C3 LEUKOTACTIC FACTORS PRODUCED BY A TISSUE PROTEASE*

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(Received for publication 29 April 1969)

Certainly the most common type of tissue injury to the body involves the almost daily insults induced nonspecifically by mechanical means. Tissue injury, whether it be in the form of an abrasion, a laceration, or a large area of severely damaged tissue, almost invariably is associated with the onset of the acute inflammatory response, which not only provides a protective shield against infection but also marks the first in a series of events leading to the intricate process of healing. The reason for the development of the acute inflammatory response under such conditions is completely obscure.

Hurley in 1964 presented evidence that when various tissues of the rat were minced and individually incubated with rat serum, a factor chemotactic for neutrophilic granulocytes appeared (1). He postulated that the interaction of a substance in tissue with a factor in serum resulted in the generation of a chemotactic product, the nature of which was not defined. The following studies indicate that many tissues of the rat contain a protease, trypsin-like in nature, which can cleave the third component of complement (C'3) into chemotactically active fragments. Not only does this provide a possible explanation for the pathogenesis of the acute inflammatory response to injured tissue, but it emphasizes once again a role for the complement system in the generation by nonimmunological means of phlogistic factors capable of triggering the acute inflammatory response.

Materials and Methods

Chemotaxis.—The micropore filter method was used, with chambers that have been described previously (2). Rabbit neutrophils from a glycogen-induced peritoneal exudate were

* This work was supported in part by grant AI-07291-AIA from the National Institutes of Health, Bethesda, Md. 20014, under the auspices of Universities Associated for Research and Education in Pathology, Inc. Sections of this paper were presented at the 53rd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April, 1969. *Fed. Proc.* 1969. **28:** 485.

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‡ Supported by the Ohio Division of the American Cancer Society.

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

Tissue Minces.—150 g adult male Wistar rats were anesthetized with ether and exsanguinated from the inferior vena cava. Wet fragments of various organs or tissues were rinsed in saline, weighed, and diced with a razor blade on a glass plate. Unless otherwise indicated, tissue minces were incubated in saline, fresh rat serum, or preparations of purified human complement components for the appropriate interval of time at 37°C. The tissue was then removed by centrifugation, and the supernatant was tested for chemotactic activity.

Complement Preparations.—Preparations of highly purified human complement consisting of the third (β_{1C}-globulin or C3) and fifth (β_{1F}-globulin or C5) components were obtained according to the methods of Nilsson and Müller-Eberhard (3). Antibody to rat C3 (anti-C3 or anti-β_{1C}) was obtained by appropriate immunization of rabbits (4). Rat serum was treated with KSCN according to the method of Dalmasso and Müller-Eberhard (5) in order to deplete C3 and C5 activity. When ethylenediaminetetraacetate (EDTA) (Aldrich Chemical Co., St. Louis, Mo.) was employed, appropriate dilutions were made from a 0.02 M stock solution, pH 7.3.

Ultracentrifugal Analysis.—Purified human C3 treated with heart tissue was analyzed by ultracentrifugation in a sucrose density gradient. Centrifugation was performed at 55,000 rpm for 15 hr (4°C) in sucrose (5–35%) dissolved in phosphate buffer (pH 7.3, ionic strength 0.05). Details of this procedure are described elsewhere (2, 6).

Gel Filtration.—The same tissue-treated C3 preparation used for density gradient analysis was also eluted from Sephadex G-75 (Pharmacia, Piscataway, N.J.) with phosphate buffer (pH 7.3, ionic strength 0.05), and the fractions were scanned for chemotactic activity. Protein or peptide markers in the density gradients included bovine serum albumin, cytochrome *c* (both from Mann Research Laboratories, New York, N. Y.), and glucagon (Eli Lilly and Co., Indianapolis, Ind.).

