Complement Receptor 1 (CD35) on Human Reticulocytes: Normal Expression in Systemic Lupus Erythematosus and HIV-Infected Patients

Estelle Lach-Trifilieff, Jutta Marfurt, Sybille Schwarz, Salima Sadallah and Jürg A. Schifferli

*J Immunol* 1999; 162:7549-7554; http://www.jimmunol.org/content/162/12/7549

**References**
This article cites 36 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/162/12/7549.full#ref-list-1

**Subscription**
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Complement Receptor 1 (CD35) on Human Reticulocytes: Normal Expression in Systemic Lupus Erythematosus and HIV-Infected Patients

Estelle Lach-Trifilieff, Jutta Marfurt, Sybille Schwarz, Salima Sadallah, and Jürg A. Schifferli

The low levels of complement receptor 1 (CR1) on erythrocytes in autoimmune diseases and AIDS may be due to accelerated loss in the circulation, or to a diminished expression of CR1 on the red cell lineage. Therefore, we analyzed the expression of CR1 on reticulocytes (R) vs erythrocytes (E). Healthy subjects had a significantly higher CR1 number per cell on R (919 ± 99 CR1/cell) than on E (279 ± 30 CR1/cell, n = 23), which corresponded to a 3.5- ± 1.3-fold loss of CR1. This intravascular loss was confirmed by FACS analysis, which showed that all R expressed CR1, whereas a large fraction of E was negative. The systemic lupus erythematosus (SLE), HIV-infected, and cold hemolytic Ab disease (CHAD) patients had a CR1 number on R identical to the healthy subjects, contrasting with a lower CR1 on their E. The data indicated a significantly higher loss of CR1 in the three diseases, i.e., 7.0- ± 3.8-., 6.1- ± 2.9-., and 9.6- ± 5.6-fold, respectively. The intravascular loss was best exemplified in a patient with factor I deficiency whose CR1 dropped from 520 CR1/R to 28 CR1/E, i.e., 18.6-fold loss. In one SLE patient and in the factor I-deficient patient, the FACS data were consistent with a loss of CR1 already on some R. In conclusion, CR1 is lost progressively from normal E during in vivo aging so that old E are almost devoid of CR1. The low CR1 of RBC in autoimmune diseases and HIV-infection is due to a loss occurring in the circulation by an active process that remains to be defined. The Journal of Immunology, 1999, 162: 7549–7554.

Complement receptor 1 (CR1)\(^3\) (CD35, C3b/C4b receptor) is an intrinsic membrane glycoprotein found on several cell types, including erythrocytes (E), most leukocytes, and glomerular podocytes (1). CR1 binds C3b-coated particles and serves as a cofactor for the degradation of C3b by factor I (2, 3). On E, CR1 participates in the transport and processing of bacteria and immune complexes (IC), despite a very low number of receptors per E (between 100 and 500 CR1/E) (4–6).

In normal subjects, the quantitative expression of CR1 on E is determined by a biallelic codominant polymorphism that correlates with a HindIII restriction fragment length polymorphism and thus occurs in a trimodal fashion (7). However, there is a high overlap in the expression of CR1/E within the three populations. The number of CR1/E is determined by other factors as well, such as the age of the E as shown in many studies in which young and old E were compared. Old E had 40–50% less CR1 than young E (8–10). However, most of these studies were done by separating young and old E by density gradients. Because E are not well separated by this method, the real loss of CR1 could not be defined. The mechanisms responsible for the loss of CR1 include proteolytic cleavage and possibly vesiculation (9, 10). It has been shown by immunofluorescence that the expression of CR1 is extremely heterogeneous among E from healthy subjects (down to not measurable) (11). This might reflect a large degree of variation in the intercellular synthesis and expression, but also the large difference in the E age, i.e., from some h to 120 days.

