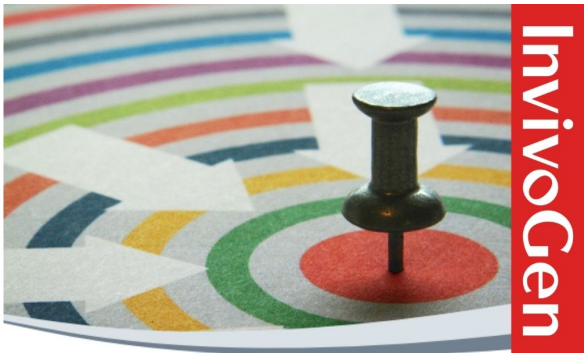

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PRODUCTION AND CHARACTERIZATION OF SUPERNATANTS WITH HIGH TITERS OF HUMAN LEUKOCYTE INHIBITORY FACTOR (LIF)

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Studies were performed with an indirect agarose microdroplet migration inhibition assay to maximize the production of leukocyte inhibitory factor (LIF) from peripheral blood mononuclear cells (MN) stimulated with PPD. LIF activity was evaluated by the ability of various dilutions of culture supernatants to inhibit migration of polymorphonuclear cells from agarose droplets. Supernatants with titers of $>10^4$ to 10^6 of LIF activity were produced by culturing MN at high cell concentrations with PPD in conical tubes (2×10^7 cells in 50-ml tubes and 5×10^5 cells in Microfuge tubes), whereas supernatants from round-bottom tubes containing low (5×10^6) or high (2×10^7) MN numbers or those from 50-ml conical tubes with low (5×10^6) MN numbers failed to produce LIF titers higher than 4 to 10^2. Generation of high-titered LIF in conical tubes was dependent on stimulation with antigen, and only tuberculin skin test-positive donors responded. The mechanisms underlying the ability to generate such remarkably high titers of LIF activity by simple changes in culture conditions are not clear, but the results point toward the importance of optimal cell-cell contact. Sephadex G-200 column fractionation of high-titered supernatants revealed that the active fraction(s) had a m.w. of slightly less than 68,000, which is consistent with previous reports for LIF. Additional physicochemical studies demonstrated that LIF activity in high-titered supernatants was blocked by 10^{-6} M α-L fucose and 10^{-4} M *N*-acetylglucosamine, but not by 10^{-2} M α-D fucose, glucose, or other sugars. The development of this miniaturized method for production of supernatants with high titers of LIF should facilitate quantitative assessment of cell-mediated immunity and further physicochemical studies on LIF.

Reactivity of human peripheral blood leukocytes in *in vitro* direct and indirect leukocyte migration inhibition (LMI)[1] assays of human peripheral blood leukocytes generally correlates with delayed cutaneous hypersensitivity reactions to recall antigens such as tuberculin (1, 2), and reactivity in LMI assays also has been detected against antigens of human tumors (3). The major lymphokine involved in these reactions, designated leukocyte inhibitory factor (LIF) (4) and made by T and B lymphocytes (5) after stimulation by nonspecific mitogens including concanavalin A (Con A) or recall antigens such as purified protein derivative (PPD) inhibits the migration of polymorphonuclear leukocytes (PMN). LIF has been partially characterized (6, 7) and has a m.w. of approximately 68,000 (4). It has serine esterase activity (8, 9), and its effects on migration of PMN can be inhibited by sugars including α-L-fucose (10) and *N*-acetylglucosamine (11).

We have been interested in using LMI assays for serial immunologic monitoring of anti-tumor reactivity of cancer patients and for more extensive characterization of the physicochemical properties of LIF. One major problem that has hampered these studies is the lack of quantitation in the assays and the small quantity of LIF that is generally produced during the interaction of mononuclear cells with antigens or mitogens. Frequently, supernatants will rapidly lose their activity with minimal dilution and at times may even require concentration in order for the activity to be adequately measured. It would, therefore, be desirable to have access to sufficient quantities of high-titered LIF supernatants for the purpose of isolation and purification.

Recently, Philp *et al.* (12) studied production of macrophage migration inhibitory factor (MIF) by guinea pig lymphoid cells in response to tuberculin and found that MIF activity could be amplified markedly by performing the cultures in conical tubes.

The present study describes the adaptation of the methodology of Philp *et al.* (12) in the generation of LIF by human peripheral blood mononuclear cells in response to the antigen, PPD. It has been possible to develop an indirect agarose microdroplet LMI assay in which small numbers of mononuclear cells can be used to produce high titers of the lymphokine. Finally, we also examined some of the properties of the factor(s) responsible for the inhibition of PMN migration with particular emphasis on comparison with the previously described characteristics of conventionally generated LIF.

