Chemotactic and Anaphylatoxic Fragment Cleaved from the Fifth Component of Guinea Pig Complement

Abstract. *The fifth component of guinea pig complement, with a sedimentation coefficient 7.8S, is cleaved by sensitized sheep erythrocytes treated with the first four components of complement into two fragments with sedimentation coefficients of 7.4S and 1.5S. The smaller fragment, with a molecular weight of about 15,000, possesses chemotactic activity for rabbit polymorphonuclear leukocytes, as well as anaphylatoxic activity for guinea pig ileum.*

As part of the accumulating evidence on the role of complement in the inflammatory processes initiated by antigen-antibody reactions, it has been reported that anaphylatoxins can be generated from C'3 (*1*), as well as from C'5 (*2*), and that treatment of human serum with antigen-antibody aggregates produces a trimolecular complex of C'5, C'6, and C'7 which is chemotactic for rabbit polymorphonuclear leukocytes (*3*). We now find that treatment of C'5 with EAC'1a,4,2a,3 cleaves a fragment that has a molecular weight of about 15,000 and possesses chemotactic activity for rabbit polymorphonuclear leukocytes, as well as anaphylatoxic activity for guinea pig ileum. Neither C'6 nor C'7 is required for generation of this inflammatory factor.

Functionally and chemically homogeneous guinea pig C'3 was prepared according to the method of Shin and Mayer (*4*). C'5 was prepared according to the method of Cook *et al.* (*5*). The functional purity of the product was demonstrated by showing that it contained less than 1 unit of any other complement component in 10,000 units of C'5. The C'3 was not detectable with rabbit antiserum to guinea pig C'3. The product was chemically homogeneous, as ascertained by chromatography and electrophoresis. Methods for obtaining the other complement components in a functionally pure form, as well as procedures for their titration, have been described (*6*). Purified C'5 was labeled with an average of 0.1 atom of I^{125} per molecule of C'5 (*7*), with substantial loss of hemolytic activity which could be attributed to the manipulation rather than the iodination. Preparations of EAC'1a,4 were made (*8*) and stored in glucose-gelatin-barbital buffer with Ca^{++} and Mg^{++}; this buffer is designated $DGVB^{++}$ (*6*). The EAC'1a,4,2a were prepared by incubating EAC'1a,4 (1.5×10^8 cell/ml) with an equal volume of the buffer con-

