A PLASMIN-SPLIT FRAGMENT OF C'3 AS A NEW CHEMOTACTIC FACTOR*

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When the interaction of complement components takes place, as, for example, upon addition of an antigen-antibody complex to fresh serum, a factor is generated that is chemotactic for polymorphonuclear leukocytes (1, 2). The factor has been characterized as a trimolecular complex consisting of the fifth (C'5), sixth (C'6), and seventh (C'7) components of complement (2, 3).

Recently it has been reported that when rabbit serum is treated with human plasmin, a chemotactic factor also appears (4). This paper now records the characterization of this new chemotactic factor as a fragment of the third component of complement (C'3, β1C-globulin). As such, this newly described chemotactic factor of low molecular weight is completely different from the previously described complement-associated chemotactic factor, in terms of requirements for generation as well as in physical-chemical characteristics.

That C'3 is involved in the generation of this new chemotactic factor is of interest in view of the other known functions of C'3. These include its central role in immune adherence (5), immune phagocytosis (5, 6), and conglutination (7). Of particular interest are the reports by Dias da Silva and Lepow (8) that a fragment of C'3 is biologically active in causing contraction of smooth muscle, and by Müller-Eberhard that a factor in cobra venom when added to serum can cause cleavage of C'3.[1] The appearance of cleavage products of C'3, at least one of which has biologic activity, has an obvious parallel in the present report.

Materials and Methods[2]

Reagents.—Streptokinase (Sk) and highly purified human plasminogen (Plm), prepared as recently described (9), were supplied by Dr. Fletcher B. Taylor, Jr. (University of Pennsyl-

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[1] Müller-Eberhard, H. J. Isolation from cobra venom of an inactivator of the third component of complement and its mode of action. Manuscript in preparation.

[2] The "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research were observed during this study.

vania School of Medicine). The highly purified C'3 was supplied by Dr. Hans J. Müller-Eberhard (Scripps Clinic and Research Foundation, La Jolla, Calif.). In some experiments, C'3 labeled with ^{131}I was used as a convenient marker in preparatory and analytical procedures. Incubation of complement components with Sk-Plm was carried out in a phosphate buffer, pH 7.5, ionic strength 0.2, at 37°C for 2 hr.

Chemotaxis.—Chambers were used that were separated into upper and lower compartments by a micropore filter. These chambers, which are modifications of Boyden's originally described model (1), were employed in all chemotaxis experiments. Details of these chambers and the technique for measuring chemotaxis have been presented in recent reports (2, 3). The system operates on the principle that a chemotactic substance in the lower compartment of a chamber can cause rabbit polymorphonuclear leukocytes (PMN's) to move in a specific and directional manner toward the increasing gradient of concentration of chemotactic factor. By the use of micropore filters, the migration of cells in the filter can be visualized and quantitated.

Activation of Rabbit Serum.—In experiments in which the chemotactic factor in serum was used, the factor was generated by the addition of rabbit antibody to bovine serum albumin (anti-BSA) with antigen added at equivalence. 100 μg N anti-BSA, purified as IgG by elution from an ion exchange cellulose (10), was used for each milliliter of serum and incubated for 2 hr at 37°C. The precipitate was then removed by centrifugation.

Electrophoresis.—Preparative electrophoresis was carried out in the supporting medium Pevikon with barbital buffer, pH 8.6, ionic strength 0.04. Duration of electrophoretic separation of starting material was 18 hr.

Ultracentrifugation.—Ultracentrifugal separation of various substances was attained by use of a sucrose density gradient (10–40% sucrose in phosphate buffer, pH 7.3, ionic strength 0.02) in a Beckman (Spinco) model L ultracentrifuge. Depending on the rotor employed (SW-39 or SW-50), the duration of centrifugation at 35,000, 40,000, or 50,000 rpm varied from 12 to 19 hr. In one experiment, the density gradient was made up in 0.15 M sodium chloride in acetate buffer (0.01 M) at a pH of 5.0. Details of these procedures are found elsewhere (2).

Column Chromatography.—Ion exchange chromatography was employed for the isolation of the chemotactic factor of complement that consists of a trimolecular complex of the fifth (C'5), sixth (C'6), and seventh (C'7) components of complement (2, 3). This material was obtained by eluting rabbit euglobulin from triethylaminoethyl cellulose (TEAE) with a salt gradient. The resulting material ("TEAE fraction") was then interacted with the first four reacting components of complement to activate the C'5, C'6, C'7 trimolecular complex to a chemotactically active substance. This procedure is described in detail in a prior report (2).

Chromatography utilizing a gel filter ("molecular sieve") was carried out in Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) with 0.15 M sodium chloride in acetate buffer at a pH of 5.0. The gel column was calibrated with blue dextran (Pharmacia), glucagon (Eli Lilly and Co., Indianapolis, Ind.), protamine sulfate (Sigma Chemical Corp., St. Louis, Mo.), and cytochrome *c* (Mann Research Laboratories, Inc., New York, N. Y.) in order to obtain an elution sequence that could be plotted according to molecular weights (11). When the treated C'3 preparation was eluted, the column was calibrated immediately before and afterwards with the known compounds. The resultant elution positions for each compound were found to be in excellent agreement, not varying by more than 1.0 ml.

Immunoelectrophoresis.—An LKB-Produkter (Stockholm, Sweden) immunoelectrophoretic apparatus was used with a barbital buffer (pH 8.2, ionic strength 0.04). Antibodies produced in rabbits or guinea pigs to human and rabbit C'3, respectively, were obtained by the method described recently by Mardiney and Müller-Eberhard (12). These preparations were utilized in immunoelectrophoretic and Ouchterlony double-diffusion systems to assess changes in C'3 and also to detect C'3-related antigenic material in preparative electrophoretic fractions.