Enzyme Studies.—Crystallized trypsin and chromatographically purified soybean trypsin inhibitor were products of Mann Research Laboratories. Each was dissolved in phosphate-buffered saline (pH 7.3) immediately before use. Organophosphonate compounds of the type previously used in chemotaxis experiments (7) and employed under a variety of experimental conditions in which inhibition of serine esterases is desired (8) were kindly supplied by Dr. Elmer L. Becker (Walter Reed Army Institute of Research). The homologous phosphonate series used included the ω-chloroalkyl *p*-nitrophenyl ethyl phosphonates containing alkyl chains of 3, 4, 5, or 6 carbon atoms with the chloro group in the terminal carbon atom. It is well known that these compounds inhibit many types of esterases, including trypsin, chymotrypsin, and choline esterases, each of which has a serine atom at or near the active site of the enzyme (8). Heart tissue was pretreated with the appropriate phosphonate dissolved in phosphate-buffered saline (pH 7.3) in a volume of 0.5 ml. After incubation at room temperature for 20 min, tissues were diluted to 8 ml with buffered saline and washed twice before incubation with rat serum.

Competitive substrate inhibition was studied using heart tissue, which contains a protease capable of hydrolyzing rat or human C3. In order to determine whether a defined ester could competitively inhibit this reaction, various synthetic amino acid esters were added with the substrate (consisting of purified human C3 or C3 in rat serum), mixed with heart tissue, and incubated at 37°C for 90 min. To provide adequate controls in this experiment, esters were added either at the *beginning* or at the *end* of the incubation period. The tissue was then re-

moved by centrifugation, and the supernatant was tested for chemotactic activity. The following synthetic esters, obtained from Mann Research Laboratories, New York, N. Y., were used: *p*-tosyl-L-arginine methyl ester HCl (TAMe), M.A. (Mann assayed); benzoylarginine methyl ester hydrochloride (BAMe), M.A.; *N*-acetyltyrosine ethyl ester, M.A.; glycylglycine ethyl ester; and glycine ethyl ester.

RESULTS

Ability of Various Tissues to Generate Chemotactic Activity in Serum.—Six different tissues from rats were tested for their ability to generate chemotactic activity in fresh autologous rat serum. In the experiments to be described, any activity found in untreated rat serum or in supernatants of tissue incubated in

TABLE I

Ability of Various Rat Tissues to Generate Chemotactic Activity in Rat Serum

Tissue*	Chemotactic activity‡	
	Exp. A	Exp. B
Heart	300	300
Lung	120	35
Kidney	0	180
Liver	200	0
Spleen	320	100
Skeletal muscle	0	

* 10 mg tissue/0.1 ml normal rat serum, 37°C, for 90 min.

‡ Chemotactic values have been corrected for background activity due to tissue by itself and serum by itself.

saline was subtracted from the values obtained by incubating tissue in serum. In general, such control values were less than 20% of activity found in serum incubated with tissue. In two different experiments it became evident that most tissues were capable of generating chemotactic activity in rat serum (Table I). There was some variability between liver and kidney from one experiment to the next, but in several subsequent experiments both tissues generated chemotactic activity in rat serum, kidney tending to be more active. Heart tissue has consistently been the most effective tissue for generation of chemotactic activity in serum, whereas skeletal muscle has shown little or no ability. As a general rule, it can be stated that most tissues of the rat are able to generate chemotactic activity in homologous serum.

Chemotactic Activity as a Function of Dose of Heart Tissue and Rat Serum.—In order to define the features of this chemotactic factor–generating system, the amount of serum was varied, maintaining a constant reaction volume of 0.3 ml (phosphate-buffered saline used as necessary) with 25 mg heart tissue. Fig. 1 shows that the amount of chemotactic activity appearing in the supernatant

was related to the amount of serum used. Maximal activity was achieved at 0.2 ml, with a drop-off in chemotactic activity when 0.3 ml serum was used. Fig. 2 indicates that with a constant amount of serum, the chemotactic activity appearing was a function of the amount of heart tissue used, 40 mg/0.1 ml serum giving maximal activity, whereas the largest dose of heart tissue (50 mg/0.1 ml serum) resulted in considerably less chemotactic activity. The reason for this reduction in activity is not known.

