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Use of Complement Receptor 1 (CD35) Assay in the Diagnosis and Prognosis of Immune Complex Mediated Glomerulopathies

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SUMMARY **Complement Receptor 1 (CR1) is a polymorphic glycoprotein expressed on erythrocytes, leukocytes and glomerular podocytes and has a major role in immune complex processing. In addition, it regulates the complement cascade activation by preventing formation of classical and alternative pathway convertases and by acting as a cofactor for Factor I mediated cleavage of C3. In this study, we have examined the expression of erythrocyte CR1 (E-CR1) and glomerular CR1 (G-CR1) in different kinds of nephropathies using ELISA and immunofluorescence microscopy to understand their role in immune complex (IC) mediated renal diseases. E-CR1 was significantly reduced in all categories of lupus nephritis in comparison to normal subjects and non-IC renal diseases. However, other IC mediated diseases like IgA nephropathy and membranoproliferative glomerulonephritis had normal E-CR1 levels. G-CR1 showed distinct differences between IC and non-IC mediated diseases. G-CR1 was virtually absent in lupus kidneys. In other IC mediated diseases, there was a correlation of G-CR1 expression to the IC and complement fragment deposition. G-CR1 serves as a useful diagnostic marker for IC mediated diseases while E-CR1 is useful as a prognostic marker to monitor the course of disease after the treatment has initiated.**

Complement receptor 1 (CR1) is a polymorphic glycoprotein ranging in molecular weight from 160 to 250 kDa which plays a critical role in the erythrocyte immune complex (IC) clearing mechanisms.[1,2] Four allotypes of CR1 have been defined based on their structural polymorphism.[3] Kumar *et al.*[4] have demonstrated polymorphism by RFLP using *Hind* III restriction enzyme, which correlates with the level of expression of CR1. In blood, CR1 is expressed on erythrocytes (ECR1), neutrophils, eosinophils, monocytes and some T lymphocytes. Besides preventing the formation of classical and alternate pathway convertases, CR1 serves as a cofactor for Factor I mediated cleavage of C3 into iC3b and C3dg and C4b into C4c and C4d.[5] This prevents immune complexes bearing fixed iC3b and IgG from triggering neutrophil degranulation.[5] Bacteria and circulating immune complexes that activate complement are rapidly bound to E-CR1 and are transported to the liver where they are removed by the reticulo-endothelial system.

In the normal kidney, CR1 is present exclusively in the podocytes (G-CR1), distributed homogeneously on the plasma membrane.[6] IC mediated renal diseases are associated with activation of the complement cascade leading to renal injury. Complement regulatory proteins such as complement receptor 1, decay accelerating factor (CD55), membrane cofactor protein (CD46), CD59 and C8bp help to prevent tissue damage by inactivating the cascade at convertase and assembly points. Studies in our laboratory have shown that rheumatoid arthritis patients with high levels of circulating immune complexes have significantly reduced number of CR1 on their erythro-

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cytes.[4] The aim of our study was to determine CR1 expression on erythrocytes and podocytes in various types of glomerulopathies. We wanted to establish the utility of CR1 as a diagnostic and prognostic marker, its ability to differentiate between IC mediated and non-IC mediated renal injury and the correlation of E-CR1 and G-CR1 expression to the severity of immunoinflammation.

MATERIALS AND METHODS

Patients admitted in the nephrology ward of All India Institute of Medical Sciences, New Delhi, undergoing kidney biopsy for routine histological diagnosis for renal diseases were included in this study. The healthy controls were the staff and students of the All India Institute of Medical Sciences.

Samples

Heparinized blood was collected and plasma was separated by centrifugation at 800 x *g* for 10 minutes at 4°C and stored at -70°C. Serum was separated from plain blood and stored at -70°C. Erythrocytes were washed three times with phosphate buffered saline (PBS, 100 mM, pH 7.4) at 800 x *g* for 10 minutes and resuspended in PBS. Cell counting was done using a hemocytometer and the final concentration was adjusted to 5 x 10^7 cells/ml.