RESULTS

Comparison of Complement-Associated Chemotactic Factor and Plasmin-Generated Chemotactic Factor.—A striking difference in the behavior of the two chemotactic factors in sucrose density gradients was demonstrated. When fresh rabbit serum that had been treated with an immune precipitate to activate the C'5, C'6, C'7 complex was analyzed, the chemotactically active fractions, although activity was low, were found in the region of C'6 (Fig. 1, upper frame). On the other hand, fresh rabbit serum treated with Sk and Plm for 15 or 90 min acquired chemotactic activity that was confined to fractions from the top portion of the sucrose gradient (Fig. 1, middle and lower frames). Both the C'6 and the protein profiles were shifted toward the top of the gradient as the duration of incubation with the Sk-Plm mixture increased from 15 to 90 min.

Because of the possibility that C'3 or C'5 (but not C'6)[3] was involved in the appearance of the plasmin-generated chemotactic factor, TEAE fractions rich in these components were treated with Sk-Plm and analyzed in sucrose density gradient. When the untreated TEAE fraction was incubated with Sk and Plm at 37°C for 2 hr, chemotactic activity was sharply confined to the upper portion of the gradient (Fig. 2, upper frame, fraction 19). The protein in this gradient was distributed over a wide zone, although a peak was found in the upper portion of the gradient (Fig. 2, upper frame). Very little hemolytic activity of C'6 remained. When the TEAE fraction was "activated" by interaction with the first four reacting components of complement in order to generate the complement-associated chemotactic factor, and then treated with Sk-Plm, little chemotactic activity remained (a chemotactic value of 58 vs. 185 for the material before treatment). Of the little C'6 activity remaining, a discrete biphasic distribution with areas of rapidly and slowly sedimenting C'6 was observed (Fig. 2, middle frame). When the TEAE fraction that had been "activated" to generate the complement-associated chemotactic factor was analyzed in the gradient but was not subjected to treatment with Sk-Plm, chemotactic activity was confined, as expected, to the area occupied by the more rapidly sedimenting C'6 fraction (Fig. 2, lower frame). These results revealed the striking difference in the velocity of sedimentation of the plasmin-generated and the complement-associated chemotactic factors. In addition, dissociation of the C'5, C'6, C'7 complex and loss of C'6 hemolytic acitivity as a result of treatment with Sk-Plm was noted.

Electrophoretic Changes in Rabbit C'3 after Treatment with Sk-Plm.—When the TEAE fraction containing approximately 35 mg total protein, which is rich in rabbit C'3, was incubated for 2 hr at 37°C with 10 mg Sk and 4 mg Plm,

[3] In a previous paper (4), C'6 was shown not to be required inasmuch as rabbit serum genetically deficient in C'6 supported generation of the chemotactic factor.

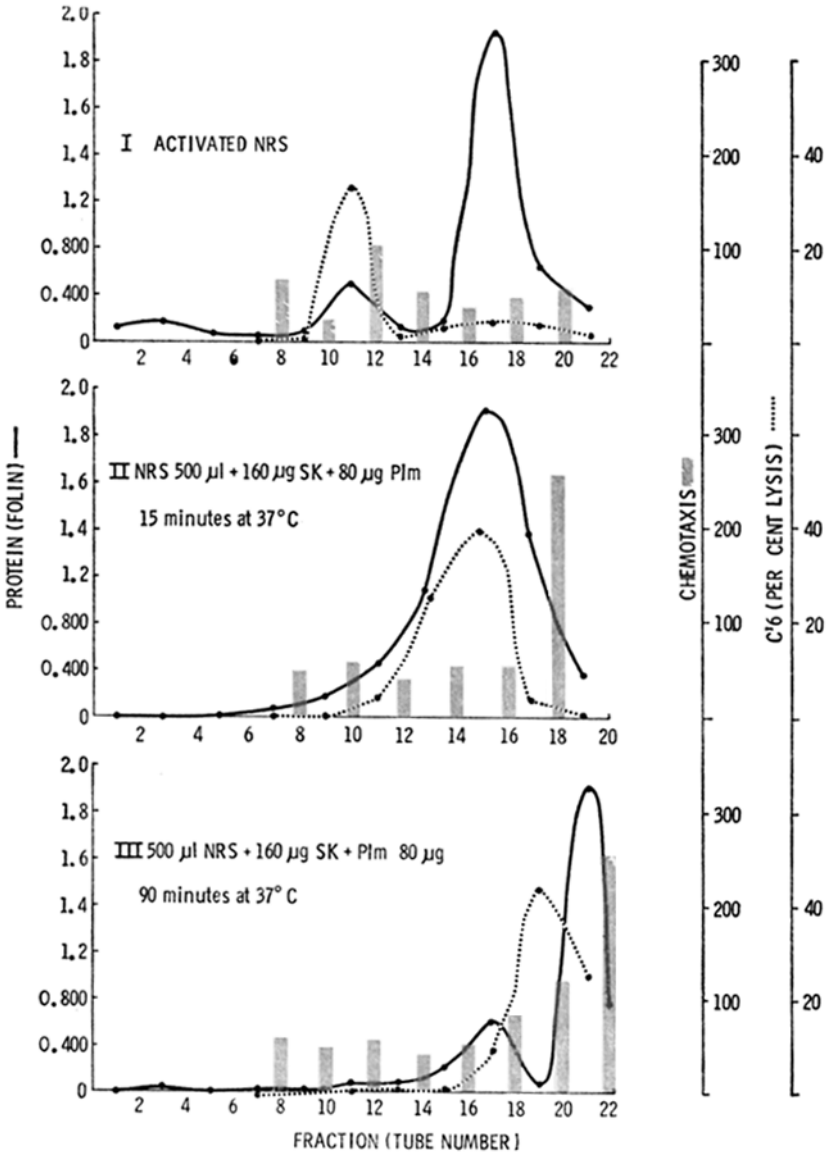

FIG. 1. Analysis of sucrose density gradient of rabbit serum (NRS) treated with streptokinase (Sk) and plasminogen (Plm). Activated serum has been incubated with immune complexes. (See text.)