Chemotactic Activity as a Function of Duration of Incubation.—The amount of chemotactic activity generated by the interaction of heart tissue with serum was directly related to the duration of incubation of heart tissue with rat serum

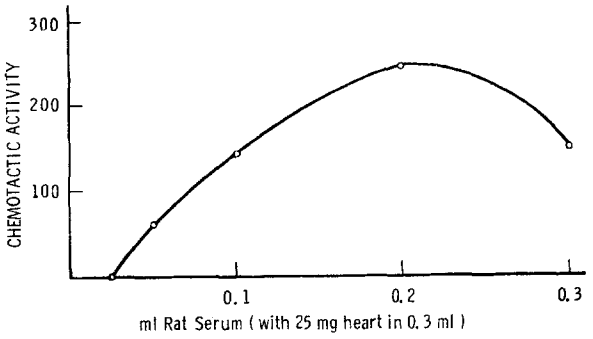

FIG. 1. Chemotactic activity as a function of the amount of serum (milliliters), using 25 mg heart tissue in a total volume of 0.3 ml.

at 37°C (Fig. 3, experiments A and B). Maximal activity appeared in the supernatant after an incubation period of 60–90 min. Shorter periods of incubation resulted in less activity, and prolongation of the period beyond 90 min did not result in additional chemotactic activity. Also shown in this figure is the ability of heart tissue to react with human C3 to give chemotactic activity. In this case, a fairly abrupt reduction in activity occurred if the period of incubation was extended beyond 90 min. The ability of C3 to substitute for serum is discussed in following sections.

Requirements of Serum in Generation of Chemotactic Activity.—In order to find out more about the way in which heart tissue interacted with rat serum to result in chemotactic activity, several manipulations were performed. The controls, consisting of supernatants from heart tissue incubated in saline or serum, and serum by itself, as well as heart-serum-EDTA, are listed in Table II. Heating of rat serum before or after incubation with heart tissue precluded the appearance of, or resulted in the disappearance of, chemotactic activity. Compelling evidence that the chemotactic factor was related to C3 was provided

by the finding that anti-C3 (β_{1C}-globulin) abolished chemotactic activity regardless of whether the antibody was added at the beginning or the end of the incubation period. However, the fact that EDTA in serum caused a 3-fold

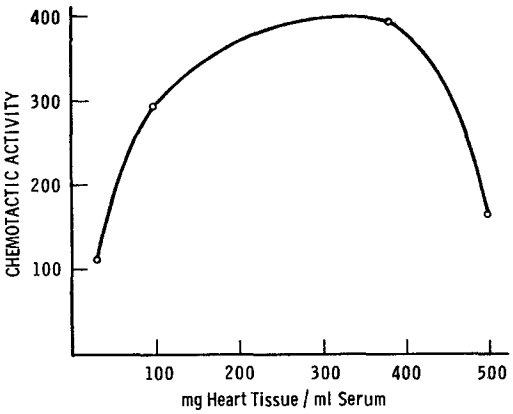

FIG. 2. Chemotactic activity as a function of the amount of heart tissue, with a constant amount of rat serum. 0.1 ml serum was tested for chemotactic activity.

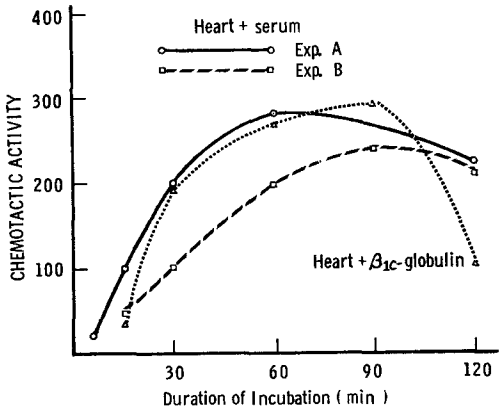

FIG. 3. Chemotactic activity as a function of the duration of incubation of 10 mg heart tissue in 0.1 ml serum or 0.1 ml (100 μg) human C3 (β_{1C}-globulin).

enhancement in chemotactic activity indicated that conventional triggering of the complement system (requiring the first and the second components of complement) was not occurring. The mechanism of enhancement of chemotactic activity by EDTA has not been determined. It also became apparent that the presence of trypsin inhibitor in the system precluded generation of the chemotactic factor (Table II), suggesting the role of a proteolytic enzyme.

