic heart disease

There were eleven sera from non-Aboriginal subjects with rheumatic heart disease (mean age 51 years). Most of these had valve replacements performed together with coronary artery bypass grafting.

Some of these subjects had their initial episode of rheumatic fever diagnosed up to fifty seven years prior. Details of the method of diagnosis

were unavailable. A common feature however, was that most of these subjects could remember when their last episode of rheumatic fever was. Histology was performed on all resected valve tissue but was generally less discriminatory than that done on the younger aboriginal subjects. This was largely due to calcification of the valves.

Serum 1

From a 61 year old non-Aboriginal woman with a history of rheumatic fever at the age of 21 years. Mitral valve replacement performed.
ASOT - 96, ADB - <128

Serum 4

From a 73 year old non-Aboriginal male with a history of rheumatic fever at the age of 16 years. Mitral valve replacement performed.
ASOT - 128, ADB - <128

Serum 5

From a 60 year old non-Aboriginal female with a history of rheumatic fever at the age of 26 years. Mitral valve replacement performed.
ASOT - 128, ADB - <128

Serum 9

From a 62 year old non-Aboriginal woman with a history of rheumatic fever at the age of 30 years. Mitral valve replacement performed.
ASOT - 1536, ADB - <128

Serum 10

From a 70 year old non-Aboriginal man who had an aortic valve replacement done for rheumatic heart disease.

Serum 19

From a 25 year old non-Aboriginal woman who had a past history of rheumatic fever as a child. The time of her first episode was uncertain, but was at least ten years prior.

Serum 20

From a 15 year old non-Aboriginal female with a past history of rheumatic fever as a child. This was at least eight years prior.

Serum 29

From a 26 year old non-Aboriginal male with a past history of rheumatic fever. The first episode was approximately twelve to fifteen years prior.

Serum 54

From a 52 year old non Aboriginal female with previous rheumatic heart disease. She was unable to recall when the last episode of rheumatic fever was. Mitral valve replacement performed.

ASOT - 128, ADB - 192

Serum 62

From a 59 year old non-Aboriginal female with previous rheumatic heart disease. She was unable to recall when the last episode of rheumatic fever was. Mitral valve replacement performed.

ASOT - 192, ADB - <128

Serum 98

From a 62 year old non-Aboriginal female who first had rheumatic fever at the age of 16 years.

ASOT - 64, ADB - 128

2.5 Control sera from non-Aboriginal persons with no history of rheumatic fever

The remaining twenty three sera were from non-Aboriginal controls (mean age 59 years). All of these patients were reviewed by the author to clinically exclude rheumatic heart disease or a past history of rheumatic fever.

These included the following :

Nine subjects with coronary artery disease.

Four subjects with non-rheumatic valvular heart disease.

Eight subjects with Group A streptococcal sepsis of undetermined M type.

One subject with trauma.

One normal subject.

2.6 Study population - conclusions

It was not possible to obtain sera from non-Aboriginal subjects with acute rheumatic fever, nor was it possible to obtain a closely matched group of non-Aboriginal subjects with recent rheumatic fever. This demonstrated the fact that this disease has disappeared largely from the non-Aboriginal population in the last forty years. A broad spectrum of control sera were obtained, in particular from young Aboriginal subjects who would have been exposed to streptococcal infection without evidence of rheumatic fever. Where possible, subjects with proven group A streptococcal disease were also included.

Of these sera, thirty one were tested by Western blot and eighty two were tested by enzyme immunoassay. Seven sera were tested by Western blot alone (sera 10, 11,19,20,22,29 and 39) due to insufficient volume of specimen.

Chapter 3

Materials and Methods

3.1 Bacterial strains

Organisms used were *Streptococcus pyogenes* M18 PHLS J17C (OF-) and *Streptococcus pyogenes* M24 PHLS C98/97 (OF-). These were provided by Dr A. Goodfellow, of the Menzies School of Health Research, Darwin.

3.2 Culture

The organisms were inoculated into human blood and incubated overnight at 35°C to maximise M protein production. They were then subcultured onto Columbia based horse blood agar (Oxoid) and incubated at 37°C overnight. Single colonies were inoculated into 1 litre volumes of Todd-Hewitt broth containing 0.2% yeast extract. They were incubated at 37°C for 18 hours in a shaker.

3.3 Extraction of M-Protein by mild pepsin digestion

The method of Beachey et al (1977) was used with slight modifications.
Following overnight culture, the organisms were pelleted by centrifugation at 3,800 x g for 5 minutes. They were washed twice in ice cold 0.067 M phosphate buffer pH 5.8. On each occasion being pelleted by centrifugation as before.
The pellets were then weighed and suspended in prewarmed (37°C) 0.067 M sodium phosphate buffer pH 5.8, containing 50 μg/ml of pepsin (Sigma). A final pH of 5.8 was then confirmed.

On average, 2.5 g of cells (wet weight) were obtained per litre of broth and this was added to 5 ml of pepsin/phosphate buffer solution as above. This was incubated for 45 minutes at 37°C with shaking. The effect of pepsin was terminated by the addition of enough 7.5% sodium bicarbonate to raise the pH to 7.4 and the immersion of the container into ice. The mixture was then centrifuged at 3,800 x g for 15 minutes to remove unwanted particulate matter and the supernatant filtered through a 0.2 μm cellulose acetate filter. Protein was precipitated using saturated ammonium sulphate to achieve a final saturation of between 50-60% and was kept at 4 °C overnight before being centrifuged at 10,000 x g for 30 minutes. The sediment was dissolved in 1 ml of 0.1 M phosphate buffer pH 8 and dialysed against 0.01 M phosphate buffer overnight at 4°C. The volume was reduced by vacuum-centrifugation (Speedi-Vac) and the protein stored at -70°C until used.

The protein concentration of the pepM24 as extracted above, was 0.25 g/L (Colorimetric method using a centrifugal analyser with Coomassie blue).

3.4 Polyacrylamide gel electrophoresis of extracted M protein

M proteins extracted from M serotypes 18 and 24 (PepM 18 and PepM 24) were examined by sodium dodecylsulphate-12% polyacrylamide gel electrophoresis (SDS-PAGE), run at 100 V for 18 hours (Hames 1981 *In* Gel Electrophoresis of proteins). Gels were stained with 0.275% Coomassie Blue in 10% methanol/10% ethanol 7% acetic acid and destained in 7% acetic acid 10% methanol/10% ethanol. Standard molecular mass markers (Biorad Laboratories, USA) ranging from 14.4 kDa to 97.4 kDa were run concurrently with the M protein samples so that size estimates could be made.

3.5 Western Blotting

Western blotting was done using the method of Towbin et al (1979) with slight modifications. PepM18 and PepM24 were run on SDS-PAGE and blotted onto nitrocellulose filters using a Tris/glycine/methanol buffer. The filters were then soaked in 5% skimmed milk for 20 minutes to block non-specific protein binding sites. Test sera were diluted 1:10 using 0.05% Tween 20 in 20 mM Tris buffered saline 0.9% (TTBS) and 0.02% skimmed milk, added to the filters and these were incubated overnight with agitation at room temperature. They were washed 3 times in TTBS with agitation the next day. Secondary antibody (rabbit anti-human IgG-horseradish peroxidase conjugate, Dako) was diluted 1:1000 in TTBS and added. The filters were again incubated overnight at room temperature with agitation. Repeated washes were done using TTBS three times and 20 mM Tris buffered saline 0.9% (TBS) twice. Substrate (4-chloro-1-naphtol) was then added with hydrogen peroxide. The filters were allowed to develop in the dark and the presence and position of bands if present were noted.

3.6 Sera used in the Western blot using pepM24 and pepM18 as antigens

Sera from thirty one subjects were studied. This included eighteen patients with clinically proven rheumatic heart disease or rheumatic fever. Of these, ten were Aboriginal and eight were non-Aboriginal patients. The mean age of the Aboriginal subjects was 32 years (range 13-56), while that of the non-Aboriginal subjects was 49 years (range 15-73). Sera from thirteen subjects were used as controls. Four patients had coronary artery bypass grafting, two had valve replacements for non-rheumatic indications, two

had severe group A streptococcal sepsis, one had group G streptococcal sepsis, three had pathology unrelated to streptococcal disease and one was a normal subject. Of these thirteen, eight were non-Aboriginal subjects with a mean age of 57 years (range 26-80) and five were Aboriginal subjects with a mean age of 33 years (range 14-61).

3.7 ASOT determination

Antistreptolysin O titres (ASOT) were determined on all sera using a standard method (Ayoub et al *In* Manual of Clinical Laboratory Immunology) and a commercial kit (Wellcome, UK).

Reagents:-

1. Reduced streptolysin O MR23 (Wellcome).
2. Streptolysin O buffer MR45 (Wellcome).
3. 2.5% sheep red cell suspension in Wellcome buffer.

Test sera were inactivated by heating to 56°C for 30 minutes. Serial dilutions were made ranging from 1:64 to 1:3072. Standard positive and negative controls were run in parallel. The endpoint was taken as the last well of serum dilution containing enough antibody to prevent complete haemolysis.

3.8 ADB determination

The ADB titres were determined on all sera using a standard method (Ayoub et al *In* Manual of Clinical Laboratory Immunology)

Reagents:-

1. Streptonase B enzyme.
2. Streptonase B substrate.
3. Streptonase B buffer concentrate.

Test sera dilutions were made ranging from 1:128 - 1:3072 after being inactivated by heating as before. Appropriate controls were used.
The endpoint was read as that dilution which showed the last definite colour change (violet), indicating enzyme inhibition by antibody.
Both the ASOT and ADB determinations were performed at the Institute for Medical and Veterinary Science, Adelaide, as part of routine serologic testing.

3.9 Enzyme immunoassay using biotinylated peptides

Synthetic peptide antigens which are hydrophilic and contain less than 20 amino-acids, are difficult to immobilize on plastic surfaces in sufficiently reliable quantities for a direct enzyme immunoassay (EIA) (Fischer et al 1990). The binding of these peptides to microtiter plates has been achieved by the use of peptide - polymer rod combinations (Geysen et al 1987), conjugation to carrier proteins with covalent binding to chemically activated surfaces (Twinning et al 1979), the use of high pH carbonate buffers (Pruksakorn et al 1994) and the capture of biotinylated peptides by immobilized avidin or streptavidin (Fischer et al 1990; *In* "Pinpoints" Chiron Mimotopes 1992). The attachment of biotin to synthetic peptides is done by specific introduction at the amino terminus after solid - phase synthesis (Scott et al 1984). Antibody detection by enzyme immunoassay can be enhanced by the use of the biotin - avidin system in which biotinylated peptides are captured by immobilised avidin or streptavidin on microtiter plates. Amplification occurs because each avidin molecule has several biotin binding sites (Fischer et al 1990).
Eighty two, sixteen-mer, biotinylated peptides corresponding to the 539 amino-acid sequence of M24 were obtained from Chiron Mimotopes, Melbourne, Australia. Details of the peptide sequences are given in Table

3.1. This bank was derived from the published sequence of Mouw et al (1988). Peptides were offset by 6 amino acids, covering all 10 - mers. They were numbered from peptide 44 to 96 (N terminal) and 99 to 127 (C terminal). Peptides 97 and 98 are control peptides unrelated to M-protein. The sequence SGSG at the N terminal of each peptide is used for biotin binding. The signal sequence of the protein was not included as this is cleaved in the production of the mature protein. The peptides were dissolved in phosphate-buffered saline (PBS pH7.2) and dimethyl formamide. The working dilution of the peptides was 0.028 mg/ml.

Table 3.1

Biotinylated peptide amino acid sequences numbered from 44 (N-terminus) to 127 (C-terminus).

Peptide number	Sequence	Peptide number	Sequence
44	SGSGVATRSQTDTLEKVQER	64	SGSGALEGAMNFSTADSAKI
45	SGSGTDTLEKVQERADKFEI	65	SGSGNFSTADSAKIKTLEAE
46	SGSGVQERADKFEIENNTLK	66	SGSGSAKIKTLEAEKAALEA
47	SGSGKFEIENNTLKLKNSDL	67	SGSGLEAEKAALEARQAELE
48	SGSGNTLKLKNSDLSFNNKA	68	SGSGALEARQAELEKALEGA
49	SGSGNSDLSFNNKALKDHND	69	SGSGAELEKALEGAMNFSTA
50	SGSGNNKALKDHNDELTEEL	70	SGSGLEGAMNFSTADSAKIK
51	SGSGDHNDELTEELSNAKEK	71	SGSGFSTADSAKIKTLEAEK
52	SGSGTEELSNAKEKLRKNDK	72	SGSGAKIKTLEAEKAALAAR
53	SGSGAKEKLRKNDKSLSEKA	73	SGSGEAEKAALAARKADLEK
54	SGSGKNDKSLSEKASKIQEL	74	SGSGLAARKADLEKALEGAM
55	SGSGSEKASKIQELEARKAD	75	SGSGDLEKALEGAMNFSTAD
56	SGSGIQELEARKADLEKALE	76	SGSGEGAMNFSTADSAKIKT
57	SGSGRKADLEKALEGAMNFS	77	SGSGSTADSAKIKTLEAEKA
58	SGSGKALEGAMNFSTADSAK	78	SGSGKIKTLEAEKAALEARQ
59	SGSGMNFSTADSAKIKTLEA	79	SGSGAEKAALEARQAELEKA
60	SGSGDSAKIKTLEAEKAALA	80	SGSGEARQAELEKALEGAMN
61	SGSGTLEAEKAALAARKADL	81	SGSGLEKALEGAMNFSTADS
62	SGSGAALAARKADLEKALEG	82	SGSGGAMNFSTADSAKIKTL
63	SGSGKADLEKALEGAMNFST	83	SGSGTADSAKIKTLEAEKAA

Peptide number	Sequence	Peptide number	Sequence
84	SGSGIKTLEAAKAALEAEKA	107	SGSGLAALEKLNKELEESKK
85	SGSGEKAALEAEKADLEHQS	108	SGSGLNKELEESKKLTEKEK
86	SGSGEAKADLEHQSQVLNAN	109	SGSGESKKLTEKEKAELQAK
87	SGSGEHQSQVLNANRQSLRR	110	SGSGEKEKAELQAKLEAEAK
88	SGSGLNANRQSLRRDLDASR	111	SGSGLQAKLEAEAKALKEKL
89	SGSGSLRRDLDASREAKKQL	112	SGSGAEAKALKEKLKAQAEE
90	SGSGDASREAKKQLEAEHQK	113	SGSGKEKLAKQAEELAKLRA
91	SGSGKKQLEAEHQKLEEQNK	114	SGSGQAEELAKLRAGKASDS
92	SGSGEHQKLEEQNKISEASR	115	SGSGKLRAGKASDSQTPDAK
93	SGSGEQNKISEASRQSLRRD	116	SGSGASDSQTPDAKPGNKAV
94	SGSGEASRQSLRRDLDASRE	117	SGSGPDAKPGNKAVPGKGQA
95	SGSGLRRDLDASREAKKQLE	118	SGSGNKAVPGKGQAPQAGTK
96	SGSGASREAKKQLEAAHQKL	119	SGSGKGQAPQAGTKPNQNKA
97	SGSGEEAEKITVQAAIDYIG	120	SGSGAGTKPNQNKAPMKETK
98	SGSGIDGTVFDSTEKAGKP	121	SGSGQNKAPMKETKRQLPST
99	SGSGKQLEAEHQKLEEQNKI	122	SGSGKETKRQLPSTGETANP
100	SGSGHQKLEEQNKISEASRQ	123	SGSGLPSTGETANPFFTAAA
101	SGSGQNKISEASRQSLRRDL	124	SGSGTANPFFTAAALTVMAT
102	SGSGASRQSLRRDLDASREA	125	SGSGTAAALTVMATAGVAAV
103	SGSGRRDLDASREAKKQVEK	126	SGSGVMATAGVAAVVKRKEE
104	SGSGSREAKKQVEKALEEAN	127	SGSGMATAGVAAVVKRKEEM
105	SGSGQVEKALEEANSKLAAL		
106	SGSGEEANSKLAALEKLNKE		

3.10 Titration of streptavidin concentration for coating wells

Strategies to reduce high background absorbance values:

A recognised problem with using avidin to coat plates is the higher background absorbance readings obtained. Streptavidin (from *Streptomyces avidinii*) has been suggested as an alternative (*In* "Pinpoints" Chiron Mimotopes 1992). One mole of streptavidin is reported to bind four moles of biotin. Alternatively, one unit of active streptavidin will bind 1.0 µg of biotin (*In* Sigma Biochemicals).
The concentration of streptavidin recommended for coating plates is 5 µg/ml or 0.5 µg/well (*In* Chiron Mimotopes 1992).

