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# Inherited Structural and Quantitative Polymorphisms of C3b Receptor (CR1) in Normals and Patients with Glomerular Diseases

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**SUMMARY** The erythrocyte C3b receptor (CR1) has been studied for its structural and quantitative polymorphisms in normal Indian individuals and in patients with glomerular diseases. In the normal Indian population, purification of CR1 by immunoprecipitation or C3b-Sepharose affinity column and subjecting it to electrophoresis showed the existence of two types of structural polymorphic patterns with $M_r$ of 190 kDa and 220 kDa, and with gene frequencies of 0.975 and 0.025, respectively. The gene frequencies of these alleles remain unaltered in the patient population. Evaluation of CR1 levels in the normal Indian population revealed a trimodal distribution of CR1 number suggesting a co-dominant allelic pattern (L and H alleles) for the quantitative expression of CR1 with gene frequencies of 0.523 and 0.477, respectively. In our earlier study we have shown that there is a decreased expression of CR1 on the erythrocytes of patients with acute glomerulonephritis. Since this decrease in the CR1 level in patients is an acquired characteristic, it may not be the level controlled by the LL homozygous alleles. The discrepancy in the gene frequencies of the structural and quantitative polymorphic alleles in normal individuals show that they are not linked to each other. In our earlier study, we showed that the affinity constant of C3b-CR1 binding in different individuals remains the same irrespective of the number of CR1 on the erythrocyte surface. Comparison of this result with the present investigation shows that there is no functional difference among various structural polymorphic forms of CR1 and the susceptibility to glomerular diseases is not associated with any of the CR1 polymorphic patterns.

The immune adherence receptor of the complement system (C3b receptor, CR1) exhibits four types of genetically inherited structural polymorphic forms that differ in $M_r$ on SDS-PAGE.[1] Four different autosomal co-dominant alleles encode CR1 of varying $M_r$ ranging from 160-250 kDa. Biosynthetic studies have shown that these variations in $M_r$ are due to differing lengths of polypeptide but not due to post-translational modifications such as glycosylation.[2] Subsequently, incremental differences of 1.3-1.5 kb were also observed in the CR1 transcripts from various allotypes.[3,4] The numerical expression of human erythrocyte CR1 varies from 100 – 1200 per erythrocyte and it has been proposed that it is controlled by two polymorphic alleles, namely L (low) and H (high) in Caucasian populations.[5] High and low level CR1 were shown to be associated with allelic 7.4 and 6.9 kb genomic *Hind* III fragments, respectively.[6] Linkage disequilibrium was observed between this RFLP and CR1 structural allotypes suggesting that this polymorphism is located within or near the CR1 gene. Reduced levels of CR1 have been reported in various disease states manifested by the accumulation of immune complexes.[7,8] Since CR1 is involved in the clearance of circulating immune complexes, the efficiency with which it performs this function may be correlated with the numerical expression of CR1. However, the role of the structural polymorphic patterns of CR1 is also not well understood. Our earlier studies in subjects with glomerulonephritis showed that the CR1 level goes

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down with increasing levels of circulating immune complexes (unpublished observation). In the present investigation, we determined the CR1 levels in healthy Indian individuals which shows a wide range of 200–1100. We also investigated structural and numerical variation of CR1 to analyze their relationship both in the normal Indian population and patients with various glomerular diseases.

## MATERIALS AND METHODS

### Subjects

Blood samples collected from healthy individuals mainly from the northern region of India ranging in age from 12–40 years have been used as normal controls. Blood samples from patients with glomerulonephritis were collected from the Outpatient Department and the Nephrology Ward of the All India Institute of Medical Sciences. Based on the clinical and immunohistopathological diagnosis, the glomerulonephritis patient population comprising patients of non-SLE nephritis was classified into three groups : (i) Acute glomerulophritis type (AGN), (ii) Chronic glomerulonephritis type (CGN) and (iii) Recovering AGN type. AGN, recovering AGN and CGN were defined mostly on the basis of history (the time of onset of the disease), and acuteness of the symptoms. The presence of active sediment abnormality, ie plenty of RBC and RBC casts were considered acute, a designation which was later confirmd by the kidney biopsy. The recovering AGN patients are those who had onset of symptoms two to four weeks previously and whose clinical symptoms were improving. There was improvement in the clinical and biochemical parameters (decrease in proteinuria, absence of RBC or RBC casts and improvement of the serum creatinine) and the signs of inflammation were less in the kidney biopsy.

