The Journal of Immunology

Macrophage production of fibronectin, a chemoattractant for fibroblasts.

Y Tsukamoto, W E Helsel and S M Wahl

J Immunol 1981; 127:673-678; ;
http://www.jimmunol.org/content/127/2/673

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

0022-1767/81/1272-0673$00.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 127, No. 2, August 1981
Printed in U.S.A.

MACROPHAGE PRODUCTION OF FIBRONECTIN, A CHEMOATTRACTANT FOR FIBROBLASTS

YOSHIO TSUKAMOTO,[1] WILLIAM E. HELSEL, AND SHARON M. WAHL[2]

From the Laboratory of Microbiology and Immunology, NIDR, National Institutes of Health, Bethesda, Maryland 20205

Activation of macrophages results in the production of numerous enzymes and effector molecules. One of these monokines released by macrophages can cause directed migration of connective tissue fibroblasts *in vitro*. Production of this macrophage-derived chemotactic factor for fibroblasts requires activation of the macrophages either *in vivo* or *in vitro* and *de novo* protein synthesis. The chemotactic activity in the macrophage supernatants could be removed by a fibronectin-specific affinity column and was inhibited in the presence of antibodies to fibronectin. Furthermore, chemotactic activity in the depleted macrophage supernatants could be restored by the addition of exogenous fibronectin. Fibronectin was identified in activated macrophage supernatants by an enzyme-linked immunoassay for fibronectin. From these findings it was concluded that activated macrophages release a chemoattractant for fibroblasts and that the primary chemoattractant molecule is fibronectin. The production of fibronectin by activated macrophages may thus serve as an inflammatory mediator that in addition to its other functions can recruit fibroblasts to an area of damaged tissue, where they can proliferate and form the scar tissue necessary for tissue repair. Furthermore, in chronic inflammation, the prolonged activation of macrophages may be related to the extensive fibroblast infiltration and fibrosis that can accompany these lesions.

As most inflammatory responses progress, the appearance of fibroblasts becomes evident. These fibroblasts, through the production of collagen, are responsible for repair of the tissue injury caused during the inflammatory process. This collagen production, that may be extensive in chronic inflammation, may also result in fibrotic organ damage and replacement of functional parenchymal cells. Thus, the fibroblast plays an important role in the final outcome of many inflammatory lesions. What attracts the fibroblasts to these inflammatory sites and regulates their functions is poorly understood. Lymphocytes have recently been shown to produce a chemotactic factor for fibroblasts (1, 2). An additional mechanism for stimulating fibroblast chemotaxis may be through the generation of a chemotactic factor in serum by the activation of complement (3). At the site of an inflammatory lesion, especially in nonspecifically induced inflammation, one of the most obvious cells is the macrophage. Because the appearance of the fibroblasts is preceded by macrophages, it was of interest to determine whether macrophages could also recruit fibroblasts into an inflammatory site through the release of soluble mediators. It was found that a monokine is released by activated macrophages that causes directed migration of fibroblasts, suggesting a molecular link between the inflammatory process and the subsequent healing and scar formation. During the course of characterizing the macrophage-derived fibroblast chemotactic factor, it became clear that this molecule was distinct from other previously identified monokines such as lymphocyte activating factor (4), colony stimulating factor (5), or fibroblast activating factor (6–8). Recent studies have reported that macrophages were capable of synthesizing the high m.w. glycoprotein, fibronectin (9–11). Because of the involvement of fibronectin in cellular adhesion, spreading and locomotion (12), we investigated the possibility that macrophage-derived fibronectin was involved in the migration of fibroblasts induced by macrophage supernatants. Here we report that macrophage-derived fibronectin is itself a stimulant of fibroblast chemotaxis.

Received for publication March 6, 1981.
Accepted for publication April 30, 1981.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Present Address: Department of Pharmacology, Osaka Dental University, Kyobashi, Higashiku, Osaka 540, Japan.
[2] To whom all correspondence should be addressed.

MATERIALS AND METHODS

Macrophage culture. Peritoneal exudate cells were harvested 4 days after i.p. injection of guinea pigs with sterile mineral oil as described previously (7). The washed cells were routinely cultured at 6×10^7 cells/10 ml Dulbecco Vogt's medium (Grand Island Biological Co., Grand Island, NY) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA). After a 2 hr incubation, the nonadherent cells were removed by washing, leaving a population of >95% phagocytic cells. These adherent monolayers were cultured with or without stimulants and the cellfree supernatants were harvested at various time points as indicated for each experiment. Stimuli included lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, MI) and N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP)[3] (Boehringer Mannheim Biochemicals, Indianapolis, IN). Additional populations of adherent peritoneal cells were obtained from noninjected guinea pigs and from guinea pigs injected i.p. with thioglycollate (20 ml) or 1% shellfish glycogen (30 ml) (NIH Media Unit). The culturing and processing of these cells was identical to that of oil-induced peritoneal cells.

Fibroblast chemotaxis assay. Guinea pig dermal fibroblasts were obtained and subcultured as reported (13). Fibroblasts were harvested between 5 and 20 passages by a brief (2 to 3 min) trypsinization (0.1% trypsin in Dulbecco's phosphate-buffered saline without Ca^{++} and Mg^{++}, containing 2% dextran and ethylene diaminetetraacetate, NIH Media Unit). The cells were suspended in Dulbecco Vogt's medium containing 10% fetal calf serum (FCS) (Flow Laboratories, Rockville, MD), washed, and preincubated for 20 min at 37°C in serum supplemented medium. After being washed twice in serum-free medium at 4°C and resuspended in serum-free medium at 2.5×10^5 cells/ml, the fibroblasts were added to blind-well chemotactic chambers (Neuroprobe, Rockville, MD) containing appropriate chemotactic stimuli diluted in Dulbecco-Vogt's medium and polycarbonate filters with 8-μ pores (Neuroprobe) as described by Postlethwaite *et al.* (1). The polycarbonate filters were either coated with gelatin (1) or additional filters that were polyvinylpyrrolidone (PVP)-free (Neuroprobe) were not coated with gelatin before being used in the fibroblast chemotaxis assay. After a 150 min incubation at 37°C in a humidified 5% CO_2 incubator, the filters were removed from the chambers, stained with Diff-Quik (Harleco, Gibbstown, NJ) and quantitated. Data represent the mean ± 1 SE of fibroblasts per high power field (20 fields counted) for triplicate filters.

