CONTROL OF THE FUNCTION OF SUBSTRATE-BOUND C4b-C3b BY THE COMPLEMENT RECEPTOR CR1

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It has been recently reported (1–3) that the complement receptor for C3b (CR1) has a unique role in the degradation of C3b bound to the cell membrane or to immune complexes (*C3b). CR1 can be more effective (4) than serum factor H in promoting the 1-mediated transformation of *C3b into the hemolytically inactive intermediate *iC3b (5). Moreover, under physiological conditions, CR1, but not H, serves as a cofactor for the further cleavage of *iC3b into C3c and *C3dg, the ligand for the C3d receptor (CR2) (1–3). These findings, in conjunction with other evidence (6–10), have been interpreted as meaning that the processing of *C3b-containing antigen-antibody complexes in vivo occurs on the surface of red cells (which bear most of the CR1 in human peripheral blood), and as indicative that the CR1 deficiencies observed in patients with systemic lupus erythematosus and rheumatoid arthritis (11–15) might be involved in the pathogenesis of these immune complex diseases.

When the complement cascade is triggered by the classic pathway, both *C3b and *C4b bind covalently to the activating substrate and participate in formation of the enzymes C3-convertase (C4b,2a) and C5-convertase (C4b,2a,3b), which in turn augment the cascade reaction (reviewed in 16). Since *C4b, which regulates the activity of both enzymes, is also a ligand for CR1 (17, 18), and CR1 can inhibit both enzymes (19), we study here the effects of the interaction between red cell intermediates bearing C3 and C5 convertases and CR1 in the presence of serum factor I. We present evidence not only that CR1 may be a principal cofactor responsible for the physiological degradation of *C4b, but also that the inactivation of *C4b can be greatly enhanced when sufficient *C3b is deposited in its vicinity. Likewise, the inactivation of *C3b is enhanced by adjacent *C4b.

Materials and Methods

Buffers, Reagents, and Proteins. Isotonic veronal-buffered saline (DGVB++)1 used in most studies contained 2.5 mM veronal, pH 7.4, 73 mM NaCl, 2.5% dextrose, 0.15 mM CaCl₂, 1 mM MgCl₂, and 0.1% gelatin. Isotonic veronal buffer (GVB++) used in some

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1 Abbreviations used in this paper: DGVB++, isotonic veronal-buffered saline containing 2.5 mM veronal, pH 7.3, 75 mM NaCl, 2.5% dextrose, 0.15 mM CaCl₂, 1 mM MgCl₂, and 0.1% gelatin; E hu, human erythrocytes; GVB++, isotonic veronal buffer prepared as DGVB++ except with 146 mM NaCl instead of 73 mM NaCl and dextrose; KSCN, potassium thiocyanate; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; SFU, site-forming unit; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
experiments contained 146 mM NaCl instead of 73 mM NaCl and 2.5% dextrose. Metal-chelating veronal buffer (GVB-EDTA), used in hemolytic assays and for washing cells, contained 10 mM EDTA and lacked CaCl₂ and MgCl₂.

C₄ and C₃ were purified from DEAE-Sepharose fractions of pooled human plasma by polyethylene glycol precipitation followed by DEAE-Sepharose rechromatography or Sephadex G-200 gel filtration, respectively (20). C₄ contained no C₅ or C₇ hemolytic activity, and its specific activity was 1.1 x 10⁶ C₄ site-forming units (SFU) per mg protein immediately after purification. It was stored at 4°C in sterile tubes containing 25 mM benzamidine. C₃ was depleted of residual C₅ and H activities by adsorption with Sepharose anti-human C₅ and anti-human H, respectively. The specific activity of C₃ was 8 x 10⁵ SFU/mg. It was stored frozen in aliquots at -70°C. C₂ (21) and C₅ (20) were purified as described, and in some experiments C₂ was used in an oxidized form (°C₂) (22). C₁ (23), C₅₉ (24), and C-EDTA were prepared from guinea pig serum. C₄b was prepared by "spontaneous" conversion of C₄ upon removal of benzamidine by dialysis.

I (25), C₄bp (26), H (20), and CR1 (5, 19) were purified as described. I was also obtained commercially (Cordis Laboratories Inc., Miami, FL). C₄bp was depleted of contaminating IgM and H by adsorption with Sepharose anti-IgM and anti-H, respectively. The final product consisted predominantly of the higher molecular weight form (26). H was depleted of contaminating IgA by adsorption with Sepharose anti-IgA. For the purification of CR1, 0.1% Nonidet P-40 (NP-40) extracts of human erythrocyte (Eₜₜ) stroma were chromatographed on Biorex-70 (Bio-Rad Laboratories, Richmond, CA) as described (5). After salt gradient elution, the fractions containing CR1 (as determined by their inhibitory activity on C₅-convertase, C₄b₂a₃b) were pooled, concentrated, and filtered through Sepharose-anti-CR1 prepared with two monoclonal antibodies (44D and 57H) (12, 19). After washing, the bound CR1 was eluted with 3 M potassium thiocyanate (KSCN). Excess KSCN was removed by passage of the eluate through Sephadex G-25 equilibrated in phosphate-buffered saline (PBS) containing 0.1% NP-40. The final product contained negligible impurities as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions followed by silver staining (Bio-Rad Laboratories).