Basic enzyme immunoassay (EIA) method used for determining optimal streptavidin concentration:

Ninety six well microtitre plates (Nunc Maxisorp) were coated with 100 µl per well of streptavidin at varying concentrations as detailed below. The plates were dried overnight at 37°C then washed with PBS/0.1%Tween 20 (pH 7.2). The wells were blocked with 2% casein-10 mmol Tris-HCl/PBS (pH 7.0) for 30 minutes and washed. If peptides were to be used at this stage, they were added to each well at the recommended concentration of 0.28 µg peptide /well (*In* "Pinpoints" Chiron Mimotopes 1992). The plates were put on a shaker for one hour at room temperature. Test sera were either pre-absorbed with streptavidin as described below or used unabsorbed and diluted to 1 in 500 with 0.5% casein -Tris-HCl/PBS. The plates were incubated at 37°C for one hour. Secondary antibody conjugate (rabbit anti-human IgG horseradish peroxidase, Dako laboratories) at a dilution of 1 in 1000 in 0.5% casein -Tris-HCl/PBS, was added.

Substrate (o-phenylenediamine-2HCl) was added and the resulting colour reaction stopped with 1N sulphuric acid. All washes between steps were done with PBS/0.1%Tween 20 (pH 7.2). Absorbance values were read on a MR 7500 using a test wavelength of 490 nm (A_{490}) and a reference wavelength of 630 nm.

Sera

Sera from eight patients with clinically proven rheumatic fever or rheumatic heart disease and matched controls were obtained and used in the EIA assay as previously described.

Streptavidin test variables

Streptavidin (Sigma) stock solution was made up from affinity purified, lyophilized powder to a concentration of 1 mg/ml in distilled water. This gave approximately 14 units streptavidin activity per mg of protein

(1) Varying streptavidin concentration in the wells
(Without biotinylated peptides):
Streptavidin was added to the wells at the following concentrations
0.5 μg/well (recommended), 0.05 μg/well and 0.01 μg/well.

(2) Pre-absorption of sera with streptavidin
(Without biotinylated peptides):
Streptavidin at a concentration of 0.1 μg/ml was added to the sera with the diluent of 0.5% casein -Tris-HCl/PBS and incubated at 37°C for 1 hour. The final dilution of sera remained 1 in 500.

(3) A comparison using three different concentrations of streptavidin to coat wells (0.5 μg/well, 0.05 μg/well and 0.01 μg/well) together with biotinylated peptides. Serum from a subject with known rheumatic fever was used.

Peptides

Five biotinylated peptides, corresponding to part of the C-terminal end of M24 protein were used. Three of these were noted to be uniformly reactive with sera from Aboriginal adults with rheumatic fever (peptides 89,102 and 103) and two were uniformly non-reactive (peptides 56 and 80). They were prepared and used as described earlier. The working dilution of the peptides was 0.028 mg/ml.

3.11 Determination of optimal peptide and sera concentrations

The recommended biotinylated peptide concentration for use in an EIA is 0.28 μg/well (*In* "Pinpoints" Chiron Mimotopes 1992). To determine optimal peptide and sera concentrations to be used in the EIA, the following concentrations of peptide 89 and dilutions of sera were used in a chequerboard assay. Peptide 89 was used as it was uniformly reactive with sera from Aboriginal adults with rheumatic fever

Concentrations of peptide 89 tested:
0.56 μg/well, 0.28 μg/well (recommended) and 0.14 μg/well.
These were tested in duplicate.
Two sera were used. One was serum 47 from an Aboriginal subject with no evidence of rheumatic fever and the other was serum 25 from an

Aboriginal subject with previous rheumatic fever. Dilutions of sera used were as follows: 1 in 62.5, 1 in 125, 1 in 250, 1 in 500 and 1 in 1000. Each of these dilutions was tested against each concentration of peptide 89 used.

3.12 Enzyme immunoassay of test sera

Following the determination of EIA parameters as described in the previous sections, the following modification of the assay was used to map antigenic epitopes and to screen sera.

Ninety-six well Nunc Maxisorp microtitre plates were coated with 0.01 μg/well of streptavidin (Sigma) diluted in water. This concentration was determined by titration as described earlier. These were incubated at 37°C overnight and then washed with PBS/0.1%Tween 20 (pH7.2). The wells were blocked with 2% casein-10mmol Tris-HCl/PBS (pH7.0) for 30 minutes. Single peptides were added to each well to achieve a final concentration of 0.28 μg/well. The plates were put on a shaker for one hour at room temperature. Test sera were diluted to 1 in 500 with 0.5% casein-Tris-HCl/PBS, and incubated at 37°C for one hour. Secondary antibody conjugate (rabbit anti-human IgG horseradish peroxidase, Dako laboratories) at a dilution of 1 in 1000 in 0.5% casein-Tris-HCl/PBS, was added. Substrate (o-Phenylenediamine-2HCl) was added and the resulting colour reaction stopped with 1N sulphuric acid. All washes between steps were done with PBS/0.1%Tween 20 (pH 7.2). Absorbance values were read on a MR 7500 using a test wavelength of 490 nm (A_{490}) and a reference wavelength of 630 nm.

3.13 Peptide epitope mapping of M24 protein

All 82 peptides of the M24 bank were screened against ten selected sera using the EIA previously described. Three were from Aboriginal subjects with acute rheumatic fever (sera 31, 32 and 57), two each from Aboriginals and non-Aboriginals with previous rheumatic fever (sera 25, 46, 1 and 4 respectively) and three from Aboriginal controls (sera 17, 48 and 67). These were randomly chosen from the serum bank of the representative groups as previously described.

3.14 Screening of test sera with universally reactive peptides

As a result of the peptide epitope mapping of M24 as described above, five universally reactive peptides (89, 95, 102, 103 and 105) and two commonly non-reactive peptides (56 and 80) were identified and used as a panel for the wider screening of test sera.

The eighty two sera from the five subject groups as described in Chapter 2, were each tested against this panel of seven peptides. Each serum tested also had a serum only control. The absorbance value of this was subtracted from the readings obtained with each of the seven peptides. This was to correct for non specific serum binding to streptavidin.

3.15 The use of the five reactive peptides identified, in combination at varying concentrations

An attempt was made to see if reactivity with a combination of the five commonly reactive peptides resulted in an improved discrimination between subjects with rheumatic fever and those without.

Peptides 89, 95, 102, 103 and 105 were pooled at the following final concentrations of total peptide: 0.28 μg/well, 0.14 μg/well, 0.07 μg/well and 0.035 μg/well. Individual concentrations of each peptide in each pool were similar. The negative peptides 56 and 80 were pooled at concentrations of 0.14 μg/well and 0.07 μg/well.

The following sera were tested : 25, 34 and 57 (Aboriginal subjects with previous rheumatic fever and acute rheumatic fever); 70, 49 and 7 (Aboriginal and non-Aboriginal controls).

These were tested in the EIA system described with modifications as above.

3.16 A comparison of two 20-mer related peptides with peptides used in this study using an alternative EIA method.

A previous study using related 20 amino acid peptides in an EIA, showed no difference between both Aboriginal and Thai controls and subjects with rheumatic fever (Pruksakorn et al 1994). There was considerable amino acid sequence homology between these peptides and the five reactive 16-mer peptides of this study. One of these, the peptide designated p-145, differed most in that the sequence VEKALE is at the C-terminus when compared with both peptides 89 and 95 used in this study. Another peptide p-146 has significant homology with peptides 103 and 105 used in this study.

These peptide sequences are given below.

Peptide 145:- LRRDLDASREAKKQVEKALE

Peptide 146:- AKKQVEKALEEANSKLAALE

These peptides were obtained (Prof. M. Good, Queensland Institute of Medical Research, Brisbane) and used in the EIA system described below, against sera from the five groups of subjects as previously described in section 6.2. These peptides were not biotinylated.

Enyme immunoassay - method as per Prof. M. Good, Queensland Institute of Medical Research, Brisbane

1. Non-biotinylated peptides 145 and 146 were diluted to 5 μg/ml in carbonate-bicarbonate buffer at pH 9.6 and coated onto polyvinyl chloride microplates (Flow Laboratories Inc), in a total volume of 100 μl per well overnight at 4°C or 2 hours at 37°C.

2. Antigen was flicked off the plate and the wells were blocked with 200 μl of 5% skim milk in 0.05% PBS/Tween 20 overnight at 4°C or 1 hour at 37 °C.

3. Plates were washed 3 times with 0.05% PBS-Tween 20. Serum dilutions of 1 in 1000 were prepared in 0.5% skim milk in 0.05% PBS/Tween 20, in a final volume of 100 μl per well and incubated at 37C for 90 minutes.

4. Plates were washed 5 times with 0.05% PBS-Tween 20. Goat anti-human IgG Horseradish peroxidase (BioRad) was diluted 1 in 3000 in 0.05% PBS-Tween 20 and 100μl was added to each well and incubated at 37°C for 90 minutes.

5. Plates were washed 5 times with 0.05% PBS-Tween 20. 100 μl of OPD substrate (OPD FAST, Sigma) were added to each well and incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 450 nm.

6. Absorbance values obtained by this method were compared with those obtained using the streptavidin biotin method as described earlier for the same panel of aboriginal sera.

3.17 Statistical analysis

Statistical analysis was done using the unpaired t-test, to compare mean absorbance values. The non-parametric Mann-Whitney test was also used as this did not assume equal standard deviations between the populations from which the data were sampled. A biostatistics software program was used for data analysis (InStat, GraphPad Software, San Diego, USA).
A one-tailed p value was calculated based on the null hypothesis that the mean absorbance values would be similar for the control sera and for sera from subjects with rheumatic fever.

Chapter 4

Use of the M protein extracts in a Western blot serological survey of the study group

Pepsin extracts of M24 and M18 streptococcal proteins were prepared and purified as described. These were used as antigens in a Western Blot transfer against thirty one sera from subjects with rheumatic fever and controls. This was to determine whether antibodies to M protein from the two "rheumatogenic" serotypes could be detected in sera from subjects with rheumatic fever.

4.1 Polyacrylamide gel electrophoresis (PAGE)

The pepsin extracts of M-protein were run on SDS-PAGE prior to Western blot transfer. The profiles of pepM24 and pepM18 when run on SDS-PAGE, are shown in Figure 4.1. These profiles were reproducible with repeated extracts when the same strains were used.

The distinctive feature of pepM24 was a triplet of bands between 40 kDa and 42 kDa which was reproducible. This region was subsequently found to be more immunogenic by Western Blotting than another band at approximately 32 kDa.

PepM18 had a major band at 30 kDa which was immunogenic and a number of bands around 40 kDa which were also noted to be immunogenic.

Pepsin used in the experiment was also run on the gel and shown to be quite distinct from bands seen with the M protein extracts.

4.2 Western blots

Western blot reactions of test sera were subjectively quantified as follows: (The results are tabulated in Tables 4.1 - 4.4).

(1) Negative - No bands seen.

(2) +- Weak, indistinct bands seen at expected positions.

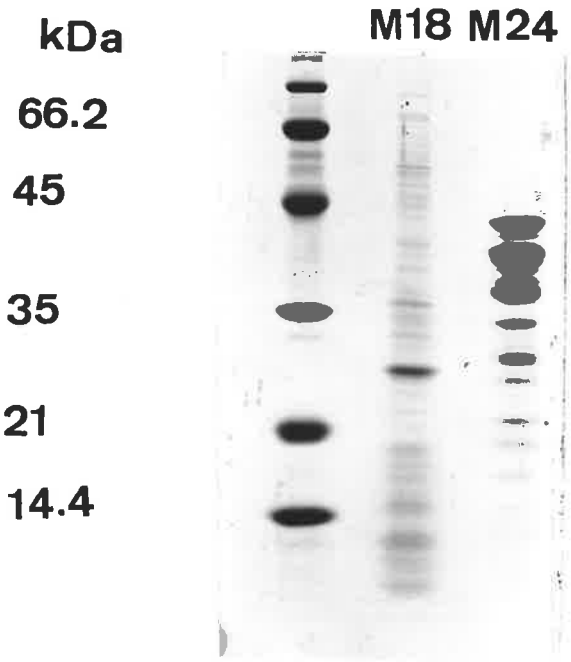
(3) + Weak but definite bands seen.

(4) ++ Clear bands seen.

(5) +++ Strong bands seen with no background staining.

Examples of immunologically significant Western blot bands are shown in Figure 4.2.

Figure 4.1. PAGE of pepM18 and pepM24

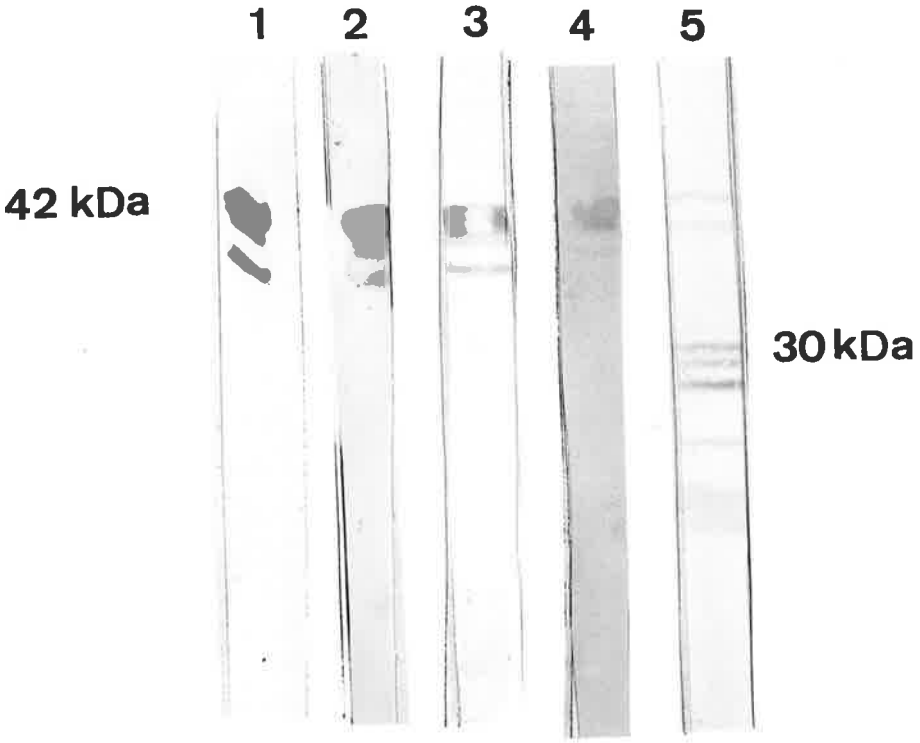


Pepsin extracts of M18 and M24 protein were run on 12% PAGE.

