QUANTITATIVE VARIATIONS OF THE C3b/C4b RECEPTOR (CR1) IN HUMAN ERYTHROCYTES ARE CONTROLLED BY GENES WITHIN THE REGULATOR OF COMPLEMENT ACTIVATION (RCA) GENE CLUSTER

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The human C3b/C4b receptor (CR1) is a single polypeptide chain glycoprotein, first isolated from the erythrocyte membrane (1), that plays an important role in the regulation of the activation of the complement system. It is a cofactor of Factor I in the cleavage of C3b and C4b, and a potent inhibitor of the C3/C5 convertases of both the classical and the alternate pathways (1, 2). In addition, CR1 has a major role in the processing and clearance of circulating immune complexes (3, 4). Genetically, CR1 is encoded by C3bR, a polymorphic locus that determines at least four alleles, C3bR-A, C3bR-B, C3bR-C and C3bR-D, the products of which differ in their M, on SDS-PAGE (5-8).

Linkage analysis of the loci encoding C4-binding protein (C4BP), CR1 (C3bR), and Factor H (FH)1 in informative families demonstrated that these three genes are closely linked in humans (9, 10). The C4BP locus is so closely linked to the C3bR locus that no recombinants have been observed thus far, and complete linkage disequilibrium exists between the C4BP-2 and C3bR-B alleles (9, and our unpublished observations) in studies comprising 130 families and several hundred unrelated individuals. The distance between C4BP-C3bR and FH has been estimated in the range of 5-10 cm (10 and our unpublished observations). We have named this linkage group the regulator of complement activation (RCA), and we have shown (10) that it segregates independently of HLA and of the C3 locus. Recently, the gene encoding CR1 has been cloned (11) and mapped to the long arm of chromosome 1 in humans (12), thereby establishing the chromosomal location of all the genes within the RCA gene cluster.

In addition to the electrophoretic variation, differences have been encountered in the number of CR1 molecules per red cell expressed by different individuals, which are also believed to be under genetic control (13-15). The nature and the mode of inheritance of this regulatory control are unclear. It has been proposed (15), for example, that these quantitative variations are controlled by a diallelic locus, designated C3bRQ. Under this model, the phenotypic frequencies of the

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1 Abbreviations used in this paper: Ch, child; EACA, e-aminocaproic acid; Fa, father; FH, factor H; Mo, mother; RCA, regulator of complement activation; TN buffer, buffer with Tris and NaCl.
quantitative variants should fit a trimodal distribution in the population, a proposition that has recently been questioned (16). Quantitative variation has also been encountered in heterozygotes for structural gene alleles. In these people, it is possible to measure the relative quantities of CR1 produced by each allele. The proportion is constant for each individual but varies substantially among different qualitatively identical heterozygotes (5, 7).

In this paper we analyze the genetic relationships of structural and quantitative variations of CR1. Our results indicate that the putative C3bRQ locus is located within the RCA gene cluster and closely linked to the C3bR structural locus. In addition, we show the existence of multiple alleles at the C3bRQ locus that control the levels of expression of the C3bR structural alleles by a cis-acting mechanism.

Materials and Methods

**Materials.** e-aminocaproic acid (EACA), NP-40, 2-ME, PMSF, EDTA, Coomassie Brilliant Blue R, and neuraminidase type V (from Clostridium perfringens) were from Sigma Chemical Co., St. Louis, MO. Ampholines (pH 3.5-10, 4-6, and 6-8) from LKB Produkter, Bromma, Sweden; N,N'-methylene-bis-acrylamide, acrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, and SDS were obtained from Bio-Rad Laboratories, Richmond, CA; and ultrapure urea was from Schwarz-Mann Biotech, Cambridge, MA.

**Samples.** Human blood samples were obtained by venipuncture and collected in standard ACD (acid/citrate/dextrose, formula A) anticoagulant. Tests were done on fresh specimens whenever possible. Otherwise, samples were frozen and kept in the gas phase above liquid nitrogen until used. Additionally, samples of EDTA-anticoagulated and clotted blood were used to allotype for C4BP and FH. EDTA-plasma and serum were separated from each specimen within 2 h of collection and stored at −80°C until use.

