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J Clin Invest. 1979;64(5):1379-1385. https://doi.org/10.1172/JCI109595.

Research Article

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Generation of a Fibroblast Chemotactic Factor in Serum by Activation of Complement

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ABSTRACT When serum complement is activated by either the classical or alternative pathways, a factor with an apparent 80,000 mol wt is generated that is chemotactic for human dermal fibroblasts. The origin of this serum-derived chemotactic factor (SDCF) is not known; however, it may be a cleavage product from C5 because it is inactivated by monospecific antiserum to human C5, and it is not generated when the complement system is activated in human serum deficient in C5. SDCF is not chemotactic for human neutrophils or monocytes.

Because SDCF is generated when serum complement is activated, it may function in vivo to attract connective tissue fibroblasts to sites of inflammatory reactions in which the complement system participates.

INTRODUCTION

Fibroblasts accumulate at sites of tissue injury and inflammation and effect repair by synthesizing new connective tissue elements (collagen, fibronectin, and glycosaminoglycans). The realization that fibroblasts participate in virtually all types of inflammatory lesions has prompted us to look for different sources of chemoattractants for these specialized effector cells. We have recently developed an assay that measures human fibroblast chemotaxis in vitro (1).

Thus far, two different types of fibroblast chemoattractants have been recognized, (*a*) a lymphokine with a 22,000 mol wt produced by antigen- or mitogen-stimulated human peripheral blood lymphocytes, and (*b*) collagen and collagen-derived peptides (1–3).

In this report we have examined human serum as a possible source of a chemoattractant for fibroblasts. Normal human serum itself is not chemotactic for fibroblasts. However, activation of either the classical or alternative pathways of complement in serum is accompanied by production of a factor that is chemotactic for fibroblasts. Characterization studies suggest that this factor may be derived from the fifth component of complement.

METHODS

Fibroblast chemotaxis assay. Human dermal fibroblasts obtained from punch biopsy of the skin of adults and maintained in monolayer cultures served as responding cells in the in vitro chemotaxis assay. Details regarding the methods employed in maintaining the cultures and harvesting the fibroblasts by trypsinization for use in the assay have been given in a previous report (1). Blind-well modified Boyden chemotaxis chambers (Duke University Surgical Instrument Shop, Durham, N. C.) were used to measure fibroblast chemotaxis. Polycarbonate filters with 8-μm pores were used in the chemotaxis chambers. Before being used in the assay, filters were treated with a dilute gelatin solution to change surface properties to facilitate fibroblast adherence, as previously described (1).

Substances being tested for fibroblast chemotactic activity (CTX)[1] were brought up to a volume of 0.4 ml with 0.015 M glycylglycine/0.14 M NaCl (GGBS) at pH 7.2 and were mixed with 0.35 ml of Eagle's minimum essential medium (MEM) supplemented with nonessential amino acids, ascorbic acid (50 μg/ml), and streptomycin (100 μg/ml). Aliquots (0.2 ml) of this mixture were loaded into the lower compartment of the chemotaxis chambers. Prepared polycarbonate filters (dull side up) were placed over the lower compartments. The chambers were assembled, and the upper compartments were filled with fibroblasts suspended at a concentration of 2.5 $\times$ 10^5 cells/ml of serum-free MEM.

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Received for publication 7 September 1977 and in revised form 21 June 1979.

[1] *Abbreviations used in this paper*: CTX, chemotactic activity; GGBS, 0.015 M glycylglycine/0.14 M NaCl; HAGG, heat-aggregated human gamma globulin; LPS, lipopolysaccharide; MEM, minimum essential medium; SDCF, serum-derived chemotactic factor.

Loaded chambers were placed in a humidified incubator in an atmosphere that contained 5% CO_2 at 37°C for 150 min. After incubation, the filters were removed, stained with hematoxylin, and mounted on glass slides as described previously (1). Fibroblast CTX was quantitated by counting in 20 oil immersion fields (magnification: 1,000) nuclei of fibroblasts that migrated to the lower surface of the filters. All samples were assayed in triplicate, and final activity was expressed where indicated as the mean±SEM of the replicates.

