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Structural optimisation of a conformational protective epitope allows expression as a recombinant fusion protein

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Abstract

A conformationally restricted B-cell epitope has been identified from the major group A streptococcal virulence factor, the M protein, as a potential safe vaccine candidate. To maintain alpha-helical secondary structure, the minimal epitope is flanked with heterologous sequences to produce the chimeric vaccine candidate J14. As a strategy towards developing an affordable multivalent GAS vaccine, we have expressed J14 recombinantly with a second GAS protective antigen H12 (rJ14H12). When administered to mice sub-cutaneously, the fusion protein stimulated a strong serum IgG response to the H12 component, but J14 was poorly immunogenic. To increase the immunogenicity of J14 when expressed with the model fusion partner, amino acid modifications were made to the initial recombinant construct to produce rJJo. These changes stabilised the alpha-helical conformation of the recombinant antigen as assessed by circular dichroism. Mice immunised with rJJoH12, the fusion protein incorporating JJo, effectively stimulated a humoral response to both of the included antigens. These data support the feasibility of developing a multivalent vaccine incorporating the conformationally restricted protective antigen J14.

1.0 Introduction

Streptococcus pyogenes (group A streptococcus, GAS) inflicts upon human kind the second highest mortality rate for a single bacterial species after *Mycobacterium tuberculosis*, with an annual rate exceeding 500,000 . Much of this mortality is accounted for by invasive diseases and post-infectious sequelae, rheumatic fever (RF) and rheumatic heart disease (RHD). The upper respiratory track and skin are the primary sites of GAS infections . Hence an effective vaccine against GAS diseases, which also prevents bacterial transmission, should induce mucosal and systemic immune responses.

A comprehensive dissection of the conserved coiled-coil region of the major streptococcal antigen, the M protein, identified a minimal B cell epitope as a safe vaccine candidate . The two peptides encompassing this epitope, J8-i and J14-i, contain 12 and 14 amino acid residues of the M protein respectively. Initial immunological characterisation of these peptides revealed that they were conformationally restricted and the induction of a protective immune response requires their presentation as a coiled coil. However, at the length of 12 or 14 amino acid residues, these peptides did not assume an alpha-helical conformation in aqueous solution To promote their native secondary structure, helix-promoting residues from a heterologous protein (yeast DNA binding protein, GCN4) were added on either side of the M-protein derived sequences . The resulting chimeric peptides retain the necessary conformation to elicit protective immune response that would react with M protein . One chimeric peptide, J14, has been extensively studied *in vitro* and in animal models as a potential vaccine candidate . Antibodies to J14 are opsonic and thereby prevent systemic spread of GAS and disease. J14 specific antibodies have also been shown to reduce mucosal colonisation of GAS in mice following intranasal challenge . Furthermore, the J14-i sequence is highly conserved between GAS strains and offers the advantage of a broadly protective immune response .

Other streptococcal proteins such as C5a peptidase and fibronectin binding proteins have also been considered as potential GAS vaccine candidates.

SfbI, a fibronectin binding protein expressed by a majority of GAS strains is a major adhesin molecule . Intranasal immunisation of mice with SfbI results in both a systemic and mucosal humoral response which is protective against intranasal GAS challenge . Further studies revealed that the adhesion properties and protective epitopes of SfbI reside within the fibronectin binding domain, defined as H12 . As a strategy toward inducing an efficacious vaccine against GAS based on multiple mechanisms of protection, it would be advantageous to combine J14 with H12. Furthermore, fusion of J14 with H12 as a single recombinant protein would obviate the need for conjugating J14 peptide to a carrier protein, such as Diphtheria Toxoid (DT). This approach overcomes the high synthesis and production costs associated with peptide based conjugate vaccines, an important consideration for a GAS vaccine for developing countries. In addition, the multivalent approach reduces potential of immune evasion through selective pressure.

With this in mind we made a recombinant fusion protein, rJ14H12, containing the chimeric peptide J14 and the H12 fragment of SfbI. To assess the feasibility of this multivalent approach, the immunogenicity of J14 when expressed with the fusion partner was compared to that of J14 when expressed recombinantly on its own (rJ14) and as a dimer (rJJ). The immunogenicity of the recombinant constructs was also compared with the synthetic peptide conjugated to DT (J14-DT). While both rJ14 and rJJ were as immunogenic as J14-DT, the immunogenicity of the J14 moiety within J14H12 was greatly diminished. We reasoned that the decreased immunogenicity of J14 in the fusion protein is due to a disruption of its alpha-helical secondary structure. With the aim of recovering the immunogenicity of J14 when expressed as a fusion protein, we created an additional recombinant protein containing the J14 epitope which has been optimised for alpha-helical conformation (JJo). The modifications in JJo overcame detrimental effects that H12 had on the immunogenicity of the J14 entity, with the optimised fusion protein, JJoH12, stimulating a strong J14 and H12 specific immune response. These studies confirm the importance of retaining alpha-helical conformation of the minimal B cell epitope within J14 based vaccines and can

provide the backbone for the development of multivalent GAS vaccines containing J14 as one of the components.

