

# UNIQUE ROLE OF THE COMPLEMENT RECEPTOR CR<sub>1</sub> IN THE DEGRADATION OF C3b ASSOCIATED WITH IMMUNE COMPLEXES\*

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Receptors for C3b and C4b (CR<sub>1</sub>)<sup>1</sup> are found on the membrane of phagocytes where they promote the binding of C3b- or C4b-bearing particles and immune complexes (IC) (reviewed in 1). The majority of CR<sub>1</sub> in the vascular system, however, is present on erythrocytes in primates and platelets in other species. Furthermore, CR<sub>1</sub> is found on B lymphocytes (2, 3) and on the epithelial cells of kidney glomeruli (4). The function of CR<sub>1</sub> in these locations is unknown.

In the present paper, we describe a new activity of CR<sub>1</sub>; that is, its participation in the breakdown of C3b associated with IC. This investigation was prompted by previous observations that, when soluble IC were added to a mixture of normal human serum and autologous unseparated blood cells, they fixed complement and bound predominantly to the erythrocytes. The binding was mediated principally via C3 incorporated into the IC (5, 6). After complement fixation, the binding could not be prevented by preincubation of the IC with the serum enzyme C3b/C4b inactivator (I) and cofactor  $\beta$ 1H (H), but after binding, the IC could be released by treating the erythrocytes with I alone (7, 8). During this treatment, C3b in the IC was degraded beyond the iC3b stage, a fragment the size of C3c was released, and the IC became more reactive with cells bearing C3d receptors (CR<sub>2</sub>). This was unexpected, as previous reports indicated that the interaction of fluid phase C3b with I in the presence of H (9, 10) or of purified CR<sub>1</sub> (11) generated only the iC3b intermediate.

Here, we study the nature of the human erythrocyte factor involved in the

\* Supported by grant AI-08499 from the National Institutes of Health, and by a grant from the SLE Foundation of New York. This paper was presented in part at the meeting of the Federation of American Societies of Experimental Biology, April 1982.

<sup>1</sup> *Abbreviations used in this paper:* A, antibody to sheep erythrocytes; Ag:Ab, antigen:antibody complexes prepared with a soluble antigen; BSA, bovine serum albumin; C, complement; C3b, major fragment of C3; iC3b, cleavage product of C3b; C3c and C3d, not well defined cleavage products of iC3b by plasmin, trypsin or elastase; C3b\*, iC3b\*, C3d\*, cell-bound or immune-complex-bound forms of the corresponding C3 fragments; CR<sub>1</sub>, membrane receptor for C3b and C4b; CR<sub>2</sub>, membrane receptor for C3d; CR<sub>3</sub>, membrane receptor for iC3b; DVB, isotonic veronal buffered saline containing 2.5% dextrose, 0.15 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>; DOVB, DVB supplemented with 0.1% ovalbumin; DGVB, DVB supplemented with 0.1% gelatin; E, sheep erythrocytes; H,  $\beta$ 1H; I, C3b,C4b-inactivator; IC, immune complexes; K76COOH, K76 monocarboxylic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

sequential breakdown of C3b bound to IC in the presence of I and further characterize the C3-derived products of this process. We show that this factor is CR<sub>1</sub> itself. In addition, we present suggestive evidence that iC3b bound to IC is also a ligand for CR<sub>1</sub>. To facilitate exposition, C3 fragments bound to IC after fixation of C3b, initial cleavage of  $\alpha'$  chain, and release of C3c will be designated with asterisks: C3b\*, iC3b\*, and C3d\*, respectively.

### Materials and Methods

**Buffers.** Isotonic veronal-buffered saline containing 2.5% dextrose, 0.15 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub> (DVB) was used for all studies. DVB was supplemented with 0.1% ovalbumin (DOVB) for studies with soluble IC, and with 0.1% gelatin (DGVB) for studies with cellular intermediates.

**Proteins and Antibodies.** Four monoclonal antibodies to CR<sub>1</sub> (57H, 44D, 57F, and 31D) were obtained as described elsewhere (12). Polyclonal antibodies to C3c and C3d were a gift from Dr. Brian Tack (Scripps Clinic and Research Foundation, La Jolla, CA). These antisera were raised in rabbits by injecting them with C3c and C3d purified from elastase digests of C3 (13).

CR<sub>1</sub> was purified from human erythrocytes as described in ref. 11, except that the last two steps, that is, affinity chromatography on Sepharose-lentil lectin and Sepharose-C3, were substituted by affinity chromatography on Sepharose-anti-CR<sub>1</sub> prepared with monoclonal antibodies (14).

H (15), I (16), C4-bp (17), and C4 and C3 (13) were purified from human serum or plasma as described. C3 provided by Dr. John Atkinson (Washington University School of Medicine, St. Louis, MO) was used in some studies. C2 was purified as described (13), followed by chromatography on CM-Sephadex C-50 and DE-52, and was used in an oxidized form (<sup>oxy</sup>C2) (18). C5 was purchased from Cordis Laboratories, Miami, FL. C3b was prepared by incubating C3 with factors B (19) and D (20) and isolated as described (16). Plasminogen was purified from human serum (13) and activated by urokinase (Sigma Chemical Co., St. Louis, MO) (21). C1 (22) and C6-9 (23) were prepared from guinea pig serum.

**Cellular Intermediates and Hemolytic Assay.** Sheep erythrocytes (E) were sensitized with rabbit hemolysin (A), (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), and complement components C1, C4, and <sup>oxy</sup>C2 were added sequentially to yield 300 hemolytic sites per cell. Then radiolabeled C3 was added in an appropriate dilution to yield between three and five hemolytic sites per cell. After incubation, the EAC1423b cells were washed and resuspended in DGVB. To prepare EAC1423bi, the EAC1423b cells (10<sup>8</sup>/ml) were incubated at 30°C for 60 min with I (2  $\mu$ g/ml) and H (5  $\mu$ g/ml). The remaining C3 hemolytic activity on these cells was measured by incubating cells with excess C5 at 30°C for 5 min and an additional 60 min at 37°C with C6-9. In some experiments, EAC14 cells were prepared with radiolabeled C4.

