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Generation of a unique fibroblast-activating factor by human monocytes

J. G. DOHLMAN, D. G. PAYAN & E. J. GOETZL *Howard Hughes Medical Institute Laboratories and Department of Medicine, University of California Medical Center, San Francisco, California, U.S.A.*

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Summary. Purifed human monocytes incubated with phytohemagglutinin (PHA), bacterial lipopolysaccharide (LPS), or serum-opsonized zymosan particles (OZ) generate human dermal fibroblast-activating activity, as assessed by increased fibroblast incorporation of [^{3}H]-thymidine. A maximum concentration of fibroblast-activating activity was attained within 4 hr with OZ, whereas similar maximum levels required 12 hr with LPS and PHA. Sonicates of unstimulated monocytes had only minimal activity and the protein synthesis inhibitor cycloheximide suppressed significantly the appearance of fibroblast-activating activity, suggesting that the factors are generated prior to release. Filtration of supernates from OZ-stimulated monocytes on Sephadex G-75 yielded polydisperse fibroblast-activating activities, of which the major factors exhibited a mol. wt. of approximately 60,000 and 10,000. The supernates from PHA-stimulated monocytes had one predominant factor, termed fibroblast-activating factor of monocytes (FAF-M), with an apparent mol. wt. of 38,000 and a minor activity with a mol. wt. of 10,000. FAF-M was composed of two principles with isoelectric points of 5·1–5·2 and 4·0–4·2 and was free of interleukin-1, as determined by the absence of thymocyte-activating activity. FAF-M and other fibroblast-activating factors may contribute to wound healing and fibrosis in lesions characterized by mononuclear phagocyte infiltrates.

Abbreviations: PHA, phytohaemagglutinin: LPS, lipolysaccharide; OZ, opsonized zymosan; FAF-M, fibroblast-activating factor of monocytes; IL 1, interleukin 1; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; EDTA, ethylenediamine tetracetate; DME, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; M199-HPS, medium 199 containing 20 mM HEPES, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Correspondence: Dr E. J. Goetzl, Howard Hughes Medical Institute Laboratories and Department of Medicine, University of California Medical Center, San Francisco, CA 94143, U.S.A.

INTRODUCTION

Mononuclear phagocytes accumulate at sites of tissue injury and contribute both to the clearance of damaged connective tissue and to the recruitment of fibroblasts that are required for repair (Leibovich & Ross, 1975). That mononuclear phagocytes have the capacity to activate fibroblasts has been suggested by the production *in vitro* of diverse factors which augment fibroblast functions, including chemotaxis, proliferation and the secretion of collagen, collagenase, plasminogen activator, and prostaglandins (Leibovich & Ross, 1978; Leibovich, 1978; Tsukamoto, Helsel & Wahl, 1981; Glenn & Ross, 1981; Martin *et al.*, 1981; Laub & Vaes, 1982; Postlethwaite *et al.*, 1983). Characterization of human mononuclear phagocyte-derived fibroblast-activating factors was hampered initially by the lack of sufficient numbers of purified monocytes or macrophages and by the contamination of most preparations of mononuclear phagocytes with lymphocytes that also generate fibroblast-activating factors (Wahl, Wahl & McCarthy,