2.0 Materials and Methods

2.1 Construction of recombinant cassettes containing J14.

Three recombinant constructs (pQEJ14, pQEJJ and pQEJJo) encoding the chimeric sequence, J14 (KQAEDKVKASREAKKQVEKALEQLEDKVK) were produced in the expression vector pQE-30 (Qiagen). All molecular techniques were carried out following standard procedures as outlined in Sambrook et al . Two overlapping 71mer oligonucleotides, J14F-BamHI (GGGGATCCAAACAGGCGGAAGATAAAGTGAAAGCGAGCCGTGAAGCGA AAAAACAGGTGGAAAAAGCGCTG) and J14R-SacI (CCGAGCTCTTT CACTTTATCTTCCAGCTGTTCCAGCGCTTTTTCCACCTGTTTTTTCGCTTCA CGGCTCGC) (restriction sites underlined) were annealed and extended (65^{o}C) using 10:1 ratio of *taq:pfu* polymerase to produce a fragment encoding the chimeric sequence J14. The DNA fragment was sub-cloned into pGEM-T (Promega) to produce pGEM-J14bs. The sequence corresponding to J14 was released following digestion with BamHI and SacI and cloned into appropriately digested pQE-30 to produce pQE-J14. A second J14 fragment with flanking PstI and SacI sites was assembled using the same strategy described above and cloned into pGEM-T to produce pGEM-J14ps. The PstI-SacI fragment was excised from pGEM-J14ps and ligated downstream of the first J14 entity at the SacI site in pQE-J14bs to produce the J14 dimer, pQE-JJ. An additional J14 cassette, JJo, which incorporates sequence changes which promote alpha-helical conformation (outlined in section 3.2), was chemically synthesised as a single gene fragment and provided in a commercial vector (DNA2.0, USA). Primers JF-SacI (TCGGGCCTTTTGA GCTCAGCAGCAGC) and JR-BamHI (AAGCTCGTCGGGGATCCAAA CAGGCT) containing the restriction enzyme sites SacI and BamHI respectively (underlined) were used to amplify JJo from the supplied vector. The 255bp JJo fragment replaced the J14 fragment in pQE-J14 to produce pQE-JJo.

To assess whether the conformationally constricted antigen J14 could be expressed with other structurally and immunologically distinct antigens as a fusion protein, two additional constructs with H12 as the fusion partner were made. The H12 fragment was amplified from the *sfbI* gene of *S. pyogenes* NS192 using primers H12F (GGGAGCTCCCTGAAAAACCTAGTGTTGAC) and H12R (GGCTGCAGGGTCACTGTTCACTGAACC), containing SacI and PstI restriction sites respectively (underlined). The SacI and PstI digested amplicon (632bp) was directionally cloned into appropriately digested pQE-J14 and pQE-JJo downstream of the J14 cassettes, to obtain pQE-J14H12 and pQE-JJoH12 respectively. All recombinant plasmids were confirmed by DNA sequencing and transformed into *E.coli* for expression. Schematic diagrams representing the fusion proteins encoded by the recombinant plasmids are shown in figure 1.

2.2 Protein expression and purification

E.coli containing recombinant plasmids was grown in LB broth in the presence of Ampicillin (100µg/ml) until the culture reached an optical density at 600nm (OD_{600}) of 0.5. Expression of the recombinant proteins was induced with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture grown for a further 4 hours. Cells were collected by centrifugation at 4000*g* for 20 minutes and stored at -20ºC. Recombinant hexa-histidine tagged proteins were purified from the bacterial pellet under native conditions as follows . Briefly, *E. coli* pellets were thawed on ice and resuspended in 10ml of native lysis buffer containing 5mM imidazole. Lysozyme was added to the suspension at a final concentration of 1mg/ml, to promote cell lysis. The suspension was subject to three cycles of freezing in dry ice, and then thawing in a cold water bath. DNase (10µg/ml) and RNase (5µg/ml) were added to the lysate and incubated for 30 minutes with end to end rotation at 4ºC. The cellular debris was collected from the cleared lysate by centrifugation at 10000*g* for 20 minutes. Talon cobalt resin (BD Biosciences, USA) pre-equilibrated with lysis buffer was added to the cleared lysate and incubated for 1 hour with end to end rotation at 4ºC to promote protein resin interaction. Resin and bound protein complexes were transferred into a clean 5ml chromatography column, and washed with ice cold wash buffer containing

10mM imidazole. Bound recombinant proteins (rJ14, rJJ, rJJo, rJ14H12, rJJoH12) were eluted from the resin in ice cold equilibration buffer containing 250mM imidazole. Eluates were collected in 500µl fractions, pooled and dialysed overnight in phosphate buffer solution (PBS) at 4ºC. The protein concentration of the samples was determined using the BCA colorimetric detection kit (Pierce, USA) as per the manufacturer's instructions.

2.3 Circular Dichroism

Far UV spectra were collected at room temperature, with a JASCO J715 CD spectropolarimeter. Protein samples were diluted to a concentration of 50µg/ml in 10mM sodium phosphate buffer with 20mM NaCl, pH 7.5. Spectra were acquired at 1nm intervals from 260nm to 190nm, and were the average of 3 individual scans. CD spectra were also collected in the presence of 2, 2, 2-trifluoroethanol (TFE) in varying concentrations (5 – 60%). Data were corrected for buffer baseline and reported as mean residue ellipticity [Θ]. The secondary structure for each protein was predicted with spectrum deconvolution using a linear combination of three Fasman prototype spectra (α-helical, β-strand and random coil) as implemented in the program ACDP .

2.4 Murine immunisation and sample collection

Female BALB/c mice, 4-6 weeks of age, were obtained from the Animal Resource Centre (Perth, WA) and maintained in the QIMR holding facility. Mice were immunised as per standard protocols previously described . Primary immunisations of 25µg of antigen emulsified with complete freunds adjuvant (CFA) were administered at the base of the tail, on day 0. Mice received booster injections of 10µg of antigen in PBS, on days 21, 28 and 35. Blood samples (10µl) were taken before each immunisation by a snip at the end of the tail, on days 0, 20, 27 and 34. A full tail bleed (~200µl) was taken 7 days after the final boost on day 42. Mice were sacrificed on day 48 by CO_2 asphyxiation and cardiac punctures were carried out to collect larger volumes of blood. Diluted and neat blood samples were allowed to rest at room temperature for 1 hour following collection. Red blood cells were then collected by centrifugation for 10 minutes at 800*g*. Serum was aspirated from the cell pellet and stored at -20ºC.