**Radiolabeling.** C3, C3b, and C4 were labeled with <sup>125</sup>I using Enzymobeads, (Bio-Rad Laboratories, Rockville Center, NY) or lactoperoxidase beads (Worthington Biochemical Corp., Freehold, NJ) according to the instructions of the manufacturer.

**Analysis of Membrane-bound C3 Fragments.** C3 peptides from erythrocyte membranes were prepared as described by Law et al. (24). Briefly, 6  $\times$  10<sup>7</sup> of EAC1423b or EAC1423bi were lysed with distilled water containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and ghosts were collected by centrifugation. The pellets were solubilized with 25  $\mu$ l of 0.1% Triton-X in 15 mM Tris buffer (pH 7.8) containing 1 mM PMSF and incubated with 25  $\mu$ l of 1 M hydroxylamine in 2 M sodium carbonate buffer (pH 9.5) for 30 min at 37°C. After dialysis against phosphate-buffered saline (pH 7.4) the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by radioautography.

**Isolation of Human Erythrocytes.** Human erythrocytes were isolated by incubation of heparinized blood with dextran (25), followed by centrifugation of sedimented cells through Ficoll-Hypaque (d = 1.094) and washing and resuspension of the pellet. Separated cells contained

$<4 \times 10^4$  white blood cells per  $5 \times 10^9$  erythrocytes.

*Preparation and Isolation of Soluble Antigen (Ag)-Antibody (Ab)-Complement (C) Complexes (Ag:Ab:C).* Ag:Ab complexes were prepared from bovine serum albumin (BSA) and heat-inactivated guinea pig antiserum to BSA. Antibody was raised and the antigen/antibody ratio at equivalence was determined as previously described (5). Anti-BSA was diluted sufficiently that when mixed with BSA at equivalence, no precipitate formed. Ag:Ab were prepared in fourfold antibody excess, so that, after reaction of Ag:Ab with serum, Ag:Ab:C gave maximum percentage binding ( $\sim 75\%$ ) to human erythrocytes (5, 6).

$^{125}\text{I}$ -C3-labeled Ag:Ab:C were prepared by mixing one vol of Ag:Ab with an equal volume of 1:16 diluted normal human serum supplemented with  $^{125}\text{I}$ -C3 (10 ng/ $\mu\text{l}$  of 1:16 serum) and with the I-inhibitor K76 monocarboxylic acid (K76COOH) (26), a gift from Dr. Kozo Inoue, Osaka University, Japan, at a final concentration of 4 mM. After incubation at  $37^\circ\text{C}$  for 30 min, the reaction mixture was diluted twofold with ice-cold DOVB.

Ag:Ab:C bearing  $^{125}\text{I}$ -C3 were separated from free  $^{125}\text{I}$ -C3 or C3b and K76COOH by gel filtration through Biogel A1.5m (Bio-Rad Laboratories) equilibrated with DVB at  $4^\circ\text{C}$ . A typical elution profile is shown in Fig. 1, together with an elution profile of  $^{125}\text{I}$ -BSA-labeled Ag:Ab:C for comparison. Ag:Ab:C eluted as a single symmetrical peak at the void volume. 80% of added label was recovered in this peak in the case of the  $^{125}\text{I}$ -BSA-labeled preparation. 50  $\mu\text{l}$  of individual column fractions was added to suspensions of human or sheep erythrocytes ( $2 \times 10^8$  cells in DOVB), the mixtures were incubated at  $37^\circ\text{C}$  for 15 min, and the cells were washed. Fractions containing Ag:Ab:C, giving high binding to human and low binding to sheep erythrocytes, were pooled and used immediately.

*SDS-PAGE.* SDS-PAGE was performed as described by Laemmli (27). 3.75% stacking and 5–15% gradient running gels were used in most cases. A pool of proteins from a commercial kit (Bethesda Research Laboratories, Inc., Rockville, MD) was used to estimate the molecular

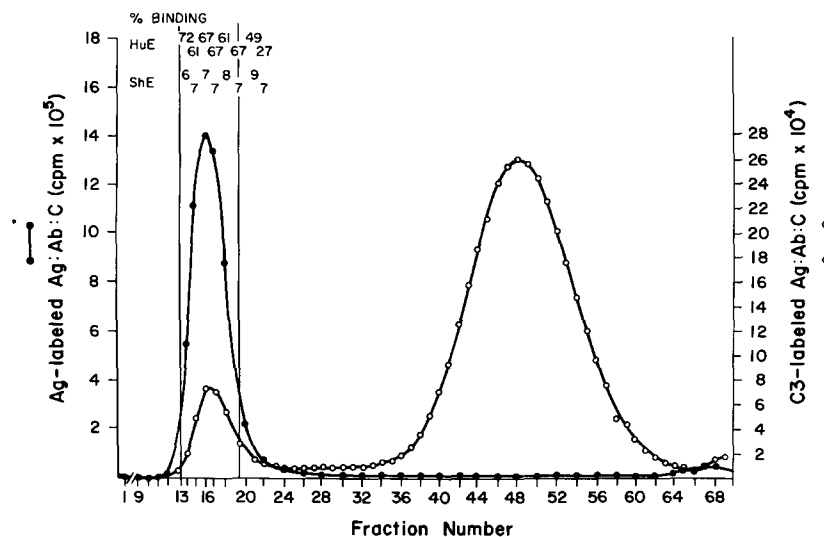


FIG. 1. Purification of Ag:Ab:C by gel filtration and analysis of their binding properties. The reaction mixture obtained after incubation of unlabeled Ag:Ab with 1:16 diluted serum supplemented with  $^{125}\text{I}$ -C3 and K76COOH, as described in Materials and Methods, was applied to a  $2 \times 100$ -cm column containing Biogel A1.5m equilibrated with DVB, and 500- $\mu\text{l}$  fractions were collected. The elution pattern (○) is shown along with the elution pattern (●) of a control reaction mixture obtained after identical incubation of  $^{125}\text{I}$ -BSA-labeled Ag:Ab with untreated 1:16 diluted serum. In the latter case,  $>80\%$  of the BSA label was recovered in the void volume. 50  $\mu\text{l}$  aliquots of individual column fractions of  $^{125}\text{I}$ -C3-labeled Ag:Ab:C were incubated at  $37^\circ\text{C}$  for 10 min with human or sheep erythrocytes (HuE, ShE) and, after washing, percentage binding was determined.

weights of samples run under reducing conditions. Rabbit IgG, F(ab')<sub>2</sub>, Fab, and bovine albumin were used as molecular weight markers when samples were analyzed under nonreducing conditions. After electrophoresis, gels were stained with Coomassie blue or with the silver staining kit (Upjohn Diagnostics, Kalamazoo, MI). Radioautography was carried out at -70°C using X-Omat R film (XR-5, Eastman Kodak Co., Rochester, NY). In some instances, proteins were eluted from bands excised from the acrylamide gels, using the technique described in ref. 28.