Fibroblast proliferation. Guinea pig dermal fibroblasts plated in plastic TC24 culture dishes (Costar, Cambridge, MA) at 5×10^4 cells/ml were incubated 4 hr in Dulbecco-Vogt's medium containing 10% FCS (13). The cultures were washed with serum-free medium and incubated overnight in the absence of serum. After aspirating this medium, diluted serum-free macrophage supernatants were added to the fibroblast cultures for 48 hr. The fibroblasts were pulsed 4 hr with 1 μCi/ml tritiated thymidine (TdR^3H, sp. act. 6.0 Ci/mM, Schwarz/Mann, Orangeburg, NY), trypsinized and harvested with a modified Skatron automated harvester (Flow Laboratories) (13). Data represent mean counts per minute ± 1 SE of triplicate cultures.

Heat and enzymatic treatment of macrophage supernatants. Aliquots of

[3] Abbreviations used in this paper: MDP, *N*-acetyl-muramyl-L-alanyl-D-isoglutamine; ELISA, Enzyme-linked immunoassay; FAF, Fibroblast activating factor; PVP, Polyvinylpyrrolidone.

supernatants from macrophage cultures were heated to 56°C for 30 min or to 100°C for 10 min to determine the heat stability of the chemotactic activity. Additional aliquots were treated with trypsin (0.39 U/ml) conjugated to polyacrylamide beads (Sigma Chemical Co.) at 37°C for varying time periods. The insoluble trypsin was removed by centrifugation (400 × G, 4°C) and the samples were then tested for fibroblast chemotaxis. The serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) (Calbiochem, La Jolla, CA) was added to macrophage supernatants at a final concentration of 10^{-4} M for 1 hr, and these supernatants were dialyzed extensively against PBS and finally Dulbecco-Vogt's medium before assay for chemotactic activity.

Sephadex gel filtration chromatography. Macrophage supernatants were dialyzed against 30 μm Tris (pH 7.45) and concentrated by lyophilization. The concentrated preparations were resuspended in phosphate-buffered saline (PBS) and applied to a precalibrated Sephadex G-75 column (Pharmacia Fine Chemicals) or a Sephadex G-200 column. The absorbance (280 nm) was determined on the individual fractions that were then dialyzed against Dulbecco-Vogt's medium before assay for fibroblast chemotactic activity.

Collagen sepharose affinity chromatography. Type I collagen was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway) by described methods (14, 15) (a gift from Dr. Hynda Kleinman, NIDR). After equilibration of the packed column with 0.05 M Tris (pH 5.3) containing 0.025 M 6-aminohexanoic acid, 20 ml guinea pig serum were added to the column, incubated 90 min at 21°C, and the unbound protein washed through the column. Bound protein was eluted with 6 M urea and the protein peak (OD_{280}) pooled, dialyzed, lyophilized, and analyzed by polyacrylamide gel electrophoresis in the presence of SDS (16).

A similar procedure was used to prepare fibronectin and fibronectin-free preparations from macrophage culture supernatants. The collagen Sepharose affinity column was equilibrated with Dulbecco-Vogts medium containing antibiotics and glutamine before the supernatants were added. Bound and unbound macrophage products were pooled, aliquoted, and frozen until assayed.

Preparation of antibodies to fibronectin. Fibronectin was isolated from guinea pig serum by collagen Sepharose affinity chromatography. The resultant fibronectin-containing preparation was further purified by preparative polyacrylamide slab gel electrophoresis (16). The fibronectin band was cut from the gel, minced in PBS, and emulsified with an equal volume of complete Freund's adjuvant (Difco). Rabbits were immunized by subcutaneous injections of this preparation as described (17) and boosted at 2 to 3 wk intervals with fibronectin in Freund's incomplete adjuvant. Sera were screened by Ouchterlony to determine if the animals were immunized and if additional booster injections were required. Final sera were analyzed by enzyme-linked immunoassay (see below). Additionally, human fibronectin and goat antiserum to human fibronectin were purchased from Calbiochem-Behring Corp. (San Diego, CA).

Enzyme-linked immunoassay (ELISA) for fibronectin. The ELISA for determination of fibronectin in the macrophage supernatants was carried out according to the method of Rennard *et al.* (18). Briefly, suspensions containing antifibronectin aniibodies preincubated with various dilutions of macrophage supernatants (1:1 to 1:512) were added to fibronectin-coated microtiter plate wells (Dynatech Diagnostics, Inc., South Windham, ME). After a 1 hr incubation, the plates were washed, and a peroxidase-labeled anti-immunoglobulin was added. Finally, the substrate, O-phenylene-diamine was added to the wells for 1 to 6 hr and the reaction was stopped with 8 M H_2SO_4. The enzyme reaction product was measured by its absorbance at 492 nm and the fibronectin concentrations were calculated from a standard curve of known amounts of fibronectin.

RESULTS

Macrophage induced fibroblast chemotaxis. Oil-induced inflammatory exudate macrophages were cultured with or without LPS for 24 hr, and the supernatants from these cultures were tested for their ability to initiate fibroblast migration. Significant numbers of fibroblasts migrated toward the LPS-activated macrophage supernatants (Fig. 1). At increasing concentrations of the nonstimulated macrophage supernatants, fibroblast migration was also frequently seen (Fig. 1). Because of the activity seen in the oil-induced macrophage cultures that had not been stimulated *in vitro*, experiments were undertaken to establish whether macrophages spontaneously released a fibroblast chemoattractant or whether these cells might have been activated *in vivo*.