2.5 ELISA

A standard direct ELISA protocol was followed as previously described . Each well of a 96 well micro titre plate (MP Biomedical, USA) was coated with 100µl of capture antigen, diluted in carbonate coated buffer to a final concentration of 5µg/ml and incubated for 90 minutes at 37ºC or overnight at 4ºC. Each well was then blocked with 200µl of 5% skim milk in PBS- 2% Tween20 (PBS-T), and incubated for either 90 minutes at 37ºC or overnight at 4ºC. Primary antibody (murine hyper-immune sera) was added to the first row of each plate at a 1:200 dilution in 0.5% skim milk/PBS-T and titrated down the plate in 1:2 dilutions. The primary antibody was incubated for 90 minutes at 37ºC. Plates were washed before the secondary antibody, anti-mouse IgG-HRP conjugate (Invitrogen, USA), diluted 1:3000 in 0.5% skim milk/PBS-T, was added to each well and incubated for 90 minutes at 37ºC. After washing the reactions were developed with o'Phenylenediamine Dihydrochloride substrate (100µl/well) for 30 minutes at room temperature and the absorbance measured at 450nm using Biorad Benchmark spectrometer. Endpoint antibody titre was determined as the lowest dilution that had an absorbance higher than that of the average + 3 standard deviations of negative control serum.

2.6 Immunofluorescence

Antibody binding to the surface of streptococci was visualised by immunofluorescent microscopy . Cells from overnight streptococcal cultures were collected by centrifugation at 1800*g* for 5 minutes and washed with sterile filtered PBS. The cell pellet was then resuspended in 5 times the original culture volume in sterile filtered PBS, to an approximate OD_{600} of 0.1. Poly-lysine slides (Biolabs Scientific, New Zealand) were smeared with 10µl of the bacterial suspension and fixed with 3% paraformaldehyde (w/v) for 10 minutes. Fixed slides were washed in PBS then blocked with 10% foetal calf serum (FCS) in a humidified chamber. Potential Fc-IgG binding by streptococcal surface proteins was blocked by the addition of non-specific human polyclonal IgG (AbD Serotec, UK) diluted 1:200 in 0.5% skim milk PBS-T or 0.1% FCS, in a humidified chamber. Excess non-specific IgG was rinsed off with PBS, and 200µl of the primary antibody (diluted 1:150 in 0.1%

FCS) was added. The primary antibody was removed by extensive washing in PBS before addition of the secondary antibody (anti mouse-IgG-FITC labelled) diluted in 0.1% FCS. The secondary antibody was incubated for 2 hours in the dark, at room temperature. Slides were washed three times for 10 minutes in PBS before a drop of Vecta Shield (Vector Laboratories Inc, USA) was added to each sample, covered with a cover slip and sealed with clear nail polish. Slides were viewed immediately under a Leica Confocal microscope at a wavelength of 520nm, or stored at 4ºC in the dark for viewing at a later period.

2.7 Statistical analysis

The mean and standard error were calculated using standard formulae. Mann Whitney tests were used to compare the antibody titre between two groups. P values were corrected using the Bonferroni method for multiple comparisons. A two tailed $P<0.05$ was considered statistically significant. Statistical comparisons were made using GraphPad Prism Software (GraphPad Software, Inc., USA) and SPSS (Version 15.1 for Windows, USA).

3.0 Results

3.1 Immunogenicity of rJ14, rJJ and fusion protein rJ14-H12.

To assess whether the recombinant vaccine antigens containing J14 could stimulate a humoral response that recognises the parent peptide (pJ14), we tested their immunogenicity in a murine model. Following sub-cutaneous delivery with adjuvant CFA, rJ14 and rJJ were immunogenic, stimulating a strong pJ14 specific serum IgG response. They both elicited a similar J14-specific antibody titre as J14-DT (figure 2a). As J14 is a chimera of streptococcal M-protein and the yeast GCN4-protein sequences, it is essential to assess whether the antibody response observed in the above experiments were indeed directed to the M-protein derived sequences. To test this, we determined the ability of hyper immune sera to bind to p145, a 20-residue peptide from the C-repeat region of the M-protein containing the minimal B-cell epitope defined by J14. P145 does not contain any GCN4 derived sequences. Both rJ14 and rJJ stimulated an antibody response that recognises p145 suggesting that immunisation with these recombinant

chimeric sequences successfully induced an M-protein specific immune response (figure 2b). Unlike the synthetic peptide, this immune response was induced without the presence of a carrier molecule such as DT, suggesting additional residues present in the rJ14 and rJJ, such as hexa-histamine tag, may offer a source of T-cell help in this mouse strain.

In an attempt to design a polytope vaccine against GAS, we combined J14 with a fragment (H12) from the virulence factor SfbI. H12 contains the fibronectin repeat domain . Previous work has shown that the H12 is immunogenic when expressed as a recombinant protein and is protective against intra-nasal GAS challenge . We constructed the fusion protein rJ14H12 and tested its antigenic characteristics in mice. Interestingly, only H12 within the fusion protein is immunogenic following sub-cutaneous immunisation (figure 3) stimulating a strong H12 specific serum IgG response. However, the same cohort of mice responded poorly to the J14 epitope with average pJ14 specific titre of 1000. A possible explanation for this diminished immunogenicity of the J14 entity is that its secondary conformation was altered when expressed with the larger protein H12.

