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TRYPSIN-ACTIVATED COMPLEX OF HUMAN FACTOR B WITH COBRA VENOM FACTOR (CVF), CLEAVING C3 AND C5 AND GENERATING A LYTIC FACTOR FOR UNSENSITIZED GUINEA PIG ERYTHROCYTES. I. GENERATION OF THE ACTIVATED COMPLEX

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SUMMARY A complex formed between cobra venom factor (CVF) and isolated human factor B (B) was found to be converted by trypsin to a stable enzyme, CVF-$\overline{B}$ which cleaved the third component (C3) and the fifth component (C5) of human complement. The formation of CVF-$\overline{B}$ by trypsin required divalent cations, whereas the formation of the lytic factor from human serum occured even in the presence of EDTA. CVF-$\overline{B}$ purified by gel filtration could initiate the hemolysis of unsensitized guinea pig erythrocytes when incubated with human complement components C5 to C9 in 0.01 M EDTA buffer. C3 was not required for the lysis of guinea pig erythrocytes initiated by CVF-$\overline{B}$ because the β1C precipitation line formed between human serum and anti-β1C antibody did not inhibit the hemolysis by CVF-$\overline{B}$ in agarose gel. Treatment of β1C and β1F globulins in whole human serum with CVF-$\overline{B}$ in the presence of 0.01 M EDTA converted them to components with higher mobilities on immunoelectrophoresis.

INTRODUCTION

Recently, the association of the cobra venom factor with an alternate pathway of complement activation has been described (Götze et al., 1971, Hunsicker et al., 1973). In this pathway the cobra venom factor (CVF) combines with a heat labile serum factor B (B) and the resulting complex CVF-B is converted to stable $\overline{B}$ by the enzymatic action of activated factor D. $\overline{B}$ can initiate the activation of C3, like C3-convertase $C\overline{42}$, and consequently leads to full activation of C5 to C9. From studies on the mechanism of activation of C3 to C9 by $\overline{B}$, the direct effect of $\overline{B}$ on C5 has been postulated by Müller-Eberhard (1974).

In studies on the antigenic conversions of B by trypsin (Miyama et al., 1975), we found that

hemolytically active conversion of purified B to $\bar{B}$ could be catalyzed by trypsin only when a complex between CVF and B had been formed. The ability of trypsin-activated complex (CVF-$\bar{B}$) to generate a lytic factor from whole human serum could easily be detected by measuring lysis of unsensitized guinea pig erythrocytes in the presence of EDTA.

This report presents evidence that trypsin-activated CVF-B complex (CVF-$\bar{B}$) can activate C5 without the incorporation of C3 into the lytic system, thereby generating a potent lytic factor, which is probably a complex containing C5 to C9.

MATERIALS AND METHODS

1. *Buffers*

Mg^{++} buffer was isotonic veronal-NaCl (pH 7.4, ionic strength 0.147) containing 0.1% gelatin and 1.0 mM Mg^{++}. EDTA buffer was prepared by mixing isotonic veronal-NaCl buffer with 0.1 M ethylenediaminetetraacetic acid (EDTA) solution (pH 7.4).

2. *Erythrocytes*

Guinea pig erythrocytes were separated from fresh blood obtained by cardiac puncture and stored at 4 C in the presence of 0.02 M EDTA. Cells which had been stored for more than a week were not used for the hemolytic reaction, because non-specific hemolysis increased on cell aging. For preparation of the standardized cell suspension, 0.5 ml of cell suspension were lyzed with 7.0 ml of distilled water, and the optical density of the clear supernatant was measured in a cuvette of 1 cm light path at 541 nm. An optical density of 0.775 corresponded to approximately 4×10^8 cells per ml of cell suspension in terms of hemoglobin concentration.

3. *Complement sera and components of human complement*

Fresh human serum was separated from blood obtained by venous puncture after clotting at room temperature for 60 min. It was ultracentrifuged at 30,000 rpm for 60 min to remove lipid and stored at −70 C. Human C-EDTA was prepared by mixing 9 parts of fresh serum with 1 part of 0.1 M EDTA solution (pH 7.4) and it was stored at −70 C. Unless otherwise mentioned, the C-EDTA used for the hemolytic reaction was diluted 1:4 with 0.01 M EDTA buffer. Human serum deficient in B activity (RB) was prepared by heating fresh serum at 50 C for 30 min. Hydrazine-treated serum was obtained by treating 9 parts of fresh serum with 1 part of 0.3 M hydrazine at 37 C for 30 min and then dialyzing it against 0.01 M EDTA buffer. The components of human complement, C3, C5, C6, C7, C8 and C9 were purchased from Cordis Laboratories, Miami, Fla. Reconstituted components were used at higher concentrations than those recommended by the manufacturer for lysis of sheep intermediate cells. For example, the reagent used for C5 assay was composed of a mixture of 0.5 ml of undiluted C6 and C7, 0.1 ml of undiluted C8 and C9, and 1.2 ml of 0.02 M EDTA buffer.

4. *Human factor B (B), glycine-rich γ-glycoprotein (GGG) and glycine-rich α-glycoprotein (GAG)*

Human B and GGG were isolated by the methods of Boenisch et al. (1970a, 1970b). Purified B was proved to be a single protein by polyacrylamide gel electrophoresis and immunoelectrophoresis at a concentration of approximately 30 mg per ml. Human GAG was purified by the method of Miyama et al. (1975).