Eₜₜwere purified from acid/citrate/dextrose-treated venous blood samples from healthy volunteers. Cells were washed five times with large volumes of GVB-EDTA and buffy coats aspirated to remove white blood cells. The remaining Eₜₜwere washed twice in DGVB ++ and resuspended to appropriate concentration.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (27). Stacking gels of 3% and running gels of 7.5% were used. Samples were reduced by the addition of 20% 2-mercaptoethanol to the sample buffer. Molecular weights were estimated by comparison with a set of commercially available standards (Bethesda Research Laboratories, Rockville, MD). Gels were fixed and stained with methanol/acetic acid containing Coomassie Blue. Radioautographs were exposed at -70°C using X-Omat R film (XR-5; Eastman Kodak Co., Rochester, NY).

Radiolabeling. C₄ and C₃ were labeled with ¹²⁵I or ¹³¹I using Iodogen (Pierce Chemical Co., Rockford, IL) as per the manufacturer's instructions. Unbound ¹²⁵I or ¹³¹I was eliminated by gel filtration through Sephadex G-25 QS-2A Quick-Sep columns (Isolab, Inc., Akron, OH) followed by dialysis.

Cellular Intermediates and Hemolytic Assays. Sheep erythrocytes (E) were sensitized with rabbit anti-sheep hemolysin (A) (Gibco Laboratories, Grand Island, NY) as per the manufacturer's instructions. EA were washed and adjusted to 1 x 10⁶ cells/ml in DGVB ++. An equal volume of C₁ at a dilution sufficient to provide 500 SFU was added and the mixture incubated 15 min at 30°C. After washing, the intermediates (EAC₁) were incubated for 20 min at 30°C with an equal volume of an appropriate dilution of C₄ to yield cells (EAC₁₄) bearing the amounts of *C₄b specified in the text or figure legends. EAC₁₄, 1 x 10⁶/ml in DGVB ++, were incubated for 5 min at 30°C with 300 SFU/ml of C₂ (or °C₂) and the intermediates (EAC₁₄₂ or EAC₁₄°C₂) washed, readjusted to 1 x 10⁶/ml in DGVB ++, and incubated with an equal volume of C₃ at a dilution sufficient to yield cells (EAC₁₄₂₃ or EAC₁₄°₃) bearing the specified amount of *C₃b. C₄b hemolytic sites were determined by sequential
incubation of EAC14 with 300 SFU of °C2 for 5 min at 30°C and C-EDTA for 1 h at 37°C. C3b hemolytic sites were determined by incubation of EAC1423 (or EAC14°C23) with 300 SFU of C5 for 5 min at 30°C and C6-9 for 1 h at 37°C. The number of *C4b or *C3b molecules deposited on the erythrocytes was calculated from the percentage of uptake of samples containing 1251-C4 or 1251-C3.

*C4c and C3c Release. EAC14 bearing 1251-labeled *C4b were added in 25-μl aliquots to reaction tubes containing 25-μl volumes of I (or DGVB ÷) as control) and 50 μl volumes of different dilutions of C4bp, CR1, or E hu (or DGVB ÷) as control). The final concentrations of the reactants are given in the text or figure legends. The tubes were incubated at either 37 or 30°C in a water bath and agitated periodically. The reaction was stopped by transfer of the tubes to an ice bath and addition of 900 μl of ice-cold DGVB ÷. The cells were pelleted and 500 μl of supernatant was removed. The percentage of released C4 label was calculated from the counts present in the separated 500-μl fraction and those remaining in the tube. Studies on the release of C4c or C3c from EAC1423 bearing 1251-labeled *C3b or bearing both 1251-labeled *C4b and 131I-labeled *C3b were performed as described above.

Results

Ability of CR1 to Promote I-mediated Degradation of Substrate-bound *C4b

Previous studies (28) have indicated that the enhancing activity of C4bp on the I-mediated degradation of *C4b is observed only when the *C4b density reaches at least 3 × 10³ molecules per cell. To determine the effect of *C4b density on CR1 activity, release of C4 label by I plus CR1 was studied using EAC14 prepared with different amounts of C4. As shown in Fig. 1, the cofactor activity of CR1 was not observed at low *C4b densities, but increased progressively when the number of *C4b molecules surpassed 3 × 10³ per cell.

