THE PHLOGISTIC ROLE OF C3 LEUKOTACTIC FRAGMENTS IN MYOCARDIAL INFARCTS OF RATS*

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The role of the complement system in the production of mediators of the acute inflammatory response is now well established. Two general classes of mediators have been identified: the chemotactic factors for neutrophils (reviewed in 1, 2) and the anaphylatoxins (reviewed in 3). Considering both categories, it is possible in an inflammatory reaction, where complement plays a role, to account for the two cardinal features of the acute inflammatory response, i.e., accumulation of neutrophils and increased vascular permeability. The usual view regarding the generation of these complement-derived mediators is that they result from sequential interactions of the complement system. In recent years it has become apparent that certain enzymes can act directly on individual complement components, and in so doing generate mediators in a manner that bypasses all other components of the complement system (2, 4).

The work to be presented here relates to the "bypass" concept and involves a tissue protease found in myocardium (as well as many other normal tissues [5]). This enzyme cleaves the third component of complement (C3) into chemotactically active fragments. In the course of "nonspecific" tissue injury, namely anoxia induced by ligation of a coronary artery in the rat, C3-cleavage products with chemotactic activity have been found in the damaged heart tissue. It seems probable that these products reflect the release of the tissue protease followed by its action on the substrate C3. The finding of C3 chemotactic products, together with the results from certain manipulations of the animal model, suggest that the acute inflammatory response to this type of nonspecific tissue injury is a complement-dependent reaction due to the release of the C3-cleaving enzyme, the production of C3 fragments, and the accumulation of neutrophils. These data provide further evidence for a central role of the complement system in the generation of phlogistic mediators.

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Materials and Methods[1]

Surgical Procedure.—Under general anesthesia (Fluothane), and with externally assisted breathing, 250 g female outbred Wistar rats were subjected to left lateral thoracotomy in the eighth intercostal space. After retraction of ribs the left coronary artery was tied off close to its origin with a 4-0 silk ligature (6). The chest was then closed in two layers with interrupted silk sutures. Monitoring of the myocardial infarcts was accomplished by electrocardiographic tracings before and after arterial ligation to insure that the electrical changes associated with infarction were produced. Rats were sacrificed at appropriate intervals after coronary artery ligation by general anesthesia (see above) and exsanguinated from the inferior vena cava. The infarcted area of myocardium was characterized by the usual gross morphologic changes of paleness and peripheral hemorrhage. Fresh myocardial tissue was obtained for extraction of chemotactic activity. For routine light microscopy tissue sections were fixed in formalin buffered at neutral pH.

Tissue Extraction Procedures.—Blocks of tissue 200–400 mg were finely minced, and then, after the addition of 2.0 ml of phosphate-buffered saline (pH 7.3), homogenized in a Virtis 45 instrument (The Virtis Co., Inc., Gardiner, N. Y.) at 37,000 rpm for a total of 9 min at 5°C. A supernate was separated from the sedimentable fraction by centrifugation at 80,000 *g* for 20 min.

Gel Filtration.—Soluble tissue extracts were fractionated by filtration through Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) using a phosphate buffer (pH 7.3, $\frac{\tau}{2} = 0.05$). Standard protein markers including bovine serum albumin (BSA), cytochrome c, and glucagon were employed. These procedures are described in detail elsewhere (5).

Ultracentrifugation.—An International B60 ultracentrifuge (International Equipment Company, Needham Heights, Mass.) was employed at 55,000 rpm for 14 hr at 4°C, using an SB405 rotor. The sucrose gradient consisted of 7.5–35% sucrose in the phosphate buffer (pH 7.3) listed above.

Assays for Chemotactic Activity.—Using neutrophilic granulocytes obtained from a glycogen-induced rabbit peritoneal exudate, chemotactic activity was assayed with the modified Boyden chambers which contain micropore filters of pore size 0.65 μ (7, 8). Chemotactic activity of a particular sample is that sum of counts of cells which have migrated in five fields in the filter (at high power magnification) in response to a chemotactic agent.

Antibody Preparations.—Antibody to rat C3 and antibody to human C5, which cross-reacts with rat C5 (9), were employed. These preparations are described in an earlier report (5).

Sources of C3.—Human C3 was purified from serum by the procedures of Nilsson and Müller-Eberhard (9). These involve isoelectric precipitation, ion-exchange chromatography in diethylaminoethyl (DEAE)-cellulose, and chromatographic elution from hydroxyapatite. The radiolabeled C3 was a gift from Doctors Irwin H. Lepow and Sheldon Taubman (Farmington, Conn.).

C3 Inactivator.—Lyophilized venom from *Naja naja* (obtained from Ross Allen Reptile Institute, Silver Springs, Fla.) was fractionated by ion-exchange chromatography on DEAE-cellulose (Mann Laboratories, New York) according to the directions of Cochrane (10). The appropriate fractions containing C3 inactivator were devoid of lethal cobra toxin (as assayed by intraperitoneal injection into mice). Rats were injected with 150–250 units of C3 inactivator (10) by multiple intraperitoneal injections over an 8 hr period. This treatment ablated detectable serum levels of C3 as measured by immunochemical techniques with double diffusion in gel.

