Title: Seroreactivity against streptococcal DRS (distantly related to SIC) protein is a predictor for ESRF

Short Title: DRS protein is linked with renal failure

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Abstract
We hypothesised that immunoreactivity against antigens from nephritic strains of *Streptococcus pyogenes* may be elevated in patients with ESRF. Additionally, we investigated whether a difference in seroreactivity exists between non-Indigenous and Indigenous patients. To examine these possibilities, antibodies against potentially nephritogenic proteins, streptokinase (Ska1, from M1), streptococcal pyrogenic exotoxin type B (SpeB, from M1), the streptococcal inhibitor of complement mediated cell lysis (SIC, from M1) and its two variants, closely related to SIC (CRS, from M57) and distantly related to SIC (DRS, from M12) were determined by ELISA in 66 patients and 31 healthy controls. A significantly higher proportion of patients compared to controls were seropositive to Ska1 (*P*=0.004), DRS (*P*=0.0003), CRS (*P*=0.001) and SIC (*P*=0.018). Regression analysis showed seroreactivity to DRS (*r*²=0.85, *P*=0.001) predicted the development of ESRF, being diabetic was positively associated with being an ESRF patient (*r*²=0.37, *P*<0.0001) and being Indigenous (*r*²=0.47, *P*<0.0001). These results suggest that these ESRF patients were exposed to strains of *S. pyogenes* that secrete Ska1, DRS, CRS and SIC, and may have a pathological significance. No significant difference was observed between Indigenous patients and the non-Indigenous patients.

Introduction
Post-streptococcal glomerulonephritis (PSGN) is an autoimmune sequelae that occurs in a minority of patients following infection with *Streptococcus pyogenes*. Only strains of *S. pyogenes* with certain M types have historically been associated with PSGN and much research has been conducted to identify the nephritogenic antigens responsible. Several antigens are suspected to be nephritogenic: streptococcal inhibitor of complement mediated cell lysis (SIC) and its variants, closely related to SIC (CRS), and distantly related to SIC (DRS); extracellular products including M protein; nephritis-strain associated protein; pre-absorbing antigen; nephritis-associated plasmin receptor and a zymogen encoded by streptococcal pyrogenic exotoxin type B gene (5,12,13,15,16,18). However, further large-scale epidemiological research is required to establish cause and effect relationships for candidate nephritogenic antigens.

PSGN has been shown to be associated with long-term renal complications (17) and is a risk factor for end-stage renal failure (ESRF). However, the exact number of cases of
ESRF that can be attributed to PSGN is unknown. To date, all research conducted on nephritogenic antigens has focused on animal models or studies in those exhibiting acute PSGN. Therefore, in this study we investigated responses to several putative nephritogenic antigens in patients treated with hemodialysis for ESRF and focused on two main questions: 1) antibody responses to the antigens in those with established disease compared with healthy controls and 2) the difference in antibody response between Indigenous and non-Indigenous patients. Five antigens were investigated in this study; streptokinase (Ska1), the streptococcal pyrogenic exotoxin type B in its zymogen form (SpeB), the SIC protein and its two variants (CRS and DRS). All of these antigens have been implicated in the pathogenesis of PSGN.

Streptokinase is produced by all strains of *S. pyogenes* and group C and G streptococci however; it has been shown that different alleles of the gene, *ska*, are associated with differing disease manifestations (12). *Ska1* is a nephritic allele of streptokinase from the M1 type strain which is associated with outbreaks of PSGN and it has been shown in a mouse model that the development of PSGN is largely dependent on the allele present (12).

Interest in SpeB’s role in the pathogenesis of PSGN is due to three observations. Firstly, nephritis-associated strains of *S. pyogenes* preferentially secrete SpeB (14). Secondly, there is serological reactivity against SpeB in the sera of PSGN patients (5,13). Thirdly, the cationic protein, SpeB, has been detected in a large proportion of PSGN biopsies (5).