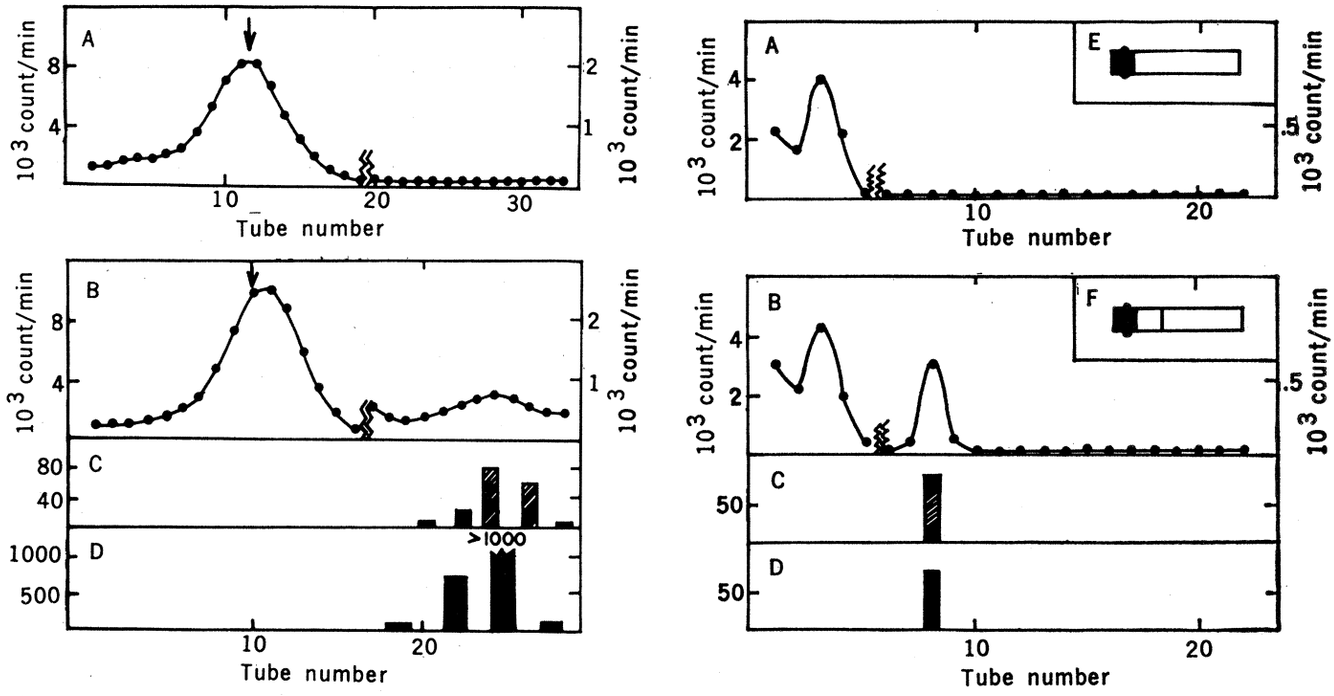

Fig. 1 (left). Ultracentrifugation in a sucrose density gradient was performed (*16*) at 27,000 rev/min at 3° to 4°C for 22 hours in the SW-39 rotor. The sucrose concentration ranged between 5 and 20 percent in 0.05*M* acetate buffer, *p*H 5.0, containing 0.10 mole of NaCl per liter. Two drops were collected per fraction. (A) I^{125}-Labeled C'5 (100 μg) plus 1 μg of unlabeled C'5 as marker. Radioactivity is indicated by solid circles. The arrow shows the peak of hemolytic activity. No biological activity was found in any fraction. (B) I^{125}-Labeled C'5 (100 μg) treated with EAC'1a,4,2a,3 mixed with 1 μg of unlabeled and untreated C'5 as marker. Symbols as above. The first radioactivity peak is displaced relative to arrow. The wiggle represents break in curve to indicate shift to radioactivity scale shown on right-hand ordinate. (C) Anaphylatoxic activity of C'5 treated with EAC'1a,4,2a,3. The entire content of each fraction was used for the assay. (D) Chemotactic activity of C'5 treated with EAC'1a,4,2a,3. Two fractions were pooled and diluted in 3 ml of Hanks's solution for the assay (Table 1). Top of the gradient is at right. Fig. 2 (right). Disc electrophoresis on acrylamide gel was performed at acid *p*H (*10*) for 3 hours at 50 volts, with a small-pore gel of 7.5 percent acrylamide. (A) I^{125}-Labeled C'5 (50 μg). Solid circles, radioactivity; no biological activity was found in any fraction. (B) I^{125}-Labeled C'5 treated with EAC'1a,4,2a,3. Solid circles, radioactivity; wiggle represents break in curve to indicate shift to radioactivity scale shown on right-hand ordinate. (C) and (D) Anaphylatoxic and chemotactic activities (respectively) of C'5 treated with EAC'1a,4,2a,3 (Table 1). Entire eluate of each fraction was used for testing anaphylatoxic activity. For chemotactic assay, eulates were diluted 50-fold in Hanks's solution containing 0.1 percent bovine serum albumin in order to fill the test chamber. (E) Radioautography of control C'5; (F) radioautography of C'5 treated with EAC'1a,4,2a,3 (cathode at right).