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

RESULTS

Preparation of Leukotactic Factor-Producing Substance from Normal Rat Heart.—In the previous study tissue minces were used as a source for material, presumably an enzyme, that cleaves C3 into leukotactic fragments (5). An improved extraction procedure involving tissue homogenization as described above was utilized. Tissue minces obtained by the previous method (5) were interacted with rat serum or purified human C3 to produce chemotactic activity (Table I). When the tissue homogenate of normal rat heart was used no chemotactic factor-generating activity was found in the sedimentable fraction, but high activity was present in the supernatant fraction (Table I). These results

TABLE I

Chemotactic Factor Generating Activity in Heart Tissue Mince, and Homogenate

Sample tested	Chemotactic activity
Tissue mince	
5 mg + ----	10
5 mg + 100 μl rat serum	210
5 mg + 100 μg C3	160
100 μl rat serum	0
100 μl C3	0
Virtis homogenate	
5 mg sediment + ----	0
5 mg sediment + 100 μl rat serum	10
5 mg sediment + 100 μg C3 (human)	0
100 μl supernate + ----	0
100 μl supernate + 100 μl rat serum	170
100 μl supernate + 100 μg C3	60

indicate that the extraction procedure permits solubilization of the C3-cleaving factor (previously termed tissue protease [5]) and suggest that the enzyme may be associated with soluble cytoplasmic components of heart cells.

Characterization of the C3-Cleaving Factor and its C3-Cleavage Products.—The supernate of the tissue homogenate described above was eluted from Sephadex G-100 and from the resulting fractions 50 μl were incubated with 50 μg of C3 for 30 min at 37°C. The mixture was then assayed for chemotactic activity. While some chemotactic factor-generating activity eluted in the void volume, most was found in fractions eluting immediately after the BSA marker (Fig. 1). These results suggest that the putative C3-cleaving enzyme has a molecular weight slightly less than that of BSA.

A supernatant sample of 150 μl from the heart homogenate was incubated with 150 μg of C3 in a volume of 300 μl at 37°C for 30 min, followed by elution of the mixture from Sephadex G-75. The resulting fractions assessed for

chemotactic activity showed a heterogeneity in distribution (Fig. 2) with some activity in the void volume, but the bulk of activity appeared in areas beyond the cytochrome c marker. The distribution of this activity in the retarded zones

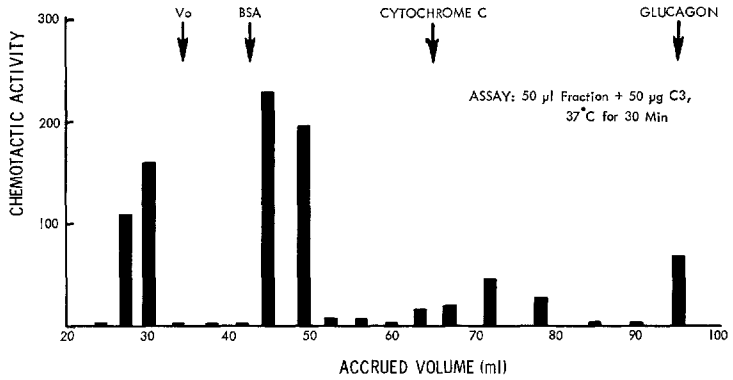

FIG. 1. Localization of chemotactic factor-generating activity in fractions of rat heart homogenate eluted from Sephadex G-100. Activity appears near the void (Vo) volume as well as just beyond the albumin (BSA) marker.

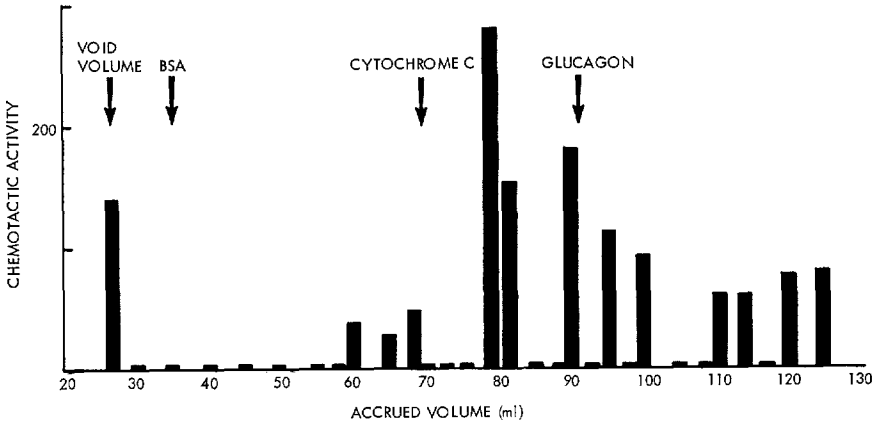

FIG. 2. Elution from Sephadex G-75 of C3 treated with an extract of normal rat myocardium. Vertical bars represent chemotactic activity for neutrophils.

of the chromatogram is in contrast to the larger C3 fragments produced when a mince of heart tissue was used as the enzyme source (5).