A triplet of bands between 40 kDa and 42 kDa is seen with pepM24. This triplet was consistently reproducible.

With pepM18, a major band is seen at 30 kDa

Figure 4.2 Representative reactive Western Blots.



Examples of immunologically significant bands with a subjective quantification as below

Lane 1: pepM24 +++ reactivity

Lane 2: pepM24 ++ reactivity

Lane 3: pepM24 + reactivity

Lane 4: pepM24 +- reactivity

Lane 5: pepM18 + reactivity

Table 4.1

Results of Western blots on sera from Aboriginal patients (n=10) with clinically proven rheumatic fever or rheumatic heart disease.

Serum No.	sex	age (years)	ASO	ADB	M18 30-35 kDa	M18 40-42 kDa	M24 30-32 kDa	M24 40-42 kDa	Comments
6.	F	33	N\A	N\A	-	+	-	++	Mitral valve replacement
22.	M	53	96	2048	-	+	-	-	Cardiac failure
24.	F	24	256	1024	-	-	-	+-	Mitral valve replacement
25.	M	16	512	128	-	+	-	+	Mitral valve replacement
31.	M	25	256	512	-	-	-	++	Acute rheumatic fever
33.	F	13	128	>3072	-	-	-	++	Acute rheumatic fever
34.	F	24	192	256	+	+	-	+	Rheumatic heart disease
35.	F	30	256	512	+++	-	-	++	Rheumatic fever
42.	M	56	256	384	++	-	-	-	Mitral valve replacement
45.	M	46	256	3584	-	-	-	-	Chorea, no heart disease

Table 4.2

Results of Western blots done on serum from non-Aboriginal patients (n=8) with clinically proven rheumatic heart disease or rheumatic fever.

Serum No.	sex	age (years)	ASO	ADB	M18 30-35 kDa	M18 40-42 kDa	M24 30 -32 kDa	M24 40-42 kDa	Comments
1.	F	61	96	<128	-	-	-	+++	Mitral valve replacement. Rheumatic fever at age 21. Commisural fusion of valve leaflets.
4.	M	73	128	<128	-	-	-	+++	Mitral valve replacement. Rheumatic fever at age 16.
5.	F	60	128	<128	++	-	-	-	Rheumatic fever at age 26, Mitral valve replacement. Commisural fusion of valve leaflets.
9.	F	62	1536	<128	-	-	-	-	Mitral valve replacement. Rheumatic fever at age 30.
10.	M	70	128	<128	-	-	-	+	Aortic valve replacement for rheumatic valvular disease.
19.	F	25	384	1024	+	-	-	++	Rheumatic fever as a child.
20.	F	15	64	1024	+	-	-	+++	Rheumatic fever as a child.
29.	M	26	256	3072	-	+	-	+	Previous Rheumatic fever.

Table 4.3

Results of Western blots done on serum from non-Aboriginal patients (n=8) with no clinical evidence of rheumatic fever or rheumatic heart disease.

Serum No.	sex	age (years)	ASO	ADB	M18 30-35 kDa	M18 40-42 kDa	M24 30 -32 kDa	M24 40-42 kDa	Comments
7.	M	68	<64	<128	-	-	-	-	Coronary artery bypass grafting.
11.	F	52	192	<128	-	-	-	-	Mitral valve replacement. Non-rheumatic
28.	F	51	512	512	-	-	-	++	Group A streptococccal septicemia. Isolate not M-typed.
36	M	26	192	2048	-	-	-	-	Severe group A pharyngitis with retropharyngeal abscess formation.
37.	M	70	64	128	-	-	-	-	Coronary artery bypass grafting.
38.	F	71	<64	<128	-	-	-	-	Coronary artery bypass grafting.
39.	F	80	256	<128	-	-	-	-	Coronary artery bypass grafting.
75	M	39	256	<128	-	-	-	-	Normal control

Table 4.4

Results of Western blots done on serum from Aboriginal patients (n=5) with no clinical evidence of rheumatic fever or rheumatic heart disease.

Serum No.	sex	age (years)	ASO	ADB	M18 30-35 kDa	M18 40-42 kDa	M24 30 -32 kDa	M24 40-42 kDa	Comments
17.	F	61	<64	<128	-	-	-	-	Coronary artery bypass grafting.
47.	F	26	128	512	-	-	-	-	Head injury.
48.	M	14.	>3072	256	-	-	-	-	Cerebral abscess.
49.	M	18.	64	256	-	-	-	-	Multiple trauma.
50.	M	46.	256	<128	-	-	-	-	Group G streptococcal septicemia.

The Western blots showed reactivity with either pepM18 or pepM24, in all but two of the eighteen sera from Aboriginal and non-Aboriginal subjects with rheumatic fever. There was considerable overlap in reactivity between the extracts of M24 and M18 proteins, suggesting common antigenic epitopes.

Three of the ten sera from Aboriginal subjects with previous rheumatic fever showed reactivity with pepM24 while two showed reactivity with pepM18. Four sera showed reactivity with both peptide extracts. Serum from one Aboriginal subject with chorea (serum 45), was unreactive to both M protein extracts. This may represent a misdiagnosis clinically, as rheumatic chorea is rare in this age group.

Of the eight sera from non-Aboriginal subjects with rheumatic heart disease, three showed reactivity with pepM24, one showed reactivity with pepM18 and three sera were reactive with both peptides. Serum from one non-Aboriginal subject (serum 9), who had rheumatic fever 30 years prior, was unreactive with both peptides. There were a total of thirteen control sera tested. All five sera from aboriginal controls were unreactive with either of the peptide extracts. Only serum 28, from a non-Aboriginal subject with group A streptococcal septicemia, was reactive with pepM24 alone.

The pattern of bands seen on Western blot using pepM24 as the antigen, correlated with two bands seen at 40 and 42 kDa on the SDS-PAGE. This pattern was seen in all positive samples. No other bands were seen on Western blot using this antigen. When pepM18 was used as the antigen, a number of reactive bands were seen on Western blot. These were at 30-35 kDa and at 40-42 kDa. The main band on SDS-PAGE however was at 32

kDa. This discrepancy between bands seen on PAGE and those reactive in the Western blot for pepM18 with patient sera, could be due to the relatively uncontrolled pepsin extraction process. The reactive component of M18 protein may have undergone incomplete peptic digestion resulting in poor differentiation on SDS-PAGE. Generally, the pepsin extract of M protein as used in the Western blot here, could distinguish between subjects with rheumatic fever and those without. Due to the relatively crude extraction process, it is uncertain as to whether the point of cleavage of the M-protein molecules by pepsin was consistent. It is possible that multiple cleavage points involving both the N and C-amino acid terminals of M protein, were involved. It is also possible that reaggregation of fragments may have occurred after digestion. Whether the reactive epitopes of M18 and M24 proteins are situated at the N-terminal or more proximally at the C-terminus of the protein, can only be determined by peptide epitope mapping.

4.3 Western Blot serological survey of the study group - conclusions

The results in this chapter demonstrate that there may be common reactive epitopes between M18 and M24. It also suggests that given the number of sera used, there may be commonly reactive epitopes in all rheumatogenic M-types. It is unlikely that all positive sera are the result of infection with either M-types 18 or 24 only. Since pepM24 appeared to react in a stronger fashion with more sera, from both Aboriginal and non-Aboriginal subjects with rheumatic fever, M24 protein was therefore chosen in preference to M18 for the peptide mapping studies.

Chapter 5

Determination of optimal parameters for the assay

Prior to use of the EIA against the complete panel of sera, a number of optimal assay parameters were determined. These included concentrations of peptides and sera used. The titration of optimal streptavidin concentration for coating microtitre plate wells was also necessary because of high background values obtained when the manufacturer's recommended streptavidin concentration was used.

5.1 Determination of optimal peptide and sera concentrations

The asssay was carried out as described in Chapter 3, with the variations described below incorporated.

No significant difference in optical densities between the three peptide concentrations (0.56 μg/well, 0.28 μg/well, and 0.14 μg/well) of universally reactive peptide 89 (see Chapter 6), was shown at all sera dilutions tested ($p=0.9405$, Mann-Whitney). This is shown in Figures 5.1 and 5.2. In view of this, peptides were used at the recommended concentration of 0.28 μg/well in subsequent enzyme immunoassays.

A progressive fall in absorbance values was noted with the increasing sera dilutions tested (1 in 62.5, 1 in 125, 1 in 250, 1 in 500 and 1 in 1000). This appeared to "level out" around the 1 in 250 and 1 in 500 dilutions with both positive and negative sera. There remained a ten fold absorbance value difference between positive and negative sera which was highly significant ($p<0.0005$, Mann-Whitney). This is shown in Figures 5.1 and 5.2. All sera were subsequently used at a dilution of 1 in 500.

Figure 5.1: The titration of peptide and sera concentrations using reactive peptide 89 (see Chapter 6) and serum 25 (Positive). Concentrations of peptide 89 used were 0.56 μg/well, 0.28 μg/well, and 0.14 μg/well. Serum concentrations used were 1 in 62.5, 1 in 125, 1 in 250, 1 in 500 and 1 in 1000.

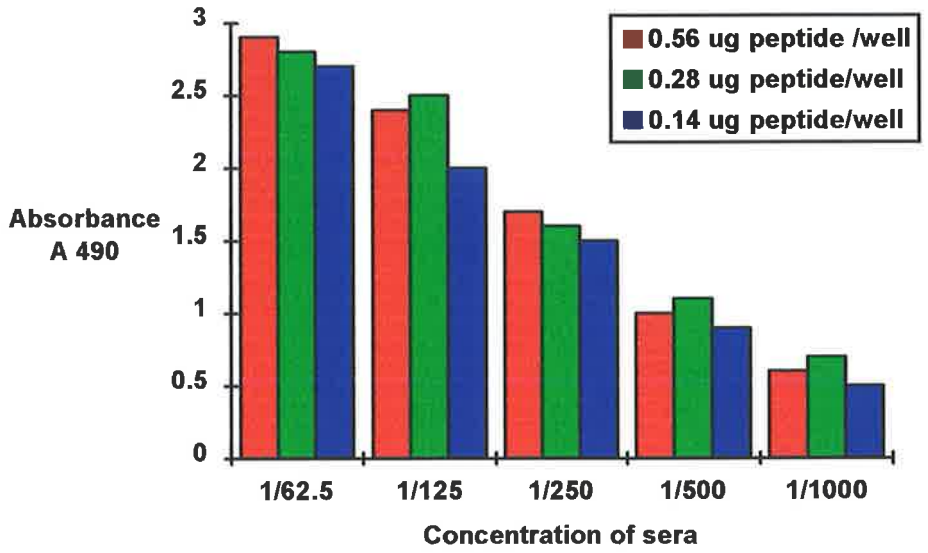
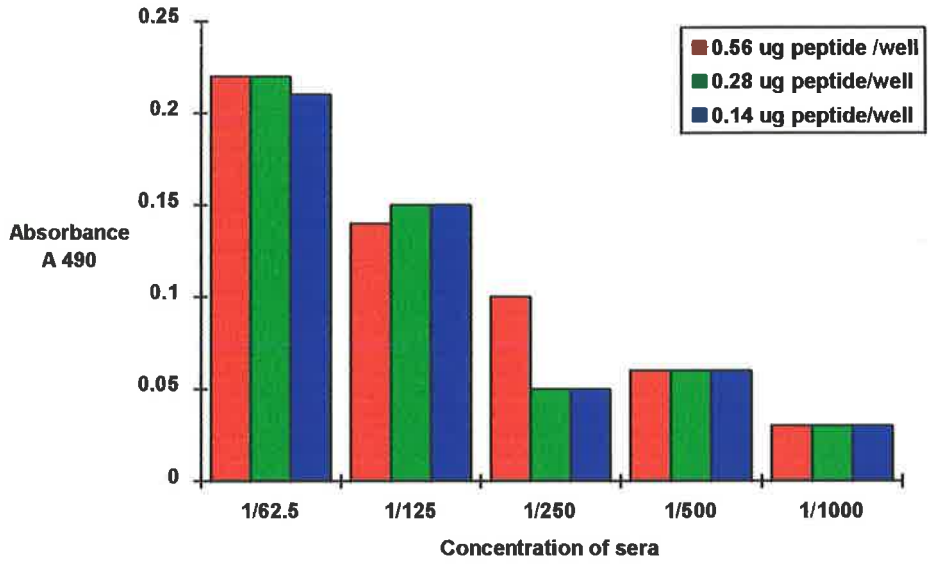


Figure 5.2: The titration of peptide and sera concentrations using peptide 89 (see Chapter 6) and serum 47 (Negative). Concentrations of peptide 89 used were 0.56 μg/well, 0.28 μg/well, and 0.14 μg/well. Serum concentrations used were 1 in 62.5, 1 in 125, 1 in 250, 1 in 500 and 1 in 1000.



5.2 The use of the five reactive peptides identified, in combination at varying concentrations

The five reactive peptides 89, 95, 102, 103 and 105 (see Chapter 6), were combined in varying concentrations to determine if this would enhance their ability to screen for reactive epitopes in sera from subjects with rheumatic fever. The mean absorbance values obtained, using these peptides in combination however, were significantly lower than if the reactive peptide 89 were used alone. These values are shown in Table 5.1.

Table 5.1: Mean absorbance values obtained using the five reactive peptides (89,95,102,103,105) in combination

		Concentration of combined five peptides (μg/well)				
Serum No.	**Clinical diagnosis**	**0.28 μg**	**0.14 μg**	**0.07 μg**	**0.035 μg**	**Pep 89 (0.28 μg)**
25	16 yr M, Prev RF	0.15	0.265	0.361	0.4	1.537
34	24 yr F, Prev RF	0	0	0.005	0.025	0.429
57	25 yr M, Acute RF	0.04	0.20	0.32	0.380	2.527
70	Control	0	0	0	0	0.041
49	Control	0	0	0	0	0.016
7	Control	0	0	0	0	0.01

5.3 Titration of streptavidin, for coating wells

The recommended concentration of streptavidin for use with biotinylated peptides is 0.5 μg/well (*In* "Pinpoints" Chiron Mimotopes 1992). To reduce high background absorbance values, due to non-specific serum binding to streptavidin, two methods were investigated. The first involved titrating the concentration of streptavidin used to coat wells and the second involved pre-absorbing test sera with streptavidin. It was also necessary to determine whether reducing the concentration of streptavidin in the wells, led to a corresponding reduction in reactivity of peptides used.

The effect of varying the streptavidin concentration used in coating wells is shown in Figure 5.3. This was done in the absence of peptide, as only serum/streptavidin binding was being investigated. A significant difference ($p<0.0001$, Mann-Whitney) in mean absorbance values between the concentrations 0.01 μg/well, 0.05 μg/well and 0.5 μg/well used, is demonstrated. These were 0.077, 0.9 and 1.225 respectively.