**Allotyping of C4BP and FH.** C4BP and FH phenotyping were performed as previously described (17) using fresh or fresh-frozen (−80°C) EDTA-plasma or serum. Samples (90 μl) were treated with 1 U of neuraminidase (in 10 μl of 1 M phosphate buffer, pH 7.0, containing 0.2 M EDTA, 50 mM EACA, 0.5% NaN₃, and 1 mM PMSF) for 2 h at 37°C. C4BP and FH were immunoprecipitated using rabbit polyclonal monospecific antisera (vol/vol), and the immunoprecipitates were washed twice in 0.1 M acetic acid buffer, pH 4.0, and 0.1 M borate buffer, pH 8.0, both buffers containing 1 M NaCl. The immunoprecipitates were then dissolved in 8 M urea containing 1% NP-40, 10% 2-ME, and 4% ampholines (pH 3.5-10, 4-6, 6-8; 1:4:4) and analyzed by IEF under completely denaturing conditions on vertical 4.5% polyacrylamide slab gels. A mixture of ampholines (pH 3.5-10, 4-6, 6-8; 1:4:4) were used to generate the pH gradient. Gels were run for 15 h at room temperature at 10 mA constant current, reaching the maximum voltage of 500 V. After focusing, the gels were fixed in methanol, acetic acid, and water (40:10:100, vol/vol/vol), and stained with Coomassie Brilliant Blue (0.15 g/liter of methanol, acetic acid, and water, 70:14:100, vol/vol/vol).

**Allotyping and Quantitative Determinations of C3bR Alleles by Western Blot Analysis.** Red cells were washed six times with PBS (pH 7.2) and the packed red cells were hemolyzed by quick-freezing and thawing. 300 μl of hemolysate (3 × 10⁹ cells) were then mixed with an equal volume of PBS containing 1% NP-40, the mixture was made 1 mM in PMSF and allowed to stand for 45 min at 4°C. Insoluble materials were removed by centrifugation at 12,000 g at 4°C. Aliquots of each of the supernatants, (solubilized red cells) were transferred to fresh tubes and incubated at room temperature for 2 h with an excess of either an mAb against CR1 (57F) or purified rabbit IgG anti–human CR1 (both reagents were generous gifts of Dr. V. Nussenzweig, Department of Pathology, New York University, New York). Immune complexes were precipitated by incubating overnight at 4°C with an excess of Sepharose-conjugated goat anti–mouse IgG or goat anti–rabbit IgG (Dynatech Laboratories, Inc., Alexandria, VA) respectively. The beads were carefully washed three times with 5 ml of PBS containing 1% NP-40, resuspended in 100 μl of 0.1
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M Tris (pH 6.8) containing 2% SDS, 10% glycerol, and 0.01% bromophenol blue, and boiled for 2 min. The total content of each tube was loaded on 5% SDS-polyacrylamide gels with a 3% stacking gel, and electrophoresed using the discontinuous buffer system of Laemmli (18). Electrophoretic transfer was performed in Tris-glycine (25 mM Tris and 192 mM glycine) containing 20% (vol) methanol by using a Trans-Blot cell (Bio-Rad Laboratories) for 7–9 h at 4°C and 250 mA, blotting onto nitrocellulose membranes (BA 85; Schleicher and Schuell, Keene, NH). The blots were first blocked overnight at 4°C in 50 mM Tris, 150 mM NaCl, pH 8 (TN buffer) containing 20% FCS and 0.05% NaN₃, and then incubated in 10 ml of TN buffer containing 2% FCS, 0.05% Tween-20, and 125I-labelled antibodies against human CRI for 2 h at room temperature with gentle mixing. Rabbit polyclonal antibodies and 57F mAb were used with similar results. 125I-labelled antibodies were prepared using Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions.

After incubation with 125I-labelled antibodies, the nitrocellulose membranes were washed three times with 100 ml of TN buffer containing 0.05% Tween-20, dried, and exposed for autoradiography at −80°C using Kodak XAR-5 film and Cronex Lightning-plus intensifying screens (DuPont Co., Wilmington, DE). A scanning densitometer, (Bio-Rad Laboratories) was used for the quantitation of the intensity of the autoradiographic bands.

