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Establishment of Gene Copy Number-Specific Normal Ranges for Serum C4 and its Utility for Interpretation of Patients with Chronically Low Serum C4.

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Objective. **To establish gene copy number (GCN)-specific normal ranges for serum C4 and determine their utility with respect to interpretation of chronically low serum C4 in clinically quiescent systemic lupus erythematosus (SLE) patients.**

Methods. **Complement C4 serum concentrations were estimated by automated turbidimitry and C4 GCN were determined using the Taqman® real-time PCR assay in 184 unselected individuals and 12 T1DM (type 1 diabetes mellitus) patients selected for having only two copies of the C4 gene. C4 GCN was also determined in 12 clinically quiescent SLE patients with chronically low serum C4 concentrations.**

Results. **33% of the variation in serum C4 concentration could be accounted for by C4A and C4B GCN (r^2 = 0.30; $p \leq 0.0001$). Median C4 GCNs were two at the C4A locus (53.8%) and two at the C4B locus (58.7%). The median total number of C4 genes was four (55.4%). C4 GCN-specific normal ranges were established. Chronically low serum C4 concentration was explained by low C4 gene copy number in three of eleven patients tested.**

Conclusion. **This study establishes the feasibility of establishing C4 GCN-specific normal ranges using the Taqman real-time PCR assay. Chronically low serum C4 concentration in SLE patients is sometimes explained by low C4 gene copy number.**

INTRODUCTION

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A number of studies have established that the C4 proteins are produced by two distinct loci referred to as C4A and C4B (1). The majority of individuals have two C4A and two C4B genes (2). However, some individuals have more than two or less than two copies of C4A and C4B genes due to duplications/deletions of one of the chromosomal segments encoding C4 genes, or partial deletions resulting in pseudo genes (3). In most individuals, all C4 genes produce C4 protein resulting in a correlation between C4 gene-copy-number (GCN) and total serum C4 concentration. The C4A and C4B proteins differ by a few amino acids and have distinct functional activities (2). Conventional assays of serum C4 concentration estimate the protein from both loci.

Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies, particularly against double stranded DNA (anti-ds-DNA). Immune complexes between the anti-ds-DNA antibody and double stranded DNA result in the activation and consumption of C4 protein resulting in lowered serum C4 concentrations (Reviewed in 4). Treatment of SLE patients with immunosupressive drugs results in diminished anti-ds-DNA titres and a return of serum C4 to normal concentrations. In some SLE patients, who respond to treatment as assessed clinically and by monitoring anti-ds-DNA, the complement C4 concentrations remain low. Given that serum C4 concentration is influenced not only by disease activity but also by the number of copies of the C4 gene (GCN) (1), it was postulated that persistently low serum C4 may be explained by a low C4 GCN in some patients. Thus, the availability of C4 GCN-specific reference ranges and the C4 GCN of a patient may assist in interpreting their serum C4 estimations.

Until recently, C4 GCN determinations required the use of cumbersome methods such as DNA hybridization/Southern blotting (2) or inferences based on

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electrophoretic allotyping of the C4 protein. Thus Uko et al., (5) over two decades ago used C4 allotyping to show that in normal individuals, the presence of null C4 alleles results in lower serum C4 concentrations. More recently, a Taqman® (Applied Biosystems, Carlsbad, CA) real-time PCR assay has been developed for estimating the number of C4 genes present (6). This assay uses probes specific for each of the C4 paralogues. It is calibrated against a standard reference gene (RNase P component H1, chromosome 14) that is also amplified in the assay. This assay has the potential to provide a method suitable for routine analysis. The objective of this study was to assess the feasibility of setting up C4 GCN-specific reference ranges for serum C4 concentration (7). Additionally, the assay was used to determine whether persistently low serum C4 concentration in patients with clinically quiescent SLE could be explained by a low C4 GCN.

METHODS

Reproducibility of the Taqman C4 GCN assay. Over the course of this study, each time the C4 GCN assay was performed, standard DNA samples known to have different numbers (zero to four) of C4A and C4B genes were included. The Life Technology Copy Caller® software (Applied Biosystems, Carlsbad, CA) estimates the number of C4A and C4B genes present as a decimal number, which is then rounded up or down to the nearest whole number. Figure 1 shows the decimal estimates for each of the standard DNA samples. It can be seen that the assay gives a reproducible and accurate result for samples with zero, one and two copies of C4A and C4B but is less reliable for samples with three and four copies. For the purpose of this study, an assay was repeated if any of the standards fell outside the acceptable ranges as shown in Figure 1. Similarly, if an individual test sample

produced a decimal result that fell outside the ranges established using the standards, it was repeated in another run.