Direct Demonstration of Cleavage of C3 by Factor in Heart Homogenate.—10 µl of radiolabeled C3 (approximately 2 µg) together with 100 µl (50 µg) of nonlabeled C3 were incubated with 50 µl of supernate from the heart homogenate for 30 min in a total volume of 300 µl containing 0.025 M phosphate buffer, pH

7.3. After this the material was placed in a sucrose density gradient. A cleavage product from C3 was found in the slowly sedimenting region of the gradient (Fig. 3, upper frame, fractions 14–16) coinciding with a peak of chemotactic activity (lower frame). Additional peaks of chemotactic activity were also found

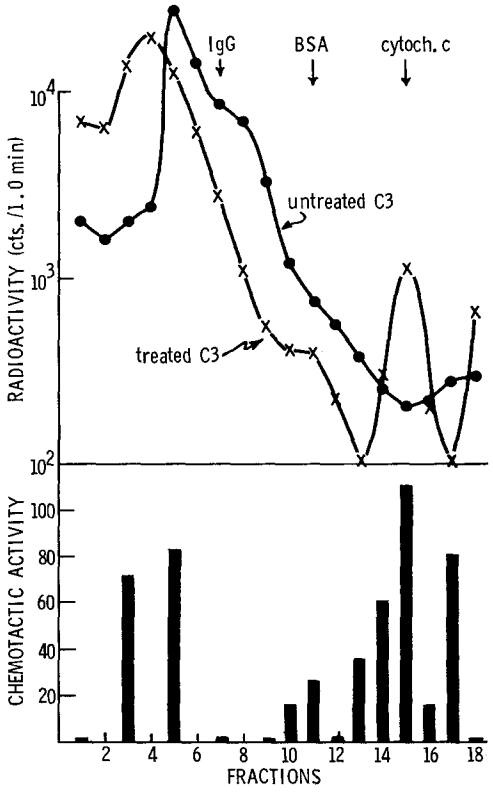

FIG. 3. Cleavage of human C3 by treatment with extract from normal rat myocardium. The cleavage product, as measured by radioactivity (upper frame, broken line (X——X), fraction 14–16), coincides with a peak of chemotactic activity (lower frame). Cytoch. c stands for cytochrome c.

in fraction 17, as well as in fractions 3 and 5. The latter may represent an altered but, as yet, uncleaved C3 molecule. The untreated C3 preparation failed to show a peak of radioactivity in the upper region of the gradient (Fig. 3, upper frame).

Time Course in Appearance of Chemotactic Activity in Infarcted Myocardium. —Extracts from infarcted heart tissue were obtained at various intervals after coronary artery ligation (Fig. 4). Each point represents the mean of chemotactic activity in 100 μl extracts from each of four different hearts. The standard devia-

tion is shown by the vertical bars. Minimal activity was found in tissues 2 hr after ligation of the coronary artery, whereas maximal activity appeared at 3–4 hr. Thereafter, activity diminished over the next 2 hr and was barely detectable after the 6th hr. Several extracts were examined for susceptibility of chemotactic activity of inhibition by antibody to rat C3 (anti-C3). 50 μl of antibody was added to 100 μl of extract, incubated at room temperature for 15

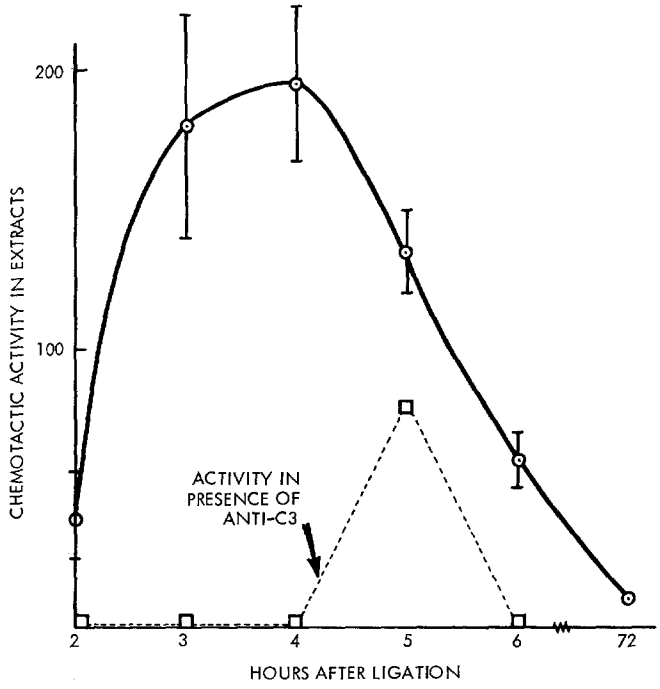

FIG. 4. Time course in appearance of extractable chemotactic activity in infarcted rat myocardium, as a function of hours after ligation of coronary artery. Most of the activity can be suppressed with antibody to C3.

min, then assayed for chemotactic activity. It is apparent that, with the exception of the 5 hr sample, all activity could be suppressed with anti-C3 (Fig. 4, dotted line).