taining 200 units of C'2 per milliliter for 10 minutes at 30°C. The EAC'1a, 4,2a,3 were made in the same way, except for addition of purified C'3 to the reaction mixture to a final concentration of 3 μg/ml. The EAC'1a,4,2a and EAC'1a,4,2a,3 were washed three times with DGVB^{++} and standardized to 1.54×10^{10} cell/ml in this buffer. In order to cleave C'5, we incubated this buffer containing 500 μg of purified C'5 per milliliter and I^{125}-labeled C'5 at a concentration not exceeding 5 μg/ml with an equal volume of EAC'1a,4,2a,3 for 10 minutes at 37°C. The cells were removed by centrifugation, and the supernatant was acidified with 1*M* acetic acid to *p*H 3.5 in order to stabilize the biological activities of the reaction mixture. The reaction mixture was then centrifuged at 8000*g* and 0°C for 10 minutes to remove the cell membranes. After these treatments, the solution was neutralized without loss of the biological activities.

When C'5 was treated with EAC'1a, 4,2a,3 in the manner just described, chemotactic as well as anaphylatoxic activity appeared in the fluid phase (Table 1). At high concentration, the reaction mixture caused the migration of more than 90 percent of all polymorphonuclear leukocytes. On ultracentrifugation in a sucrose density gradient (Fig. 1) the reaction mixture yielded two radioactive peaks corresponding to apparent sedimentation coefficients of 7.4*S* and 1.5*S*, respectively. By comparison, the sedimentation coefficient of native C'5 is 7.8*S* (*5*). The 1.5*S* fragment exhibited both anaphylatoxic and chemotactic activity (Fig. 1). Next, the reaction mixture was put through a Sephadex G-75 column (*9*) in 0.05*M* acetate buffer, *p*H 4.0, containing 0.10*M* NaCl. Two radioactive peaks appeared. One of these was in the excluded region and the other coincided with peaks of anaphylatoxic and chemotactic activity. This material, which corresponds to the 1.5*S* fragment, had a molecular weight of about 15,000, estimated in relation to both cytochrome c and myoglobin from horse heart.

The homogeneity of the 1.5*S* fragment was analyzed by disc electrophoresis of the reaction mixture on 7.5 percent acrylamide gel at acid *p*H (*10*). The small-pore gel, measuring 4.4 cm in length, was sliced longitudinally into two equal halves. One of these halves was cut into 22 equal segments and each of these was eluted with DGVB^{++} (adjusted to *p*H 3.5 with 1*M* acetic acid) for measurement of radioactivity and of biological activities. The other half of the gel was stained with amido black in 7 percent acetic acid and then destained and dried for radioautography (contact exposure for 3 days with Kodak NS-54T film). The results (Fig. 2) indicate two radioactive fractions containing about 90 and 10 percent of the total radioactivity. The smaller peak presumably represents the 1.5*S* fragment, and this fraction exhibited anaphylatoxic as well as chemotactic activity. Most of the radioactivity as well as biological activity was confined to a single gel segment of 2 mm. In accord with these measurements, radioautography showed a single narrow band in the area of the 1.5*S* fragment. Essentially the same pattern was obtained by disc electrophoresis on 15 percent acrylamide gel. In addition, a disc electrophoresis with 7.5 percent acrylamide gel was performed at alkaline *p*H with tris-glycine buffer (*11*). Conditions for this experiment were the same except that the eluates were dialyzed against Hanks's solution in order to eliminate material which interferes in the chemotactic assay. In this experiment the 7.4*S* and 1.5*S* fragments did not separate, but rather a broad band of radioactivity appeared. Both biological activities were found in a narrow region about 1 cm from the cathodal end of the small-pore gel (electrophoresis was terminated when the bromphenol blue used as tracking dye emerged from the anodal end of the small-pore gel).

Since the anaphylatoxic and chemotactic activities did not separate from one another on ultracentrifugal and electrophoretic fractionation as well as gel filtration, they may reside on the same molecular species, namely, the 1.5*S* fragment with a molecular weight of about 15,000 (*12*). We do not yet know the relation of this fragment to the chemotactic factor composed of a high-molecular-weight complex of C'5, C'6, and C'7, as reported (*3*), nor do we know its relation to the low-molecular-weight chemotactic factor generated in guinea pig serum by endotoxin (*13*).