3.2 Optimisation of recombinant J14 for increased alpha-helical conformation. In an effort to increase the conformational stability of J14 when expressed recombinantly, the construct JJo was designed. The primary sequence of JJo has continuous heptad repeats throughout the molecule (figure 4), a typical characteristic of coiled-coil proteins . Situated in positions a and d of the heptad repeat are hydrophobic amino acids which are involved in the stabilisation of two alpha-helical proteins to produce the coiled-coil . Heptad repeats are present throughout nearly the entire length of the M protein and are critical for its function and antigenicity . Accordingly, when fusing the GCN4 heterologous sequence to the J14 sequence, care was taken to ensure that the heptad was maintained throughout the chimera (figure 4) . The continuity of the heptad repeat was also maintained in rJ14, but disrupted in rJJ due to cloning artefacts. We then removed three amino acids (VDK) from rJJ, introduced a restriction enzyme site and the first lysine residue of the second J14 moiety. This enabled a continuous heptad repeat

throughout both of the J14 entities (figure 5). Furthermore, a four residue alanine spacer was introduced at the C-terminus as a flexible hinge when linking a downstream fusion peptide. The resulting optimised sequence was termed JJo.

3.2 Alpha-helical content of recombinant J14 antigens. The ability of each of the recombinant peptides (rJ14, rJJ and rJJo) to assume alpha-helical conformation was experimentally assessed in aqueous solution and in the presence of co-solvent TFE, by circular dichroism. TFE is regularly used to stabilise the alpha-helical structure of proteins in solution . CD spectra in the far UV range were collected for each of the peptides in the presence of 0 – 60% TFE. When dissolved in aqueous solution, the acquired CD spectra for each of the peptide samples tested suggested that they were largely unordered (data not shown). Upon deconvolution of the CD spectra, only JJo was found to contain any alpha-helical conformation in aqueous solution (table 1). Eight percent of JJo were predicted to be alpha-helical while the remaining 92% of the protein were predicted to be unordered. In comparison, rJ14 and rJJ were completely unordered. Similarly, the synthetic peptide pJ14 used as a control was also found to be largely unordered however, deconvolution revealed a small proportion of beta-strand structure (8%).

In the presence of TFE, marked differences were seen in the secondary structure of each of these recombinant antigens. At low concentrations of TFE (10%) the CD spectrum of rJJo was that of a typical alpha-helical protein (figure 5a). Two negative minima of similar magnitude were seen at 209nm and 222nm and a positive band at 190nm. However, at the same TFE concentration, the spectra for rJ14 and rJJ, were shallower at 222nm and the minima seen at 209nm shifted towards the lower wavelengths. A similar spectrum was observed for the synthetic peptide pJ14. Accordingly, deconvolution analysis of the CD spectra seen in figure 5b (10% TFE), yielded 100%, 31% and 14% alpha-helical conformation for rJJo, rJJ and rJ14, respectively. In contrast, pJ14 did not adopt an alpha-helical structure at 10% TFE.

The amount of TFE required to stabilise the alpha-helical structure of each of the samples is represented by TFE helix induction curves (figure 6). At low concentrations of TFE (5-10%), rJJo assumed a complete alpha-helical conformation, whereas recombinant rJJ assumed alpha-helical conformation at 40% TFE. In contrast, both rJ14 and pJ14 ~~were~~ never reached 100% alpha-helical conformation, even in the presence of high concentrations of TFE (60%). The presence of TFE did not alter the alpha-helical conformation of H12 which remained unstructured throughout the TFE titration series.

3.4 Immunological characterisation of optimised J14 constructs.

Given that rJJo has a stronger propensity for alpha-helical conformation we expected this molecule to tolerate the addition of H12 better than rJ14. We therefore constructed a recombinant fusion protein containing JJo at the N-terminus of H12 (rJJoH12). The immunogenicity of rJJo and rJJoH12 were assessed in a murine model and compared to that of the original fusion construct rJ14H12. Both of the recombinant fusion proteins were immunogenic stimulating a whole protein specific *(not quite sure about this phrase)* humoral response (data not shown). However, the immunogenicity of each of the included antigens varied ~~between them~~. rJ14H12 elicited a low J14 specific serum IgG response as previously observed. By contrast, rJJoH12 induced a 200 fold higher average J14-specific titre compared to the original fusion protein ($P > 0.001$)(figure 3). No difference was seen in the ability of each of the fusion proteins to stimulate a H12-specific response. rJJo expressed on its own induced a pJ14 response similar to synthetic peptide conjugate J14-DT (figure 2a) and there was no significant difference in the pJ14 specific response induced by rJJo or rJJoH12. Thus, maintaining the heptad repeat sequence through the tandem J14 entities and the addition of an alanine-spacer in rJJo-H12 has resulted in the elicitation of strong immune responses to both the vaccine antigens (J14 and H12) when expressed as a single recombinant fusion protein.

3.3 JJo antibody cross-reacts with M protein. Previous work has shown that recombinant J14 induced an~~d~~ antibody response that is cross-reactive with the native M-protein. As the changes made in the design of JJo affected

its alpha-helical confirmation as determined by circular dichroism we wanted to confirm that these changes didn't have a detrimental effect on the cross-reactive antibody response against the M protein. The M-protein positive GAS strain, M1, and *emm* negative GAS strain JRS145 were incubated with rJJo and J14-DT antiserum. rJJo antibodies bound strongly to the surface of M1 GAS and not to GAS strain JRS145. Antisera raised to J14-DT also bound strongly to the surface of M1. The fact that rJJo antisera did not react with JRS145 indicated that any fluorescence observed was due to specific binding to the M-protein (figure 7). Similar results were seen when rJJo antiserum was tested by Western blot analysis against multiple GAS serotypes (data not shown) suggesting that rJJo antiserum is broadly cross-reactive with multiple M proteins.