Additional evidence that C3 was involved in generation of chemotactic activity is presented in Table III. Here the enhancing effect of EDTA in development of chemotactic activity is again seen. Rat serum treated with

TABLE II

Conditions for Generation of Chemotactic Activity in Serum

Preparation*	Chemotactic activity
Heart + saline	3
Heart + rat serum	125
Rat serum	35
Rat serum + EDTA (0.02 M)	16
Heart + heated rat serum (56°C, 30 min)	35
Heart + rat serum, then heated	45
Heart + rat serum + 50 μl anti-rat C3 added:	
Before incubation	20
After incubation	35
Heart + rat serum + EDTA (0.02 M)	350
Heart + rat serum + trypsin inhibitor (50 μg)	40

* 20 mg heart tissue, 0.1 ml serum, incubation for 90 min at 37°C.

TABLE III

Evidence That C3 is Substrate for Tissue Protease

Preparation tested	Chemotactic activity
Experiment A	
Heart + saline	0
Heart + serum	160
Heart + serum + EDTA (0.02 M)	490
Serum	0
Heart + KSCN serum	0
Heart KSCN serum + 100 μg C3	190
Experiment B	
Heart + 50 μg C3	270
50 μg C3	0
Heart + 50 μg C3 + trypsin inhibitor (50 μg)	0
Heart + 50 μg C3 + 0.02 M EDTA + trypsin inhibitor:	
At beginning	17
At end	400
Heart + 50 μg C5	0

KSCN in order to inactivate C3 and C5 failed to support generation of activity (experiment A). However, the addition of 100 μg human C3 corrected this deficiency. That C3 alone, but not C5, was able to support generation of chemotactic activity is shown by experiment B, Table III. The addition of trypsin inhibitor to the tissue-C3 mixture blocked the appearance of chemo-

tactic activity, as was the case when serum instead of C3 was used (Table II). And, just as EDTA enhanced the amount of chemotactic activity in serum produced by heart tissue, it also enhanced the ability of heart tissue to generate chemotactic activity in purified C3. Using the heart-C3-EDTA system, trypsin inhibitor was effective only if added at the beginning of the incubation period. Little effect was found if the inhibitor was added at the end of the incubation time (Table III). In line with the effect of anti-C3 on chemotactic activity, the specificity for C3 in the reaction became apparent when it was found that incubation of human C5 (β_{1F}-globulin) with heart tissue was unproductive of chemotactic activity (Table III, experiment B).

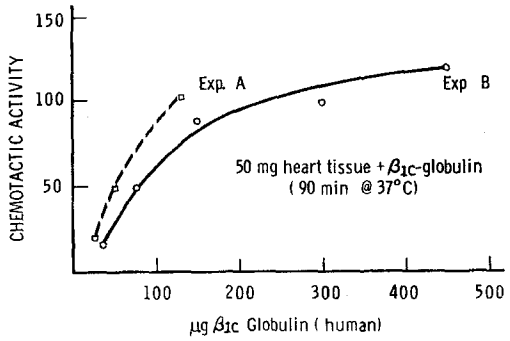

FIG. 4. Dose response of chemotactic activity appearing in heart tissue–human C3 (β_{1C}-globulin) mixture.