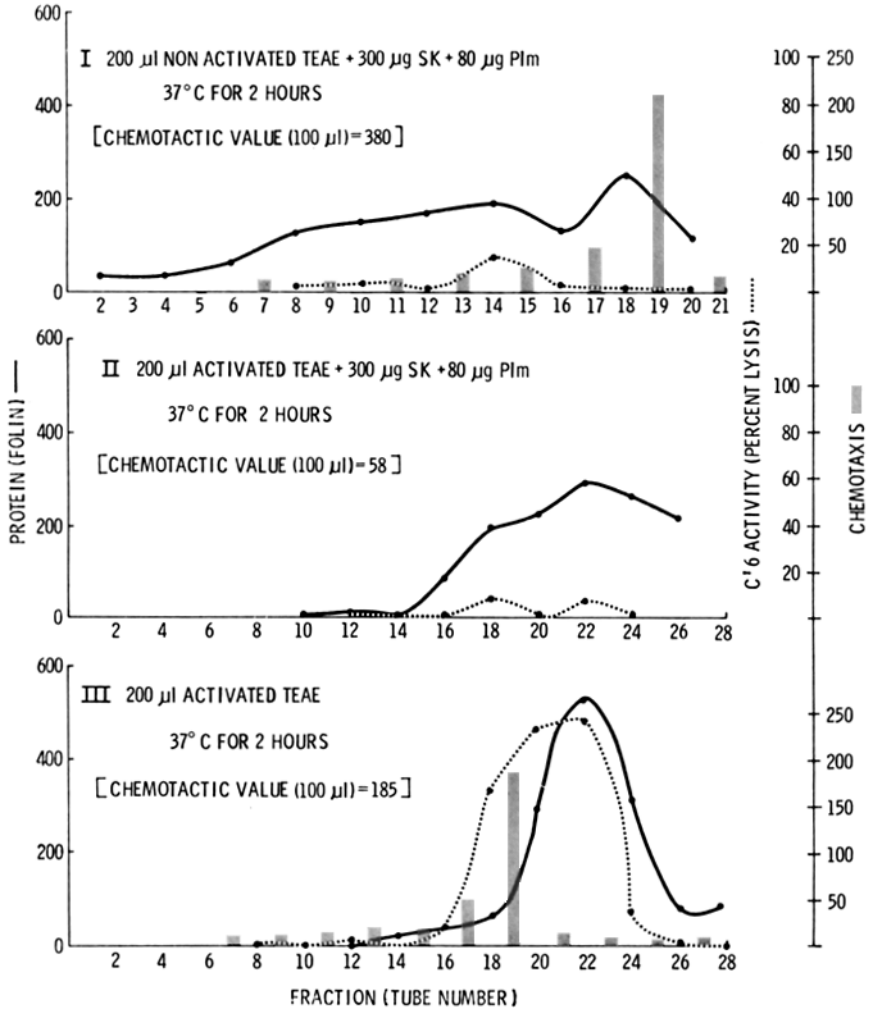

FIG. 2. Comparison of complement-associated and plasmin-generated chemotactic factors in sucrose density gradient ultracentrifugation. The plasmin-generated factor is slowly sedimenting (upper frame), whereas the complement-associated factor sediments relatively rapidly (lower frame).

a definite change in the C'3 was noted. An antigenically different, fast-migrating band appeared in the anodal region (Fig. 3). The main C'3 band was more intense and extended beyond the take-off point of the anodal band. This finding indicated the presence of antigenic material from the main C'3 molecule, which migrates electrophoretically more rapidly than the parent molecule.

Electrophoretic Position of Plasmin-Generated Chemotactic Factor in Rabbit

TEAE Fraction.—Using the same material examined in immunoelectrophoresis (described above), electrophoretic fractionation was carried out at a pH of 8.6. No chemotactic activity was noted in fractions containing C'3 and the main protein peak (Fig. 4. fractions 6–9), but substantial chemotactic activity was contained in anodal fractions 10–14, a region that possessed antigenic material of C'3. In Ouchterlony studies not picture here, the anodal fractions 11–13 contained C'3 antigenic material that was antigenically deficient when com-

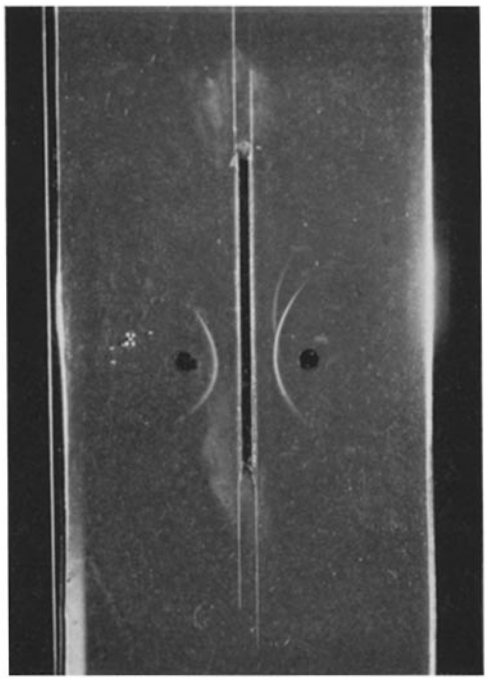

FIG. 3. Immunoelectrophoretic analysis for rabbit C'3 in TEAE fraction before (left) and after (right) treatment with Sk-Plm. Anode is at top.

pared with fractions 8–9. In TEAE fractions not treated with Sk-Plm, no such fast-migrating zone with antigenic material of C'3 was found; all of the C'3 remained with the main protein peak.

