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S Ruddy, ... , K F Austen, E J Goetzel

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Research Article

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Chemotactic Activity Derived from Interaction of Factors \bar{D} and B of the Properdin Pathway with Cobra Venom Factor or C3b

SHAUN RUDDY, K. FRANK AUSTEN, and EDWARD J. GOETZL

*From the Departments of Medicine, Harvard Medical School and
Robert B. Brigham Hospital, Boston, Massachusetts 02120*

ABSTRACT Interaction of \bar{D} (the activated form of D) and B, factors of the properdin pathway, with C3b (the major cleavage fragment of C3) generates a convertase, C3 \bar{B} , which cleaves C3 and initiates the terminal complement sequence C5–C9. A functionally analogous more stable C3 convertase, CoVFB, is formed by substituting cobra venom factor (CoVF) for C3b. Mixtures of highly purified CoVF, B, and \bar{D} were chemotactic for human neutrophil polymorphonuclear leukocytes as assessed in Boyden chambers either by microscopic enumeration of migrating cells or by counting of ^{51}Cr -labeled cells. Control mixtures containing CoVF, B, and \bar{D} , reacted in the absence of Mg^{++} , were hemolytically inactive and devoid of chemotactic activity. Over a range of doses, the chemotactic activity of mixtures yielding CoVFB correlated with their hemolytic activity. Pretreatment of neutrophils with mixtures containing CoVFB rendered them unresponsive to subsequent chemotactic stimulation by kallikrein or C5a, indicating cross-deactivation to other chemotactic factors. Similar neutrophil deactivation occurred after exposure to a mixture of C3b, B, and \bar{D} in which C3 \bar{B} was formed; with short incubation times and high cell concentration C3 \bar{B} also exhibited some chemotactic activity. The chemotactic activity of C3 \bar{B} and CoVFB is an example of a biologic function arising from interactions among factors of the properdin pathway per se, as distinguished from the capacity of this pathway to activate C3 and the terminal complement sequence.

Dr. Ruddy is the former recipient of a Research Career Development Award (AM 70233) from the National Institutes of Health; his present address is Department of Medicine, Medical College of Virginia, Richmond, Va. 23298. Dr. Goetzl is an Investigator of the Howard Hughes Medical Institute.

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INTRODUCTION

Activation of the third component of complement (C3)¹ and the terminal sequence of complement components (C5–C9) yields fragments of components (C3a and C5a) and complexes of components (C5 $\bar{67}$) with chemotactic activity for polymorphonuclear leukocytes (1–3). In the classical complement pathway, formation of these factors is initiated by C4 $\bar{2}$, a labile C3 convertase produced by the action of activated first component (C1) on its two natural substrates, the fourth (C4) and second (C2) complement components (4). In the properdin pathway, interaction of C3b, the major cleavage fragment of C3, with factor B and activated factor D (\bar{D}) generates C3 \bar{B} , an enzyme analogous to C4 $\bar{2}$ in its lability and its capacity to cleave C3 with initiation of the terminal complement sequence (5). Substitution of cobra venom factor (CoVF)² for C3b yields CoVFB, a convertase which functions similarly to C3 \bar{B} , but is more stable (6). The present report provides evidence that mixtures of factors B, \bar{D} , and either C3b or CoVF, in which the corresponding en-

¹ The symbols for sheep erythrocyte (E), rabbit antibody (A), and the components of the classical pathway (C1, C4, C2, C3, C5, C6, C7, C8, and C9) are those agreed upon by the WHO Committee on the nomenclature of complement (1968. *Bull. W. H. O.* 39: 935–938). The symbols for the properdin pathway conform to an interim nomenclature agreed upon at the 2nd International Congress of Immunology held in Brighton, England, in July 1974. A bar over a number (e.g., C1) or a letter (e.g., \bar{B}) indicates the activated form of the complement component or properdin factor.