Behavior in Gel Filtration of Chemotactic Activity Extractable from Infarcted Myocardium.—An extract of infarcted myocardium obtained 3 hr after ligation of the coronary artery was eluted from Sephadex G-75. Considerable heterogeneity of activity was found. Two areas of chemotactic activity were found at and beyond the position of the void volume; a third area of activity was present in fractions eluting immediately after the glucagon marker (Fig. 5). When the extract of myocardium obtained 5.5 hr after coronary artery ligation was ex-

amined by gel filtration, the pattern of chemotactic activity was somewhat different. No significant activity was found in the void volume, low grade activity appeared near the cytochrome c marker, but most was found just before or just after the glucagon marker (Fig. 6). These results suggest that the later (5.5 hr) infarcts contain smaller chemotactically active molecules than infarcts obtained at the 3 hr interval.

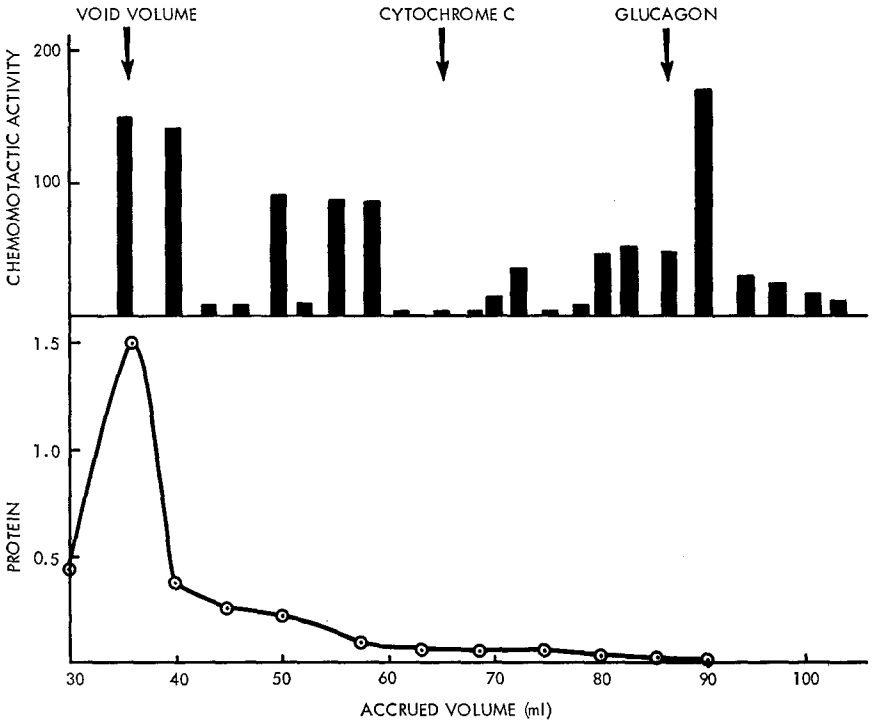

FIG. 5. Elution from Sephadex G-75 of chemotactic activity in 3-hr old infarcted rat myocardium. The activity is heterogenous.

Also shown in Fig. 6 is the susceptibility of chemotactic activity in the various fractions to inhibition by the addition of anti-C3. This experiment was performed by adding 50 μl of anti-C3 to 100 μl samples from the Sephadex column. Little inhibition of chemotactic activity in fractions 11, 13, and 14 was found, whereas in fractions 20, 21, 25, 26, and 27 half to two-thirds of the activity was suppressed by this treatment. These data provide the most direct evidence that chemotactic activity in extracts from infarcted myocardial tissue is attributable to cleavage products of C3.

Ultracentrifugal Behavior of Chemotactic Activity in an Extract of Infarcted Myocardium.—The same sample analyzed in Fig. 6 was subject to ultracentrifugal analysis (Fig. 7). As anticipated, chemotactic activity appeared in the upper portion of the gradient, forming a heterogeneous pattern, with activity