Monocyte chemotaxis assay. Human monocyte chemotaxis was measured with blind-well modified Boyden chemotaxis chambers equipped with polycarbonate filters with 5-μm pores (Wallabs, Inc., San Rafael, Calif.) by methods previously described (4, 5).

Neutrophil chemotaxis assay. Human neutrophil chemotaxis was quantitated with blind-well modified Boyden chemotaxis chambers equipped with polycarbonate filters with 3-μm pores (Bio-Rad Laboratories, Richmond, Calif.) as previously described (4).

Serum. Blood obtained from normal donors or twin sisters deficient in C5 was allowed to clot in glass tubes, and serum was collected by centrifugation at 400 *g* at 4°C.

Serum activators. Heat-aggregated human gamma globulin (HAGG), zymosan, and bacterial lipopolysaccharide (LPS) were used to activate serum complement.

HAGG was prepared by heating immune serum globulin (Armour Pharmaceutical Co., Phoenix, Ariz.) at 56°C for 10 min. HAGG was pelleted by centrifugation at 2,000 *g* for 30 min at 4°C. The pellet was resuspended in 0.9% NaCl at a concentration of 5 mg/ml. Zymosan A (Sigma Chemical Co., St. Louis, Mo.) was boiled for 15 min in 0.9% NaCl. The pellet was collected after centrifugation for 10 min at 2,000 *g* and resuspended in 0.9% NaCl at a concentration of 9 mg/ml. LPS W from *Serratia marcescens* (Difco Laboratories, Detroit, Mich.) was dissolved in 0.9% NaCl at a concentration of 100 μg/ml. Normal human serum was incubated with each of these activators in a vol/vol of two parts serum to one part activator for 1 h at 37°C and then at 56°C for 30 min to stop further activation of complement.

Molecular sieve chromatography. A single column 2.5 cm in diameter and 100 cm in length packed with Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and equilibrated with GGBS was employed in serum fractionation studies. The column was calibrated with proteins of known molecular weight. A drop of tritiated water (New England Nuclear, Boston, Mass.) was mixed with each sample applied to the column to serve as an internal marker for column volume.

Serum (2.5 ml) was incubated with 1.25 ml of 0.9% NaCl-containing zymosan (11.2 mg) for 1 h at 37°C, and applied to the G-150 column. Gel filtration was performed at 4°C, and the column effluent was continuously monitored for absorbance at 280 nm with a Uvicord III recording spectrophotometer (LKB Instruments, Inc., Rockville, Md.).

Ion exchange chromatography. A column 2.5 cm in diameter and 60 cm in length was packed with DEAE-Sephadex A-50 equilibrated with 0.0075 M glycylglycine/0.01 M NaCl at pH 8.0. Fractions (67 ml, containing 56 mg of protein) from gel filtration of zymosan-activated serum that contained fibroblast CTX were dialyzed against this same buffer and loaded onto the column. The column was washed with the buffer until the optical density of the effluent returned to the base line. The absorbed proteins were eluted from the column by a linear salt gradient at 4°C with 0.0075 M glycylglycine/1.0 M NaCl, pH 8.0, as the limiting buffer. Eluted fractions were dialyzed extensively against GGBS at pH 7.2. Volumes of dialyzed fractions were equalized by adding GGBS as necessary, and aliquots of each fraction were assayed for fibroblast CTX.

Antisera to human C3 and C5. Goat anti-human C3 and anti-human C5 were purchased from Meloy Laboratories Inc. (Springfield, Va.). The antisera were dialyzed at 4°C against GGBS to remove preservatives.