² *Abbreviations used in this paper:* A, rabbit antibody; CoVF, cobra venom factor; CP4 min, counts per 4 min; E, sheep erythrocyte; HBSS, Hanks' balanced salt solution; hpf, high power field; M199, medium 199; VBS, veronal-buffered saline; DGV \bar{B}^{++} , VBS containing 5% dextrose, 0.1% gelatin, 0.00015 M Ca^{++} , and 0.0005 M Mg^{++} .

TABLE I
Chemotactic Activity of Mixtures Containing
CoVF, B, and \bar{D}

Reactants	Chemotactic effect	
	Modified Boyden assay	Radio-chemotaxis
	neutrophils/hpf	net % radioactivity
CoVF, B, \bar{D}	17	6.2
B, \bar{D}	2	-1.7
\bar{D}	1	0.5
B	5	0.5
CoVF	3	-1.5
C5a*	32	8.4
Kallikrein*	24	6.8

* C5a and kallikrein were incubated in DGVB⁺⁺ for 30 min at 37°C, and diluted 1:10 before testing.

zymes C3 \bar{B} and CoVF \bar{B} have formed, activate human polymorphonuclear leukocytes in vitro as assessed by chemotaxis and cross-deactivation to structurally unrelated chemotactic factors.

METHODS

Acrylic radiomigration chambers (Neuro Probe, Inc., Bethesda, Md.) and disposable polystyrene modified Boyden chambers (Adaps, Inc., Dedham, Mass.) were assembled with micropore filters (Millipore Corp., Bedford, Mass.) as previously described (7, 8). Hanks' balanced salt solution (HBSS) and Medium 199 (M199) with or without phenol red (Microbiological Associates, Inc., Bethesda, Md.); ovalbumin five times recrystallized (Miles Laboratories, Inc., Elkhart, Ind.); dextran, Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.); sodium diatrizoate (Hypaque, Winthrop Laboratories, New York); two times recrystallized trypsin previously treated with diphenylcarbamyl chloride (Sigma Chemical Co., St. Louis, Mo.), hexadimethrine bromide (Aldrich Chemical Co., Inc., Milwaukee, Wis.); and ⁵¹Cr-sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.) were obtained as noted.

The low molecular weight anticomplementary CoVF and human factor B were purified as described (9). Factor D was isolated from the acid euglobulin fraction of human serum in the fully activated state (\bar{D}) (10). Human C3 was purified (11) and converted to C3b by exposure of 3.5 mg C3 to 10¹⁰ EAC14⁺⁺ cells at 37°C for 60 min in a volume of 1.3 ml DGVB⁺⁺. The C3b preparation was devoid of hemolytic C3 activity but contained significant chemotactic activity. The chemotactic activity was removed on Sephadex G-200 in veronal-buffered saline, pH 7.5, and appeared in a pool of fractions containing proteins of less than 50,000 mol wt. Fractions containing C3b as assessed by reaction with anti-C3 antibody were pooled and shown to be devoid of chemotactic activity. Functionally pure guinea pig C1 (12), human C4 (13), and oxidized C2 (14) were prepared as described (15).

Veronal-buffered saline (VBS) containing 5% dextrose, 0.1% gelatin, 0.00015 M Ca⁺⁺, and 0.0005 M Mg⁺⁺ (DGVB⁺⁺), and VBS containing 0.04 M EDTA were made

according to published methods (12). DGVB⁺⁺ from which divalent cations had been omitted and which had been made 0.002 M in EDTA by the addition of a stock solution of 0.086 M EDTA was designated DGVB-EDTA. Measurements of C1, C4, C2, and C3 (15) and factor \bar{D} hemolytic activity (5) were performed as described. Guinea pig erythrocytes and human complement were substituted for sheep erythrocytes (E) and guinea pig complement in the CoVF-induced indirect lysis assay (9), resulting in an approximate 40-fold increase in sensitivity. CoVF activity was defined by the inhibition of immune hemolysis (16), and factor B was measured by radial immunodiffusion (17).

