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Distribution of the *Hin*dIII restriction fragment length polymorphism among patients with systemic lupus erythematosus with different concentrations of CR1

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Abstract

Sixty six patients with systemic lupus erythematosus (SLE) were genotyped using a *Hin*dIII restriction fragment length polymorphism identified by CR1.1 cDNA, then were followed up for an average of 50 months to evaluate the stability of their CR1 activities. The gene frequencies for the two alleles which correlate with the numeric expression of CR1 on the erythrocytes were not significantly different between 66 patients with SLE and 52 normal controls. A discrepancy between homozygosity for a high allele and a negative CR1 activity was found in many patients. These patients, however, had significantly lower concentrations of serum complement than did patients with a positive CR1, and some were in an active state of the disease. Furthermore, there were several patients in whom the CR1 activities changed from negative to positive together with an increase in serum complement. Our results suggest that the decreased expression of CR1 on erythrocytes in patients with SLE is not inherited, rather it is a consequence of the disease processes.

The human receptor for complement fragments C3b and C4b (complement receptor type 1: CR1, CD35) is expressed on a variety of blood and tissue cells.[1–5] Despite the low number of CR1 per erythrocyte, these cells carry on their surfaces more than 90% of the circulation pool, because they are present in large numbers compared with other circulating cells.[1] Recent evidence suggests that CR1 plays an important part in the processing and clearance of circulating immune complexes.[6–8] Thus CR1 on the erythrocytes may protect against diseases mediated by deposition of circulating immune complexes.

Patients with systemic lupus erythematosus (SLE) were found to have reduced numbers of CR1 on erythrocytes. It remains controversial, however, whether the decreased expression is linked to inherited[9–13] or to acquired factors.[14–20]

A quantitative polymorphism of CR1 on erythrocytes was also noted in a normal group, and initial family studies suggested that this event was controlled by an autosomal locus with two codominant alleles.[10] Wilson *et al*[21] discovered a *Hin*dIII restriction fragment length polymorphism (RFLP) identified by a partial complementary DNA (cDNA) of the CR1 molecule (called CR1.1), which correlates with the number of CR1 on normal erythrocytes—the 7·4 kb and 6·9 kb bands corresponding to high and low expression respectively. This approach makes feasible determination of whether or not the CR1 deficiency in patients with SLE is linked to these genotypes.

In a previous study we discussed the defective CR1 in patients with SLE as the genetic factor because of their stability during over two years' follow up.[22] We have followed up these same patients for an additional three to five years and, using Southern blot analysis, examined the relation between genotype and phenotypic data.

Patients and methods

STUDY GROUP

Sixty six unselected Japanese patients with SLE, from a previous study,[22] who were receiving continuous care in the clinic of the first department of internal medicine of Kyushu University, were studied between July 1984 and March 1989. The diagnosis of SLE was based on the revised criteria of the American Rheumatism Association.[23] They were classified as having active disease if one or more of the following were present: rash, fever, arthritis, alopecia, serositis, glomerulonephritis, and central nervous system lupus, and/or two or more laboratory test results were abnormal, such as blood cell count, serum complement, anti-DNA antibody, immune complex, and erythrocyte sedimentation rate. Hypocomplementaemia was defined as a decrease in CH50 and either C3 or C4.

IMMUNE-ADHERENCE HAEMAGGLUTINATION

The CR1 concentrations were measured every month by immune adherence haemagglutination.[22] Erythrocytes obtained from EDTA treated blood were suspended in gelatin-veronal buffer. Human IgG was prepared from pooled serum samples by salt precipitation and chromatography on diethylaminoethyl-cellulose. A solution of IgG in phosphate buffered saline (10 mg/ml) was heated at 65°C for 30 minutes. After centrifugation at 15 000 *g* for 15 minutes the supernatant was used as the aggregated human γ globulin. Serial twofold dilutions of aggregated human γ globulin starting from 100 μg/ml were prepared in gelatin-veronal buffer in a polystyrene U bottomed microtitre plate. Complement was prepared by dilution of guinea pig serum in gelatin-veronal buffer containing 3

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CH50 U/ml, 25 μl of which was added to each well. The plate was incubated at 37°C for 45 minutes. Thereafter, 25 μl of dithiothreitol solution (3 mg/ml) was added to protect the generated C3b sites from decay. Subsequently, 25 μl of erythrocytes (2×10^8 cells/ml in EDTA-gelatin-veronal buffer) suspension was transferred to each well and the plate was incubated at 24°C for a further 60 minutes. When the haemagglutination exceeded 2^5 dilution a positive value was recorded.