5. *Cobra venom factor (CVF)*

CVF was isolated from 900 mg of lyophilized venom of the cobra Naja Naja (Ross Allen Reptile Institute, Silver Springs, Fla.) by the method described by Ballow et al. (1969). CVF activity was assayed during the purification procedure by measurement of lysis of guinea pig erythrocytes on microtitration plates. Mixtures of one drop each of serially diluted fraction, of guinea pig serum (1:10) and of guinea pig erythrocytes (1×10^8 cells/ml) were incubated in Mg^{++} buffer at 37 C for 30 min. Fractions which caused complete lysis in the third row of wells were collected. Analytical polyacrylamide gel electrophoresis showed that the purified CVF contained a faster moving contaminant protein. CVF units were determined by the method of Cochrane et al. (1970).

6. *Antisera*

Anti-B and anti-GGG antisera were obtained from rabbits immunized with four injections of purified B and GGG, respectively. To prepare these, 2 mg

of antigen in complete Freund's adjuvant were injected (in a total volume of 2 ml) into 2 footpads. Seven days later each animal was immunized with 1 mg of antigen in complete Freund's adjuvant into another footpad. Twenty days later each animal received 0.5 mg of antigen in complete Freund's adjuvant by subcutaneous injection. The subcutaneous injection was repeated one week later and rabbits were bled 10 to 15 days after this last injection. The anti-GGG antiserum contained contaminant anti-IgG antibody, so it was absorbed with solidified human IgG prepared by the method of Avrameas et al. (1969). Anti-GAG antiserum was obtained by the method of Miyama et al. (1975). These antisera were stored at −20 C without preservatives or prior heating. Anti-β1C/β1A globulin, anti-human C5 and anti-human C9 antisera were purchased from Boehringwerke A. G., Marburg-Lahn, Germany.

7. *Chemicals*

The following chemicals were used: trypsin (Mochida Pharmaceutical Co., Tokyo); soybean trypsin inhibitor (Sigma Chemical Co.); hydrazinium-hydroxide (Merk, Darmstadt, Germany); glutaraldehyde and sodium laurylsulfate (SDS) (Nakarai Chemicals Ltd., Kyoto).

8. *Electrophoretic procedures and Ouchterlony's gel precipitation*

Immunoelectrophoresis was performed in 1.2% agarose containing veronal buffer, pH 8.6, of 0.05 ionic strength and 0.002 M EDTA. Analytical and SDS polyacrylamide gel electrophoresis were performed by the methods of Davis (1964) and Weber et al. (1969). Immunofixation of B in polyacrylamide gel after electrophoresis was performed by immersing the gel in 10% anti-B solution in 0.01 M EDTA buffer at room temperature for 24 hr, washing it with 0.15 M NaCl for 48 hr, and staining it with 0.05% Coomassie blue. Ouchterlony's double diffusion was performed in 1.2% agarose containing 0.01 M EDTA.

9. *Gel filtration*

Preparative Sephadex G-200 gel filtration was performed in a 2×60 cm column at a rate of 8 to 10 ml/hr with either Mg^{++} buffer or 0.01 M EDTA buffer. Both the void volume (60±2 ml) and fraction volume of effluents (4.2 ml) were kept constant through the experiments.

RESULTS

1. *Formation of CVF-$\bar{B}$ on interaction between B, CVF and trypsin*

A mixture of 0.5 ml of B (2 mg/ml) and 0.5 ml of trypsin (100 μg/ml) in Mg^{++} buffer was incubated at 37 C. At intervals, 20 μliters samples were transfered to tubes containing 20 μliters of trypsin inhibitor (300 μg/ml). All samples were analyzed by immunoelectrophoresis against anti-B. As shown in Fig. 1, two kinds of products of B appeared within 2 min and the conversion was completed 10 min after the addition of trypsin. These patterns of conversion markedly resembled the conversion of components in serum treated with zymosan. However, immunoelectrophoretic analysis showed that trypsin-digested B could not split C3 when tested by measuring conversion of β1C to β1A in fresh human serum, unless it had combined with CVF before addition of trypsin. Therefore, trypsin

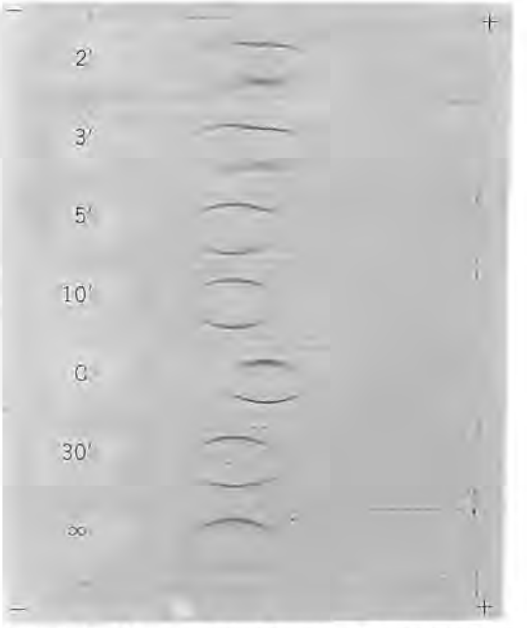


FIGURE 1. *Immunoelectrophoresis of trypsin-treated B. B treated with trypsin for 2 min to 30 min was analyzed against anti-B added to all troughs. Controls without trypsin (C) and without trypsin inhibitor (∞) were also tested.*