### Enzyme linked immunosorbent assay (ELISA) for C3b receptor

Quantitative estimation of CR1 was performed with modifications of the method described by Thomsen *et al.*[9] Increasing concentrations of cells ranging from 106–107 were run simultaneously to construct a standard curve from which the receptor number for an individual sample could be obtained using its absorbance at 405 nm. The receptor number of the standard RBC had already been established by radio-receptor assay[8] so that the receptor number/cell of any given sample can be obtained directly from the standard curve.

### Structural allotyping of CR1

The structural polymorphism of CR1 was analyzed by SDS-PAGE and subsequent autoradiography of CR1 isolated using either C3-Sepharose affinity column or immunoprecipitation with anti-CR1 monoclonal antibodies.[1]

### Surface labelling and solubilization of erythrocytes

Blood was collected in EDTA (final concentration 5 mM), centrifuged at 800 × g for 8 minutes, after which the plasma and the buffy coats were removed. The packed erythrocytes were washed three times in PBS pH 7.4, and an approximately 10% suspension was prepared. Four ml of the suspension was iodinated with a modification of the lactoperoxidase method described by Kulczycki *et al.*[10] The following reagents were added serially to 4 ml suspension of erythrocytes: lactoperoxidase-23 μl (420 units/ml), glucose oxidase-21 μl (10 times diluted the stock of 1,400 units/ml), potassium iodide-12 μl (5 mg/100 ml), $^{125}$I-250 μCi, dextrose-125 μl (50 mg/ml). The reaction mixture was incubated for 15 minutes at room temperature with occasional shaking. After incubation, iodination was stopped by cooling on ice. The cells were washed three times with PBS-BSA (30 mg/ml). Erythrocytes were chilled at 4°C and lysed with 15 ml of ice cold distilled water for 1 minute followed by addition of 15 ml of 0.3 M NaCl. Both distilled water and NaCl contained 2 mM PMSF, 3 mM EDTA, 20 mM iodoacetamide, 1 μm pepstatin and 0.33 trypsin inhibitor unit/ml of aprotinin. The stroma were isolated by centrifugation at 33,000 × g for 20 minutes and the pellet was solubilized with 1 ml PBS containing 1% Nonidet P-40 (NP-40) containing the same protease inhibitors.

### CR1 purification by affinity chromatography

Cyanogen bromide (CNBr) activation of Sepharose-6B and coupling of C3, BSA and IgG were done according to the method described by Parkhouse.[11] Solubilized membranes from $1 \times 10^{10}$ erythrocytes were incubated with constant mixing for 30 minutes at room temperature with 0.4 ml of BSA-Sepharose and centrifuged at 600 × g for 6 minutes. Then the supernatant was diluted with two parts of distilled water containing 1% NP-40 and incubated with 0.4 ml of IgG-Sepharose for 40 minutes and then with 0.4 ml of C3-Sepharose for 1 hour. After incubation the affinity beads were transferred to a 0.7 × 4 cm plastic column (Bio-Rad) with 6 ml of 1/3 PBS (PBS diluted with two parts of distilled water) containing 1% NP-40. The column was washed with 4 ml of 1/3 PBS containing 1% NP-40 and eluted with four successive 1 ml aliquots of 0.4 M NaCl/1% NP-40. The eluates were dialysed against distilled water at 4°C, lyophilized and precipitated with 95% acetone to remove NP-40. Then the precipitate was dissolved in 80 μl of electrophoresis sample buffer containing 0.25 M Tris-HCl, pH 6.9/2% SDS/10% glycerol/0.001% bromophenol blue.