1978; Wahl & Gately, 1983). Human mixed mononuclear leukocytes produced a 60,000 mol. wt. factor that stimulated fibroblast proliferation *in vitro* (Postlethwaite & Kang, 1983). Human alveolar macrophages produced an 18,000 mol. wt. fibroblast-activating factor, which was free of mitogenic activity for thymocytes and physically distinct from interleukin-1 (IL-1) (Bitterman *et al.*, 1982). Partially purified IL-1 from human monocytes also enhanced fibroblast proliferation *in vitro* in the presence of 10% foetal calf serum (Schmidt *et al.*, 1982) and stimulated the production of collagenase and prostaglandin E_2 (Postlethwaite *et al.*, 1983). Unlike other fibroblast-activating factors, IL-1 did not stimulate the proliferation of quiescent fibroblasts in the presence of only low concentrations of serum (Bitterman *et al.*, 1982). The results of the present study indicate that highly purified human peripheral blood monocytes have the capacity to generate multiple fibroblast-activating activities, the relative quantities of which are determined by the specific monocyte stimulus. The predominant fibroblast-activating factor produced by monocytes stimulated with phytohaemagglutinin is shown to be structurally distinct from both IL-1 and lymphocyte-derived fibroblast-activating factors.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium, medium 199, foetal calf serum, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) stock solution (100 mM), penicillin and streptomycin solution (5000 U/ml and 5000 μg/ml, respectively), trypsin and disodium ethylenediamine tetracetate (EDTA) solution (0·5 g/litre and 0·2 g/litre, respectively), (M.A. Bioproducts, Walkersville, MD), 75 cm^2 tissue culture flasks, 100 mm diameter plastic Petri dishes (Costar, Cambridge, MA), [^{3}H]-thymidine (New England Nuclear Corp., Boston, MA), Grade I porcine heparin, cycloheximide (Sigma Chemical Co., St. Louis, MO), Sephadex G-75 and G-10, 6 g% (w:v) macromolecular dextran in saline (Macrodex) (Pharmacia Fine Chemicals, Piscataway, NJ), phytohaemagglutinin (PHA) (Grand Island Biological Co., Grand Island, NY), *E. coli* lipopolysaccharide (LPS) (Difco Inc., Detroit, MI), PM10 Diaflo ultrafiltration membranes (Amicon Inc., Lexington, MA), Acrodisc 0·2 μm pore filters (Gelman, Ann Arbor, MI), Ampholine pH 3·5–10 ampholytes (LKB, Bromma, Sweden), PHD Cell Harvester (Cambridge Technologies, Cambridge, MA), and a GT series isoelectric focusing apparatus (Hoefer Scientific Instruments, San Francisco, CA) were obtained from the designated suppliers. IL-1 activity in the effluents from Sephadex G-75 columns was quantified by the activation of thymocytes, as described (Mizel, 1980), by Drs James Jakway and Ethan Shevach (National Institutes of Health, Bethesda, MD).

Maintenance of fibroblast cultures

Normal adult human skin fibroblasts were obtained frozen from the cell culture facility of UCSF Medical Center (passage 7 of line 6M 2912) and from the American Tissue Culture Collection (Rockville, MD; passage 2 of line 5428 and passage 1 of line 5441). The fibroblasts were thawed, washed, and resuspended in Dulbecco's modified Eagle's medium (DME) containing 10% (v:v) heat-inactivated foetal calf serum (FCS), 20 mM HEPES (pH 7·4), 100 U/ml of penicillin, and 100 μg/ml of streptomycin and were cultured in 75 cm^2 plastic tissue culture flasks in an atmosphere of 5% CO_2 in air at 37°. The media were changed three times weekly. In order to subculture the contents of each flask every 3–5 days, the fibroblasts were detached from the flasks by treatment for 3 min at 25° with 4 ml of a solution of 0·5 g/litre of trypsin and 2 g/litre of EDTA, washed and resuspended in HEPES-buffered DME-10% (v:v) FCS with penicillin and streptomycin, and distributed into three separate flasks.

Assessment of fibroblast proliferation

Fibroblasts in confluent subcultures at passages 4–15 were detached from the flasks by treatment with the trypsin-EDTA solution for 1 min at 25°, washed once with DME-10% (v:v) FCS, and resuspended in DME-0·5% (v:v) FCS at a concentration of $2{\cdot}5 \times 10^4$/ml. Two hundred microlitre aliquots of fibroblast suspension were added to 9 mm diameter flat-bottom wells of microtitre plates and incubated for 24 hr at 37° in 5% CO_2 in air. Medium then was removed from each well by aspiration and replaced with dilutions of test materials in DME-0·4% (v:v) FCS, and the plates were incubated at 37° in 5% CO_2 in air. After 60 hr, 1 μCi of [^{3}H]-thymidine was added to each well and the incubation continued for 12 hr at 37°. After a total of 72 hr of incubation, the medium was removed from the wells by aspiration and the fibroblasts in each well were detached by treatment with 100 μl of the solution of trypsin-EDTA for 15 min at 37°. The detached fibroblasts were aspirated onto a glass-fibre filter and washed with an automated har-

vesting system for the quantification of radioactivity in a liquid scintillation counter as described (Wahl & Gately, 1983).