Neutrophil migration. Human peripheral leukocytes obtained from normal donors by previously detailed methods (7) were suspended in M199, layered on Ficoll-Hypaque cushions, and centrifuged to separate the mononuclear cells from the neutrophils (18). Purified neutrophil pellets containing 95-98% neutrophils were then washed twice with M199, made 0.4 g per 100 ml in ovalbumin (M199-ovalbumin), and resuspended in the same medium at a concentration of $2.0 \pm 0.4 \times 10^6$ cells per ml (range) for the modified Boyden assay (7). Neutrophil suspensions utilized for the radiochemotactic assay were labeled with ⁵¹Cr-sodium chromate, washed three times, and resuspended in M199-ovalbumin at a concentration of $3.0 \pm 0.3 \times 10^6$ cells per 0.5 ml (range) with 30-50,000 counts per 4 min (CP4 min) per 0.5 ml (8). Modified Boyden assays were carried out for 2-2½ h at 37°C in duplicate chambers and quantitated as the mean number of neutrophils per high power field (hpf) which had migrated through the 3-μm micropore filter after correction for the background counts without a stimulus. Radiochemotactic assays were analyzed in duplicate after 3 h at 37°C and the leukocyte responses expressed as net percent radioactivity = $(CP4 \text{ min } B - CP4 \text{ min } b) / (CP4 \text{ min } i - CP4 \text{ min } b) \times 100$, utilizing the CP4 min in the bottom filters of chambers with (B) or without (b) a chemotactic factor and in the initial cell suspension (i). The chemotactic factors utilized were C5a and C3a made by tryptic digestion (2, 19) of highly purified C5 and C3 (20), respectively. The digestion mixtures were heated for 30 min at 61°C to inactivate the trypsin and were then centrifuged to separate precipitated protein from the supernatant solution of C5a or C3a. Kallikrein purified from human plasma was used at a concentration able to generate 0.2-μg bradykinin per ml from 0.2-ml heated plasma (21).

Chemotactic assays were modified to allow investigation of the interaction between neutrophils and unstable chemotactic principles. Modified Boyden chemotaxis employed 8-μm micropore filters, neutrophil suspensions of $5.0 \pm 0.5 \times 10^6$ cells per ml, and 50-60-min incubation periods, and the results were determined by enumerating the cells per hpf at 80 μm from the cell source at the top of the filter. A comparable rapid radiochemotactic assay utilized a layer of two 8-μm pore micropore filters, neutrophil suspensions of $6.0 \pm 0.5 \times 10^6$ cells per 0.5 ml with 50-80,000 CP4 min per 0.5 ml and incubation periods of 90-120 min; results were expressed as CP4 min in the bottom filter of stimulated chambers per 50,000 CP4 min in the initial cell suspension corrected for background counts in control chambers.

RESULTS

Mixtures containing CoVF \bar{B} . The ability of mixtures of purified CoVF, B, and \bar{D} to attract human neutrophils was assessed in the following experiment. 6 U of CoVF, 32 μg of B, and 2 U of \bar{D} were incu-

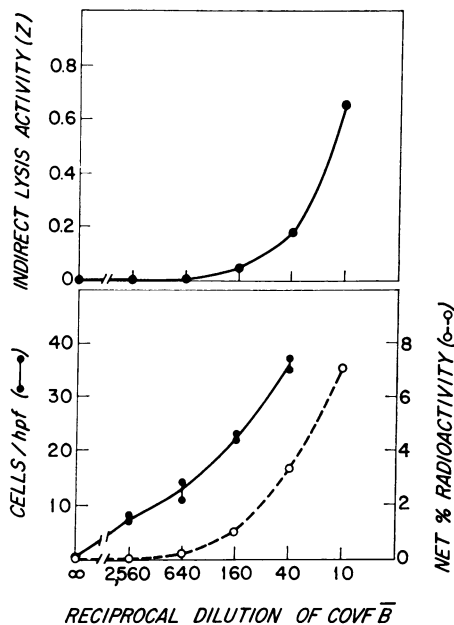


FIGURE 1 Indirect lysis (upper panel) and chemotactic activity (lower panel) of varying dilutions of a mixture in which CoVFB was generated.