The CR1 number on E is low in a series of diseases, the best-characterized of these are systemic lupus erythematosus (SLE), immune hemolytic anemias, AIDS, and lepromatous leprosy (12–18). After an initial debate over the origin of the low number of CR1/E, it now appears that acquired factors related to disease activity play a major role. Patients with AIDS have a progressive decrease of CR1 on their E, and genetic studies showed unequivocally that the defect was acquired (12). Data concerning SLE patients have been more difficult to interpret, but recent genetic studies indicated an acquired loss of CR1 as well (19, 20).

The mechanism responsible for the acquired loss of CR1 from E might be a down-regulation of the CR1 synthesis in the bone marrow and/or an enhanced peripheral catabolism. The first hypothesis has not yet been investigated, although several reports suggest such a mechanism. A reduced CR1 number on R has been described in some SLE patients (21). Low CR1 has been reported on neutrophils and B lymphocytes of SLE patients (22, 23), as well as neutrophils of AIDS patients (14), supporting the hypothesis of an altered synthesis or surface expression.

However, multiple studies suggest that reduced CR1 expression in disease is due to accelerated intravascular catabolism. First, low CR1 was mainly observed in patients with ongoing complement activation in the circulation, as found in SLE, AIDS, or cold hemolytic Ab disease (CHAD). Ross et al. (17) found an inverse correlation between E CR1 number and C3dg deposition. Then, Walport et al. (24) reported that transfused E lost their CR1 rapidly in patients with SLE and hemolytic anemia. Next, Barbosa et al. (25) described the presence of CR1 stumps on E of a patient with CHAD, as if the extracellular portion of CR1 had been proteolytically cleaved. Pascual et al. (10) showed that small CR1 fragments are found in higher abundance on E of AIDS patients than on
normal E. Also, studies performed in humans and nonhuman primates showed that the acute formation of large amounts of immune complexes in the circulation was followed by a rapid loss of CR1/E. The same observation was made in a chronic model of immune complex disease in nonhuman primates (26, 27). A model explaining the accelerated CR1 loss has been proposed on the basis of a general proteolytic cleavage of CR1 following complement activation and the transfer of E bound IC to phagocytic cells.

Although the hypothesis of enhanced proteolytic degradation of CR1 on E in autoimmune diseases and AIDS is well sustained, it is based on a very limited number of patients and data. Some evidence has been challenged. For instance, Cohen et al. (21) repeated the experiments of E transfusions in SLE patients and could not find a significant loss of CR1. The immediate loss of CR1 at the time of sudden in vivo IC formation is followed after some hours already by a partial restoration toward preexperimental values. Finally, even if enhanced peripheral catabolism of CR1 participates in the CR1 loss, it does not exclude that other mechanisms, such as down-regulation of CR1 synthesis in the bone marrow, are not crucial as well.

Reticulocytes (R) are the youngest E observed in the circulation (0–2 days). Using positive selection to purify R, we analyzed the expression of CR1 on R vs E in healthy subjects, as well as in patients with diseases in which CR1 is known to be low.

Materials and Methods

Blood samples from healthy subjects and patients

We tested blood from 35 patients, among whom 10 had SLE, 3 had CHAD, 22 were infected with HIV, and 1 patient with factor I deficiency (patient 22 in Ref. 31). All 10 SLE patients fulfilled the American Rheumatism Association criteria for the disease. Three of them had Coombs-positive and four had Coombs-negative hemolytic anemia, one had unclassified and two had no anemia. Three of the patients did not receive any treatment at the time of blood sampling. The seven other patients received prednisolone, combined in three cases with cyclophosphamide or hydroxychloroquine. All had active disease at the time of blood sampling, and none was transfused in the previous 2 wk.

Of the three CHAD patients, two were hospitalized because of severe hemolytic crisis and were treated with prednisolone. The blood samples were taken before transfusion or at least 2 wk after the last transfusion. Samples from a fourth patient with severe hemolytic anemia and CHAD were used for FACS analysis.

A total of 22 HIV-infected persons were recruited consecutively on a volunteer basis. They fulfilled the criteria for stage A2 (n = 3), A3 (n = 1), B2 (n = 2), B3 (n = 4), C2 (n = 1), and C3 (n = 11) of HIV infection, according to the Center for Disease Control and Prevention classification. One patient had not been classified. None had a transfusion in the 6 wk preceding the blood sampling. Informed consent was obtained from the patients and the healthy subjects.