Elicited and nonelicited populations of macrophages were obtained and cultured. The presence of fibroblast chemotactic activity was then compared in the supernatants of mineral oil, thioglycollate, or glycogen-induced macrophage populations. Additionally, resident macrophages were collected from noninjected guinea pigs. Whereas essentially no fibroblast chemotactic activity was seen in the cultures of resident populations of macrophages (Fig. 2), varying degrees of chemotactic activity were apparent in the elicited populations without additional *in vitro* stimulation (Fig. 2). Thioglycollate-induced exudates were especially effective in elaborating the fibroblast chemotactic factor. Repetitive analysis of supernatants from these various populations at concentrations of 1 to 100% in the chemotaxis assay demonstrated similar findings. Figures 2 and 3 are representative data in which the supernatants from resident, oil-induced, glycogen-induced, and thioglycollate-induced macrophages were all compared simultaneously. Thus, it appeared that the elicited populations were adequately triggered in the *in vivo* inflammatory response to elaborate this chemotactic activity. Attempts were made to further activate each of these populations *in vitro*. Although LPS had little additional effect on the

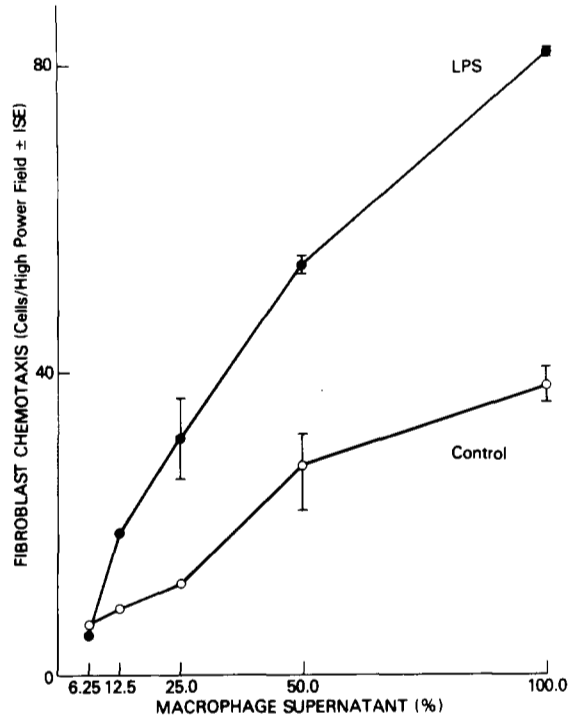

Figure 1. Dose-dependent fibroblast chemotaxis to macrophage supernatants. LPS (30 μg/ml) stimulated and nonstimulated oil-induced peritoneal macrophage supernatants (24 hr) were diluted 1:4 and tested for fibroblast chemotaxis. Data represent the mean ± 1 SE of triplicate filters (20 high power fields/filter).

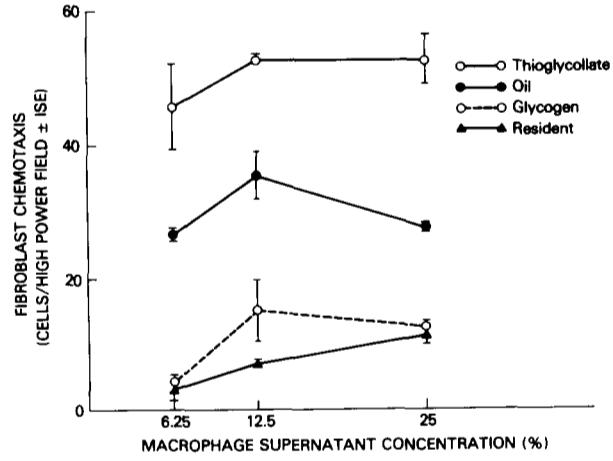

Figure 2. Fibroblast chemotaxis induced by supernatants of nonstimulated peritoneal exudate cells. Peritoneal exudates were induced with thioglycollate, 1% shellfish glycogen, or mineral oil. Resident peritoneal cells were obtained by peritoneal lavage of noninjected guinea pigs. The adherent cells obtained from these populations were cultured in the absence of additional stimulation for 24 hr, and the cellfree supernatants harvested and tested for fibroblast chemotactic activity. Data represent the mean ± 1 SE of triplicate filters (20 high power fields/filter).

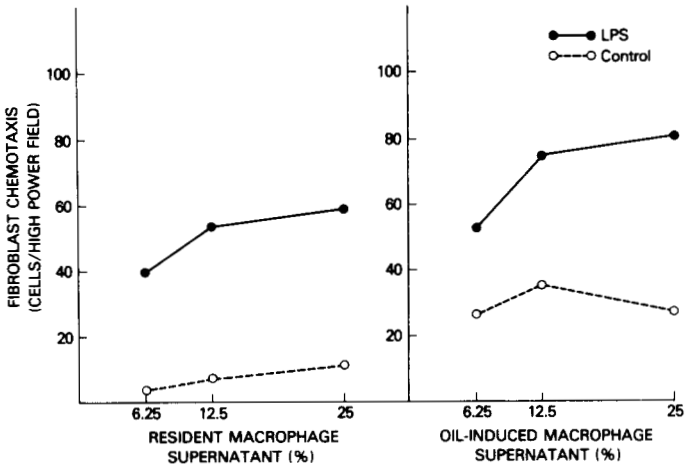

Figure 3. Enhanced production of fibroblast chemotactic factor by LPS-activated macrophages. Resident (nonelicited) (*left*) or oil-induced (*right*) adherent peritoneal cells were stimulated with 30 μg/ml LPS or not (control) and the 24-hr supernatants tested for fibroblast chemotactic activity. Data represent the mean of triplicate filters (20 high power fields/filter).

production of the chemoattractant by the thioglycollate induced populations, significantly higher levels of activity could be achieved by stimulating oil induced peritoneal cells in vitro with LPS (Fig. 3, right panel). Furthermore, the nonelicited (resident) populations of macrophages that did not spontaneously release the fibroblast chemotactic mediator *in vitro* were readily stimulated to do so with LPS (Fig. 3, left panel) or with *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (data not shown). Reconstitution of the inactive macrophage supernatants with LPS or MDP did not cause fibroblast migration.