Purification of human monocytes

Four hundred millilitres of blood from normal subjects was mixed with 200 U/ml of heparin and 8 ml of macromolecular dextran (Macrodex, 6 g/100 ml of saline) per 50 ml of blood. After 45 min at room temperature, the supernatant leucocyte-rich plasma was removed sterilely by aspiration, mixed with an equal volume of medium 199 (M199) containing 20 mM HEPES, 100 U/ml of penicillin and 100 μg/ml of streptomycin (M199-HPS), and centrifuged at 182 ***g*** for 10 min at room temperature. The pellet of mixed leucocytes was resuspended in 10 ml of M199, layered onto an equal volume of Ficoll-Hypaque, and centrifuged at 400 ***g*** for 35 min at room temperature. The mixed mononuclear leucocytes were recovered sterilely from the buffer-Ficoll Hypaque interface, washed twice, resuspended in 5 ml of M199-HPS, and incubated with approximately 2×10^9 freshly prepared sheep erythrocytes for 15 min at 37°. The suspension was centrifuged for 5 min at 46 ***g*** and held at 4° for 1 hr in order to allow the formation of T lymphocyte sheep erythrocyte rosettes. The cells were resuspended gently and the suspension was layered on an equal volume of Ficoll-Hypaque and centrifuged at 400 ***g*** for 35 min at 4° to pellet the T lymphocyte sheep erythrocyte rosettes. The monocytes and non-T lymphocytes were recovered from the buffer-Ficoll Hypaque interface by aspiration, and washed and resuspended in 1 ml of DME per 1×10^7 leucocytes. Five millilitres of suspension were added to each 100 mm diameter plastic petri-dish and incubated for 1 hr at 37° in 5% CO_2 in air. Non-adherent lymphocytes were removed by aspirating the medium and washing the layer of adherent leucocytes twice with 5 ml of DME at room temperature. The adherent monolayers consisted of $1{\cdot}5$–$2{\cdot}5 \times 10^7$ monocytes of greater than 97% purity, as assessed with a standard esterase stain (Koski, Poplack & Blaese, 1976).

Generation of monocyte-derived fibroblast-activating factors

Adherent layers of purified monocytes were covered with 5 ml of serum-free DME and incubated at 37° in 5% CO_2 in air without or with 10 μg/ml of PHA, 10 μg/ml of LPS, or 25×10^7 zymosan particles that had been opsonized by incubation with fresh human serum as described (Koski *et al.*, 1976). After every 12 hr of incubation, the medium was removed, the monocytes were covered again with 5 ml of fresh DME, and the incubation continued at 37°. Medium that had been harvested from 2×10^8 monocytes and pooled was concentrated by pressure filtration using Amicon UM10 filters.

Purification of monocyte-derived fibroblast-activating factors

Three millilitres of concentrated monocyte supernates were filtered on a 120 ml bed volume ($1{\cdot}5$ cm $\times$ 68 cm) column of Sephadex G-75 that was equilibrated and developed with 15 mM sodium phosphate–0·135 M NaCl (pH 7·4) at a flow rate of 8 ml/hr. The effluent was collected in portions containing 1% of the bed volume. The portions of effluent were assessed by optical density at 280 nm and every other portion was passed though a micropore filter and assayed for fibroblast-activating activity.

Pools of the most active portions from the Sephadex G-75 columns were concentrated to a volume of 1 ml by PM10 pressure filtration and mixed with glycerol and ampholytes (pH 3·5–10) to achieve respective final concentrations of 33% (v:v) and 2 g/100 ml. Each sample was applied to the middle of a 20 ml column containing a 10–60% (v:v) glycerol gradient in distilled water with 2 g/100 ml of ampholytes that had been prefocused for 36–48 hr at 900 V to a milliamperage of 5 at 8°. After an additional 24 hr of focusing, the gradient was aspirated in 1·2 ml portions, each of which was dialysed for 48 hr at 4° against three changes of 10 mM NaCl buffered with 5 mM HEPES (pH 7·4). Samples then were concentrated to 200–500 μl using a Speed Vac Concentrator apparatus (Savant Inc., Hicksville, NY), sterilized by micropore filtration, and assayed for fibroblast-activating activity.