Enzyme linked immunosorbent assay for quantification of erythrocyte CR1

The assay was done in triplicates as described by Kumar *et al.*[4] RBCs were prepared as described above. All steps were performed at room temperature. One hundred microliters of poly-L-lysine (PLL; 0.1 mg/ml in PBS) was placed in a 96-well flat bottomed microtiter plate (flat bottomed high binding EIA plate, Costar, New York, U.S.A.) and incubated for 30 minutes. The plate was emptied by turning it upside down and blotting on a filter paper. To each well, 100 µl of erythrocyte suspension (containing 5 x 10^6 cells) was added. After 30 minutes, 100 µl of 0.05% glutaraldehyde was gently added and left for an additional 30 minutes. The glutaraldehyde was discarded and wells were washed thrice by adding 300 µl of PBS-T (PBS with 0.1% Tween). The plate was inverted to empty it and then blotted on an absorbent sheet for drying. The residual binding capacity of the polystyrene was blocked by incubating with 300 µl of PBS-containing 1% BSA (Fraction V, Sigma Chemical Co., Mo., U.S.A.) for 1 hour and unbound albumin was removed by washing.

One hundred microliters of mouse monoclonal antibody to human C3b receptor (Dako M710, Dakopatts, Glostrup, Denmark) diluted 1:1,000 in PBS was added to each well and incubated for 1 hour. Wells were washed and 100 µl of alkaline phosphatase conjugated rabbit anti-mouse IgG (Dakopatts) diluted 1:500 in PBS was added. After washing thrice with PBS-T and finally with PBS, 250 µl of freshly prepared substrate solution containing 1 mg/ml of p-nitrophenyl phosphate in carbonate buffer (0.1 M, pH 9.6 with 1 mM $MgCl_2$) was added to each well. The enzymatic reaction was allowed to take place for one hour. Two hundred microliters of the reaction product of individual wells was transferred to the corresponding wells of another plate prefilled with 100 µl of 1 N NaOH. The absorbance was measured at 405 nm in an ELISA reader (Anthos Instruments, Austria). A standard curve was plotted by using serial dilutions of RBCs (10^6-10^7 cells/well) isolated from the blood of a healthy individual having no symptom of rheumatic or infectious diseases. The E-CR1 level in this individual had been estimated in the laboratory using a radioreceptor assay and ELISA.

Kidney specimens

Seventy-five kidney tissue sections were obtained from the patients undergoing biopsy for routine histopathological diagnosis from the Departments of Nephrology and Pathology, AIIMS. The tissues were classified according to the final diagnosis and comprised 30 cases of systemic lupus erythematosus associated glomerulonephritis (LN; classified under WHO criteria).[11] The 48 cases of non-lupus nephritis included both IC associated glomerulopathies like IgA nephropathy (IgAN), membranous glomerulonephritis (MGN) and membrano-proliferative glomerulonephritis (MPGN) non-IC associated glomerulopathies like minimal change nephrotic syndrome (MCNS), focal segmental glomerulosclerosis (FSGS) diabetic glomerulosclerosis (DGS), polyarteritis nodosa (PAN) and ischemic glomerulopathy.

Antibodies

Fluorescein isothiocynate (FITC) labeled goat antibodies against IgG, IgM and IgA, mouse monoclonal antibodies to human CR1 (Dako M710), FITC-labeled rabbit anti-mouse IgG (Dako F313) and alkaline phosphatase conjugated rabbit anti-mouse IgG (Dako D314), rabbit anti-human C3c (Dako A062) and HRP-rabbit IgG

to human C3d (Dako P387) were obtained from Dakopatts.

Immunofluorescent staining

The processing and staining procedures were based on the method of Endoh *et al.*[8] In brief, the fixed 6 μm sections were incubated with the first antibody diluted in PBS-BSA. In parallel, control tissues were incubated with corresponding amounts of irrelevant mouse monoclonal antibodies of IgG class which had no specificity for the renal tissues. After three washes with PBS, sections were incubated with second antibody diluted in PBS-BSA for 45 minutes to one hour. The washed sections were then mounted in glycerol buffer and examined under Nikon epifluorescence microscope.

