GENERATION OF CHEMOTACTIC ACTIVITY IN RABBIT SERUM BY PLASMINOGEN-STREPTOKINASE MIXTURES*

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(Received for publication 6 February 1967)

When plasminogen and streptokinase interact, a stable complex is formed which consists of plasminogen and streptokinase or the modified subunit plasmin and streptokinase (1). Depending on the ratio of plasminogen to streptokinase, two general enzyme activities may appear: the proteolytic or "plasmin" activity directed against substrates such as casein and fibrinogen (at mole ratios of 10:1), or "activator" activity directed against substrates such as plasminogen and synthetic amino acid esters (at mole ratios of 1:1 to 1:2) (2). Inasmuch as both activities exist in a given mixture of plasminogen and streptokinase and are presumed to arise from the same active site on plasmin (3), the overall or combined activities will be generally referred to as "plasmin" activity.

The effects of plasmin on the early reacting components of the complement system (C'1, C'4, C'2, C'3) were described in 1958 by Lepow, Ratnoff, and Levy (4). Because of the wide spectrum of enzyme activities attributed to plasmin, it was considered of interest to determine if plasmin had any effect on the complement system, particularly in terms of generation of chemotactic activity in serum. This paper describes the capacity of plasmin (activator form) to generate a chemotactic factor which can be readily distinguished from the previously described chemotactic factor appearing after interaction of the complement components (5, 6).[1] In addition, it was found that plasmin (activator form) destroys the biologic activity of the trimolecular complex.

* Supported by United States Public Health Service Grant HE 10, 907-01 and National Institutes of Health Grant AI 07291-01 AIA.

[1] In this paper the chemotactic factor which is generated by interaction of the first seven reacting components of complement is referred to as the "activated trimolecular complex," consisting of the fifth (C'5), sixth (C'6), and seventh (C'7) components of complement.

Materials and Methods

Plasminogen.—The proenzyme plasminogen was prepared from Cutter euglobulin plasminogen (lot #690-44) as described previously (3). The activity of this preparation was 140–150 Remmert and Cohen units per mg N. The material was stored as a lyophilized powder and reconstituted to a concentration of 1 mg/ml in 0.0025 N HCl and stored at 4°C. This reconstituted material was used within 5 days of preparation. On the day of each experiment the plasminogen was converted to plasmin with 100 μg (0.001 mg) streptokinase (SK) per 1 mg of plasminogen.

Streptokinase.—Highly purified streptokinase was prepared from Varidase (lot #107, 478 kindly supplied by Lederle Laboratories, Pearl River, N.Y.) by sequential chromatography and gel filtration on diethylaminoethyl (DEAE) cellulose and Sephadex G-100 (7). The DEAE, which was obtained from Carl Schleicher and Schuell Co., Keene, N. H., had a 0.91 meq/g binding capacity. The Sephadex G-100 was supplied by Pharmacia, Uppsala, Sweden. The specific activity of the streptokinase after purification was between 150 and 180 thousand Christensen units per mg protein. This material was stored as a lyophilized powder and reconstituted to a concentration of 1 mg/ml in 0.15 M NaCl and stored at 4°C for no longer than 5 days. The material used in the initial studies was less pure and less active (80,000 Christensen units per mg protein) than that described above. However, the streptokinase used in the studies involving the plasmin-SK ratio and sedimentation gradient analysis was of the grade described above.

Rabbit Euglobulin Fraction Rich in C'3, C'5, C'6, and C'7 (TEAE fraction).—In order to obtain a fraction rich in the third (C'3), fifth (C'5), sixth (C'6), and seventh (C'7) components of complement, rabbit euglobulin was eluted from triethylaminoethyl (TEAE) cellulose with a phosphate buffer as described elsewhere (5). The material then was concentrated by ultrafiltration. In some experiments the "activated" TEAE fraction was used. This consists of material which had been interacted with the first four reacting components of complement, thus rendering the trimolecular complex (C'5, C'6, C'7) chemotactically active (6, 7). The protein concentration of the TEAE fraction was 760 μg N/ml. In the experiments where the TEAE fraction was treated with the plasmin-SK complex before testing for chemotaxis or density gradient analysis, 0.5 ml at pH 7.3 was mixed with 160 μg SK and 80 μg plasmin, followed by incubation at 37°C for 2 hr.