TABLE 1. *Formation of CVF-$\bar{B}$ from mixtures of CVF, B and trypsin*

Reactions in Mg^{++} buffer		Reactions in EDTA buffer	
1st stage	2nd stage	3rd stage	Degree of lysis[b]
B+trypsin[a]	Mg^{++} buffer	Trypsin inhibitor	0
B+trypsin	CVF	,,	0
B+CVF	Mg^{++} buffer	,,	0
B+CVF	Trypsin	,,	1.0
B+CVF	Trypsin inhibitor	Trypsin	0
CVF+trypsin	B	Trypsin inhibitor	0

[a] 20 μliters of each reactant were mixed at each stage.
[b] 0.1 ml each of guinea pig erythrocytes and human C-EDTA were added after the 3rd reaction stage.

TABLE 2. *Activation of the CVF-B complex by various concentrations of trypsin*

	Trypsin (μg/ml)	Trypsin inhibitor (μg/ml)	Degree of lysis[c]
CVF-B in Mg^{++} buffer	400	1,200	0.946
,,	200	600	0.960
,,	100	300	0.960
,,	50	150	0.950
,,	25	75	0.930
,,	12.5	37.5	0.910
,,	400	1,200	0[a]
CVF-B in EDTA buffer	400	1,200	0[b]

[a] The order of additions of trypsin and trypsin inbibitor was reversed.
[b] The CVF-B complex was treated with trypsin in 0.01 M EDTA buffer.
[c] The degree of lysis was determined after the reaction mixture was incubated with 0.1 ml each of unsensitized guinea pig erythrocytes and of C-EDTA at 37 C for 60 min.

converted purified B to inactive fragments, which resembled $\bar{B}$ electrophoretically but were not identical with it. However, when CVF was added to the system, trypsin converted B to active $\bar{B}$.

Twenty μliters each of B (2 mg/ml), CVF (200 units/ml) and trypsin (100 μg/ml) were mixed in two stages in various orders, as indicated in Table 1. The mixtures were incubated at 37 C for 10 min and then 20 μliters of trypsin inhibitor (300 μg/ml) and 0.12 ml of 0.02 M EDTA buffer were added. Finally 0.1 ml of human C-EDTA and 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml) were added and after incubation at 37 C for 60 min, 3 ml of cold 0.01 M EDTA buffer were added. The mixtures were centrifuged and the optical densities of supernatants were measured at 413 nm. Lysis of guinea pig erythrocytes could be seen only in the tube in which B and CVF had been mixed in the first stage and then subjected to trypsin digestion.

Cooper demonstrated that the conversion of B to $\bar{B}$ by $\bar{D}$ required the presence of Mg^{++} (1973). Similarly, as shown in Table 2, the conversion of preformed CVF-B complex to activated CVF-B complex (CVF-$\bar{B}$) by trypsin did not occur when EDTA was present in the first and second stages. The formation of a lytic factor from human serum by CVF-$\bar{B}$, on

the other hand, could proceed in the absence of divalent cations, as revealed in the last reaction step where EDTA was present. Thus, the mechanism of B activation by trypsin was quite similar to that of $\bar{B}$ formation initiated by factor D.

To see how much trypsin was required, a mixture of 0.2 ml of B (4 mg/ml) and 0.2 ml of CVF (200 units/ml) was incubated in Mg^{++} buffer at 37 C for 15 min. Then 20 μliters volumes of the resulting CVF-B complex were treated with 20 μliters of trypsin at concentrations of 12.5 μg/ml to 400 μg/ml at 37 C for 3 min. After incubation, 20 μliters of solution containing trypsin inhibitor in 3-fold weight excess over the amount of trypsin were added to each tube. In the control, the order of additions of trypsin and trypsin inhibitor was reversed. Then the total volume of each mixture was adjusted to 0.2 ml with 0.02 M EDTA buffer, and 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml) and 0.1 ml of human C-EDTA were added to all tubes and the mixtures were incubated at 37 C for 60 min. As shown in Table 2, lysis of unsensitized guinea pig erythrocytes was complete with all concentrations of trypsin employed.

2. *Kinetic analysis of the formation of CVF-$\bar{B}$ by trypsin*

A volume of 0.2 ml of CVF-B complex, prepared by mixing equal volumes of B (4 mg/ml) and CVF (200 units/ml), was treated with 0.2 ml of trypsin (12.5 μg/ml) at 37 C. At the times indicated in Fig. 2, 20 μliters of samples were mixed with 20 μliters of trypsin inhibitor (37.5 μg/ml). A portion of each mixture was removed for immunoelectrophoretic analysis, and then 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml), 0.1 ml of C-EDTA and 0.16 ml of 0.02 M EDTA buffer were added to the remaining reaction mixture. The results showed that CVF-$\bar{B}$ was formed rapidly and then gradually decayed (Fig. 2). On immunoelectrophoretic analysis a precipitation line was observed only in the position of the original B against anti-B antiserum during the generation of CVF-$\bar{B}$, though a faint line of a slow moving component (GGG) was sometimes observed.