Electrophoretic fractions 8, 10, and 12 were examined in sucrose density gradient to determine the relative sedimentation velocity of the chemotactic activity (Fig. 5). Fraction 8, chemotactically inactive, possessed protein with a relatively rapid rate of sedimentation (upper frame), whereas both fractions 10 and 12 contained protein and chemotactic activity with a relatively slow rate of sedimentation (middle and lower frames). In summary, these findings indicated that the plasmin-generated chemotactic factor was, electrophorec-

tically, a rapidly migrating material with a slow velocity of sedimentation in the sucrose density gradient. There were also indications that this material was a split product of C'3.

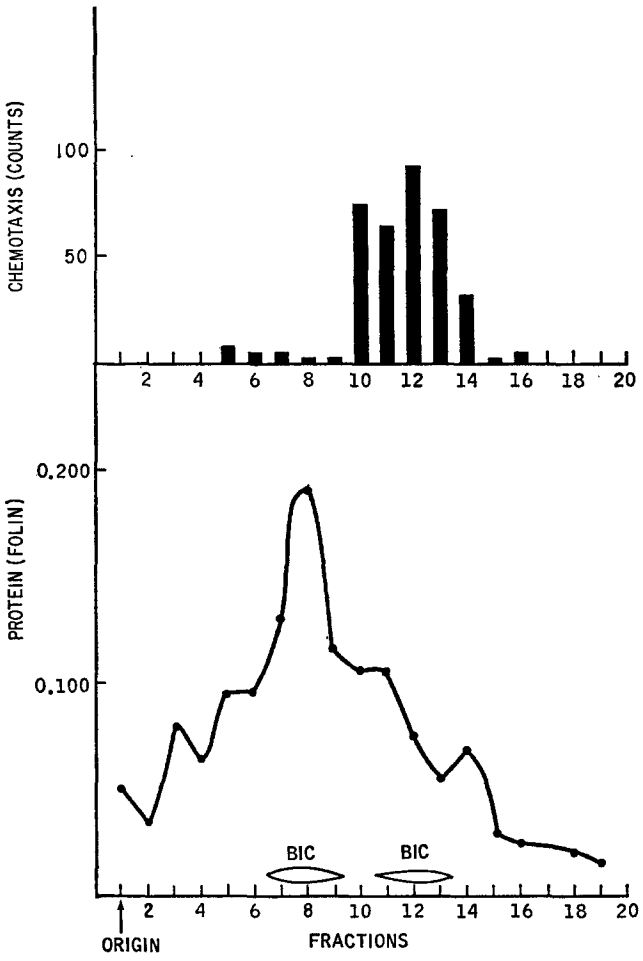

FIG. 4. Electrophoretic separation of TEAE fraction (rich in rabbit C'3, C'5, C'6, C'7) treated with Sk-Plm and analyzed for rabbit C'3 (β1C) and chemotactic activity.

Plasmin Generation of Chemotactic Activity from Highly Purified Components of Human Complement.—Since the TEAE fraction is rich in C'3 and C'5, highly purified components of human complement were tested for ability to support generation of chemotactic activity after incubation with Sk-Plm. With C'3, substantial chemotactic activity could be generated when neither the purified,

untreated component nor the Sk-Plm mixture per se possessed chemotactic activity (Table I). In contrast, treatment of human C'5 with Sk-Plm failed to result in the appearance of chemotactic activity. As anticipated, chemotactic