Diagnostic classification and clinical parameters

The cases were classified on the basis of the histopathological diagnosis under light and immunoflourescence microscopy.

Statistical analysis

A two sample Student *t*-test was used to compare the means of CR1 receptor numbers on erythrocytes in the different groups.

RESULTS

E-CR1 in normal subjects and patients with various immune complex and non immune complex associated renal diseases are shown in Table 1. The mean CR1/E in normal controls ± standard deviation were 646 ± 260, with no significant age and sex differences. Non-immune complex associated renal diseases, namely, MCNS, MGN, DGS and PAN did not show any significant variation from the controls. The mean CR1/E in MCNS ± standard deviation were 602 ± 127. Diabetic glomerulosclerosis and polyarteritis nodosa also did not show any significant difference, however, the sample size in these cases was insufficient.

All categories of lupus nephritis classified according to the WHO criteria showed significant reduction in E-CR1. The mean E-CR1 in Type III was 198 ± 18 while that in Type IV was 176 ± 4.5. In contrast, other IC mediated diseases like IgAN and MPGN had near normal levels of CR1/E.

Table 1 A correlation of CR1 expression in erythrocytes and kidney biopsies with complement deposition in IC and non-IC mediated renal diseases

Diagnosis	No.	IgG	IgM	IgA	C3b	C3d	G-CR1	E-CR1
Normal controls	46	nd	nd	nd	nd	nd	nd	646 ± 260
Non-IC associated*								
MCNS	20	-	-	-	-	-	+++	602 ± 127
Chronic TIN	2	-	-	-	-	-	++	646, 600
DGS	3	-	-	-	-	-	+++	466 ± 64
PAN	4	-	-	-	++	-	++	614 ± 45
IC associated								
IgAN	6	-	-	+++	++	++	++	648 ± 138
MGN	4	+	+	-	++	+	+	496 ± 67
MPGN	6	+	-	-	+	+	+	676 ± 20
Lupus Nephritis**								
Type III	8	+++	-	+	++	+++	-	196 ± 18
Type IV	12	+++	+	+	+	+++	-	176 ± 5
Type V	6	+	-	-	+	++	+	267 ± 86
Type VI	4	+++	++	++	++	++	-	123 ± 48

Intensity grading: (-) negative, (+) moderate, (++) strong, (+++) intense, nd: not determined.
MCNS:minimal change nephrotic syndrome; TIN: tubulo-interstitial nephritis; DGS: diabetic glomerulosclerosis; PAN: polyarteritis nodosa; IgAN: IgA nephropathy; Type III: focal segmental lupus nephritis; Type IV: diffuse proliferative lupus nephritis; G-CR1: glomerular complement receptor 1; E-CR1: erythrocyte CR1.
Immunofluorescent grading was done with the most fluorescent segment of the glomerulus as the most positive. Other segments were graded accordingly.
*No significance
**$p < 0.05$.