RESULTS

Characteristics of the generation of fibroblast-activating activity by human monocytes

In four preliminary experiments, monocytes from different subjects were incubated for 16 hr at 37° with medium containing no stimulus, 10 μg/ml of PHA, 10 μg/ml of LPS, or 25×10^7 particles of opsonized zymosan, and the supernates were assayed for fibroblast-activating activity. The uptake of [^{3}H]-thymidine by human fibroblasts in medium alone was increased $188\% \pm 83\%$ (mean$\pm$SD) by a 1:2 dilution of supernate from control monocytes and $472\% \pm 80\%$

($P<0.01$ compared to the control value), 432%±140% ($P<0.025$), and 492%±9% ($P<0.01$) by 1:2 dilutions of supernates from monocytes stimulated with PHA, LPS, and opsonized zymosan, respectively. The mean increase in [^{3}H]-thymidine uptake by fibroblasts incubated with 1:8 dilutions of the same supernates was 85%, 108%, 312% and 286%, respectively. In subsequent experiments, the fibroblast-activating activity was assessed for a range of concentrations of each supernate and the values reported are for the level of optimal stimulation. In order to examine whether the fibroblast-activating activity was present preformed in unstimulated monocytes, replicate dishes of adherent monocytes from three of the same donors were washed twice, covered with 5 ml of DME, and sonified for 2 min at 4°. The mean increases in [^{3}H]-thymidine uptake by fibroblasts incubated with 1:4 dilutions of the sonicates was −25%, 19%, and 149% ($P>0.1$ relative to control values), suggesting a requirement for synthesis prior to release of the activity. This requirement was confirmed by the finding that preincubation of replicate plates of monocytes for 1 hr at 37° with the protein synthesis inhibitor cycloheximide, prior to the addition of PHA, suppressed the appearance of fibroblast-activating activity. The supernates were filtered on 20 ml columns of Sephadex G-10 in DME and concentrated to the original volume prior to testing of activity in order to remove residual cycloheximide that might have interfered with the expression of fibroblast-activating activity. The mean increase in [^{3}H]-thymidine uptake by fibroblasts incubated with PHA alone was 340%, as contrasted with 216% and 71% in the presence of 1 μg/ml and 5 μg/ml of cycloheximide, respectively ($n=2$). Cycloheximide thus suppressed significantly ($P<0.02$ for 1 μg/ml and $P<0.0004$ for 5 μg/ml) the generation of fibroblast-activating activity by monocytes stimulated with PHA.

In time-course studies, the incubation of adherent human monocytes in serum-free medium resulted in the release of fibroblast-activating activity that was detectable by 4 hr, and by 6 hr reached a plateau which was maintained for up to 72 hr (Fig. 1). When 10 μg/ml of PHA was added at the beginning of the incubation interval, a 10-fold increase in the generation of fibroblast-activating activity was achieved by 12 hr ($P<0.01$ compared to control value), maintained for up to 48 hr, and declined thereafter. Stimulation of monocytes with 10 μg/ml of LPS resulted in a significant increase in the generation of fibroblast-activating activity, which was observed by 4 hr ($P<0.01$ relative to control), and reached a plateau at 12–48 hr which was similar in magnitude to that seen with PHA stimulation. The addition of opsonized zymosan particles to the monocytes stimulated an increased production of fibroblast-activating activity that was significantly greater than the control level by 2 hr ($P<0.02$) and at 4–24 hr reached a maximum level equivalent to that observed with PHA or LPS stimulation. The mean viability of the monocytes at 36, 48, and 72 hr, respectively, as assessed by trypan blue dye exclusion, was 97%, 95% and 96% with PHA, 98%, 98% and 96% with LPS, 95%, 83% and 93% with opsonized zymosan, and 95%, 98% and 97% with buffer alone ($n=3$).

Purification of monocyte-derived fibroblast-activating factors

In order to resolve the factors which contributed to the observed fibroblast-activating activity, monocytes were stimulated with opsonized zymosan and PHA for 36 and 48 hr, respectively, in order to obtain in the supernates maximal quantities of each activity without including products of damaged monocytes. The protocol for the generation of fibroblast-activating activities to be purified was modified to limit the possibility of extracellular proteolysis of the factors by harvesting and freezing the supernates every 12 hr and adding back to the monocyte layers 5 ml of DME without a stimulus. In two experiments performed in triplicate, the activity generated at 0–12, 12–24, 24–36, and 36–48 hr, respectively, increased fibroblast uptake of [^{3}H]-thymidine by a mean of 208, 263, 182 and 159% with PHA, and 350, 295, 149 and 147% with opsonized zymosan. Supernates of opsonized zymosan-stimulated monocytes were concentrated 100-fold by pressure filtration and 2 ml of the concentrate were filtered on a column of Sephadex G-75. Numerous fibroblast-activating activities were observed in the effluent (Fig. 2). Two major peaks eluted at 43–45% bed volume (approximately 60,000 mol. wt.) and 71–76% bed volume (approximately 10,000 mol. wt.). The same pattern of activity was seen when supernates from two other preparations were filtered on Sephadex G-75. IL-1 activity eluted at 32–33% and 45–61% bed volume.