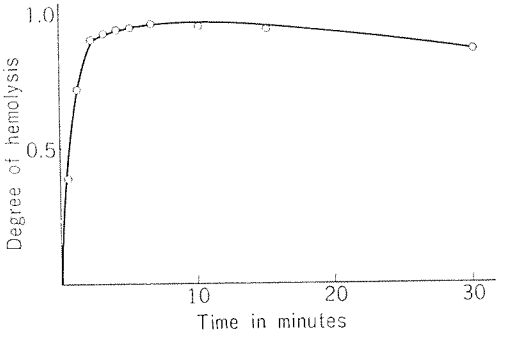


FIGURE 2. *Kinetics of CVF-$\bar{B}$ formation from the CVF-B complex by trypsin. For experimental conditions and procedures, see text.*

3. *Analysis of CVF-$\bar{B}$ formation by gel filtration*

Cooper demonstrated that a complex of equimolar amounts of B and CVF was formed reversibly, and that on analytical ultracentrifugation, the CVF-B complex alone sedimented as a 8–9S peak, whereas after treatment with factor D it sedimented as a 9S peak (1973). The concentration of B was in excess of that of CVF in the experiments described above, so it was expected that the mixture of CVF and B should contain both the CVF-B complex and free B, and that trypsin digests of the mixture should contain not only hemolytically active CVF-$\bar{B}$ but also inactive products of B. To confirm this, the gel filtration patterns of B, CVF, CVF-B complex and CVF-$\bar{B}$ on Sephadex G-200 were compared.

Nine mg of purified B in 2 ml of Mg^{++} buffer were chromatographed on a Sephadex G-200 column (2×60 cm) equilibrated with Mg^{++} buffer. Then single drops of serially diluted fraction were incubated with one drop of diluted CVF (200 units/ml) in Mg^{++} buffer at 37 C for 15 min and treated with one drop of trypsin (12.5 μg/ml) at 37 C for 7 min. Finally, one drop of reagent containing guinea pig erythrocytes (1×10^8 cells/ml), human C-EDTA and trypsin inhibitor (37.5 μg/ml) in 0.02 M EDTA