Quantitative Determination of Total Erythrocytic CRI by Immunoradiometric Assays. The number of CRI molecules on erythrocytes was measured by a specific immunoradiometric assay using monoclonal and polyclonal anti-CRI antibodies. This assay is a modification of methods previously described (13, 20) for studies on SLE. Briefly, 96-well microtiter plates (Dynatech Laboratories, Inc.) were coated with anti-human CRI mAb (57F). Solubilized red cells, prepared as described above for the quantitative determination by Western blot analysis, were diluted 1:20 or 1:40 in PBS, adjusting the final concentration of NP-40 to 0.1%. 50 μl were then added to triplicate wells and incubated overnight at 4°C. The wells were then washed four times with PBS containing 1% BSA and 0.1% NP-40, and the amount of bound CRI was quantitated by the addition of radiolabelled anti-human CRI rabbit polyclonal antibody. Each determination includes the standard of a titration of purified CRI for comparison, as well as extracts from known erythrocyte preparations. The number of CRI molecules was estimated by assuming an average M, 200,000.

Results and Discussion

Variations in the number of CRI molecules on human erythrocytes was first shown by Miyakawa et al. (14) using immune adherence hemagglutination, which involves the agglutination of erythrocytes by C3b-bearing immune complexes. This initial observation was soon independently confirmed by other studies involving direct binding of dimeric C3b (15), mAb against CR1 (13, 16), or rabbit polyclonal anti-CRI (15) to human erythrocytes, and immunoradiometric assays using C3b-coated (13) or anti-CR1–coated (20) microtiter plates and 125I–anti-CRI antibodies. All of these methods for the quantitation of CR1 yield equivalent results, and in fact, direct comparison of erythrocytic CR1 levels determined by these different methods showed excellent correlation (13, 15, 16, 20). In this study, we have performed the quantitative determinations of erythrocytic CR1 with similar immunoradiometric assays and the same reagents used in these previous studies (13, 16, 20).

As reported previously for other population samples (15–16, 20), an almost continuous 10-fold range variation existed in the total population sample we studied (not shown). Also in agreement with previous studies (15), no association was found between levels of CR1 and individual CR1 structural alleles. Since
erythrocytic CR1 levels have been shown (13-15) to be genetically determined, we undertook the analysis of the segregation of quantitative and structural variations of CR1 within families.

Families were selected for this study if they had at least one parent heterozygous for the RCA system, and if members of the pedigree exhibited significantly different numbers of CR1 molecules per cell. The five sibships that satisfied both criteria are shown in Table I. Because of the tight linkage between the structural loci C3BR, C4BP, and FH (the RCA cluster of genes), parents heterozygous for at least one of these were informative for joint segregation with CR1 levels. In family BRU, the three children differed in at least one RCA haplotype, and expressed very different amounts of CR1. The combination of the paternal (Fa) a with the maternal (Mo) c genes (child [Ch 3]) resulted in the highest number of CR1 molecules per cell, while the opposite combination, b, d (Ch 2) produced the lowest. The number of CR1 molecules per cell expressed by Ch 1 (a, d) was intermediate between the other two, and similar to those in the parents. According to the codominant hypothesis of Wilson et al. (15), both parents should be heterozygotes for alleles at the C3bRQ locus determining high and low expression. The paternal a and maternal c RCA haplotypes segregated with the C3bRQ high allele, while the other two (b and d) RCA-haplotypes segregated with the C3bRQ low allele. Family BAC had two sets of RCA-identical sibling pairs that differed in one haplotype. Overall, this family had lower numbers of CR1 molecules than family BRU; both BAC parents were in the range of the lowest expression of CR1 in BRU, Ch 2. Each set of RCA-identical sibling pairs expressed similar numbers of CR1 that differed significantly from the other set, the lower being significantly below those in Ch 2 in BRU. These two families, taken together, indicate that the number of alleles at the quantitative control locus exceeds the two suggested in the earlier literature (15). Family PRE was similar. Again, there are two sets of RCA-identical sibling pairs each with very much the same numbers of CR1 molecules. The broad range of these quantitative phenotypes further indicates the existence of multiple C3bRQ alleles.