The effects of C4bp, of purified CR1, and of intact E hu on the I-mediated breakdown of cell-bound *C4b into C4c and *C4d were then compared. In view of the results shown in Fig. 1, these studies were performed using a cell intermediate EAC14 bearing 6 × 10³ 1251-*C4b molecules per cell. Cells (5 × 10⁷/ml in DGVB ÷) were mixed with I (16 μg/ml) plus CR1, E hu, or C4bp, in increasing concentrations, or with each factor alone as control. After incubation at 37°C for 60 min, the reactions were stopped and the percentage of C4

![Figure 1](image-url)
radiolabel released into the supernatant was determined. CR1, Ehu, and C4bp all enhanced I-mediated release of radiolabel in a dose-dependent fashion (Fig. 2). As little as 50 ng/ml of purified CR1 or 6.2 × 10^7 Ehu/ml (containing ~60 ng/ml of CR1) caused significant enhancement. In contrast, as much as 30 µg/ml of C4bp had substantially less effect. Minimal release occurred in the presence of CR1, Ehu, or C4bp alone.

To verify whether the release observed in the presence of CR1 or Ehu was mediated in fact by CR1 rather than by some other factor, additional studies were performed in which a pool of purified monoclonal anti-CR1 antibodies (44D, 31D, 57F) was added to the reaction mixtures. Parallel studies were also performed in which Ehu were replaced by erythrocytes from animal species known not to mediate immune adherence of EAC1423. As also shown in Fig. 2, the CR1- or Ehu-mediated release of C4 label was strongly inhibited by monoclonal antibodies to CR1. Minimal release was observed in the presence of as many as twofold higher numbers of erythrocytes from guinea pigs or sheep (not shown).

Modulation by *C3b of I plus CR1-mediated *C4b Degradation. In the presence of C3, *C4b,2a-bearing substrates can rapidly fix *C3b. Since CR1 binds to *C3b in addition to *C4b, we next studied the effect of deposited *C3b on the degradation of substrate-bound *C4b by I plus CR1. EAC14 (5 × 10^7/ml) prepared with radiolabeled 125I-C4 and bearing ~3 × 10^3 molecules of *C4b per cell were incubated with excess C2. Then increasing amounts (3–81 SU/ml) of unlabeled C3 (or DGVB++ as control) were added. The number of *C3b molecules deposited at each C3 input was calculated from parallel experiments in which EAC14 were prepared with unlabeled C4 and the cells incubated with 125I-C3. The resulting cells bearing 3 × 10^3 molecules of 125I-labeled *C4b and from 0 to 5 × 10^4 molecules of *C3b were then incubated at 37°C for 60 min with I (16 µg/ml) and increasing concentrations of CR1 (100–800 ng/ml). In a second set of tubes, cells were incubated with the same concentration of I and increasing concentrations of C4bp in comparable blood proportions (2.5–20 µg/
The percentages of C4 radiolabel released from the red cells are shown in Fig. 3. Clearly, *C3b fixation enhanced the I plus CR1-mediated C4 release in a dose-dependent fashion. Maximum enhancement of CR1 activity (5-10-fold in different experiments) was obtained with cells bearing 2 × 10^4 molecules of *C3b at a *C3b/*C4b molar ratio of ~8:1. In contrast, in this and other experiments, *C3b suppressed the I plus C4bp-mediated release of C4 radiolabel. Maximum suppression of approximately two- to fourfold was observed with cells bearing 5 × 10^4 molecules of *C3b at a *C3b/*C4b molar ratio of 17:1.

The release of C4 label by I alone was also inhibited by *C3b.

The *C3b-mediated enhancement of the activity of CR1 on *C4b degradation had two interesting and perhaps physiologically significant consequences. First, this enhancement was also observed when *C4b densities were low. In Fig. 4, we show the effect of I plus CR1 on EAC1423 bearing only 1 × 10^3 molecules of *C4b but increasing numbers of *C3b per cell. Clearly *C3b promoted C4 release from the EAC1423 cells and the effect was proportional to the amount of *C3b fixed. This result should be contrasted with those of Fig. 1 and of Fujita and Tamura (28) showing that CR1 and C4bp only enhance the activity of I on EAC14 if the density of *C4b is >3 × 10^3 molecules per cell.

Second, we found that after *C3b fixation, CR1 was active on *C4b even in the presence of C2, which, in previous studies (19), has been shown to competitively inhibit the binding of CR1 to *C4b. Our studies were performed as follows. EAC142 bearing 3 × 10^3 molecules of ^125I-*C4b per cell and saturating amounts of C2a were incubated with DGVB++ or with C3 at a dilution sufficient to yield

![Graph](image_url)