Stimulation of chemotaxis, not chemokinesis, by macrophage supernatants. To determine whether the fibroblast migration was merely representative of hyperkinesis or was indeed directed migration towards a positive macrophage derived stimulus, varying concentrations of the macrophage supernatants were added to either the upper well, the bottom well, or both wells, and fibroblast migration determined under these conditions. Table I demonstrates the results of such an experiment in which fibroblasts migrated only in the presence of a positive stimulatory gradient.

Kinetics of macrophage elaboration of fibroblast chemotactic activity. After the addition of LPS or MDP to macrophage cultures, the supernatants were harvested at various time points and assayed for fibroblast chemotactic activity. No significant activity was detected in the supernatants of these oil-induced cells within the first 2 hr after stimulation. However, by 12 hr, the macrophages had elaborated enhanced levels of this mediator that remained relatively constant for the next 24 to 48 hr (Fig. 4). In some experiments, activity continued to increase throughout the 48 hr macrophage culture period. Elaboration of this factor required protein synthesis since its appearance was inhibited 93% by the addition of 1 μg/ml cycloheximide to the macrophage cultures (Table II) coincidently with the LPS. This inhibition was not attributed to toxicity since macrophages remained viable as determined by the exclusion of trypan blue in the presence of 1 μg/ml cycloheximide.

Characterization of the macrophage-derived fibroblast chemotactic factor. In initial studies, it was found that the factor produced by activated macrophages which was a chemoattractant for fibroblasts was heat labile (Table III) and trypsin sensitive (Table III). The chemotactic properties were not significantly inhibited in the presence of PMSF, a serine protease inhibitor (Table III). These observations indicated that the fibroblast chemotactic factor was a protein and that its activity was not dependent upon a trypsin-like enzymatic interaction with the fibroblasts. Elution profiles of concentrated macrophage supernatants from a Sephadex G-75 column revealed the primary chemotactic activity in the exclusion volume. The major peak of activity was again found in the exclusion volume of a Sephadex G-200 column, suggesting that the chemoattractant was a molecule of >200,000 daltons (data not shown).

Depletion of macrophage-derived fibroblast chemotactic activity by collagen Sepharose affinity columns. Supernatants were obtained from adherent macrophage monolayers 48 hr after stimulation with muramyl dipeptide. The cellfree supernatants were incubated on a Type I collagen Sepharose affinity column that selectively removed fibronectin (15) and the nonadsorbed macrophage products were washed through the column. Fractions were pooled and assayed for their ability to initiate fibroblast chemotaxis. Nontreated MDP activated macrophage supernatants contained significant levels of chemotactic activity for fibroblasts (Table IV).

TABLE I

Dependence of fibroblast migration on macrophage supernatant concentration gradient

Macrophage Supernatant in Lower Well[a]	Fibroblast Chemotaxis (Cells/HPF ± 1 SE)[b] Macrophage Supernatant in Upper Well					
	0	1:32	1:16	1:8	1:4	1:2
0	2 ± 0	1 ± 1	2 ± 1	2 ± 1	2 ± 1	3 ± 0
1:32	4 ± 1	2 ± 2	2 ± 1	3 ± 2	3 ± 1	2 ± 1
1:16	7 ± 1	5 ± 1	4 ± 1	3 ± 2	2 ± 1	1 ± 1
1:8	9 ± 1	6 ± 2	4 ± 2	5 ± 1	4 ± 1	6 ± 2
1:4	41 ± 3	29 ± 7	26 ± 18	14 ± 2	6 ± 1	7 ± 4
1:2	50 ± 3	48 ± 11	35 ± 5	18 ± 2	14 ± 3	8 ± 1

[a] LPS-activated macrophage supernatants were added to the lower well or to the upper well (with fibroblasts) of the chemotaxis chambers at the indicated concentrations.

[b] Fibroblast chemotaxis was then assayed according to procedure outlined in *Materials and Methods.* Data represent the mean number of fibroblasts per high power field (20 fields quantitated) on the lower surface of triplicate filters ± 1 SE.

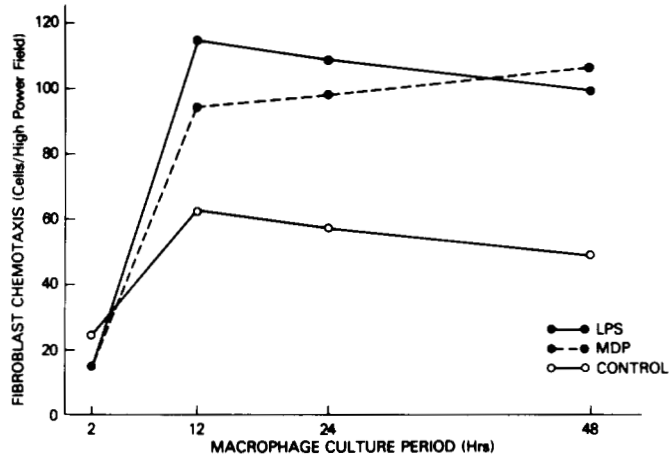

Figure 4. Kinetics of macrophage-derived fibroblast chemotactic factor production. Adherent oil-induced peritoneal cells were cultured with LPS (30 μg/ml), MDP (5 μg/ml), or no stimulant for varying time periods (2 to 48 hr) and the supernatants collected, diluted (1:4), and tested in the fibroblast chemotaxis assay.