Activated Rabbit Serum.—When the complement-associated chemotactic factor of whole serum was used, it was generated in fresh rabbit serum by the addition of an immune complex consisting of antibody to bovine serum albumin with antigen added to equivalence. 100 μg antibody (as IgG) was added for each 0.1 ml of rabbit serum. After incubation at 37°C for 1 hr, the precipitate was removed by centrifugation. The supernate constituted the "activated rabbit serum." The details of the procedure are described elsewhere (5).

C'6-Deficient Rabbit Serum.—In one series of experiments, rabbit serum genetically deficient in the sixth component was employed. This was kindly supplied by Dr. Klaus Rother, New York University School of Medicine, New York, N.Y.

Chemotaxis.—Stainless steel chambers with a micropore filter separating the two compartments were used as described previously (5). This is a modification of Boyden's original chamber (8). Rabbit polymorphonuclear leukocytes were obtained from the glycogen-induced peritoneal exudate. Quantitation of chemotaxis was determined by counts of cell numbers migrating through the micropore filter (5).

Assay for C'6.—As described in an earlier publication (5), rabbit C'6 was assayed by the use of rabbit serum genetically deficient in C'6. In this assay, a sample of material to be tested was added to a suspension of sensitized sheep erythrocytes, and a 1:15 dilution of C'6-deficient rabbit serum in Veronal buffer was added. C'6 is thus defined by its restorative capacity in this hemolytic system.

Dialysis.—When serum was to be dialyzed after treatment with plasmim-SK or immune complexes, 0.2 ml to 1.0 ml was dialyzed in 200 ml of medium 199 at 5°C for 18 hr.

Ultracentrifugation.—A 10–40% sucrose density gradient was used to study the velocity of sedimentation of the plasmin-SK generated chemotactic factor. The sucrose was dissolved in phosphate buffer at pH 7.3, ionic strength 0.02 and ultracentrifugation of the 250 μl sample (TEAE fraction treated with plasmin-SK) carried out at 35,000 rpm for 18 hr. This procedure has been described previously (5, 6).

TABLE I

Generation of Chemotactic Activity in Rabbit Serum by Plasminogen-Streptokinase (SK)

Experiment*	SK	Plasminogen	Serum	Chemotactic value
	μg	μg	100 μl	
A	—	—	—	10
	—	—	+	37
	100	20	+	145
	100	20	—	58
	100	—	+	49
	—	20	+	46
B	—	—	—	38
	—	—	+	5
	200	20	+	288
C	—	—	+	33
	160	—	+	49
	—	80	+	46
	160	80	+	209
	160	80	+‡	75

* Reaction volumes of 0.3 ml were incubated for 30 min at 37°C before testing for chemotaxis.

‡ ϵ-Aminocaproic acid (100 μg/ml serum) was dissolved in serum before addition of plasminogen-streptokinase.

RESULTS

Generation of Chemotactic Activity in Rabbit Serum by Plasmin.—When streptokinase and plasminogen in weight ratios of 5:1 and 10:1 were added to fresh rabbit serum, chemotactic activity appeared which could not be ascribed to the serum alone or the streptokinase-plasminogen mixture (Table I, A, B). It was also clear that chemotactic activity could not be generated in serum if either streptokinase or plasminogen was missing (Table I). ϵ-Amino caproic acid (EACA), if present as 100 mg/ml in the reaction mixture, could prevent generation of more than 60% of the chemotactic activity (Table I, C). In data not presented here, it was found that this compound had no effect on the activity of the preformed chemotactic factor generated after the addition of streptokinase and plasminogen to serum.

Generation of Chemotactic Activity as a Function of the Ratio of Streptokinase and Plasminogen.—Varying the ratio of streptokinase to plasminogen was found to have a significant effect on the generation of chemotactic activity. A 2:1 ratio of streptokinase to plasminogen (mole ratio of 2.8:1) was optomal for this effect (Table II). Progressive changes of this ratio in either direction resulted in a progressive diminution in the appearance of chemotactic activity. When 100 μg streptokinase was added with 1 μg plasminogen, little activity was found (chemotactic value of 60), indicating little activation of any endogenous plasminogen in the rabbit serum by streptokinase.[2] At the other extreme, with 1 μg of streptokinase and 100 μg of plasminogen, only about 20% of the peak chemotactic activity was generated.