SOUTHERN BLOT ANALYSIS OF GENOMIC DNA

DNA was obtained from peripheral white blood cells by sodium dodecyl sulphate-proteinase K digestion, followed by phenol-chloroform extraction. Then the DNA was digested with *Hin*dIII restriction enzyme (Nippon Gene, Tokyo) at a concentration of 3 U/μg DNA. The DNA fragments were separated according to size by electrophoresis in 0·7% agarose gels and transferred to a nylon membrane (Zeta-probe: Bio-rad, Richmond, CA), by the method of Southern.[24] Prehybridisation and hybridisation were carried out, at 42°C in the presence of 50% formamide, according to the manufacturer's instructions. A 0·79 kb *Eco*RI insert from pBR327 plasmid called CR1.1, which is related to the CR1 gene,[25] was used as the probe and was labelled with α-(^{32}P)-dCTP by multiprime DNA labelling systems (Amersham Japan, Tokyo). After hybridisation the filters were washed under stringent conditions at 65°C. Autoradiography was performed at −70°C with a Kodak XAR-5 film.

LABORATORY ASSESSMENT

Complement activity (CH50) was assessed by a modification of Mayer's method. Serum C3 and C4 were determined by single radial immunodiffusion until August 1987, and after that by laser nephelometry. Anti-DNA antibody was measured by radioimmunoassay and immune complex was measured by a C1q binding enzyme linked immunosorbent assay (ELISA).

STATISTICAL ANALYSIS

Variables were compared by χ^2 test or Student's *t* test.

Results

HINDIII RFLP PATTERN IN PATIENTS WITH SLE AND CONTROLS

A CR1 cDNA probe was used to study the *Hin*dIII RFLP in 66 patients with SLE and in 52 normal controls. As reported previously,[21] polymorphic bands at 7·4 kb and 6·9 kb were obtained. Among 66 patients with SLE, 40 had only the 7·4 kb band, three had only the 6·9 kb band, and 23 patients were heterozygous for the 7·4 kb and 6·9 kb bands. The gene frequencies were 0·78 for the 7·4 kb and 0·22 for the 6·9 kb band and did not differ significantly from findings in the unselected control group (table 1).

Table 1 Distribution of the HindIII *restriction fragment length polymorphism (RFLP) among patients with systemic lupus erythematosus (SLE) and normal subjects*

Subjects	HindIII RFLP			Total
	7·4/7·4 kb	7·4/6·9 kb	6·9/6·9 kb	
SLE	40	23	3	66
Normal	34	15	3	52

Gene frequencies did not differ significantly (p>0·5)

CHANGES IN CR1 CONCENTRATIONS IN PATIENTS WITH SLE DURING LONG TERM FOLLOW UP

CR1 concentrations on erythrocytes from 66 patients with SLE were examined by immune-adherence haemagglutination for 35 to 55 months. At the initial study no CR1 activity was evident in 56 patients. Among these 56 patients, 38 were persistently negative. The CR1 activity varied in 18 patients during the follow up and eight became constantly positive. On the other hand, among 10 patients with positive CR1 at the initial study, three became negative, but seven patients were persistently positive.

DISTRIBUTION OF HINDIII RFLP AMONG PATIENTS WITH SLE WITH DIFFERENT CR1 CONCENTRATIONS

Table 2 shows the relation between CR1 concentrations and the *Hin*dIII RFLP patterns in the patients with SLE. Three patients with only the 6·9 kb band showed a persistently negative CR1 and the seven with a persistently positive CR1 were all homozygous for the 7·4 kb band. For the other 56 patients, however, there was a discrepancy between CR1 concentrations and RFLP patterns.