bated in a final volume of 1.2 ml DGVB⁺⁺ for 30 min at 37°C; parallel reaction mixtures from which one or more of the reactants had been omitted were similarly incubated. The mixtures were chilled, diluted 1:10, and assayed for chemotactic activity with purified human neutrophils in both the modified Boyden and radiochemotactic assays. The mixture containing CoVF, B, and \bar{D} resembled the positive controls of C5a and kallikrein in attracting neutrophils, whereas none of the mixtures from which one or two of the reactants had been omitted were chemotactically active (Table I). In six other experiments employing three different lots of CoVF, four of \bar{D} , and two of B, mixtures of CoVF, B, and \bar{D} yielded a chemotactic stimulus which averaged $84 \pm 12\%$ (mean \pm SEM) of that observed with kallikrein or C5a which attracted an average of 24 neutrophils per hpf or 8% ⁵¹Cr-labeled cells; control mixtures from which one or more of the reactants were omitted consistently yielded less than 8 cells per hpf or 3% ⁵¹Cr-labeled cells.

The dose response of mixtures containing CoVFB in the two chemotactic assays and in indirect lysis is shown in Fig. 1. 40 μ g B, 2 U \bar{D} , and 8 U CoVF were incubated in a final volume of 1.5 ml DGVB⁺⁺ for 30 min at 37°C and chilled. Aliquots were initially diluted 1:10 and then serially fourfold in DGVB⁺⁺ for testing in indirect lysis and in M199-ovalbumin for chemotactic assays. Increasing amounts of reaction mixture yielded incremental increases in both indirect

lysis and chemotactic activity. Further, chemotactic activity was observed at dilutions containing insufficient complex to produce indirect lysis.

Since assembly of the CoVFB enzyme requires Mg⁺⁺, the effect of chelating divalent cations on the generation of chemotactic activity was examined. Identical reaction mixtures containing 6.4 μ g B, 0.3 U \bar{D} , and 1.4 U CoVF were prepared in 0.6-ml final volumes of DGVB⁺⁺ or DGVB-EDTA, and incubated for 60 min at 37°C. Samples of each mixture were tested for indirect lysis and for chemotactic activity. 0.45 U of hemolytic activity was present in the mixture prepared in DGVB⁺⁺ whereas no hemolytic activity was formed in EDTA. At a 1:25 final dilution, the DGVB⁺⁺ reaction mixture attracted 20 neutrophils per hpf in the micropore filter assay, compared to 6 neutrophils per hpf for the EDTA mixture. DGVB-EDTA alone yielded 2 neutrophils per hpf; and DGVB-EDTA did not inhibit the chemotactic activity of a standard kallikrein preparation which attracted 25 cells per hpf.

The ability of CoVFB to render cells unresponsive to subsequent chemotactic stimulation by kallikrein was examined with a reaction mixture containing amounts of CoVF, B, and \bar{D} identical to those used above for the chelation experiment. Controls included the same reactants in DGVB-EDTA and mixtures in DGVB⁺⁺ from which one or more reactants had been omitted. 3×10^6 neutrophils were exposed to dilutions of each reaction mixture for 30 min at 37°C, washed twice in HBSS, and then tested for their ability to respond to the standard dose of kallikrein in the modified Boyden assay. Only the active CoVFB mixture rendered the cells unresponsive to the subsequent kallikrein stimulus (Table II). Similar results were obtained in three additional experiments, including one in which the chemotactic response was assayed by both the radiochemotactic and modified Boyden techniques.

Mixtures containing C3B. Since C3b, the major

TABLE II
Deactivation of Neutrophils by Mixtures Containing
CoVF, B, and \bar{D}

Treatment	Deactivation to kallikrein*
	Modified Boyden assay
	%
CoVF, B, \bar{D} (1:25)	100
CoVF, B, \bar{D} (1:50)	56
CoVF, \bar{D} (1:25)	16
B, \bar{D} (1:25)	0
CoVF, B, \bar{D} in EDTA (1:25)	0
DGVB-EDTA (1:25)	4

* Percent decrease from 25 neutrophils per hpf.