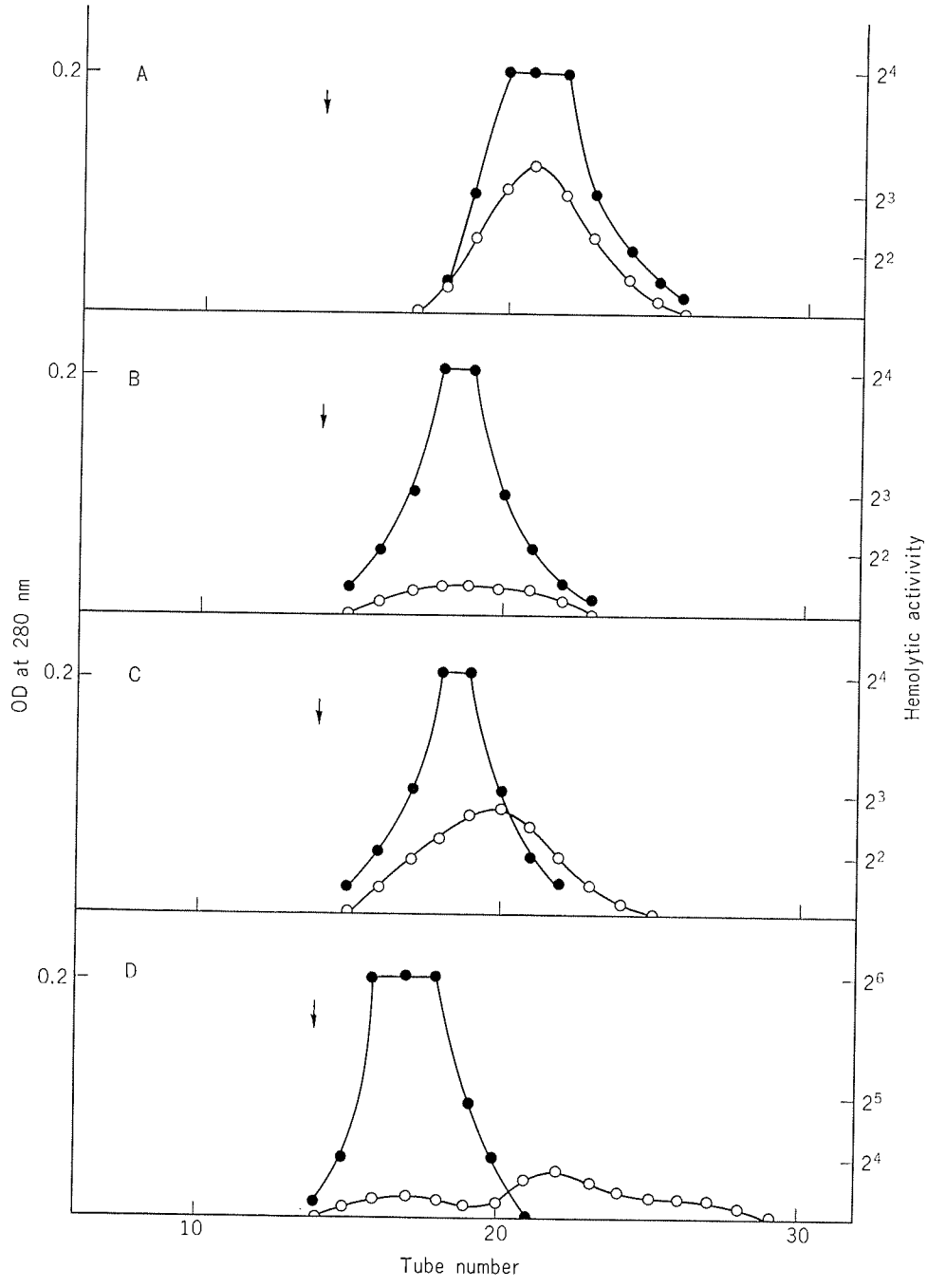


FIGURE 3. *Gel filtration patterns of B, CVF, CVF-B complex and CVF-$\bar{B}$ on a column (2×60 cm) of Sephadex G-200 (A, B, C and D, respectively). The optical density at 280 nm (○) and hemolytic activity (●) were measured. The arrow indicates the void volume of the column.*

buffer was added. As shown in Fig. 3A, the position of the hemolytic activity of B was completely superimposed on that of protein. Next, 1,000 units of CVF in 2.0 ml of Mg^{++} buffer were chromatographed on the same Sephadex column. The biological activity of CVF was measured as described above, except that CVF was replaced by diluted B (100 μg/ml) (Fig. 3B). The hemolytic activity of CVF was also eluted in the same position as protein. These chromatographed samples of B and CVF were concentrated by ultrafiltration using a UP-2 filter (Toyo Roshi Co.) and incubated together in Mg^{++} buffer at 37 C for 15 min to allow formation of the CVF-B complex. The CVF-B complex was subjected to gel filtration on the same column. Assay of the CVF-B complex in each fraction was carried out as B and CVF assays, except that both CVF and B were omitted. The hemolytic activity of the CVF-B complex was found as a shoulder of the protein peak (Fig. 3C). Fractions with hemolytic activity were pooled, concentrated to 2 ml and digested with 60 μliters of trypsin (2 mg/ml) at 37 C for 7 min. Finally, 0.18 ml of trypsin inhibitor (2 mg/ml) and 0.25 ml of 0.1 M EDTA were added. The reaction mixture was subjected to gel filtration on the same Sephadex column equilibrated with 0.01 M EDTA buffer. CVF-$\bar{B}$ activity, detected on a microtitration plate by the reaction of one drop of the fractions with one drop of human C-EDTA and of guinea pig erythrocytes (1×10^8 cells/ml), was found to move faster than either CVF or the CVF-B complex (Fig. 3D). A small peak of protein corresponding to CVF-$\bar{B}$ activity was detected, whereas the major peak of protein was found to contain material without hemolytic activity derived from B by Ouchterlony's gel precipitation against anti-B. In a control experiment the trypsin-trypsin inhibitor complex and free trypsin inhibitor were shown to be eluted later than the major protein peak. The peak fractions of hemolytic activity of B, CVF, CVF-B complex and CVF-$\bar{B}$, respectively, were analyzed by polyacrylamide gel electrophoresis. The gel filtration pattern of the CVF-B complex could not distinguish from that of CVF, but CVF-B seemed to move more slowly than CVF alone on polyacrylamide gel. Figure 4 also shows that the peak fraction of CVF-$\bar{B}$ activity stained as a single band and migrated at the slowest rate. On the other hand, the peak fraction of protein in the CVF-$\bar{B}$ fractionation column separated into 6 bands which were found to be antigenically identical with each other by immunoelectrophoresis and by immunofixation analysis against anti-B.

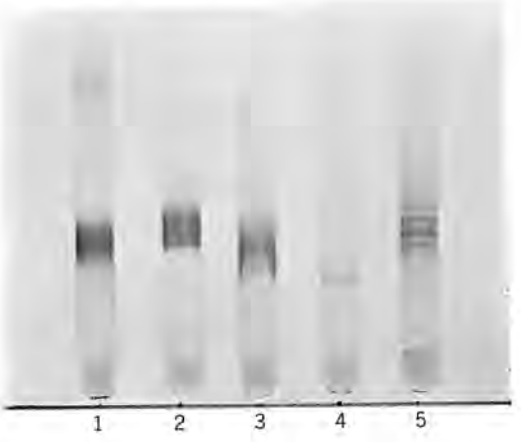


FIGURE 4. *Analytic polyacrylamide gel electrophoresis of B, CVF, CVF-B complex and CVF-$\bar{B}$ separated by gel filtration on Sephadex G-200. Gel 1: CVF (concentrated Fr. #18, in Fig. 3B). Gel 2: B (Fr. #21, in Fig. 3A). Gel 3: CVF-B complex (Fr. #19, in Fig. 3C). Gel 4: CVF-$\bar{B}$ (Fr. #17, in Fig. 3D). Gel 5: inactive products of trypsin-digested CVF-B complex (Fr. #22, in Fig. 3D). Migration was towards the anode (top of the gels). Electrophoresis was performed in 7.7% gel at 3 ma/gel in Tris-glycine buffer, pH 8.6. The gel was stained with 0.05% Coomassie blue.*

These results clearly indicate that pure CVF-$\bar{B}$ is still associated with CVF and that its molecular weight is more than that of the CVF-B complex. Its molecular weight was estimated as approximately 300,000 by Sephadex gel filtration. Its exact molecular weight and the complex nature of its molecule are under investigation.

