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-deoxy-ribonuclease B (AS0 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...
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.

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: SLRRLDASREAKKQL
95: LRRDLNARREKQLE
102: ASRQLRRDLNARRE
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.
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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