*Two-Dimensional Electrophoresis.* A modified O'Farrell technique (29) was used as described (30). Electrofocussing was performed in the first dimension. A mixture of Ampholines, pH 3-10, 5-8, and 9-11 (LKB Instruments, Inc., Rockville, MD), was used to obtain a pH range of from 5.1 to 9.1. The second dimension (SDS-PAGE) was carried out in a 5-15% gradient gel. The slab gels were then subjected to radioautography.

## Results

*Ability of Purified CR<sub>1</sub> + I to Release C3 Fragments from EAC1423b.* Because previous studies had shown that C3 fragments produced during release of Ag:Ab:C from human erythrocytes by I differed from those obtained after exposure of fluid phase C3b to I + H or to I + CR<sub>1</sub>, the effects of each of these factors on C3b\* were compared using the cell intermediate EAC1423b prepared with <sup>125</sup>I-C3 and bearing five hemolytic sites per cell. Cells (10<sup>8</sup>/ml in DGVB, containing ~0.13 μg of C3b\*/ml) were mixed at 30°C with I (2 μg/ml), CR<sub>1</sub> (100 ng/ml), or H (5 μg/ml), or with mixtures of I + H or I + CR<sub>1</sub> at the same final concentrations. At various time intervals (from 5 to 50 min) duplicate 100-μl samples of each mixture were removed, mixed with 2 ml of ice-cold DGVB, and centrifuged at 600 g for 10 min. In one set of samples, the radioactivity in the pellets and supernatants was counted in a gamma counter. In the other set, the residual hemolytic activity of EAC1423b was measured. The results (Fig. 2 a) show that within a few minutes of incubation of EAC1423b with I + H or with I + CR<sub>1</sub> or CR<sub>1</sub> alone, C3 hemolytic activity was abolished. The results agree with previous findings that I + H can transform EAC1423b into hemolytically inactive EAC1423bi (9, 15) and that CR<sub>1</sub> alone can inhibit EAC1423b hemolytic sites (31).

In contrast, as shown in Fig. 2 b, only I + CR<sub>1</sub> released large amounts of C3 fragments from the cells. At 30 min of incubation with I + CR<sub>1</sub> ~70% of the C3 radioactivity was found in the supernatant, whereas <10% was specifically released by I or by I + H. Under the same conditions, we incubated EAC14 cells bearing five hemolytic sites per cell and prepared with radiolabeled C4, with I (2 μg/ml) + CR<sub>1</sub> (100 ng/ml), or with I + C4bp (1 μg/ml). The amount of radioactivity released in the supernatant during 1 h of incubation at 30°C was negligible and not significantly different from that released from cells incubated with buffer alone.

In a separate experiment (not shown), we studied the effects of the concentration of I and CR<sub>1</sub> on the release of C3 fragments from the EAC1423b cells. The extent of release was higher as the concentration of either reagent was increased. Moreover, when the dose of CR<sub>1</sub> was diminished, the release could be enhanced by an increase in the dose of I and vice versa. 12% of the C3b\* counts were released specifically from cells incubated for 30 min at 30°C with 12.5 ng/ml of CR<sub>1</sub> and 4 μg/ml of I. About 9% release was observed in the presence of 125 μg/ml of H and 12.5 μg/ml of I. Considering that the molecular weights of the subunits of CR<sub>1</sub> and H are ~200,000

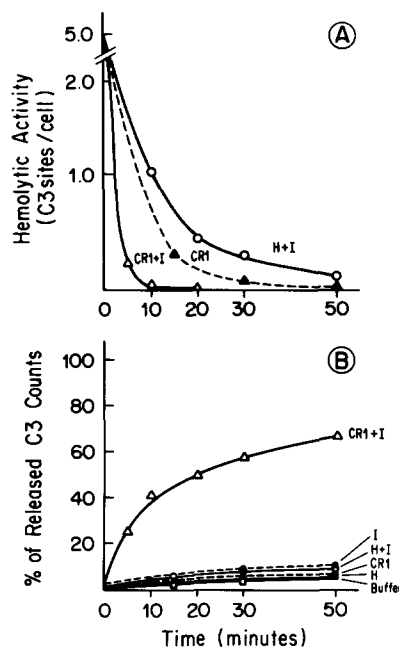


FIG. 2. Interaction of CR<sub>1</sub>, I, and H with EAC1423b. Time-course of inactivation of hemolytic activity (A) and of release of C3 fragments (B). EAC1423b ( $10^8$  cells/ml), prepared with radiolabeled C3 and bearing five hemolytic sites per cell, were mixed with I ( $2 \mu\text{g/ml}$ ), CR<sub>1</sub> ( $100 \text{ ng/ml}$ ), H ( $5 \mu\text{g/ml}$ ), or mixtures of I + CR<sub>1</sub> or I + H at the same final concentrations. At timed intervals, duplicate samples of  $100 \mu\text{l}$  were withdrawn from each tube and mixed with 2 ml of ice-cold DGVB. One set of samples was used to test the hemolytic activity remaining on EAC1423b. The other tubes were centrifuged, and the radioactivity in the supernatants and pellets was counted in a gamma counter. The results indicate that while CR<sub>1</sub> + I, H + I, or CR<sub>1</sub> alone inhibited the hemolytic activity of erythrocyte intermediates, only CR<sub>1</sub> + I released large amounts of C3 fragments from the cells.

and 150,000, respectively, on a molar basis, the CR<sub>1</sub> activity is at least  $10^4$  greater than that of H.