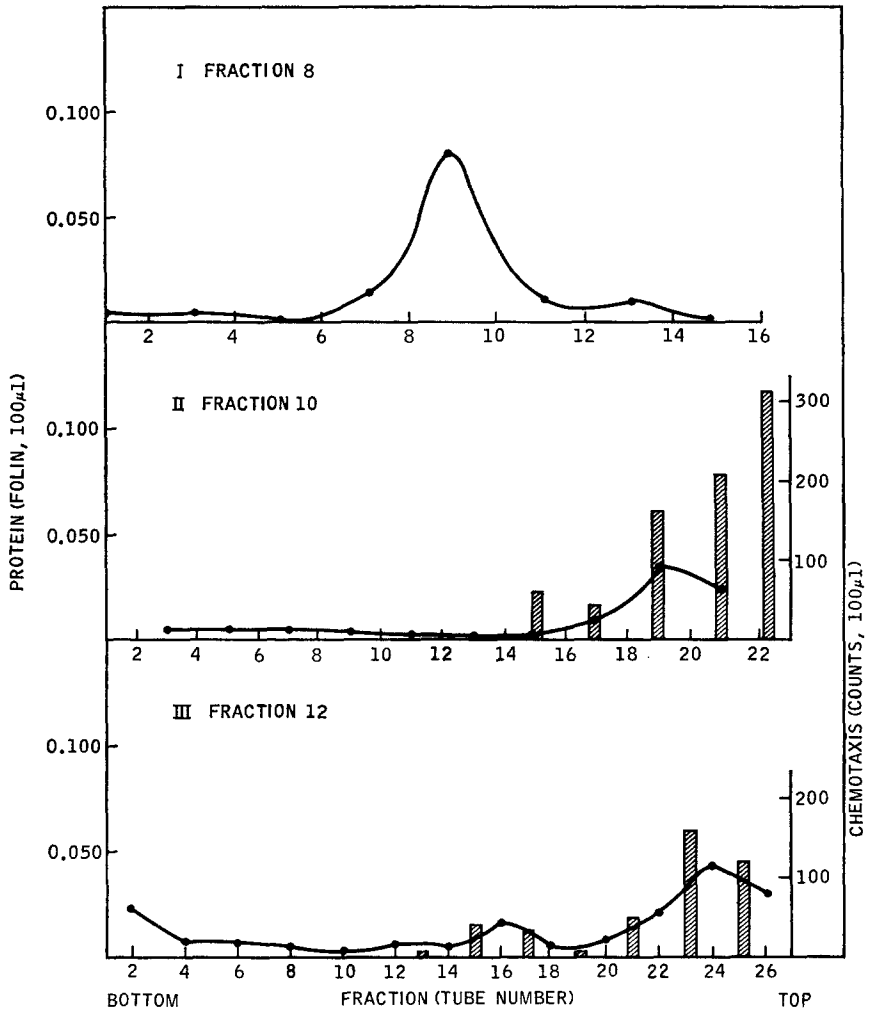

FIG. 5. Analysis in sucrose density gradient of electrophoretic fractions described in Fig. 4.

activity could be generated by treatment of fresh human serum. These data, together with the finding that chemotactic activity could be generated in rabbit serum genetically deficient in C'6, precluded the role of C'5 and C'6 in generation of the factor and indicated a requirement for C'3 in the generation of this chemotactic factor.

Immunologic Investigations of Human C'3 Treated with Sk-Plm.—In order to investigate in detail the plasmin-generated chemotactic factor, 6.88 mg of highly purified human C'3, with 160 μg C'3-^{131}I added as a tracer, was incubated with 5 mg Sk and 2.5 mg Plm at 37°C for 2 hr. Immunoelectrophoretic analysis of this material revealed a marked change in the electrophoretic mobility of C'3 after treatment (Fig. 6). Whereas the untreated material was demonstrable as a single band slightly cathodal to the point of origin (left), the treated material separated into three discrete bands (right): a faint residual band similar in position to the untreated C'3, a main band of electrophoretically "converted" faster migrating material, and a rapidly migrating anodal fraction that was antigenically deficient when compared with the intermediate band.

TABLE I

Ability of Plasmin to Generate Chemotactic Activity from Purified Components of Human Complement

Material used	Amount	Sk-Plm*	Chemotactic value
C'3 (β1C-globulin)	32 μg	Present	20
	65 μg	Present	98
	65 μg	Absent	13
	133 μg	Present	130
C'5 (β1F-globulin)	40 μg	Present	48
	40 μg	Absent	58
Human serum	100 μl	Present	119
	100 μl	Absent	15
None	—	Present	46

* For each sample, 200 μg streptokinase (SK) and 100 μg human plasminogen (100 μg) were added in a final volume of 200 μl, followed by incubation for 2 hr at 37°C, pH 7.5, in phosphate buffer, ionic strength 0.2.

Reduction in Sedimentation Velocity of C'3 after Treatment with Sk-Plm.—It was shown by density gradient ultracentrifuged analysis that treatment of human C'3 with Sk-Plm resulted in a reduction in sedimentation velocity, implying a reduction in molecular weight (Fig. 7). It could be calculated that the sedimentation rate fell from a value for the untreated molecule of 9.5S to 9.0S. It was also apparent that with the treated C'3 a discrete zone of radioactivity (which was not present in the gradient containing the untreated material) appeared in the upper regions of the gradient, implying cleavage products of low molecular weight (See upper frame). Approximately 10% of the total radioactivity appeared in this zone. When tests for chemotactic activity were carried out on various fractions of the gradient, activity was rather broadly distributed over the upper region of the gradient (also upper frame). Little chemotactic activity was associated with the main peak of radioactivity.

Electrophoretic Separation of Plasmin-Generated Chemotactic Factor from Human C'3.—The treated human C'3 described above was electrophoretically fractionated in barbital buffer at a pH of 8.6. In a manner similar to that used for the rabbit preparation (Fig. 4), the human C'3 was separated into a main

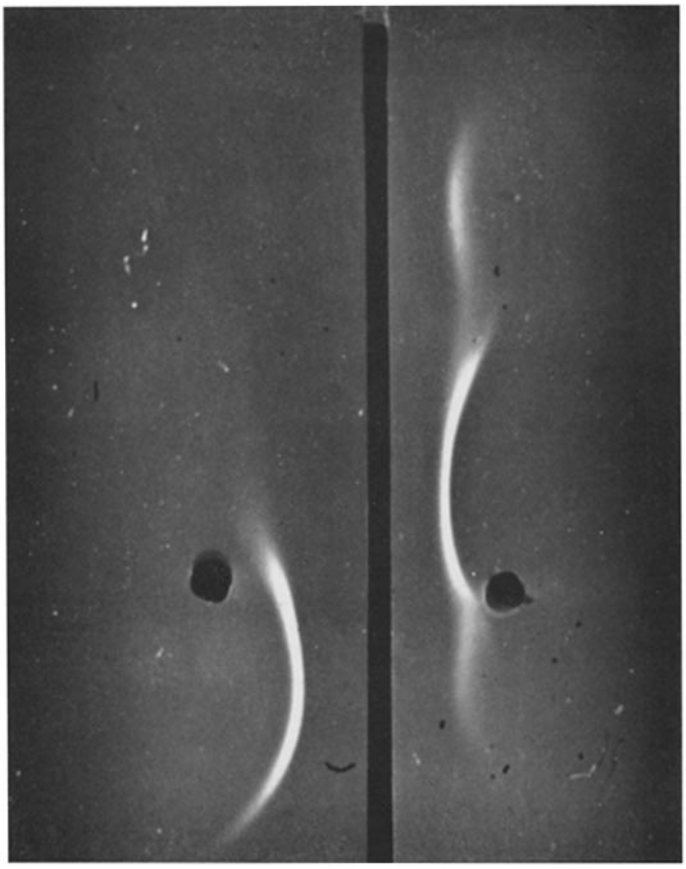

FIG. 6. Immunoelectrophoretic analysis of human C'3 before (left) and after treatment with Sk-Plm (right). Anode is at top.