Dose Response of C3 Incubated with Heart Tissue.—Much in accord with what was found with rat serum (Fig. 1), the amount of human C3 (β_{1C}-globulin) added to heart tissue determined the amount of chemotactic activity appearing. In two different experiments, parallel dose-response curves were obtained (Fig. 4, experiments A and B). In experiments not reported here, kidney, spleen, lung, and liver also had the ability to generate chemotactic activity when incubated with human C3, indicating that this is a common feature of the tissue studied. We have also had the opportunity to study fresh human tissues from the various organs and have found them also to be active in generating chemotactic activity either in autologous serum or in purified C3.

Identification of Chemotactic Factor as a Split Product of C3.—The foregoing studies strongly suggested that the chemotactic factor generated by interaction of heart tissue with serum was a product of C3, possibly a cleavage product produced by the action of a proteolytic enzyme in heart tissue. In order to assess this possibility, C3 was incubated with heart tissue (in the same manner described in Fig. 4), and the supernatant was subjected to analysis by gel filtration

and ultracentrifugation. Sucrose density gradient analysis of the treated C3 revealed a heterogeneity of chemotactic factors produced by incubation with heart (Fig. 5, middle frame) or heart-EDTA (Fig. 5, bottom frame), with chemotactic activity appearing in the general position determined by a cytochrome c marker (Fig. 5, top frame) and a zone of more rapidly sedimenting

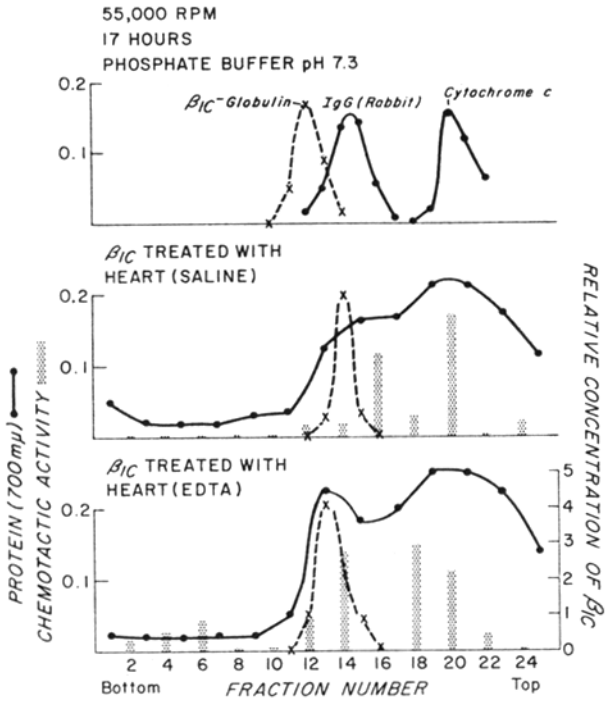

FIG. 5. Sucrose density gradient analysis of human C3 treated with heart tissue, showing positions of chemotactic activity (vertical bars), C3, and protein standards. C3 was quantitated by immunodiffusion in agar gel.

activity which varied according to the preparation. It also became apparent that the appearance of chemotactic activity could be related to reduction in the velocity of sedimentation of C3. Calculations revealed a drop in the known sedimentation rate of 9.5 for C3 to values between 8.5 (bottom frame of Fig. 5) and 7.4 (middle frame of Fig. 5). This reduction seems to depend upon particular conditions of the experiment and is in line with the concept that the chemotactic factor is a fragment split from the native C3 molecule.

More rigorous study of the C3 cleavage products with chemotactic activity was carried out by filtration of the treated C3 in Sephadex G-75 (Fig. 6). Heterogeneity of chemotactic activity was confirmed with the finding of zones

of chemotactic activitv coinciding with the cytochrome marker as well as activity in a position between the bovine serum albumin and cytochrome c markers. Using the Andrews plot to estimate molecular weight (9), the approximate weights of these C3 fragments were 30,000 and 14,000. In repeated experiments of this kind, chemotactic activity has been found consistently in the vicinity of the cytochrome marker, whereas the other zone, representing a less retarded factor in gel filtration, has varied between the void volume and the