Treatment of fibroblast chemotactic factor with insoluble trypsin. It was of interest to determine what effect trypsin would have on the biologic activity of the fibroblast chemotactic factor. Normal serum was treated with zymosan and fractionated on Sephadex G-150 as described above. Fractions from the region of the chromatogram with maximal fibroblast CTX were pooled. Aliquots (1 ml) of the pooled fractions were mixed with 0.680 U of trypsin conjugated to polyacrylamide beads (Sigma Chemical Co.). The beads were preswollen and washed with RPMI 1640 medium before they were added to aliquots of the column fractions. Samples were incubated for 0–120 min with the trypsin-polyacrylamide conjugate at 30°C. After incubation, the trypsin-polyacrylamide conjugate was removed from samples by centrifugation at 400 *g* at 4°C. Samples were then tested for fibroblast CTX.

RESULTS

Generation of fibroblast CTX by complement activators. In preliminary studies, the ability of fresh normal human serum to act as a chemoattractant for human fibroblasts was evaluated. Fibroblasts did not recognize normal serum as a chemotactic stimulus at concentrations ranging from 1 to 50% (data omitted).

Serum complement was activated by HAGG, zymosan, and LPS to determine whether such treatment would lead to the generation of fibroblast CTX. When serum was incubated with HAGG, zymosan, or LPS for 1 h at 37°C and then heated to 56°C for 30 min it became chemotactic for fibroblasts (Table I). Incubation of serum with saline produced minimal stimulation of fibroblast migration that was approximately twofold greater than that observed with saline alone (Table I). If serum were heated to 56°C for 30 min before being incubated with the activators, only minimal fibroblast CTX was generated (Table I). The activators, when incubated with saline, were not chemotactic for fibroblasts (Table I). These data are consistent with the notion that serum-derived chemotactic factor (SDCF) for fibroblasts is derived from a complement component or is generated when complement is activated.

Molecular sieve chromatography. In an effort to determine the molecular weight of SDCF, zymosan-activated serum was fractionated on Sephadex G-150, and column fractions were assayed for fibroblast CTX. A major peak of CTX eluted from the column in advance of the albumin peak (Fig. 1). Based on this elution profile, the molecular weight of SDCF is calculated to be ≅80,000. In additional gel filtration studies not shown, we found that activation of serum complement by HAGG or LPS was associated with production of a similar size chemotactic factor for fibroblasts, but no chemotactic factor for fibroblasts was found when

TABLE I

Generation of Fibroblast CTX by Agents that Activate Complement

Condition*	Incubation	CTX‡
		fibroblasts/20 OIF§
Normal serum		
+ Saline	37°C for 1 h, then 56°C for 30 min	13±2
+ Zymosan	37°C for 1 h, then 56°C for 30 min	66±5
+ LPS	37°C for 1 h, then 56°C for 30 min	52±6
+ HAGG	37°C for 1 h, then 56°C for 30 min	60±7
Heated serum		
+ Saline	56°C for 30 min, then 37°C for 1 h	5±1
+ Zymosan	56°C for 30 min, then 37°C for 1 h	11±2
+ LPS	56°C for 30 min, then 37°C for 1 h	10±1
+ HAGG	56°C for 30 min, then 37°C for 1 h	12±2
Normal saline		
+ Saline	37°C for 1 h, then 56°C for 30 min	6±1
+ Zymosan	37°C for 1 h, then 56°C for 30 min	9±2
+ LPS	37°C for 1 h, then 56°C for 30 min	6±1
+ HAGG	37°C for 1 h, then 56°C for 30 min	4±1

* 1-ml aliquots of normal serum, heated serum, or normal saline were mixed with 0.5-ml aliquots of stock saline, zymosan, LPS, or HAGG solutions and incubated as indicated (see Methods). Aliquots from each mixture were then assayed in triplicate for fibroblast CTX.

‡ Mean±SEM.

§ OIF, oil immersion field.

serum previously incubated with saline was fractionated on the Sephadex column.