Reticulocyte preparation

We adapted an isolation procedure based on the specific cell surface expression of transferrin receptor by R (28). To avoid interference with monocytes or other leukocytes expressing transferrin receptors during the R isolation process, white cells were first removed from the whole blood. Freshly drawn EDTA blood was mixed with an equal volume of PBS-EDTA and allowed to sediment on 4% Dextran. The E recovered were extensively washed and resuspended in PBS-BSA. The efficacy of white blood cell removal exceeded 99% as assessed by direct staining. Anti-transferrin receptor mAb-coated beads (CD71, Dynabeads M-450; Dynal, Oslo, Norway) were incubated with the leukocyte depleted E at room temperature for 15 min with gentle mixing to allow binding of cells with transferrin receptors to the beads. The beads with attached R were washed, and the R were displaced by addition of the autologous plasma, known to contain soluble transferrin receptor (29). The final suspension consisted of 98–100% R verified by staining with brilliant cresyl blue. The yield of pure R isolated varied between 5 and 10%.

Quantitation of CR1 per cell

Erythrocytes or R were washed, lysed, and their respective membranes solubilized with 0.5% Triton X-100 in the presence of 5 mM EDTA and 1 mM PMSF. The solubilization was performed at 4°C for 20 min, followed by a centrifugation at 13,000 rpm for 20 min.

The ELISA for the CR1 number determination was made as previously described (30). Briefly, a mAb (3D9) (31) against CR1 was coated on the wells. After incubation of the sample to be tested, CR1 bound to the first mAb was revealed with a biotinylated second mAb (J3D3) (31) recognizing another epitope of CR1. Recombinant soluble human CR1 (rCR1 kindly provided by T-Cell Sciences, Cambridge, MA) was used as a standard. The absorbance values obtained in the ELISA by using the solubilized E or R membranes were compared with those obtained with known amounts of rCR1. The possible contamination of R by leukocytes was assessed by incubating the R suspension with beads coated with the Pan Leukocyte Ag (CD45, Dynabeads M-450; Dynal), which were then removed. The R CR1 number was not modified significantly by the removal of putative leukocytes (<3% difference in CR1 determination in three different experiments). To assess the solubilization procedure, 3.6 × 10⁹ E/ml were solubilized, and the lysate as well as the remaining pellet were analyzed at different dilution by SDS-PAGE and immunoblotting using anti-CR1 mAb J3D9.

Analysis of E and R by FACS

Whole RBC population from healthy subjects was double stained for FACS analysis. The RBC were incubated with biotinylated anti-CR1 mAb J3D3 and labeled with streptavidin-PET to stain for CR1 expression and with thiazole orange reagent from Reti-count TM Kit (Becton Dickinson, Mountain View, CA) to identify R. Control experiments were performed with an irrelevant isotype-matched biotinylated mAb, the anti-complement factor D mAb 72-96-25 (IgG1) (32). The data were analyzed on a FACScan with the CellQuest program (both from Becton Dickinson).

CR1 analysis by immunoblotting

E or R solubilized membranes were electrophoresed under nonreducing conditions on a 5% SDS-PAGE. Proteins were blotted onto nitrocellulose membranes. After blocking with 5% BSA PBS, blots were incubated overnight at 4°C with anti-CR1 mAb E11 (31). Biotinylated sheep IgG against mouse IgG at 1/1000 was used as a second Ab. Streptavidin-HRP at 1/2000 was added and the blots revealed with enhanced chemiluminescence detection reagents (Amersham, Arlington Heights, IL).

Statistical analysis

Student’s t test and Spearman’s rank correlation test were performed with the StatView SE+ graphics program (Synergy Software, Reading, PA). The CR1 measurements of E and R of a given patient were done in the same batch.

