# Chapter V

## Discussion and Conclusion

### The Technique

C4 allotyping was carried out in EDTA-plasma in which the EDTA chelates $Mg^{2+}$ and $Ca^{2+}$, inhibits the activation and conversion of complement components. Special care should be taken during collection and storage of samples for complement testing because of the instability of these proteins at room temperature , 4°C and even at -20°C. Samples should be placed immediately on wet ice, at 4°C and processed within 24 hr of collection. Multiple aliquots should be made for each sample in order to minimize repeated freezing and thawing that could alter the proteins. Complement proteins appear to be stable when stored at -80°C. Fresh frozen samples were the best for C4 typing since typing of aged plasma samples is not reliable (95).

Neuraminidase treatment prior to electrophoresis removes sialic acid and reduces the heterogeneity of C4 yielding good resolution(40). It was noted that excess enzyme was essential for a reproducible result (93) since obscure patterns of C4 proteins were observed following the use of inappropriate enzyme concentration. Adequate neuraminidase enzyme was reached at a final concentration of 5 mU/ul plasma at 4°C for 18 hr.

Although neuraminidase treatment of plasma samples has improved the resolution, one major problem in assigning the pattern of several allotypes in an individual still exist. C4 patterns of neuramindase - treated plasma obtained on agarose gel electrophoresis,

with three bands for each allotype , are attributed to the difference in the amount of charged amino acid (especially arginine) at the carboxy-terminal of the alpha and beta chain. Consequently, pancreatic carboxypeptidase B specific for C-terminal arginine and lysine residue was utilized to remove charged residues giving rise to mobility shift thereby eliminating the difference in charged residues within this protein in the minor bands. The complex multiple-band pattern of C4 was then converted into a more informative pattern with a single, sharp, distinct band for each allotype (94).

To further improve the technique of C4 typing, Udomsopagit (96) adopted a new system combining horizontal electrophoresis system with electrotransfer and immunoblotting. This not only facilitates C4 allotyping but also provides a higher sensitivity. The use of immunodetection on blotted membranes effectively reduces both sample and antibody requirements and allows visualization of C4 typing even in samples with low C4 level (99), when immunofixation yields only very faint bands. Immunoblotting in our study required only one microlitre of sample and minute amount of antibody (1:750) while immunofixation required 3 microlitres or more of sample and a higher concentration of anti-C4 (1 : 4).

In samples with overlapping or unusual migrating types,the C4A or C4B assignment was determined by the hemolytic overlay technique. In general, the C4A allotypes show considerably less or no hemolytic activity in comparison with C4B allotypes. Hydrazine - treated guinea pig serum was utilized as a C4 depleted serum in the hemolytic

functional overlay (93,95). Since hydrazine bonds convalently to the polypeptide chain containing the active site thiolester of C4, inhibits covalent binding of activated C3 leading to depletion of C4 functional activity. Hemolytic detections was selectively produced from C4B protein. Recently, with the use of C4A and C4B monoclonal antibodies, C4 allotypes with overlapping migration patterns can be readily identified (96,100, 101).

The detection of the heterozygous null allele at the C4A and C4B has been one major drawback in C4 phenotyping. Besides two-dimensional crossed immunoelectrophoresis (102) , densitometric evaluation of relative band intensity has been employed to overcome this problem (76). In our study, judgement on heterozygous null allele of C4A and C4B was attempted both from visual inspection and densitometric determination of C4A/C4B ratio (Figure 9). The latter provides much better evidence for assignment of C4 null alleles. Nevertheless, it should be noted that this method can not distinguish the phenotypes containing no null allele from those containing one null allele at each locus. This distinction can usually be resolved by family studies.

In the present study, the most common C4A and C4B allotypes were found to be C4A3 and C4B1 respectively. The distribution of C4A and C4B in this study is comparable to that reported by Udomsopagit and Tokunaga (Table 11). The frequency of C4A null alleles (C4A*QO) and C4B null alleles (C4B*QO) in Thai population were 14% and 7%, respectively. This finding is comparable to the results of Kay (103)

and Udomsopagit (96) in which the frequency of C4A*QO was between 10-27% and 2.24% respectively and of C4B*QO was 13-34% and 7.46% respectively in Thai/Thai Chinese. The genotype frequencies of C4A null alleles (0.0727) and C4B null alleles (0.036) in this study was similar to those in various population as reported in the IX$^{th}$ International Histocompatibility Workshop (4) (Table 12).

