THE CHEMOTACTIC ATTRACTION OF HUMAN FIBROBLASTS TO A LYMPHOCYTE-DERIVED FACTOR

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Fibroblasts play a critical role in inflammatory reactions and wound healing by synthesizing new connective tissue substances (collagen and mucopolysaccharides) and constructing scar tissue. However, the mechanisms by which fibroblasts are attracted to sites of inflammation are unknown.

In the present report we describe a quantitative technique that measures fibroblast chemotaxis in vitro in modified Boyden chambers. While other workers have demonstrated that fibroblasts are capable of migrating in vitro and in vivo (1–4), this is the first description of a quantitative assay that measures fibroblast chemotactic response.

Using this assay we have found that human lymphocytes stimulated by specific antigen or nonspecific mitogen in vitro produce a heat stable proteinaceous substance that is chemotactic for fibroblasts. This substance will be referred to as "lymphocyte-derived chemotactic factor for fibroblasts" (LDCF-F).[1] LDCF-F is distinguishable from human "lymphocyte-derived chemotactic factor for monocytes" (LDCF-M).

Materials and Methods

Fibroblast Chemotaxis Assay. Dermal fibroblasts obtained from punch biopsy of the skin of normal adults were grown in monolayer culture by standard techniques. Cultures were maintained in 32 oz. Brockway bottles (Brockway Glass Co., Inc. Brockway, Pa.) in a humidified atmosphere containing 5% CO_2. Eagle's minimal essential media supplemented with nonessential amino acids, ascorbic acid (50 μg/ml), $NaHCO_3$, and HEPES buffers (pH 7.2), penicillin (100 U/ml), streptomycin (100 μg/ml), and heat-inactivated fetal calf serum (15%) was used as maintenance media. A major portion of the cells in each bottle was harvested every 3–5 days for use in the chemotaxis assay.

Fibroblasts were dispersed routinely by pouring off the maintenance media, washing the

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[1] *Abbreviations used in this paper:* CTX, chemotactic activity; GGBS, 0.015 M glycylglycine/0.14 M NaCl, pH 7.2; [^{3}H]TdR, tritiated thymidine; LBTI, lima bean trypsin inhibitor; LDCF-F, lymphocyte-derived chemotactic factor for fibroblasts; LDCF-M, lymphocyte-derived chemotactic factor for monocytes; MNL, mononuclear leukocyte(s); OIF, oil immersion field; PBS, 0.015 M phosphate/0.135 M NaCl; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin; RPMI 1640 + PSH, RPMI 1640 media containing penicillin (100 U/ml), streptomycin (100 μg/ml) and HEPES buffer (0.001 M), pH 7.4; SKSD, streptokinase-streptodornase.

monolayers three times with 30–40 ml of 0.015 M phosphate/0.135 M NaCl (PBS), pH 7.4, and by then adding 1.5 ml trypsin (0.25%) in PBS. During trypsinization each bottle was frequently agitated to facilitate detachment of the cells. Fibroblasts were exposed to trypsin for up to 3 min. Trypsin was inactivated by adding 10 ml maintenance media containing serum. Detached cells were collected from the Brockway bottles, centrifuged at 4°C at 300 *g* for 10 min, and washed two times with serum-free maintenance media. Fibroblasts were suspended at a concentration of 2.5×10^5 cells/ml of serum-free media at 4°C before being used in the assay.

Blind-well-modified Boyden chemotaxis chambers (Duke University Surgical Instrument Shop, Durham, N. C.) and polycarbonate filters, 13-mm diameter, containing 8-μm pores (Wallabs Inc., San Rafeal, Calif.) were used to measure fibroblast chemotaxis. The polycarbonate filters as obtained from the manufacturer were not suitable for use in the assay because fibroblasts would not adhere to and spread-out on the upper surface of the filters or migrate through the filter pores in response to a chemotactic stimulus (Fig. 1). We found that fibroblasts would adhere to and migrate through filters previously treated with a dilute gelatin solution so as to change the surface properties of the filters (Fig. 2). Therefore, filters used in all experiments were treated as follows: filters were placed in wire staining baskets (Duke University Surgical Instrument Shop), heated at 50°C in 0.5% acetic acid solution for 20 min, rinsed two times in glass-distilled water at 25°C, placed in a beaker containing gelatin in glass-distilled water (5 mg/liter) at 100°C for 1 h, dried with a hair dryer, and heated in an oven (100°C) for 1 h.

Substances being assayed for fibroblast chemotactic activity (CTX) were mixed with serum-free maintenance media (0.4 ml/0.35 ml). Aliquots of this mixture were placed in the lower compartment of blind-well chemotaxis chambers. Prepared polycarbonate filters (dull side up) were placed in the chambers so as to cover the filled lower compartment, and chamber caps containing the upper compartment were screwed into the chambers to effect a water tight seal around the periphery of the filter. The upper chamber compartments were then loaded with the fibroblast suspension, prepared as described above.

Loaded chambers were incubated at 37°C for 150 min in a humidified atmosphere containing 5% CO_2. After the incubation period, chambers were disassembled, filters removed, placed in staining baskets, fixed for 15 s in absolute ethanol, stained with hematoxylin, and mounted on glass cover slips. During incubation, fibroblasts responding to a chemotactic stimulus migrate from the upper filter surface through the pores and adhere to the lower filter surface (Fig. 2). Fibroblast CTX was quantitated by counting nuclei of fibroblasts on the lower surface of the filters in 20 oil immersion fields (× 1,000). All samples were assayed in triplicate, and final activity was expressed as the mean ± SEM of the replicates.

Monocyte Chemotaxis Assay. Monocyte chemotactic activity was assayed in certain samples by methods previously described (5). CTX was quantitated by determining the number of monocytes migrating per oil immersion field (× 1,000) after counting 20 fields.