### Immunoprecipitation of CR1

Solubilized membranes from approximately 0.5 × $10^9$ erythrocytes were incubated for 1 hour at 4°C with 50 µl (20 µg/ml) of mouse monoclonal antibody against C3b receptor (DAKO-C3bR) and then with rabbit anti-mouse IgG for 1 hour. It was further mixed with 150 µl suspension of protein A-Sepharose and incubated for 1 hour at room temperature with constant mixing. The mixture was centrifuged and the pellet was washed 3 times with 1 ml of PBS/1% NP-40 at 4°C. The protein bound to the pellet was removed by resuspending the pellet in 80 µl of electrophoresis loading buffer and heating at 80°C for 15 minutes. The supernatant collected by centrifugation at 12,000 rpm for 10 minutes was subjected to SDS-PAGE.

### SDS-PAGE and autoradiography

Sample containing C3b receptor, which was obtained by affinity purification or by immunoprecipitation was loaded on a 6% polyacrylamide gel with 3% stacking gel in non-reducing conditions. The electrophoresis was performed as described by Laemmli.[12] A set of molcular weight markers (Bio-Rad) were also run along with the samples. The gel was dried and exposed to X-ray films (INDU, India) for 5 days in a cassette containing intensifying screen. The the film was developed and the bands of CR1 were compared to that of the molecular weight markers for the calculation of $M_r$ of CR1.

### Statistical analysis

The gene frequencies of the polymorphic alleles were compared using Chi-square ($\chi^2$) analysis.

## RESULTS

### Quantitative polymorphism of CR1 in normals

The number of CR1 sites/cell has been represented as a histogram of frequency distribution, taking 100 sites as the interval in 66 normal individuals of healthy Indian population. Fig. 1 shows the frequency distribution histogram of CR1 sites in a normal Indian population and it clearly represented a trimodal distribution of CR1 which formed the basis for the proposal of the existence of two co-dominant alleles, H (high) and L (low) controlling the CR1 quantitative expression. Accordingly, a HH homozygous genotype yielded 800-1100 sites, LL homozygous genotype yielded 0-400 sites and LH heterozygous genotype has expressed an intermediate range of 400-800 sites/cell. Table 1 shows

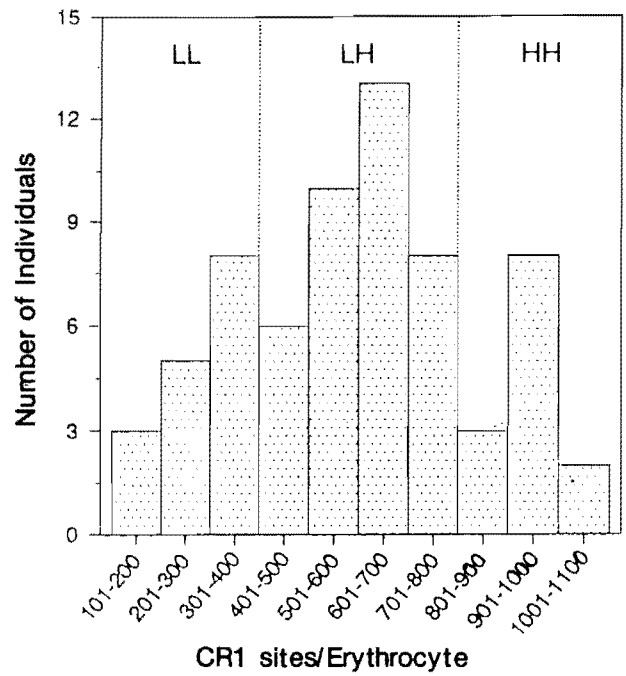


**Fig. 1** Frequency distribution histogram of the number of CR1/erythrocyte of normal individuals. CR1 levels in 66 normal individuals have been plotted taking 100 sites as the interval. LL-low CR1 level controlled by LL homozygous alleles, HH-high CR1 level controlled by HH homozygous alleles, LH-intermediate CR1 level controlled by LH heterozygous alleles.