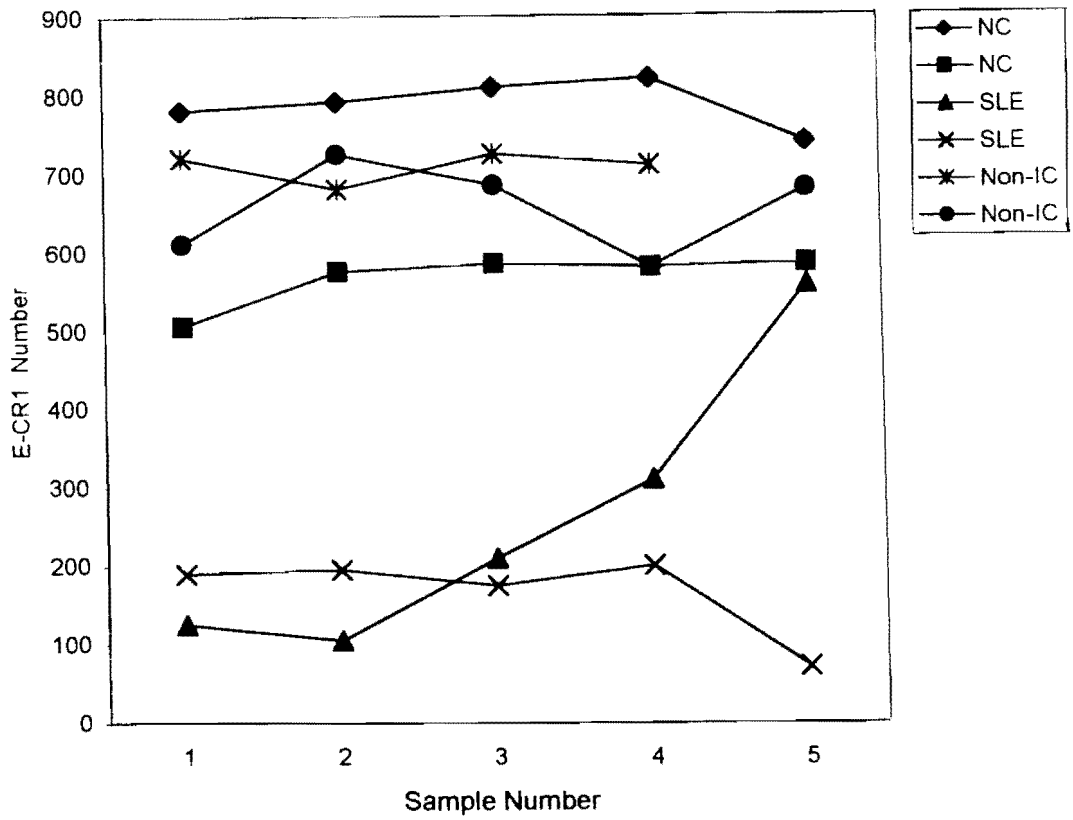

Fig. 1 Number of CR1/E in normal controls and patients at different time intervals (the patients were on treatment

Glomerular CR1 (G-CR1) showed clear difference between immune complex and non immune complex associated diseases (Table 1). G-CR1 was virtually absent in lupus kidneys. In other immune complex mediated diseases, the extent of decrease correlated with the degree of deposition of immune complexes (Table 1). IC mediated diseases also showed high levels of complement fragment deposits i.e. C3b and C3d.

Follow-up studies of E-CR1 levels were carried out in normal controls, non-IC disorders and lupus nephritis (Type IV) patients. The CR1 levels were assayed at the time of diagnosis and after the initiation of treatment at regular intervals. Mean levels of E-CR1 is represented in Fig. 1

DISCUSSION

The complement system protects against antibody and immune complex mediated renal diseases by a variety of mechanisms as exemplified by the occurrence of lupus like syndrome in individuals with genetic deficiencies of complement components.[7] Under certain circumstances, the kidney becomes a victim of the friendly fire that emanates from activation of the complement system.[6,8,9]

Complement mediated binding of circulating immune complexes (CIC) to E-CR1 protects against IC mediated renal diseases. E-CR1 levels are decreased as a result of damage to CR1 from IC transport and metabolism. Catabolism of E-CR1 may also be related to CR1 phenotype, age of the erythrocyte and age of the individual in addition to the CIC load.

On the other hand, the role of CR1 on the podocyte is not clear. Fischer *et al.*[10] have shown that each podocyte bears approximately 200,000 CR1 molecules. It has been suggested that podocyte CR1 may be involved in the transport of immune complexes. This appears unlikely, since significant amounts of immune complexes do not cross the glomerular filtration barrier in the physiological state.