Lymphocyte Cultures. Peripheral blood mononuclear leukocytes (MNL) were isolated from venous blood of normal human donors by the Ficoll-Hypaque technique. Isolated MNL were washed three times in RPMI 1640 media containing penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES buffer (0.01 M), pH 7.4 (PSH) at 4°C. Culture studies designed to measure production of LDCF-F were performed with MNL at a concentration of 6×10^6 cells/ml of RPMI 1640 + PSH and 5% AB+ heat-inactivated (56°C for 30 min) serum. Serum was omitted from cultures used to generate LDCF-F for gel filtration studies. Streptokinase-streptodornase (SKSD), 100 U/ml (American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.) and purified protein derivative of tuberculin (PPD), 100 μg/ml (Ministry of Agriculture, Fisheries, and Food, Surrey, England) were used as stimulating antigens. Phytohemagglutinin (PHA), 1 μg/ml (Burroughs-Wellcome, Research Triangle Park, N. C.) was used as a mitogen. Controls for each study consisted of antigen or mitogen added to harvested supernates from culture of nonstimulated lymphocytes.

Lymphocyte transformation was measured by culturing MNL at a concentration of 1×10^6 cells/ml RPMI 1640 + PSH containing 10% AB+ heat-inactivated human serum with and without antigen or mitogen. Four aliquots (0.2 ml) from each sample were placed in flat bottom wells of microtiter tissue culture plates (Linbro Plastic Co., Hamden, Conn.). Cultures were pulsed 20 h before harvest with 0.5 μCi tritiated thymidine ([^{3}H]TdR, New England Nuclear, Boston, Mass.) in 0.01 ml of media. Cells from all microcultures were harvested onto glass fiber filters (Reeve Angel Corp., Clifton, N. J.) with a multiple sample harvester (MASH III; Microbiological Asso-

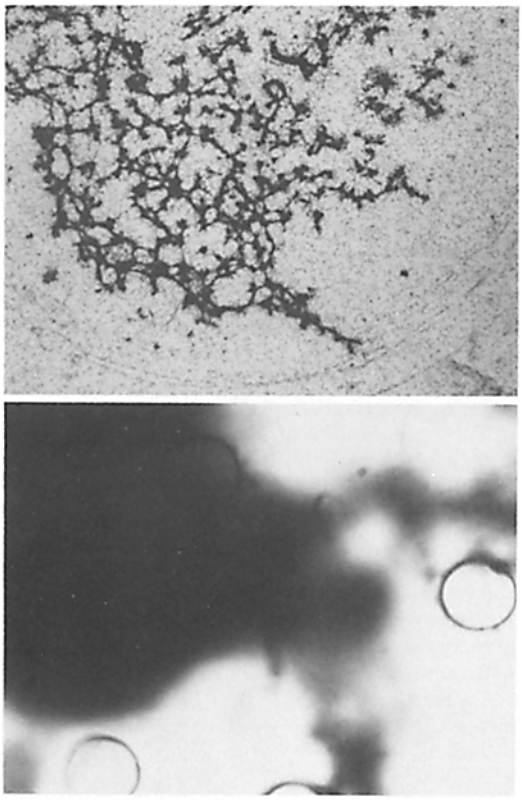

FIG. 1. The upper photograph is a low power view (× 8) of the upper surface of an untreated polycarbonate filter taken from a chemotaxis chamber after incubation for 150 min with an aliquot of supernate from 96-h culture of PHA-stimulated lymphocytes (see Materials and Methods). There is poor adherence of fibroblasts to the upper surface. The lower photograph is a view of the lower surface of the same filter (× 1,000). There is no fibroblast migration to the LDCF-F-rich supernate. The blurred images in the background represent fibroblasts on the upper surface of the filter.

ciates, Bethesda, Md.) followed by precipitation of incorporated label with 5% cold trichloroacetic acid (TCA).

Glass fiber filter disks containing TCA-precipitated material were placed in vials containing 5 ml Aquasol (New England Nuclear) and chilled at 4°C for 4 h. The radioactivity of each disk was measured in a Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). All lymphocyte cultures were incubated in a humidified atmosphere containing 5% CO_2 for specified lengths of time.

Gel Filtration Studies. Columns 2.5 cm in diameter and 100 cm in length containing either Sephadex G-100 or Sephadex G-75 superfine (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and equilibrated with 0.015 M glycylglycine/0.14 M NaCl (GGBS) at pH 7.2 were employed to isolate LDCF-F and LDCF-M from lyophilized lymphocyte culture supernates. All lyophilized supernates were dissolved in 3 ml GGBS and cleared of insoluble material by centrifugation before being loaded on the columns. Gel filtration was performed at 4°C. The column effluent was continuously monitored for absorbance at 280 nm with a Uvicord III recording spectrophotometer (LKB Instruments, Inc., Rockville, Md.). Columns were calibrated with proteins of known molecular weight, and a drop of tritiated water was mixed with each sample to serve as an internal marker for column volume.

Heat Treatment of LDCF-F. Heat stability of LDCF-F was evaluated by heating an aliquot of

an active Sephadex fraction to 56°C for 30 min and assaying heated and unheated aliquots for fibroblast CTX.

Trypsin Treatment of LDCF-F. To determine whether LDCF-F was susceptible to proteolytic digestion by trypsin, a 0.3-ml aliquot of a Sephadex fraction containing fibroblast CTX was added to trypsin (0.06 μg) (Sigma Chemical Co., St. Louis, Mo.), and the mixture was incubated at 37°C for 2 h. Lima bean trypsin inhibitor (LBTI) (2.7 μg) (Worthington Biochemical Corp., Freehold, N. J.) was added to inactivate trypsin after the 2-h incubation. As controls, aliquots of the Sephadex fraction with fibroblast CTX were incubated for 2 h with LBTI alone and with LBTI to which trypsin was added. Fibroblast CTX was then determined in all samples.