**Figure 3.** Effect of fixed *C3b on the I plus CR1- and I plus C4bp-mediated release of substrate-bound C4 fragment. EAC14 (5 × 10^7/ml) bearing 3 × 10^3 molecules of ^125I-*C4b per cell were incubated stepwise with 300 SU of C2 and then either with DGVB++ or with increasing amounts of C3. The resulting cellular intermediates bearing a constant amount of ^125I-*C4b and the respective numbers of (unlabeled) *C3b molecules indicated in the figure were then incubated at 37°C for 60 min with 16 µg/ml I plus increasing concentrations of CR1 or with the same concentration of I plus comparable proportions of the normal blood content of C4bp. The percentage of release of C4 radiolabel is shown as a function of CR1 or C4bp input. Release by I plus CR1 increased as the amount of deposited *C3b was increased, whereas release by I plus C4bp generally decreased.
FIGURE 4. Ability of CR1 to augment the I-mediated *C4b fragmentation at low *C4b density when *C3b is present on the same cells. EAC14 (5 × 10⁷/ml) bearing 1 × 10⁸ molecules of ¹²⁵I-*C4b per cell were incubated with 300 SFU of C2 and then with increasing amounts of ¹²⁵I-labeled C3. The resulting EAC1423 bearing either 0, 4 × 10⁵, or 1.1, 2.4, 4.7, or 7.2 × 10⁶ molecules of *C3b per cell were then incubated at 37°C for 30 min with I (16 μg/ml) and CR1 (800 ng/ml). Release of ¹²⁵I-C4 radiolabel is shown as a function of density of ¹²⁵I-*C3b on the cells. The release increased in a dose-dependent fashion with *C3b density. The release in the presence of I (16 μg/ml) alone was not significantly greater than that in the presence of DGVB++.

EAC1423 bearing an eightfold molar excess of unlabeled *C3b (2.4 × 10⁴ molecules per cell). After decay of original C2a from the DGVB++-treated EAC142 and from the EAC1423, the cells were reincubated with multiple dilutions of C2. The resulting intermediates, EAC142 and EAC1423 (5 × 10⁷/ml) bearing various amounts of new C2a, were then incubated at 37°C for 60 min with I (16 μg/ml) and CR1 (200 ng/ml) and the percentage of release of C4 radiolabel determined. The results are shown in Fig. 5. Reincubation with large amounts of C2 (2,500 SFU) significantly inhibited the release of C4 label from EAC142. Prior presence of *C3b, however, at a *C3b/*C4b molar ratio of 8:1, reversed the C2-mediated inhibition.

It is also of interest to note that in previous studies (28-30) relatively high concentrations of the control proteins I and C4bp and 1-2 h of incubation at 37°C have been required to release significant amounts of C4c from EAC14. After fixation of optimal amounts of *C3b, however, we observed significant release of C4 label after incubation for only 30 min at 30°C in the presence of as little as 2 μg/ml of I and 25 ng/ml of CR1. As expected, under these conditions there was minimal release of C4 fragments in the presence of I alone or I plus C4bp.

Evidence that *C4b Can Enhance the I plus CR1-mediated Degradation of *C3b on the Cell Intermediate EAC1423. To examine the influence of *C4b on the CR1-mediated degradation of neighboring *C3b, we used guinea pig C4 (4 SFU) and C2 (300 SFU) and a concentration of ¹²⁵I-human C3 predetermined to yield the intermediate EAC14⁴P₂⁴PGP₃hù bearing limited amounts of C4⁴P and C3₃hù (1.5 × 10⁵ molecules/cell). The cells were washed and after decay of C2a and incubation with additional C1, different portions of the EAC14⁴P₃hù cells were reincubated with increasing amounts of human C4. The EAC143, bearing a constant and limited amount of both ¹²⁵I-*C3b₃hù and *C4b⁴P, but increasing amounts of *C4b₃hù, were then incubated with I plus CR1 as above. As can be seen in Fig. 6, the release of C3c was enhanced in a dose-dependent fashion by the addition of
FIGURE 5. Inhibitory effect of C2a on the degradation of *C4b and reversal of the C2a effect by *C3b. EAC142 (5 \times 10^7/ml) bearing 3 \times 10^3 molecules of 125I-*C4b per cell and prepared with 100 SFU of C2 were incubated with either DGVB+ or with 30 SFU of unlabeled C3. The resulting cells (5 \times 10^7/ml) bearing the same number of 125I-*C4b molecules and either 0 or 2.4 \times 10^4 molecules of *C3b were then decayed at 30°C for 60 min to remove residual C2a, reincubated for 5 min at 30°C with multiple dilutions of fresh C2, and, immediately after washing, incubated for 60 min at 37°C with I (16 \mu g/ml) and CR1 (200 ng/ml). The percentage of release of C4 radiolabel is shown as a function of the amount of new C2 added in the absence or presence of *C3b on the cells. In the absence of *C3b, C2a inhibited I plus CR1-mediated release of C4 radiolabel in a dose-dependent fashion. The presence of 2.4 \times 10^4 molecules of *C3b not only enhanced release but also reversed the C2a effect.

FIGURE 6. Enhancing effect of *C4b on *C3b fragmentation by I plus CR1 at low *C3b density and comparative activities of human and guinea pig *C4b. EAC1423 were prepared with 5 SFU of C4gp, 300 SFU of C2gp, and sufficient human 125I-C3 (20 SFU) to deposit 1.5 \times 10^3 molecules per cell of 125I-*C3b. The EAC1423 were then decayed by incubation in DGVB++ for 60 min at 30°C to remove C2a and reincubated with additional C1 (300 SFU) and then increasing amounts of C4 ha or C4 gp. The resulting cells bearing a small amount of *C4bgp and a constant amount of 125I-*C3b but increasing amounts of *C4bha or additional *C4b were then incubated at 30°C for 30 min with I (2 \mu g/ml) and CR1 (300 ng/ml). The percentage of release of C3 radiolabel is shown as a function of the amount of C4ha or additional C4gp added to the cells. The release increased with C4 input and release from cells bearing human *C4b was greater than that from cells bearing guinea pig *C4b.
A similar experiment was performed in which the same EAC14\textsuperscript{pC4b} were reincubated with increasing amounts of C4\textsuperscript{pC4b}. In this case the enhancement of release was less marked, most likely because C4\textsuperscript{pC4b} reacts less efficiently with human CR1.