Supernates of PHA-stimulated monocytes were concentrated 88-fold by pressure filtration and 3 ml were filtered on the same Sephadex G-75 column (Fig. 3). One predominant peak of monocyte-derived fibroblast-activating factor activity, designated FAF-M, was found at 45–53% bed volume

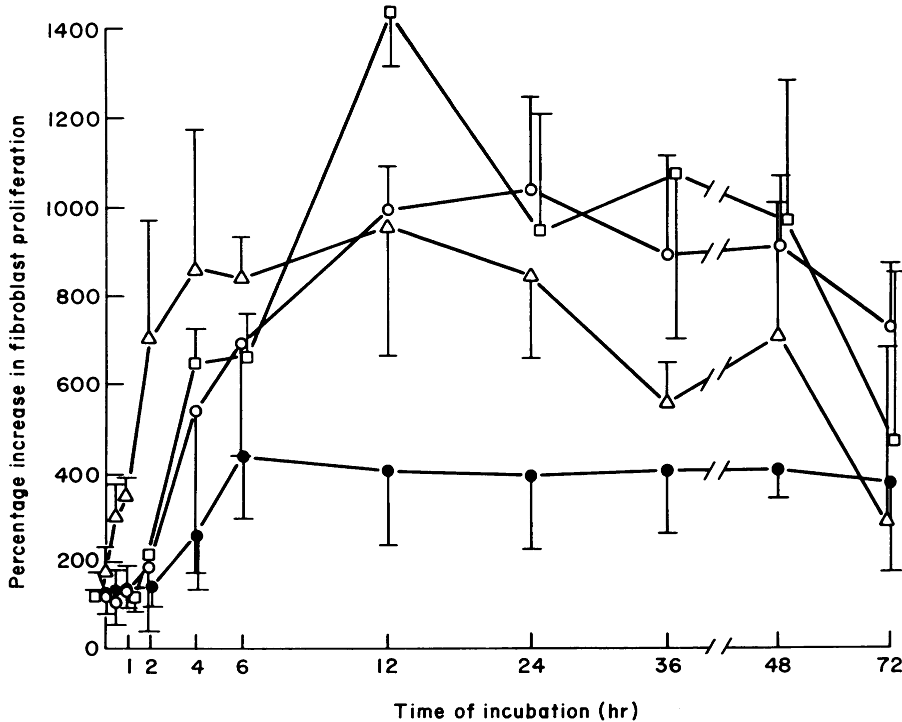

Figure 1. Time course of the production of fibroblast-activating activity by monocytes. Adherent monocytes were incubated for different times with and without PHA, LPS, and opsonized zymosan. The monocyte supernates were assessed at a dilution of 1 : 2 for stimulation of fibroblast uptake of [^{3}H]-thymidine. Each point and bracket represents the mean ± SD of the results of two experiments. The uptake of [^{3}H]-thymidine by control fibroblasts incubated in DME-0·4% foetal calf serum alone (0% increase) was 1027 ± 498 c.p.m. (mean ± SD). (●) No stimulus; (○) PHA; (△) opsonized zymosan; (□) LPS.