The *sic* gene is present in the *mga* regulon of M1 strains, the M57 strains possess a homologous protein, CRS, located elsewhere in the genome and a gene with partial homology to *sic*, called *drs* (distantly related to *sic*) has been found in M12 and M55 strains (3). While SIC interferes with complement function *in vitro* by inhibiting binding of the membrane attack complex to the cell membrane (6), DRS binds C6 and C7, but has no significant effect on complement activity (4). Despite this fact, a possible relationship has been suggested between antibodies against DRS and PSGN (16). The proteins SIC, CRS and DRS are expressed only by M types of *S. pyogenes* suggested to be nephritogenic.
First, given the link between PSGN and ESRF, we hypothesised that patients being treated for ESRF would have elevated levels of specific anti-streptococcal antibodies. Second, since streptococcal skin infections and PSGN are widespread amongst Indigenous communities (7), and rates of ESRF are as much as ten times that of non-Indigenous Australians, it was hypothesised that this population would have elevated levels of antibodies compared to non-Indigenous patients. A finding of elevated antibody levels in Indigenous patients would reflect repeated exposure to nephritogenic antigens resulting in progressive renal disease and ultimately ESRF.

Materials and Methods

Patients and controls

Patients currently being treated for ESRF with hemodialysis at The Townsville Hospital (QLD, Australia) were recruited for participation in the trial. Sera from 66 patients (age range: 18-68) being treated with hemodialysis were investigated with a male/female ratio of 1.28 and 56% of patients identifying as being of Aboriginal and/or Torres Strait Islander (i.e. Indigenous) descent. Serum samples were collected prior to the commencement of dialysis and stored at -80°C until used. Antibodies were also measured in sera collected from 31 age-matched (based on mean; range 20-75) healthy controls with no known history of kidney disease: male/female ratio=0.94, and 48% identified as being of Aboriginal and/or Torres Strait Islander descent. Four of the Indigenous controls were diabetic, 27 of the Indigenous ESRF patients were diabetic and 6 non-Indigenous ESRF patients were diabetic. This study was based on informed consent and received ethical approval (James Cook University: H2394, Townsville hospital: 03-04).

Bacterial strains and DNA

*S. pyogenes* strain 2031 (*emm1*) was obtained from the Group A Streptococcus Reference Laboratory in Prague, Czech Republic. NS488 (*emm12*) and BSA5 (*emm57*) are Northern Territory isolates and were obtained from the Queensland Institute of Medical Research. All GAS strains were routinely propagated at 37°C on horse blood agar (BioMérieux) or in liquid cultures of Todd-Hewitt broth (Oxoid), supplemented with 1% w/v yeast extract. *Escherichia coli* BL21 strains were cultured at 37°C on Luria Burtani
(LB) agar or in LB broth with agitation at 200 rpm. Where appropriate, *E. coli* strains were grown in the presence of kanamycin (25 µg/mL) and ampicillin (100 µg/mL).

Template preparation for PCR was performed using the alkali lysis procedure previously described (8).

Cloning, expression and purification of recombinant proteins

Using polymerase chain reaction, the *ska1, sic1, crs57, drs12* and *speB* genes were amplified with specific primers (Table 1). The amplified products obtained with the SIC, DRS and CRS primers were subsequently cloned into the pBAD-TOPO-TA (thiofusion) expression system (Invitrogen, Australia), while the amplified Ska1 and SpeB products were cloned into the pQE30 vector (Qiagen) upstream of the His6 tag. The pQE30 vector was used to produce Ska1 and SpeB, because use of the pBAD-TOPO-TA vector resulted in the formation of inclusion bodies during protein production. The pQE30 constructs were transformed into BL21 cells harbouring the pREP4 repressor plasmid (Qiagen). All transformants were screened by PCR and sequence analysis was performed (Macrogen, Korea) to confirm positive clones.

One-litre cultures of BL21 containing the recombinant plasmid were grown until the optical density at 600 nm reached 0.5. Protein production was induced over 4 hours using 0.02% L-arabinose in the pBAD-TOPO-TA vector and 1M IPTG in the pQE30 vector.

To purify the recombinant thioredoxin-fusion proteins and SpeB protein, under non-denaturing conditions, cell pellets from induced cultures were sonicated to lyse the cells and centrifuged to remove the insoluble cellular debris. The expressed recombinant proteins were isolated from the resultant cleared lysate by using a column with Ni-nitrilotriacetic acid matrix (Qiagen). The recombinant proteins were eluted with an imidazole (ICN Biomedical) gradient (20 to 300 mM) and dialyzed against phosphate-buffered saline (PBS). Clones expressing only the thioredoxin fusion protein were also obtained as controls and the protein was purified as above.