To obtain further evidence that C4\textsuperscript{b} enhances the degradation of C3\textsuperscript{b}, we studied the effects of C2 (which interacts with C4\textsuperscript{b}) and of antibodies to C4. EAC1423 bearing unlabeled C4\textsuperscript{b} and labeled C3\textsuperscript{b} were prepared in a conventional fashion using 300 SFU C4\textsuperscript{bu} and 5 SFU \textsuperscript{125}I-labeled C3\textsuperscript{bu}. The cells were incubated at 30°C for 60 min to decay residual C2a. Then the resulting EAC143 were incubated with increasing amounts of C2 (100–2,500 SFU), or alternatively, with increasing amounts of IgG from a goat antiserum to human C4. After washing, the cells (5 × 10\textsuperscript{7}/ml) were incubated at 30°C for 30 min with I (2 μg/ml) and CR1 (100 ng/ml) and the percentage of release of C3 radiolabel measured. As shown in Fig. 7, treatment of EAC143 with C2 or exposure to anti-C4 antibody inhibited C3c release. Assuming that 10% of the immunoglobulin in the antiserum is anti-C4, we calculate that 1–2 antibody molecules per C4\textsuperscript{b} molecule were sufficient to inhibit C3b fragmentation.

The effect of C4\textsuperscript{b} on I plus CR1-mediated fragmentation of EAC1423\textsuperscript{i} was next examined. The rationale for these experiments was that C3\textsuperscript{b} has low affinity for CR1, and therefore C3\textsuperscript{b} degradation might also be greatly enhanced by neighboring C4\textsuperscript{b}. EAC1423 were prepared bearing limited C4\textsuperscript{b} (4 SFU of C4) and \textsuperscript{125}I-C3\textsuperscript{b} (3.5 × 10\textsuperscript{5} molecules/cell) and incubated at 30°C for...
30 min with I (2 μg/ml) and H (10 μg/ml) to obtain EAC1423i. Hemolytic assays revealed >90% abrogation of the initial C3b hemolytic activity on these cells. The cells were then reincubated with excess C1 (300 SFU) and increasing amounts of C4. The cells were subsequently mixed with I (2 μg/ml) and increasing concentrations of CR1 or intact Ehu, containing an equivalent amount of CR1. Following incubation at 30°C for 30 min, the percentage of release of C3 radiolabel was determined (Fig. 8). Release of C3 radiolabel increased in a dose-dependent fashion as the amount of human C4 added to the cells was increased, whether the release was supported by purified CR1 (Fig. 8A) or by membrane-associated CR1 on intact Ehu (panel B). Minimal release occurred in the presence of I alone, regardless of the C4 concentration used to prepare the cells.

**Kinetics of C4 and C3 Fragmentation.** Kinetic analyses were next performed to determine the temporal relationship between *C4b* and *C3b* breakdown and release. EAC1423 bearing 3 × 10³ molecules of 125I-*C4b* and 2 × 10⁴ molecules of 125I-labeled *C3b* (*C3b/*C4b molar ratio of 7:1), or EAC142 bearing the same number of 125I-labeled *C4b* molecules, were mixed with I (16 μg/ml) and CR1 (200 ng/ml). After incubation at 37°C for increasing times (2–64 min), the reactions were stopped and the percentages of released C4 and C3 labels were determined. As shown in Fig. 9, although the presence of *C3b* accelerated the release of C4 label from EAC1423 (compared with that from EAC142), almost 60% of C3 label had already been released before 10% of C4 label was found in the supernatant.

Additional kinetic analyses were performed using cells bearing different *C3b/*C4b ratios. Release of C3 label preceded that of C4 label in all cases. At very high densities of bound *C3b* (>10⁵ molecules/cell) and *C3b/*C4b ratios of >50:1, the initial rate of release of C4 label from EAC1423 was less than that.