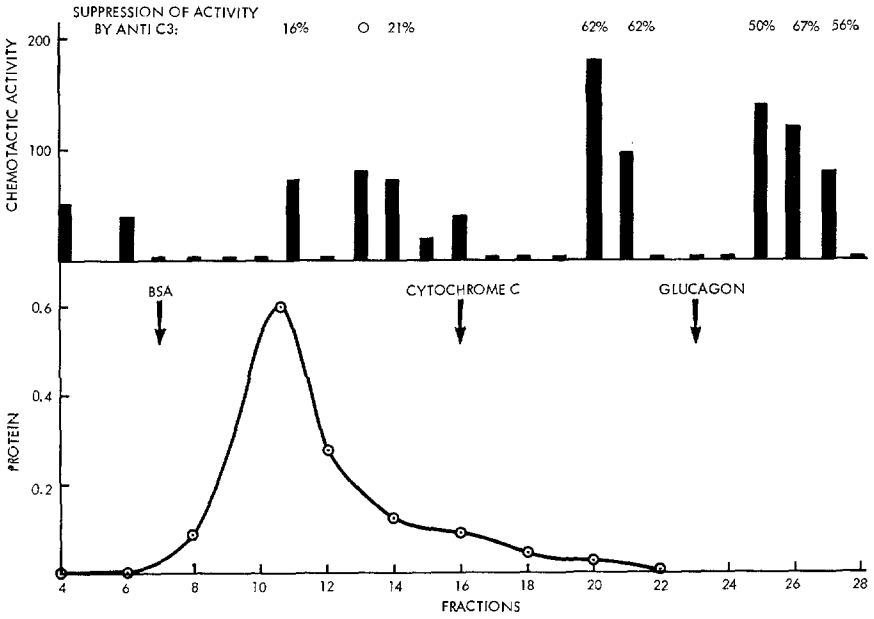

FIG. 6. Elution from Sephadex G-75 of an extract of a 5.5-hr old myocardial infarct. The activity is later eluting, compared with Fig. 5, and much of the activity is C3-related.

TABLE II

C3 Dependence of Leukotactic Activity in Myocardial Infarcts of Rats

Treatment	Serum $C'H_{50}$	Serum C3*	Chemotactic activity in tissue extract‡
	(*units/ml*)		
None			
Rat 1	25	Present	130
Rat 2	>18	Present	220
Rat 3	26	Present	250
C3 inactivator			
Rat 4	<6	Absent	10
Rat 5	<6	Absent	60
Rat 6	<6	Absent	0
Rat 7	<6	Absent	0
Rat 8	<6	Absent	0

* As determined by immunochemical assay (see Fig. 8).

‡ 4 hr myocardial infarct.

just above and just below the cytochrome c marker. This provided additional evidence for C3 products of low molecular weight.

The Effect of C3 Inactivation on Appearance of Chemotactic Activity in Extracts of Myocardial Infarcts in Rats.—Rats were treated 1.5 days before coronary artery ligation with C3 inactivator (purified from cobra venom) in amounts sufficient to completely abolish serum C3, as measured by Ouchterlony analysis.

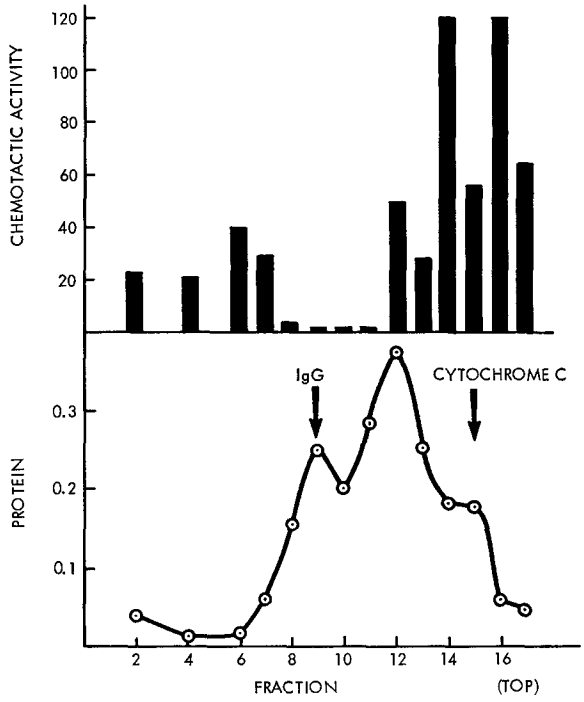

FIG. 7. Ultracentrifugal separation in a sucrose density gradient of chemotactic activity in an extract of a 5.5-hr old infarct, the same as in Fig. 6. As expected, the chemotactic activity sediments slowly.

Serum from rats Nos. 1 and 3, which were not treated with the C3 inactivator, possessed normal levels of hemolytic activity for complement 5 hr after coronary artery ligation (Table II). As expected, immunodiffusion showed the typical precipitin bands for C3 (Fig. 8). Tissue extracts from the infarcted myocardium of these control rats revealed abundant chemotactic activity (Table II). On the other hand, five rats treated with C3 inactivator had no serum complement as measured by total hemolytic complement activity (Table II). Their sera failed to show C3 by immunodiffusion (Fig. 8). With the exception of a single rat (Table II, No. 5), none had extractable chemotactic activity in the heart

tissue. The data are in accord with the findings presented above and support the conclusion that chemotactically active C3 fragments appear in infarcted myocardium.

Histologic Features of Acutely Infarcted Rat Hearts.—During the first 24–48 hr after ligation of the coronary artery, the myocardium underwent the characteristic changes of coagulation necrosis, hemorrhage, and an intense accumulation of neutrophils. In Fig. 9 the typical extravascular accumulation of red cells and neutrophils is seen adjacent to the infarcted muscle. In contrast, those rats depleted of C3 showed the changes of coagulation necrosis accompanied by hemorrhage but few if any neutrophils accumulated in the infarcted tissue (Fig. 10). Rats treated with the C3 inactivator did not develop a leukopenia or neutropenia, ruling out the depletion of neutrophils from the circulation as an explanation for the failure of these cells to accumulate in the infarcted tissue. These findings are in concert with the hypothesis that neutrophils accumulate in infarcted heart tissue in response to the local production of C3 leukotactic products after release of the C3-cleaving enzyme from damaged tissue.