The effect of pre-absorbing test sera with varying streptavidin concentrations in an attempt to reduce non-specific binding is shown in Figure 5.4. As above, this was done in the absence of peptide. The mean absorbance values for each of the streptavidin concentrations used (0 μ g/ml, 0.05 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml) were 0.02, 0.02, 0.01, 0.01 and 0.02 respectively. No significant difference was demonstrated between the groups tested ($p=0.8934$, Mann-Whitney).

In Figure 5.5, a comparison is made of the use of streptavidin at 0.5 μ g/well, 0.05 μg/well and 0.01 μg/well, with previously described reactive and non-reactive biotinylated peptides, to determine if a reduction in

positive peptide reactivity is seen with reduction of streptavidin concentration. As the concentration of streptavidin in the wells is decreased, there is a corresponding fall in absorbance values. This is most evident at the streptavidin concentration of 0.01 μg/well. This is particularly so with the non-reactive peptides 56 and 80 where the absorbance values were 1.75, 1.1 and 0.05 for the streptavidin well concentrations of 0.5 μg/ml, 0.05 μg/ml and 0.01 μg/ml respectively. The reactive peptides 89, 102 and 103 however, remained positive with absorbance values of 2.5, 1.75 and 1.5 for the same streptavidin concentrations, suggesting that the reactivity of positive peptides was not significantly affected by the reduction in streptavidin concentration used to coat wells.

Figure 5.3: The effect of varying streptavidin concentration in the absence of peptide, for coating wells in an EIA using 8 different sera from subjects with previous rheumatic fever. This was done to reduce background non-specific serum binding to streptavidin. Streptavidin was used at concentrations of 0.01µg/well, 0.05µg/well and 0.5µg/well (manufacturer's recommendation).

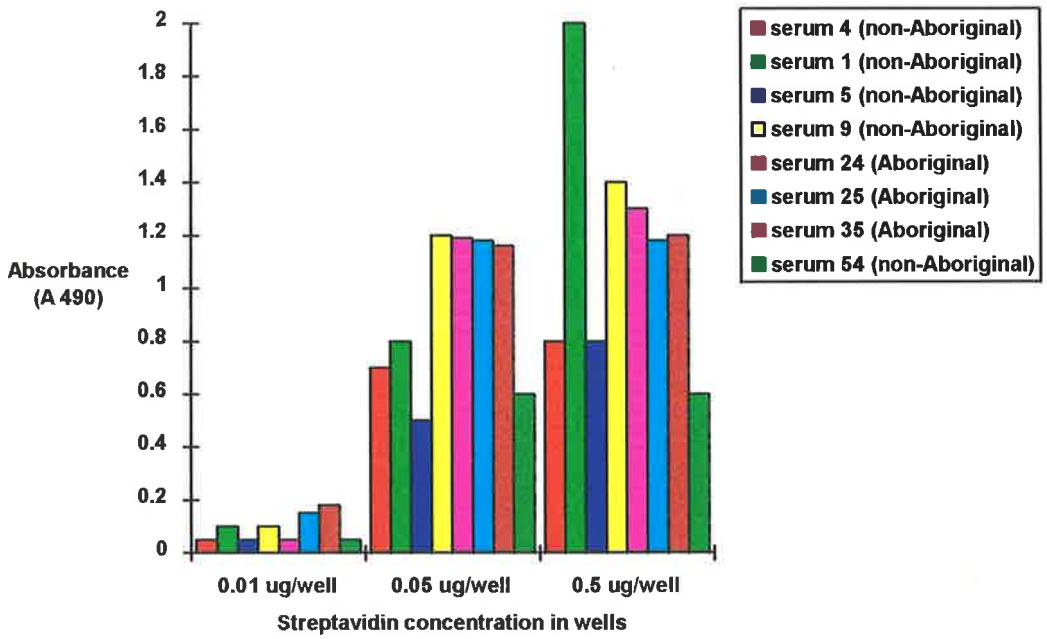


Figure 5.4: The effect of pre-absorbing test sera (n=4) from subjects with previous rheumatic fever, with varying concentrations of streptavidin (0µg/ml, 0.05µg/ml, 0.1µg/ml, 0.2µg/ml and 0.5µg/ml), to determine if this can reduce non-specific serum binding.

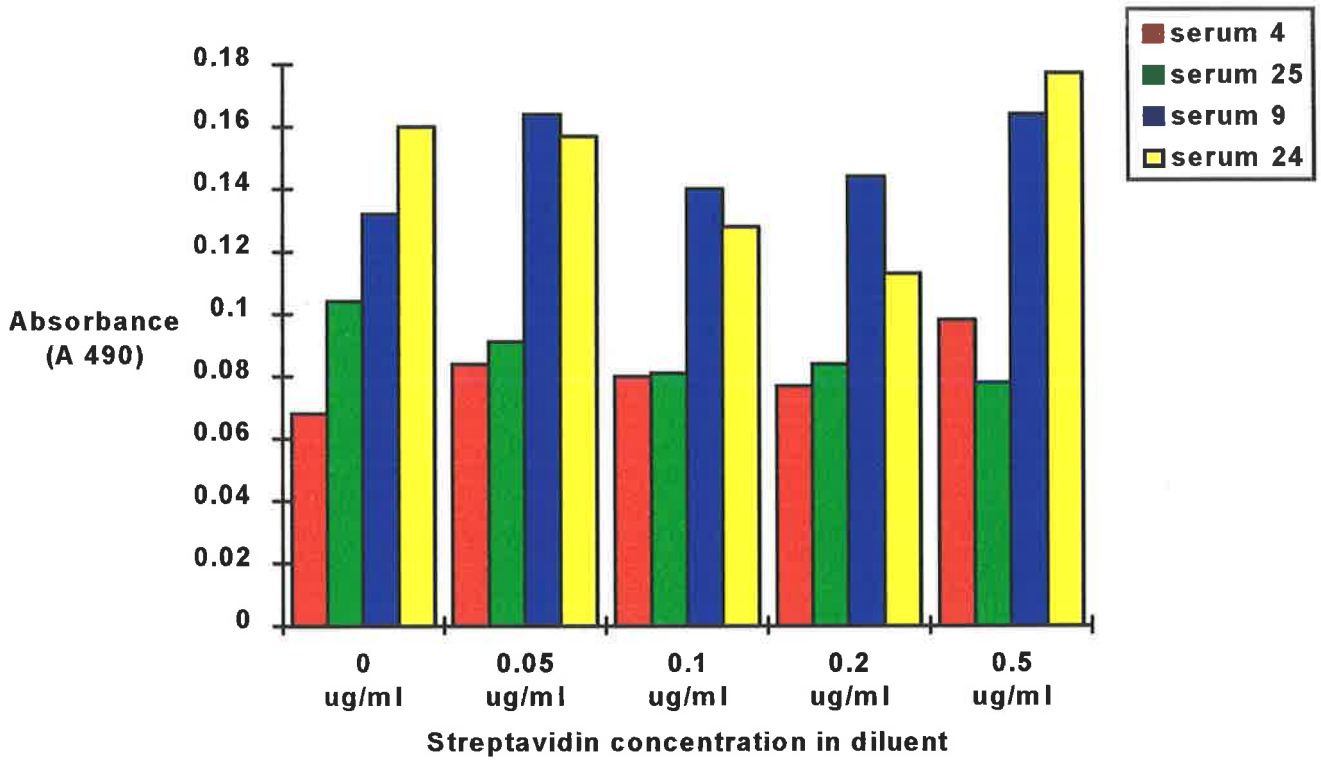
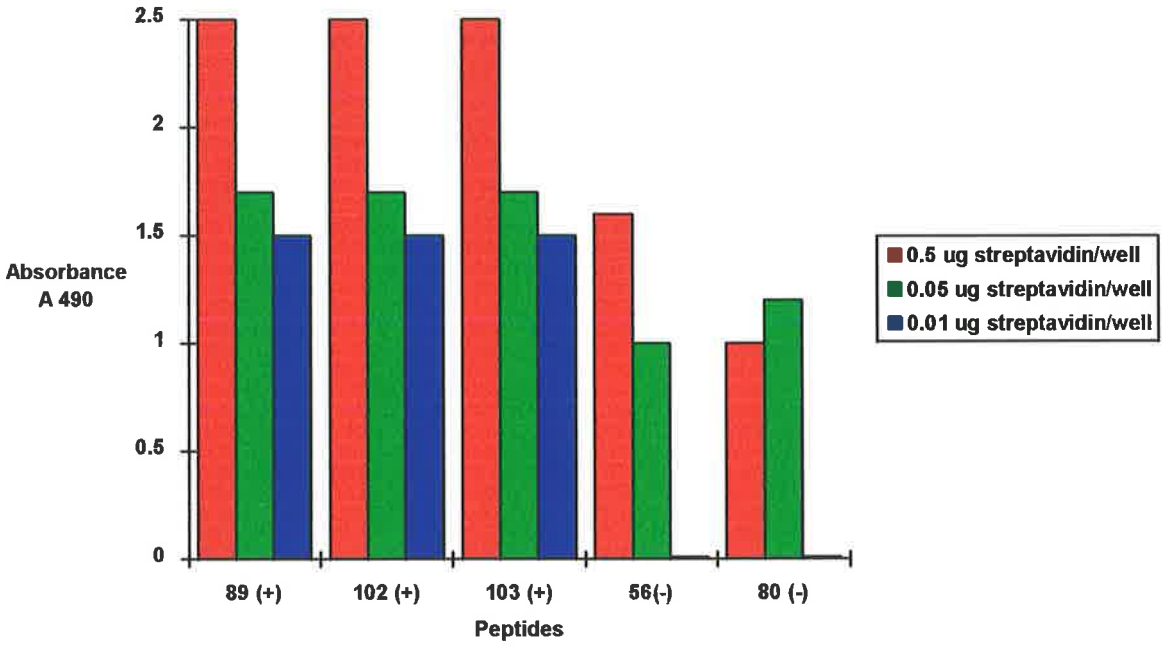


Figure 5.5: A comparison of the use of streptavidin at three different concentrations (0.5 µg/well, 0.05 µg/well and 0.01 µg/well) to coat wells, using reactive and non-reactive biotinylated peptides, to determine if a reduction in positive peptide reactivity is seen with the reduction of streptavidin. Used with serum 57 (A 25 year old Aboriginal with acute rheumatic fever).



5.4 Determination of optimal parameters for the assay - conclusions

In the light of these results, the assay parameters to be used are as follows:
Streptavidin concentration: 0.01 μg/ml
Serum concentration: 1 in 500
Peptide concentration: 0.28 μg/well

Non-specific binding of streptavidin to serum is a significant problem when using biotin labelled peptides. Not using this system has the disadvantage of not knowing the retained peptide concentration in the wells. This however may not be critical as shown in Figure 5.1, where no significant difference in optical densities is obtained with different peptide concentrations. This experiment used only limited numbers of peptides. Whether this phenomenon would be observed for all peptides is not known. Subsequent epitope mapping of the entire peptide bank therefore, utilised the assay conditions above. It is also clear from Figure 5.5 that considerable streptavidin non-specific binding to serum is observed using the manufacturer's recommended concentration. Reduction of this 50 fold to 0.01 μg/ml maximises the difference between the optical density obtained from reactive and non-reactive peptides.

Chapter 6

Peptide epitope mapping

The first part of this study showed that pepsin extracts of streptococcal M24 and M18 proteins when used in a Western blot, could distinguish between sera from subjects with rheumatic fever and controls. To determine whether this reactivity was confined to the Amino (N) or Carboxy (C) amino acid terminals, peptide epitope mapping was carried out using an overlapping peptide bank as potential antigens to define potential epitopes. As pepM24 appeared to react with most sera and more strongly than pepM18 (Chapter 4), it was decided to use the amino acid sequence of M24 protein to construct the peptide bank.

Preliminary screening of all peptides against a limited number of sera was first done to determine if there were universally reactive peptides within the peptide bank.

6.1 Preliminary screening of the peptide bank

Eighty two, 16-mer, overlapping, biotinylated peptides, based on the entire amino acid sequence of M24 protein (excluding the signal sequence), were used in an EIA against ten sera.

These sera were from five Aboriginal subjects with rheumatic fever, two non-Aboriginals with previous rheumatic fever and three Aboriginal controls.

Preliminary screening of the eighty two peptides with these sera, to identify significant B-cell epitopes showed that reactivity was confined to peptides from the C-terminal end. This was particularly true of the five Aboriginal subjects with rheumatic fever where the mean A_{490} values for the two most reactive peptides 89 and 95, was 1.36 and 1.38, respectively.

Three of these subjects had acute rheumatic fever (sera 31, 32 and 57) and two had previous histories of rheumatic fever (sera 25 and 46). The mean age of this group was 26 years. Other peptides found to be reactive, but to a lesser extent in this group, were peptides 102, 103 and 105. This is shown in Figure 6.1.

The mean A_{490} values for the three Aboriginal controls (sera 17, 48 and 67), were 0.08 and 0.04 for peptides 89 and 95 respectively. The standard deviations (SD) were 0.038 and 0.012, respectively. This is shown in Figure 6.2. The mean age of this group was 31 years.

The A_{490} values for the two non-Aboriginals with previous rheumatic fever (sera 4 and 1), was 0.07 and 0.06 for peptides 89 and 95 respectively. This is shown in Figure 6.3. Their mean age was 67 years and is a reflection of the present rarity of rheumatic fever among non-Aboriginals.
The amino acid sequences of these five commonly reactive peptides are as follows.

89 : SLRRDLDASREAKKQL
95 : LRRDLDASREAKKQLE
102 : ASRQSLRRDLDASREA
103 : RRDLDASREAKKQVEK
105 : QVEKALEEANSKLAAL

Although the mean A_{490} values for peptides 121 and 122 at the extreme C-terminal end, were modestly elevated at 0.21 and 0.36, respectively, when tested against sera from Aboriginal subjects with rheumatic fever, this was due to one subject only (serum 32). This was a 24 year old female with acute rheumatic fever. These peptides were therefore not used to screen the larger serum bank as they did not appear to be universally reactive.