Study populations. DNA samples from 184 individuals who participated in the 1994-95 Busselton Health Survey were used to establish the C4 GCN-specific normal ranges for serum C4 concentration. This population has been used as a normal population in various research studies since 1995 (7). The 184 individuals were a subset of 200 individuals randomly selected to represent the Busselton population including 20 males and 20 females in each decade of age between 30-80 years. Sixteen individuals of non-European ancestry were excluded. Ethics approval was obtained for this study from Curtin University and Royal Perth Hospital. In order to compare the results of the C4 GCN real-time PCR assay with those of C4 protein allotyping, twelve type 1-diabetes (T1DM) patients (males and females) for whom prior allotyping data was available were tested (8). Two SLE patients suspected of having a low C4 GCN based on low anti-ds-DNA, and being clinically well but with C4 concentrations consistently below normal were also included in the study. In addition, nine SLE patients identified from a database search for SLE patients with persistently low serum C4 concentration despite normal anti-ds-DNA antibodies were tested.

Complement C4A, C4B and total C4 gene copy number and serum C4 concentration. The Busselton samples and 12 T1DM samples were assayed for serum C4 concentration by turbidimetry by the Royal Perth Hospital Biochemistry Department using an Abbott Architect® C16000 (Abbott Laboratories, Abbott Park, IL) according to the manufacturer's instructions. Samples were first thawed at 4°C and centrifuged at 3000 RPM following a brief vortex. Serum C4 concentrations of SLE patients, previously determined for disease management, were measured by

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automated turbidity using Roche Hitachi 917 (Roche Diagnostics, Australia). DNA was extracted by different methods over a number of years at Royal Perth Hospital, using three Qiagen proprietary methods (Quiagen N.V, Netherlands): columns kits (prior to 2006), MagAttract DNA Blood Mini M48 kit (2006 – 2009) and the automated Qia Symphony DNA Investigator kit (2010 – present). The gene copy number for C4A and C4B were assayed by real-time PCR using Life Technology Taqman® (Applied Biosystems) probes and primers on a HT 7900 thermocycler using Taqman® Genotyping master mix, Taqman® C4A and C4B gene copy number assay and the Taqman® copy number reference assay (RNase P component H1). Assays were performed in 96-well plates containing 4μl test DNA, together with 10μL of 2× Taqman® Genotyping master mix, 1μL of 20× Taqman® gene copy number assay, 1μL of 20× Taqman® copy number reference assay and 4μL of nuclease-free water for a total volume of 20μL per reaction. PCR assays were performed with cycles parameters set to 95°C for 10 minutes (hold), and 40 cycles consisting of 95°C for 15s followed by 60°C for 60s. Data collected were analyzed using Life Technology Copy Caller® version 2.0 software (Applied Biosystems).

CT insertion in exon 29 of C4A. The CT insertion in exon 29 of the C4A gene was detected by a real-time PCR assay using Sybr Green (Applied Biosystems) on an Applied Biosystems 7900 thermal cycler (Applied Biosystems). The insertion was detected using the primers described in Paakkanen et al (9). (Forward: CTC TTC TCC CTG CCT TCC T, Reverse: GCT CTG AGA ACC AGT GAC TGA GAG). The number of cycles required to detect the CT insertion PCR product was compared with that required to produce a PCR product for human growth hormone (Forward primer: CAG TGC CTT CCC AAC CAT TCC CTT A, Reverse primer: ATC CAC TCA CGG

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ATT TCT GTT GTG TTT C). The thermal cycling profile used was 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 sec), 60°C (60 sec).

Statistical analysis. SPSS Statistics (IBM Computershare Trust company, N.A, Providence, RI) version 20 and JMP version 10 (SAS Institute Inc, Cary, NC) software were used to perform bivariate and multivariate regression analyses to investigate the relationship between serum C4 concentration and C4A and C4B gene GCN, age and sex parameters in the Busselton population. One-way analysis of variance was used to compare the mean differences of serum C4 concentration stratified by GCN together with a Tukey HSD p*ost hoc* test with significance set at 0.05. Graphical results were generated using the Excel package (Microsoft®, Redmond, WA).