TABLE II

Inhibition of macrophage production of fibroblast chemotactic factor by cycloheximide

Macrophage Supernatant	Fibroblast Chemotaxis (Cells/HPF ± 1 SE)	
	0	Cycloheximide
LPS[a]	28 ± 1[b]	2 ± 1
Control	2 ± 1	6 ± 1

[a] Six × 10^7 guinea pig peritoneal exudate cells were incubated 4 hr, washed to remove nonadherent cells, and stimulated with 30 μg/ml LPS in the presence or absence of 1 μg/ml cycloheximide for 24 hr.

[b] Macrophage supernatants were diluted 1:2 and assayed for fibroblast chemotactic activity. Data represent the mean ± 1 SE of triplicate filters.

However, these same supernatants after collagen Sepharose affinity chromatography were completely depleted of chemotactic activity for fibroblasts (Table IV). The inhibition appeared to be specific for the fibroblast chemotactic factor since the macrophage-derived fibroblast activating factor (FAF) also present in these supernatants (7) was not affected by the collagen Sepharose affinity column (Table IV). The activity of FAF, which has a molecular size of 40 to 50,000 daltons (8), was slightly enhanced after passage through the column whereas the chemotactic factor was completely lost.

Restoration of fibroblast chemotactic activity by fibronectin. Because these data suggested that fibroblast migration might be related to fibronectin in the macrophage supernatants, an attempt was made to restore fibroblast migration in the depleted supernatants by adding exogenous fibronectin. When purified fibronectin was added to affinity column-purified macrophage supernatants depleted of activity at concentrations as low as 0.1 μg/ml, fibroblast chemotactic activity was restored (Fig. 5). Maximum migration occurred at 10 μg/ml and at higher concentrations, inhibition was frequently noted (Fig. 5). Since fibronectin is an attachment protein (12, 19, 20), the possibility existed that fibronectin might be serving as a cofactor in the macrophage supernatants that, together with a chemoattractant, was responsible for fibroblast migration. To determine whether fibronectin was necessary for attachment of the fibroblasts to the gelatin coated filter, and an additional macrophage product was the actual attractant, fibronectin was added directly to fresh Dulbecco-Vogt's tissue culture medium in the absence of any additional macrophage products and then tested for fibroblast chemotaxis. Fibronectin was independently capable

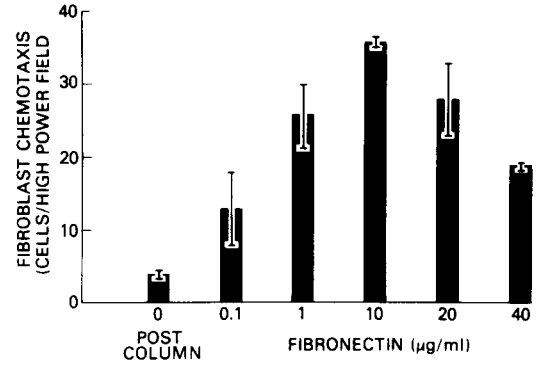

Figure 5. Restoration of chemotactic activity in macrophage supernatants by exogenous fibronectin. Macrophage supernatants were depleted of fibronectin on a collagen Sepharose affinity column and tested for fibroblast chemotactic activity (*postcolumn*). Aliquots of these fibronectin-depleted supernatants were then reconstituted with 0.1 to 40 μg/ml fibronectin and assayed for fibroblast chemotactic activity. Data represent the mean of triplicate filters ± 1 SE.

of stimulating fibroblast chemotaxis (Fig. 6), suggesting that the production of fibronectin by activated macrophages can serve as a direct chemoattractant for fibroblasts.

Furthermore, this fibronectin-mediated fibroblast migration was not dependent upon a fibronectin-collagen interaction. Fibronectin caused fibroblast chemotaxis whether PVP-free membranes that are not coated with gelatin or gelatin-coated membranes were used in the chemotaxis assay (Table V).

Inhibition of macrophage-mediated fibroblast chemotaxis by antifibronectin antibody. Further evidence that macrophage-derived fibronectin was involved in the fibroblast chemotactic response was obtained by the addition of anti-fibronectin antibodies to the macrophage supernatants. Preincubation of chemotactic factor-containing macrophage supernatants with varying dilutions of an antiserum to guinea pig fibronectin prepared in rabbits resulted in a significant loss of the chemotactic activity (Table VI). No fibroblast chemotactic activity was evident in the dilutions of antiserum tested alone. Similarly, the fibroblast chemotactic activity could also be blocked when an anti-human fibronectin immunoglobulin preparation was used (Table VI).

Detection of fibronectin in macrophage supernatants by enzyme-linked immunoassay (ELISA). Because of these data indicating that activated macrophages release a chemoattractant for fibroblasts, and that this attractant appeared to be the glycoprotein, fibronectin, an ELISA was established to quantitate fibronectin in the macrophage supernatants. In activated macrophage cultures, 1 to 2 μg/ml fibronectin was found in the medium after a 48 hr culture period. This amount of fibronectin accounted for 2 to 5% of the total amount of protein generated in the macrophage supernatants after LPS or MDP stimulation. Fibronectin was also detected by ELISA in those fractions obtained by gel filtration that contained fibroblast chemotactic activity. Thus the presence of fibroblast chemotactic activity was coincident with the presence of fibronectin.

TABLE III

Heat and enzyme sensitivity of macrophage-derived fibroblast chemotactic factor

Supernatant Treatment	Fibroblast Chemotactic Activity (Cells/HPF ± 1 SE)		
	Macrophage supernatants		
	LPS	MDP	Control
None[a]	39 ± 1	33 ± 7	10 ± 1
56°, 30 min[b]	21 ± 2	9 ± 1	7 ± 1
100°, 10 min[c]	5 ± 1	4 ± 1	2 ± 1
Trypsin[d]	2 ± 1		8 ± 1
PMSF[e]		26 ± 1	9 ± 2

[a] Peritoneal macrophages were stimulated or not with LPS (30 μg/ml) or MDP (1 μg/ml) for 24 hr. The cellfree supernatants were collected and tested for fibroblast chemotactic activity.