Figure 6.1: Peptide epitope mapping using an overlapping, biotinylated, 82 peptide bank, based on Streptococcal M24 protein. Mean optical densities obtained with sera from Aboriginal subjects with rheumatic fever (n=5)

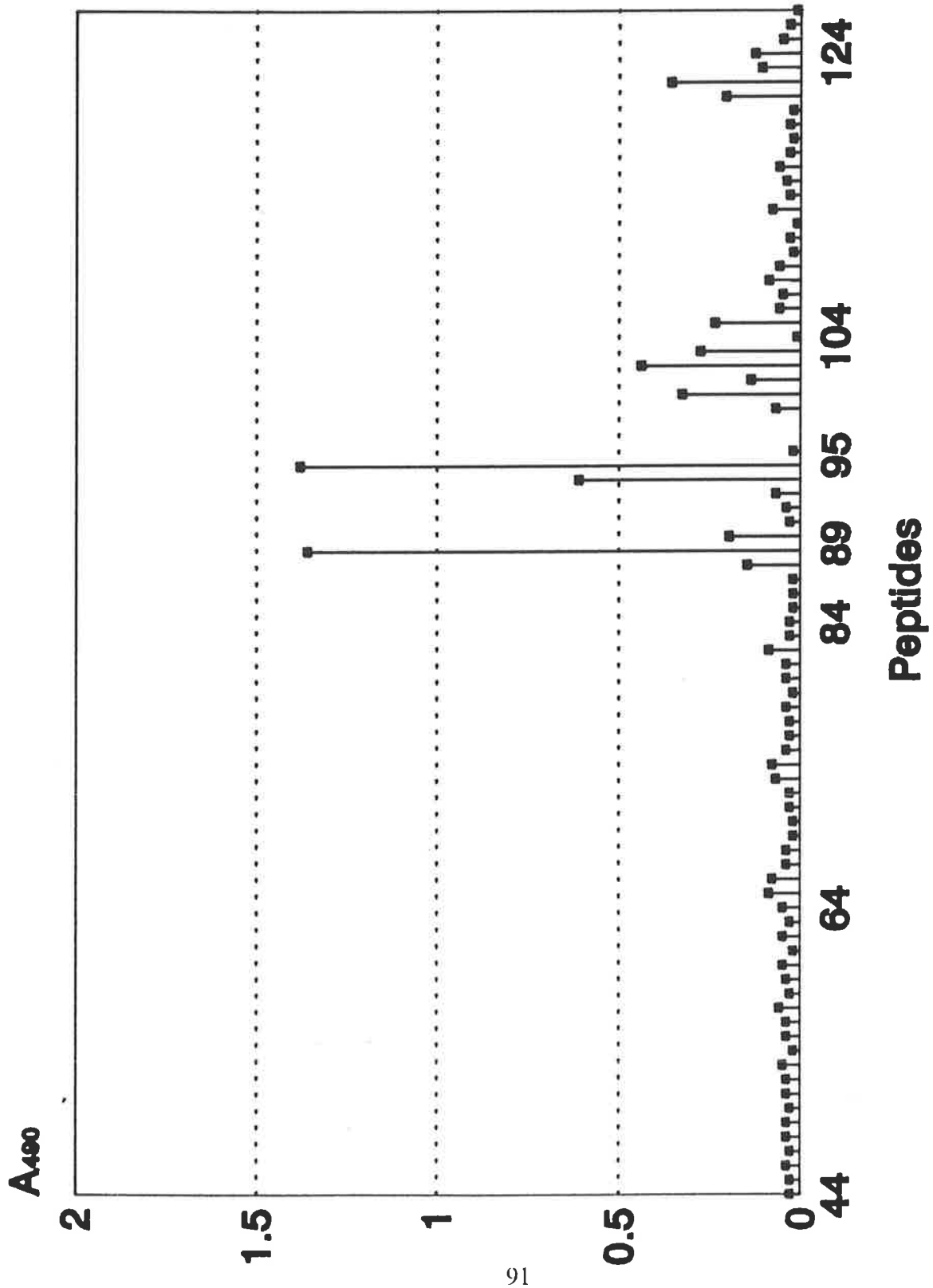


Figure 6.2: Peptide epitope mapping using an overlapping, biotinylated, 82 peptide bank, based on Streptococcal M24 protein. Mean optical densities obtained with sera from Aboriginal controls (n=3)

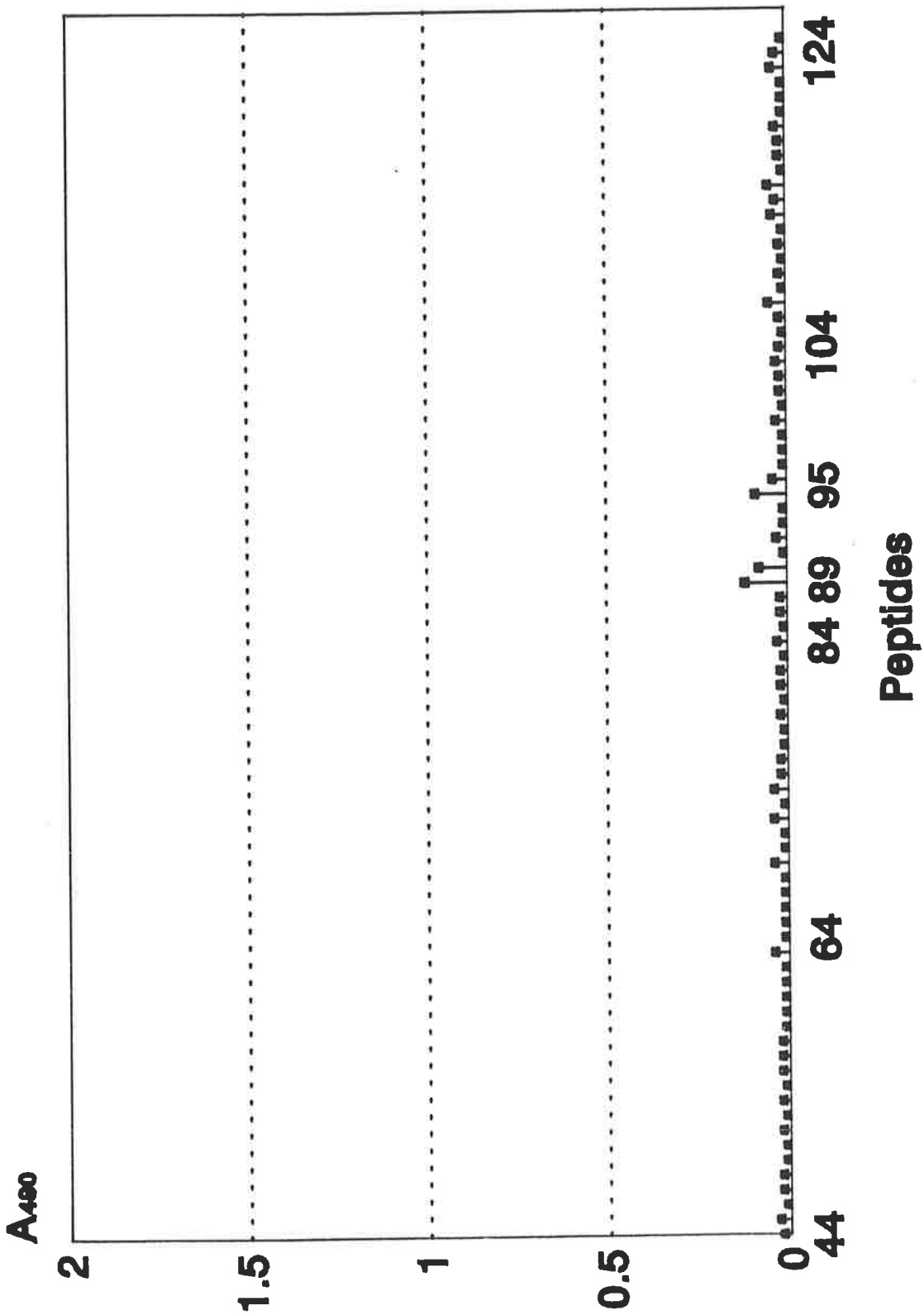


Figure 6.3: Peptide epitope mapping using an overlapping, biotinylated, 82 peptide bank, based on Streptococcal M24 protein. Mean optical densities obtained with sera from non-Aboriginal subjects with previous rheumatic fever (n=2)

6.2 Screening of test sera with universally reactive peptides

Screening of the eighty two test sera using the commonly reactive peptides 89, 95, 102, 103 and 105 showed that peptides 89 and 95 could distinguish between Aboriginals with acute rheumatic fever and Aboriginal controls. The mean A_{490} values for sera from the four Aboriginal subjects with acute rheumatic fever were 1.393 and 1.073 for peptides 89 and 95 respectively. Corresponding mean A_{490} values for the fourteen Aboriginal subjects with previous rheumatic fever were 0.56 and 0.385 respectively. Sera from the thirty six Aboriginal controls had mean A_{490} values of 0.112 and 0.08, for peptides 89 and 95 respectively. Included in this group were two Aboriginal subjects with post-streptococcal glomerulonephritis.

Sera from the seven non-Aboriginal subjects with previous rheumatic fever, had mean A_{490} values of 0.141 and 0.077, for peptides 89 and 95 respectively. As previously mentioned, some of these subjects had their initial episode of rheumatic fever up to fifty years prior. A fall in antibody levels over time, may account for the non-reactivity. Control sera from the twenty one non-Aboriginal subjects showed mean A_{490} values of 0.056 and 0.03 for peptides 89 and 95 respectively.

The actual A_{490} values of the five subject groups against the seven peptides are presented in Tables 6.1 - 6.5. Mean values are shown in Figure 6.4 with standard error bars.

Table 6.1: Mean A_{490} values obtained using the universally reactive peptides 89, 95, 102, 103 and 105 and the unreactive peptides 56 and 80, in an EIA with sera from Aboriginal subjects (n=4), with acute rheumatic fever (ARF).

		Peptide Number								
Serum No.	**Clinical Diagnosis**	**89**	**95**	**102**	**103**	**105**	**56(-)**	**80(-)**	**ASOT**	**ADB**
31	25 yr M ARF	0.908	0.860	0.015	0.842	0	0	0.044	256	512
32	24 yr F ARF	0.811	0.836	0.067	0.454	0.013	0	0	192	1024
33	13 yr F ARF	1.325	0.410	0.218	0.878	0.678	0	0	128	>3072
57	25 yr M ARF	2.527	2.184	0.200	0.012	0.006	0.008	0	1536	>3584
	Mean Abs	1.393	1.073	0.126	0.547	0.174	0.002	0.011		

Table 6.2: Mean A_{490} absorbance values obtained using the universally reactive peptides 89, 95, 102, 103 and 105 and the unreactive peptides 56 and 80, in an EIA with sera from Aboriginal subjects with previous rheumatic fever (n=14).

		Peptide Number								
Serum No.	**Clinical Diagnosis**	**89**	**95**	**102**	**103**	**105**	**56(-)**	**80(-)**	**ASOT**	**ADB**
6	33 yr F, Mitral valve replacement	0.245	0.163	0.242	0.139	0.205	0.021	0.006	N/A	N/A
24	24 yr F, Mitral valve replacement	0.185	0.191	0.237	0.112	0	0	0.024	256	1024
25	16 yr M, Mitral valve replacement	1.537	0.615	1.488	1.481	0.334	0	0	512	128
34	24 yr F	0.429	0.054	0.005	0.322	0.239	0.010	0.005	192	256
35	30 yr F	0.168	0.035	0.035	0.031	0.006	0.009	0.004	256	512
42	56 yr M,Mitral valve replacement	0.351	0.251	0.492	0.349	0	0	0	256	384
45	46 yr M, Chorea	0.232	0.175	0.167	0.146	0.164	0.004	0.010	256	3584
46	39 yr M	0.709	0.568	0.075	0.004	0	0	0	192	256
52	23 yr F, Rheumatic fever at 14 years	0.102	0.031	0.148	0.030	0.033	0.04	0.03	64	128
64	24 yr M, Mitral valve replacement	0.546	0.195	0.058	0.379	0.472	0.313	0	64	512
65	22 yr F, Mitral valve replacement	0.395	0.404	0.307	0.297	0.412	0.515	0.616	512	768
71	17 yr M, Mitral valve replacement	0.091	0.072	0.040	0.010	0.004	0.005	0	256	768
76	49 yr F	2.10	1.90	0.397	1.614	0	0	0	256	256
85	57 yr F, Mitral valve replacement	0.764	0.734	1.00	0.605	0.571	0	0	128	192
	Mean Absorbance	0.561	0.385	0.318	0.382	0.175	0.057	0.048		

Table 6.3: Mean A_{490} absorbance values obtained using the universally reactive peptides 89, 95, 102, 103 and 105 and the unreactive peptides 56 and 80, in an EIA with sera from Aboriginal controls (n=36)

		Peptide Number								
Serum No.	**Clinical Diagnosis**	**89**	**95**	**102**	**103**	**105**	**56(-)**	**80(-)**	**ASOT**	**ADB**
17	61 yr Coronary artery grafting	0	0	0.002	0.043	0.011	0.004	0	<64	<128
21	12 yr M, Post streptococcal glomerulonephritis	0.300	0.260	0.430	0.210	0	0	0	256	1024
23	10 yr M, Post streptococcal glomerulonephritis	0.290	0.200	0.390	0.200	0	0	0	128	2048
47	26 yr F, Head injury	0.012	0.037	0.055	0.053	0.026	0	0.005	128	512
48	14 yr M, cerebral abscess	0.033	0	0.015	0.030	0.023	0.002	0.005	>3072	256
49	18 yr M, Trauma	0.016	0.045	0.051	0	0.031	0	0	64	256
50	46 yr M, Gp G strep sepsis	0.027	0.032	0.072	0.053	0.048	0.007	0	256	<128
51	38 yr M, Liver disease	0.261	0.233	0.320	0.161	0	0.004	0	256	>3072
58	61 yr M, Gp A strep sepsis	0.219	0.223	0.638	0.136	0.012	0.008	0.003	128	>3584
67	18 yr F, Head injury	0.07	0.078	0.141	0.05	0.034	0	0	192	>3072
68	34 yr F, Staphylococcal sepsis	0.277	0.108	0.479	0.388	0	0.009	0.001	384	1536
69	27 yr M, Trauma	0.007	0.003	0.015	0.030	0	0	0	<64	256
70	36 yr M, Mitral valve replacement (non-rheumatic)	0.041	0.044	0.086	0.016	0	0.006	0.003	256	1536
72	28 yr M, Fracture of jaw	0.039	0.043	0.028	0.040	0	0	0	256	1024
73	58 yr M, Ca larynx	0.076	0.047	0.073	0.023	0.084	0.051	0	192	384
74	44 yr M, Quadraplegic	0.042	0	0.024	0	0.065	0	0	384	1024
77	39 yr M, cerebral abscess	0.016	0.001	0.009	0	0	0	0.033	192	>3072
78	32 yr M, Fracture of jaw	0.004	0.012	0	0.002	0.074	0	0.009	96	256
79	61 yr M, Liver disease	0.453	0.49	0.061	0.048	0.063	0.033	0.005	96	192
80	60 yr M, Cerebral abscess	0.078	0.087	0.179	0.071	0	0	0	96	<128
81	38 yr M, Coronary Artery grafting	0.072	0.022	0	0	0	0	0	128	384
82	35 yr M, Head injury	0.200	0.110	0.043	0.160	0.085	0	0	96	192
83	16 yr M, Lymphoma	0.022	0.138	0.132	0.135	0.122	0.111	0.170	64	192
84	19 yr F, Fracture mandible	0.110	0.085	0.082	0.085	0	0	0	128	512
86	27 yr F, Pulm.TB	0	0.05	0.115	0.016	0.022	0.020	0.005	192	768
87	24 yr F, Sub arachnoid haemorrhage	0.080	0.140	0.068	0.070	0.014	0	0	192	384
88	54 yr M, Staphylococcal sepsis	0.335	0.280	0.276	0.264	0.141	0	0	192	>3072
89	35 yr M, Campylobacter sepsis	0.200	0.250	0.160	0.160	0.030	0	0	512	>3072

Table 6.3: Aboriginal control sera (Continued)

		Peptide Number								
Serum No.	**Clinical Diagnosis**	**89**	**95**	**102**	**103**	**105**	**56(-)**	**80(-)**	**ASOT**	**ADB**
90	19 yr M, Empyema	0.140	0.114	0.145	0.07	0.015	0.011	0	128	2048
91	37 yr M, Neutropenic	0.100	0.106	0.060	0.060	0	0	0	256	<128
92	28 yr F, Syphilis	0.046	0.012	0.080	0.035	0.033	0	0	<64	1024
94	68 yr M, Nocardiosis	0.100	0.09	0.06	0.08	0.007	0.07	0	128	128
95	53 yr M, Endocarditis	0.033	0.016	0	0.014	0	0	0	64	256
96	44 yr F, Head injury	0.25	0	0	0	0	0	0	192	768
97	58 yr F, Biliary obstruction	0.010	0.011	0.010	0.010	0.010	0.011	0.015	512	192
99	53 yr F, Ca Tonsil	0.076	0.055	0.090	0.041	0.005	0.174	0.014	512	768
	Mean Absorbance	0.112	0.08	0.101	0.067	0.040	0.010	0.010		