Neuraminidase Treatment of LDCF-F. In an attempt to determine whether an intact glycoprotein structure was essential for retention of the biological activity of LDCF-F, an active Sephadex fraction was treated with *Vibrio cholera* neuraminidase (Worthington Biochemical Corp.). Neuraminidase (0.6 and 6.0 μg) with 0.3-ml aliquots of buffer or active Sephadex fraction at pH 5 were incubated for 30 min at 37°C and then heated to 56°C for 30 min to partially inactivate the enzyme. The pH was then adjusted to 7.4, and all samples were assayed for fibroblast CTX. This enzyme preparation caused release of sialic acid from bovine maxillary mucin (Worthington Biochemical Corp.) in a preliminary experiment.

Role of Macrophage in LDCF-F Production. Since it has been demonstrated that macrophages are required for lymphocyte production of some lymphokines (6, 7), it was of interest to determine their role in LDCF-F production. MNL were prepared by Ficoll-Hypaque separation of venous blood obtained from a healthy donor. This population of cells contained 24% monocytes as determined by nonspecific esterase staining (8). A population of highly purified lymphocytes containing approximately 1% monocytes was prepared by placing the MNL in glass Petri dishes for 2 h and passing the nonadherent cells through a glass bead column as previously described by Horton et al. (7). A portion of the purified lymphocytes was mixed with fresh sheep erythrocytes to allow spontaneous rosettes to form (9). The rosetted and nonrosetted cells were separated from each other by isopyknic centrifugation on a Ficoll-Hypaque gradient (10). The denser rosetted cells (T lymphocytes) were collected from the pellet on the bottom of the centrifuge tube and were washed five times with RPMI 1640 + PSH. Less than 2% of this population of cells exhibited positive immunofluorescent staining with polyvalent rabbit anti-human immunoglobulin antisera, and only 0.6% exhibited staining for nonspecific esterase.

Autologous macrophages were prepared by the method described by Horton et al. (7). 86% of these cells stained positively for nonspecific esterase. The macrophage population was irradiated with 2,500 rads to kill contaminating lymphocytes and suspended in RPMI 1640 + PSH containing 20% AB+ serum at a concentration of 6×10^4 cells/ml. Aliquots (1 ml) were added to wells of Falcon 3008 multiwell tissue culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and incubated in a humidified atmosphere containing 5% CO_2 for 18 h to allow macrophages to attach to the plastic surfaces. The macrophage monolayers were then washed three times with RPMI 1640 + PSH to remove serum.

MNL, purified lymphocyte, and T-lymphocyte populations were each separately suspended in RPMI 1640 + PSH containing 5% heat-inactivated AB+ serum at a concentration of 4×10^6 cells/ml. Aliquots (1 ml) of each population were separately placed in wells of Falcon 3008 tissue culture plates and cultured with or without PHA (1.0 μg). Aliquots (1 ml) of purified lymphocytes and T lymphocytes were separately cultured with the macrophage monolayers with or without the addition of PHA. Supernates from all cultures were harvested after 48 h and were cleared of debris by centrifugation. As a control, PHA (1.0 μg) was added to supernates harvested from unstimulated cultures. All supernates were then assayed for fibroblast CTX.

Results

Lymphocyte Transformation and Fibroblast Chemotactic Factor Production. The amount of fibroblast CTX detectable in supernates of SKSD- and PHA-stimulated lymphocyte cultures at a given time was correlated with the degree of [^{3}H]TdR incorporation (Fig. 3). PHA-stimulated cultures exhibited maximal fibroblast CTX and [^{3}H]TdR incorporation at 72 h. SKSD-stimulated cultures contained maximal fibroblast CTX at 96 h, and this correlated with peak [^{3}H]TdR incorporation.

