Seroreactivity against Streptococcal DRS (Distantly Related to SIC) Protein Is a Predictor for End-Stage Renal Failure

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We hypothesized that immunoreactivity against antigens from nephritogenic strains of Streptococcus pyogenes may be elevated in patients with end-stage renal failure (ESRF). Additionally, we investigated whether a difference in seroreactivity exists between nonindigenous and indigenous (Aboriginal/Torres Strait Islander) patients. To examine these possibilities, antibodies against potentially nephritogenic proteins, streptokinase (Skα1) (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 in 66 patients and 31 healthy controls by enzyme-linked immunosorbent assays. A significantly higher proportion of patients compared to controls were seropositive to Skα1 (P = 0.004), DRS (P = 0.0003), CRS (P = 0.001), and SIC (P = 0.018). Regression analysis showed that seroreactivity to DRS (r² = 0.85, P = 0.001) predicted the development of ESRF and that being diabetic was positively associated with being an ESRF patient (r² = 0.37, P < 0.0001) and being indigenous (r² = 0.47, P < 0.0001). These results suggest that these ESRF patients were exposed to strains of S. pyogenes that secrete Skα1, DRS, CRS, and SIC and may have pathological significance. No significant difference was observed between the indigenous patients and nonindigenous patients.

Poststreptococcal glomerulonephritis (PSGN) is an autoimmune sequela 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; preabsorbing antigen; and 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: (i) antibody responses to the antigens in those with established disease compared with healthy controls and (ii) the difference in antibody response between indigenous (Aboriginal/Torres Strait Islander) and nonindigenous patients. Five antigens were investigated in this study; streptokinase (Skα1), the streptococcal pyrogenic exotoxin type B in its zymogen form (SpeB), and 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. First, nephritis-associated strains of S. pyogenes preferentially secrete SpeB (14). Second, there is serological reactivity against SpeB in the sera from PSGN patients (5, 13). Third, the cationic protein, SpeB, has been detected in a large proportion of PSGN biopsy specimens (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 drc (distantly related to sic) has been found in M12 and M55 strains (2). While SIC interferes with complement function in vitro by inhibiting binding of the membrane attack complex to the cell membrane (7), DRS binds C6 and C7 but has no significant effect on complement activity (3). Despite this fact, a possible relationship has been suggested between

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antibodies against DRS and PSGN (16). The proteins SIC, CRS, and DRS are expressed only by M types of \textit{S. pyogenes} suggested to be nephritogenic.

First, given the link between PSGN and ESRF, we hypothesized that patients being treated for ESRF would have elevated levels of specific antistreptococcus antibodies. Second, since streptococcal skin infections and PSGN are widespread among indigenous communities (6) and rates of ESRF are as much as 10 times that of nonindigenous Australians, it was hypothesized that this population would have elevated levels of antibodies than the levels in nonindigenous 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 (Queensland, Australia) were recruited for participation in the trial. Sera from 66 patients (age range, 18 to 68 years) being treated with hemodialysis were investigated. For these patients, there was a male/female ratio of 1.28, and 56% of patients were identified 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 (range, 20 to 75 years) healthy controls with no known history of kidney disease. For the controls, there was a male/female ratio of 0.94, and 48% were identified as being of Aboriginal and/or Torres Strait Islander descent. Four of the indigenous controls were diabetic, and 6 nonindigenous ESRF patients were diabetic. This study was based on informal consent and received ethical approval (James Cook University ethical approval reference number, H2394; Townsville Hospital ethical approval reference number, 03-04).

**Bacterial strains and DNA.** \textit{S. pyogenes} strain 2031 (emm1) was obtained from the Group A Streptococcus Reference Laboratory in Prague, Czech Republic. NS488 (emm12) and BSAS5 (emm57) are Northern Territory isolates and were obtained from the Queensland Institute of Medical Research. All group A streptococcus strains were routinely propagated at 37°C on horse blood agar (BioMérieux) or in liquid cultures of Todd-Hewitt broth (Oxoid), supplemented with 1% (wt/vol) yeast extract. \textit{Escherichia coli} BL21 strains were cultured at 37°C on Luria-Bertani (LB) agar or in LB broth with agitation at 200 rpm. Where appropriate, \textit{E. coli} strains were grown in the presence of kanamycin (25 \(\mu\)g/ml) and ampicillin (100 \(\mu\)g/ml). Template preparation for PCR was performed using the alkaline lysis procedure previously described (8).