In family FRI, the mother's RCA haplotypes are structurally indistinguishable, but the inheritance of the paternal RCA haplotypes is easily discernible. Thus, the father's a haplotype, in Ch 4, 5, and 6 resulted in levels of CR1 significantly higher than those present on the erythrocytes of Ch 1, 2, and 3, who inherited the paternal RCA haplotype b.

In family CYP, duplicate or triplicate examples of all four possible RCA genotypes were observed, each set characterized by a common quantitative phenotype.

These data, therefore, indicate that the number of CR1 molecules per cell is inherited, and that it segregates together with the RCA gene cluster. After completion of this work, we learned of results by Wilson et al. (19), who used a cloned cDNA probe to detect human CR1-restriction fragment length polymorphisms, and obtained data consistent with this conclusion. Additionally, the quantitative control gene (C3bRQ) segregating with the RCA haplotypes must be highly polymorphic (i.e., it must have multiple discrete alleles). Because of the reproducibility of C3bRQ phenotypes in siblings, this gene appears to be solely responsible for the control of CR1 expression. This conclusion further suggests
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TABLE I
Segregation of RCA Haplotypes and Erythrocytic CR1 Levels

| Family | Members | RCA typing* | CR1 levels (molecules per cell) |
|--------|---------|-------------|-------------------------------|
| BRU    | Fa      | (a) 1, B, 1; (b) 2, B, 1 | 548 |
|        | Mo      | (c) 1, A, 1; (d) 1, A, 2 | 463 |
|        | Ch1     | a, d         | 421 |
|        | Ch2     | b, d         | 203 |
|        | Ch3     | a, c         | 741 |
|        |         |              |     |
| BAC    | Fa      | (a) 1, A, 1; (b) 3, A, 1 | 164 |
|        | Mo      | (c) 1, A, 1; (d) 1, A, 2 | 180 |
|        | Ch1     | b, d         | 53  |
|        | Ch2     | b, d         | 59  |
|        | Ch3     | b, c         | 206 |
|        | Ch4     | b, c         | 296 |
|        |         |              |     |
| FRI    | Fa      | (a) 1, A, 1; (b) 1, B, 1 | 490 |
|        | Mo      | (c) 1, A, 1; (d) 1, A, 1 | 70  |
|        | Ch1     | b, c or d    | 200 |
|        | Ch2     | b, c or d    | 125 |
|        | Ch3     | b, c or d    | 120 |
|        | Ch4     | a, c or d    | 380 |
|        | Ch5     | a, c or d    | 360 |
|        | Ch6     | a, c or d    | 300 |
|        |         |              |     |
| PRE    | Fa      | (a) 1, A, 1; (b) 1, B, 2 | 456 |
|        | Mo      | (c) 1, A, 1; (d) 1, D, 1 | 78  |
|        | Ch1     | a, d         | 250 |
|        | Ch2     | a, d         | 210 |
|        | Ch3     | b, c         | 160 |
|        | Ch4     | b, c         | 153 |
|        |         |              |     |
| CYP    | Fa      | (a) 1, B, 1; (b) 1, A, 1 | 270 |
|        | Mo      | (c) 1, A, 2; (d) 1, A, 1 | 600 |
|        | Ch1     | b, d         | 695 |
|        | Ch2     | b, d         | 500 |
|        | Ch3     | b, c         | 603 |
|        | Ch4     | b, c         | 595 |
|        | Ch5     | b, c         | 419 |
|        | Ch6     | a, d         | 419 |
|        | Ch7     | a, d         | 390 |
|        | Ch8     | a, d         | 388 |
|        | Ch9     | a, c         | 262 |
|        | Ch10    | a, c         | 258 |

* RCA typing is indicated for each paternal (a, b) or maternal (c, d) RCA haplotypes, i.e., (a) 1, B, 1 is (a) C4BP-T, C3bR-B, FH-I. Typings were performed as described in Materials and Methods.
† CR1 levels were determined by immunoradiometric assays (see Materials and Methods). Number of CR1 molecules were estimated by assuming an average $M_r$ for CR1 of 200,000. Duplicate measurements from the same blood sample agree within 5%; from different samples, assayed independently, within 10%.
that the same control mechanism may account for the observation that CR1 heterozygotes often express different amounts of CR1 in each of their two electrophoretically separated allelic gene products.