RESULTS

The distribution of serum C4 concentration in the Busselton population cohort is approximately normal. Serum C4 concentration in the Busselton population ranged from 0.105 g/L to 0.566 g/L with a mean of 0.290 g/L (SD = 0.084; n = 184) (see Table 1). Eleven (5.6%) of the 184 Busselton samples had serum C4 concentrations outside the accepted normal range (0.16 – 0.52 g/L). A small but significant positive correlation was observed between serum C4 concentration and age ($p \leq 0.006$, $R^2 = 4\%$). It was noted also that serum C4 concentration showed a small but significant positive correlation with age in males ($R^2 = 0.076$; $p \leq 0.0045$)

that was not present in females (R^2 = 0.0865; p ≤ 0.2619). However, the correlation coefficients for males and females were not themselves significantly different to each other and the differences observed are likely idiosyncratic.

C4 gene frequencies and the relationship between serum C4 concentration and C4 gene copy number in the Busselton population. The median number of C4 genes was two (53.8%) for C4A, two (58.7%) for C4B and four (55.4%) for total C4, respectively. 1.1% and 3.8% of individuals had zero C4A genes and C4B genes, respectively. Regression analysis and analysis of variance showed that serum C4 concentration was positively correlated with total C4 GCN (R^2 = 0.33; p<0.001). Regression analysis of serum C4 concentration versus C4A and C4B GCN also generated significant correlation coefficients (C4B: p < 0.001; C4A: p = 0.007) For C4A, the *post hoc* Tukey analysis revealed significantly different mean serum C4 concentrations for one versus two loci (p = 0.034), whereas for C4B, the presence of three loci resulted in a significantly different mean serum concentration than for zero, one or two loci (p < 0.001). Additionally, the mean serum C4 concentration for two genes differed significantly from C4 concentration for four and five total C4 genes (p = 0.002 and < 0.001) but not with three total GCN (p = 0.303).

Since only seven Busselton individuals had a total of two C4 genes, 12 T1DM patients were included in the study. These samples were chosen because they had previously been shown to have only two C4 genes by protein allotyping (8). The mean serum C4 concentration in the 12 T1D patients (7 males and 5 females; mean age (±SD) 46.33± 10.96 years) with two C4 genes was 0.200g/l ± 0.08g/L. Since this value does not differ significantly to the mean of the seven Busselton subjects with only two copies of C4 (0.202g/l ± 0.06g/L), subsequent analyses included the twelve T1DM patients.

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GCN-specific serum C4 concentration normal reference ranges. Figure 2A shows the mean and 95% range for serum C4 concentration for the Busselton cohort versus total numbers of C4 GCN. Figure 2B shows the same data as Figure 2A with the twelve T1DM patients included. It is evident that a C4 concentration of 0.16 g/l, previously considered within the normal range, is actually abnormally low for individuals with four copies of the C4 gene but normal for individuals with two or three copies. Similarly, a C4 concentration of 0.10 g/l, previously considered abnormally low, is actually well within the normal range for individuals with only two copies of C4. The mean serum C4 concentration for individuals with five copies of C4 is surprisingly high given the means for individuals with two, three and four copies of C4. We have observed however that the accuracy of the Taqman® (Applied Biosystems) assay diminishes when the total GCN exceeds four copies. It is therefore possible that some of the points plotted as five copies will actually represent six copies. Based on the mean concentrations and 95% range, we have derived new GCN-specific serum C4 reference ranges. Thus, for individuals with 2, 3, 4 and 5 copies of the C4 gene, the normal ranges are 0.059 - 0.343g/L, 0.117 - 0.383g/L, 0.159 - 0.439g/L and 0.279 - 0.571g/L, respectively.

Thirty seven percent of the variation in serum C4 concentration in is accounted for by C4A or C4B GCN and age. The data collected were analyzed by multiple regression analysis. A model for serum C4 concentration was tested based on C4A and C4B GCN, age and sex of the individuals, and interactions between these factors. Multivariate linear analysis showed that only C4A-GCN, C4B-GCN and age significantly influenced serum C4 concentration. Variation in serum C4 concentration explained by total C4 GCN was ≈ 33% with each of the C4A and C4B loci contributing independently. In this model, variation in age contributed a further ≈

4%. Hence, these factors together accounted for ≈ 37% of the total variation in serum C4 concentration. No interactions between the factors tested were observed, including between the C4A and C4B loci. The results of this analysis are shown in Table 2. It was noted that serum C4 concentration in males was significantly associated with the number of C4A loci (R^2 = 0.10; p ≤ 0.002), whereas in females the number of C4B loci had a greater effect (R^2 = 0.24; p ≤ 0.002). However, the correlation coefficients for males and females were not significantly different from each other.