It was of interest to determine whether SDCF was also chemotactic for human monocytes or neutrophils. To resolve this issue, zymosan-activated serum was chromatographed on Sephadex G-150, and each column fraction was assayed for fibroblast, monocyte, and neutrophil chemotactic activity. Aliquots from a column fraction that contained SDCF (80,000 mol wt) were not chemotactic for monocytes or neutrophils (Table II). As expected, the major amount of chemotactic activity for monocytes and neutrophils was found in a column fraction from the 15,000-mol wt peak that contained C5a (Table II). Although the column fraction that contained C5a was chemotactic for monocytes and neutrophils, it was not chemotactic for fibroblasts (Table II).

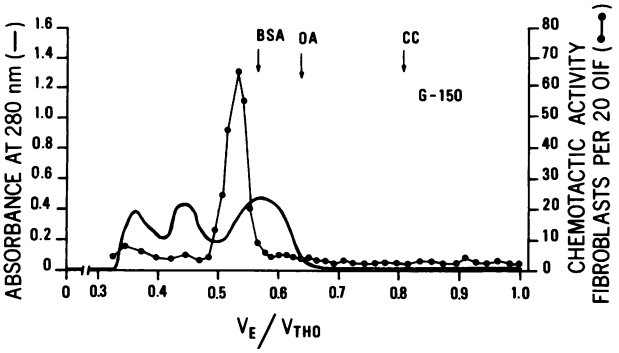

FIGURE 1 Serum (2.5 ml) from a normal volunteer was incubated with 1.25 ml of stock zymosan solution (9 mg/ml of saline) at 37°C for 1 h and fractionated on Sephadex G-150 as described in Methods. Column fractions were assayed for fibroblast CTX. OIF, oil immersion fields; BSA, bovine serum albumin; OA, ovalbumin; CC, cytochrome *c*.

Ion exchange chromatography. SDCF recovered from Sephadex G-150 was further purified by ion exchange chromatography. Fractions that contained SDCF from two column runs of zymosan-activated serum were pooled together and passed through a column packed with DEAE-Sephadex A-50 (Fig. 2). Retained proteins were eluted from the column by a linear gradient of NaCl. A small amount of CTX was found in the major protein peak, but the majority of activity eluted from the column after most of the proteins when the conductivity of the eluting buffer was between 8 and 12 mMHO (Fig. 2). SDCF recovered from this ion exchange column was used in additional characterization studies.

Nature of fibroblast migration induced by the SDCF. It was of interest to determine whether the fibroblasts were recognizing the SDCF as a chemotactic stimulus or as a stimulator of random cell migration. To resolve this question, different amounts of a single sample of DEAE-Sephadex-purified SDCF

TABLE II

Comparison of the Chemotactic Response of Fibroblasts, Monocytes, and Neutrophils to SDCF and C5a

Condition*	CTX‡		
	fibroblasts/ 20 OIF§	*monocytes/ 20 OIF*	*neutrophils/ 20 OIF*
Column buffer	6±1	8±1	10±2
SDCF	83±7	11±1	12±1
C5a	7±1	56±5	69±7

* Serum (2.5 ml) from a normal volunteer was incubated with 1.25 ml of stock zymosan solution (9 mg/ml of saline) at 37°C for 1 h, cleared of zymosan particles by centrifugation and fractionated on Sephadex G-150 as described in Methods. Data are shown for the CTX for each cell type obtained in a column fraction from the peak that contained SDCF (80,000 mol wt) and the peak that contained C5a (15,000 mol wt). Aliquots (0.4 ml) of column fractions or column buffer were mixed with 0.35 ml of Eagle's MEM (for fibroblast chemotaxis assay) or with 0.35 ml of Gey's balanced salt solution (for monocyte or neutrophil chemotaxis assay). Column fractions were then tested in triplicate for fibroblast, monocyte, and neutrophil CTX.

§ Mean±SEM.