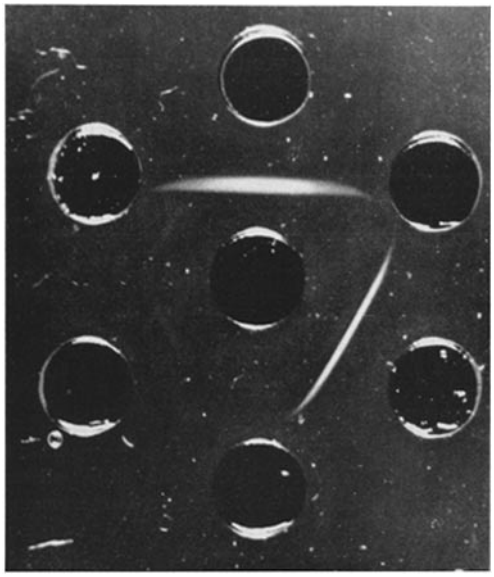

FIG. 8. Analysis by double diffusion in gel of rat C3. Sera are in peripheral wells, anti-C3 in center well. Wells at 12 and 4 o'clock contain control sera from rats whose coronary arteries were ligated 4 hr earlier. The other four wells contain sera from rats with ligated arteries (4–7, Table II) pretreated with C3 inactivator. No C3 activity is present.

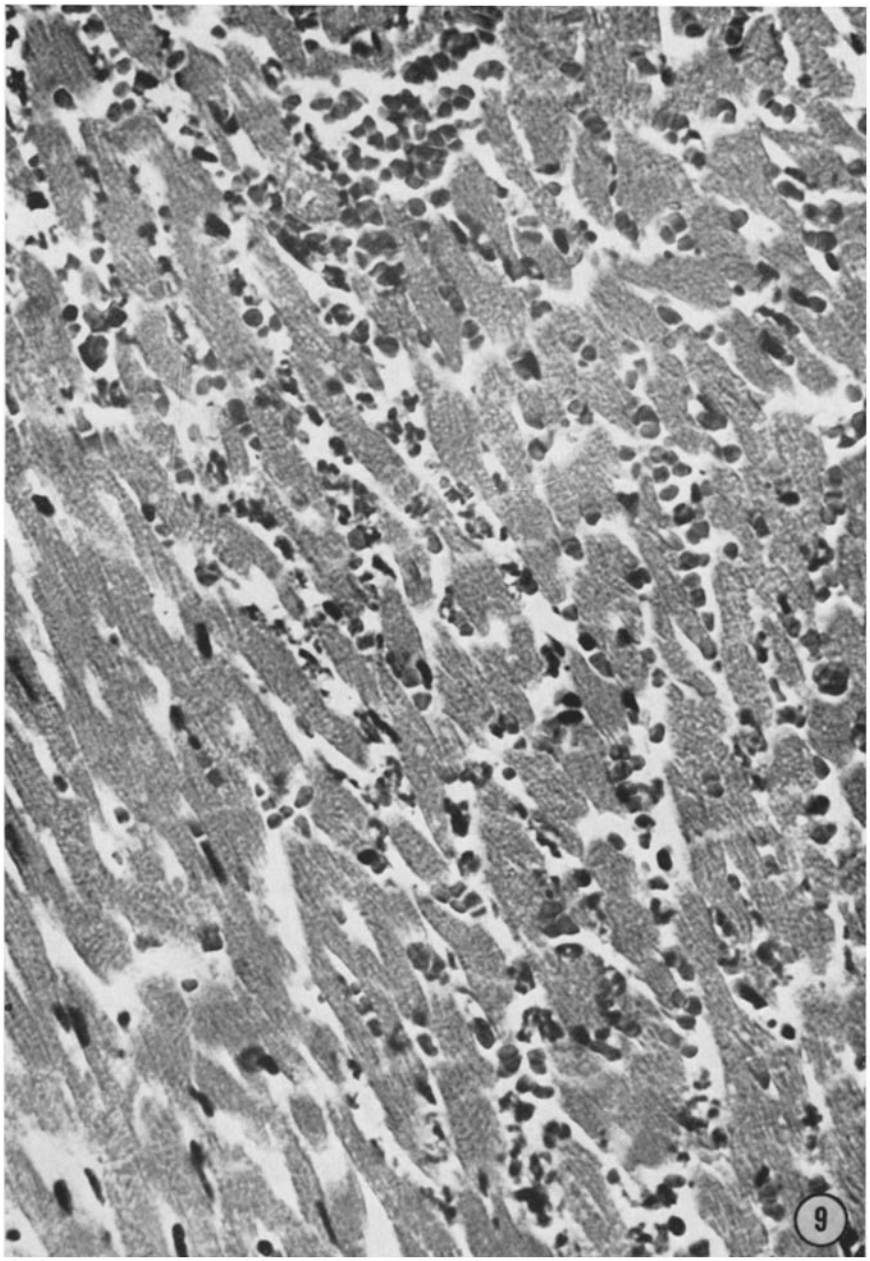

FIG. 9. Photomicrograph from control rat heart 24 hr after ligation of coronary artery. The infarcted myocardium contains numerous red cells as well as neutrophilic granulocytes. Hematoxylin and eosin; × 395.