**Table 1.** Distribution of polymorphic alleles controlling the quantitative expression of CR1 in normal Indian population

| | | Common phenotypes | | | Gene frequencies | |
|---|---|---|---|---|---|---|
| | No. | LL (%) | LH (%) | HH (%) | L | H |
| Observed | 66 | 16 (24.2) | 37 (56) | 13 (19.7) | 0.523 | 0.477 |
| Expected | 66 | 18 (27.3) | 33 (50) | 15 (22.7) | | |

$\chi^2 = 1.008$, $p > 0.50$

the observed and expected frequencies of high, low and intermediate ranges of CR1 sites which followed Hardy-Weinberg equilibrium. Chi-square analysis yielded no significant difference between the observed and expected frequencies of various quantitative polymorphic alleles of CR1 ($\chi^2$ = 1.008, $p > 0.50$) consistent with the co-dominant inheritance of these alleles.

**Structural polymorphism of CR1**

CR1 was purified from iodinated erythrocyte membranes either by affinity chromatography on C3-Sepharose column or by immunoprecipitation and subjected to SDS-PAGE on a 6% gel. Autoradiography of these gels demonstrated the existence of two different CR1 polymorphic patterns A and B (Fig. 2, lanes a and b). The A type indicated a $M_r$ of 220 kDa. Immunoprecipitation of CR1 using anti-CR1 monoclonal antibody and protein A-Sepahrose yielded an identical result (Fig. 2, lane c) confirming that the bands obtained are C3b receptor bands only. The A pattern was due to the homozygous AA alleles and the AB pattern was due to the heterozygous AB alleles in the CR1 locus. However, we did not obtain any BB homologous pattern of CR1.

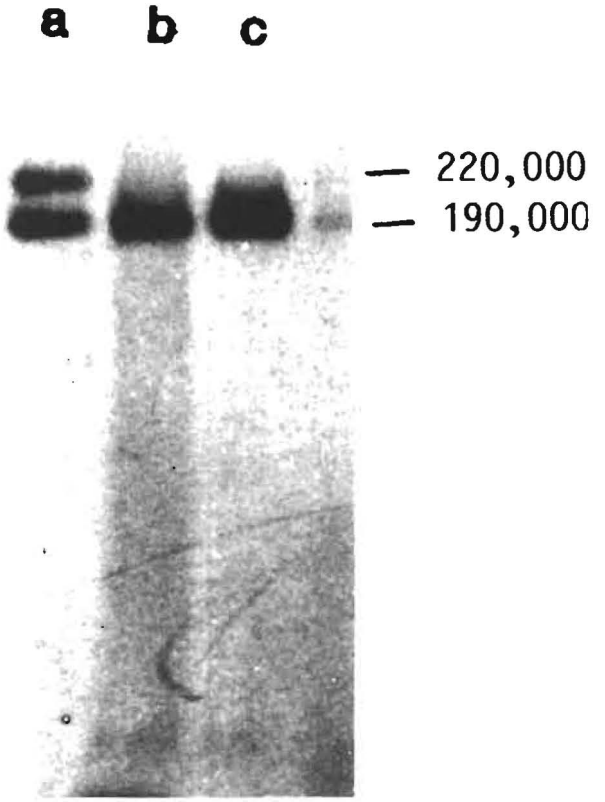


**Fig. 2** SDS-polyacrylamide gel electrophoresis of CR1 isolated. **Lane a:** heterozygous pattern AB of CR1 isolated using C3-Sepharose, **lane b:** homozygous pattern A of CR1 isolated using C3-Sepharose, **lane c:** homozygous pattern A precipitated by anti-CR1 monoclonal antibody.