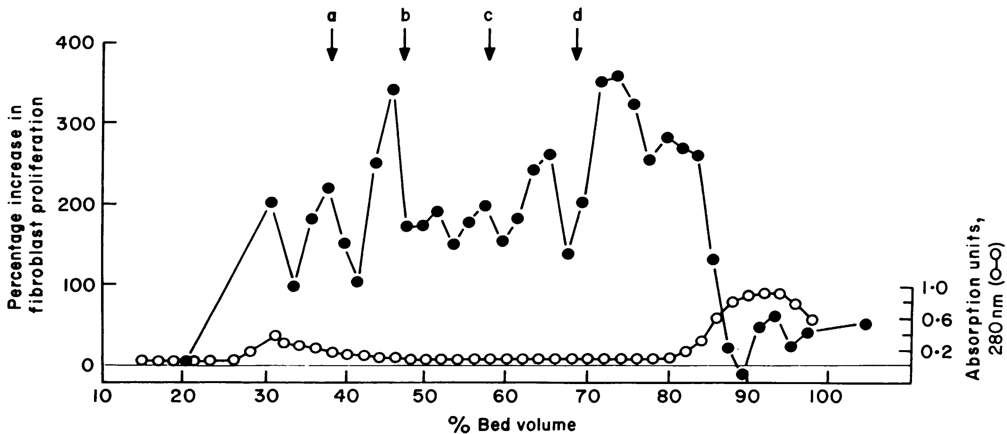

Figure 2. Purification of fibroblast-activating activity derived from opsonized zymosan-stimulated monocytes by filtration on Sephadex G-75. The mol. wt. standards shown on this representative chromatogram are: (a) dextran blue; (b) ovalbumin (43,000 mol. wt.); (c) chymotrypsin (25,000); and (d) ribonuclease (13,700). Portions of the effluent were assayed at a dilution of 1 : 4. The mean uptake ± SD of [^{3}H]-thymidine by control fibroblasts in DME-0·4% foetal calf serum alone (0% increase) was 669 ± 176 c.p.m. IL-1 activity was found in the effluent at 32–33% bed volume and 45–61% bed volume.

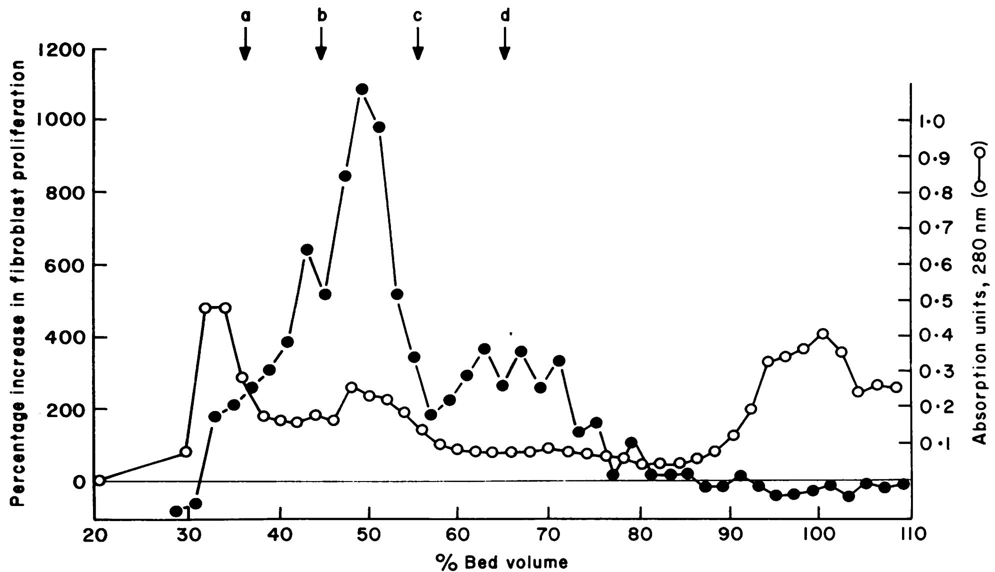

Figure 3. Purification of fibroblast-activating activity derived from PHA-stimulated monocytes by filtration on Sephadex G-75. The mol. wt. standards on this representative chromatogram are the same as in Fig. 2. Portions of the effluent were assayed at a dilution of 1/10. The mean uptake ± SD of [^{3}H]-thymidine by control fibroblasts in DME-0·4% foetal calf serum alone (0% increase) was 682 ± 419 c.p.m. IL-1 activity was found in the effluent at 53–56% bed volume.

(peak = 49% bed volume), that exhibited an apparent mol. wt. of approximately 38,000. A peak which corresponded in size to the larger of the major activities seen with opsonized zymosan stimulation (Fig. 2) was observed on the ascending limb of FAF-M. Lesser peaks were also seen at 63–74% bed volume. IL-1 activity eluted at 53–56% bed volume. The same pattern of activity was seen when supernates from three other preparations were filtered on Sephadex G-75. Four millimetres of three similarly concentrated supernates from unstimulated monocytes were filtered on Sephadex G-75 and the effluents were assessed for fibroblast-activating activity. An increase in the uptake of [^{3}H]-thymidine by fibroblasts was stimulated by the effluent at a bed volume of 65–69% (122% ± 17% increase, mean ± SD, $n = 3$), 75% (123%) and 79% (115%), but not by the effluent corresponding to the mol. wt. of FAF-M or the activities in the supernates from monocytes stimulated by opsonized zymosan.