**Figure 8.** Enhancing effect of *C4b* on the degradation of bound *iC3b* by I plus CR1 or I plus Ehu. EAC1423 were prepared with limited human C4 (4 SFU) and sufficient 125I-C3 to yield an uptake of 3.5 × 10³ *C3b* molecules per cell. The cells (5 × 10⁷/ml) were then incubated at 30°C for 30 min with I (2 μg/ml) and H (10 μg/ml). Titrations revealed >90% loss of C3b hemolytic activity. The EAC142im/3i (5 × 10⁷/ml) were then reincubated at 30°C for 20 min with C1 (300 SFU) and then with 10, 30, 100, or 300 SFU of (human) C4. After washing, the cells (5 × 10⁷/ml) bearing increasing amounts of *C4b* and a constant amount of 125I-*C3b* were incubated at 30°C for 30 min with I (2 μg/ml) plus increasing concentrations of CR1 or increasing numbers of Ehu. The percentage of release of C3 radiolabel is shown as a function of CR1 or Ehu input in A and B, respectively. Addition of *C4b* enhanced the release of C3 label whether purified CR1 or Ehu were used.
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Figure 9. Kinetics of fragmentation of *C4b and *C3b by I plus CR1. EAC142 (5 x 10⁷/ml) bearing 3 x 10⁵ *C4b molecules and either 0 or 2 x 10⁴ molecules of *C3b were incubated at 37°C with I (16 μg/ml) plus CR1 (200 ng/ml) and the reaction stopped after different times. The percentage of release of 125I-C4 label from EAC142 and of 125I-C4 and 131I-C3 labels from EAC1423 are shown as a function of incubation time. The presence of *C3b accelerated the release of C4 label. Nevertheless, release of C3 label preceded release of C4 label.

from EAC142. Nevertheless, release of C4 label after 60 min at 37°C from EAC1423 in all cases studied exceeded that from EAC142 (not shown). Even when the *C3b/*C4b ratio was reversed and cells bearing low densities of *C3b (1 x 10⁵ molecules/cell) and high densities of *C4b (8 x 10⁵ molecules/cell) were used, release of C3 label still preceded that of C4 label.

Analysis of the Products of I plus CR1-mediated Degradation of *C4b on Cells Bearing *C4b and *C3b. Previous studies have shown that the C3b fragments generated by I plus CR1 are the same whether *C3b is deposited on the substrate by the classic (1) or by the alternative pathways (2, 3, 31), indicating that the presence of nearby *C4b molecules does not alter the pattern of *C3b breakdown. To determine whether the presence of neighboring *C3b molecules influences the pattern of *C4b breakdown, EAC14 and EAC1423 were prepared with 125I-C4 and treated with I plus CR1, E₉₅, or C4bp. The supernatants and stroma of each reaction mixture were isolated and structural analyses performed by SDS-PAGE and radioautography. As shown in Fig. 10, (lanes 1–3, 5, 7) I, CR1, E₉₅, or C4bp alone had negligible activity.

The major fragments released from EAC14 or EAC1423 by I plus CR1, I plus E₉₅, or I plus C4bp were identical. They had molecular weights of 140,000 (140 K) under nonreducing conditions, and after reduction (Fig. 10, lanes 4, 6, 8), they yielded peptides of 75 K and 31 K mol wt, corresponding to β and γ chains, and of ~25 K and 17 K mol wt, corresponding to α'3 and α'4 segments of α' chain. The amount of released C4c was much smaller in the presence of I plus C4bp (lane 8).

The SDS-PAGE of the stroma of EAC1423, isolated after the various treatments (lanes 9–13) revealed the presence of remaining *C4b α', β, and γ chains.
Figure 10. SDS-PAGE analysis of C4b fragments generated by I plus CR1, E, or C4bp in the presence of *C3b. EAC1423 (5 x 10^7/ml), bearing 5 x 10^5 molecules of *C4b and 2 x 10^5 molecules of (unlabeled) *C3b, were incubated at 37°C for 60 min with DGVB**, I alone (8 μg/ml), CR1 alone (400 ng/ml), E alone (1 x 10^9/ml), C4bp alone in equivalent blood proportion (30 μg/ml), or with I plus CR1, I plus E, or I plus C4bp at the same concentrations. Samples of supernatants and cell stroma in each reaction mixture were subjected to SDS-PAGE under reducing conditions and radioautographs prepared. A small amount of polypeptides with molecular weights corresponding to α', β, and γ chains of uncleaved C4b were released into the supernatant by DGVB**, I, CR1, E, or C4bp alone (lanes 1, 2, 3, 5, and 7 respectively). Polypeptides with molecular weights corresponding to β and γ chains, and α'3 and α'4 fragments, were released in small amounts in the presence of I alone (lane 2) or I plus C4bp (lane 8) but in much larger amounts in the presence of I plus CR1 or I plus E (lanes 4 and 6). The C4b polypeptides released by I plus CR1 or I plus E were identical to those generated in the absence of *C3b (not shown).

Polypeptides corresponding to the α', β, and γ chains of uncleaved C4b were seen in all samples of stroma (lanes 9-13). Additional polypeptides of 75 K mol wt were prominent only in samples treated with I alone (lane 10) and in the absence of *C3b (not shown) except that small amounts of 45 K mol wt peptides were also seen in samples treated with I plus C4bp.