TABLE III
Deactivation of Neutrophils by Mixtures Containing
C3b, B, and \bar{D}

Treatment	Deactivation to kallikrein*	
	Modified Boyden assay	Radiochemotaxis
	%	%
C3b, B, \bar{D} (1:20)	70	108
C3b, B, \bar{D} (1:40)	64	65
C3b (1:20)	19	0
C3b (1:40)	9	28
C3b, B, \bar{D} in EDTA (1:20)	33	7
C3b, B, \bar{D} in EDTA (1:40)	30	20
DGVB-EDTA (1:20)	36	13
DGVB-EDTA (1:40)	0	41
DGVB ⁺⁺	3	5

* Percent decrease from 33 neutrophils per hpf in micropore filter and 8850/CP4 min in radiochemotaxis.

cleavage fragment of C3, functions as a normal serum homologue of CoVF, inducing the formation of a labile C3-cleaving enzyme, C3B, from factor B in the presence of active \bar{D} , the chemotactic activity of this enzyme was assessed. Under the standard conditions of the micropore filter and radiochemotactic assays, C3B generated with varying amounts of C3b and concentrations of B and \bar{D} comparable to those used in the CoVF experiments appeared to have no chemotactic activity. That C3B had in fact been generated during the incubation of the reactants was demonstrated by the presence of the electrophoretically converted fragments of B (Bb and Ba) on immunoelectrophoretic analysis of the incubation mixtures and by the complete inactivation of 1,000 U of native C3 which had been introduced into a replicate mixture at the beginning of the incubation.

Since the half-life of C3B at 37°C is 15 min compared to 5 h for CoVFB, it seemed likely that the evanescent presence of C3B did not provide a sufficiently prolonged stimulus to chemotaxis in the conventional assay systems. As an alternative, C3B was examined for its ability to render neutrophils unresponsive to a second chemotactic stimulus, a phenomenon which requires only brief exposure of the cells to the deactivating agent (22). A mixture of 40 μ g C3b, 32 μ g B, and 1 U \bar{D} in a final volume of 0.3 ml was prepared in DGVB⁺⁺ at ice bath temperature, together with controls consisting of the same reagents in DGVB-EDTA, C3b alone, and DGVB-EDTA alone. The mixtures were brought to 37°C for 5 min, and then diluted 1:20 and 1:40 in HBSS. 4.6×10^6 purified neutrophils were exposed to 1 ml of each of the diluted reaction mixtures at 37°C for 30 min, washed twice in

HBSS, and assessed for their ability to respond to a standard dose of kallikrein in the micropore filter and radiochemotactic assays. The C3B mixture prepared in the presence of divalent cations did render the neutrophils unresponsive to the kallikrein stimulus, C3b alone being much less active in this regard, and the mixtures prepared in DGVB-EDTA exhibited the same effect as the DGVB-EDTA buffer alone (Table III). In three other experiments, performed with different preparations of C3b and factor \bar{D} , the maximal effect of C3B was 64, 53, and 112% deactivation.

Since C3B resembled a chemotactic principle in its ability to render the neutrophil unresponsive to a subsequent chemotactic stimulus, the standard assay systems were modified to accommodate the lability of C3B by employing higher cell concentrations, larger pore filters, and shorter incubation times. With such modified assay systems and concentrations of C3b, B, and \bar{D} as described above for deactivation, stimulation of chemotaxis to an average of 1.5 times background was observed in both the micropore filter and radiochemotaxis systems in three additional experiments.

Reaction mixtures of C1, C4, and ⁵¹C2, 5,000 U each in a final volume of 0.5 ml DGVB⁺⁺, did not have chemotactic activity when tested in either the standard or the modified assay system, even though C42 had been formed in the reaction mixture as indicated by inactivation of the hemolytic activity of 1,000 U of native C3 during 1 h at 37°C. These mixtures of C1, C4, and ⁵¹C2 also did not have deactivating activity when interacted with neutrophils which were subsequently exposed to kallikrein.