4.0 Discussion

One of the major targets of GAS vaccine research is the highly abundant M protein. The M protein plays a major role in disease pathogenesis, with its coiled-coil conformation essential for the binding of host proteins and also responsible for molecular mimicry with myosin and tropomyosin, the mechanism believed to be responsible for the auto-immune sequelae . Therefore, much consideration has been given to the mapping of the highly conserved, protective epitopes devoid of deleterious human-tissue cross-reactive epitopes. Relf et al proposed that the identified B cell epitopes needed to be folded into a coiled-coil structure to be an effective vaccine candidate . Previously, the minimal B-cell epitope was flanked with sequences from yeast alpha-helical protein GCN4, resulting in the chimeric vaccine candidate J14. While these flanking regions are enough to promote alpha-helical conformation of the 29mer chimeric peptide, in this study we show that the intrinsic forces that maintain this conformation are too weak to maintain the secondary structure when combined with a larger and structurally distinct antigens such as H12. This would be problematic for the development of any recombinant multivalent GAS vaccines aimed at combining the conformationally constrained B-cell epitope with other streptococcal virulence factors.

In this current study, we successfully expressed the conformationally restricted vaccine candidate J14 as a recombinant protein. Both rJ14 and rJJ were immunogenic in mice, stimulating a strong serum IgG response that recognised both the peptide J14 and the parent M-protein derived peptide p145. However, when we expressed J14 as a fusion protein with the H12 fragment of SfbI; we observed a drastic decrease in the ability of the recombinant protein to induce antibodies that recognised pJ14. As it has previously been demonstrated that the M-protein derived B-cell epitope within J14 is conformationally restricted , we hypothesised that H12 was affecting the ability of J14 to assume an alpha-helical conformation, either through its large size and/or independent secondary structure. As rJJ is twice the length of rJ14, it may be a better fusion partner, hence ~~to~~ increasing the alpha-helical propensity of rJJ. Three internal amino acids were removed and a C-terminal alanine spacer was added to produce the structurally optimised antigen JJo. We believe that these changes would render JJo recalcitrant to the effects of H12 when expressed as a fusion partner.

Circular dichroism indeed confirmed that JJo had a higher propensity for alpha-helical conformation. This was supported by TFE helix induction curves. The increased propensity of JJo to adopt alpha-helical structure compared to the original rJJ could be attributed to the continuous heptad periodicity throughout the entire molecule. The fact that rJJ did assume 100% helical conformation in 20% TFE compared to 5% TFE required for rJJo, suggests that rJJ requires more assistance to fold. This is most likely because of less stabilising interactions for an alpha-helical structure in rJJ, due to disturbances in the heptad repeat sequence. In native proteins, these non-optimal sequences are referred to as stutters or slides, which can result in loops or knobs in an alpha-helix . Within the native M protein, the flanking regions provide assistance to overcome the disruption of the heptad repeat and the entire molecule can assume conformation. Most importantly, the lack of alpha-helical structure displayed by synthetic pJ14 may have implications on its efficacy as a vaccine candidate. Previous studies describing the structure of the chimeric peptide found that it was alpha-helical only in the presence of 50% TFE . While these data indicate pJ14 is not alpha-helical in

aqueous solution, the peptide can still capture antibodies against conformational epitopes when it is coated on an ELISA plate. Similarly, mice immunised with pJ14 in aqueous solution can induce an antibody response that recognises the native M-protein . As a consequence, this suggests that a complete alpha-helical secondary structure in aqueous solution is not required to present the M-protein derived conformation epitope. However, we believe that the propensity for alpha-helical conformation is a pre-requisite for an immune response. The ability of JJo to assume an alpha-helical conformation in aqueous solution and complete helical conformation at low levels of TFE suggests it can tolerate external disruptive forces better than rJJ or rJ14.

The structural evidence in favour of JJo is supported by the experimental studies using the murine immunisation model. Following sub-cutaneous immunisation of mice with JJoH12, high titres were observed to both pJ14 and also H12. In comparison to the original J14H12 fusion protein, JJoH12 induced a 200 folder higher response to pJ14. While the increased stability of the alpha-helical conformation of JJo is an essential factor contributing to the immunogenicity of JJoH12, the presence of the alanine spacer domain may also be critical. Commonly considered an alpha-helical breaker, we assume that the alanine spacer acts as a structural hinge, allowing the J14 antigen to assume secondary conformation independent of the larger H12 fibrillar domain.

We confirmed that antisera raised against JJo and JJoH12 recognised pJ14 and M-protein derived p145 by ELISA. In addition, JJo specific antibodies bound the surface of multiple GAS strains. The ability of antibodies raised against JJo to bind to the surface of GAS was re-assuring, in that the stronger alpha-helical propensity of JJo did not alter the antigen characteristics of the minimal B-cell epitope in J14. Recently, McNamara et al similarly observed that the sequence optimisation of the N-terminal end of the M-protein for an ideal alpha-helical conformation, resulted in a decrease in protein functionality and host protein cross reactivity, but had no affect on antigenicity .

In conclusion, we have constructed a modified GAS vaccine antigen JJo, based on the conformationally restricted peptide vaccine J14 that has an

increased propensity for alpha-helical conformation. Using H12 as a model fusion partner, we have demonstrated that JJo is recalcitrant to the structural influences of flanking antigens when expressed as fusion protein. These results re-iterate the importance of considering the conformation of vaccine antigens based on M-protein and demonstrate the feasibility of constructing a recombinant multivalent GAS vaccine containing the safe and highly conserved conformationally restricted B-cell epitope J14.