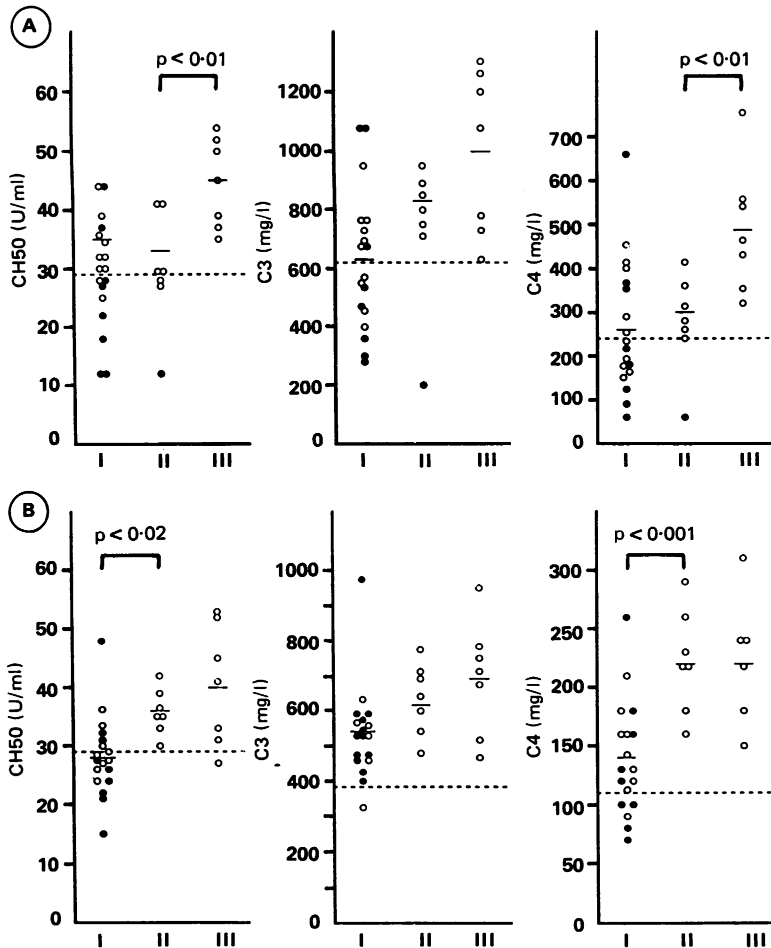
RELATION BETWEEN CR1 CONCENTRATIONS AND DISEASE ACTIVITIES

To evaluate the existence of acquired factors which influence CR1 concentrations we examined the patients with SLE who only had the 7·4 kb bands and separated them into three groups based on CR1 activity (group I: persistently negative, group II: varied, group III: persistently positive). Eight patients in group II with frequently changing CR1 concentrations were excluded from analysis.

In group I 13 patients had episodes of active disease, but they had a negative CR1 even during the inactive state. The other five were inactive during follow up. In group II changes in CR1 concentrations accompanied by remission were evident in only one patient. The other six were inactive and the CR1 changed from negative to positive but with no change in the disease activities. All seven patients in group III were inactive during follow up.

Table 2 Distribution of the HindIII *restriction fragment length polymorphism (RFLP) among patients with systemic lupus erythematosus with different CR1 activity*

CR1 activity	HindIII RFLP			Total
	7·4/7·4 kb	7·4/6·9 kb	6·9/6·9 kb	
Persistently negative	18	16	3	37
Varied	15	7	0	22
Persistently positive	7	0	0	7
Total	40	23	3	66



Serum complements, CH50, C3, and C4, in patients with systemic lupus erythematosus. (A) Initial study: CH50 and C4 concentrations in group II patients who were negative CR1 at that time were similar to those in group I patients with persistently negative CR1 and significantly lower than those in group III patients with a persistently positive CR1 (p<0·01, p<0·01 respectively). (B) Follow up study: CH50 and C4 concentrations in group II patients who were positive CR1 at that time were similar to those in group III and significantly higher than those in group I (p<0·02, p<0·001 respectively). Bars depict the mean for each group; broken lines depict the normal mean; ●=active disease; ○=inactive disease. Group I=persistently negative, group II varied, and group III persistently positive CR1.

RELATION BETWEEN THE CR1 CONCENTRATIONS AND SERUM COMPLEMENT

The figure shows serum complement concentrations in these three groups at the initial and follow up study. At the initial study (A) the mean value of CH50 in group II with a negative CR1 at that time was 30 (SD 10) U/ml, a value close to the 29 (9) U/ml in group I and significantly different from the 45 (8) U/ml in group III (p<0·01). Similarly, the mean (SD) value of C4 in group II was 270 (110) mg/l, a value similar to the 260 (150) mg/l in group I and significantly different from the 490 (140) mg/l in group III (p<0·01).

In contrast, at the follow up study (B) the mean value of CH50 in group II with a positive CR1 at that time was 36 (4) U/ml—that is, significantly different from the 28 (7) U/ml in group I (p<0·02) but similar to the 40 (10) U/ml in group III. Similarly, the mean value of C4 in group II was 220 (40) mg/l, which significantly differed from the 140 (50) mg/l in group I (p<0·001) and was similar to the 220 (50) mg/l in group III.

The mean value of C3 was significantly different between groups I and III in both studies, but values in group II were not statistically different from those for each group.