[b] Stimulated and control macrophage supernatants were incubated at 56°C for 30 min and tested for fibroblast chemotactic activity.

[c] Stimulated and control macrophage supernatants were heated at 100°C for 10 min and tested for fibroblast chemotactic activity.

[d] Stimulated and control macrophage supernatants were incubated with 0.39 U/ml insoluble trypsin for 1 hr, centrifuged, and assayed for fibroblast chemotactic activity.

[e] Stimulated and control macrophage supernatants were incubated with 10^{-4} PMSF, dialyzed, and tested for chemotactic activity.

TABLE IV

Effect of collagen Sepharose affinity chromatography on macrophage-derived fibroblast chemotactic factor and fibroblast-activating factor

Macrophage Supernatants[a]	TdR^{3}H Incorporation (cpm)[b]	Chemotactic Activity (Cells/HPF)[c]
Precolumn		
1:2		50 ± 3
1:5	6,492 ± 327	37 ± 1
1:10	7,729 ± 175	21 ± 2
Postcolumn		
1:2		5 ± 3
1:5	10,840 ± 1,212	3 ± 1
1:10	9,044 ± 224	2 ± 1

[a] MDP-activated macrophage supernatants were tested for FAF and fibroblast chemotactic activity before (*precolumn*) and after (*postcolumn*) incubation on a collagen-Sepharose affinity column.

[b] Macrophage supernatants were diluted and added to 5 × 10^4 serum-free fibroblasts for 48 hr. The fibroblast cultures were pulsed 4 hr with 1 μCi/ml TdR^{3}H and the amount of radioactivity incorporated determined. Data represent the mean ± 1 SE of triplicate cultures.

[c] Macrophage supernatants were diluted and tested for fibroblast chemotactic activity. Data represent the mean ± 1 SE of triplicate filters.

DISCUSSION

Synthesis and secretion of fibronectin by activated macrophages appears to serve as a chemotactic stimulus for fibroblasts. That the chemoattractant in the macrophage supernatants is fibronectin is supported by several observations. The chemotactic activity in the macrophage supernatants can be removed on a collagen Sepharose affinity column that specifically binds fibronectin. Chemotactic activity in these defibronectinized supernatants can be restored by the exogenous addition of physiologic quantities of fibronectin. Fibronectin levels equivalent to those that cause fibroblast migration can be measured by enzyme- linked immunoassay in the macrophage supernatants. And finally, the chemotactic activity in the macrophage supernatants can be blocked by antibodies to fibronectin.

Fibronectin is a large m.w. glycoprotein originally identified in plasma (reviewed in 12, 19, 20). Although once considered a product primarily of connective tissue cells such as fibroblasts,

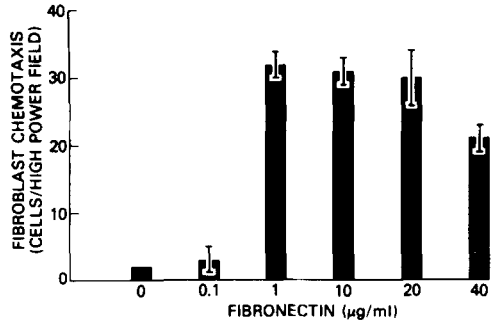

Figure 6. Fibroblast chemotaxis to fibronectin. Fibronectin was diluted (0.1 to 40 μg/ml) in fresh Dulbecco-Vogt's medium and assayed for fibroblast chemotactic activity. Data represent the mean of triplicate filters ± 1 SE.

TABLE V

Fibroblast migration on chemotaxis filters coated or noncoated with gelatin

Macrophage Supernatant	Fibroblast Chemotaxis (Cells/HPF)	
	Gelatin coated[a]	Nongelatin coated[b]
Control	3 ± 1	6 ± 4
LPS	48 ± 1	49 ± 14

[a] Standard polycarbonate 8 μ pore filters were coated with 0.1% gelatin (1), before use in the fibroblast chemotaxis assay.

[b] Polyvinylpyrrolidone-free polycarbonate 8 μ pore filters were not treated with gelatin before use in the fibroblast chemotaxis assay.

TABLE VI

Inhibition of macrophage-mediated fibroblast chemotaxis by fibronectin antibody

Stimulant	Fibroblast Chemotaxis (Cells/HPF)	
Control	1 ± 1	2 ± 1
Macrophage supernatant	33 ± 1	47 ± 2
Macrophage supernatant + fibronectin antibody	Anti-human Fibronectin[a]	Anti-guinea pig Fibronectin[b]
1:200	1 ± 1	11 ± 1
1:400	14 ± 4	
1:500		9 ± 1
1:800	13 ± 4	
1:1000		8 ± 1
Fibronectin Antibody		
1:200	1 ± 1	2 ± 1
1:400	2 ± 1	
1:500		1 ± 1
1:800	1 ± 1	
1:1000		1 ± 1

[a] Goat anti-human fibronectin immunoglobulin at the indicated concentrations was preincubated with the macrophage supernatants for 45 min, centrifuged, and the supernatants assayed for fibroblast chemotactic activity.

[b] Rabbit anti-guinea pig fibronectin serum at the indicated concentrations was preincubated with macrophage supernatants for 45 min, centrifuged, and the supernatants assayed for fibroblast chemotactic activity.

fibronectin has been found to be produced by various cell types including astroglial cells (21), Schwann cells (22), and endothelial cells (23–25). Initially, fibronectin production was not attributed to peritoneal macrophages (26), but subsequent investigations have found that human monocytes (9) and murine and guinea pig peritoneal macrophages (10, 11) synthesize fibronectin. Although macrophages synthesize and secrete this glycoprotein, they do not appear to have significant amounts of fibronectin attached to their cell surfaces nor do they form an intercellular matrix containing fibronectin (9) as do fibroblasts and connective tissue cells in culture.