§ OIF, oil immersion fields.

were added to the upper and/or lower compartments of the chemotaxis chambers, and fibroblast migration to the lower filter surface was determined (Table III). The diagonal lines enclose values for the number of cells migrating when zero concentration gradient was established by adding equal concentrations of SDCF to both compartments of the Boyden chambers. Fibroblast migratory responses to positive gradients appear to the right of these lines; responses to negative gradients to the left. Fibroblasts migrated through the filter when an excess of SDCF was present in the lower

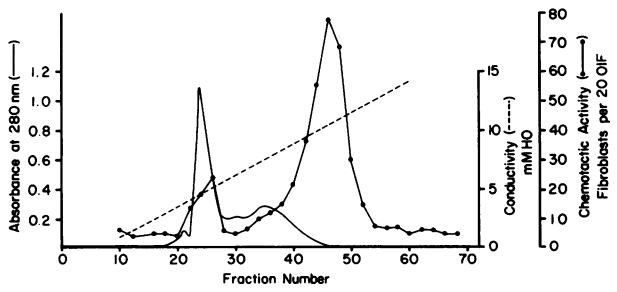

FIGURE 2 Fractions (67 ml) from two different gel filtration fractionations of zymosan-treated serum were applied to a 2.5 × 60-cm column packed with DEAE-Sephadex A-50. Absorbed proteins were eluted from the column by a linear salt gradient as described in Methods. Eluted fractions were dialyzed extensively against GGBS and tested for fibroblast CTX. Fibroblast CTX eluted from the column just after the major contaminating proteins when conductivity of the eluting buffer was between 8 and 12 mMHO. OIF, oil immersion fields.

TABLE III

*Effect of Varying Concentration Gradients of SDCF on Fibroblast Migration**

SDCF fibroblast concentration in upper compartment	SDCF concentration in lower compartment				
% ...	0	10	20	30	40
%			%		
0	4±1	15±1	29±2	45±7	65±8
10	4±1	3±1	22±3	39±4	51±5
20	9±2	5±1	5±1	21±3	34±4
30	7±1	5±1	4±1	5±1	24±2
40	7±2	9±1	13±2	8±2	14±1

* Number of fibroblasts in 20 oil immersion fields, mean±SEM of triplicate assay determinations.

chamber compartment relative to the upper compartment. In other words, fibroblasts migrated in response to a positive gradient of SDCF, fulfilling criteria of Zigmond and Hirsch (6) for a chemotactic stimulus. A small amount of stimulated random cell migration was observed when SDCF at 40% concentration was present in the upper compartment (Table III).

Chemotactic response of different fibroblast lines. Six different human fibroblast lines were tested for their ability to migrate in response to DEAE-Sephadex-purified SDCF (Table IV). All six fibroblast lines migrated in response to the SDCF (Table IV). This suggests that all human dermal fibroblasts recognize SDCF as a chemotactic stimulus.

Chemotactic response of fibroblasts dispersed with EDTA. Trypsin may alter plasma membrane recep-

TABLE IV

Response of Different Human Dermal Fibroblast Lines to SDCF

Fibroblast line	CTX*	
	SDCF‡	Buffer
	fibroblasts/OIF§	
D 21	90±11	10±1
T 57	63±7	8±1
T 50	61±4	7±2
T 2	89±8	12±2
S 1	63±5	6±1
Y 51	74±6	13±2

* Mean±SEM.

‡ SDCF from zymosan-activated serum was purified by Sephadex G-150 and DEAE-Sephadex A-50 chromatography as described in Methods.

§ Oil immersion field.

tors on cells. Its proteolytic action may lead to temporary modification of surface proteins so that previously unexposed receptors may become accessible. It may also destroy receptors, as has been demonstrated for the insulin receptors on the adipose cell (7, 8). The use of trypsin to harvest fibroblasts from monolayer cultures could theoretically alter the plasma membrane and expose an artificial receptor for SDCF. Therefore, it was essential to determine whether fibroblasts harvested with EDTA would respond chemotactically to SDCF. Fibroblasts grown in monolayer culture were dispersed with 5 mM EDTA so as not to alter surface membrane receptors. Fibroblasts harvested in this manner also migrated in response to the SDCF (data omitted), which suggests that the membrane recognition site for the factor is readily accessible and does not have to be uncovered or altered by trypsin.