Fig. 1 and Table II demonstrate these quantitative variations at the allelic level. In each of the families in Table II there are two RCA-identical C3bR-heterozygous sibling pairs who fail to share RCA haplotypes with the other pair. In each pedigree, the fraction of the total CR1 contributed by each C3bR allele is constant in RCA-identical siblings.

These data, taken together with those in Table I, are consistent with the suggestions that C3bRQ exclusively determines the quantity of CR1 produced by the corresponding haplotype, and that the total number of molecules per cell is equal to the sum of the molecules contributed by the two haplotypes under their respective controls. To test this hypothesis, we determined the absolute level of expression of six individual C3bR alleles, inherited by at least three family members each, using quantitative Western blot analysis. The results, summarized in Table III, demonstrate that, within experimental error, the number of molecules of CR1 for erythrocyte is in fact inherited together with the corresponding structural gene, and that it is independent of the quantitative expression of the paired allele.

We conclude, therefore, that quantitative variations of CR1 on human erythrocytes are controlled by a multiallelic locus, designated C3bRQ, closely linked to the CR1 structural gene and involving a rigorously cis-acting mechanism. This quantitative control system appears to play a major, if not the sole, role in the genetic determination of the total amounts of CR1 on normal human erythrocytes. Fig. 1 shows the CR1 patterns of two pedigrees that support this conclusion. Data from these families show that the absolute levels of expression of the individual C3bR alleles remain constant, independently of those that characterize the paired allele. The mother in family FRI is homozygous for the structural allele C3bR-A, but could well be heterozygous at the quantitative C3bRQ locus.
TABLE II
Distinct Expressivity of C3bR Alleles in Heterozygous Individuals

| Family | Members | RCA typing* | Percentage of total CR1$ |
|--------|---------|-------------|-------------------------|
|        |         |             | C3bR-A | C3bR-B | C3bR-D |
| PRE    | Fa      | (a) 1, A, 1; (b) 1, B, 2 |
|        | Mo      | (c) 1, A, 1; (d) 1, D, 1 |
|        | Ch1     | a, d        | 88     | 12     |
|        | Ch2     | a, d        | 89     | 11     |
|        | Ch3     | b, c        | 31     | 69     |
|        | Ch4     | b, c        | 36     | 64     |
| PED    | Fa      | (a) 2, B, 1; (b) 1, A, 2 |
|        | Mo      | (c) 1, A, 1; (d) 1, B, 1 |
|        | Ch1     | a, d        | 58     | 42     |
|        | Ch2     | a, d        | 60     | 40     |
|        | Ch3     | b, c        | 41     | 59     |
|        | Ch4     | b, c        | 37     | 63     |
| PRI    | Fa      | (a) 1, B, 1; (b) 1, A, 2 |
|        | Mo      | (c) 1, A, 1; (d) 1, B, 1 |
|        | Ch1     | a, c        | 46     | 54     |
|        | Ch2     | a, c        | 45     | 55     |
|        | Ch4     | b, d        | 75     | 25     |
|        |         |             | 71     | 29     |
| FRI    | Fa      | (a) 1, A, 1; (b) 1, B, 1 |
|        | Mo      | (c) 1, A, 1; (d) 1, A, 1 |
|        | Ch1     | b, c or d   | 57     | 43     |
|        | Ch2     | b, c or d   | 32     | 68     |
|        | Ch3     | b, c or d   | 26     | 74     |

* As in Table I.

$Percentages of CR1 in each C3bR allele were determined by scanning densitometry of Western blots as described in Materials and Methods.

because the C3bR-A,B heterozygous siblings in this family show two different levels of expression of the maternally inherited C3bR-A allele. The differences in the total number of CR1 molecules among these C3bR-A,B heterozygous children in family FRI (Table I) are thus attributable to the different expressions of the C3bR-A allele encoded by each of the maternal RCA haplotypes shown in this figure. Therefore, the RCA-linked C3bRQ locus is sufficient to account for the variations observed in the total erythrocytic CR1-levels.