*Effect of Membrane-associated CR<sub>1</sub> on C3b\* in the Presence of I.* The effects of isolated CR<sub>1</sub> and membrane-associated CR<sub>1</sub> on EAC1423b are compared in Fig. 3. EAC1423b ( $10^8/\text{ml}$ ) and I ( $2 \mu\text{g/ml}$ ) were incubated at  $30^\circ\text{C}$  for 30 min with increasing numbers of washed human erythrocytes or with the equivalent amounts of purified CR<sub>1</sub> ( $400 \text{ ng}/10^9$  human erythrocytes) (12), and the reaction was terminated as described above. As shown, C3 fragments were released from EAC1423b by both human erythrocytes and purified CR<sub>1</sub> in a dose-dependent fashion. Moreover, the specific activities of the intact erythrocytes and purified CR<sub>1</sub> were almost identical. That the human erythrocyte activity was mediated exclusively by CR<sub>1</sub> was shown by its complete inhibition in the presence of  $2 \mu\text{g/ml}$  of pooled purified monoclonal antibodies to CR<sub>1</sub>.

*Evidence That iC3b\* Is a Ligand for CR<sub>1</sub>.* Previous observations have shown that CR<sub>1</sub> + I or H + I cleave the  $\alpha'$  chain of fluid-phase C3b and generate iC3b. One trivial explanation for the finding that CR<sub>1</sub> + I but not H + I can fragment C3b\* beyond the iC3b\* stage is that CR<sub>1</sub> is contaminated by a proteolytic enzyme. Alternatively, the reaction between C3b\*, CR<sub>1</sub>, and I could proceed in two steps,

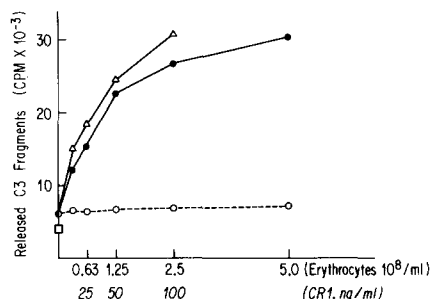


FIG. 3. Inhibitory effect of anti-CR<sub>1</sub> on the release of C3 fragments from EAC1423b by human erythrocytes and I. EAC1423b prepared with radiolabeled C3 and bearing three hemolytic sites per cell ( $10^8$  cells/ml) were incubated with I ( $2 \mu\text{g/ml}$ ) and human erythrocytes (●) or purified CR<sub>1</sub> (Δ), in the presence (○) or absence of monoclonal anti-CR<sub>1</sub> ( $2.5 \mu\text{g/ml}$ ) for 30 min at  $30^\circ\text{C}$ . The final concentrations of CR<sub>1</sub> or human erythrocytes in the reaction mixtures are indicated on the abscissa. The input of C3 counts per tube was  $4.4 \times 10^4$ . The results show that the erythrocyte activity was inhibited by the monoclonal antibodies.

both requiring CR<sub>1</sub>.

To distinguish between these possibilities, we studied the activity of CR<sub>1</sub> + I on the intermediate EAC1423bi prepared with radiolabeled C3. To prepare EAC1423bi, we incubated EAC1423b with I + H, as described in Materials and Methods, and washed the cells by centrifugation. Hemolytic assays showed no residual C3b\* sites, and direct analysis by SDS-PAGE of the hydroxylamine-treated membranes of these cells demonstrated that the  $\alpha'$  chain of C3b\* had been cleaved (inset of Fig. 4).

The EAC1423bi were then incubated at  $37^\circ\text{C}$  in the presence of buffer, I, CR<sub>1</sub>, or CR<sub>1</sub> + I. In the presence of CR<sub>1</sub> + I (but not CR<sub>1</sub> or I alone) C3 fragments were released in the supernatant (Fig. 4). Moreover, release also occurred when the cells were first exposed to CR<sub>1</sub> alone, washed thoroughly, and then incubated with I. That the release was CR<sub>1</sub> mediated was shown by its complete inhibition when monoclonal antibodies to CR<sub>1</sub> ( $5 \mu\text{g/ml}$ ) were added to the incubation medium. As an additional control, other tubes containing EAC1423bi were incubated with plasmin. In this instance, the release of C3 fragments could not be inhibited by the monoclonal antibodies. These results demonstrate that EAC1423bi has binding affinity for CR<sub>1</sub> and that the further degradation of iC3b\* in the presence of I is CR<sub>1</sub> dependent.

*Structure of the Fragments Released by CR<sub>1</sub> + I from EAC1423b.* The C3 fragments released in the supernatant of the reaction between EAC1423b with CR<sub>1</sub> + I or with human erythrocytes + I were analyzed by SDS-PAGE. No significant variation was observed in their structure when the time of incubation at  $30^\circ\text{C}$  varied from 5 to 75 min. As shown in Fig. 5, their apparent molecular weight was  $\sim 135$  K under nonreducing conditions. After reduction, two main labeled polypeptides were detected:  $\beta$  chain and a fragment with an apparent molecular weight of 31,000, most likely derived from the  $\alpha'$  chain (Fig. 6). When gels were overexposed, weak 70-K and 40-K bands were observed. The structure of the fragments released in small amounts by H + I was identical (data not shown).

The 135-K fragment was further identified as C3c because it could be specifically immunoprecipitated with an antiserum to C3c but not to C3d. Moreover, the 31-K polypeptide isolated from bands excised from the slab gels also reacted with anti-C3c.