Neutralization of the hemolytic activity of CVF-$\bar{B}$ by anti-B was tested on a Ouchterlony gel precipitation plate. Anti-B was placed in

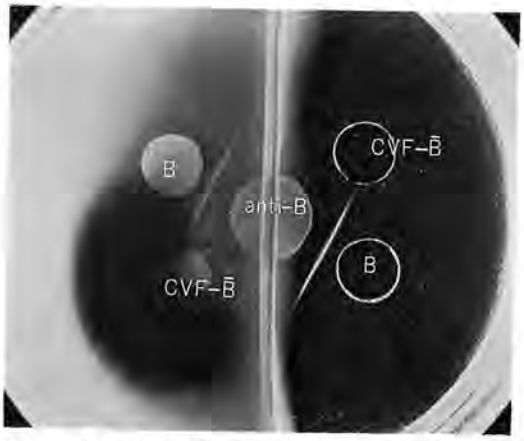


FIGURE 5. *Neutralization of CVF-B̄ activity by anti-B antibody in agarose gel. B (400 μg/ml) and CVF-B̄ (Fr. #17, in Fig. 3D) were placed in symmetric positions in the Ouchterlony's plate. When the precipitation lines became visible, half the gel surface (left side) was covered with 1 ml of 1.0% agarose, containing 5% guinea pig erythrocytes and 0.25 ml C-EDTA (1: 1). Then the plate was incubated at 37 C for 1 hr and at 4 C for 24 hr.*

the center well and B (400 μg/ml) and undiluted CVF-B̄ (Fr. #17, in Fig. 3D) were put in symmetrical positions in the surrounding 4 wells, and the plate was incubated at 0 C for 24 hr. Then half the surface of the plate was covered with 1 ml of 1.0% agarose, containing 5% guinea pig erythrocytes and 0.25 ml of human C-EDTA (1: 1). The plate was incubated at 37 C for 1 hr and then at 4 C for 24 hr. As seen in Fig. 5, the hemolytic zone induced by CVF-B̄ was completely inhibited by anti-B serum.

4. *Titration of CVF-B̄*

A sample of 0.2 ml of CVF-B̄ which had been purified by Sephadex G-200 gel filtration was diluted with 0.01 M EDTA buffer and incubated with 0.1 ml of human C-EDTA and 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml). As shown in Fig. 6, the average number of damaged sites per guinea pig erythrocyte was proportional to the concentration of CVF-B̄ added and 0.2 ml of CVF-B̄ diluted 1: 200 (1 unit) could produce damage of 63% of the

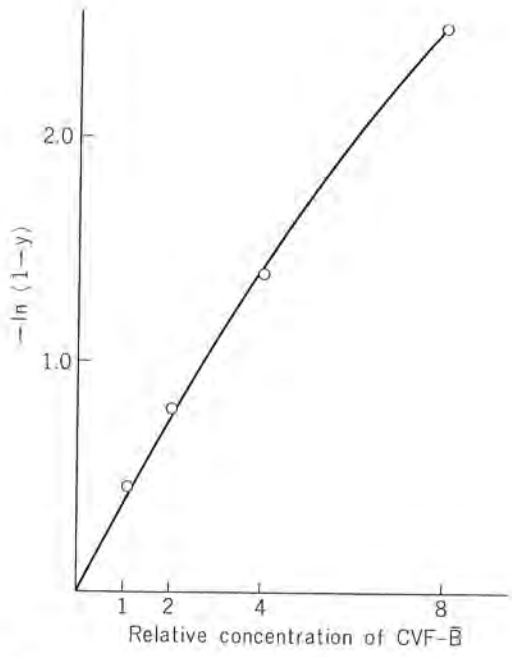


FIGURE 6. *Titration of purified CVF-B̄. -ln (1-y) indicates the average number of damaged sites per guinea pig erythrocyte. For experimental conditions and procedures, see text.*

guinea pig erythrocytes employed. This strongly suggests that formation of a lytic factor by CVF-B̄ is a single hit reaction.

5. *Interaction of CVF-B̄ with late acting components of human complement*

Preliminary experiments showed that hydrazine-treated serum and RB, instead of fresh human serum, were useful for generation of a lytic factor by CVF-B̄. These observations strongly suggested that C3 was not necessary for generation of a lytic factor by CVF-B̄. Thus it was necessary to find out whether C3 was required for generation of a lytic factor by CVF-B̄ in a system containing purified components C3 to C9.