Ska1 was purified under denaturing conditions. Briefly, following centrifuge of the cultured broth, the insoluble pellet was solubilised in 8M urea for 1 hour and the resulting lysate centrifuged at 10, 000 x g for 20 minutes. The supernatant was mixed with the Ni-
NTA matrix at room temperature for 1 hour, eluted using urea at pH gradients (6.3-4.5) and dialysed against PBS.

Analysis of Proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on all protein eluates as previously described (9). The majority of the SIC and SIC-variant thioredoxin fusion proteins were intact, and migrated between 30 and 50 kDa. Ska1 was observed at a band corresponding to approximately 47 kDa and SpeB was purified in its inactive zymogen form at a band of approximately 37 kDa. Figure 1 shows the expression of the recombinant thioredoxin-fusion proteins and the recombinant Ska1 and SpeB proteins on the Coomassie blue-stained gel (Figure 1).

Western blot

Following SDS-PAGE analysis the fractionated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) by using a wet Western transfer apparatus (Bio-Rad) at 50V for 2 hours. The membrane was then removed from the transfer tank and allowed to air-dry overnight at room temperature, thus preventing the requirement for a blocking step. For the identification of the SIC and SIC variant recombinant proteins, the blotted membrane was probed with CRS and DRS specific rabbit antiserum at 1 in 1000 dilution (IMVS, Gilles Plains, South Australia) by immunising rabbits with recombinant M1 SIC (pQE30, Qiagen) from 2031 (anti-CRS) or recombinant M12 DRS (pQE30) from NS488, as previously reported (4). Goat anti-rabbit (Heavy + Light chains) horseradish peroxidase (HRP)-conjugate IgG (Pierce Biotechnology, Australia) was used as the secondary antibody at 1 in 2000 dilution. For the identification of Ska1 and SpeB, mouse anti-(His)$_4$ antibody (Qiagen) was used at a 1 in 1000 dilution to detect the His-tag. Goat anti-mouse (Heavy + Light chains) HRP-conjugate IgG (Pierce biotechnology) was used as the secondary antibody diluted 1 in 2000. Membranes were washed three times between each step and prior to visualisation using the 3,3’-diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (Figure 2).

ELISA studies
An indirect ELISA was used to detect streptococcal antibodies in sera. Unless otherwise stated, all steps were performed using 100 µl of each reagent with a reaction period of 1 hour at room temperature (28°C±2°C) and between each step the plate was washed five times with wash buffer (TropBio, Australia). U-bottom immunoplates (Sarstedt, Australia) were coated by adding recombinant protein (100 µg/ml) in ELISA diluent (TropBio) to each well and incubating overnight. After blocking with 150 µl of post-coating buffer (TropBio) for 2 hours, the bound proteins were allowed to react with a 1 in 300 dilution of human serum in ELISA diluent. After treating with a secondary goat anti-human IgG (H+L) HRP-conjugate (Pierce Biotechnologies), the reactions were detected with 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (TropBio). The optical density (OD) was measured using dual absorbance of 414 and 492 nm after incubation in the dark for 1 hour.

Statistical methods

Normal population seroreactivity for the investigated proteins has not been established; therefore we used a cut-off for positive serology based on three times the average of the lowest quartile of the studied population’s absorbance values, as previously described (16). Fisher’s exact test (2-tailed) was used to calculate significance levels for relationships between positive serology to various streptococcal proteins and having ESRF. The data in this study was transformed to ensure a Gaussian distribution. For the ELISA studies comparing the level of antibodies amongst different groups of the population (Indigenous controls, non-Indigenous controls, Indigenous patients and non-Indigenous patients), the one-way analysis of variance was used followed by the Tamhane’s post-hoc test with an α=0.05. The null hypothesis assumes that all groups have similar levels of antibodies against each of the streptococcal antigens. The Pearson’s rank correlation test was used for analysis of correlations. All the statistical calculations were done using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA).

Results

Comparative antibody levels between ESRF patients (n=66) and healthy controls (n=31) A large proportion of patients and controls had detectable antibodies against Ska1, DRS, CRS, SIC and SpeB. In general patients had higher antibody levels than controls (Figure...
3). The proportion of ESRF patients with a seropositive result for Ska1 ($P=0.004$), DRS ($P=0.0003$), CRS ($P=0.001$) and SIC ($P=0.018$) was significantly greater compared to the proportion seropositive in the controls but no significant difference was observed for SpeB ($P=0.0538$).