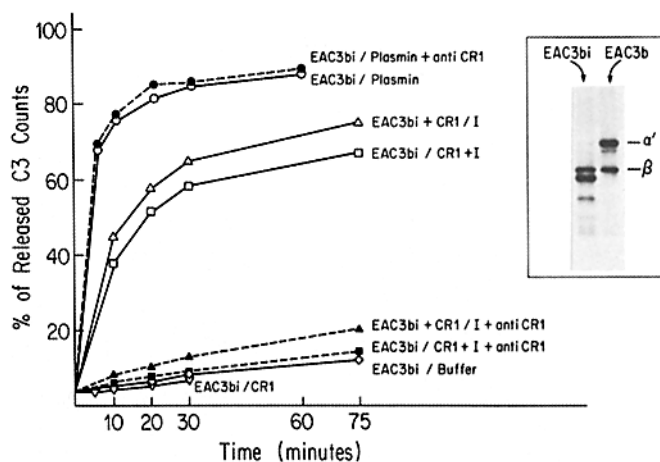


FIG. 4. Release of C3 fragments from EAC1423bi by CR<sub>1</sub> + I or plasmin. EAC1423bi ( $10^8$  cells/ml) were incubated with plasmin ( $10 \mu\text{g/ml}$ ), or CR<sub>1</sub> ( $400 \text{ ng/ml}$ ) or CR<sub>1</sub> + I ( $4 \mu\text{g/ml}$ ) in the presence or absence of anti-CR<sub>1</sub> ( $5 \mu\text{g/ml}$ ) at  $30^\circ\text{C}$ . Alternatively, EAC1423bi were first incubated with CR<sub>1</sub> for 30 min at  $30^\circ\text{C}$ , washed thoroughly, and used for the kinetic studies of C3 release by I alone. At timed intervals,  $100 \mu\text{l}$  of reaction mixtures were withdrawn into tubes with 2 ml ice-cold DGVB, centrifuged, and the radioactivity in pellets and supernatants counted. In the figure, the symbol / represents a washing step before the addition of reagent(s) to the erythrocyte intermediate. The results demonstrate that CR<sub>1</sub> interacts with EAC1423bi and that C3bi\* can be further cleaved in the presence of I. That the activity is mediated by CR<sub>1</sub> itself and not a contaminant enzyme is shown by the inhibition of the reaction with monoclonal antibodies to the receptor. As expected, the monoclonals did not interfere with the activity of plasmin. The inset of this figure contains the SDS-PAGE analysis of the membranes of the EAC1423b and EAC1423bi cells, prepared as described in Materials and Methods. After treatment with H + I, the  $\alpha'$  chain of C3b was cleaved.

(These antisera were directed against fragments purified from elastase-digests of C3.)

Possible explanations for the discrepancy between the molecular weight of C3c under nonreducing conditions (135,000) and the molecular weights of the major observed bands (75 K and 31 K) under reducing conditions are that the 31-K band represents two polypeptide chains or that C3c contains a weakly labeled peptide. The 31-K peptide could not be resolved by two-dimensional electrophoretic analysis, and its pI was found to be 8.6. In other experiments, we analyzed supernatants originating from EAC1423b cells prepared with 100 times more C3. Silver stain of the gels revealed two C3-derived bands at 39 K and 42 K (not shown).

The breakdown products released by plasmin are also shown in Figs. 5 and 6 because this enzyme has been used frequently by others to generate C3c and C3d fragments. Clearly, the C3c fragments obtained with plasmin and with CR<sub>1</sub> + I differ greatly in structure. Under nonreducing conditions, some free  $\beta$  chain was detected in the plasmin C3c, but the main fragment had an  $\sim 66\text{-K}$  mol wt. Under reducing conditions, three peptides were detected of 75 K ( $\beta$  chain), 60 K, and 27 K mol wt. It should be pointed out that plasmin cleaves the  $\beta$  chain of fluid-phase iC3b (32). That plasmin probably also cleaves the  $\beta$  chain of fluid-phase iC3b\* is shown in Fig. 6: the intensity of the  $\beta$  chain band in track CR<sub>1</sub> + I is stronger than that of plasmin + H + I.

In addition, Fig. 6 shows the products obtained by incubation of radiolabeled fluid-phase C3b with I + CR<sub>1</sub> or I + H. As expected (10, 11), the 70-K and 40-K fragments

**CLEAVAGE PRODUCTS OF  
CELL-BOUND C3b  
(nonreducing conditions)**

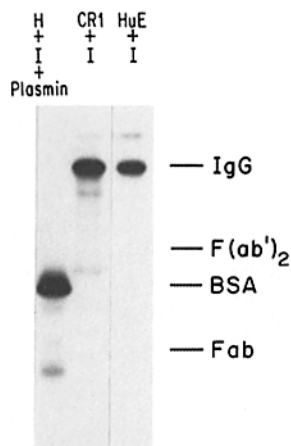


FIG. 5. SDS-PAGE analysis under nonreducing conditions of the cleavage products released from cell-bound C3b\*. EAC1423b ( $10^8$  cells/ml), prepared with radiolabeled C3 and bearing three hemolytic sites per cell, were incubated with I ( $2 \mu\text{g/ml}$ ) + H ( $5 \mu\text{g/ml}$ ) for 60 min at  $30^\circ\text{C}$ , washed, and further incubated with plasmin ( $10 \mu\text{g/ml}$ ) for 60 min. The EAC1423b cells were also incubated with I ( $2 \mu\text{g/ml}$ ) + CR<sub>1</sub> ( $100 \text{ ng/ml}$ ) or with I + human erythrocytes ( $2.5 \times 10^6$  cells/ml) for 60 min at  $30^\circ\text{C}$ . The cells were centrifuged and the supernatants were analyzed by SDS-PAGE under nonreducing conditions and radioautography. The positions of rabbit IgG and its fragments and of BSA in the stained gel are indicated. It is apparent that the main fragment released by CR<sub>1</sub> + I or human erythrocytes + I, has a molecular weight of  $\sim 135,000$ . Moreover, its structure differs from that of the fragment resulting from the activity of plasmin on EAC1423bi.

of the  $\alpha'$  chain were generated. When the radioautographs were overexposed, faint 39-K and 31-K bands were observed in the tracks containing the product of the reaction between CR<sub>1</sub> + I and C3b. The 39-K radiolabeled peptide and the 39-K silver stained band found upon SDS-PAGE of C3c may be identical. This peptide may be poorly labeled when C3 rather than C3b is radioiodinated (33).