Empirical estimates of C4 protein synthesis in C4A and C4B deficient Busselton samples and 12 type I diabetes individuals. In the combined sample cohort, a small number of individuals manifested C4A or C4B deficiency (i.e. GCN = 0). Nine individuals were C4A deficient, (mean C4 concentration = 0.208; mean GCN = 2) and nine individuals were C4B deficient, (mean C4 concentration = 0.218; mean GCN = 3.11). These data permitted an empirical estimate of how much of the serum C4A and C4B protein is synthesized by each C4 gene. Estimates for each C4 deficient isotype were based on serum C4 concentration synthesized by its counterpart gene, assuming that the deficiency at one locus results in a complete deficiency of the corresponding protein. Hence, the regression line for serum C4 concentration versus contributions from only one of the two C4 gene types should pass through zero with any remaining serum C4 concentration intercept on the Y-axis being due to the alternative C4 gene. This analysis estimated that each copy of the C4A gene produces 0.067g/L of C4A protein and each copy of the C4B gene produces 0.104g/L of C4B protein.

Chronically low serum C4 concentration in SLE patients is generally not explained by low C4 GCN. In order to determine whether low C4 GCN explains a

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persistently low serum C4 concentration in some SLE patients despite responding to treatment, we studied two groups of patients. The first group was known SLE patients selected solely on serological criteria of a persistently low or borderline C4 concentration with concurrently negative or stable low ds-DNA-antibody results (n=8). A minimum of four consecutive serum C4 concentration measurements spanning an interval of at least 12 months with at least two concurrent ds-DNA antibody results were used for patient selection. Serum C4 concentration ranged from <0.02g/L to 0.24g/L. In all patients, C4 concentrations were stable during the lower point of concurrent consecutive ds-DNA antibody levels. Three patients had single instances of a positive concurrent ds-DNA antibody level (normal cut-off < seven units) but none of these was higher than ten units. One of these eight patients had only two copies of the C4 genes that probably explained her low serum C4 concentration. All seven other patients had at least three copies of the C4 genes. Two additional patients were referred for the GCN assay by clinicians seeking an explanation for low serum C4. One patient was a known SLE patient whose serum C4 remained < 0.07 g/L despite being clinically stable and having negative anti-ds-DNA antibody. The second patient was a new patient suspected of having SLE due to strongly positive anti-nuclear antibody, lymphopaenia and low serum C4 (persistently less than 0.13g/L) but negative anti-ds-DNA antibody. Both of these patients had at least three copies of the C4 gene suggesting that low C4 GCN was not responsible for their low serum C4 concentration.

The real-time PCR assay does not discriminate between expressed and non-expressed genes. C4 protein allotyping data were available for 12 T1DM samples, which permitted an independent estimate of expressed C4 genes. When compared to the C4 GCN results obtained by the Taqman® assay, ten of the 12

T1DM samples gave concordant results. However, two discordant results showed that the Taqman® (Applied Biosystems) assay detected C4A genes whereas the C4 allotyping assay detected zero C4A genes. One T1DM patient was shown by allotyping to have a C4A*Q0 and C4B*01/C4B*02. The real-time PCR accounted for 1 C4A copy and 2 C4B copies. A second T1DM patient was shown by allotyping to have a C4A*Q0 and C4B*02. The real-time PCR accounted for 2 C4A copy and 2 C4B copies. Interestingly, both discrepant samples were from patients exhibiting the HLA–B40 and HLA-DRB*13 alleles, a MHC haplotype known to include a C4A gene with an inactivating dinucleotide insertion in exon 29. The first patient in whom the Taqman® assay detected one C4A gene was subsequently demonstrated to have the exon 29 insertion that would inactivate the patient's single C4A gene. In the second patient, the Taqman® assay detected two C4A genes. This patient was subsequently shown to have two copies of the exon 29 CT insertion that would inactivate both C4A genes thereby reconciling the Taqman® result with the protein electrophoresis.

DISCUSSION

The Life Technology Taqman® (Applied Biosystems) real-time PCR assay was reliable, technically straightforward and suitable for routine assessment of C4A and C4B locus quantification. However, the assay was sensitive to the quality of the extracted DNA samples used. This observation supports the assay validation by Wu et al., (10) who found that partially degraded DNA samples yielded conflicting results. As the assay tests for the presence of a specific DNA sequence in exon 26 of the C4 gene, it does not discriminate between expressed and non-expressed genes. The frequency of most non-expressed C4 genes is very low (<1%) with the exception of the CT insertion in exon 29 that is present in 3-5% of Caucasian populations (1, 3, 11). Indeed, two of twelve TIDM patients tested illustrated this point. Both discordant

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individuals carried HLA-B40 and HLA-DRB1*13 alleles and it is likely therefore that they possess the haplotype described by Barba et al., (3) for which the C4A gene is present but non-functional due to the CT-insertion mutation in exon 29 (CT*ins*).