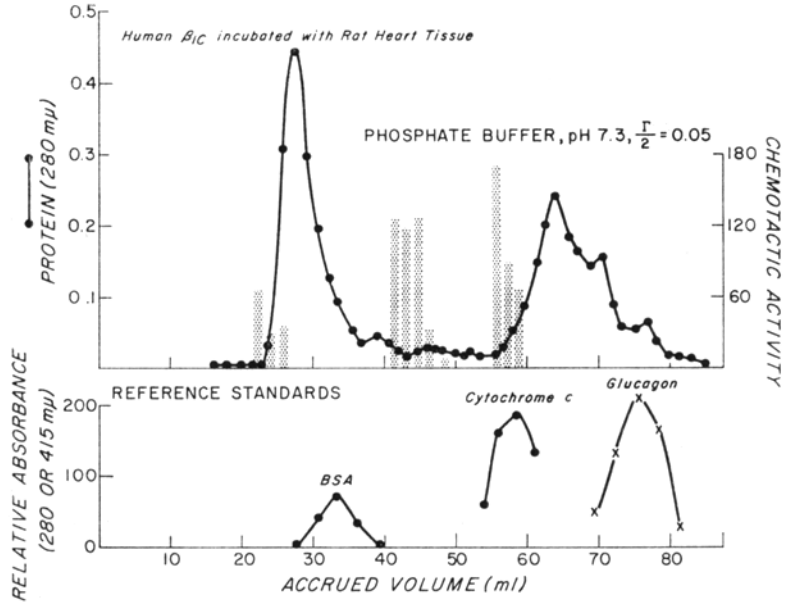

FIG. 6. Behavior of chemotactic factors in gel filtration. Human C3 was pretreated with rat heart tissue, then eluted from Sephadex G-75. Two major zones of chemotactic activity are present. BSA is bovine serum albumin.

cytochrome position. Chemotactic activity has thus far not been found in a more retarded position (i.e. beyond the cytochrome marker).

Characterization of a Protease in Heart Tissue.—It appeared likely that a trypsin-like enzyme in heart tissue was involved in the generation of chemotactic factors from C3. In order to determine this possibility, heart tissue was pretreated with saline or with trypsin inhibitor or the chloroalkyl phosphonate series (inactivators of serine esterases), since both types of compounds would be expected to inhibit a trypsin-like enzyme. After a 20 min preincubation at room temperature, heart tissues were washed twice and then added to rat serum and incubated for 90 min. As is evident in Table IV, pretreatment with trypsin

inhibitor completely blocked the ability of heart tissue to interact with serum and generate a chemotactic factor. In varying degrees, from 55 to 100%, the various chloroalkyl phosphonates also inhibited the capacity of heart tissue to generate chemotactic activity in rat serum.

Ability of Esters to Interfere with Action of Tissue Protease on C3.—It appeared possible to obtain information on the nature of the heart enzyme by the addition of amino acid esters that would compete with the C3 substrate. Experiments listed in Table V were devised to test this possibility. Both rat serum and purified human C3 were used as the source of substrate, and esters were added

TABLE IV

Inhibition of a Protease in Heart Tissue by Trypsin Inhibitor and Phosphonate Compounds

Pretreatment of heart tissue	Reduction in chemotactic activity generated in rat serum
	%
None*	0
3-Chloroalkyl phosphonate‡	94
4- " " ‡	100
5- " " ‡	55
6- " " ‡	78
Trypsin inhibitor (200 μg in 0.4 ml)	100

* Controls included 25 mg heart tissue incubated in saline for 20 min at room temperature, washed twice in saline, then incubated in rat serum for 90 min at 37°C. Respective negative control (tissue alone) and positive control (heart + serum) chemotactic values were 30 and 180.