peak of radioactivity and a minor, fast-moving peak, the latter accounting for about 4% of the total radioactivity (Fig. 8). Chemotactic testing revealed that chemotactically active fractions were sharply confined to a narrow region with rapidly migrating radioactive material (See fractions 15 and 16). The presence of radioactivity in these fractions indicated the presence of some altered portion of C'3, since untreated C'3 never gave such rapidly migrating material. Frac-

tions 9 and 15 were tested both for the ability to enhance vascular permeability in the skin of the rat and to cause the accumulation of leukocytes in rat skin. In Table II it is shown that fraction 15 induced vascular permeability as well as the accumulation of polymorphonuclear leukocytes (PMN's) in the rat, whereas fraction 9 possessed neither biologic activity.

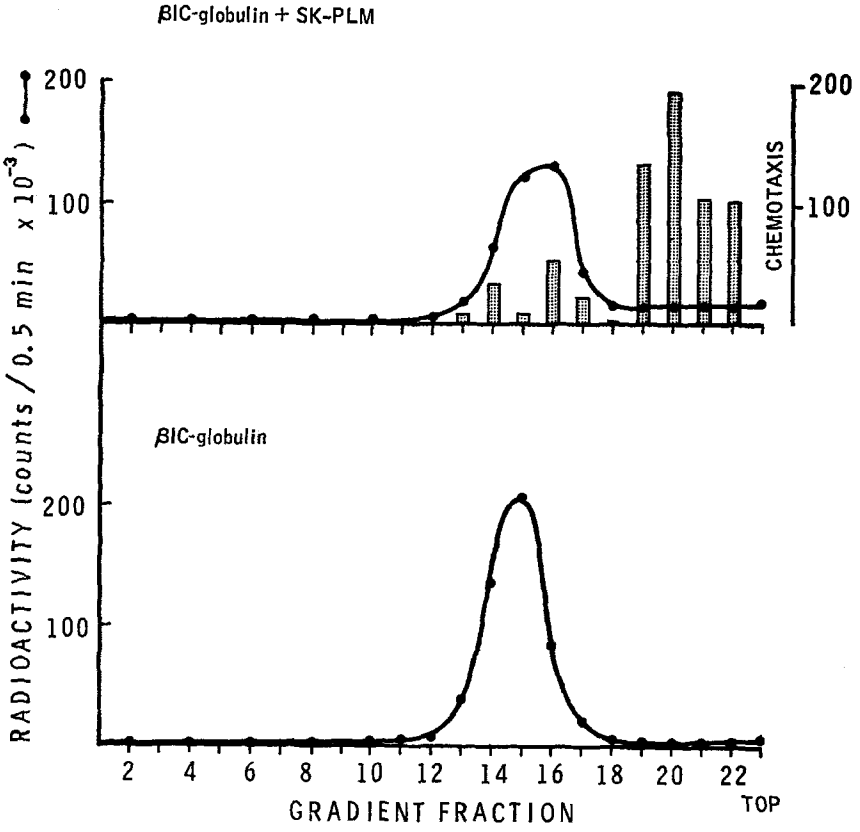

FIG. 7. Alteration in sedimentation pattern of C'3 (β1C-globulin) and location of chemotactic activity in density gradient fractions after treatment of C'3 with Sk-Plm.

Characterization of C'3 Fragments by Elution from Sephadex G-50.—An amount of Sk-Plm-treated C'3 with a radioactive marker similar to that described above was eluted from a Sephadex G-50 column with an acetate buffer at pH 5.0. This Sephadex column was initially calibrated for the elution profiles using cytochrome *c*, protamine, and glucagon. Immediately thereafter the treated C'3 was eluted. After this, and on the same day, the elution positions of cytochrome *c* and protamine were again determined and were found to correspond

within 0.7 ml to the initial values for elution positions. Fractions from the column containing C'3 were counted for radioactivity, neutralized, and tested for chemotactic activity. Radioactivity measurements indicated most of the material to be eluting in the void volume, followed by two discrete areas of radioactivity (Fig. 9). The first of these two areas was eluted with approximately 70 ml of buffer. The extrapolated molecular weight of this material was 12,000. The second, slightly biphasic peak, eluting in the position between 100 ml and 115 ml, contained material with an estimated molecular weight of 6,000.