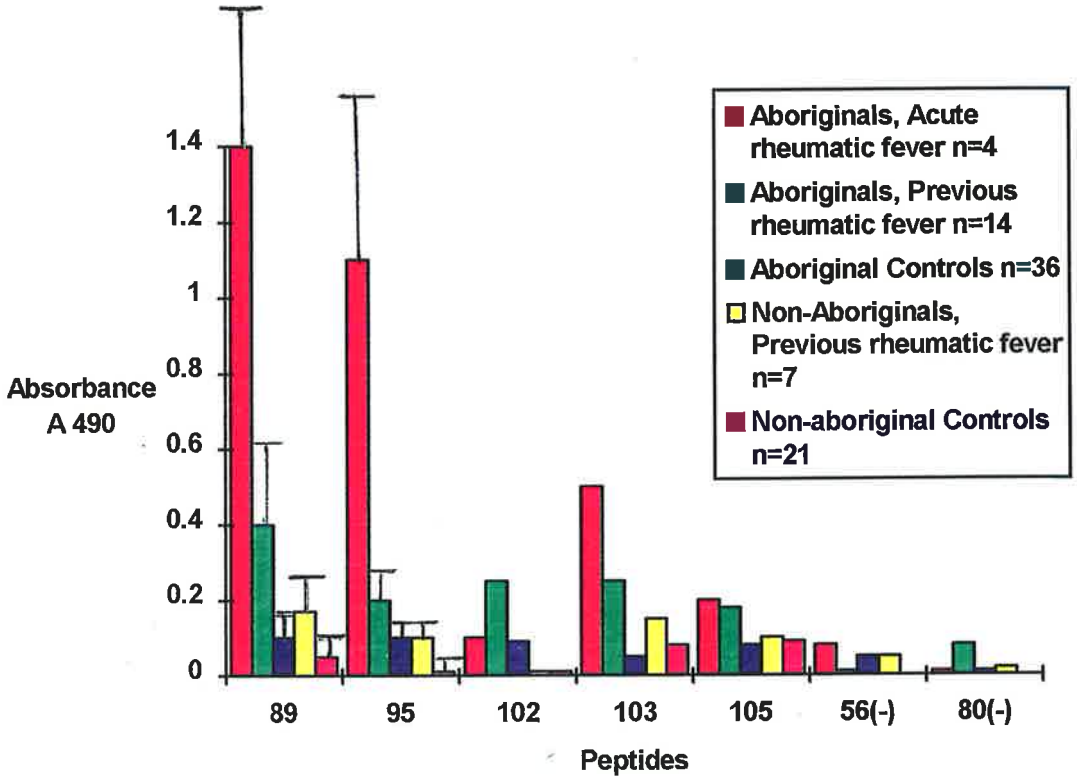
Table 6.4: Mean A_{490} absorbance values obtained using the universally reactive peptides 89, 95, 102, 103 and 105 and the unreactive peptides 56 and 80, in an EIA with sera from non-Aboriginal subjects with previous rheumatic fever (n=7)

		Peptide number								
Serum No.	**Clinical Diagnosis**	**89**	**95**	**102**	**103**	**105**	**56(-)**	**80(-)**	**ASOT**	**ADB**
1	61 yr F, Mitral valve replacement. RF at 21 yrs	0.047	0.067	0.019	0.041	0.252	0.042	0	96	<128
4	73 yr M, Mitral valve replacement. RF at 16 yrs	0.131	0.072	0.047	0.091	0.006	0.013	0	128	<128
5	60 yr F, Mitral valve replacement. RF at 26 yrs	0.101	0.043	0.006	0.029	0.003	0	0	128	<128
9	62 yr F, Mitral valve replacement. RF at 30 yrs	0.033	0.036	0.058	0.002	0.010	0	0	1536	<128
54	52 yr F, Mitral valve replacement.	0.491	0.250	0.021	0.508	0.020	0.046	0	128	192
62	59 yr F, Mitral valve replacement.	0.131	0.036	0	0.129	0.130	0.065	0	192	<128
98	62 yr F, RF at 16 yrs	0.05	0.040	0.155	0.01	0	0	0.01	64	128
	Mean Absorbance	0.141	0.077	0.043	0.115	0.07	0.028	0		

Table 6.5: Mean A_{490} absorbance values obtained using the universally reactive peptides 89, 95, 102, 103 and 105 and the unreactive peptides 56 and 80, in an EIA with sera from non-Aboriginal controls (n=21).

		Peptide Number								
Serum No.	**Clinical Diagnosis**	**89**	**95**	**102**	**103**	**105**	**56(-)**	**80(-)**	**ASOT**	**ADB**
2	73 yr F, Aortic valve replacement	0	0	0	0	0	0	0	512	128
7	68 yr M, Coronary artery grafting	0.010	0	0	0	0	0	0	<64	<128
8	72 yr M, Coronary artery grafting	0.061	0.060	0.030	0.054	0.012	0.007	0	128	<128
12	65 yr M, Coronary artery grafting	0.026	0.033	0.021	0.029	0.040	0.058	0.063	96	<128
13	72 yr F, Coronary artery grafting	0.048	0.053	0.036	0.165	0.058	0.059	0.061	64	<128
16	65 yr M, Coronary artery grafting	0.003	0	0	0	0	0.001	0.014	<64	<128
28	51 yr F, Gp A sepsis	0.238	0	0	0	0.239	0	0.262	512	512
30	10 yr M Trauma	0.018	0.005	0.001	0.009	0.010	0.020	0.008	256	3072
36	26 yr M, Gp A sepsis	0.050	0.021	0.064	0.054	0.072	0.076	0.076	192	2048
37	70 yr M, Coronary artery grafting	0.269	0.011	0.034	0.205	0.088	0.227	0.034	64	128
38	71 yr F, Coronary artery grafting	0.144	0.011	0	0.102	0.004	0.101	0.003	<64	<128
41	71 yr M, Coronary artery grafting	0.018	0.011	0.032	0.019	0.091	0.028	0.037	<64	<128
43	65 yr M, Gp A sepsis	0.010	0.100	0	0.050	0	0	0	N/A	N/A
53	60 yr F, Gp A sepsis	0	0	0	0	0.016	0	0.006	<64	<128
55	52 yr F, Mitral valve replacement	0	0.008	0	0.016	0.075	0	0	<64	<128
56	35 yr F, Gp A sepsis	0.270	0.200	0.340	0.200	0.032	0.010	0.014	N/A	N/A
60	73 yr F, Gp A sepsis	0	0	0	0	0	0	0	<64	<128
61	48 yr M, Gp A sepsis	0.010	0.010	0.035	0.014	0.007	0.020	0.007	N/A	N/A
63	58 yr F, Mitral valve replacement	0.003	0	0	0	0.013	0	0	<64	<128
66	56 yr M, Gp A sepsis	0.002	0.036	0.018	0.002	0.002	0.004	0.004	<64	1024
75	39 yr M Normal	0	0.04	0	0.019	0.253	0.026	0	256	<128
	Mean Absorbance	0.056	0.03	0.03	0.045	0.05	0.03	0.028		

Figure 6.4: Screening of eighty-two sera against the five commonly reactive peptides 89, 95, 102, 103 and 105 with peptides 56 and 80 as negative controls. The sera are from five subject groups: Aboriginals with acute rheumatic fever (n=4), Aboriginals with previous rheumatic fever (n=14), Aboriginal controls (n=36), Non-Aboriginals with previous rheumatic fever (n=7) Non-Aboriginal controls (n=21).



6.3 ASOT and ADB results

The ASOT and ADB tests are not specific for rheumatic fever and are used to provide supporting evidence of streptococcal infection when rheumatic fever is clinically suspected. An ASOT or ADB titre of >256 is considered elevated (Ayoub, 1992, *In* Manual of Clinical Laboratory Immunology).

A summary of the ASOT and ADB titres obtained from sera used in this study follow:
9/54 (16.6%) Aboriginal subjects had an ASOT >256
33/54 (61%) Aboriginal subjects had an ADB titre >256
3/28 (10.7%) non-Aboriginal subjects had an ASOT >256
4/28 (14.3%) non-Aboriginal subjects had an ADB titre >256

11/18 (61%) of Aboriginals with rheumatic fever had an ADB >256
22/36 (61%) of Aboriginal controls had an ADB >256
This demonstrates that streptococcal infections are common in Aboriginal communities as shown by the high percentage of this group with elevated ADB titres. This is unrelated to the presence of rheumatic fever.

In this study, only one of the four Aboriginal subjects with acute rheumatic fever, had an ASOT of >256 which would have supported the diagnosis of rheumatic fever. All four had elevated ADB titres of >256. In comparison, six of the thirty six aboriginal controls (16.6%) had ASOTs of >256 and twenty two (61%) had ADBs >256.
This confirms the limited usefulness of the ASOT and ADB tests when used in Australian Aboriginal communities to support the diagnosis of rheumatic fever.

6.4 Comparison of two 20-mer peptides (p145, p146) with peptides used in this study

During the concluding stages of this study, a publication by Pruksakorn et al (1994), described two peptides of similar sequence to peptides 89 and 95 derived from the M5 protein sequence. These peptides (peptides 145 and 146) had been shown to not distinguish between Thai and Aboriginal subjects with rheumatic fever and those without . When used in the EIA system in this study, against sera from the five subject groups, they were found to be less discriminatory than peptides 89 and 95. Sera from the Aboriginal subjects with acute rheumatic fever and previous rheumatic fever, were relatively non-reactive with p146 (Mean A_{490} value : 0.358 and 0.198 respectively). Peptide 145 (p145) was equally as reactive as peptides 89 and 95 with this group of sera. Unlike peptides 89 and 95, p145 was also reactive with Aboriginal control sera. When used with sera from non-Aboriginal subjects with rheumatic fever and controls, both p145 and p146 had higher mean absorbance values than those obtained with peptides 89 and 95. The absorbance values were between five and six times greater in the control groups using p145 as antigen, than when peptide 89 was used. This had the effect of reducing the discriminatory potential of p145 as an antigen in the serodiagnosis of rheumatic fever.

A comparison of the mean absorbance values between the larger peptides p145 and p146, and peptides 89 and 95, are given in Table 6.6.

Table 6.6. A comparison of mean absorbance values obtained using the larger peptides p145 and p146, with the universally reactive peptides 89 and 95 in an EIA. Peptides were tested aginst all eighty two sera of the study population. The absorbance values for peptides 89 and 95 are taken from a different dataset than that reported in the earlier screening of sera.

	Aboriginal Acute rheumatic fever (n=4)	**Aboriginal Previous rheumatic fever (n=14)**	**Aboriginal Controls (n=36)**	**Non-aboriginal Previous rheumatic fever (n=7)**	**Non-aboriginal Controls (n=21)**
p 145	1.315	0.512	0.460	0.268	0.364
p 146	0.358	0.198	0.164	0.268	0.133
Peptide 89	1.393	0.553	0.095	0.141	0.059
Peptide 95	1.073	0.368	0.08	0.077	0.03

6.5 Peptide epitope mapping - conclusions

Peptide epitope mapping of M24 protein has demonstrated that peptides based on epitopes at the C-terminal of M24 protein can be used to distinguish between Aboriginal controls and Aboriginal subjects with past rheumatic fever or acute rheumatic fever. This difference is not seen with non-Aboriginal subjects with previous rheumatic fever. It is also significant given the common nature of streptococcal infection amongst Aboriginals, that this test using peptides 89 and 95, is capable of distinguishing acute rheumatic fever and previous rheumatic fever from Aboriginal controls.

Chapter 7

Discussion and Conclusions

The aim of this study was to investigate whether the detection of antibodies to specific linear epitopes of a streptococcal M protein, could be used to distinguish subjects with rheumatic fever from controls. These epitopes could form the basis of an improved serological test for rheumatic fever. Early and reliable diagnosis would allow the institution of penicillin prophylaxis and the prevention of subsequent rheumatic heart disease.

7.1 Pepsin extraction of M protein and PAGE

A preliminary step to epitope mapping was the extraction and use of streptococcal M protein as an antigen, to detect antibodies specific for rheumatic fever. The choice of group A streptococcal M types 18 and 24 for M protein extraction was essentially arbitrary. They represented two "rheumatogenic" serotypes, which could carry common epitopes specific for rheumatic fever.

Passaging streptococci through human blood prior to large scale broth culture, was done to increase the yield of M protein. There would have been no components of human blood in the final broth culture as they were inoculated with single colonies obtained from sub-culture of the original blood enriched broth culture.

The crude nature of the pepsin extracts of M18 and M24 proteins is demonstrated by the multiple bands seen on PAGE. This may also be due to multiple points of pepsin cleavage of streptococcal surface proteins. The

difference in fragment sizes between peptide extracts of different M-proteins has been described before (Beachey et al 1977, 1986). These were reproducible with different batches of pepsin extracts of M protein (pepM).

7.2 Screening of sera by Western blot

The pepsin extracts of streptococcal M24 and M18 proteins were used in a Western blot to detect reactivity in sera from patients with clinical evidence of rheumatic heart disease. This was done as a preliminary approach to the broader study of epitope mapping of M protein in rheumatic fever. At the commencement of this part of the study, it was thought that reactivity if present, would be confined to the hypervariable N-terminal end of M protein.

The mean age of aboriginal subjects with rheumatic fever, in this Western blot study, was significantly lower than that of non-Aboriginals (32 vs 49 years respectively). This reflects the diminishing incidence of rheumatic fever in non-Aboriginals.

Of the 18 subjects with proven rheumatic fever, sera from 16 showed distinct bands to pepM24 and/or pepM18 on Western blot. The two exceptions were serum number 45 from an Aboriginal subject who had a clinical diagnosis of chorea, with no evidence of rheumatic heart disease and serum number 9 from a non-Aboriginal subject who had rheumatic fever 30 years ago.

Six sera reacted solely with pepM24 in the Western blot, three reacted solely with pepM18 while seven reacted with both.

In most of these, background staining was not significant. There was no significant difference between Aboriginals and non-Aboriginals with regard to specific M-type reactivity. There was also no correlation between the degree of reactivity and the presence of acute or previous rheumatic fever.

In the control group of 13 sera, only one serum showed reactive bands to pepM24. This was serum number 28 from a 51 year old female who had a documented group A streptococcal septicemia. The isolate from this patient was not M-typed. It was therefore unclear whether or not this reactivity was M-type specific.

In this part of the study it was shown that the pepsin extracts of M18 and M24 proteins could distinguish between sera from subjects with rheumatic fever and those unaffected. This was unrelated to M type specificity which is N-terminal dependent. As the N-terminals of both M24 and M18 proteins do not share any significant amino acid homology, it was felt that the degree of cross-reactivity seen between pepM18 and pepM24 was due to specific peptides in the B or C repeat blocks which share significant homology. It is possible that the pepsin extraction process resulted in peptide fragments from this region of M protein which reacted preferentially with sera from subjects with rheumatic fever. One possible way to confirm this would have been with M-type specific antisera. Bands obtained using this antisera in the same Western blot, would possibly be quite different from the patterns obtained using the rheumatic fever positive sera as in this study. It is also possible that the denaturing conditions used in the PAGE may affect Western blot reactivity.