**CR1 structural polymorphism in a normal healthy Indian population**

Normal, healthy individuals (n=79) in Indian population were studied for the allotypes of CR1. Seventy five had only the A form of CR1 identifying an AA homozygous genotype and 4 showed an AB form of CR1 denoting AB heterozygous genotype. No BB homozygous individuals were identified. The gene frequencies of A and B alleles based on the polymorphic studies were 0.975 and 0.025, respectively. $\chi^2$ analysis of the observations showed a good probability of fitness with Hardy-Weinberg equilibrium ($\chi^2 = 0.054$, $p > 0.950$) demonstrating no significant difference between the observed and expected phenotypic frequencies (Table 2). The data are consistent with an autosomal co-dominant inheritance pattern of two alleles encoding the different CR1 structural forms. However, there is a discrepancy in the gene

**Table 2.** Distribution of structural polymorphic alleles of CR1 in normal Indian population.

| | | Common phenotypes | | | Gene frequency | |
|---|---|---|---|---|---|---|
| | No. | AA (%) | AB (%) | BB (%) | A | B |
| Observed | 79 | 75.0 (94.9) | 4.00 (5.1) | 0.00 (0) | 0.975 | 0.025 |
| Expected | 79 | 75.1 (95.06) | 3.85 (4.87) | 0.05 (0.06) | | |

$\chi^2 = 0.0536$, $p > 0.95$

**Table 3.** Distribution of structural polymorphic alleles of CR1 in normal and GN patients.

| | | Common phenotypes | | | Gene frequency | |
|---|---|---|---|---|---|---|
| | n | AA (%) | AB (%) | BB (%) | A | B |
| Normals | 79 | 75 (94.9) | 4 (5.1) | 0 (0) | 0.975 | 0.025 |
| AGN | 14 | 13 (92.9) | 1 (7.1) | 0 (0) | 0.964 | 0.036 |
| CGN | 23 | 22 (95.6) | 1 (4.4) | 0 (0) | 0.978 | 0.022 |

Normals *vs* AGN $\chi^2 = 0.992$, $p > 0.50$
Normals *vs* CGN $\chi^2 = 0.00102$, $p \geq 0.995$

frequencies of CR1 structural alleles and the H and L alleles determining the level of CR1 expression on erythrocytes (see Tables 1, 2) indicating that the quantitative expression of CR1 is not determined by the observed protein polymorphisms.

### CR1 structural polymorphism in GN patients

Glomerulonephritis patients were studied for their CR1 allotypes. The pattern of CR1 polymorphism (Table 3) both in AGN as well as CGN patients followed Hardy-Weinberg equilibrium. $\chi^2$ analysis of the gene frequencies of CR1 alleles in normals and AGN population (Table 3) showed no significant difference between them ($\chi^2 = 0.992$, $p > 0.50$). CGN patients showed a gene frequency of 0.978 and 0.022 for the A and B alleles, respectively (Table 3). $\chi^2$ analysis showed no significant difference between the gene frequencies in normals and CGN patients ($\chi^2 = 0.00102$, $p > 0.995$). Thus, there was no difference in the gene frequencies of the normal and patient populations with various types of glomerulonephritis as far as CR1 structural allotypes were concerned. These results suggest that the susceptibility to glomerular diseases is not associated with any of the observed CR1 structural polymorphisms.