*Ability of CR<sub>1</sub> + I to Cleave C3 Bound to Soluble Ag:Ab:C.* Soluble BSA-anti-BSA-C complexes bearing radiolabeled C3 fragments were purified as described in Materials and Methods. About 80% of the C3 associated with these complexes bound to the surface membrane of human erythrocytes but not to sheep erythrocytes. The binding was CR<sub>1</sub> mediated because treatment of the human erythrocytes with 50 ng/ml of pooled monoclonal anti-CR<sub>1</sub> antibody before addition of Ag:Ab:C completely abolished binding, and treatment with  $4 \mu\text{g/ml}$  anti-CR<sub>1</sub> after binding released  $>50\%$  of Ag:Ab:C.

The following experiment was performed to determine whether isolated CR<sub>1</sub> could serve the function of intact erythrocytes (7) in the I-associated cleavage of the C3 bound to the Ag:Ab:C and to compare its effect to that of H. Aliquots of  $^{125}\text{I}$ -C3-labeled Ag:Ab:C were incubated at  $37^\circ\text{C}$  for 30 min with DOVB, I, H, CR<sub>1</sub>, H + I, CR<sub>1</sub> + I, or human erythrocytes + I. Samples of each reaction mixture were then subjected to SDS-PAGE under reducing conditions, and the radioautograph patterns were compared (Fig. 7).



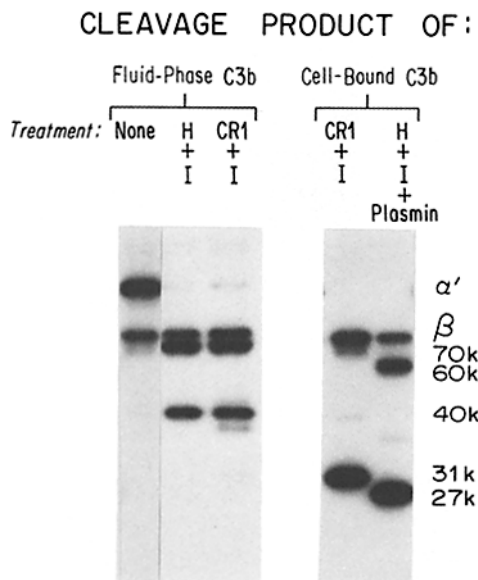


FIG. 6. SDS-PAGE analysis under reducing conditions of cleavage products released from cell-bound C3b\*. The supernatants, obtained as described in the legend of Fig. 4, are analyzed here under reducing conditions. In addition, for comparison, we analyzed the cleavage products of fluid phase C3b by I + H and I + CR<sub>1</sub>. For this purpose, radiolabeled C3b (0.25 μg/ml) was incubated for 3 h at 37°C with I (2 μg/ml) + CR<sub>1</sub> (200 ng/ml) or with I + H (20 μg/ml). The results show that the fragment released from EAC1423b by CR<sub>1</sub> + I contains mainly radiolabeled β chain and a 31-K peptide. The structure of the fragment released by plasmin is quite different, and contains some β chain, a 60-K, and a 27-K peptide. The intensity of the β chain band is reduced, suggesting that it was broken down by the activity of plasmin. The treatment of fluid-phase C3b resulted in the cleavage of the α' chain into 70-K and 40-K polypeptides. 39-K and 31-K polypeptides could be seen in the radioautographs of the tracks containing the reaction products of CR<sub>1</sub> + I with fluid-phase C3b when the radioautograph was overexposed (data not shown).

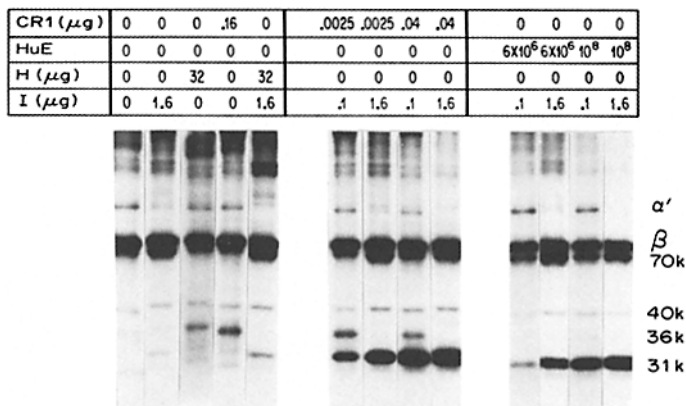


FIG. 7. SDS-PAGE analysis of cleavage products of C3 after treatment of soluble immune complexes with H, I, CR<sub>1</sub>, and human erythrocytes. <sup>125</sup>I-C3-labeled Ag:Ab:C were incubated at 37°C for 30 min with DOVB, I alone (1.6 μg), H alone (32 μg), or CR<sub>1</sub> alone (0.16 μg) or I (1.6 μg) + H (32 μg) or CR<sub>1</sub> (0.0025 μg or 0.04 μg) + I (0.1 μg or 1.6 μg) or HuE (6 × 10<sup>6</sup> or 10<sup>8</sup>) + I (0.1 μg or 1.6 μg) in a total volume of 150 μl. Samples of each reaction mixture were assessed by SDS-PAGE 5–15% gradient gels under reducing conditions, followed by radioautography.

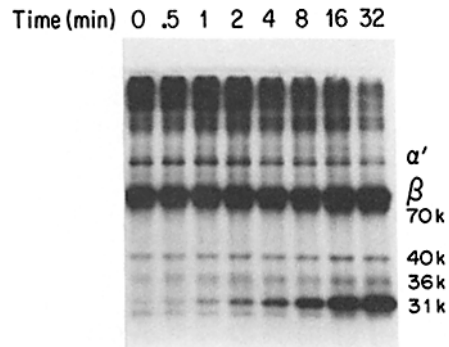


FIG. 8. Time-course of cleavage by CR<sub>1</sub> + I of C3 associated with soluble immune complexes. Aliquots of <sup>125</sup>I-C3-labeled Ag:Ab:C were added to equal volumes of a solution of I (2 μg/ml) + CR<sub>1</sub> (50 ng/ml) and incubated at 37°C. Reactions were stopped after various times by addition of K76COOH (4 mM) and transfer to ice. Samples were subjected to SDS-PAGE 5–15% gradient gels under reducing conditions, followed by radioautography.