EAC1423 stroma treated with I and the different cofactors additionally yielded peptides of 45 K mol wt presumably representing covalently bound α'2 (*C4d) fragments, as well as peptides of 75 K mol wt, which appear fused with the β chain band. In lane 14 we show a repeat of the radioautography of lane 13 with
less exposure time, which permits the distinction between the \( \alpha'75 \) and \( \beta \) peptides.

A comparison of the patterns seen in samples derived from the stroma of cells treated with \( \text{I plus C4bp} \) with those in samples treated with \( \text{I plus CR1 or I plus E}^{\text{hu}} \) showed that they differed in that the proportion of the 45 K mol wt peptide relative to the 75 K mol wt peptide was much smaller in the samples treated with C4bp plus I (lane 13). The high molecular weight bands seen in lanes 9–13 probably represent \( \alpha' \) chain fragments covalently bound to macromolecules from the cell membrane.

**Discussion**

The mechanisms regulating the function of *C4b* have remained incompletely understood. Although C4bp is required for cleavage of fluid phase C4b by I, the \( \alpha' \) chain of *C4b* can be cleaved successively at two or more sites by I alone and C4c subsequently released from the substrate (19, 30). Relatively high concentrations of I and prolonged incubation are necessary, however, and degradation is usually incomplete. One study (29) shows that C4bp can enhance this reaction, while another (19) reports that C4bp has no effect. Recently, Fujita and Tamura (28) have resolved this controversy by showing that an enhancing action of C4bp can be demonstrated only at high *C4b* density (>3 \( \times \) \( 10^5 \) molecules/cell).

Initial experiments of ours (1) indicated that CR1 also did not appear to substantially alter I-mediated release of C4c. These earlier studies were performed with low concentrations of I and CR1 and a temperature of incubation of 30°C; that is, under reaction conditions similar to those which we used to study degradation of *C3b*. In the present paper we present the results of additional studies, performed under different experimental conditions. We show here that in the presence of higher concentrations of I, CR1 does promote the degradation of *C4b* on EAC14 cells provided that the density of *C4b* is >3 \( \times \) \( 10^3 \) molecules per cell, and that the incubation is carried out at either 37°C or alternatively for longer periods of time at 30°C. Under these conditions, on a weight basis, CR1 is ~10³-fold more effective than C4bp in promoting fragmentation of *C4b* on EAC14 (Fig. 2). Since it could be argued that the behavior of the detergent-extracted and purified CR1 in an aqueous environment might differ from that of membrane-associated CR1, these and other experiments (Figs. 2, 8, 10) were also performed using intact human erythrocytes as a source of receptor. The results showed that the CR1 activity on E\( ^{\text{hu}} \) and in purified form was comparable. In addition, the experiments revealed that the cofactor activity of erythrocytes was almost exclusively mediated by CR1, since it was strongly inhibited by monoclonal antibodies to the receptor (Fig. 2).

Perhaps of greater significance is the finding that the breakdown of *C4b* by I plus CR1 was enhanced by the presence of *C3b* on the cellular intermediates (Fig. 3). On EAC1423 cells, CR1 supported the *C4b* breakdown even at low *C4b* densities and low concentrations of I and CR1, under incubation conditions comparable to those required for *C3b* breakdown (1). It appears, therefore, that in the physiological situation, before *C3b* uptake, *C4b* is less vulnerable to attack by I. Once optimal *C3b* uptake has taken place, binding to CR1 is
enhanced and the activities of the *C4b- and *C3b-dependent enzymes are inhibited.

Despite the marked difference in efficiency between CR1 and C4bp in promoting I-mediated *C4b fragmentation, similar degradation products were obtained from EAC14 or EAC1423 cells (Fig. 10). The C4c fragment released into the supernatant, after incubation with I and either cofactor, had a molecular weight of 140 K and consisted of polypeptides of 75 K, 31 K, 25 K, and 17 K mol wt, corresponding to \( \beta \) and \( \gamma \) chains and \( \alpha'3 \) and \( \alpha'4 \) segments, respectively. The bands remaining associated with the cell had molecular weights of about 75 K and 45 K, the latter most likely representing *C4d (28, 30). As shown by Fujita and Tamura (28), the 75 K band derives from an intermediate product of degradation of C4b (iC4b) first described by Nagasawa et al. (32). The results of multiple experiments, one of them represented in Fig. 10, showed that the 45 K/75K ratio was much higher in *C4b samples treated with I plus CR1 than in those treated with I plus C4bp, suggesting that the cleavage of *iC4b by I is promoted by CR1 more effectively than by C4bp. These findings indicate, therefore, that the efficiency of the I-mediated conversion of *C4b into C4c and *C4d is substantially greater in the presence of CR1 than of C4bp. This difference in the effects between I plus CR1 and I plus C4bp parallels the differential effects of I plus CR1 and I plus H on *C3b (1, 4).