7.3 Preliminary screening of the peptide bank by EIA

This part of the study used a streptavidin - biotinylated EIA system to map rheumatic fever specific linear epitopes of M-protein. Preliminary screening of the eighty two peptide bank using selected sera, showed that commonly reactive epitopes were located at the conserved carboxy-terminal. There was significant amino-acid sequence homology between the five universally reactive peptides 89,95,102,103 and 105. In particular, peptides 89 and 95, only differed by two amino acids. The C-terminus of streptococcal M protein is highly conserved among different M-types. This is demonstrated in Figure 1.2 where the amino acid sequences of nine M-type proteins, are compared. This homology is not confined to "rheumatogenic" M-types, as some "non-rheumatogenic" types M41, M49 and M52, also share closely related sequences with the reactive peptides.

The lack of reactivity at the N-terminus, further supports the earlier Western blot findings in that reactivity was frequently not M-type specific. The clustering of reactive epitopes at the C-terminal confirms the findings of Bessen et al (1995) in relation to the C-terminal repeat region of M6 protein. This region is identical to that of M24 protein. Interestingly,this region of M-protein (C-terminal), has also been shown to share antigenic epitopes with components of heart tissue (Pruksakorn 1992).

7.4 Screening of test sera with commonly reactive peptides

Wider screening of sera using these peptides, suggest that peptides 89 and 95 can distinguish between Aboriginals with acute rheumatic fever and Aboriginal controls. Using peptide 89, this difference was significant ($p< 0.0001$, Mann-Whitney, $p=0.0238$ unpaired t-test). For peptide 95, the

difference was similar, ($p<0.0001$, Mann-Whitney and $p=0.0416$, unpaired t-test).

The difference between A_{490} values in Aboriginal subjects with previous rheumatic fever and Aboriginal controls was also significant. The values were 0.561 for peptide 89 ($p<0.0001$,Mann-Whitney and $p=0.0067$, unpaired t-test) and 0.385 for peptide 95 ($p<0.0001$, Mann-Whitney and $p=0.0214$, unpaired t-test).
There was a less significant difference between non-Aboriginal subjects with rheumatic fever and non-Aboriginal controls. The mean A_{490} values for peptide 89 in these groups, were 0.141 and 0.056 respectively ($p=0.0139$, Mann-Whitney and $p=0.1130$, unpaired t-test).

There was also a significant difference in mean A_{490} values for peptide 89 between Aboriginal subjects and non-Aboriginal subjects with previous RF. These were 0.561 and 0.1406 respectively ($p=0.0037$, Mann-Whitney and $p=0.0114$, unpaired t- test). This may be due to the longer interval between the original episode of rheumatic fever in non-aboriginals and the collection of serum for this study. In some subjects this period was up to 50 years. Another potential reason might be the higher incidence of recurrent streptococcal infections in Aboriginal communities resulting in anamnestic antibody stimulation. The high ADB titres in these subjects confirm the high level of streptococcal skin sepsis commonly seen in this population. This would suggest that while recurrent streptococcal infection may be a feature in Aboriginal communities, the antibody response to peptides 89 and 95 can still be used to distinguish between Aboriginals with rheumatic fever and unaffected individuals.

Sera from non-Aboriginal controls who had documented group A streptococcal sepsis were also unreactive with peptides 89 and 95. Serum number 28, from a non-Aboriginal with group A streptococcal sepsis, which was reactive on Western blot with pepM24, was less reactive with peptide 89 (absorbance value 0.238) in the EIA. This would further support the specificity of reactivity with these peptides for rheumatic fever. Using a threshold A_{490} value of 0.30 for peptide 89, (Limit of detection = mean A_{490} value of aboriginal controls for peptide 89 + 2 standard deviations) this peptide would have a sensitivity of 100% and a specificity of 91% for Aboriginals with acute rheumatic fever. This compares with a sensitivity of 57% and a specificity of 91% in Aboriginals with previous rheumatic fever.

There were six "false-negative" results in Aboriginal subjects with previous rheumatic fever (sera 6,24,35,45,52 and 71). The mean A_{490} values of these six sera, for peptide 89 was 0.170 whereas the mean for the negative Aboriginal control sera was 0.112. There were no clear clinical differences between this group and other Aboriginals with rheumatic fever whose sera were strongly reactive. There was a single "false positive" result in a 61 year old Aboriginal man with chronic liver disease (A_{490} = 0.45).

The overall low reactivity of sera from non-Aboriginals with previous rheumatic fever, may be due to some subjects having had their initial episode of rheumatic fever up to 57 years earlier, resulting in a fall in antibody levels. Non-Aboriginals are also less likely to be continuously challenged by streptococcal antigens, as occurs with Aboriginals. Another reason may be different B-cell reactive epitopes for rheumatic fever in non-Aboriginals. The trend of greater reactivity at the C-terminal end of M

protein remains consistent, although at a lower level for non-Aboriginals with previous rheumatic fever.

7.5 A comparison of the two 20-mer related peptides (peptides 145 and 146) with peptides used in this study

The C-terminal aminoacid sequence, from position 530 of M24 protein, is virtually identical to that of M5 (Figure 1.2). The detection of antibodies to the C-terminal region of M5, as previously described by Pruksakorn et al (1994), did not show any difference between both Aboriginal and Thai controls and subjects with rheumatic fever. Two of the peptides used in that study (peptides 145 and 146), when used in an EIA with sera from this study, could not reliably differentiate between Aboriginal subjects with previous rheumatic fever and controls. While the difference in mean absorbance values between Aboriginal subjects with acute rheumatic fever and controls was significant for peptide 145 ($p=0.0168$, Mann-Whitney), it was not as significant as when peptide 89 was used ($p<0.0001$, Mann-Whitney). There was no significant difference between Aboriginal subjects with previous rheumatic fever and controls ($p=0.8060$) using peptide 145. This is in contrast to peptide 89 which could discriminate within this group ($p=0.0067$).

Differences in the methodologies between the two EIA systems used in this study, are largely related to one group of peptides being biotinylated. Both use horseradish peroxidase detection systems although the detection antibodies used are different. The serum dilutions used also differ with a 1 in 500 dilution used with the biotinylated peptides compared with a 1 in 1000 dilution used with peptides 145 and 146. It is unlikely that these methodological differences are responsible for the different reactivities

shown here between the 20-mer peptides 145, 146 and the reactive 16-mer biotinylated peptides used.

The 20-mer peptide 145 differs from the 16-mer peptide 89, by six amino acids at the C-terminal end (VEKALE). This sequence is also present in peptide 105. Interestingly, this peptide (pep 105) also does not reliably distinguish between aboriginal subjects with rheumatic fever and controls (Figure 6.4). It is possible that the larger peptide 145 contains two adjacent epitopes, which may explain the lack of discrimination between sera from the groups mentioned. Alternatively, the structure of peptide 145 could allow the binding of antibody that is less specific for rheumatic fever.
Peptide 146, which is the other 20-mer peptide tested, also has some homology with the 16-mer peptide 105. It differs by three amino acids at the N-terminal and one amino-acid at the C-terminal. Both these peptides contain the sequence VEKALE as mentioned previously and were less discriminatory than peptide 89. It is possible that the presence of this sequence causes a reduction in reactivity specific for rheumatic fever in aboriginal subjects. A less likely alternative reason would be that the SGSG spacer region in the biotinylated peptides, or the biotin, may affect binding due perhaps to steric hindrance.

7.6 The use of the five reactive peptides in combination

Attempts to use the reactive peptides in combination to improve their discriminatory power, were unsuccessful. Rather than enhancing absorbance values, this procedure resulted in a progressive lowering of absorbance values with increasing peptide concentration. This would be most likely due to steric interference between individual peptides.

7.7 Titration of streptavidin concentration for coating wells

A problem encountered with this system, was high absorbance values due to non-specific serum binding to streptavidin. This was addressed by reducing the concentration of streptavidin used for coating wells, from the recommended 0.5 μg/well to 0.01 μg/well. Preabsorbing sera with streptavidin did not reduce absorbance values, suggesting that anti-streptavidin antibodies in sera, were not a reason for this binding. This would also suggest that non-biotinylated peptides would need to be used in any further development of this assay if reliable binding to microtitre plate surfaces could be demonstrated.

7.8 A comparison of the Western blot and EIA results

Although both Western blots and EIAs were only carried out on twenty four sera (seven sera had only a Western blot performed due to insufficient specimen), both methods were able to distinguish between Aboriginal subjects with rheumatic fever and those without. The EIA however, was less likely to distinguish between non-Aboriginal subjects with previous rheumatic fever and controls, whereas the Western blot could reliably distinguish between these groups. There was no correlation between degrees of reactivity in both methods.

Six sera reacted solely with pepM24 in the Western blot. Two of these were from Aboriginal subjects with acute rheumatic fever (sera 31 and 33) and were strongly reactive with peptide 89 in the EIA. Of the remaining four subjects with previous rheumatic fever (sera 1, 4, 10 and 24) three were tested by EIA as well and were negative (serum 10 was not tested by EIA because of insufficient specimen).

Three sera from subjects with previous rheumatic fever, were reactive with pepM18 alone by Western blot (sera 5, 22 and 42). Of these, two were tested by EIA and one (serum 42) was reactive.

Of the seven sera from subjects with previous rheumatic fever which were reactive with both pepM18 and pepM24 on Western blot, four were tested by EIA (sera 6, 25, 34 and 35). Two of these were positive (sera 25 and 34).

This discrepancy between the two methods may be due to different, possibly conformational reactive epitopes, found in the Western blot antigen used when compared with the linear peptide epitopes. The reason for the selective reaction of some sera in the Western blot to either pepM24 or pepM18 is less clear. This may be related to denatured or partially exposed epitopes following the extraction process or during PAGE, which may in turn lead to variable reactivity.

All control sera (Aboriginal and non-Aboriginal) except one were negative by both Western blot and EIA. The only "false-positive" Western blot was in serum 28. This was a 51 year old non-Aboriginal woman with proven group A streptococcal septicemia (M-type not known). The EIA result while negative was relatively high (A_{490} value - 0.238) when compared with the cutoff value of 0.30. This would suggest a degree of cross-reactivity which may be due to sequence homology sufficient to result in a positive Western Blot yet dissimilar so as not to react with the less sensitive EIA.

The two "false negative" Western blot results with sera 45 and 9, were also negative by EIA. One was a non-Aboriginal subject who had the original

episode of rheumatic fever at least 30 years prior. Waning of antibody levels may be a reason for poor reactivity. The other was a 46 year old Aboriginal male who had chorea. As this is an unlikely age group to develop Sydenham's chorea, therefore the clinical diagnosis of rheumatic fever must be in doubt.

7.9 A comparison between the peptide based EIA as described here and current serological tests used to confirm streptococcal infection (ASOT and ADB)

An ASOT or ADB titre of >256 is considered elevated (Ayoub, 1992 *In* Manual of Clinical Laboratory Immunology). As mentioned in section 6.3, only one of the four Aboriginal subjects with acute rheumatic fever, had an ASOT of >256 which would have supported the diagnosis of rheumatic fever. The high ADB titres seen in the aboriginal group as a whole, demonstrates the limited usefulness of this test when used in Australian Aboriginal communities, which have high rates of streptococcal infection.

The peptide based EIA as used here, does not have these limitations. All streptococcal M-types, in which M-proteins have been sequenced, have homologous regions at the C-terminal (Miller et al 1988). There are minor amino acid differences between M types in this region. M49 for example has three single amino acid variations in the region corresponding to peptides 89 and 95 (Figure 1.2). It is this region that has been shown to be reactive with sera from aboriginal subjects with rheumatic fever. The use of peptides 89 and 95 in a serologic test for rheumatic fever, would therefore not be affected by different M-types of the organism initiating the episode of rheumatic fever. As has also been demonstrated, the

presence of non-rheumatic group A streptococcal sepsis, does not affect reactivity with the C-terminal peptides used.

7.10 Possible role of the peptide epitopes identified in this study, in the pathogenesis of rheumatic fever

As discussed in Section 1.13, epitopes of streptococcal M protein which cross react with heart or articular tissue, have been defined.
The C-terminal region has been identified as a common cross-reactive region in some studies (Pruksakorn et al 1992, Baird et al 1991).
The reactive peptide 89 used in this study, is very similar to peptide 145 used in the study by Pruksakorn et al which was cross reactive with porcine heart myosin. Peptide 105 is also closely related to peptide 146 used by Pruksakorn et al, which cross-reacted with both human atrial tissue and porcine heart myosin (Pruksakorn et al 1992). This would suggest that antibodies to these specific epitopes, generated in subjects with rheumatic fever, cross-react with cardiac tissue. As described earlier, peptides 145 and 146 differ from peptides 89 and 105 by 6 and 4 amino acids respectively. To confirm this cross-reactivity, peptides 89 and 105 would need to be assessed in a similar study.

The N-terminal has generally not demonstrated this cross reactivity (Dale et al 1983, Dale et al 1986a, Beachey et al 1986, Sargent et al 1987). The exceptions being the study by Bronze et al, which showed that a synthetic peptide representing amino acids 11-24 of the N-terminal of M19, cross reacted with sarcolemmal membranes of human myocardium.

The study by Cunningham et al looked at a pepsin extract of M5. Reactivity to this was mapped and found to be confined to a region at the

C-terminal end of this protein. Peptides further down towards the C-terminal end of the entire M protein were not tested (Cunningham et al 1989).

Peptide sequences of the cross-reactive epitopes of M5 and M19 so mapped are quite dissimilar. These being QKSKQ (Cunningham et al 1989) and KLKKIIDDLDAKENC (Bronze et al 1988).

The importance of determining the site of cross-reactivity has implications both for the serodiagnosis of rheumatic fever and in vaccine development. Current literature would suggest that this is at the C terminal end, although as has been discussed above, opinion is divided. This study addresses this by peptide epitope mapping of the entire M24 protein molecule. This has shown that linear epitopes at the C-terminal end of M24 protein, react selectively with sera from Aboriginal subjects with rheumatic fever. These epitopes are highly conserved throughout M proteins of different serotypes. The fact that these epitopes are located in the same region of M proteins which have been shown to be cross-reactive with heart and joint tissue is highly significant. Rheumatogenicity of group A streptococcal isolates has traditionally been associated with specific M types. Variability among M types is related to the hypervariable N-terminal of streptococcal M protein. The combination of a lack of immunoreactivity to this region, with tissues implicated in the pathology of rheumatic fever and with sera from subjects with rheumatic fever, confirms that the N-terminal is not involved in the pathogenesis of this disease. Conversely, the C-terminal reactivity demonstrated by peptide epitope mapping in this study, provides added weight to the hypothesis that the pathogenesis of rheumatic fever is related to C-terminal epitopes.

7.11 Conclusions

The first part of this study showed that in the thirty one sera tested, the use of pepsin extracted M24 and M18 proteins as the antigen in a Western blot, distinguished between subjects with a history of rheumatic fever and those without such a history. Significant cross-reactivity between the two M-proteins used (pep M18 and pep M24) was also noted. This suggested that immunoreactivity detected was not type specific and unlikely to be N-terminal related.

In the second part of the study, reactive linear epitopes at the conserved C-terminal end of M24 protein were identified. The peptides 89, 95, 102, 103 and 105 showed significant reactivity with sera from Aboriginal subjects with acute rheumatic fever and to a lesser extent, those with previous rheumatic fever.