TABLE II

Effect of Varying Ratios of Plasminogen to Streptokinase on the Generation of Chemotactic Activity from Normal Rabbit Serum

SK	Plasminogen	Ratio	Chemotactic value
μg	μg		
1	100	1:100	80
10	100	1:10	54
50	100	1:2	66
75	100	1:1.25	91
100	100	1:1	120
100	100	1:1	122
100	75	1.25:1	158
100	50	2:1	279
100	10	10:1	170
100	1	100:1	60
—	—	—	5*

* Value of normal rabbit serum control (untreated).

Serum Factors Involved in Generation of Chemotactic Activity by Plasminogen-Streptokinase.—In an attempt to define the factors in serum responsible for generation of chemotactic activity by plasminogen-streptokinase, several preparations were used. Serum which had been heated for 1 hr. at 56°C did not support generation of chemotactic activity, indicating the requirement for a heat-labile material (Table III, B). As far as could be determined, C'6 was not a requirement, in that rabbit serum genetically deficient in C'6 supported generation of the chemotactic factor (Table III, D). (The fact that the count of 251 for this preparation is below the count of 444 for the normal serum is difficult to evaluate, since serums vary in their ability to generate chemotactic activity.) The TEAE fraction of rabbit euglobulin rich in C'3, C'5, C'6, and C'7 served

[2] Streptokinase activates human plasminogen, but does not normally activate plasminogen of other animal species.

as a suitable material for generation of the chemotactic activity, suggesting the possible role of C'3, C'5, or C'7 as a substrate of plasmin in this reaction (Table III, C).

Physical-Chemical Characteristics of Two Chemotactic Factors.—The complement associated chemotactic factor was generated by incubation of fresh rabbit serum with immune complexes, leading to activation of the trimolecular complex (5). Serum from the same donor was also tested for activity after generation of chemotactic activity by plasmin. Dialysis or heating the sample containing the activated trimolecular complex had no effect on the activity, whereas

TABLE III

Requirement of Serum Factors for Plasminogen-Streptokinase (SK) Generation of Chemotactic Activity

Material tested*	Plasminogen	SK	Chemotactic value
	μg	μg	
A. Normal rabbit serum			
100 μl	—	—	48
100 μl	40	100	444
B. Normal rabbit serum (heated 56°C for 1 hr)			
100 μl	—	—	29
100 μl	40	100	16
C. TEAE fraction‡ (rich in C'3, 5, 6, & 7)			
100 μl (72 μgN)	—	—	22
100 μl (72 μgN)	40	150	385
D. C'6-Deficient rabbit serum			
100 μl	—	—	48
100 μl	40	100	251

* Reaction volumes of 0.3 ml were incubated at 37°C for 1 hr prior to testing for chemotactic activity.

‡ Eluate of rabbit euglobulin from TEAE cellulose, rich in the third, fifth, sixth, and seventh components of rabbit complement (7).

the same treatment of the plasmin-generated chemotactic factor caused 42-48% loss of activity (Table IV). On the basis of these data, there appeared to be clear differences in the two chemotactic factors.

Ultracentrifugal Studies of the Plasmin-Generated Chemotactic Factor.—In order to compare the plasmin-generated chemotactic factor with the activated trimolecular complex, ultracentrifugation of plasmin-treated rabbit serum after plasmin treatment in a sucrose density gradient was carried out. Chemotactic activity was sharply confined to the uppermost portion of the gradient (Fig. 1, tubes 20, 22), indicating a slow velocity of sedimentation for this factor, in sharp contrast to the much more rapid sedimentation of the activated trimolecular complex (5, 6). It was of interest to note that little C'6 activity remained in any

TABLE IV

Comparison of Two Chemotactic Factors for Heat Stability and Dialyzability

Substance tested	Treatment	Chemotactic value	% Reduction of chemotactic activity
NRS	—	44	
NRS + SK + Plasmin*	—	287	
Act. NRS‡	—	209	
NRS	Dialysis vs. medium 199 for 18 hr	71	None
NRS + SK + Plasmin		180	42
Act. NRS		228	None
NRS	Heated at 56°C for 1 hr	80	None
NRS + SK + Plasmin		165	48
Act. NRS		227	None

* 1 ml of normal rabbit serum (NRS) plus 1 mg SK plus 500 μg plasminogen were incubated at 37°C for 2 hr. 100 μl of serum were used for chemotactic testing.