In analogy to the above findings showing that *C3b enhances degradation of *C4b, studies with cells bearing limited amounts of *C3b demonstrated a parallel potentiating effect of *C4b on *C3b degradation. That this effect was indeed mediated by *C4b was shown by several observations. For example, binding of C2a or anti-C4 antibody to *C4b on EAC14231im inhibited the I plus CR1-mediated C3c release (Fig. 7). Reduction of *C4b density on the cells or replacement of human *C4b by guinea pig *C4b (which most likely has a lower binding affinity for human CR1) had a similar effect (Fig. 6). Uptake of additional *C4b onto EAC143i increased I plus CR1-mediated C3c release in a dose-dependent fashion (Fig. 8). The most plausible mechanism of these effects is that *C4b promotes interaction of *C3b or *iC3b with CR1.

Provided that the kinetics of C3c and C4c release from EAC1423 treated with CR1 plus I (Fig. 9) parallel the kinetics of the underlying proteolysis of the \( \alpha' \) chains of *C3b and *C4b, the enzymatic attack on *C3b proceeds faster than that on *C4b. The simplest explanation for this observation is that CR1 has greater affinity for *C3b than for *C4b. It is also conceivable that the spatial arrangement of *C3b and *C4b on the substrate could influence the outcome of the reaction if multiple bonds have to be formed between CR1 and the substrate-bound ligands before the inactivator I can fully exert its effect.

The finding that *C4b can enhance *C3b interaction with CR1, taken together with the results above that the converse is also true, argue that clusters of *C4b*3b can serve as a ligand for CR1, which is itself probably multivalent (35, 34). This notion is consistent with previous observations that *C3b can enhance the rosetting of EAC14 with E \( \text{hu} \), and that excess *C4b can enhance rosetting of EAC14231im (18). A greater ability of *C4b*3b clusters than of *C4b or *C3b monomers alone to interact with CR1 is also compatible with more recent findings (35) that C3b dimer binds to CR1 with high affinity, while C3b monomer
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does not. The formation of multiple bonds between ligand and CR1 can likewise account for the observation (4) that the activity of CR1 in the inactivation of classical pathway C5-convertase can be much greater than that of H, and that CR1 activity can increase progressively with increasing *C3b density on the substrate. This latter result, in conjunction with longstanding observations by many investigators that rosetting between EAC14 and E hu occurs only at very high *C4b densities, and the multiple data showing cooperation between *C4b and *C3b in the present study, suggests that effective interaction with CR1 requires a multivalent ligand which can consist of clusters of *C4b, *C3b, *iC3b, *iC4b, or of these fragments, in various combinations.

In summary, the present results strongly suggest that *C4b is more effectively inactivated when *C3b is fixed, and that the inactivation of *C4b*3b by I is greatly enhanced by CR1, but not by C4bp. The greater ability of CR1 than C4bp to serve as a cofactor of I in this reaction can be explained by the lack of high binding affinity of C4bp for *C3b (30), but steric considerations arising from the multimeric nature of the interaction between substrate and cofactor may also be important. The findings of this paper also have implications in the pathophysiology of complement deficiency diseases. For example, they predict that in C2-deficient individuals, *C4b should persist and thereby accumulate to a greater extent on substrates, since in these individuals *C3b cannot be deposited by the classical pathway and *C4b inactivation therefore should be less efficient. On the other hand, when CR1 levels on erythrocytes are diminished, as, for example, can occur in patients with systemic lupus erythematosus (11-15), *C4b and *C4b*3b clusters will be less subject to I-mediated control and complement consumption by the classical pathway will increase.

Summary

The complement fragments C3b and C4b are the main ligands for the membrane receptor CR1. We showed elsewhere that CR1 functions as an essential cofactor for the factor I-mediated enzymatic breakdown of membrane-bound C3b (*C3b) into C3c and *C3dg. One of the main findings of the present paper is that CR1 also promotes the degradation of bound C4b (*C4b) into C4c and *C4d. On a weight basis, the cofactor activity of CR1 in the cleavage of *C4b present on the cell intermediate EAC14 is 10³-fold greater than that of the serum cofactor C4-binding protein (C4bp).

An additional finding is that the effect of CR1 on either *C3b or *C4b is modulated by the presence of the other ligand in its vicinity; that is, *C4b degradation by CR1 plus I is enhanced by neighboring *C3b and vice versa. For example, upon uptake of optimal amounts of *C3b onto EAC142 and the assembly of the C3-convertase EAC1423, the activity of CR1 in generating C4c is enhanced 5-10 times further. Conversely, when the number of *C3b molecules on EAC1423 is relatively small (or when EAC1423 has been converted by I plus H into EAC 1423i), the presence of neighboring *C4b enhances the conversion of *C3b (or *iC3b) into C3c plus *C3dg.

The enhancing effect of *C3b on the cleavage of *C4b by I is observed only if the cofactor of this reaction is CR1. Indeed, the activity of I or I plus C4bp on *C4b is significantly inhibited when *C3b is fixed and the main product of the
reaction is *iC4b. Taken together, these findings suggest that degradation of
*C4b will be more effective when enough C3b molecules are fixed nearby, thus
facilitating the interaction of *C4b*3b clusters with CR1-bearing cells, and that
under physiological conditions, *C4b activity can be efficiently controlled by
CR1.

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