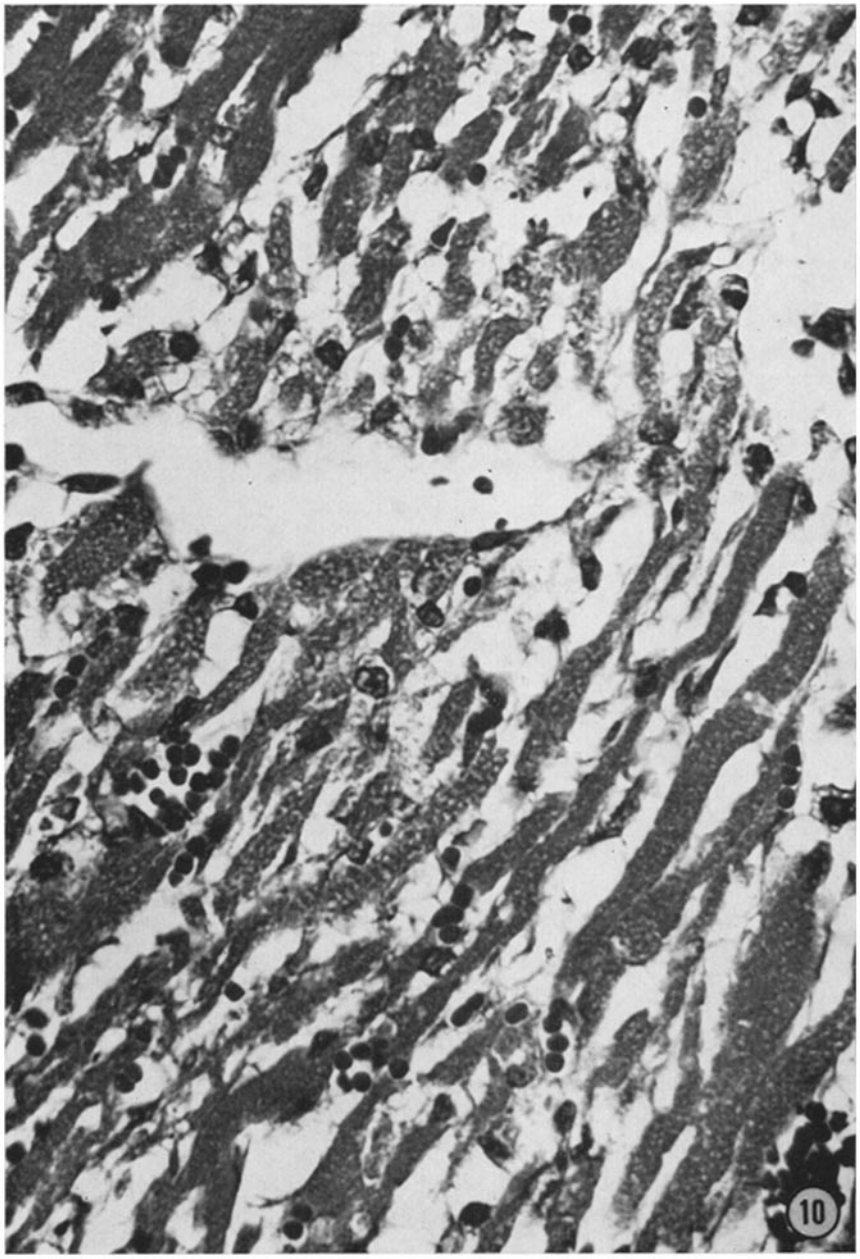

FIG. 10. Photomicrograph of infarcted rat myocardium at 48 hr after ligation of coronary artery. This rat was C3-depleted. Myocardium shows typical changes of coagulation necrosis and some hemorrhage, but even 2 days after the infarct no neutrophils are seen. Hematoxylin and eosin; × 395.

DISCUSSION

The necessity of C3 for the accumulation of neutrophils in response to acutely infarcted rat myocardium is a logical sequel to the recent findings of a factor (presumably an enzyme) in most normal tissues, including myocardium (5). This enzyme has the capacity to cleave C3 into leukotactic fragments, and it seemed reasonable to consider that in nonspecific tissue injury, such as a myocardial infarct, this enzyme would be released from its residence in tissue (the localization of which, whether parenchyma or stroma, is not known), after which contact with the substrate would lead to cleavage of C3 and generation of chemotactic activity. The logical outcome would be the accumulation of neutrophils.