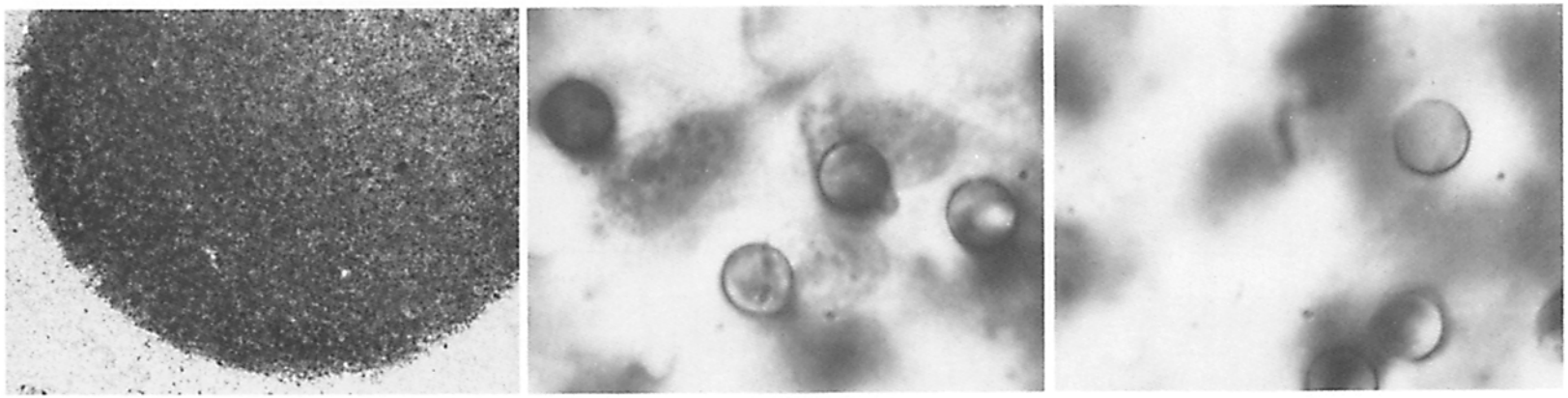

FIG. 2. Polycarbonate filters from the same lot used in Fig. 1 were treated with gelatin (see Materials and Methods) and placed in chemotaxis chambers containing aliquots of the same supernate from PHA-stimulated lymphocytes used in Fig. 1. The chemotactic response of fibroblasts to PHA-stimulated and PHA control supernates was determined. Illustrated on the left is a low power view (× 8) of the upper surface of a gelatin-treated filter taken from a chemotaxis chamber after incubation for 150 min with an aliquot of the same supernate from 96-h culture of PHA-stimulated lymphocytes used in Fig. 1. Note uniform adherence of fibroblasts to the upper surface of the filter. In the center is a view (× 1,000) of the lower surface of the same filter shown to the left. Nuclei from three fibroblasts migrating in response to PHA-stimulated supernate are visible on the lower surface of the filter. To the right is a view of the lower surface of a gelatin-treated filter taken from a chemotaxis chamber containing PHA control supernate. No fibroblasts are visible on the lower filter surface. Fibroblast chemotaxis was measured with untreated (Fig. 1) and gelatin-treated (Fig. 2) filters in the same experiment. The same fibroblast suspension was loaded into upper compartments of chambers containing untreated or treated filters.

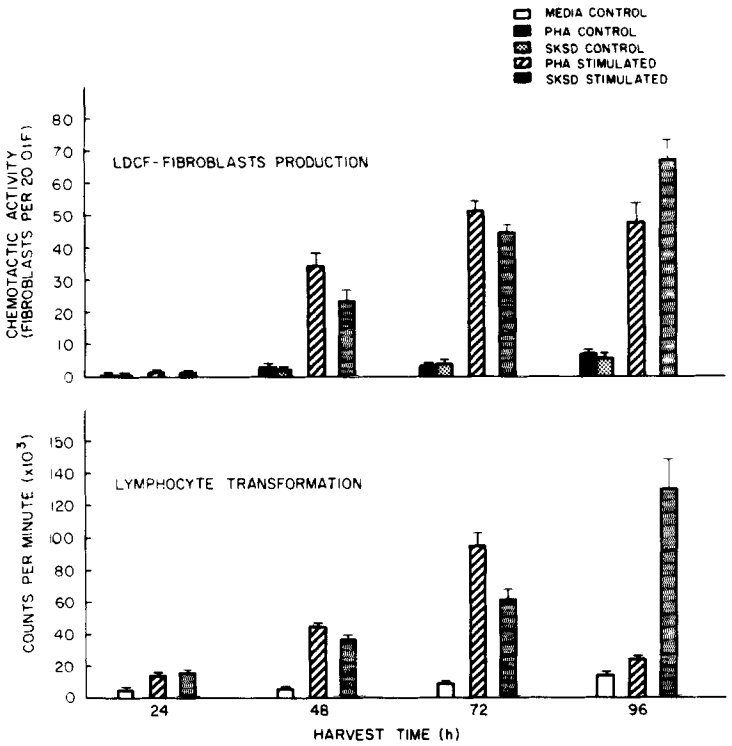

FIG. 3. Lymphocytes were cultured from a donor having a positive delayed skin test to SKSD. CTX for fibroblasts (upper panel) and lymphocyte transformation (lower panel) were measured in control, PHA- (1 μg/ml), and SKSD-stimulated (100 U/ml) cultures. Cultures were harvested daily on four successive days. Production of LDCF-F was synchronous with [^{3}H]TdR incorporation.

Gel Filtration Studies. Gel filtration of lyophilized supernate from a culture of SKSD-stimulated lymphocytes from a SKSD skin test-positive donor yielded a single peak of fibroblast CTX that eluted from the Sephadex G-100 column after excluded proteins (Fig. 4). Supernate from nonstimulated lymphocytes from the same donor, to which SKSD was added after culture, when subjected to gel filtration on the same column did not yield active fractions. The mol wt of LDCF-F as determined by gel filtration on G-100 is approximately 22,000 daltons (Fig. 5).

Additional studies revealed that LDCF-F was clearly distinguishable from LDCF-M. When concentrated supernate from a culture of PHA-stimulated lymphocytes was subjected to gel filtration on a 2.5 × 100 cm column of Sephadex G-75 superfine, a discrete peak of fibroblast CTX eluted in advance of the monocyte CTX peak (Fig. 6).

Physicochemical Characterization of LDCF-F. Physicochemical studies were performed on partially purified LDCF-F isolated by Sephadex G-100 chromatography. LDCF-F was found to be heat stable since its biological activity was unchanged after incubation at 56°C for 30 min (Table I).

LDCF-F is protein in nature, being susceptible to digestion by trypsin (Table II). LBTI used to inactivate trypsin after its incubation with LDCF-F did not effect the fibroblasts' chemotactic response to LDCF-F, and it was not chemotac-