RELATION BETWEEN THE CR1 CONCENTRATIONS AND OTHER MANIFESTATIONS

We further examined 18 patients in group I with a persistently negative CR1. Nine patients were in an active state of the disease and among the nine patients with inactive disease, seven had leucocytopenia, or hypocomplementaemia, or high levels of antibody to DNA. Consequently, there were only two patients (11%) with negative CR1 and no apparent disorder. On the other hand, among 13 patients in groups II and III with positive CR1 at follow up, one patient had leucocytopenia and three had slightly high levels of antibody to DNA. Therefore in nine (69%) of 13 patients with positive CR1 there were no abnormal findings.

Discussion

In this study we investigated the distribution of RFLPs, which correlate with the numeric expression of CR1, on erythrocytes from 66 Japanese patients with SLE, using the CR1.1 cDNA probe and *Hin*dIII restriction enzyme. Wilson *et al* first reported a smaller proportion homozygous for the 7·4 kb band among patients with SLE and their relatives than in controls.[13] Moldenhauer *et al*[18] and Cohen *et al*,[19] however, reported no difference between them. As table 1 shows that the frequencies of each genotype did not differ significantly between patients with SLE and normal controls.

Cohen *et al* also suggested that homozygosity of the 6·9 kb band could be a protective genetic factor for SLE. The data pooled from these studies[13 18 19] indicate that only one of the 132 patients with SLE showed homozygosity of the 6·9 kb band. Among our patients, however, we found this genotype in three of 66 patients with SLE and in three of 52 normal controls. Although the gene frequencies of each band did not differ among these reports, ethnic differences may play some part.

Correlations between the erythrocyte CR1 numbers and immunological indices such as serum complement, antibody to DNA, and circulating immune complex were examined,[12 14–16 20] but these results were also controversial perhaps because the genotype had not been taken into account. In those of our patients homozygous for the 7·4 kb allele it was noteworthy that all subjects with active disease were among those with a negative CR1. There were patients homozygous for the 7·4 kb allele who had negative CR1, however, despite their inactive state. As shown in the figure the concentration of serum complements in patients with a negative CR1 was significantly lower than those in patients with a positive CR1, and in group II, the change in CR1 from negative to positive was accompanied by an increase in serum complement. The existence of potential complement activation has been described even in patients with SLE in clinical remission.[26 27] Moreover, Tokiyama *et al* showed that complement activation played an important part in

persistent hypocomplementaemia in patients with inactive SLE.[28] The erythrocyte CR1 may have a functional role in the removal of circulating immune complexes and this process would depend on complement consumption. Therefore, even if the genotypes are homozygous for the 7·4 kb allele, the CR1 activities may be negative as long as immunological disorders remain and the complement concentrations are low.

In conclusion, our results support the notion that a decreased CR1 expression on erythrocytes in patients with SLE is not inherited, rather it is the consequence of the disease process.