This problem was directly approached by isolating and identifying C3 leukotactic products in infarcted tissue. By manipulating the model, depletion of C3 in rats led to the failure to detect chemotactic activity in extracts of infarcted tissue. Coincidentally, ablation of C3 in the serum by the C3 inactivator precluded the appearance of neutrophils in the infarcted tissue.

This model seems to be a prototype in which, through a bypass of the conventional complement sequence (i.e. sequential interaction of complement components is not operational), an enzyme can act directly on one intact complement (C3) to generate factors with phlogistic potential. This emphasizes the potential role of complement in the generation, by nonimmunologic means, of mediators of the acute inflammatory response. The findings of other enzymes such as trypsin, plasmin, and thrombin (4), each of which can cleave C3 with the liberation of C3a possessing chemotactic as well as anaphylatoxic properties, simply emphasizes the various ways in which C3 can be cleaved into biologically active fragments.

In terms of the bypassing of the conventional interaction of the complement sequence, a very similar story has now begun to unfold for C5. C5a can be produced directly by the action of trypsin (11) and by a neutral protease extractable from lysosomal granules of the neutrophil (11). Even C1 can be activated by trypsin or plasmin (see review in reference 12), but in this case its activation results in the interaction with its natural substrates of the complement system, C4 and C2. It is not completely clear if the activation of C1 by these enzymes is productive of biologically active fragments from C3 and C5, although such products would be anticipated.

Relating the work in this case to previous studies requires some comments. To begin with, the C3-cleavage products extractable from infarcted rat myocardium are heterogeneous, but this is not surprising in view of the fact that incubation of tissue minces gives at least two C3-cleavage products with estimated molecular weights of 14,000 and 30,000 (5). In the present study, however, C3 fragments of considerably lower molecular weight have been found when the enzyme preparation (obtained in soluble form from the heart homog-

enate) is incubated with C3 or when C3-cleavage products from infarcted tissues are extracted and eluted from Sephadex. Such differences can be accounted for by a more active enzyme preparation. This is not surprising, since contact of C3 with the enzyme present in the tissue mince would probably be very limited (5). Thus, it appears that the action of the enzyme prepared from the tissue homogenate, rather than from the tissue mince, more closely reflects what happens locally to C3 in the course of a myocardial infarct.

It is of interest that not all the extractable chemotactic activity in infarcted myocardium is related to C3, at least at 5 hr after coronary ligation (Fig. 4). Other candidates for chemotactic mediators include C5a (13) and collagen-breakdown products (14). The former could be due to the release of the C5-cleaving enzyme from lysosomal granules of neutrophils (13) that have responded to the initial chemotactic stimulus in the infarcted myocardium. For this to occur, however, it would be necessary to postulate either the damage or destruction of neutrophils responding to the infarcted tissue, or the specific release of the lysosomal product through a phagocytic mechanism. The possible role of collagen-breakdown products as another chemotactic stimulus also has to be considered, although the relative lack of collagen (substrate) and fibroblasts (source of collagenase) in the heart reduce the likelihood of this explanation. The data in Fig. 4 certainly suggest that C3 products are the earliest and the predominant chemotactic factors. In the absence of C3 little other chemotactic activity appears (Table II).

Finally, these findings must be put into the perspective of clinical medicine. In the first place, although we know that human myocardium (as well as many other tissues) contains a C3-cleaving enzyme (5), there are no data with human material to indicate if, in fact, C3-cleavage products are produced in infarcted myocardium. In the second place, the experimental studies in the rat do not allow for any definite statement regarding the outcome of the infarct in the absence of neutrophils. The exclusion of these cells in the inflammatory reaction has not been associated with any obvious defect in the formation of granulation tissue in the infarcted heart.[2] C3-depleted rats seem to lay down a dense fibrous scar in the infarcted tissue in the absence of neutrophils and C3, just as did the control rats. There are no obvious effects of the C3 depletion, either in the intensity of the myocardial damage, or in the healing of the infarcted heart, but both parameters are very difficult to measure objectively.

SUMMARY

An improved method is described for extraction from rat myocardium of an enzyme that cleaves the third component of complement (C3) into leukotactic

[2] Hill, J. H., and P. A. Ward. Personal observations.

fragments. Direct cleavage of C3 has been shown by the use of a radiolabeled preparation of human C3. The enzyme elutes slightly beyond (retarded to) an albumin marker in gel filtration.

After surgical ligation of a coronary artery, leukotactic activity can be extracted from infarcted rat myocardium. Described in terms of its time of appearance in infarcted tissue, the leukotactic activity has been identified as cleavage products of rat C3. The C3 fragments are of low molecular weight and heterogenous in size.

Rats, if treated with the C3 inactivator (isolated from cobra venom) so as to ablate serum C3, fail to develop leukotactic activity in the infarcted myocardium. In turn, few if any of the usual intense accumulations of neutrophils in response to infected myocardium are seen during the first 48 hr after coronary artery ligation.

These studies suggest a nonimmunologic role for C3 in the mediation of the acute inflammatory response in nonspecific tissue injury.

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