Antibodies against streptococcal proteins between groups

As predicted, Indigenous patients ($n=37$) had elevated levels of antibodies to each of the streptococcal proteins compared to the non-Indigenous patients ($n=29$), however these differences were not statistically significant (Figure 4). Overall ($n=97$) there was a significant difference between groups for Ska1 ($P=0.001$), SIC ($P=0.024$), CRS ($P=0.016$) and DRS ($P<0.0001$) but not SpeB ($P=0.16$). Indigenous patients had significantly higher antibodies than both the Indigenous ($n=15$) and non-Indigenous ($n=16$) controls for Ska1, SIC, CRS and DRS.

Correlations between antibodies in the different groups

Relationships between antibody levels were investigated for the patient group ($n=66$, Table 2). In the patient group all antibodies were closely correlated and were significant at the $\alpha=0.0001$ except SIC and SpeB. In the control group ($n=31$), only antibody levels to CRS correlated with those of SIC, Ska1 and SpeB and antibody levels to SIC correlated with those against DRS and SpeB.

Relationship between antibody levels, diabetes and ESRF

Being diabetic was positively associated with being an ESRF patient ($r^2=0.37$, $P<0.0001$) and being Indigenous ($r^2=0.47$, $P<0.0001$). Regression analysis showed antibodies to DRS ($r^2=0.85$, $P=0.001$) predicted ESRF development. No other associations was found between streptococcal antibodies and ESRF at $\alpha=0.05$ level.

Discussion

Four M types of S. pyogenes are classified as ‘nephritic’ based on an association with epidemics of acute glomerulonephritis. Recent studies have shown that certain streptococcal proteins are associated with the development of PSGN as demonstrated by elevated titres and glomerular antigen deposition in PSGN patients. These findings suggest that these antigens play a role in the development of acute glomerulonephritis.

Although it has been proposed that exposure to these proteins is associated with long-term
renal complications, the specific serological responses in ESRF patients remains uncertain. In the present study, we used ELISA to evaluate the level of serum antibodies against 5 nephritic streptococcal antigens in patients with established ESRF. We observed that ESRF patients had specific elevated antibodies to Ska1, SIC, CRS and DRS compared to healthy controls. Further no significant difference was observed between Indigenous and non-Indigenous patients. This is the first study to indicate that exposure to these streptococcal proteins is associated with long-term renal complications.

We hypothesised that Indigenous compared with non-Indigenous patients would have significantly higher antibody levels. In the current study, Indigenous patients had elevated levels of antibodies to all five streptococcal antigens; however the difference was not significant. It is possible that in a larger sample a difference would be observed because on post-hoc analysis Indigenous patients differed from all controls for Ska1, DRS, CRS and SIC. Non-Indigenous patients had a significant difference only for DRS compared to Indigenous controls. Additionally, Indigenous patients had significantly elevated antibodies compared to Indigenous controls. In this study, Indigenous patients and controls were from the same geographical area, where streptococcal infections are endemic, therefore it is unlikely that this difference represents an increased incidence of streptococcal skin infections in childhood but more likely represents an increased infection rate with nephritic strains of group A streptococcus.

This study provides evidence to support the hypothesis that the current epidemic of renal failure in the Australian Indigenous population may be a result of recurrent streptococcal skin infections and sporadic episodes of PSGN. It is postulated that complications of PSGN may manifest clinically later in life as progressive renal disease and ultimately ESRF. Furthermore the results are not limited to the Australian Indigenous population as a proportion of non-Indigenous patients in this study had specific elevated streptococcal antibodies, which may be implicated in their disease pathology. This finding was expected given the research was conducted in a tropical location where streptococcal skin infections such as impetigo are common (10). The M types isolated from streptococcal skin infections are associated with the development of PSGN.

Despite the fact that antibodies titres to SpeB in its zymogen form are currently the best-known marker for *S. pyogenes* infections associated with acute PSGN (12), in this study
no difference was observed between any of the groups. Furthermore, high titres to SpeB are also associated with group A streptococcal infections in general (1). Therefore, it is possible that SpeB titres are a marker of acute infections and glomerulonephritis, but the antibodies are not long lasting. It has previously been shown that anti-SpeB titres tend to rise and peak within the first two weeks of onset of disease and then lower with time (13). The group of patients analysed in this study were adults with established ESRF and therefore it would be years since a possible childhood episode of PSGN.