Results

CR1 ELISA

The CR1 ELISA has previously been used to quantify sCR1 from plasma. Use of this ELISA was extended to measure CR1 from solubilized E and R membranes. Control experiments were performed to analyze whether all CR1 was recovered in the lysate

FIGURE 1. ELISA for detecting sCR1 in solubilized E membranes. Wells coated with 3D9 received increasing concentrations of solubilized E membranes or rCR1. The number of CR1/E was calculated to be 274 ± 15.
after the solubilization procedure. Using immunoblots, the solubilization was shown to be almost complete (≤2% of CR1 remaining in the pellet; data not shown). rCR1 was used to standardize the CR1 ELISA (Fig. 1).

**Loss of CR1 between R and E in healthy subjects**

Expression of CR1 was quantified on healthy human R and E. The number of receptors was $919 \pm 99$ CR1/cell (mean ± SEM) on R and $279 \pm 30$ CR1/cell on E ($n = 23$) (Fig. 2). All except one individual displayed at least a 2-fold reduction in the CR1 number from R to E, the average being $3.5 - 1.3$-fold (mean ± SD). The number of CR1/R was directly proportional to that of CR1/E ($r_s = 0.74; p < 0.001$). The CR1/E and CR1/R levels in three normal controls were quantified two to three times over a period of 3–6 mo. No significant difference was found in the consecutive samples.

The maturation of R to E in 1–2 days induces a loss of specific receptors, such as the transferrin receptors. To see whether a similar mechanism might be responsible for the loss of CR1, FACScan was performed on RBC from eight healthy subjects using a double staining procedure, the first for R, thiazole orange, and the second for CR1. Fig. 3 illustrates the results obtained: R, cells positive for thiazole orange, had high and homogeneous fluorescence for CR1. There was no reduction in CR1 within the R population between young and old cells (high and low thiazole orange fluorescence, respectively). In addition, all R expressed CR1, contrary to a large fraction of E, which was negative. The CR1 fluorescence of R was not higher than that of E, suggesting that the CR1 heterogeneity among E does not arise at the time of R maturation into E but at a later stage. The results were identical in the seven other controls.

**Loss of CR1 between R and E in SLE, HIV-infected, and CHAD patients**

The CR1 expression per E in SLE ($120 \pm 21$ CR1/cell, $p < 0.005$), HIV-infected ($198 \pm 34$ CR1/cell, $p < 0.005$), and CHAD ($122 \pm 16$ CR1/cell, $p < 0.05$) patients was significantly lower than the expression on healthy subjects. By contrast, we found no statistical difference between the CR1 expression on healthy and on SLE, HIV-infected, or CHAD R, although the CR1 value measured on SLE patients was slightly below the controls (Fig. 4). As for healthy subjects, the CR1/R correlated with the CR1/E for SLE ($r_s = 0.76; p < 0.01$) and HIV-infected ($r_s = 0.69; p < 0.001$) patients. The average loss of CR1 was $7.0 - 3.8$-fold for SLE, $6.1 - 2.9$-fold for HIV-infected patients, and $9.6 - 5.6$-fold for...
past element. The last patient to be studied had factor I deficiency (33). He had 520 CR1/R and 28 CR1/E, corresponding to a loss of 18.6-fold. Thus, all groups of patients had a significantly higher loss of CR1 than the healthy individuals.

FACS analysis was performed for some patients to define whether a CR1 loss occurring at the time of R maturation was responsible for low CR1/E (Fig. 5). Similar to healthy individuals, the R showed no change in CR1 during maturation in the CHAD patient studied. By contrast, the expression of CR1 on R was heterogeneous in the SLE and factor I-deficient patients, consistent with a loss of CR1 occurring already at this early stage. Interestingly, in the factor I-deficient patient, the expression of CR1 appeared to correlate with R age, i.e., young R had more CR1 than old R (age defined from the Thiazole orange staining).

**Immunoblotting of R CR1**

R CR1 of healthy individuals were analyzed by Western blotting and compared with their E counterpart. The size of the E structural A and B alleles of CR1 was identical to that of E CR1 (Fig. 6).