Twenty μliters each of C3, C5, C6, C7, C8 and C9 were mixed and the total volume was made up to 0.2 ml with 0.02 M EDTA buffer. Mixtures deficient in one of the components were also prepared by replacing the component

TABLE 3. *Requirement of late acting components for formation of a lytic factor by CVF-$\bar{B}$*

Components of mixture	Degree of hemolysis
C3, C5, C6, C7, C8, C9[a]	0.254
C5, C6, C7, C8, C9	0.260
C3, C6, C7, C8, C9	0.012
C3, C5, C7, C8, C9	0.063
C3, C5, C6, C8, C9	0.040
C3, C5, C6, C7, C9	0.011
C3, C5, C6, C7, C8	0.027

[a] Volumes of 0.2 ml of mixtures were mixed with 0.1 ml of CVF-$\bar{B}$ and 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml).

by 20 μliters of 0.01 M EDTA buffer. Then 0.2 ml of the mixtures were incubated with 0.1 ml of CVF-$\bar{B}$ (Fr. #17, in Fig. 3D) and 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml) at 37 C for 60 min. Table 3 shows that mixture deficient in C3 and the complete mixture containing C3 to C9 could produce lysis of guinea pig erythrocytes in the presence of CVF-$\bar{B}$, while all the other mixtures were inactive. When the mixture deficient in C5 was supplemented with purified C5, it gained the ability to make a lytic factor. The lysis of guinea pig erythrocytes was directly proportional to the amount of C5 added in the presence of excess C6, C7, C8, C9 and CVF-$\bar{B}$ (Fig. 7). These results demonstrated that C5 reacted with CVF-$\bar{B}$ as an initiating component for generation of a lytic factor.

The direct activation of C5 by CVF-$\bar{B}$ for generation of a lytic factor from whole human serum without the participation of C3 was demonstrated in the following experiments.

The center well of an Ouchterlony's plate was filled with human C-EDTA (1: 1) and the surrounding wells with anti-B, anti-β1C/β1A, anti-C5 and anti-C9, respectively. After incubation at 4 C for 24 hr to allow development of the precipitation lines, the plate was overlayed with 2 ml of 1.0% agarose, containing 5% guinea pig erythrocytes and 0.1 ml of

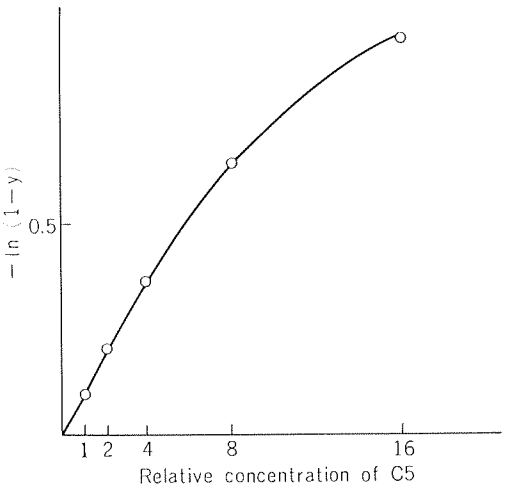
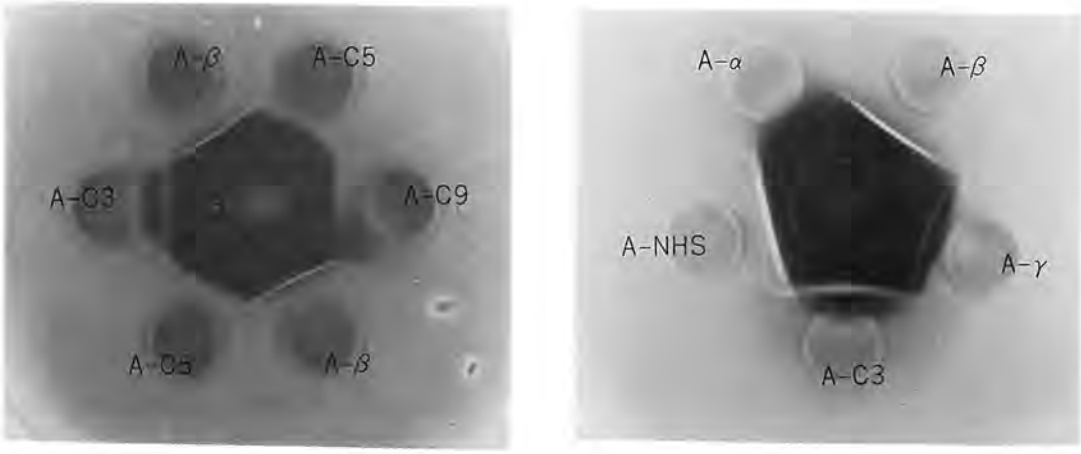


FIGURE 7. *The requirement of C5 for generation of a lytic factor. Volumes of 0.1 ml of serially diluted C5 were mixed with 0.1 ml of mixture containing C6, C7, C8 and C9, 0.1 ml of CVF-$\bar{B}$ (10 units/ml), and 0.1 ml of guinea pig erythrocytes (1×10^8 cells/ml). -ln (1-y) indicates the average number of damaged sites per cell.*

undiluted CVF-$\bar{B}$ (Fr. #17, in Fig. 3D). The plate was incubated at 37 C for 1 hr and then at 4 C for 24 hr. The hemolytic zone produced in the gel was bounded by the precipitation line developed against anti-C5 and anti-C9, whereas the β1C precipitation line did not inhibit enlargement of the hemolytic zone (Fig. 8). When the Ouchterlony's plate was overlayed with guinea pig erythrocytes only, no hemolysis was observed. This indicated that the hemolysis produced in the agarose gel was dependent on added CVF-$\bar{B}$ but not some effect of agarose.