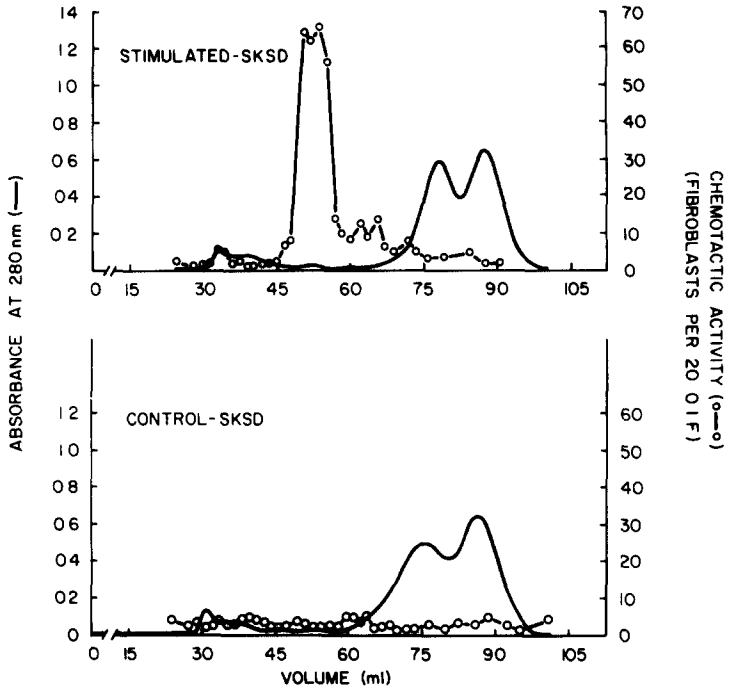

FIG. 4. MNL (4×10^6 cells/ml) from a donor with a positive delayed skin test to SKSD were cultured in serum-free RPMI 1640 + PSH for 72 h. To one-half of the cells SKSD (100 U/ml) was added before culture, and to the other half SKSD was added to the supernate after harvest at 72 h. Culture supernates were lyophilized, and 30 mg of each was dissolved in GGBS (3 ml) and chromatographed at different times on the same Sephadex G-100 column (2.5 × 100 cm). Fractions (4.9 ml) were collected from each column run and were assayed on different days for fibroblast CTX (see Materials and Methods). A single peak of fibroblast CTX was found in fractions from gel filtration of SKSD-stimulated supernate (upper panel). No CTX was found in fractions from gel filtration of control supernate. A positive control (0.35 ml unfractionated SKSD-stimulated supernate) was included in each chemotaxis experiment. Fibroblast CTX for the control was 63 ± 5 on the day "stimulated SKSD" fractions were assayed and 57 ± 6 on the day "control-SKSD" fractions were assayed.

tic itself (Table II). The biological activity of LDCF-F does not appear to depend on a glycopeptide structure involving sialic acid, since it retained its activity after neuraminidase treatment (Table III).

Effect of LDCF-F on Fibroblast Migration. It was essential to determine whether the fibroblasts were recognizing the lymphocyte-derived factor as a chemotactic stimulus or as a stimulator of random cell migration. To determine which was the case, we compared migration of fibroblasts through the chamber filters in the presence and absence of a concentration gradient of LDCF-F (Fig. 7). When LDCF-F was placed in equal concentration in the upper and lower compartments of the modified Boyden chamber, migration of fibroblasts through the filter was the same as when buffer was added in equal concentration to both compartments (Fig. 7), suggesting that random migration of the cell was not stimulated by the lymphocyte-derived factor. In contrast, fibroblasts migrated through the chamber filters in numbers greater than that observed in

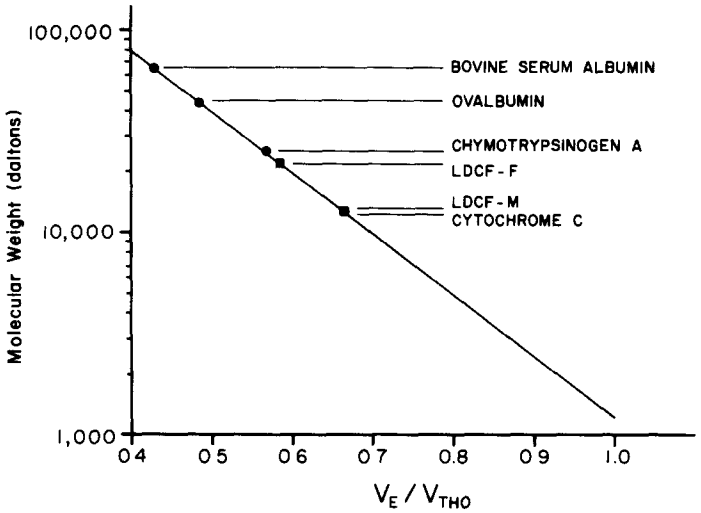

FIG. 5. The molecular weights of proteins (vertical log scale) are plotted as a function of their elution volume (V_E) relative to that of tritiated water (V_{THO}). The column (2.5 × 100 cm) was packed with Sephadex G-75 superfine. The mol wt of LDCF-F is 22,000 daltons.

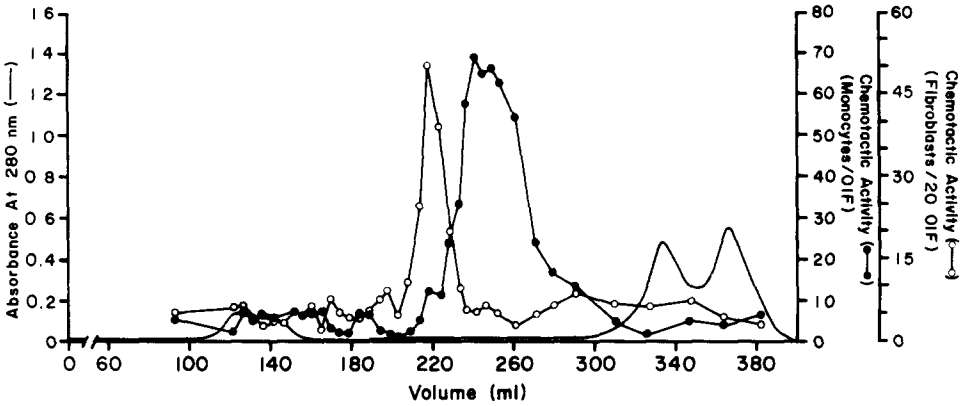

FIG. 6. MNL (4 × 10^6/ml) were cultured with PHA (1 μg/ml) for 72 h in serum-free RPMI 1640 + PSH. Lyophilized culture supernate (30 mg dissolved in 3 ml GGBS) was applied to a column (2.5 × 100 cm) packed with Sephadex G-75 superfine. Fractions (4.9 ml) were collected and assayed for monocyte and fibroblast CTX (see Materials and Methods). LDCF-F eluted in advance of LDCF-M.

chambers having the buffer control only when LDCF-F was added exclusively to the lower test compartment (Fig. 7), suggesting that it stimulates fibroblast migration by acting as a chemotactic stimulus.