Cross-reactivity between streptococcal M protein and heart or articular tissue, has been shown in previous studies to be largely related to the C terminal end. The reactivity at the C-terminal end shown in this study, provides added support for the hypothesis that at least part of the pathogenesis of rheumatic fever, is related to this part of the M protein molecule.

While the number of sera tested are small, it is suggested that peptides 89 and 95 as used here, could be the basis of an improved serodiagnostic test for acute rheumatic fever. The sequence of these peptides is found in the C-terminals of all M-types examined so far. The test would therefore not be dependent upon specific M-types causing disease.

A number of approaches can be made to further develop this work. Larger numbers of sera from subjects with acute rheumatic fever would need to be tested against these peptides to validate these findings. It would be particularly useful to obtain and test sera from non-Aboriginal subjects with acute rheumatic fever. This would help to determine whether immunoreactivity to these epitopes in subjects with rheumatic fever, is dependent upon ethnic origin.

In addition, the construction and use of a larger peptide incorporating both peptides 89 and 95, could improve the discriminatory ability of the test system.

To further confirm that these epitopes are cross-reactive with heart and articular tissue, these smaller peptides could be used in a study similar to the one using the closely related, though larger peptides 145 and 146 (Pruksakorn et al 1992).

The early diagnosis of rheumatic fever, particularly in Aboriginal communities, would lead to earlier institution of prophylaxis, reduced recurrences of rheumatic fever and a subsequent reduction in chronic valvular heart disease.

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Antigenic epitope mapping of the M24 protein of *Streptococcus pyogenes*: implications for serodiagnosis of rheumatic fever

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Abstract

Rheumatic fever continues to be a significant problem in Australian Aboriginal communities and developing countries worldwide. Early diagnosis could facilitate the institution of penicillin prophylaxis resulting in the prevention of recurrences of rheumatic fever. An overlapping biotinylated peptide bank of 82 peptides, based on the known sequence of *Streptococcus pyogenes* M24 protein, was used in a standard enzyme immunoassay. A total of 82 sera were tested from both aboriginal and non-aboriginal subjects with clinically proven rheumatic fever, rheumatic heart disease and matched controls. Two peptides with significant sequence homology at the C-terminal end were found to be discriminatory between aboriginal cases and controls. It is proposed that these peptides could be the basis of a serological test for rheumatic fever.

Keywords: Peptide; Epitope; M24 protein; Enzyme immunoassay; Rheumatic fever

1. Introduction

Rheumatic fever (RF) is one of the non-suppurative complications of Group A streptococcal (*Streptococcus pyogenes*) pharyngitis. Valvular heart disease, as a sequela of acute rheumatic fever (ARF), continues to be a significant cause of morbidity and mortality not only in the developing world but also among Aboriginal communities in Australia where the reported incidence is as high as 800 per 100 000 [1]. In Africa as many as 470 cases of RF per 100 000 population have been reported [2], whereas in the United States the figure is markedly lower at 0.63 per 100 000 population [3].

The frequent occurrence of group A streptococcal skin and throat infections increase the difficulty of interpreting the standard streptococcal serological tests, namely the anti-streptolysin O and anti-deoxyribonuclease B (ASO and ADNaseB) titres. It is now clear that following an initial episode of ARF, subsequent exposure to group A streptococci may lead to an increased risk of carditis. This could be prevented with prophylactic penicillin had the diagnosis of ARF been made earlier. A serological test with greater specificity for RF could help in making an earlier diagnosis.

Group A streptococcal strains most strongly associated epidemiologically with recent outbreaks of

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ARF belong to the well-recognised rheumatogenic M-serotypes 1, 3, 5, 6, 18 and 24 [4]. M-protein is a major virulence factor of the group A streptococcus. This is largely through its antiphagocytic effect and its role in adherence [5]. It exists as a filamentous molecule consisting of two protein chains in a coiled coil configuration extending about 60 nm above the surface of the organism [5]. It has a hypervariable N-terminal domain and a relatively conserved C-terminal end.

Immunity to group A streptococcal infection is associated with the development of opsonic antibodies to anti-phagocytic epitopes of M-protein. Immunity is type-specific and lasting [6]. The opsonophagocytic test of Lancefield (bactericidal test) has been used as a standard for M-antibody detection [7]. It is however cumbersome and not suited for a routine diagnostic laboratory.

The amino-acid sequence of M24 protein is known [8]. To determine if there are reactive linear epitopes within this sequence which might be specific for RF, a bank of 82, overlapping 16-mer, biotinylated peptides, representing the entire mature M24 protein, was used in a standard enzyme immunoassay (EIA) system with selected sera from aboriginal and non-aboriginal subjects with and without rheumatic fever. Commonly reactive peptides were identified and used in a similar EIA against a larger number of test sera to confirm these findings.

2. Materials and methods

2.1. Synthesis of peptides

Eighty-two, 16-mer, biotinylated peptides corresponding to the 539 amino-acid sequence of M24 protein were obtained (Chiron Mimotopes). Peptides were offset by 6 amino-acids, covering all 10-mers. They were numbered from peptide 44 (amino terminal) to 96 and 99 to 127 (carboxy terminal). The signal sequence was not included. The peptides were dissolved in phosphate-buffered saline (PBS pH 7.2) and dimethyl formamide. The working dilution of the peptides was 0.028 mg/ml.

2.2. Subjects

A total of 82 sera comprising 5 subject groups were tested. Four sera were from aboriginal subjects (mean age 21 years) with acute rheumatic fever (ARF), 14 from aboriginal subjects (mean age 33 years) with previous rheumatic fever (RF) or rheumatic heart disease (RHD) and 36 sera from aboriginal controls (mean age 37 years) who had no record of RF. This control group consisted of 2 subjects with non-rheumatic valvular heart disease, 2 with significant coronary artery disease, 2 with group A streptococcal sepsis, 2 with post-streptococcal glomerulonephritis, 1 with group G streptococcal sepsis, 9 with sepsis relating to a broad range of causative agents, 9 with trauma and 9 with miscellaneous conditions.

There were 7 sera from non-aboriginal subjects with RHD (mean age 61 years). Some of these subjects had their initial episode of rheumatic fever up to 57 years prior. The remaining 21 sera were from non-aboriginal controls (mean age 59 years). These included 8 subjects with significant coronary heart disease, 3 with non-rheumatic valvular heart disease, 8 with group A sepsis and 2 with trauma. The difference in mean ages between aboriginal and non-aboriginal subjects with RHD highlights the rarity of this condition in younger non-aboriginals in Australia.

All cases of ARF, RF or RHD had been confirmed clinically or by echocardiography. In those subjects who had valve replacements, there was, in addition, histologic evidence of rheumatic involvement. All controls were reviewed to exclude RHD or previous RF.

2.3. Enzyme immunoassay

Ninety-six-well Nunc Maxisorp microtitre plates were coated with 0.01 μg/well of streptavidin (Sigma) diluted in water. These were incubated at 37°C overnight and then washed with PBS/0.1% Tween 20 (pH 7.2). The wells were blocked with 2% casein–10 mmol Tris-HCl/PBS (pH 7.0) for 30 min. Single peptides were added to each well to achieve a final concentration of 0.28 μg/well. The plates were put on a shaker for 1 h at room temperature. Test sera were diluted to 1 in 500 with 0.5% casein–Tris-HCl/PBS, added to the wells and incubated at 37°C for 1 h. Secondary antibody conjugate (Dako rabbit anti-human IgG horseradish peroxidase) at a dilution of 1 in 1000 in 0.5% casein–Tris-HCl/PBS was added. Substrate (*O*-phenylenedia-

mine-2HCl) was added and the resulting colour reaction stopped with 1 N sulphuric acid. All washes between steps were done with PBS/0.1% Tween 20 (pH 7.2). Absorbance values were read on a MR 7500 using a test wavelength of 490 nm (A_{490}) and a reference wavelength of 630 nm.

Chequerboard titrations were done prior to this study to determine the optimal sera dilution and peptide concentrations as used above. Controls used included serum-streptavidin only wells, positive and negative sera and negative peptides. The A_{490} values of the respective serum-streptavidin controls were subtracted from those of the wells with peptide, to adjust for non-specific serum binding to streptavidin.

2.4. Preliminary screening of the 82 peptides to determine commonly reactive epitopes

All 82 peptides of the M24 bank were screened against 10 selected sera. Five were from aboriginal subjects with ARF or previous RF, 2 from non-aboriginals with previous RF and 3 from aboriginal controls.

2.5. Wider screening using commonly reactive peptides

The 82 sera from the 5 subject groups were each tested against the 5 commonly reactive peptides (89, 95,102,103 and 105) and 2 commonly non-reactive peptides (56 and 80) using the EIA as described.

3. Results

Preliminary screening of 82 peptides to identify significant linear B-cell epitopes showed that reactivity was confined to peptides at the carboxy (C) terminal end.

This was particularly true of the 5 aboriginal subjects with ARF or previous RF where the mean A_{490} values for the 2 most reactive peptides (89 and 95) was 1.36 and 1.38, respectively. Other peptides found to be reactive, but to a lesser extent in this group, were 102, 103 and 105. These are shown in Fig. 1.

The mean A_{490} values for the three aboriginal controls were 0.08 and 0.04 for peptides 89 and 95, respectively. These sera did not have mean A_{490} values greater than 0.08 with any of the other peptides.

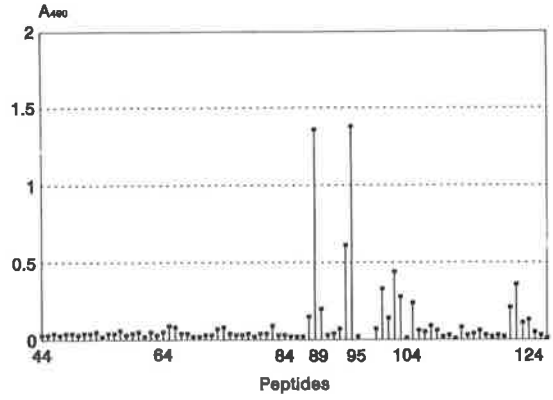

Fig. 1. Peptide epitope mapping of M24 protein. Mean optical densities (A_{490}) of sera from Aboriginal subjects with rheumatic fever ($n = 5$).

The mean A_{490} values for the 2 non-aboriginals with previous RF was 0.07 and 0.06 for peptides 89 and 95, respectively. As with the aboriginal controls, sera from this group did not have mean A_{490} values greater than 0.07 with any of the other peptides.

The amino acid sequences of these reactive peptides are as follows.

89: SLRRDLDASREAKKQL
95: LRRDLDASREAKKQLE
102: ASRQSLRRDLDASREA
103: RRDLDASREAKKQVEK
105: QVEKALEEANSKLAAL

Screening of the 82 test sera using the commonly reactive peptides 89, 95, 102, 103 and 105 with 2 non-reactive peptides (56 and 80) as controls confirmed a similar trend. The mean A_{490} values of the 5 subject groups against these 7 peptides are presented in Fig. 2.

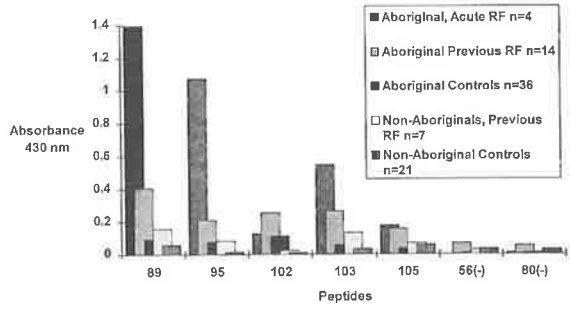

Fig. 2. Screening of 82 sera against the 5 commonly reactive peptides 89, 95, 102, 103 and 105 with peptides 56 and 80 as negative controls. Mean optical densities shown for the 5 groups.

4. Discussion

Preliminary screening of the 82-peptide bank showed that commonly reactive epitopes were located at the carboxy-terminal. There was significant amino-acid sequence homology between the 2 commonly reactive peptides (89 and 95) which only differed by 2 amino acids.

Wider screening of sera using these peptides suggest that peptides 89 and 95 can distinguish between aboriginals with acute rheumatic fever and aboriginal controls. Using peptide 89, this difference was significant ($P < 0.0001$ Mann-Whitney, $P = 0.0238$, unpaired t-test). For peptide 95, the difference was similar, ($P < 0.0001$ Mann-Whitney and $P = 0.0416$, unpaired t-test).

The difference between A_{490} absorbance values in aboriginal subjects with previous rheumatic fever and aboriginal controls were also significant. The values were 0.561 for peptide 89 ($P < 0.0001$ Mann-Whitney and $P = 0.0067$, unpaired t-test) and 0.385 for peptide 95 ($P < 0.0001$ Mann-Whitney and $P = 0.0214$, unpaired t-test).

There was a less significant difference between non-aboriginal subjects with rheumatic fever and non-aboriginal controls. The mean A_{490} absorbance values for peptide 89 in these groups were 0.141 and 0.056, respectively ($P = 0.0139$, Mann-Whitney, and $P = 0.1130$, unpaired t-test).

There was also a significant difference in mean A_{490} values for peptide 89 between aboriginal and non-aboriginal subjects with previous RF. These were 0.561 and 0.1406, respectively ($P = 0.0037$, Mann-Whitney, and $P = 0.0114$, unpaired t-test). This may be due to the longer interval between the original episode of rheumatic fever in non-aboriginals and the collection of serum for this study. In some subjects this period was up to 50 years. Another potential reason might be the higher incidence of recurrent streptococcal infections in aboriginal communities resulting in anamnestic antibody stimulation. This would suggest that while recurrent streptococcal infection may be a feature in aboriginal communities, the antibody response to peptides 89 and 95 can still distinguish between aboriginals with rheumatic fever and controls. Sera from controls who had documented group A streptococcal sepsis were also unreactive with peptides 89 and 95. This would further support the specificity of these peptides for rheumatic fever.

The C-terminal amino-acid sequence of M24 protein is virtually identical to that of M5 (another rheumatogenic strain). The detection of antibodies to this region has been described [1]. No difference was found between both Aboriginal and Thai controls and subjects with RF in that study. The peptide used in that study (peptide 146) differed most in that the sequence VEKALE is found at the C-terminus when compared with both peptides 89 and 95 used in this study. Another study by Fischetti et al. [9], however suggested that peptides at the C-terminus could distinguish between sera from subjects with rheumatic fever and those without.

The sequence VEKALE is also present in peptide 105. Interestingly, in this study, the peptide does not reliably distinguish between aboriginal subjects with rheumatic fever and controls (Fig. 1). It is possible that the larger peptide 146 used in the study by Pruksakorn et al. [1] contained 2 adjacent epitopes, which may explain the lack of discrimination of this peptide between sera from subjects with and without rheumatic fever.

Using a threshold A_{490} value of 0.30 for peptide 89 (limit of detection = mean A_{490} value of aboriginal controls for peptide 89 + 2 standard deviations), this peptide would have a sensitivity of 100% and a specificity of 91% for aboriginals with ARF. This compares with a sensitivity of 57% and a specificity of 91% in aboriginals with previous RF. There were 6 false negative results in aboriginal subjects with RF. These generally gave A_{490} values just below the threshold (0.30), whereas true negatives were much lower. There was a single false positive result in a 61-year-old aboriginal man with chronic liver disease ($A_{490} = 0.45$). From our data, it would appear that the use of peptides 89 and 95 in an EIA system can distinguish between aboriginal subjects with rheumatic fever or rheumatic heart disease and aboriginal controls.

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