On the other hand, when 0.9 ml of human C-EDTA (1: 1) was treated with 0.1 ml of undiluted CVF-$\bar{B}$ (Fr. #17, in Fig. 3D) at 37 C for 60 min and analyzed by immunoelectrophoresis against anti-β1C/β1A and anti-C5, both β1C and β1F globulins were shown to be converted to fast-moving components (Fig. 9).

These observations demonstrated that C3 and C5, independently, could be substrates of CVF-$\bar{B}$ formed by activation of the CVF-B



A B

FIGURE 8. *Detection in gel of late acting components required for generation of a lytic factor by CVF-B̄. For experimental procedures for A, see text. B: instead of anti-C5 and anti-C9, horse anti-human serum (A-NHS), anti-GAG (A-α) and anti-GGG (A-γ) were used. Experimental procedures were as for A. A-β: anti-B. A-C3: anti-human β1C|β1A. A-C5: anti-human C5. A-C9: anti-human C9. Center well: human C-EDTA (1: 1).*

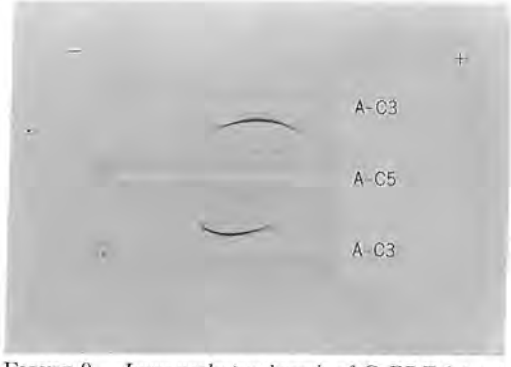


FIGURE 9. *Immunoelectrophoresis of C-EDTA treated with CVF-B̄. Upper well: C-EDTA (1: 1) treated with CVF-B̄. Lower well: C-EDTA alone. Anti-human β1C|β1A (A-C3) was added to the upper and lower troughs, and anti-human C5 (A-C5) was added to the middle trough.*

complex with trypsin, so that activation of C5 by CVF-B̄ does not require the co-operation of C3 or C3b.

DISCUSSION

It has been described (Pickering et al., 1969, Brai et al., 1972) that unsensitized guinea pig erythrocytes are sensitive to a lytic factor generated by an alternate pathway involving CVF. Using unsensitized guinea pig erythrocytes, we demonstrated the formation of CVF-B̄, an enzyme cleaving C3 and C5, in reaction mixture containing CVF, with isolated B and trypsin. On the other hand, although B seemed to be cleaved by trypsin to GGG and GAG in the absence of CVF, this cleavage did not generate any biological activity. Analysis of the reaction sequence indicated that trypsin effectively reacted only with the preformed CVF-B complex in the presence of divalent cations. The CVF-B complex itself did not lyze unsensitized guinea pig erythrocytes when mixed with human C-EDTA. As discussed in the following paper, we tested the capacities of CVF-B complexes prepared from 3 CVF preparations to generate a lytic factor from human C-EDTA. The CVF-B complexes prepared from 2 of 3 CVF preparations had a weak hemolytic activity against unsensitized guinea pig erythrocytes in the presence of C-EDTA only when tested in the nascent state. On treatment with trypsin, however, all these preparations of CVF-B complex were converted to stable, highly active

CVF-$\bar{B}$. These data are in agreement with those of Cooper (1973), who demonstrated the formation of C3 cleaving enzyme from reaction mixture containing B, CVF and factor D.

We previously reported (Miyama et al., 1975) that B in whole human serum was cleaved to hemolytically inactive GGG and GAG during alternate complement activation, and that both anti-B and anti-GGG inhibited the activation of C3 by zymosan but anti-GAG did not. Although no precipitation line was visible between CVF-$\bar{B}$ and anti-B serum (Fig. 5), neutralization of CVF-$\bar{B}$ activity must be caused by the GGG antibody in anti-B serum.

Recently, Brade et al. (1975) demonstrated that factor B combined with zymosan-properdin involving C3b could be activated by trypsin to a stable C3 cleaving enzyme in the presence of Mg^{++}. Unlike the C3 cleaving enzyme generated by the properdin pathway, CVF-$\bar{B}$ did not contain either C3 or C3b in its molecule. When C-EDTA was absorbed with an equivalent amount of anti-β1C/β1A, a lytic factor was also generated. These observations that CVF-B did not require the presence of C3 for generation of a lytic factor from human C-EDTA or isolated human complement components indicated that CVF-$\bar{B}$ could directly enter a pathway of activation of C5 to C9. Incubation of C-EDTA with CVF-$\bar{B}$, however, showed the conversions of β1C and β1F globulins to fast-moving components. Thus it seems likely that C3 and C5 can both act as substrates of CVF-$\bar{B}$.

The formation of a lytic factor from whole human serum by CVF-$\bar{B}$ was a one hit reaction, like activation of C5 ,and it was inhibited by antibodies against C5 and C9. These results suggest that the lytic factor generated by CVF-$\bar{B}$ is a complex molecule containing C5 to C9 (Kolb et al., 1973).

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