## DISCUSSION

In the present study, the analysis of CR1 distribution in normal population shows that the quantitative variation of CR1 in normal Indian population is controlled by two polymorphic co-dominant alleles L and H with gene frequencies of 0.523 and 0.477 following Hardy-Weinberg equilibrium. Accordingly 24% (LL), 56% (LH) and 20% (HH) are the frequencies of the individuals having low, intermediate and high CR1 levels in the normal Indian population. There is a mounting line of evidence available following the observations of Brown and Broom[13] to demonstrate the existence of an inherited polymorphism of CR1. Several other observations in Caucasian populations[14-16] are also in accordance with the proposal of co-dominant allele pattern for the quantitative expression of CR1. This co-dominant allele model has further been strengthened by the identification of restriction fragment length polymorphism (RFLP) using a cDNA probe for CR1 and correlating it with the numerical expression of CR1 on erythrocytes.[6] Moldenhaver *et al*[16] reported gene frequencies of 0.27 and 0.73 for the L and H alleles. Evaluation of CR1 number by radioreceptor assay using C3b monomer and the affinity of CR1-C3b binding in various glomerular diseases showed a decreased expression of CR1 on the erythrocytes of AGN patients,[8] however this decrease in CR1 number was not a genetically inherited characteristic but a secondary phenomenon associated with the disease process (our unpublished observation). Thus, the CR1 levels observed in the patients are not the actual levels which would have been present before the occurrence of the disease. If the alleles controlling the quantitative expression of CR1 in the GN patient population are presumed to have a pattern of co-dominant inheritance, the gene frequencies of the L and H alleles should be same as that of normal population.

Two structural polymorphic forms of CR1 have been identified with molecular weights 190 kDa (CR1-A) and 220 kDa (CR1-B)[1,17] Dykman et al[18] have shown the existence of two more rare allelic forms of CR1 with molecular weights 160 kDa (CR1-C) and 250 kDa (CR1-D). These four allotypic forms of CR1 are present with the gene frequencies of 0.83, 0.16, 0.01 and 0.002 respectively in Caucasian populations. Studies on CR1 polymorphism in our laboratory by analysis of C3 binding protein however, have shown only the presence of only two allelic forms of CR1 in the Indian population with molecular weights of approximately 190 and 220 kDa. Immunoprecipitation with monoclonal anti-C3b receptor also yielded identical results confirming that the protein isolated by affinity column as CR1 protein. Unlike the results reported in Caucasian populations, we observed only the AA homozygous and AB heterozygous pattern but no BB homozygous pattern. The gene frequencies of A and B alleles in the Indian population were found to be 0.975 and 0.025, respectively. A similar range has been reported in Oriental populations with gene frequencies of 0.98 and 0.02 for A

and B alleles.[19] However, the reason for the variations in the gene frequencies in various ethnic groups remains unexplained. The gene frequencies of these alleles did not differ significantly between normal Indian individuals and various GN patients groups suggesting that the susceptibility to glomerulonephritis is not associated with any of the CR1 structural polymorphisms. In addition, no relationship was observed between the level of expression of CR1 and structural polymorphism, as indicated by the difference in their respectively gene frequencies. Van Dyne *et al*[19] have reported a relatively increased incidence of the C allele in SLE patients. A decreased CR1 level was found on the erythrocytes of patients with SLE.[14,20-28] Although, a decreased CR1 level in SLE was associated with an increased incidence of the C allele of the CR1 structural polymorphism, individuals with the C allele were found to have higher CR1 levels in the normals. This observation fails to explain the association of the C allele to disease susceptibility. The absence of an influence of quantitative expression of CR1 based on the polymorphic types further suggests that products of regulatory genes are necessarily not limited to cis-effect alone.

Thus the significance of the CR1 polymorphic pattern still remains an enigma. Perhaps linkage studies on loci regulating CR1 structural and quantitative expression could throw more light on this question. This however, requires in depth genetic analysis of the families investigated with an obligatory limitation of requiring homozygous and heterozygous parental alleles.

In addition to this, there was no functional differences among the four alleles.[17,29] Our failure to observe any change between the Ka values of normals and GN patients in C3b-CR1 binding studies,[8] is in agreement with these results. Thus the present study suggests that (i) the quantitative expression of CR1 is not influenced by its structural polymorphic forms, and (ii) the susceptibility to glomerular diseases is not associated with any of the structural polymorphic alleles.

## ACKNOWLEDGEMENTS

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