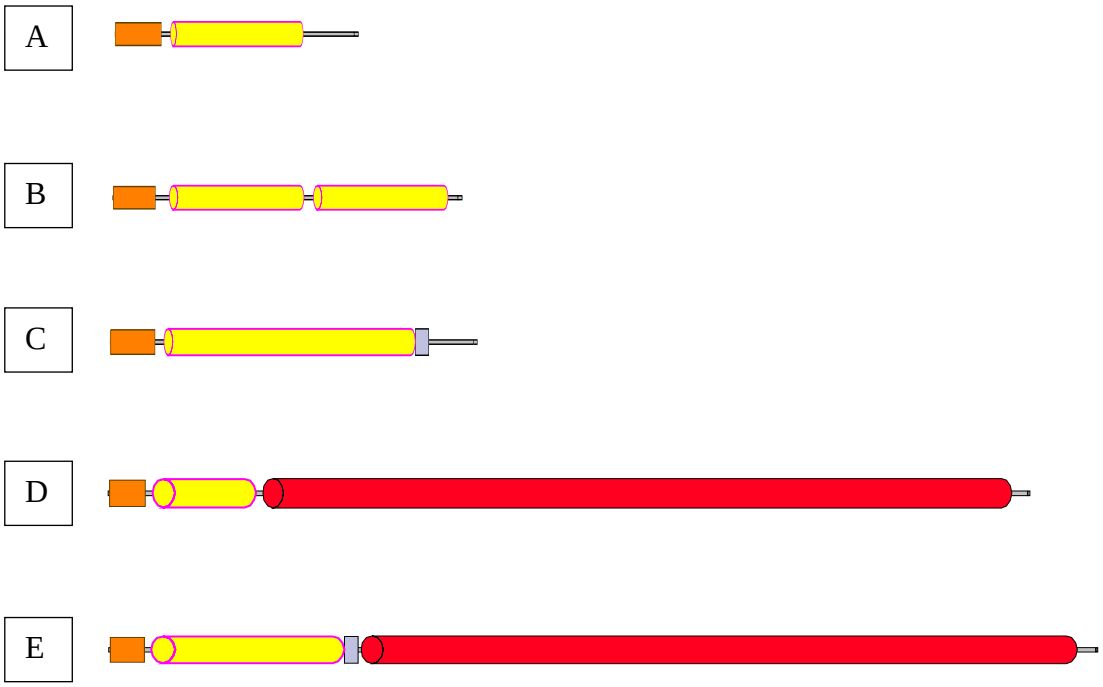


Figure 1 Schematic diagram of recombinant proteins containing the chimeric antigen J14 **A)** rJ14, **B)** rJJ, **C)** rJJo, **D)** rJ14H12, **E)** rJJoH12. The hexa-histidine tag is at the N-terminus of the recombinant proteins and is represented by an orange box. The J14 containing sequence is depicted in yellow. The four residue alanine spacer is depicted in grey, and the H12 domain in red. The diagrams are not drawn to scale.

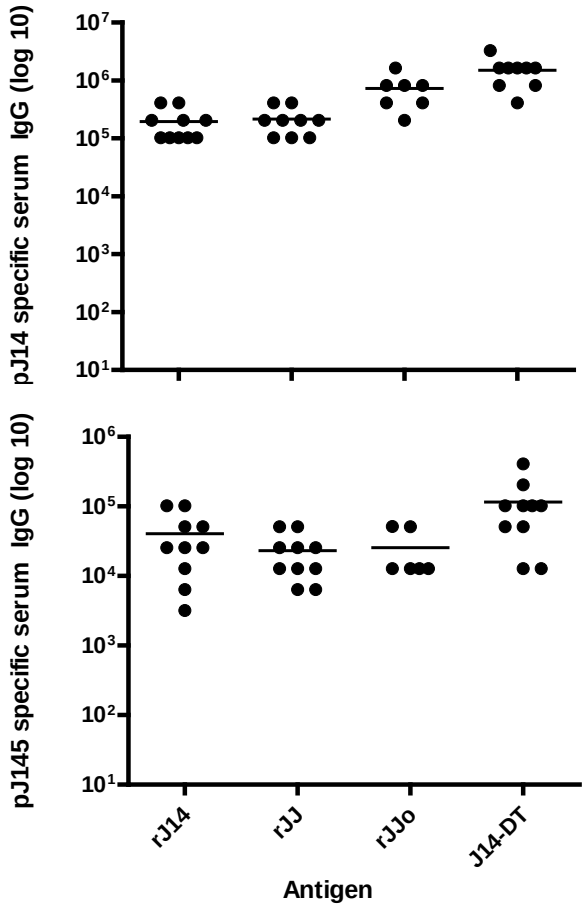


Figure 2 Immunogenicity of the J14 containing recombinant proteins rJ14, rJJ, rJJo and synthetic fusion protein J14-DT following subcutaneous immunisation in the presence of CFA (n=10). **A**: Each of the J14 recombinant proteins was immunogenic in the murine model stimulating strong serum IgG response specific for pJ14. **B:** Antisera raised against each of the J14 containing recombinant proteins recognised the parent M-protein derived peptide p145. There was no significant difference in the p145 specific IgG response between each of the groups (p> 0.05).