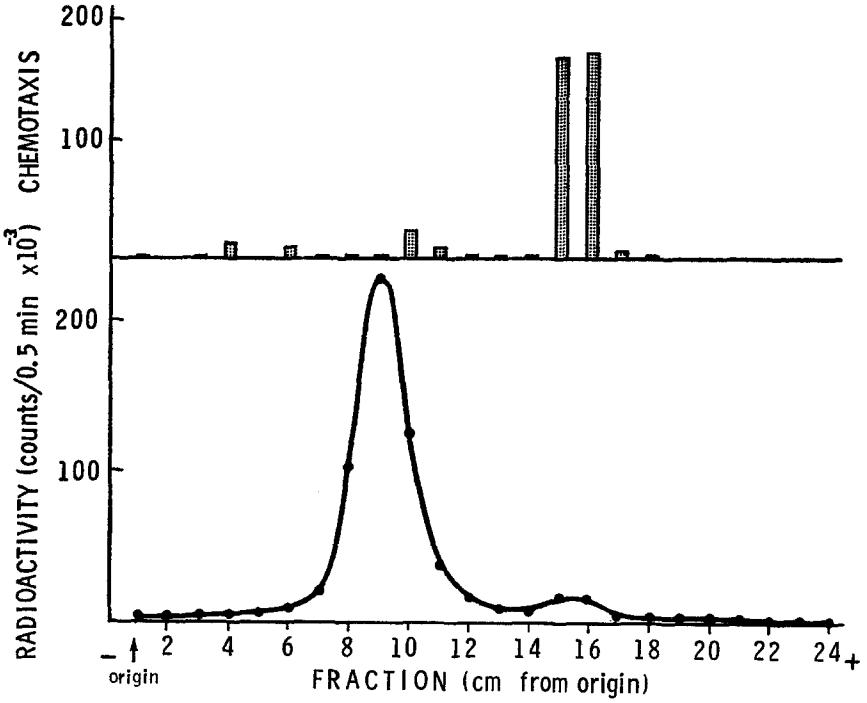

FIG. 8. Electrophoretic separation of human C'3 (lower frame) after treatment with Sk-Plm and localization of chemotactic activity (upper frame).

Approximately 4% of the radioactivity was contained in this zone. Chemotactic activity could be found *only* in the general area of this last zone of radioactivity (100 to 115 ml, Fig. 9). From these findings it was apparent that treatment of human C'3 with Sk-Plm resulted in a fragment of low molecular weight split from C'3 that was chemotactically active for rabbit PMN's.

DISCUSSION

That plasmin can cause cleavage of C'3 to release a fragment chemotactically active, both in chambers in vitro and in rat skin, indicates a property of plasmin

that has not been previously recorded. Although Ratnoff has recently reported the ability of plasmin to cause increased vascular permeability, neither the substrate nor the reaction product has been characterized (13). Nor is it clear from his data if accumulations of PMN's appeared after injection of plasmin into guinea pig skin. The present report may be relevant to both of these questions.

If the plasmin-split fragment of C'3 is important in the mobilization of PMN's in vivo, as the activated trimolecular complex of complement appears to be (3),[4] a second system for the regulation of PMN function has been defined. In animals in which one of the complement components of the trimolecular complex is genetically absent (C'5 in the mouse, reference 14, C'6 in the rabbit, reference 15), an alternative system for generating a chemotactic

TABLE II

Biologic Activities of the Plasmin-Split Fragment of Human C'3

Rat	Vascular permeability* (mm blueing)		PMN accumulation in rat skin‡	
	Fraction 9	Fraction 15	Fraction 9	Fraction 15
1	0	8	0	3+
2	3	8	±	2+
3	0	3	0	2+
4	0	7	Not done	Not done

* 0.5 ml of 1% Evans blue was injected intravenously, followed immediately by 0.1 ml of each fraction intradermally. In all experiments, electrophoretic fraction 9 (see Fig. 8) was diluted 1:3 to achieve a protein concentration comparable to that of fraction 15.

‡ Graded 0 to 4+ depending upon intensity of PMN infiltration (see reference 13).

factor would remain intact. Because of the requirement for C'3 in both systems, it would be of great interest if a genetic deficiency of C'3 were found in animals or man, in which case an additional chemotactic factor-generating system would be implied.

In Table III the two chemotactic factors are compared in terms of the requirements for generation and according to their physical-chemical characteristics. To what extent each factor is important in biological reactions in which the mobilization of PMN's occurs is not known. Under the usual conditions for activation of normal serum to produce the activated trimolecular complex, dialyzable chemotacitc activity is not found (2, 3, 4). No conclusions can be made about the situation as it applies in the intact animal.

In view of the trypsin-like activity of plasmin, the wide range of effects on the complement system is not surprising (16). In 1953, Pillemer and coworkers found that plasmin reduces the hemolytic activities of the second (C'2) and

[4] Ward, P. A. Unpublished observations.