‡ Normal rabbit serum was treated with immune complexes as described previously (7).

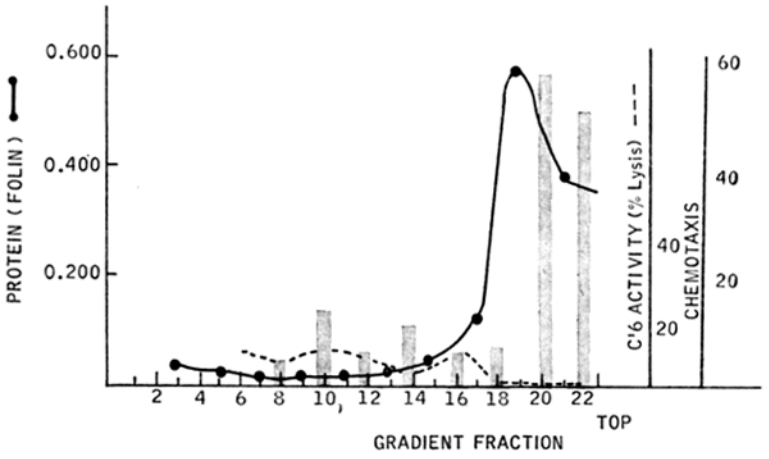

FIG. 1. Sucrose density gradient analysis of the chemotactic factor generated by addition of streptokinase-plasminogen to the rabbit euglobulin eluate from TEAE cellulose. 250 ml of sample was analyzed in the ultracentrifuge after incubation of 160 μg streptokinase and 80 μg plasminogen with 500 μl TEAE fraction (380 μgN) at 37°C for 2 hr.

portion of the gradient. In addition, most of the protein measurable by the Folin technique was confined to the upper portion of the gradient.

Destruction of Chemotactic Activated Trimolecular Complex by Plasmin.—In two different experiments the ability of plasmin to destroy the chemotactic ac-

TABLE V

Capacity of Plasminogen-Streptokinase to Inactivate the Chemotactic Factor Generated through the Complement System by Immune Complexes

Material tested	Plasminogen	Streptokinase	Chemotactic value
	μg	μg	
Activated rabbit serum Experiment A			
100 μl	—	—	117
100 μl	20	100	36
Activated rabbit serum Experiment B			
100 μl	—	—	270
100 μl	20	200	45
Activated TEAE fraction*			
100 μl (72 μgN)	—	—	185
100 μl (72 μgN)	40	150	58

* TEAE eluate of rabbit euglobulin, rich in C'3, C'5, C'6, and C'7, treated with the first four reacting components of rabbit complement in order to "activate" the trimolecular complex of complement (C'5, C'6, & C'7) to the chemotactically active material. See reference 7 for details.

TABLE VI

Capacity of Plasminogen-Streptokinase to Inactivate the Chemotactic Factor Generated through the Complement System by Immune Complexes

Plasminogen	Streptokinase*	Ratio	Chemotactic value
μg	μg		
1	100	1:100	22
10	100	1:10	22
50	100	1:2	0
75	100	1:1.25	0
100	100	1:1	15
100	100	1:1	9
100	75	1.25:1	22
100	50	2:1	40
100	10	10:1	62
100	1	100:1	126
—	—	—	146‡

* Total reaction volume for each preparation was 300 μl with incubation at 37°C for 30 min before testing.

‡ Chemotactic value of 10% rabbit serum previously activated with immune complexes. See reference 5 for details.

tivity of the activated trimolecular complex was demonstrated. Rabbit serum activated by the addition of immune complexes lost from 70 to 83% of its chemotactic activity after treatment with plasmin (Table V). A similar loss of chemotactic activity occurred in the TEAE fraction of rabbit euglobulin containing the activated trimolecular complex. From these results, it was also clear that no significant chemotactic activity was generated by plasmin in serum which had been previously incubated with an immune complex.

When investigations were undertaken to determine the optimal ratio of plasminogen to streptokinase for destruction of chemotactic activity expressed by the trimolecular complex, it was found that a ratio of streptokinase to plasminogen of 1:2 resulted in complete loss of chemotactic activity (Table VI). This was the same ratio found to be optimal for plasmin generation of chemotactic activity in normal serum (Table II).