Table 1. Biological activities of C'5 treated with EAC'1a,4,2a,3. Chemotactic activity was measured (*14*) with a Millipore filter (1.2 μ). Hanks's solution with 0.1 percent bovine serum albumin was used. Rabbit polymorphonuclear leukocytes were harvested from 3-hour peritoneal exudate [induced with saline containing 0.1 percent shellfish glycogen (Mann Research Laboratory)]. Chemotactic activity, after incubation for 3 hours at 37°C, was scored as the average number of leukocytes appearing on the bottom side of the filter, under five different microscopic fields at 450-fold magnification. Anaphylatoxic activity was measured by contraction of guinea pig ileum, according to Osler *et al.* (*15*). For the chemotactic assay, C'5 (500 μg/ml) before and after cleavage by EAC'1a,4,2a,3 was diluted 50-fold in Hanks's solution containing 0.1 percent bovine serum albumin. For anaphylatoxic assay, 20 μl of reaction mixture was diluted with 1 ml of Tyrode's solution containing 3×10^{-2} μg of atropine sulfate per milliliter, and this mixture was injected into the 10-ml bath holding the ileum. Anaphylotoxic activity is expressed as the percentage of response relative to that obtained with 2 μg of histamine.

C'5 treatment	Chemotactic activity (No. cells)		Anaphylatoxic activity (% response)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
EAC'1a,4,2a,3	79	204	60	90
EAC'1a,4,2a		42		0
None	0		0	0
Buffer alone*	0	30	0	0

* No C'5; buffer was incubated with EAC'1a, 4,2a,3.

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1. C', complement; A, antibody to boiled sheep erythrocyte stromata; E, sheep erythrocyte; C'1, first component of complement; C'1a, activated form of C'1; C'2, second component of complement; C'2a, fragment of C'2; C'3, third component of complement; C'4, fourth component of complement; C'5, fifth component of complement; EAC'1a,4, symbols for E which have reacted with A, C'1a, and C'4; EAC'1a,4,2a indicates E which have reacted with A, C'1a, C'4, and C'2a; EAC'1a, 4,2a,3 indicates E which have reacted with A, C'1a, C'4, C'2a, and C'3 [M. M. Mayer in *Protides of the Biological Fluids*, H. Peeters, Ed. (Elsevier, New York, 1967), vol. 15, p. 383].
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Sterols: Isolation from a Blue-Green Alga

Abstract. *A crystalline mixture of sterols was isolated from the filamentous blue-green alga* Phormidium luridum. *The mixture consisted of unsaturated 24-ethylcholesterols possessing* Δ^7-, $\Delta^{5,7}$-, *and* Δ^5-*bonds together with their* Δ^{22}-*derivatives and a small amount of cholesterol. The major component was 24-ethyl-*Δ^7-*cholestenol. Squalene and phytol were also evident.*

Due to their procaryotic nature, blue-green algae are thought to represent a primitive form of plant possessing a number of unusual properties (*1*) including the apparent lack of sterols (*2*). In an attempt to deduce where sterol formation is blocked, we investigated the isopentenoid constituents of the blue-green alga *Phormidium luridum* var. *olivaceae* Boresch (*3*).

Phormidium luridum was grown on an alkaline mixture of salts [Medium C of Kratz and Myers (*4*)] and flushed with air supplemented with 4 percent CO_2 (*5*). There was no contamination of our culture by eucaryots, or of our growth medium or our chemicals by sterols. Soxhlet extraction was carried out with acetone on 100 g (fresh weight) of whole cells. After saponification of the acetone-soluble material in 10 percent ethanolic KOH, the ligroin-soluble portion was chromatographed on alumina. The monohydroxyl region of the chromatogram was chromatographed again on alumina, yielding a crystalline material which proved to be sterol. Recrystallization from methanol gave 3.0 mg of colorless plates or leaflets, m.p. 136° to 141°C (Kofler hot stage).