Pascual *et al.*[9] have shown that vesicles containing CR1 released from the podocytes are excreted in normal urine. It has also been shown that most proteins of the complement cascade are synthesized in the renal glomeruli and tubules, especially C3 and C4.[11] Thus, the physiological role of podocyte CR1 might be in the prevention of complement activation in the renal tubular system. Conclusive proof awaits the study on CR1 knockout animal models.

In IC mediated glomerulonephritis such as lupus nephritis, the extent of decrease in G-CR1 was found to correlate with increased amount of immune complex deposits and reduced levels of E-CR1. However, we did not find reduction in E-CR1 and G-CR1 values in some cases of IC mediated diseases such as IgAN and MPGN. In IC mediated glomerulonephritis, complement activation by immune complexes might trigger the release of CR1 from the podocytes. In IgAN, IgA bearing complexes do not activate the complement cascade significantly,[2] and hence do not alter G-CR1 and E-CR1 levels markedly. E-CR1 also was found to be restored as the disease activity declined in lupus nephritis.

Since expression of G-CR1 was reduced in all IC mediated glomerulopathies, its quantitation can serve as a useful diagnostic marker of IC mediated disease activity. The differential loss of CR1 may be attributable to the specialized physiological roles of E-CR1 and G-CR1. Further large scale studies are required to confirm these observations and to validate the utility in diagnosis, prognostication and follow-up of patients with IC mediated glomerulonephritis (GN).

Further, on monitoring the E-CR1 expression at the onset and at different time point of treatment, it was observed that there is a dramatic increase in E-CR1 expression once the patients were kept on steroids and anti-inflammatory drugs. This makes E-CR1 expression a very reliable and easily usable diagnostic marker. Instead of performing an invasive biopsy again to ascertain if the disease has been controlled, assaying E-CR1 on blood sample from a patient will be much more convenient and cost effective.

REFERENCES

1. Fearon DT. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leucocyte, B-lymphocyte. J Exp Med 1980;152: 20-30.
2. Schifferli JA, Yin CN, Peters DK. The role of complement and its receptor in the elimination of immune complexes. N Engl J Med 1986; 315: 488-95.
3. Wong WW. Structural and functional correlation of the human complement receptor 1. J Invest Dermatol 1990; 94: 64S-7S.
4. Kumar A, Malaviya AN, Srivastava LM. Lowered expression of C3b receptor (CR1) on erythrocytes of rheumatoid arthritis patients. Immunobiol 1994; 191: 9-20.
5. Taylor RP, Burge J, Horgan C, Shasby DM. The complement mediated binding of soluble antibody/dsDNA immune complexes to human neutrophils. J Immunol 1983;130: 2656-62.
6. Kazatchkine MD, Fearon DT, Appay MD, Mandet C, Bariety C. Immunohistochemical study of the human glomerular C3b receptor in normal kidney and 75 cases of renal diseases: Loss of C3b receptor antigen in focal hyalinosis and in proliferative nephritis of systemic lupus erythematosus. J Clin Invest 1982; 69: 900-12.
7. Morgan BP, Walport MJ. Complement deficiency and disease. Immunol Today 1986; 12: 301-6.
8. Appay MD, Kazatchkine MD, Levi-Strauss M, Hinglais N, Barrety J. Expression of CR1 (CD35) mRNA in podocytes from adult and fetal human kidneys. Kidney Int 1990; 38:289-93.
9. Pascual M, Steiger G, Sadallah S, Paccaud J, Carpentier J, James R, Schifferli JA. Identification of membrane bound CR1 (CD35) in human urine. Evidence for its release by glomerular podocytes. J Exp Med 1994; 179: 889-909.
10. Fischer E, Appay MD, Cook J, Kazatchkine MD. Characterization of the human glomerular C3 receptor as the C3b/C4b complement type one (CR1) receptor. J Immunol 1986; 136: 1373-7.
11. Andrews PA, Zhou W, Sacks SH. Tissue synthesis of complement as an immune regulator. Mol Med Today 1995; 1: 202-7.