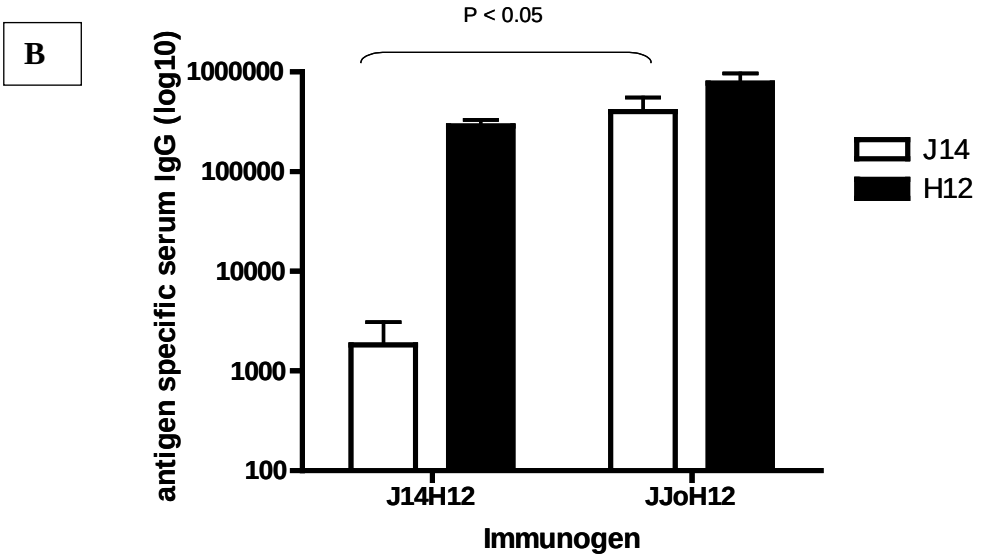


Figure 3 Comparison of the immunogenicity of the J14 and H12 recombinant fusion proteins rJ14H12 and rJJoH12. The original fusion protein rJ14H12 stimulated a strong serum IgG response to the H12 component, however J14 was poorly immunogenic. The fusion protein containing JJo stimulated a strong serum IgG response to both of the included antigens. The J14 specific response induced by rJJoH12 was statistically greater than that of rJ14H12 ($P<0.05$)

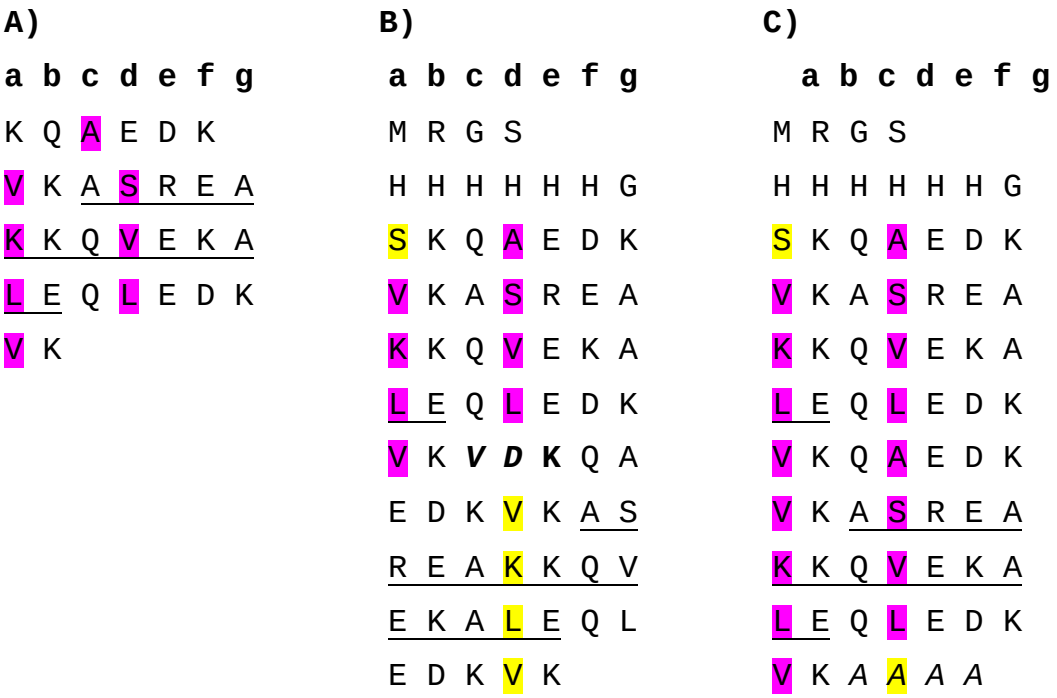


Figure 4 Amino acid sequences of M-protein derived chimeric peptide J14 **(A),** the original recombinant duplicate rJJ **(B)** and the optimised recombinant JJo **(C)**. The heptad repeat amino acid positions are depicted by the letters a-g above the single letter amino acids sequence of the proteins. Positions *a* and *d* of the heptad repeat are highlighted purple when the corresponding amino acid is hydrophobic and the same at that position in pJ14 or is highlighted yellow when the amino acid at that position is different to what is found in pJ14. If the amino acid present at positions *a* or *d* is not hydrophobic, it is not highlighted. The M-protein derived B-cell epitope is underlined in each of the protein sequences. The three amino acids removed from the recombinant J14J14 sequence are in bold. The alanine spacer at the C-terminus of the JJo sequence is italicised.

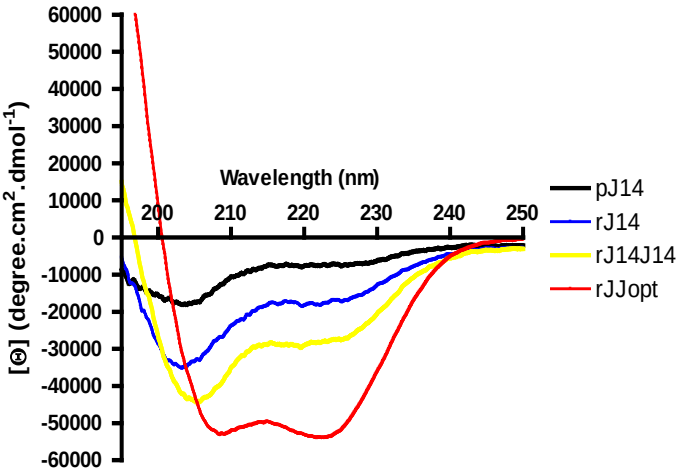
Table 1 Deconvolution[†] of the CD spectra of protein samples in aqueous solution

Protein*	**% Alpha-Helix**	**% β-strand**	**% un-ordered**
pJ14	0	8	91
rJ14	0	0	99
rJ14J14	0	1	99
rJJo	8	0	92
rH12	0	22	77

[†] Deconvolution of the CD spectra was achieved by using linear combination of three Fasman prototype spectra as implemented in the program ACDP

[*] Protein samples were diluted to a concentration of 0.05mg/ml in 10mM Sodium Phosphate Buffer containing 20mM Sodium Chloride pH 7.4.