‡ 25 mg heart tissue pretreated with 0.4 ml 8×10^{-4} M phosphonate as above, then washed twice and incubated with rat serum. The same protocol was followed with the trypsin inhibitor.

at either the *start* or the *end* of the period of incubation. Conditions included 20 mg heart tissue, 0.1 ml ester solution (0.07 M), and 0.1 ml rat serum or C3 (100 μg). After incubation, the tissue was removed by centrifugation and the supernatant was tested for chemotactic activity. When necessary, the pH of the supernatant was adjusted to neutrality before testing for chemotactic activity. It was found that esters appropriate to the functional features of an enzyme like trypsin (TAMe and BAMe) were effective inhibitors in the generation of chemotactic activity, whereas the aromatic amino acid ester *N*-acetyltyrosine ethyl ester and other esters (glycine ethyl ester and glycylglycine ethyl ester) had little or no effect. The important control in this experiment, to rule out nonspecific "toxic" effects of esters on cells, was the addition of ester at the end of the period of incubation. With a single exception, only TAMe and BAMe

inhibited the generation of chemotactic activity if added at the beginning of the period of incubation of tissue with substrate. *N*-Acetyltyrosine ethyl ester had inhibitory effects (36–43% when serum was used as the substrate) that were nonspecific; that is, the inhibition was unrelated to the period of incubation of tissue with substrate. When purified C3 was used as the substrate, the ester was ineffective regardless of the time of addition (Table V). These results are compatible with the notion that the protease in heart tissue is a trypsin-like enzyme.

TABLE V

Inhibition by Amino Acid Esters of Chemotactic Factor-Generating Capacity of Heart Tissue

Ester (0.07 M)	Ester added to serum		Ester added to C3	
	Before*	After	Before*	After
	% inhibition		% inhibition	
p-Tosyl-L-arginine methyl ester	90	0	64	0
Benzoylarginine methyl ester	79	0	91	0
N-Acetyltyrosine ethyl ester	43	36	0	0
Glycine ethyl ester	0	7	0	0
Glycylglycine ethyl ester	0	0	9	0

* "Before" and "after" refer to the presence of ester during the 90 min incubation period at 37°C.

DISCUSSION

These results seem to establish clearly that many tissues can generate chemotactic activity in serum and that, at least in the case of heart tissue, this involves an interaction between C3 and a tissue-contained, trypsin-like enzyme. The interaction of the protease and C3 results in the production of C3 fragmentation products with chemotactic activity. The relatively large amount of tissue (milligram quantities) required to cleave C3 probably simply reflects the very inefficient contact of enzyme with substrate. In most respects, these findings confirm and extend those of Hurley (1), who described an interaction between rat tissue and serum that resulted in chemotactic activity in vitro for neutrophils. It seems quite possible that these data provide a plausible explanation for the development of the acute inflammatory response in mechanically injured tissue; namely, through the release of the tissue protease, C3 in the vicinity of the injured tissue can serve as a substrate for the production of phlogistic (leukotactic) factors. Thus, in the case of burned tissue, mechanically disrupted tissue, or infarcted tissue, to name a few examples, the stage would be set for the induction of the acute inflammatory response by the generation of C3 chemotactic factors. This would provide an alternative explanation for the classical view that the appearance of neutrophilic leukocytes simply reflects

their adherence ("sticking") to altered endothelium and subsequent random migration of these cells into extravascular sites. However, in order to test the relevance of the hypothesis presented in this paper, it will be necessary to show the release of the protease from damaged tissue and the appearance of C3 fragments with chemotactic activity in the same tissue. Preliminary supporting evidence for this latter point has already been obtained.

Once again, in considering the acute inflammatory response, we come to the complement system as a mediator. It has previously been shown that immunological triggering of the complement system will result in production of factors with chemotactic (2, 6, 10) as well as vasoactive properties (11–13).

TABLE VI

Chemotactic Factors Generated from the Complement System

Factor	Requirements	References
$C\overline{567}$	C1–C7	2, 6
C3a	Plasmin	15
	Trypsin,* cobra venom factor	17, 14
	Tissue protease	This paper
C5a	C1–C5	10, 16, 17
	Trypsin	17

* Very limited activity under conditions described in reference.