Macrophage Dependence for LDCF-F Production. Culture studies revealed that lymphocytes stimulated with PHA required the presence of macrophages for production of LDCF-F. Unfractionated MNL containing 24% monocytes elaborated fibroblast chemotactic factor in response to PHA stimulation (Table IV). However, when lymphocytes were purified from MNL they did not produce chemotactic factor unless macrophages were added to the culture (Table IV). In

TABLE I
Effect of Heat on Chemotactic Activity of LDCF-F

Substance tested*	Incubation	Chemotactic activity (mean ± SEM)
		fibroblasts per 20 OIF
LDCF-F	56°C-30 min	54 ± 6
Column effluent	56°C-30 min	5 ± 1
LDCF-F	–	48 ± 5
Column effluent	–	3 ± 1

* Aliquots (0.3 ml) of a LDCF-F-rich Sephadex G-100 fraction or of column effluent were tested for fibroblast CTX with and without being heated to 56°C for 30 min.

TABLE II
Effect of Trypsin on Chemotactic Activity of LDCF-F

Substances added		Chemotactic activity (mean ± SEM)
Before incubation*	After incubation*	
		fibroblasts per 20 OIF
LDCF‡	GGBS-$CaCl_2$ (50 μl)§	42 ± 6
LDCF-F + trypsin‖ (25 μl)	LBTI¶ (25 μl)	5 ± 1
LDCF-F + LBTI (25 μl)	Trypsin (25 μl)	37 ± 3
LDCF-F + LBTI (25 μl)	GGBS-$CaCl_2$ (25 μl)	41 ± 5
Column effluent§	GGBS-$CaCl_2$ (50 μl)	4 ± 1

* Incubations were at 37°C for 2 h.
‡ Aliquots (0.3 ml) of a LDCF-F-rich column fraction or of column effluent collected before the excluded volume from a Sephadex G-100 column.
§ GGBS supplemented with 0.005 M $CaCl_2$.
‖ Trypsin (0.06 μg in 25 μl of GGBS-$CaCl_2$) was added as indicated.
¶ LBTI (2.7 μg in 25 μl of GGBS-$CaCl_2$) was added as noted.

like manner, T lymphocytes did not produce chemotactic factor unless cultures were supplemented with macrophages (Table IV).

Fibroblast Chemotactic Factor Production and Skin Test Positivity. In an effort to investigate the correlation between delayed dermal hypersensitivity and production of LDCF-F, CTX for fibroblasts was determined in supernates from cultures of lymphocytes from four PPD skin test-positive and four PPD skin test-negative healthy volunteers (Table V). PPD-positive subjects produced fibroblast chemotactic factor when their MNL were cultured with PPD, mean ± SEM CTX for PPD control and stimulated cultures being 13 ± 1 and 60 ± 4, respectively. In contrast, PPD-negative subjects did not produce chemotactic factor when their lymphocytes were cultured with PPD, mean ± SEM CTX for control and stimulated cultures being 15 ± 1 and 17 ± 2, respectively. Production of LDCF-F by MNL after PHA stimulation was similar in PPD-positive and PPD-negative groups, mean ± SEM CTX being 66 ± 7 and 57 ± 5, respectively. Mean fibroblast CTX in supernates of PHA control cultures was 13 ± 2 in tuberculin-positive and 15 ± 1 in tuberculin-negative subjects.

Chemotactic Response of Different Fibroblast Lines. Six different human dermal fibroblast lines were tested for their ability to respond to aliquots of the

TABLE III
Effect of Neuraminidase on CTX of LDCF-F

Condition*	Chemotactic activity (mean ± SEM)
	fibroblasts per 20 OIF
LDCF-F + GGBS (25 μl)	37 ± 4
LDCF-F + neuraminidase (0.6 μg/25 μl GGBS)	42 ± 6
LDCF-F + neuraminidase (6 μg/25 μl GGBS)	44 ± 5
Column effluent + neuraminidase (0.6 μg/25 μl GGBS)	4 ± 1
Column effluent + neuraminidase (6 μg/25 μl GGBS)	4 ± 1
Column effluent + GGBS (25 μl)	3 ± 1

* LDCF-F-rich Sephadex fraction or column effluent (0.3-ml aliquots of each) were incubated with or without neuraminidase at pH 5.0, 37°C for 30 min and at 56°C for 30 min. The pH was then adjusted to 7.4 and samples were assayed for chemotactic activity.

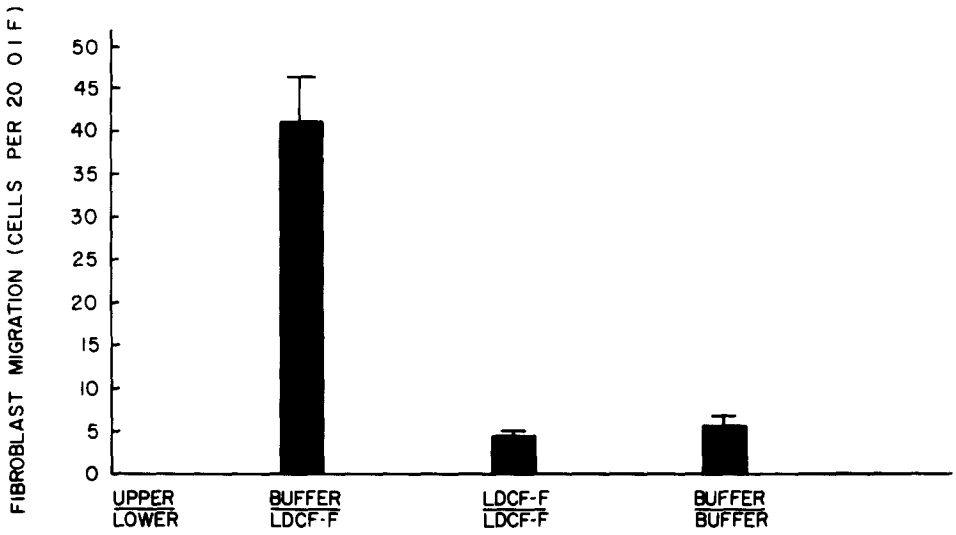

FIG. 7. LDCF-F-rich fraction or column buffer were added in equal amounts to the upper and, or lower compartments of modified Boyden chemotaxis chambers as illustrated. Fibroblast migration through chamber filters occurred only when a concentration gradient existed across the filters by placing LDCF-F exclusively in the lower chamber compartment.