DISCUSSION

The results of these studies show that generation of chemotactic activity in rabbit serum by plasmin requires both plasminogen and streptokinase. Neither of these factors alone was sufficient for development of chemotactic activity. The finding that EACA inhibited generation of the activity by plasminogen-streptokinase further supported the role of plasmin and/or "activator" activities, both of which are inhibited by EACA (3). It should be noted that plasmin can assume two activities or forms (2). Plasmin in the presence of small amounts of streptokinase (amounts which are sufficient to activate plasminogen to plasmin) exhibits a high affinity for casein and fibrinogen and a low affinity for plasminogen and synthetic substrates. This activity is defined as "plasmin" activity. However, plasmin in the presence of equimolar or greater amounts of streptokinase forms a stable complex (1) which has a low affinity for casein and fibrinogen and a high affinity for plasminogen and synthetic substrates. Because of its high affinity for plasminogen this activity or form of plasmin is called "activator" (2). It is believed that both of these activities arise from the same active site (3) and that streptokinase modifies the original plasmin activity by virtue of formation of a complex. For, it has been demonstrated that the original plasmin activity (i.e., the fibrinolytic or caseinolytic property), can be recovered upon dissociation of the plasmin-SK complex at pH 3.0. Thus plasmin appears to have a sliding scale of specificity as a function of streptokinase concentration.

The question as to which of the two activities was involved in generation of the chemotactic factor was approached by the studies described in Table II in which the ratios of plasminogen and streptokinase were varied. It became apparent that in order to generate chemotactic activity in rabbit serum, plasmin must be in the "activator" form, not in its "plasmin" form. The same relationship was found for destruction by plasmin of the preformed chemotactic factor in the form of activated trimolecular complex (Table VI).

The question as to what substance in rabbit serum was serving as a substrate for plasmin was approached by the use of heated serum and C'6-deficient serum, as well as utilizing fractions rich in certain complement components. The fact that C'6-deficient serum supported generation of chemotactic activity by plasmin stands in marked contrast to the inability of this serum to support generation of chemotactic activity upon addition of an immune precipitate (5). The identification of the critical substrate and the characterization of the reaction product and will be described in a succeeding paper.[3]

A physical-chemical study by sucrose density gradient ultracentrifugation of the plasmin-generated chemotactic factor led to the conclusion that the activity was associated with a slowly sedimenting fragment of low molecular weight (Fig. 1). This was in accord with the reduction in chemotactic activity of plasmin-treated serum after dialysis (Table IV). Rather unexpected, however, was the relative heat lability of this material (Table IV).

The observation that plasmin inactivates the trimolecular complex generated in rabbit serum following addition of immune complexes is yet another manifestation of the broad range of effects by plasmin of the complement system. Not only can the enzyme inactivate several of the complement components (4 and footnote 3) it can also activate the proesterase in the first component of complement (4). A succeeding paper[3] will describe the product of yet another action of plasmin in the complement system—generation of a chemotactic factor (4).

The above-described effects of streptokinase, a foreign protein, on plasmin constitute an observation of how a foreign protein might subvert or redirect the natural specificity of an enzyme normally present in plasma without necessarily involving an immune mechanism. However, the relevance of such an observation, either specifically or generally, to the natural in vivo condition is not known.

SUMMARY

The addition of plasminogen and streptokinase to rabbit serum results in appearance of chemotactic activity which is relatively heat-labile and dialyzable. Generation of this activity requires heat-labile factors in serum, but not the sixth component of complement. The optimal mole ratio for generation of chemotactic activity is 1:1 to 1:3 for plasminogen to streptokinase. Neither material alone is sufficient to generate the activity in fresh serum. Generation of chemotactic activity can be blocked by the presence of ϵ-aminocaproic acid. This plasmin-generated chemotactic factor sediments slowly in a sucrose density gradient during ultracentrifugation.

The addition of plasminogen and streptokinase to rabbit serum containing the *preformed* chemotactic factor (activated trimolecular complex of C'5, C'6,

[3] Ward, P. A. A plasmin-split fragment of C'3 as a new chemotactic factor. In preparation.

C'7) causes destruction of the chemotactic factor and loss of hemolytic activity of the sixth component of complement. The same mole ratio of reactants for optimal expression of the phenomenon holds as for plasmin generation of chemotactic activity in untreated serum.

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