The major radioactive C3 fragments associated with the starting Ag:Ab:C (first track on left) presumably represent intact β chains, and, in addition, on top of the gel, α' chain-derived peptides bound tightly (covalently or through hydrophobic bonds) to components of the Ag:Ab:C (24, 34–36). In addition, C3 fragments of 105-K (α'), 70-K, and 40-K mol wt and small amounts of material of lower mol wt were detected.<sup>2</sup> After incubation with H or CR<sub>1</sub>, little change was detected in the structure of C3 associated with IC, except that small amounts of 36-K peptides were formed. Incubation with I or H + I led to the appearance of a light 31-K band and a change in the pattern of the high mol wt bands.

More conspicuous modifications occurred when the complexes were treated with CR<sub>1</sub> + I: the intensity of the high mol wt C3 bands diminished, and simultaneously a 31-K band appeared. The intensity of the 31-K band was dependent on the dose of CR<sub>1</sub> and I. When the concentration of I was decreased, a 36-K band was also observed in the radioautographs, suggesting that it may be an intermediate product. The degradation of C3 was even more pronounced after incubation with human erythrocytes + I. At the highest concentration of erythrocytes + I, little radioactivity remained at the top of the gel, and the band at 31-K was most prominent (track on the right side of Fig. 7). The radioactivity remaining on top of the gel could represent C3d\* fragments of the α' chain covalently associated with constituents of the Ag:Ab:C.

Next, we studied the time-course of appearance of the 31-K peptide. Separate aliquots of <sup>125</sup>I-C3-labeled Ag:Ab:C were added to equal volumes of a solution of I (2 μg/ml) + CR<sub>1</sub> (200 ng/ml), the mixtures placed at 37°C, and reactions stopped after various times (from 0.5 to 30 min) by addition of K76COOH (final concentration, 4 mM) and transfer to ice. Samples of each reaction mixture were subjected to SDS-PAGE, and radioautograph patterns were analyzed.

As seen in Fig. 8, within 0.5 min of incubation, some 31-K peptide was generated. Its intensity as well as that of other peptides (70-K, 40-K, and 36-K) increased progressively during the incubation. At the same time, there was a decrease in intensity of the high mol wt bands.

<sup>2</sup> The degradation of C3b\* observed in Ag:Ab:C is I mediated. The Ag:Ab:C prepared with C3-reconstituted, I-deficient serum (kindly provided by Dr. C. Alper, Harvard University, Cambridge, MA) as a source of complement did not contain the 70-K and 40-K or lower mol wt C3 peptides.

In short, these experiments confirm previous observations (7, 8) that human erythrocytes and I can cleave C3\* associated with Ag:Ab:C beyond the iC3b\* stage and show that the cleavage is CR<sub>1</sub> dependent. Also, from the comparison in Fig. 7 of the effects of H (32 μg) + I (1.6 μg) with CR<sub>1</sub> (0.0025 μg) + I, (0.1 μg) we conclude that, on a molar basis, CR<sub>1</sub> is at least  $2 \times 10^4$  times more efficient than H in the degradation of C3 peptides bound to the Ag:Ab:C. As in the case of iC3b\* associated with cell membranes, a 31-K peptide is generated during this interaction. The precise pathway of degradation of the α' chain of iC3b\* is not evident from these experiments. Although the results indicate that the 31-K band is an end product of the reaction and that the 36-K peptide could represent an intermediate, this interpretation requires further investigation.

### Discussion

The mechanisms of C3b inactivation have been studied extensively. Both fluid-phase C3b and C3b\* can be cleaved by I. The former reaction requires H, and the latter can be enhanced by H (9, 10). In this process, the α' chain is cleaved into major fragments of apparent 70 K and 40 K mol wt. Both fluid-phase iC3b and iC3b\* can be further cleaved in vitro by plasmin, elastase, or trypsin, but the physiological relevance of the breakdown products has remained conjectural (32, 37, 38). It is also known that further degradation of iC3b\* occurs slowly in serum. Fearon (11) has shown that CR<sub>1</sub> can replace H in the cleavage of fluid-phase C3b to C3bi. Recently, some of us have shown (7) that release of C3c from soluble IC is mediated by I and dependent on binding to human erythrocytes. Moreover, in serum this reaction occurs independent of H. Medicus and Arnaout (39) have also reported that breakdown of iC3b\* can be I-mediated in a reaction that was greatly enhanced by human erythrocytes.

Here we show directly that the human erythrocyte factor mediating this effect is CR<sub>1</sub>. In the presence of I + purified CR<sub>1</sub> or I + human erythrocytes, C3b\* associated with EAC1423b was cleaved into C3c and C3d\*. Generation of C3c was inhibited in the presence of low concentrations of monoclonal antibodies to CR<sub>1</sub>. The C3c fragments released from EAC1423b and from Ag:Ab:C (7) have an ~135-K mol wt under nonreducing conditions. Upon reduction, major bands of 75 K (most likely β chain) and 31K (pI 8.6) were detected by radioautography.

One possible interpretation is that C3c consists of β chains, one 31-K α'-derived labeled peptide, and a second weakly radiolabeled peptide. The findings that two-dimensional electrophoresis did not resolve the 31-K band and that two other bands at 39 K and 42 K not apparent in the radiographs were revealed in the slab gels by silver staining support this possibility. However, further studies are necessary to determine the precise structure of C3c, the number of cleavages involved in its production, and whether small mol wt degradation products of C3, some with biological activity (C3e, C3f, ref. 40-42), are generated during the fragmentation of iC3b\* by CR<sub>1</sub> + I.

The studies with soluble Ag:Ab:C (Figs. 7, 8) suggest that iC3b\* degradation is complex and that it may involve more than two steps with the formation of intermediates. Bands with the mobility of degradation products of fluid-phase C3b; that is, 70 K and 40 K, were found in variable amounts in different studies with soluble IC. These fragments could arise from noncovalent C3b\*. Indeed, we found

striking differences in the susceptibility to proteolysis by CR<sub>1</sub> + I of fluid-phase C3b and C3b\*, in that C3c was effectively released only from the latter. For example, when 0.25 μg/ml of fluid-phase C3b were incubated for 3 h at 37°C with 200 ng/ml of CR<sub>1</sub> and 2 μg/ml of I, the end product was mostly iC3b; only small amounts of the 39 K and 31 K fragments were formed (Fig. 6). This is to be contrasted with the treatment of 0.13 μg/ml of C3b\* associated with cell membranes, in which case most or all of it was cleaved beyond iC3b\*, and C3c released by 100 ng/ml of CR<sub>1</sub> + 2 μg/ml of I. The reasons for this difference are not clear but may relate to the nature of the bonds between C3b\* and the IC, the clustering of C3b during the binding process (perhaps allowing the formation of multivalent bonds with CR<sub>1</sub>), or the modulating activity of neighboring molecules from the IC.