Gas-liquid chromatography on XE-60 indicated that the crystals were a mixture with two major components (> 80 percent) and four minor components. The retention times relative to cholesterol (RRT) of the major components were 1.67 and 1.96 in an abundance ratio of two to three. The RRT's of the minor components were 1.00, 1.47, 1.80, and 2.11 with the last two substances being the principal ones. Ultraviolet analysis of the crystalline mixture showed the typical spectrum (maximum absorbancy 272, 282, and 294 nm) of a $\Delta^{5,7}$-sterol, and the extinction coefficients indicated that the substance amounted to 14 percent of the total.

In the mass spectrum of the crystals there were three parent peaks at mass/charge 414, 412, and 410 (in decreasing order of peak heights). Comparison of the amounts in these various analyses indicate that the substance with RRT 1.96 had a mass of 414, that with RRT 1.67 a mass of 412, and that with RRT 1.80 a mass of 410. Ultraviolet absorption spectra indicate that this third most abundant component must have had a $\Delta^{5,7}$-system. Thin-layer chromatography of the mixture on silica gel-G gave no spot in the 4,4′-dimethyl region (such as lanosterol gives), but a strong spot was evident in the normal sterol region. Peaks at *m/e* 231 and 229 for typical steroidal loss of the side chain plus 42 mass units (*6*) confirmed the absence of 4,4′-substitution and furthermore eliminated the possibility of a 14-methyl group. Based on the molecular weights, then, the sterols must have had a 24-ethyl group and one, two, and three double bonds ($C_{29}H_{50}O$ = 414; $C_{29}H_{48}O$ = 412; and $C_{29}H_{46}O$ = 410).

The ultraviolet spectrum of our mixture after correction for 14 percent $\Delta^{5,7}$-sterol had an unusually high end absorption (extinction coefficient ε at 215 nm of 2100) which was more characteristic (*7*) of Δ^7 (ε_{215} 3000) than of Δ^5 (ε_{215} 700). This was corroborated by the infrared spectrum which showed a frequency (ν_{max}) of 1033 cm^{-1} for Δ^7 rather than ν_{max} 1050 cm^{-1} for Δ^5 (*8*). A shoulder near ν_{max} 1050 cm^{-1}, however, indicated some Δ^5-component as was also suggested by the lowered ε_{215}, and the relationship of the intensities of the gas-liquid chromatographic and mass spectral peaks. The infrared spectrum had a weak band at 968 cm^{-1} corresponding to some *trans*-Δ^{22} (*9*).

This information is consistent with the following structures: component with RRT 1.96 is 24-ethyl-Δ^7-cholestenol ($C_{29}H_{50}O$); component with RRT 1.67 is 24-ethyl-$\Delta^{7,22}$-cholestadienol ($C_{29}H_{48}O$); component with RRT 1.80 is 24-ethyl-$\Delta^{5,7,22}$-cholestatrienol ($C_{29}H_{46}O$). An authentic sample of 24-ethyl-$\Delta^{7,22}$-cholestadienol had RRT 1.67. From the known contributions (*10*) of Δ^{22}- and $\Delta^{5,7}$-unsaturation the calculated RRT's of the Δ^7- and $\Delta^{5,7,22}$-derivatives are 1.94 and 1.80. All three values agree with the structures assigned. The RRT's of the other components at 1.00, 1.47, and 2.11 similarly indicate they represent cholesterol (authentic RRT 1.00), 24-ethyl-$\Delta^{5,22}$-cholestadienol (authentic RRT 1.44) and 24-ethyl-$\Delta^{5,7}$-cholestadienol (calculated RRT 2.09). In addition, authentic 24-ethyl-Δ^5-cholestenol had RRT 1.67 and, for the reasons mentioned above, it must have occurred together with the $\Delta^{7,22}$-component (RRT 1.67).

We have also isolated and similarly identified phytol from *P. luridum* by mass spectroscopy, thin-layer chromatography, and nuclear magnetic resonance spectroscopy, and a trace of squalene was apparent by gas-liquid chromatography.

This is further evidence that the usual biosynthetic pathway to sterols is not always inoperative in blue-green algae.

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