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1 Fearon D T. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leucocyte, B lymphocyte, and monocyte. *J Exp Med* 1980; **152:** 20–30.
2 Reynes M, Aubert J P, Cohen J H M, *et al.* Human follicular dendric cells express CR1, CR2 and CR3 complement receptor antigens. *J Immunol* 1985; **135:** 2687–94.
3 Wilson J G, Tedder T F, Fearon D T. Antigenic and functional detection of C3b receptors on human peripheral blood T-lymphocytes. *Fed Proc* 1982; **41:** 965.
4 Kazatchkine M D, Fearon D T, Appay M D, Mandet C, Bariety J. Immunochemical study of the human glomerular C3b receptor in normal kidney and in seventy-five cases of renal diseases. *J Clin Invest* 1982; **69:** 900–12.
5 Ross G D. Identification of human lymphocyte subpopulations by surface marker analysis. *Blood* 1979; **53:** 799–811.
6 Schifferli J A, Ng Y C, Estreicher J, Walport M J. The clearance of tetanous toxoid/anti-tetanus toxoid immune complexes from the circulations of humans. Complement- and erythrocyte complement receptor 1-dependent mechanisms. *J Immunol* 1988; **140:** 899–904.
7 Ng Y C, Schifferli J A, Walport M J. Immune complexes and erythrocyte CR1 (complement receptor type 1): effect of CR1 numbers on binding and release reactions. *Clin Exp Immunol* 1988; **71:** 481–5.
8 Inada Y, Kamiyama M, Kanemitsu T, *et al.* In vivo binding of circulating immune complexes by C3b receptors (CR1) of transfused erythrocytes. *Ann Rheum Dis* 1989; **48:** 287–94.
9 Miyakawa Y, Yamada A, Kosaka K, Tsuda F, Kosugi E, Mayumi M. Defective immune-adherence (C3b) receptor on erythrocytes from patients with systemic lupus erythematosus. *Lancet* 1981; **ii:** 493–7.
10 Wilson J G, Wong W W, Schur P H, Fearon D T. Mode of inheritance of decreased C3b receptors on erythrocytes of patients with systemic lupus erythematosus. *N Engl J Med* 1982; **307:** 981–6.
11 Minota S, Terai C, Nojima Y, *et al.* Low C3b receptor reactivity on erythrocytes from patients with systemic lupus erythematosus detected by immune adherence hemagglutination and radioimmunoassays with monoclonal antibody. *Arthritis Rheum* 1984; **27:** 1329–35.
12 Thomsen B S, Nielsen H, Andersen V. Erythrocyte CR1 (C3b/C4b receptor) levels and disease activity in patients with SLE. *Scand J Rheumatol* 1987; **16:** 339–46.
13 Wilson J G, Wong W W, Murphy E E III, Schur P H, Fearon D T. Deficiency of the C3b/C4b receptor (CR1) of erythrocytes in systemic lupus erythematosus: analysis of the stability of the defect and of a restriction fragment length polymorphism of the CR1 gene. *J Immunol* 1987; **138:** 2706–10.
14 Iida K, Mornaghi R, Nussenzweig V. Complement receptor (CR1) deficiency in erythrocytes from patients with systemic lupus erythematosus. *J Exp Med* 1982; **155:** 1427–38.
15 Inada Y, Kamiyama M, Kanemitsu T, Hyman C L, Clark W S. Studies on immune adherence (C3b) receptor activity of human erythrocytes: relationship between receptor activity and presence of immune complex in serum. *Clin Exp Immunol* 1982; **50:** 189–97.
16 Yoshida K, Yukiyama Y, Hirose S, Miyamoto T. The change in C3b receptors on erythrocytes from patients with systemic lupus erythematosus. *Clin Exp Immunol* 1985; **60:** 613–21.
17 Ross G D, Yount W J, Walport M J, *et al.* Disease associated loss of erythrocyte complement receptors (CR1, C3b receptors) in patients with systemic lupus erythematosus and other diseases involving autoantibodies and/or complement activation. *J Immunol* 1985; **135:** 2005–14.
18 Moldenhauer F, David J, Fielder A H L, Lachmann P J, Walport M J. Inherited deficiency of erythrocyte complement receptor type 1 does not cause susceptibility to systemic lupus erythematosus. *Arthritis Rheum* 1987; **30:** 961–6.
19 Cohen J H M, Caudwell V, Levi-Strauss M, Bourgeois P, Kazatchkine M D. Genetic analysis of CR1 expression on erythrocytes of patients with systemic lupus erythematosus. *Arthritis Rheum* 1989; **32:** 393–7.
20 Yen J-H, Liu H-W, Lin S-F, Chen J-R, Chen T P. Erythrocyte complement receptor type 1 in patients with systemic lupus erythematosus. *J Rheumatol* 1989; **16:** 1320–5.
21 Wilson J G, Murphy E E, Wong W W, Klickstein L B, Weis J H, Fearon D T. Identification of a restriction fragment length polymorphism by a CR1 cDNA that correlates with the number of CR1 on erythrocytes. *J Exp Med* 1986; **164:** 50–9.
22 Kawaguchi T, Yokota E, Niho Y, Imamura T. Complement receptor 1 (CR1) on erythrocytes of patients with systemic lupus erythematosus. *Japanese Journal of Human Genetics* 1988; **33:** 451–60.
23 Tan E M, Cohen A S, Fries J F, *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25:** 1271–7.
24 Southern E M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; **98:** 503–17.
25 Wong W W, Klickstein L B, Smith J A, Weis J H, Fearon D T. Identification of a partial cDNA clone for the human receptor for complement fragments C3b/C4b. *Proc Natl Acad Sci USA* 1985; **82:** 7711–5.
26 Swaak A J G, van Rooyen A, Vogelaar C, Pillay M, Hack E. Complement (C3) metabolism in systemic lupus erythematosus in relation to the disease course. *Rheumatol Int* 1986; **6:** 221–6.
27 Charlesworth J A, Peake P W, Golding J, *et al.* Hypercatabolism of C3 and C4 in active and inactive systemic lupus erythematosus. *Ann Rheum Dis* 1989; **48:** 153–9.
28 Tokiyama K, Yokota E, Chifu Y, *et al.* Mechanism of persistent hypocomplementemia in systemic lupus erythematosus. Analysis of plasma C3a and C4a. *Japanese Journal of Clinical Immunology* 1989; **12:** 156–61.