Although some breakdown products can be released from C3b\* by I + H, and their structure is similar to those generated by I + CR<sub>1</sub>, CR<sub>1</sub> is between 10<sup>4</sup> and 10<sup>5</sup> times more efficient than H in promoting fragmentation of C3b\*. Considering that the concentration of H in blood is only about 125 times greater than CR<sub>1</sub> (250 μg/ml vs. 2 μg/ml), the physiological breakdown of C3b\* most likely takes place in solid phase; that is, on the surface of cells bearing CR<sub>1</sub>. This is supported by observations *in vitro* and *in vivo*. When soluble IC are added to human blood *in vitro*, they bind to various blood elements in proportion to their relative content of CR<sub>1</sub> and thus associate predominantly with CR<sub>1</sub> of erythrocytes (5). Incubation with serum factors (I + H) does not eliminate this interaction (8). However, exposure to I of IC bound to human erythrocytes causes C3b breakdown and IC release. Similar processing of IC in other species may occur on other cells because a large proportion of Ag:Ab:C injected into mice fix complement *in vivo* and become rapidly associated with platelets that, in this species, bear the majority of C3b receptors (43).

Of interest was the finding that CR<sub>1</sub> promotes the cleavage of iC3b\*. About 75% of the C3 label was released as C3c fragments when EAC1423bi (prepared by treating EAC1423b with H + I) was further incubated with CR<sub>1</sub> + I. The same amount of C3c was released when EAC1423bi was first treated with CR<sub>1</sub>, washed, and then incubated with I. The observations that EAC1423bi had no measurable hemolytic activity and that almost no intact α' chains were detected by SDS-PAGE on the stroma of EAC1423bi argue that the substrate of the CR<sub>1</sub> activity was not residual uncleaved C3b\* remaining on the cells. The simplest interpretation of these experiments is that CR<sub>1</sub> can bind iC3b\* and that the reaction between C3b\*, CR<sub>1</sub>, and I

proceeds in at least two steps: C3b\*  $\xrightarrow{\text{CR}_1 + \text{I}}$  iC3b\*  $\xrightarrow{\text{CR}_1 + \text{I}}$  C3c + C3d\*. A likely implication, therefore, is that CR<sub>1</sub> also recognizes particles bearing iC3b\*, a property that had been previously attributed exclusively to another membrane receptor, termed CR<sub>3</sub>, present on phagocytes as well as other cells (44). Whether CR<sub>3</sub> recognizes other C3 degradation products (42, 45) requires further investigation.

Finally, the present results support the idea that CR<sub>1</sub> is a membrane-associated regulatory protein (11, 31) whose function is to process IC *in vivo* (5-8) and are probably of relevance to the understanding of the pathology of diseases associated with circulating IC. For example, patients with certain connective tissue diseases have a deficiency in CR<sub>1</sub> on erythrocytes (12, 46) and kidney glomeruli (47, 48). Whether this represents a primary abnormality or a secondary manifestation of the disease

process, such a deficiency could lead to an impairment of inactivation of C3b\* and contribute to perpetuation of inflammation.

### Summary

The main finding of this paper is that CR<sub>1</sub>, the membrane receptor for C3b and C4b, together with C3b/C4b-inactivator (I), degrades C3b bound to immune complexes (C3b\*). Two fragments are generated: C3c, which is released from the immune complexes, and C3d\*. The C3c fragment released from the cell intermediate EAC1423b prepared with <sup>125</sup>I-C3 was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and radioautography. It has a 135,000 mol wt and contains disulfide bonded labeled polypeptide chains of 75,000 and 31,000 mol wt, which presumably represent the β and a fragment of the α'-chain of C3b\*. Silver staining of the SDS-PAGE gels revealed other C3-derived bands with 39–42,000 mol wt. Human erythrocytes + I also cleave C3b\* into C3c and C3d\*. The activity of the erythrocytes is CR<sub>1</sub> mediated because it can be totally inhibited by monoclonal antibodies to CR<sub>1</sub>.

In contrast with these results, I together with the serum protein β1H (H) transform EAC1423b into hemolytically inactive EAC1423bi and cleave the α' chain of C3b\* into fragments of 70,000 and 40,000 mol wt. Small amounts of C3c are also released at relatively high concentrations of H. On a molar basis, the efficiency of CR<sub>1</sub> in the generation of C3c and C3d is 10<sup>4</sup>–10<sup>5</sup> greater than H.

An additional observation was that C3c could be released by treating EAC1423bi with CR<sub>1</sub> + I and that this reaction was also inhibited by monoclonal antibodies to CR<sub>1</sub>. Therefore, it is likely that CR<sub>1</sub> has binding affinity for iC3b and that the degradation of C3b\* proceeds as follows: C3b\*  $\xrightarrow{I + CR_1}$  iC3b  $\xrightarrow{I + CR_1}$  C3c + C3d\*. Taken together, our findings argue that the processing of C3b\* in vivo occurs in solid phase, that is, on the surface of cells bearing CR<sub>1</sub>.

*Note added in proof:* During the preparation of this manuscript, Medicus et al. found that polyclonal anti-CR<sub>1</sub> antibody also blocks enhanced breakdown of iC3b\* by human erythrocytes in the presence of I in their system (R. Medicus, personal communication).

The authors thank Mr. Gregory Prince, Ms. Debbie Froh, and Ms. Tina Lam for their assistance, Mr. Richard Melton for technical help, Dr. Ferenc Kezdi for discussion, and Ms. Betty Walker and Me. Joanne Joseph for manuscript preparation.

*Received for publication 28 June 1982.*

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