Human monocytes in culture did not synthesize fibronectin until they became differentiated and then they released significant amounts of fibronectin *in vitro* (9). We have found that macrophages from induced peritoneal exudates produce some chemoattractant without additional stimulation *in vitro*, whereas noninduced, resident peritoneal macrophages have no detectable activity in their supernatants. The resident cells may actually be synthesizing fibronectin, but not in sufficient quantities to be detected in the fibroblast chemotaxis assay. However, whether activated *in vivo* or not, additional activity could generally be obtained following *in vitro* stimulation. Measurable chemotactic activity was found within 12 hr after stimulation of the macrophages with LPS or MDP. Since the macrophage cultures were carried out in the absence of serum, it was not possible that the phagocytic cells were taking up fibronectin from the tissue culture medium and then releasing it. Furthermore, the appearance of the fibroblast chemotactic activity in the macrophage cultures was blocked by cycloheximide, indicating that the fibronectin was synthesized after stimulation of the macrophages.

The ability of exogenous fibronectin alone to cause fibroblast chemotaxis suggests that fibronectin itself is a chemoattractant and not a necessary adhesion cofactor for an additional stimulus present in the macrophage supernatants. Although we cannot definitively exclude the possibility that other chemoattractants may be present in the activated macrophage supernatants, the major fibroblast chemotactic factor appears to be fibronectin. Additionally, it has recently been reported (27, 28) that plasma-derived fibronectin is chemotactic for fibroblasts. Whereas it was demonstrated that a collagen-fibronectin interaction was necessary for fibroblast migration (27), we have found that fibroblasts migrate toward a fibronectin gradient independently of collagen. With PVP-free filters that are not coated with gelatin, the fibroblasts migrated as well toward fibronectin as they did under identical conditions with gelatin-coated filters. That the fibronectin was a true chemoattractant and not merely initiating chemokinetic behavior was verified by using the checkerboard type of experiment in which varying concentrations of macrophage supernatants were placed on both sides of the chemotactic filter. In the absence of a chemical gradient, migration was not significant, whereas when the fibroblasts were exposed to increasing concentrations of macrophage supernatants in the lower chamber, migration of the fibroblasts through the filter was enhanced.

Thus it appears that among its many inflammatory mediators, the activated macrophage produces the glycoprotein, fibronectin. Fibronectin, in turn, can serve as a stimulus in the recruitment of fibroblasts to an inflammatory area. Although fibronectin is a constituent of connective tissue, most of this fibronectin is bound to cells and connective tissue components and not available in a soluble form to establish a chemical gradient that is required for fibroblast migration. Since macrophages appear to synthesize and release fibronectin rather than binding it to their surfaces (9), this soluble form of the molecule may be available as a chemotactic stimulus. After localization of the fibroblasts, they can be further influenced by macrophage mediators to proliferate (7, 8) and to produce the proteins including collagen (8, 29) responsible for tissue repair. In chronic inflammation, the prolonged release of these fibroblast-active agents by stimulated macrophages may result in extensive collagen synthesis causing pathophysiologic fibrosis.

In addition to the influence that fibronectin has on fibroblasts, this macrophage product may also have other functions in inflammation. Because macrophages secrete enzymes such as collagenase (30) and migrate through the tissues toward their target area, the production of fibronectin may facilitate cellular adhesion to the substratum. Furthermore, the production of fibronectin which has opsonin properties (14, 31) may promote phagocytosis of fibrin and collagen-containing material in inflammatory lesions.

Acknowledgments. The authors wish to thank Dr. Hynda Kleinman and Ms. Charlotte Wilkes for their significant contributions to these studies and Drs. A. L. Sandberg and S. E. Mergenhagen for their helpful review of this manuscript. The authors also appreciate the typing and the patience of Mrs. Carol Oesch.

REFERENCES

1. Postlethwaite, A. E., R. Snyderman, and A. H. Kang. 1976. The chemotactic attraction of human fibroblasts to a lymphocyte-derived factor. J. Exp. Med. 144:1188.
2. Postlethwaite, A. E., and A. H. Kang. 1980. Characterization of guinea pig lymphocyte-derived chemotactic factor for fibroblasts. J. Immunol. 124: 1462.
3. Postlethwaite, A. E., R. Snyderman, and A. H. Kang. 1979. Generation of a fibroblast chemotactic factor in serum by activation of complement. J. Clin. Invest. 64:1379.