Portions of the effluent from a Sephadex G-75 column that contained FAF-M activity from PHA-stimulated monocytes were pooled, concentrated to 1 ml, and subjected to isoelectric focusing. Individual fractions from the focusing column were dialysed, concentrated by vacuum centrifugation to a volume of 250 μl, and assessed for FAF-M activity (Fig. 4). Two predominant peaks of FAF-M activity were found in this and another identical experiment, which focused at pH 5·1–5·2 and 4·0–4·2, respectively, and together accounted for a mean of 26% of the FAF-M recovered from the Sephadex G-75 columns. No fibroblast-activating activity was recovered when the principles in the supernates of monocytes stimulated by opsonized zymosan were subjected to isoelectric focusing.

DISCUSSION

Human mixed mononuclear leucocytes have been shown to generate fibroblast-activating activity *in vitro* when stimulated by soluble principles (Postlethwaite *et al.*, 1983). The capacity of human monocytes to secrete fibroblast-activating activity, in the absence of lymphocytes, was demonstrated initially by stimulation with endotoxin, concanavalin A and zymosan (Glenn & Ross, 1981). Similarly, the incubation of human alveolar macrophages with phagocytosable particles or surfactant elicited the release of fibroblast-activating activity (Bitterman *et al.*, 1982). The activity from monocytes was not characterized further, but that from alveolar macrophages was shown to be

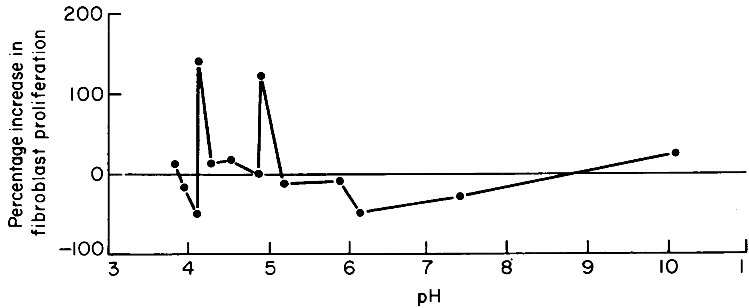

Figure 4. Purification by isoelectric focusing of the FAF-M activity recovered by filtration of the supernates from PHA-stimulated monocytes on a Sephadex G-75 column. The points represent mean values for the fibroblast-activating activity in a 1:4 dilution of fractions from a representative focussing column. The mean uptake of [^{3}H]-thymidine by control fibroblasts in DME-0·4% foetal calf serum alone (0% change) was 514 ± 111 c.p.m.

attributable to a factor of 18,000 mol. wt. (Bitterman *et al.*, 1982).

Purified human monocytes, free of lymphocytes, elaborate a substantial amount of fibroblast-activating activity. The time-course of appearance of the fibroblast-activating activity and the characteristics of the constituent factors that contribute to the net activity are both determined by the specific monocyte stimulus. Exposure of the monocytes to opsonized zymosan results in the release of optimal fibroblast-activating activity by 2–4 hr at 37°, whereas a comparable level of activity is achieved with LPS or PHA only after 6–12 hr (Fig. 1). With opsonized zymosan stimulation the monocytes generated two major fibroblast-activating factors of apparent mol. wt. 60,000 and 10,000, along with smaller amounts of numerous other factors (Fig. 2). In contrast, the supernates from monocytes exposed to PHA generated one predominant factor of apparent mol. wt. 38,000 and a much smaller amount of a factor of approximate mol. wt. 10,000. Sonicates of unstimulated monocytes had minimal or no detectable activity and the protein synthesis inhibitor cycloheximide suppressed the generation of fibroblast-activating activity by monocytes stimulated with PHA, suggesting a requirement for *de novo* production of the factors prior to release.

The factor which comprises the bulk of the fibroblast-activating activity released from monocytes stimulated with PHA was termed FAF-M. FAF-M clearly differs in mol. wt. and charge (Figs 3, 4) from the lymphocyte-derived factors with similar activity (Wahl & Gately, 1983). IL-1 activity, assessed by the stimulation of thymocyte proliferation (Mizel, 1980), eluted from Sephadex G-75 after FAF-M and did not contaminate the effluent containing FAF-M. Thus, FAF-M is a unique human monocyte-derived principle that differs substantially from lymphocyte-derived fibroblast-activating activities. FAF-M may contribute significantly to wound healing and fibrosis in tissue lesions characterized by a monocyte/macrophage-predominant infiltrate.

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