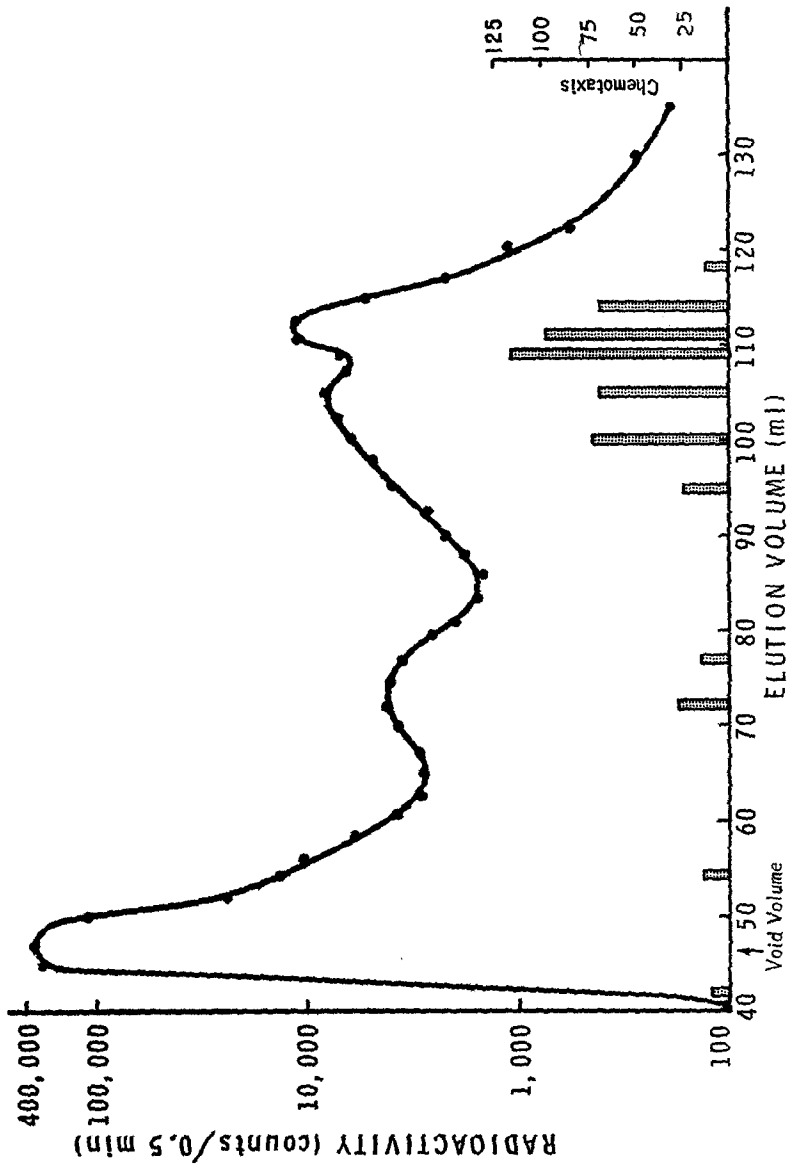

FIG. 9. Elution of human C'3 from Sephadex G-50 after treatment with Sk-Plm. Elution positions for chemotactic activity are given.

fourth (C'4) components of complement and, to a lesser extent, activities of the first (C'1) and third (C'3) components (17). In 1958, Lepow, Ratnoff, and Levy described activation of the proesterase of C'1 by plasmin (18). The present report indicates that the trimolecular complex (C'5, C'6, C'7) is destroyed through dissociation of its subunits and reduction in hemolytic activity of C'6. The susceptibility of this complex to degradation by plasmin may account for the very short persistence of the trimolecular complex in the circulation of rabbits after its generation by the injection of zymosan (3).

TABLE III

*Comparison of Two Chemotactic Factors**

Property	Complement-associated factor‡	Plasmin-generated factor
Requirement for generation	C'1–C'7	C'3
Molecular weight	>300,000	6000 (approximate)
Sedimentation velocity	rapid	slow
Antigenic composition	β1F-globulin + C'6 and C'7§	β1C-globulin fragment
Dialyzability	Does not pass through membrane	Passes through membrane
Heat stability (56°C, 1 hr)	stable	labile
Electrophoretic mobility	intermediate	fast

* This chart represents a compilation of data from this report as well as from references 2, 3, and 4.

‡ Trimolecular complex consisting of C'5, C'6, and C'7 (references 2 and 3).

§ Antigens of C'6 and C'7 have not yet been characterized.

Biologically active fragments of C'3 have recently been described that can apparently be differentiated from the plasmin-split fragment. Dias da Silva and Lepow report that anaphylatoxin is generated in free solution after interaction of the first four components of complement (8). The active material appears to be a fragment of C'3 that causes contraction of smooth muscle in guinea pig ileum and causes release of histamine from rat mast cells.[5] Müller-Eberhard has described the interaction of a cobra venom factor and serum cofactor re-

[5] Dias da Silva, W., and W. Lepow. Personal communication.

sulting in a complex that alters C'3 (19). In collaboration with Cochrane, he has found a C'3 fragment cleaved by the cobra venom factor–serum cofactor complex that causes the release of histamine from mast cells (20). In collaboration with Dias da Silva and Lepow, we have found that human anaphylatoxin lacks chemotactic activity.[6] Such a finding is not surprising in view of the earlier report that the intermediate complex of the first four reacting components of human or guinea pig complement on sensitized erythrocytes was not chemotactic (2, 3). In contrast to the properties of human anaphylatoxin, the plasmin-split fragment of C'3 is chemotactic but does not cause contraction of guines pig ileum.[5,6] Further studies to define the structural and biologic properties of these various C'3 fragments are indicated.

SUMMARY

When streptokinase and highly purified human plasminogen are added to human serum or to partly purified or highly purified preparations containing the third component of complement (C'3), either rabbit or human, a chemotactic factor is generated. This chemotactic factor is a split product of C'3 and is dialyzable, fast moving electrophoretically, slowly sedimenting in sucrose density gradient ultracentrifugation, and has an approximate molecular weight of 6000. It is calculated that this fragment accounts for approximately 4% of the intact molecule.

The C'3 fragment has the following biologic properties: It is chemotactic for rabbit PMN's in vitro, it causes accumulation of PMN's in vivo, and it increases vascular permeability in rat skin.

In addition to generating a chemotactic factor, plasmin destroys the complement-associated chemotactic factor that is a trimolecular complex consisting of the fifth (C'5), sixth (C'6), and seventh (C'7) components of complement. This has been shown by a loss of chemotactic activity, as well as a dissociation of the C'5, C'6, C'7 complex and a destruction of C'6 hemolytic activity.

The biologic significance of the plasmin-generated chemotactic factor is discussed in relation to other recently discovered biologically active fragments of C'3.

The author is indebted to Dr. Fletcher B. Taylor, Jr., University of Pennsylvania School of Medicine, who supplied the preparations of streptokinase and plasminogen.

Part of the work reported in this paper was carried out in the laboratories of Dr. Hans J. Müller-Eberhard and Dr. Charles G. Cochrane of the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. The author is indebted to Dr. Müller-Eberhard for the availability of his laboratory facilities and the supply of highly purified preparations of human C'3 and C'5, and to Dr. Cochrane for his assistance in the laboratory procedures.

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[6] Ward, P. A., W. Dias da Silva, and I. H. Lepow. Manuscript in preparation.

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