same supernates from PHA-stimulated and control lymphocyte cultures. Migration to control culture supernate ranged from 12 to 23 cells per 20 oil immersion fields (OIF) (Table VI). Migration of fibroblasts to supernate from PHA-stimulated lymphocyte culture ranged from 61 to 95 cells per 20 OIF (Table IV). Fibroblast migration to PHA-stimulated culture supernate was statistically different from migration to control supernate when analyzed by the paired Student's t test ($P < 0.0005$). These data suggest that the chemotactic attraction of dermal fibroblasts to LDCF-F is probably an inherent property of dermal fibroblasts of all normal individuals.

Chemotactic Response of Fibroblasts Dispersed with EDTA. The use of trypsin to disperse cells from monolayer cultures raised the question of altera-

TABLE IV

Macrophage Dependence for LDCF-F Production by Lymphocytes

Culture*	Chemotactic activity* (mean ± SEM)	
	fibroblasts per 20 OIF	
Mononuclear leukocytes		
Control	12 ± 2	
PHA	44 ± 5	
	With macrophages	*Without macrophages*
Purified lymphocytes		
Control	20 ± 3	3 ± 1
PHA	82 ± 12	6 ± 1
T lymphocytes		
Control	14 ± 2	2 ± 3
PHA	65 ± 7	1 ± 1
Media		
Control	11 ± 1	4 ± 1
PHA	10 ± 2	5 ± 2

* MNL, purified lymphocytes, T lymphocytes, and media were cultured with and without PHA (1 μg/ml). Macrophages were cultured with purified lymphocytes, T lymphocytes, and media as noted. See Materials and Methods for details.

‡ Culture supernates were harvested after 48 h and assayed for fibroblast CTX.

TABLE V

*LDCF-F Production by Tuberculin-Positive and -Negative Subjects**

Condition	Chemotactic activity (mean ± SEM)	
	Control	PPD
	fibroblasts for 20 OIF	
Tuberculin-positive subject		
1	10 ± 2	60 ± 9
2	16 ± 2	65 ± 8
3	12 ± 3	49 ± 4
4	14 ± 1	65 ± 7
Mean ± SEM	13 ± 1	60 ± 4
Tuberculin-negative subject		
5	15 ± 2	19 ± 1
6	13 ± 1	17 ± 2
7	16 ± 3	20 ± 3
8	14 ± 1	13 ± 3
Mean ± SEM	15 ± 1	17 ± 2

* Peripheral blood MNL were obtained from tuberculin-positive and -negative subjects and cultured as described in Materials and Methods for 4 days with or without PPD (10 μg/ml) and PHA (1 μg/ml). As controls, cells from each subject were cultured with media alone, and PPD and PHA were each added separately to harvested supernates. PHA induced production of LDCF-F to a similar degree in tuberculin-positive and -negative individuals, CTX being 66 ± 7 and 57 ± 5, respectively. Mean CTX in PHA control cultures of tuberculin-positive and -negative subjects was 13 ± 2 and 15 ± 1, respectively.

TABLE VI
*Chemotactic Response of Different Human Fibroblast Lines to LDCF-F**

Fibroblast line	Chemotactic activity (mean ± SEM)	
	Control	PHA
	fibroblasts per 20 OIF	
S-10	12 ± 2	89 ± 6
Y-51	17 ± 2	81 ± 5
S-14	23 ± 1	72 ± 8
S-19	16 ± 2	86 ± 8
D-21	14 ± 1	61 ± 6
S-21	19 ± 3	95 ± 10

* Six different fibroblast lines were tested on the same experimental day to compare their responsiveness to aliquots of the same supernates from culture of PHA-stimulated and control MNL from a normal donor (see Materials and Methods). Each culture supernate (0.15 ml) was mixed with 0.25 ml GGBS and 0.35 ml serum-free maintenance media and assayed in triplicate for fibroblast CTX.

tion of the fibroblast's membrane receptor for LDCF-F by the proteolytic action of the enzyme. Evidence accumulated from other sources has suggested that in some instances proteases may temporarily uncover hidden membrane receptors not normally exposed and in effect create a new receptor (11). To determine whether trypsin was uncovering a membrane receptor for LDCF-F not normally accessible, fibroblasts grown in monolayer culture for 4 days were dispersed with 0.0005 M EDTA and tested for their ability to respond chemotactically to LDCF-F. Fibroblasts harvested with EDTA were able to respond chemotactically to LDCF-F in supernates from culture of PHA- and SKSD-stimulated lymphocytes or in column fractions (Table VII).

Effect of Prolonged Trypsinization on Fibroblast Chemotactic Responsiveness. The length of time that fibroblasts are exposed to trypsin during dispersion from monolayer culture influences their ability to respond chemotactically to LDCF-F. Fibroblasts respond well if trypsinization is limited to 3 or 5 min (Table VIII). If trypsinization is continued for 10 min the response is greatly diminished, and if it is continued for 15 min fibroblasts do not respond chemotactically at all (Table VIII). Fibroblasts exposed to trypsin for 15 min adhered to the upper surface of the gelatin-treated polycarbonate filters just as well as those exposed for 3 or 5 min and appeared to be unaltered morphologically by light microscopy (data not shown).