This quantitative control gene (C3bRQ) extends the complexity and increases the polymorphism of the RCA system, and individuals, otherwise identical at the C3BP, C3bR, and FH loci, such as the mothers in families BAC and CYP (Table I), may now be shown to carry different RCA haplotypes because of the different expression of the C3bR alleles. The segregation of family FRI (Table I and Fig. 1), in which the mother's RCA haplotypes are structurally indistinguishable
TABLE III
Constant Expression of C3bR Alleles, Identical by Descent, in Family Members

| Family | Members | RCA haplotype* | Level of expression of C3bR allele (Molecules per cell)‡ |
|--------|---------|---------------|--------------------------------------------------------|
| FRI    | Fa      | 1, B, 1       | 113                                                   |
|        | Ch1     |               | 86                                                    |
|        | Ch2     |               | 85                                                    |
|        | Ch3     |               | 89                                                    |
|        |         |               | 96 ± 14.8§                                           |
| PRE    | Fa      | 1, A, 1       | 296                                                   |
|        | Ch1     |               | 220                                                   |
|        | Ch2     |               | 187                                                   |
|        |         |               | 234 ± 55.9                                            |
|        | Fa      | 1, B, 2       | 159                                                   |
|        | Ch3     |               | 110                                                   |
|        | Ch4     |               | 98                                                    |
|        |         |               | 122 ± 32.3                                            |
|        | Mo      | 1, A, 1       | 50                                                    |
|        | Ch1     |               | 50                                                    |
|        | Ch2     |               | 55                                                    |
|        |         |               | 51.7 ± 2.9                                            |
|        | Mo      | 1, D, 1       | 27                                                    |
|        | Ch3     |               | 30                                                    |
|        | Ch4     |               | 23                                                    |
|        |         |               | 26.7 ± 3.5                                            |
| CYP    | Fa      | 1, B, 1       | 43                                                    |
|        | Ch6     |               | 58                                                    |
|        | Ch7     |               | 66                                                    |
|        | Ch8     |               | 66                                                    |
|        | Ch9     |               | 42                                                    |
|        | Ch10    |               | 46                                                    |
|        |         |               | 53.5 ± 11.2                                           |

* Inheritance of each C3bR allele was unequivocally determined by the segregation of the RCA haplotypes.

‡ The level of expression of each C3bR allele in erythrocytes was determined for each individual by quantitative Western blot analysis and scanning densitometry. The proportion of CR1 in each band was then multiplied by the total CR1 determined immunoradiometrically, as described in Materials and Methods, to obtain the number of molecules per cell.

§ Mean ± SD.

(C4BP-1, C3bR-A, FH-1), shows that it is in fact possible to differentiate such haplotypes on the basis of C3bRQ.

Because of the functional relevance of CR1 to the processing of immune complexes (3, 4), it has been proposed (13–15) that quantitative differences of CR1 play a significant role in determining an individual's susceptibility to SLE.
The studies reported here, showing that the quantitative genetic control of erythrocytic CR1 is inherited with the RCA gene cluster, provide a new way to explore the possible association between CR1 levels and disease. We may precisely characterize the C3bRQ allele within the relevant RCA haplotypes in healthy relatives of patients unencumbered by the quantitative changes induced by the disease itself. Additionally, by following the other markers of the RCA cluster, we could use these quantitative variants as genetic markers for the study of genetic association and linkage, and potentially for the study of the pathophysiologic mechanisms.

Summary

The genetic relationships of quantitative and structural variations of the C3b/C4b receptor (CR1) in human erythrocytes have been analyzed in informative families. Our results demonstrate the existence of multiple discrete quantitative variations of CR1 controlled by a locus, C3bRQ, closely linked to the CR1 structural locus, C3bR. Since the amounts of CR1 produced by each C3bR allele are shown to be independently regulated, we propose that a cis-acting genetic mechanism controls the level of expression of the C3bR alleles, and that this quantitative control plays a major, if not the sole, role in determining the total amounts of CR1 on normal human erythrocytes.

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