Treatment of SDCF with anti-C3 and anti-C5 antisera. The generation of fibroblast CTX in serum by agents that activate the complement sequence suggested that the factor might be derived from a complement component. Because cleavage products from C3 and C5 have been reported to be chemotactic for leukocytes (5, 9–11), we wondered whether the fibroblast chemotactic factor might also be derived from either of these complement components. If C3 or C5 were the source of SDCF, then monospecific antiserum to these respective complement components might block its ability to act as a chemoattractant for fibroblasts. To explore this issue, aliquots (0.3 ml) of SDCF, obtained from fractionation of zymosan-treated serum by Sephadex G-150, were incubated with 0.05 ml of monospecific goat anti-human C3 or anti-human C5 antisera. Fibroblast CTX in treated and untreated samples was determined. The anti-C5-treated SDCF elicited no migration of fibroblasts (Table V). In contrast, anti-C3-treated SDCF retained CTX (Table V). As a control for nonspecific inhibition of fibroblast migration by the anti-C5 antiserum, collagen, a chemoattractant for fibroblasts, was incubated with the antiserum. The antiserum did not reduce migration of fibroblasts to collagen (Table V).

These data suggest that the SDCF generated by activators of complement share antigenic determinants in common with C5.

Inability of C5-deficient serum to generate SDCF. Because data obtained by the use of antisera suggested that the fibroblast chemotactic factor might be derived from C5, it was important to determine whether "activated" sera from subjects with C5 deficiency contained CTX for fibroblasts. Sera were obtained from two siblings with proven C5 deficiency and from two normal, control donors (12). Aliquots of each serum sample were incubated with saline, HAGG, or LPS and assayed for fibroblast CTX. Fibroblast CTX was not detectable in HAGG- or LPS-treated sera from the C5-deficient subjects (Table VI). Chemotactic activity for fibroblasts was generated in the normal sera by these complement activators (Table VI). These results suggest that SDCF is derived from C5 or that C5 is necessary for generation of SDCF.

TABLE V
Effect of Anti-C5 and Anti-C3 on CTX of SDCF

Condition*	CTX‡
	fibroblasts/20 OIF§
SDCF	60±3
SDCF + anti-C5	4±1
SDCF + anti-C3	57±5
Column buffer	7±1
Column buffer + anti-C5	5±1
Column buffer + anti-C3	5±2
Collagen	52±6
Collagen + anti-C5	53±4

* Zymosan-activated serum was fractionated on a column packed with Sephadex G-150. Aliquots (0.3 ml) of column buffer and the 80,000-mol wt peak that contained SDCF were separately incubated with 50 μl of heat-inactivated monospecific antiserum to human C5 or to human C3 for 1 h at 37°C. Type I chick skin collagen (1 mg/ml) served as a control. After incubation, MEM (0.3 ml) was added to each mixture, and samples were assayed in triplicate for fibroblast CTX.
‡ Mean±SEM.
§ Oil immersion field.

Effect of trypsin on SDCF. It was of interest to determine whether SDCF was a protein. Treatment of DEAE-Sephadex-purified SDCF with trypsin at 30°C resulted in destruction of fibroblast CTX (Table VII). These data suggest that an intact protein structure is essential for fibroblast CTX to be expressed by SDCF.