Discussion

A quantitative in vitro assay that measures fibroblast chemotaxis in modified Boyden chambers has been described. By employing this technique, we have found that human peripheral blood lymphocytes when stimulated by mitogen (PHA) or antigens (SKSD, PPD) produce a factor that is chemotactic for human dermal fibroblasts. We have called this substance "lymphocyte-derived chemotactic factor for fibroblasts" or LDCF-F.

Production of LDCF-F by lymphocytes obtained by isopyknic centrifugation of

TABLE VII
*Chemotactic Response of Fibroblasts Dispersed with 0.0005 M EDTA**

Substance tested	Chemotactic Activity (mean ± SEM)
	fibroblasts per 20 OIF
LDCF-F‡	36 ± 3
Buffer	5 ± 2
Lymphocyte supernates§	
Stimulated-PHA	79 ± 6
Control-PHA	13 ± 6
Stimulated-SKSD	95 ± 5
Control-SKSD	14 ± 1

* Fibroblasts were grown in monolayer culture for 4 days and harvested with 0.0005 M EDTA in PBS. Dispersed cells were washed two times in maintenance media and tested for their ability to respond chemotactically.

‡ LDCF-F was obtained from pooled active Sephadex fractions.

§ Lymphocyte supernates were harvested after 96 h of culture in RPMI 1640 media supplemented with 5% AB+ human serum (see Materials and Methods).

TABLE VIII
Effect of Duration of Trypsinization on Fibroblast Chemotactic Responsiveness

Trypsinization*	Chemotactic activity (mean ± SEM)	
	Control	PHA
min	*fibroblasts per 20 OIF*	
3	18 ± 1	88 ± 9
5	16 ± 2	96 ± 9
10	8 ± 1	19 ± 1
15	10 ± 2	10 ± 1

* Fibroblasts of the same line were grown in four Brockway bottles. Cells were dispersed by 0.25% trypsin in PBS as described in Materials and Methods except the duration of trypsinization was different for each bottle, ranging from 3 to 15 min as indicated. Cells from each bottle were tested for their ability to migrate in modified Boyden chambers to aliquots of the same control and PHA-stimulated lymphocyte culture supernates.

peripheral blood with Ficoll-Hypaque appears to be synchronous with mitogenesis induced by antigen or mitogen stimulation. In addition, production of LDCF-F in the presence of PPD correlates with delayed hypersensitivity to this antigen as measured by skin testing.

Purified T lymphocytes stimulated by PHA require macrophages for production of LDCF-F. Osteoclast-activating factor and interferon are two other lymphokines that require macrophages for their production by lymphocytes (6, 7).

LDCF-F from antigen- or mitogen-stimulated lymphocytes has a mol wt of approximately 22,000 daltons. It is clearly distinct from LDCF-M. The findings that LDCF-F is not chemotactic for human monocytes, and that LDCF-M is not

chemotactic for fibroblasts suggest that chemotactic receptors on monocytes and fibroblasts may be different. In contrast to this dichotomy between fibroblast and monocyte receptors for lymphocyte-derived chemotactic factors, we have found that native collagen, constituent α1- and α2-chains of collagen and peptides obtained by collagenase, cyanogen bromide, and pepsin degradation of collagen are chemotactic for monocytes (12) and fibroblasts.[2] Thus, it appears that similar receptors for collagen and collagen peptides are present on both fibroblasts and monocytes.

The fibroblast surface membrane receptor for LDCF-F has not been fully characterized, but our data suggest that it is readily accessible, being present on cells dispersed by EDTA or brief trypsinization. The finding that prolonged trypsinization renders the fibroblast chemotactically unresponsive to LDCF-F could be due to removal or alteration of the receptor or to an effect of trypsin on other membrane proteins involved in the chemotactic response. The demonstration that trypsin can temporarily destroy a membrane receptor has been extensively documented for the adipose cell receptor for insulin (13–16). Definitive information regarding the nature of the fibroblast receptor for LDCF-F will have to await its isolation and purification.

The finding that fibroblasts are able to respond chemotactically to a T-lymphocyte-derived factor in vitro may have significance in vivo. For example, cell-mediated immune reactions produced by tubercle bacilli and *Schistosoma mansoni* result in formation of granulomas that are characterized by the presence of increased members of fibroblasts (17, 18). Perhaps the release of LDCF-F by sensitized, stimulated lymphocytes might function to attract neighboring connective tissue fibroblasts to these granulomas. The organized connective tissue capsule that characteristically surrounds "benign" tumors may in like manner result from release of LDCF-F by host lymphocytes as they react to neoplastic cells.

Recently, humans with pulmonary fibrosis (19) and progressive systemic sclerosis (20) have been found to have evidence of cell-mediated immunity to collagen. Both of these conditions are characterized by lesions that contain increased numbers of fibroblasts and increased deposition of collagen. Perhaps T lymphocytes sensitized to collagen in these disease states release LDCF-F upon contact with collagen in vivo, and this leads to fibroblast accumulation.

This study demonstrates that lymphocytes under appropriate stimulation by antigen or mitogen in vitro produce a chemotactic factor for fibroblasts. The function and role of such a chemotactic factor in vivo remain to be defined.

Summary

A quantitative assay that measures fibroblast chemotaxis in vitro is described. Application of this technique has revealed that peripheral blood lymphocytes stimulated by antigen or mitogen in vitro produce a factor that is chemotactic for human dermal fibroblasts. This lymphocyte-derived chemotactic factor for fibroblasts (LDCF-F) is different from the lymphokine that is chemotactic for monocytes or macrophages. Macrophages are required for the genera-

[2] Postlethwaite, A. E., and A. H. Kang. Chemotactic attraction of human fibroblasts to collagen and collagen derived peptides. Manuscript submitted for publication.

tion of LDCF-F by T lymphocytes stimulated by phytohemagglutinin. The fibroblast chemotactic factor is heat stable (56°C for 30 min), trypsin sensitive, and neuraminidase resistant. LDCF-F could function to attact connective tissue fibroblasts to sites at which cell-mediated immune reactions are occurring in vivo.

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