Increasingly apparent is the fact that the complement system contains a series of substrates from which several phlogistic mediators can be generated in such a manner that only one complement component is needed, the rest of the eight components being bypassed. This is true for the production of C3 or C5 anaphylatoxin by trypsin (11–13) or cobra venom factor (14), as well as for the generation of chemotactic factors from C3 (15) or C5 (16, 17).

Table VI indicates the multiplicity of chemotactic factors capable of being generated from the complement system. These factors can be produced either by sequential interaction of the complement system, resulting in $C\overline{567}$ or a C5 fragmentation product, or by direct interaction of a single complement component with an enzyme not intrinsic to the complement system, such as plasmin or trypsin. Quite apparent in Table VI is the multiplicity of agents capable of directly interacting with C3 or C5 to give a chemotactic product. As more data of this type accumulate, one begins to wonder if the complement system is more important as a mediator for nonimmunologically triggered events leading to production of phlogistic factors, rather than in its historical (immunological) role. If experimental evidence can be obtained to suggest a relation between C3 products and the inflammatory response to "nonspecific" tissue injury, this would have therapeutic implications. In cases of inflammation in or around vital areas, such as the central nervous system, the facial, or respiratory areas, where the inflammatory response poses a threat to vital organ function, it would be

very desirable to know more about the mechanism of inflammation. The data presented in this report suggest one approach to the question.

The precise identification of the trypsin-like enzyme in heart tissue awaits further work. It seemed possible that activated plasminogen might be the critical enzyme. However, two observations militate against this possibility. Kwaan and Astrup failed to find plasmin activity in rat heart tissue (18). Furthermore, the split product of C3, at least as far as human plasmin is concerned, is of a lower molecular weight, around 6000 (15). However, neither observation rigorously precludes the possible role of plasmin. That thrombin might be involved in the reaction is under consideration, but no evidence so far completely substantiates or denies this possibility. Certainly the trypsin-like enzyme has properties different from the trypsin (beef) product used in prior experiments (17), in which this enzyme generated little chemotactic activity from C3. But that the protease closely resembles trypsin from a functional point of view is unquestioned. Whether or not the protease active on C3 is identical with any more well-defined proteases in tissue is not known at this time.

SUMMARY

When various rat tissues are incubated in homologous serum, a factor chemotactic in vitro for neutrophils is generated. The amount of chemotactic activity is a function of duration of incubation and the quantity of heart tissue or serum employed. Addition of trypsin inhibitor or antibody to the third component of complement (C3) precludes generation of chemotactic activity. In addition, antibody to C3 ablates chemotactic activity even after its formation.

Purified human C3 (β_{1C}-globulin) effectively substitutes for serum in the generation of chemotactic activity by heart tissue. The active product, as determined by gel filtration or by ultracentrifugal analysis in a sucrose density gradient, appears to be a cleavage product of C3 with a molecular weight of approximately 14,000. In addition, a larger C3 fragmentation product varying in molecular weight, depending upon experimental conditions, is also found.

The protease in rat heart tissue capable of cleaving C3 into chemotactic fragments is a serine esterase with trypsin-like properties and can be inhibited by organophophorous compounds or trypsin inhibitors. The use of amino acid esters in the manner of competitive substrate inhibition confirms the trypsin-like nature of the protease.

The presence of a protease in heart, and presumably in other normal tissues, capable of fragmenting C3 into factors with chemotactic activities may explain the development of the acute inflammatory response when tissues are nonspecifically injured. If true, this would reinforce the role of the complement system in the mediation of nonimmunologically induced inflammation.

Note added in proof: The recent publication of V. A. Bokisch, H. J. Müller-Eberhard, and C. G. Cochrane 1969, *J. Exp. Med.* **129**:1109, emphasizes the bioactive fragments originating from C3 and extends the data presented in Table VI.

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