DISCUSSION

Activation of serum complement by classical or alternative pathways is accompanied by generation of a trypsin-sensitive protein with an apparent 80,000 mol wt that is chemotactic for human dermal fibroblasts in vitro. Several lines of evidence suggest that SDCF might be derived from the 5th component of complement. Chemotactic migration of fibroblasts to the factor is negated if it is preincubated with monospecific anti-C5 antiserum. This indicates that SDCF is either C5 derived or has antigenic similarity to C5. The factor is not generated when sera from patients genetically deficient in C5 are treated with endotoxin or HAGG. This suggests that the presence of C5 in human serum is essential for generation of SDCF.

Whereas SDCF has not been purified to homogeneity in this study, a combination of gel filtration and ion exchange chromatography effectively separated SDCF

TABLE VI
Inability of C5-Deficient Serum to Generate Fibroblast CTX

Condition*	CTX‡
	fibroblasts/20 OIF§
C5-deficient subject 1	
Serum + saline	6±1
Serum + HAGG	8±2
Serum + LPS	7±1
C5-deficient subject 2	
Serum + saline	4±1
Serum + HAGG	6±2
Serum + LPS	3±1
Normal subject 1	
Serum + saline	4±1
Serum + HAGG	48±4
Serum + LPS	51±6
Normal subject 2	
Serum + saline	6±1
Serum + HAGG	44±5
Serum + LPS	56±5

* Serum obtained from normal and C5-deficient donors was activated by HAGG and LPS as described in Methods and tested at a final concentration of 20%, vol/vol for fibroblast CTX. The C5-deficient donors had no detectable C5 as measured by a hemolytic assay or by radioimmunodiffusion (11).
‡ Mean±SEM.
§ Oil immersion field.

from serum proteins detectable by UV absorbance at 280 μm. This suggests that SDCF is a potent chemoattractant for fibroblasts, perhaps similar in potency to human lymphocyte-derived chemotactic factor for fibroblasts, which we have previously characterized (1).

Fibroblasts accumulate at sites of inflammatory reactions having diverse etiologies. Fibroblasts infiltrate lesions resulting from antigen-antibody reactions, as for example in the classic Arthus reaction (13). Fibroblasts are also attracted to inflammatory reactions having nonimmune etiologies such as those resulting from chemical, thermal, or traumatic injury (14–16). Studies by others have suggested that the extent of activation of the complement system largely determines the degree of severity of Arthus reactions and inflammation induced by turpentine (17–19). Activation of complement in these and perhaps other types of inflammatory reactions might lead to generation of SDCF and provide a chemotactic stimulus for connective tissue fibroblasts. Fibroblasts are also present in lesions associated with cell-mediated immune reactions in vivo, e.g., tuberculous and Schistosoma mansoni granulomas. We have previously described a lymphokine that is chemotactic for human fibroblasts in vitro (1). The release of this lymphocyte-derived chemotactic factor for fibroblasts in vivo at sites of cell-mediated immune reactions could serve as a chemotactic signal for fibroblasts. Lymphocyte-derived chemotactic factor for fibroblasts and SDCF may function in vivo as important fibroblast chemoattractants for the cell-mediated immune and humoral host defense systems, respectively.

TABLE VII
Cleavage of SDCF by Insoluble Trypsin

Condition*	Incubation at 30°C	CTX‡
	min	*fibroblasts/20 OIF§*
SDCF	0	62±4
SDCF + trypsin	30	19±2
	60	5±1
	120	6±1

* Fibroblast chemotactic factor from zymosan-treated serum was purified by Sephadex G-150 and DEAE-Sephadex chromatography. Aliquots (1 ml) were incubated with trypsin-polyacrylamide conjugate (0.68 U) as described in Methods. Reaction products were tested for fibroblast CTX.
‡ Mean±SEM.
§ Oil immersion field.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of J. McPherson, J. DeBerry, S. Brauer, B. Deming, and S. Turner.

This study was conducted under Veterans Administration research projects 4826-01 and 4862-02; and was supported in part by grants AM-16506 from the National Institute of Arthritis, Metabolism, and Digestive Diseases and grant DE03738-3 from the National Institute of Dental Research.

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