C4 Allotype and SLE

The prevalence of putative genetic risk factor for SLE in the patients studied is of some interest. In the 76 SLE patients, only the frequency of C4A*QO was significantly increased (35.5% versus 14%,p = 0.0015, R.R =3.38)(Table 8). There was a slight, non statistically significant increase of C4B*QO among the SLE patients (14.5% versus 7%, p = 0.1). The high prevalence of null alleles of C4A and the non significant increase of C4B null allele are consistent with those in the previous studies of C4 null alleles in Caucasian, Black, Japanese and Chinese with SLE (Table 13 ). This confirms the biological relevance of C4A null alleles, crossing the racial barrier, in SLE (82). Nevertheless, according to Udomsopagit (97), the frequency of both C4A*QO and C4B*QO were significantly higher than normal in another group of Thai SLE patients. More stringent criteria for patients selection might be necessary for further study. The relationship of C4B*QO to SLE was also implicated by Wilson in three black American patients, in whom C4B was completely deficient (104).

Hitherto, the mechanism by which C4A*QO impart an increased risk of SLE remain speculative. It is possible that C4 null alleles result in complement dysfunction which may increase susceptibility to immune complex disease perhaps by interfering with the processing or disposal of complexes (7,8). Welch rationalized that if this was the case, there would be three possible mechanisms by which null gene could cause complement dysfunction (105). First, individual with null gene could have low circulation levels of C4 leading to decreased efficiency in processing immune complexes through activation of the classical pathway. Second, C4 null gene could be associated with defects of other complements of the complement system that together could impair the complement-mediated reaction. Third, the presence of C4 null gene could form a different C3 convertase affecting complement-mediated responses. In order to verify this hypothesis, complete investigation of the complement system in healthy persons with C4 null alleles have been done (105,106). It was found that C4 levels correlated with the number of C4 null alleles (105,106,107) but there was a wide range of individual values. There was no evidence of abnormalities in the concentrations of other complement components (105) nor was there any significant increases in Ig G, Ig M, or Ig A (106). Besides, the in vitro kinetics of C3 convertase formation, critical for the efficient opsonization of immune complex and for the prevention of the precipitation of potentially damaging complexes, was the same for homozygous C4A*QO and C4B*QO which argued against a direct involvement of these states in the pathogenesis of immune complex disorders. Thus, it is conceivable that C4 null allele is not

directly involved in the causation of SLE, but rather a marker for the disease susceptibility gene. Besides, Awdeh and Alper have shown that linkage disequilibrium exist between certain HLA haplotype (108). For instance, increased frequency of the extended haplotype HLA A1,B8, DR3, BfS, C2C, C4AQO, C4B1, has been reported in SLE (15). This might simply be markers for a haplotype conferring disease susceptibility. Further efforts should be made to identify the possibility of such haplotype in Thai SLE patients.

C4 Allotype and Rheumatoid Arthritis

The examination of C4 allotypes in rheumatoid arthritis in this study revealed a high frequency of C4B*21 and C4B*4 (8.8% p = 0.17, R.R = 3.13 and 2.9%, p = 0.44, R.R = 3.00). The frequency of C4B*2 was significantly decreased in rheumatoid arthritis ( 29.4% in RA versus 53% in normal, P = 0.029). We could not find significant increase of C4A*QO, C4B*QO, C4B*29 or C4B*5, all of which have been reported to be associated with RA in previous studies preliminary data for the study of RA in Thai patients. Moreover, it is widely accepted that the genetic susceptibility to rheumatoid arthritis is accounted for by genes within the MHC II region (85). This is in particular with HLA-DR 4 and HLA-DR1 depending on the ethnic origins of the population study (86) as confirmed by probe hybridization and DNA sequence analysis present at the symposium on immunogenetics of the rheumatic disease, in 1988 (87,109). It was also concluded then that more than one gene within the major histocompatibility complex contribute to disease susceptibility in RA.

Our preliminary result on C4 allotypes in Thai RA patients indicated that genetic association, if present, may be different from other ethnic groups. Further study of the full "supratype" especially MHC II in this disease in Thai population will reveal additional insights into the specific polymorphisms that may predispose to this disease.

**In summary this work has shown that**

1. Immunoblotting technique is applicable for the examination of C4 allotypes with the advantage of antisera economization. Nevertheless its value in C4 null allele identification has yet to be established. Further examination including family study should help resolve the problem.

2. The prospect of laser densitometry and the evaluation of relative densities of C4A and C4B bands for the assignment of C4 null allele is promissing but further verification should be undertaken.

3. The most common C4A and C4B allotypes in Thai were found to be C4A3 and C4B1 respectively.

4. The frequency of C4A*QO is significantly increased in SLE patients with a relative risk of 3.38. Hence there is a strong association of C4A*QO in this disease.