**Cloning, expression, and purification of recombinant proteins.** Using PCR, the \textit{skal, sicl, crs57, drs12,} and \textit{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 \textit{Ska1} and SpeB products were cloned into the pQE30 vector (Qiagen) upstream of the His, tag. The pQE30 vector was used to produce \textit{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 \textit{E. coli} BL21 cells harboring the pREP5 repressor plasmid (Qiagen). All transformants were screened by PCR, and sequence analysis was performed (Macrogen, Korea) to confirm positive clones.

**TABLE 1. Primers used in this study to amplify streptococcal genes**

| Gene     | S. pyogenes strain | M type | Primer                                           | Reference |
|----------|--------------------|--------|-------------------------------------------------|-----------|
| skal     | 2031               | M1     | Forward, 5′-AGGATATCAGAAAATACTTATCTCTTAC          | This study |
|          |                    |        | Reverse, 5′-GGGTCGACTTTGTCTTTAGGTTTACAGG         |           |
| sicl     | 2031               | M1     | Forward, 5′-CTAAGGAGCCTACAAACCA                  | 4         |
|          |                    |        | Reverse, 5′-CAAGTACCTTTTATATTCG                  |           |
| crs57    | BSAS5              | M57    | Forward, 5′-CTAAGGAGCCTACAAACC                   | 2         |
|          |                    |        | Reverse, 5′-CGTTCGATGTTGTTATAGG                  |           |
| drs12    | NS488              | M12    | Forward, 5′-CTAAGGAGCCTACAAACC                   | 3         |
|          |                    |        | Reverse, 5′-TTTAATAACCTCAGGAAATACCTCT            |           |
| speB     | 2031               | M1     | Forward, 5′-AGGATATCAGAAAATACTTATCTCTTAC          | This study |
|          |                    |        | Reverse, 5′-GGGTCGACAGTTTGTAGGTTTACAGG           |           |

One-liter cultures of \textit{E. coli} BL21 containing the recombinant plasmid were grown until the optical density at 600 nm (OD_{600}) reached 0.5. Protein production was induced for 4 h using 0.02% L-arabinose in the pBAD-TOPO-TA vector and 1 M isopropyl-\(\beta\)-thiogalactopyranoside (IPTG) in the pQE30 vector. To purify the recombinant thioredoxin fusion proteins and SpeB protein, under nondenaturing 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. Clones expressing only the thioredoxin fusion protein were also obtained as controls, and the protein was purified as described above.

\textit{Ska1} was purified under denaturing conditions. Briefly, following centrifugation of the cultured broth, the insoluble pellet was solubilized in 8 M urea for 1 h, and the resulting lysate was centrifuged at 10,000 \(\times\) g for 20 min. The supernatant was mixed with the Ni-nitrilotriacetic acid matrix at room temperature for 1 h, eluted using urea at pH gradients (6.3 to 4.5), and dialyzed against phosphate-buffered saline.

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

**Western blot.** Following SDS-polyacrylamide gel electrophoresis analysis, the fractionated proteins were transferred to polyvinylidene difluoride membrane (Millipore) by using a wet Western transfer apparatus (Bio-Rad) at 50 V for 2 h. 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 antisera at a dilution of 1 in 1,000 (Institute of Medical and Veterinary Sciences, Gilles Plains, South Australia, Australia) by immunizing rabbits with recombinant M1 SIC (pQE30; Qiagen) from strain 2031 (anti-ERSF) or recombinant M12 DRS (pQE30) from isolate NS488 as previously reported (4). Goat anti-rabbit (heavy and light chain) horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (Pierce Biotechnology, Australia) was used as the secondary antibody at a dilution of 1 in 2,000. For the identification of \textit{Ska1} and SpeB, mouse anti-His antibody (Qiagen) was used at a dilution of 1 in 1,000 to detect the His tag. Goat anti-mouse (heavy and light chain) HRP-conjugated IgG (Pierce Biotechnology) was used as the secondary antibody diluted 1 in 2,000. Membranes were washed...
three times between each step and prior to visualization using the 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (Fig. 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 h at room temperature (28°C ± 2°C). For ELISA studies comparing the level of antibodies among different groups of the population (indigenous controls, nonindigenous controls, indigenous patients, and nonindigenous patients), one-way analysis of variance was used followed by Tamhane’s posthoc test with an $\alpha$ level of 0.05. The null hypothesis assumes that all groups have similar levels of antibodies against each of the streptococccal antigens. 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).