The median C4A GCN, C4B GCN and total C4 GCN values observed in this study were two, two, and four, respectively which is consistent with distributions in European Americans (1, 2), English and Spanish (12) and in Asian Indians populations (13).

Serum C4 concentration has previously been shown to decrease with increasing numbers of C4 null alleles in T1DM patients (5). Similarly, serum C4 has been shown to increase with increasing C4 GCN in healthy Asian Indian American, European American, and Hungarian populations (1, 13, 14). We confirm a positive linear relationship between serum C4 and C4 GCN in an Australian population and provide provisional GCN-specific normal ranges. Due to the paucity of healthy individuals with only two copies of the C4 gene, we supplemented their numbers with ten T1DM patients selected for having only two copies of the C4 gene. However, this still only resulted in a total of 17 individuals with two copies of C4 and therefore the accuracy of the normal range for two copies would be greatly improved with a larger number of individuals. However, the frequency of individuals with two copies of C4 is only 2-3% and as the Taqman® assay is relatively expensive (~$30/sample), screening the population for additional examples would be prohibitively expensive. Nevertheless, it appears that a serum C4 concentration as low as 0.10 g/L, which is below the current lower limit of normal (0.16g/L,) is within the normal range for this group.

Multivariate linear modeling shows that only 33% of the variation in serum C4 concentration is accounted for by C4 loci GCN with the C4A and C4B loci behaving

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independently. In this model, age also had a small but significant positive influence on serum C4 concentration that is consistent with the report by Kramer et al. (15), who showed that C4B null alleles were less frequent in nonagenarians, especially males. As age accounted for only 4% of the population variation in serum C4 concentration, the establishment of sex-specific normal ranges is not warranted. Serum C4 concentrations appeared to be more strongly influenced by the number of C4A loci in males whereas the C4B locus appeared more influential in females but the interaction with sex did not reach significance. In the mouse, sex-specific effects on the serum concentrations of C4 and its related protein Slp are known to occur (16). The remaining variation in serum C4 concentration must therefore be accounted by other factors including variation in anabolic and catabolic rates that may be influenced by non-coding region polymorphisms and inflammation. Complement C4 genes also exhibit size variation due to the insertion of a HERV-K retrovirus (≈ 6.5 kb) in intron 9 (17). Although the retroviral element is not functional due to multiple mutations sites, it can influence complement C4 protein levels by retarding transcription, resulting in decreased rate of C4 protein synthesis (14). The role of inflammation is suggested by the demonstration that C4 proteins show hyper-catabolism in rheumatoid arthritis and SLE patients (18-20). Evidence has also been presented that allotypic variants of C4 proteins may have differing rates of catabolism in SLE patients (18-20).

The empirical relative estimates of C4 protein synthesized by each of C4A genes and C4B genes (0.067g/L of C4A protein/C4A gene and 0.104 g/L of C4B protein/C4B gene) are approximate and do not allow for C4 genes detected but not expressed. However, it is interesting that these estimates are consistent with those reported in other studies (14) that show C4B protein/C4B gene exceeds that of the

C4A protein/C4A gene in individuals with mixed genotypes. It is important to note that serum concentration of C4 does not increase as a simple function of GCN. That is, the average serum C4 concentration for individuals with four genes is not double that of individuals with two genes. This is in clear contrast to the relationship in humans between serum C2 concentration and GCN at the single diploid C2 locus (22, 23).

Persistently low serum C4 concentration despite clinical response to treatment and normalization of anti-ds-DNA antibodies presents a dilemma to the physician who may wonder whether the low C4 concentration is due to the patient having a low C4 GCN. The current data suggest that the frequency of low GCN (2 or 3 copies) in SLE patients with persistently low C4 serum concentrations (3/11 patients) is not very different to that in controls (36% in Busselton). Hence low C4 GCN does not explain the majority of SLE patients with persistently low serum C4 levels after treatment. However this assay does represent a practical method of identifying those SLE patients whose low serum C4 is a result of low GCN thereby sparing them further aggressive immunosuppression.

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