4. Gery, I., and B. W. Waksman. 1972. Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potential mediator(s). J. Exp. Med. 136:143.
5. Eaves, A. C., and W. R. Bruce. 1974. *In vitro* production of colony-stimulating activity. I. Exposure of mouse peritoneal cells to endotoxin. Cell Tissue Kinet. 7:19.
6. Leibovich, S. J., and R. Ross. 1976. A macrophage-dependent factor that stimulates the proliferation of fibroblasts *in vitro*. Am. J. Pathol. 84:501.
7. Wahl, S. M., L. M. Wahl, J. B. McCarthy, L. Chedid, and S. E. Mergenhagen. 1979. Macrophage activation by mycobacterial water soluble compounds and synthetic muramyl dipeptide. J. Immunol. 122:2226.
8. Wahl, S. M., and L. M. Wahl. 1981. Modulation of fibroblast growth and function by monokines and lymphokines. Lymphokine Reports 2:179.
9. Alitalo, K., T. Hovi, and A. Vaheri. 1980. Fibronectin is produced by human macrophages. J. Exp. Med. 151:602.
10. Johansson, S., K. Rubin, M. Höök, T. Ahlgren, and R. Seljelid. 1979. *In vitro* biosynthesis of cold insoluble globulin (fibronectin) by mouse peritoneal macrophages. F.E.B.S. Lett. 105:313.
11. Colvin, R. I., J. Lanigan, R. A. Clark, T. H. Ebert, E. Verderber, and M. E. Hammond. 1979. Macrophage fibronectin (cold insoluble globulin, LETS protein). Fed. Proc. 38:1408.
12. Yamada, K. M., and K. Olden. 1978. Fibronectins-adhesive glycoproteins of cell surface and blood. Nature 275:179.
13. Wahl, S. M., L. M. Wahl, and J. B. McCarthy. 1978. Lymphocyte-mediated activation of fibroblast proliferation and collagen production. J. Immunol. 121:942.
14. Hopper, K. E., B. C. Adelmann, G. Gentner, and S. Gay. 1976. Recognition by guinea pig peritoneal exudate cells of conformationally different states of the collagen molecule. Immunology 30:249.
15. Engvall, E., and E. Ruoslatti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer 20:1.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680.
17. Yaoita, H., J. M. Foidart, and S. I. Katz. 1978. Localization of the collagenous component in skin basement membrane. J. Invest. Dermatol. 70:191.
18. Rennard, S. I., R. Berg, G. R. Martin, J. M. Foidart, and P. G. Robey. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. Anal. Biochem. 104:205.
19. Yamada, K. M. 1980. Fibronectin: transformation-sensitive cell surface protein. Lymphokine Reports 1:231.
20. Vaheri, A., and D. F. Mosher. 1978. High m.w., cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. Biochim. Biophys. Acta 516:1.
21. Vaheri, A., E. Ruoslatti, B. Westermark, and J. Ponten. 1979. A common cell-type specific surface antigen in cultured human glial cells and fibroblasts: loss in malignant cells. J. Exp. Med. 143:64.
22. Kurkinen, M., and K. Alitalo. 1979. Fibronectin and procollagen produced by a clonal line of Schwann cells. F.E.B.S. Letters 102:64.
23. Jaffe, E. A., and D. F. Mosher. 1978. Synthesis of fibronectin by cultured human endothelial cells. J. Exp. Med. 147:1779.
24. Macarak, E. J., E. Kirby, T. Kirk and N. A. Kefalides. 1978. Synthesis of cold-insoluble globulin by cultured calf endothelial cells. Proc. Natl. Acad. Sci. 75:2621.
25. Birdwell, C. R., D-Gospodarowicz, and G. L. Nicholson. 1978. Identification, localization, and role of fibronectin in cultured bovine endothelial cells. Proc. Natl. Acad. Sci. 75:3273.
26. Pearlstein, E., S. R. Dienstman, and V. Defendi. 1978. Identification of macrophage external membrane proteins and their possible role in cell adhesion. J. Cell Biol. 79:263.
27. Gauss-Müller, V., H. K. Kleinman, G. R. Martin, and E. Schiffman. 1980. Role of attachment factors and attractants in fibroblast chemotaxis. J. Lab. Clin. Med. 96:1071.
28. Postlethwaite, A. E., J. Keski-Oja, G. Balian, and A. H. Kang. 1981. Induction of fibroblast chemotaxis by fibronectin. J. Exp. Med. 153:494.
29. Wahl, L. M., S. M. Wahl, and J. B. McCarthy. 1980. Adjuvant activation of macrophage function. *In* Regulatory Role of the Macrophage in Immunity Edited by A. Rosenthal and E. Unanue. Academic Press, New York. P. 491.
30. Wahl, L. M., C. E. Olsen, A. L. Sandberg, and S. E. Mergenhagen. 1977 Prostaglandin regulation of macrophage collagenase production. Proc. Natl. Acad. Sci. 74:4955.
31. Blumenstock, F. A., T. M. Saka, P. Weker, and R. Laffin. 1978. Biochemical and immunological characterization of human opsonic γ_2SB glycoprotein: its identity with cold insoluble globulin. J. Biol. Chem. 253:4287.

0022-1767/81/1272-0678$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 127, No. 2, August 1981
Printed in U.S.A.

PREVENTION OF TOLERANCE INDUCTION BY SIMULTANEOUS ADMINISTRATION OF ANTI-Lyb3 ANTISERUM WITH TOLEROGEN[1]

BRIGITTE T. HUBER AND YVES BOREL[2]

From the Tufts Cancer Research Center, Department of Pathology, Tufts Medical School, Boston, MA 02111, and the Division of Immunology, Children's Hospital Medical Center, Harvard Medical School, Boston, MA 02115

Lyb3 serves as a triggering receptor on a mature subset of B lymphocytes. To further investigate the nature of the activating signal received by this receptor, we examined its involvement in hapten-specific B cell tolerance induced *in vivo* with isologous IgG. We obtained the following results: injection of anti-Lyb3 serum simultaneously with low doses of tolerogen prevented tolerance induction in B cells responsive to TD and TI type-1 antigens but failed to influence B cells responding to a TI type-2 antigen. Furthermore, administration of anti-Lyb3 serum did not change the tolerogenic signal to an immunogenic one, allowing B cell maturation into plaque-forming cells. However, it did make the B cell responsive to a subsequent challenge with antigen. This effect is transient, and dependent on the dose of tolerogen. These results are discussed in terms of our knowledge of the mechanism of B cell tolerance.

Received for publication February 12, 1981.
Accepted for publication May 6, 1981.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work is supported by Grants AI-13867, AI-14910, and CA-24530 from the National Institutes of Health, and Grant No. IM-178 from The American Cancer Society.
[2] To whom correspondence should be addressed, at the Harvard Medical School.

The B cell surface antigen Lyb3 has been defined by serologic and functional means (1–3). This differentiation antigen is an isogenic specificity present on a mature subset of B lymphocytes and absent in adult *xid* defective and newborn normal mice. Thus far, the effect of administration of Lyb3 serum has been examined on immunity, as measured by either enhanced antigen activation or polyclonal triggering of B cells (4). In this report, its possible involvement in B cell tolerance to different types of antigens is investigated. Carrier-determined hapten-specific B cell tolerance was chosen as a model system, because B cells can be tolerized directly, regardless of whether the antigen is T cell dependent