Unlike streptokinase and SpeB, the SIC and SIC-variant proteins are only secreted by M types that are historically associated with PSGN. Thus, patients exhibiting higher antibodies to these antigens suggests that they have been infected with these so-called ‘nephritic’ M types of \textit{S. pyogenes}. The findings of this study support the proposal by Sriprakash \textit{et al.} (16), that seroreactivity to DRS may be of pathological significance. However, the patients in the current paper had clearly established renal disease whereas in previous papers determination of a history of PSGN has been conducted retrospectively using scarce medical records and therefore include bias. In the present study, the control group were healthy and therefore it is possible that the serological changes may reflect non-specific factors associated with kidney failure and/or dialysis treatment. However the statistical analysis used to determine a cut-off for seropositivity incorporated all sample data and not just healthy controls, therefore allowing renal failure patients to be either seropositive or seronegative depending upon the ELISA result.

This research has demonstrated that ESRF patients have significantly higher prevalence of seropositivity against Ska1, SIC, CRS and DRS, compared to controls and shown that elevated antibodies to DRS is a predictor for ESRF. This is the first study to suggest elevated antibodies to Ska1, SIC and SIC-variant proteins might be of pathological significance in ESRF. This is possible via antigen-antibody deposition, or the long-term presence of elevated streptococcal antibodies and future studies should be conducted with larger sample sizes and should include the review of renal biopsies to determine if, 1) the candidate antigens are deposited in the glomeruli and 2) if there is evidence of PSGN pathology.
Acknowledgement

This work was supported by a Biomedical Scholarship from Kidney Health Australia and by a Smart State Grant from the Queensland Government.
References


Titles and legends to figures

Figure 1. The expression of several recombinant streptococcal proteins on a Coomassie stained SDS-PAGE gel. The antigens run in each lane are: 1 is streptokinase, 2 is streptococcal pyrogenic exotoxin type B, 3 is thioredoxin, 4 is CRS, 5 is DRS, and 6 is SIC. Note: the arrows denote the purified protein band of interest, SIC: streptococcal inhibitor of complement, CRS: closely related to SIC and DRS: distantly related to SIC, protein ladder used was #SM0431 (Fermentas, Australia).

Figure 2. The antigens run in each lane are 1 is streptokinase (Ska1), 2 is streptococcal pyrogenic exotoxin type B (SpeB) in its zymogen form, 3 is CRS, 4 is DRS and 5 is SIC. Tetra-his antibody was used as a positive control for the detection of Ska1 and SpeB, polyclonal rabbit SIC antiserum was used to detect CRS and SIC, and anti-DRS rabbit polyclonal antibodies were used to detect DRS. Note: the arrows denote the reaction of the antibody with the protein of interest, SIC: streptococcal inhibitor of complement, CRS: closely related to SIC and DRS: distantly related to SIC, protein ladder used was #SM0431 (Fermentas, Australia).

Figure 3. Graph showing mean (± SEM) optical densities obtained for each streptococcal protein analysed according to group. Using an ELISA, the sera from 31 controls and 66 ESRF patients were compared for seroreactivity (measured in OD units) to 5 streptococcal recombinant antigens.

Figure 4. Scatterplots of sera from Indigenous patients show greater reactivity to Ska1, DRS, CRS and SIC streptococcal antigens than other experimental groups. Using an ELISA, the sera from 15 Indigenous controls, 16 non-Indigenous controls, 37 Indigenous patients and 29 non-Indigenous patients were compared for seroreactivity (measured in OD units) to 5 recombinant streptococcal antigens. The significant results of statistical analyses of the mean serological responses are shown (calculated using Tamhane’s post-hoc test). Solid horizontal lines represent the means of each category and dotted horizontal lines show the cut-off for seropositivity (three times the overall mean of the lowest quartile).
Table 1. Primers used in this study to amplify streptococcal genes.

<table>
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<th>Reference</th>
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Table 2. Pearson correlation coefficients ($r^2$) between antibodies to streptococcal antigens in patients ($n=66$) and controls ($n=31$). Significant correlation coefficients ($r^2$) are written in bold with associated $P$ values in parenthesis.

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Figure 1.
Figure 2.
Figure 3.
Closely Related to SIC

Streptococcal Inhibitor of Complement Mediated Cell Lysis (SIC)
Figure 4. Streptococcal Pyrogenic Exotoxin Type B

Optical Density

Indigenous Controls  Non-Indigenous Controls  Indigenous Patients  Non-Indigenous Patients

0.38