![FIGURE 4. Quantification of CR1 on R (a) and E (b) of healthy controls (n = 23), SLE (n = 10), HIV-infected (n = 22), and CHAD (n = 3) patients. No statistical difference on the mean CR1/R expression was noticed between healthy controls (919 ± 473), SLE (732 ± 454), HIV-infected (1030 ± 585), and CHAD (1220 ± 926) patients.](image)

**FIGURE 5.** Two-color flow cytometry with thiazole orange and anti-CR1 mAb on RBC from a healthy subject, SLE, CHAD, and factor I-deficient patients. Comparative staining for CR1 on the E (thin line) and R (bold line) gated populations of each individual (dashed line, negative control). Dot blot representation of the whole RBC population of the factor I-deficient patient.
The number of CR1 on normal human E measured by ELISA was within the range previously reported (34–36). A much higher level of CR1 was detected on R for each individual, and there was a striking correlation between the number of CR1 on R vs E, indicating that the process was likely to be similar for all individuals independently of the initial CR1 expression. Since it is accepted that R mature in 1–2 days into E, the possible explanation for the difference is a loss during either maturation and/or aging. The FACS analysis data indicated that the loss did not occur during the maturation process from R to E, since R’s fluorescence was not stronger than that of E. Among E, some cells (probably the younger ones) have the same fluorescence level as R and, therefore, the same CR1 expression on their cell surface. An additional observation was that all R were positive and the fluorescence profile of R was more homogeneous than that of E. Thus, the absence of CR1 on a fraction of the E would correspond to in vivo aging. Considering the mean ages of R and E to be 24 h and 30 days, respectively, and the loss of CR1 to be a continuous process, the calculated half-life of CR1 on E would be 16.2 days. This estimation suggests that very old E have very few CR1, which would explain why many E were negative by FACS. Whether the loss of CR1 might be one of the factors involved in limiting the life span of an E remains an intriguing question.

The mAbs against CR1 used here (3D9, J3D3) recognize multiple epitopes on CR1 (31). The structural alleles of CR1 have different numbers of epitopes for a given mAb. Thus, it is evident that the determination of CR1 number per E, as performed here, did not allow a definitive comparison between individuals bearing different alleles of CR1. However, in a given individual, the measurements of CR1 on R vs E and the CR1 loss were not influenced by such differences. Similar to previous reports, E CR1 were low in SLE, HIV-infection, and CHAD. By contrast, CR1 expression on R in the three groups of patients was not different from normal controls. These data appear to invalidate the hypothesis that there was a down-regulation of CR1 expression on the E lineage in the bone marrow. By contrast, the rate of loss of CR1 in the circulation was increased (7.0-fold instead of 3.5-fold). Thus, in agreement with recent genetic studies (19, 20), our data indicated that the reduced CR1/E number observed in SLE arises from an acquired process. The same held true for HIV-infected patients and CHAD. R from two SLE patients expressed very few CR1 (below 250/cell), suggesting that, in these cases, enhanced peripheral catabolism may not be the only mechanism accountable for the CR1 number. Another explanation would be that both patients belong to the low CR1 HindIII RFLP quantitative genotype population. The mechanism underlying the accelerated loss of CR1 in all three disease groups was not analyzed. That complement activation per se might generate the sufficient conditions for such a loss to occur is suggested by the patient with factor I deficiency. This patient, who also had evidence for continuous complement activation and depletion of the alternative pathway (patient 22 in Ref. 31), had a 29-fold loss of CR1 from R to E. In addition, this very fast loss of CR1 was already evident when comparing young and old R by FACS.

In conclusion, our data demonstrate unequivocally that CR1 are lost during the physiological aging of RBC. There is no lack of CR1 expression on young E (R) in various diseases in which E CR1 is known to be low. Thus, in most cases, the low CR1 on E is due to an accelerated loss occurring in the circulation. The mechanisms underlying the physiological loss of CR1 and the accelerated loss in disease have yet to be defined. They might be degraded because they fix and transport immune complexes, although anti-CR1 autoantibodies may produce a similar loss (37, 38). Further studies should be aimed at identifying the proteases responsible for the CR1 fragmentation.

References