DISCUSSION

The effect of CoVF on whole serum has provided a useful model for studies of the final steps in the properdin pathway by allowing delineation of the role of factors B and \bar{D} in the formation of the convertase, CoVFB, which cleaves C3 and activates the terminal complement sequence. The stability of the CoVF-induced C3-cleaving activity has facilitated the detection and purification of factors B (23) and \bar{D} (9) and the identification of C3b as the normal serum homologue of CoVF (24, 25). The convertase, C3B, formed by interaction of C3b, B, and \bar{D} , is labile, due to the decay of \bar{B} (5). In the present study, the utility of the CoVF-dependent convertase has been extended to include the demonstration of a biologic activity intrinsic to the properdin pathway, namely that of chemotactic activation and deactivation of human polymorphonuclear leukocytes. Qualitatively similar activity was also demonstrable for mixtures yielding the more labile C3B.

Mixtures of highly purified CoVF, B, and \bar{D} were consistently found to have chemotactic activity as assessed either in the micropore filter or in the radio-

chemotaxis assay systems (Table I). Omission of any one of the proteins involved in generation of the CoVFB convertase prevented development of both hemolytic and chemotactic activities. Indeed, the chemotactic activity of CoVFB correlated with its hemolytic activity over a range of doses (Fig. 1). CoVFB resembled other known chemotactic factors in its capacity to render leukocytes unresponsive to subsequent chemotactic stimulation by kallikrein, a phenomenon termed "deactivation" (Table II). The chemotactic activating and deactivating capacity associated with the interaction of B, \bar{D} , and CoVF required formation of the convertase, since prevention of convertase assembly by chelation of Mg^{++} suppressed the appearances of both activities (Table II).

For studies of the C3b-induced convertase, native C3 was converted to hemolytically inactive C3b by treatment with EAC14⁹⁷² cells and freed of low molecular weight chemotactic activity by gel filtration. C3b induced the cleavage and electrophoretic conversion of factor B and the formation of a C3 convertase when incubated with B and \bar{D} . Despite these demonstrable activities, reaction mixtures prepared with C3b, B, and \bar{D} had no direct chemotactic activity in the standard micropore filter or radiochemotactic assay systems. Since the generation of both CoVFB and C3B yielded the Ba fragment, yet only CoVFB had readily demonstrable chemotactic activity, it is likely that the chemotactic activity is associated with the active complex rather than with a fragment of B produced during convertase generation. It follows therefore that the failure of C3B to be chemotactic in the standard assay system might be due to its lability. When tested for its ability to render neutrophils unresponsive to a subsequent chemotactic stimulus, a technique which requires relatively brief exposure of the cells to a chemotactic principle, mixtures of C3b, B, and \bar{D} were consistently active; mixtures lacking one or more of the ingredients or prepared in the absence of Mg^{++} had no specific deactivating effect (Table III). In modified chemotactic assay systems, with short incubation times, large pore filters and high cell concentrations, some chemotactic activity of mixtures yielding C3B could be demonstrated.

These studies demonstrating that fluid-phase formation of alternative pathway convertases is associated with the appearance of chemotactic activity, attributed to the complex rather than to a released fragment, have not been performed with cell-bound factors. Nonetheless it is of interest to speculate about the latter possibility. The convertase itself could be formed secondarily by immune complexes or sensitized cells generating C3b by the classical pathway or directly in response to activation of the properdin pathway by substances such as endotoxin. Of equal interest is the

possibility that C3b formed in the fluid phase might attach to leukocytes such as monocytes, B lymphocytes, or polymorphonuclear leukocytes by immune adherence with resultant formation of the alternative pathway convertase at the membrane. It has not been clearly established that C3b bound by its immune adherence site will initiate generation of such a convertase, but the observation that lymphoid tumor cells interacted with either C3 or C3b lyse upon subsequent exposure to fresh serum (26) could be explained by such a mechanism. The formation of membrane-bound convertase might not only be associated with lysis but also influence the directional migration of the cell.

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