Table 1



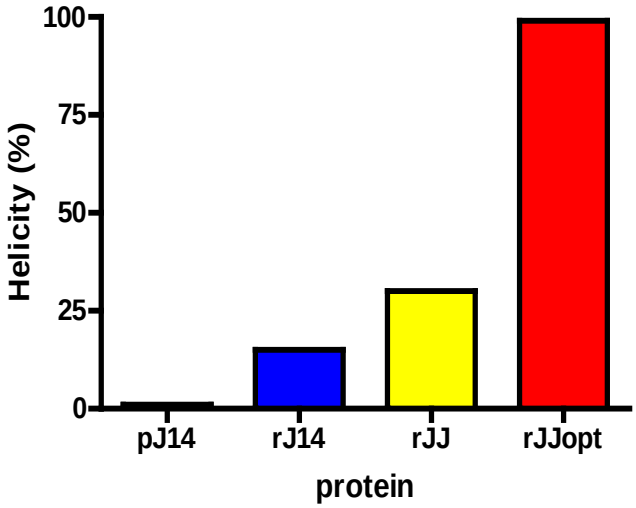


Figure 5 Smoothed CD spectra and secondary structure deconvolution for each of the peptide samples in the presence of 10% TFE. **A:** CD spectra for pJ14 (black), recombinant J14 (blue), recombinant J14J14 (yellow) and recombinant JJo (red) reported in units of mean residue ellipticity [Θ]. **B:** Histogram of the percent (%) helical content of each of the J14 derived peptides in 10% TFE, determined by deconvolution. In 10% TFE, JJo was completely alpha-helical in structure, whereas the other samples varied in their proportion of alpha-helical structure.

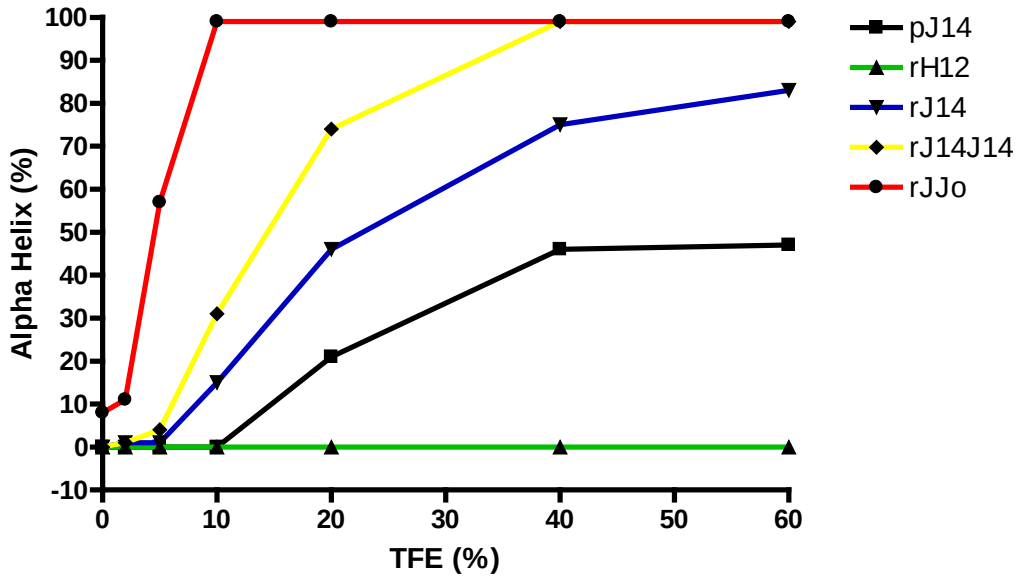


Figure 6 TFE helix induction curves of J14 containing recombinant peptides and recombinant H12. Proteins were titrated in various amounts of TFE from 0%-60% (v/v) in CD buffer (10mM sodium phosphate, 20mM NaCl, pH 7.5).

The CD spectrum acquired at each TFE concentration was deconvoluted and the predicted alpha-helix content of each of the sample was plotted. JJo became completely alpha-helical at low concentrations of TFE. pJ14 never adopted alpha-helical structure, even in the presence of 60% TFE. As expected, H12 had no propensity for alpha-helical conformation

The rJ14J14 curve appears incomplete in the printed version, as the dot at 60% is not visible.

Anti-J14-DT anti-JJo

M1

JRS 145

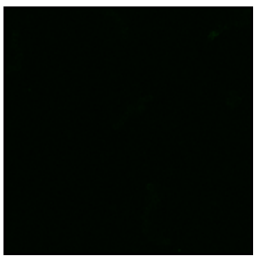


Figure 7 Immunofluorescent microscopy demonstrating binding of J14-DT and JJo antisera to the surface of GAS. Immunofluorescent images are shown on the right of each panel. The corresponding bright field micrographs are shown on the right. Both J14-DT and JJo antisera bound to the surface of M1 but not JRS145. Control sera did not react with either of the strains (data not shown). The scale bar represents 8.83µm. All images were acquired at the same level of magnification.