**RESULTS**

**Comparative antibody levels between ESRF patients ($n = 66$) and healthy controls ($n = 31$).** A large proportion of patients and healthy controls had detectable antibodies against Ska1, DRS, CRS, SIC, and SpeB. In general, patients had higher antibody levels than the healthy controls did (Fig. 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 nonindigenous patients ($n = 29$); however, these differences were not statistically significant (Fig. 4). Overall, for all patients ($n = 97$), there was a significant difference between the 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 nonindigenous ($n = 16$) controls for Ska1, CRS, SIC, 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 $\alpha = 0.0001$ level except SIC and SpeB. In the control group ($n = 31$), only antibody levels to CRS were 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 titers 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 remain uncertain. In the present study, we used an ELISA to evaluate the level of serum antibodies against five 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 nonindigenous patients. This is the first study to indicate that exposure to these streptococcal proteins is associated with long-term renal complications.

We hypothesized that compared with nonindigenous patients, 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 posthoc analysis indigenous patients differed from all controls for Ska1, DRS, CRS, and SIC. Nonindigenous patients had a significant difference only for DRS compared to indigenous controls. Additionally, compared to indigenous controls, indigenous patients had significantly elevated antibody levels. 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 nonindigenous patients in this study had specific elevated streptococcal antibodies, which may be implicated in their disease pathology. This finding was expected, given the fact that 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 antibody titers to SpeB in itszymogen 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 titers to SpeB are also associated with group A streptococcal infections in general (1). Therefore, it is possible that SpeB titers are a marker of acute infections and glomerulonephritis, but the antibodies are not long lasting. It has previously been shown that anti-SpeB titers tend to increase and peak within the first 2 weeks of onset of disease and then decrease with time (13). The group of patients analyzed 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 protein and SIC variant proteins are secreted only by M types that are historically associated with PSGN. Thus, the fact that patients exhibit higher antibody levels 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 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 was healthy, and therefore, it is possible that the serological changes may reflect nonspecific factors associated with kidney failure and/or dialysis treatment. However, the statistical analysis used to determine a cutoff 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 healthy 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

FIG. 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 nonindigenous controls, 37 indigenous patients, and 29 nonindigenous patients were compared for seroreactivity (measured in OD$_{414}$ units) to five recombinant streptococcal antigens. The significant results of statistical analyses of the mean serological responses are shown (calculated using Tamhane’s post hoc test). The solid horizontal lines represent the means for the groups, and the dotted horizontal lines show the cutoff for seropositivity (three times the overall mean of the lowest quartile).

TABLE 2. Pearson correlation coefficients between antibodies to streptococcal antigens in ESRF patients and healthy controls

| Group (n)        | Streptococcal antigen | Pearson correlation coefficient ($r^2$) between antibodies to streptococcal antigens in patients and controls* |
|------------------|-----------------------|------------------------------------------------------------------------------------------------------------------|
| Healthy controls (31) | Ska1                  | 0.17 (0.079)                                                                                                      |
|                  | SIC                   | 0.24 (0.0062)                                                                                                     |
|                  | CRS                   | 0.00057 (0.90)                                                                                                    |
|                  | DRS                   | 0.12 (0.056)                                                                                                      |
|                  |                      | 0.24 (0.0065)                                                                                                    |
|                  |                      | 0.35 (0.0006)                                                                                                    |
|                  |                      | 0.0034 (0.76)                                                                                                    |
| ESRF patients (66) | Ska1                  | 0.29 (<0.0001)                                                                                                    |
|                  | SIC                   | 0.64 (<0.0001)                                                                                                    |
|                  | CRS                   | 0.34 (<0.0001)                                                                                                    |
|                  | DRS                   | 0.67 (<0.0001)                                                                                                    |
|                  |                      | 0.45 (<0.0001)                                                                                                    |

* Significant correlation coefficients ($r^2$) are shown in bold type. The associated $P$ values are shown in parentheses.
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 biopsy specimens to determine whether (i) the candidate antigens are deposited in the glomeruli and (ii) there is evidence of PSGN pathology.

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