5. There is an increase of C4B21 and C4B4 in rheumatoid arthritis. Nevertheless, no distinct correlation of C4 allotypes with rheumatoid arthritis was observed.

6. The decrease of C4B2 in rheumatoid arthritis (29.4% in RA versus 53% in normal, p = 0.029) might signify a protective trend of this allele in rheumatoid arthritis.

Table 11. Distribution of C4A and C4B Allotypes in Japanese and Thai Normal Populations.

| Allotype | Thai I[a] (n=76) % | Thai II[b] (n=134) % | Japanese[c] (n=166) % |
|---|---|---|---|
| C4 A 1 | 0.00 | 0.00 | NR |
| 2 | 10.00 | 5.22 | 19.90 |
| 3 | 70.00 | 73.13 | 91.00 |
| 4 | 40.00 | 27.61 | 25.30 |
| 5 | 0.00 | 1.49 | NR |
| 6 | 5.00 | 8.21 | NR |
| QO | 14.00 | 2.24 | 12.00 |
| C4 B 1 | 69.00 | 75.37 | 80.70 |
| 2 | 53.00 | 50.00 | 30.10 |
| 21 | 3.00 | 0.75 | NR |
| 3 | 1.00 | 1.49 | NR |
| 4 | 1.00 | 0.74 | NR |
| 5 | 12.00 | 7.46 | 16.90 |
| QO | 7.00 | 7.46 | NR |

a = Result in this study

b = Udomsopagit 's report

c = Tokunaga et al's report

NR = No report

Table 12. Frequency of the C4 "null" alleles in various populations.

| Population | Frequency | |
|---|---|---|
| | C4A*QO | C4B*QO |
| Caucasoids | | |
| Australia | 0.168 | 0.195 |
| Finland | 0.113 | 0.175 |
| France | 0.132 | 0.164 |
| W. Germany | 0.087 | 0.138 |
| USA. | | |
| Baltimore | 0.095 | 0.111 |
| Boston | 0.191 | 0.104 |
| IX WS* | 0.126 | 0.138 |
| Negroids | | |
| S. Africa | 0.091 | 0.258 |
| USA. | | |
| Baltimore | 0.071 | 0.071 |
| IX WS* | 0.116 | 0.195 |
| Mongloids | | |
| Japan | 0.067 | 0.158 |
| China | | |
| Hongkong | 0.155 | 0.147 |
| IX WS* | 0.099 | 0.112 |
| Aborigines | | |
| Australia | | |
| Alice Springs | 0.123 | 0.178 |
| Darwin | 0.228 | 0.257 |

*IX th International Histocompatibility Workshop.

From : Hauptman et al, Complement. Inflamm., 6, 74-80, 1989

Table 13. Association of C4 null allele in SLE in various population.

| | C4A*QO | | | C4B*QO[a] | |
|---|---|---|---|---|---|
| | Control | SLE | RR. | Control | SLE |
| Howard et al 1986 (19) | | | | | |
| Caucasian | 19.0% | 41.3% | 2.93 | 20.6% | 19.8% |
| Black | 14.3% | 37.1% | 3.54 | 37.1% | 20.0% |
| Tokunaga et al 1986 (22) | | | | | |
| Japanese | 12.0% | 21.2% | 1.96 | -[b] | -[b] |
| Hawkins et al 1987 (69) | | | | | |
| Chinese | 28.6% | 52.0% | 2.95 | 27.3% | 30.8% |
| Wilson et al 1988 (78) | | | | | |
| Black | 15.3% | 32.3% | 2.6 | 23.7% | 28.8% |
| Partinen et al 1988 (81) | | | | | |
| Caucasian | 22.0% | 54.0% | - | 45.0% | 43.0% |

a : No report relative risk

b : No report

Table 14. Genetic Marker in Rheumatoid Arthritis.

| | HLA association |
|---|---|
| O'Neill et al 1982 (16) (Western Australia) | : HLA-A2, B15, Cw3, DR4 BfS, C2C, C4A3, C4B2.9 |
| Westedt et al 1986 (90) (Dutch, Netherlands) | : HLA-DR4[a] |
| Thomson et al 1986 (88) (United Kingdom) | : HLA-B44, DR4, BfS, C4A3, C4BQO[b] |
| Thomson et al 1988 (89) (United Kingdom) | : HLA-DR4, C4BQO[a] |
| Tokunaga and Takeuchi 1986 (91) (Japanese) | : HLA-Bw54, DR4, BfS, C2C, C4A3, C4B5 |
| | : BfS, C2C, C4AQO, C4B1 or C4B2 |

a = RA patients with Felty Syndrom

b = protective association