MATERIALS AND METHODS

Leukocyte donors. Donors were skin tested for delayed hypersensitivity to tuberculin by intradermal inoculation of 0.1 ml of intermediate strength PPD (5 units, Parke Davis and Co.,

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[1] Abbreviations used in this paper: LMI, leukocyte migration inhibition; LIF, leukocyte inhibitory factor; PPD, purified protein derivative; PMN, polymorphonuclear leukocytes; MIF, migration inhibitory factor; PBS, 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl; gp52, 52,000-dalton glycoprotein from mouse mammary tumor virus; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid sodium salt; MN, mononuclear cells; MI, migration indices relative to conditioned medium; (MI), migration indices relative to fresh medium.

Detroit, Mich.). Positive skin reactions were defined as ≥5 mm of induration at 48 hr. Blood was collected by venipuncture and was anticoagulated with preservative-free heparin (20 units/ml). All blood specimens used in *in vitro* assays were collected at least 12 to 24 months after the last skin test.

Antigen. For *in vitro* assays, PPD without preservatives or other additives was obtained from Parke Davis and Co., Detroit, Mich. Aliquots containing 1 mg/ml PPD in McCoy's 5A media supplemented with 10% fetal bovine serum (FBS) were stored at −70°C until use. We determined from dose response studies that a concentration of 25 μg/ml of PPD was generally optimal for eliciting LIF production. In some experiments, the major glycoprotein antigen (gp52) of mouse mammary tumor virus was used to generate supernatants with mononuclear cells from breast cancer patients.

Indirect PMN agarose droplet assay

Preparation of supernatants containing LIF. Mononuclear cells from whole blood were prepared by Ficoll-Hypaque separation, and the cells were resuspended in medium consisting of RPMI 1640 supplemented with HEPES (see Abbreviations) buffer (25 mM), pH 7.4, glutamine (1%), 10% heat-inactivated FBS, and gentamicin (50 μg/ml), and adjusted to a concentration of 2×10^7 mononuclear cells/4 ml when 50-ml plastic conical tubes (Falcon Plastics, #2070) were used, 5×10^6 mononuclear cells/ml when 12 x 75 mm round-bottom tubes (Falcon Plastics, No. 2003) were employed, or 5×10^5/0.2 ml when 4 x 46 mm Microfuge tubes (Bolab, Derry, N.H., No. BB10-450) were used. Appropriate concentrations of PPD or control medium were added to the cells, and the mixtures were incubated at 37°C in a humidified 5% CO_2 incubator for 2 hr to initiate lymphocyte stimulation. Cells were centrifuged at 200 × G for 10 min, the supernatant containing PPD was aspirated and discarded, and the cell pellet was resuspended in complete medium without PPD. The lymphocyte cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 or 48 hr to generate LIF and then centrifuged at 200 × G for 10 min. The supernatant fluid from each culture was collected and stored at −70°C until assayed for LIF. To evaluate the levels of LIF activity in each supernatant, a series of 2- or 10-fold dilutions in fresh medium was made.

Mixing of PMN and agarose and assay for LIF. PMN were prepared by sedimentation of most of the erythrocytes from the blood with the aid of Plasmagel followed by Ficoll-Hypaque separation of the leukocyte-rich plasma diluted 1:2 with phosphate-buffered saline. The PMN with some red blood cell contamination were harvested from the pellet and mixed (1.5×10^7) with a 0.1-ml aliquot of 0.2% agarose at 37°C. Two microliter droplets of the 0.2% agarose containing PMN (3×10^5 PMN/droplet) were placed into micro-test plate wells (Falcon No. 3034), and 0.1 ml of culture medium or various dilutions of PPD supernatants of cultured cells were added to four replicate wells and incubated at 37°C in a humidified CO_2 incubator for 16 to 18 hr. The areas of migration were determined by planimetry. Two methods of evaluation of migration areas were used, both of which yielded comparable results. First, as previously described (2), the inner agarose droplet and outer areas of cell migration were drawn onto paper, and measurements were determined by planimetry. Second, two perpendicular diameters of the inner agarose droplet and of the outer circle of cell migration were made, and the cell migration area was calculated directly as recently described (13). Migration indices (MI) were calculated, by using supernatants of mononuclear cell cultures without added antigen as the conditioned medium control or in some cases also by using fresh medium as the control, by the formula:

$$\text{MI} = \frac{\text{Average area of migration in the presence of cell supernatants generated with antigens}}{\text{Average area of migration in the presence of cell supernatants generated without antigens}}$$

In the tables, MI indicates indices relative to conditioned medium, and (MI) indicates indices relative to fresh medium. Migration index values of ≤0.85 were arbitrarily considered positive. The titer of a supernatant was defined as the reciprocal of the highest dilution giving a positive MI.

Gel filtration chromatography. Gel filtration chromatography of three different supernatants containing high-titered LIF activity was conducted in the descending mode on Sephadex G-200 columns at 4°C. The data in Figure 1 were obtained by using a 1.5 × 57 cm column of G-200 equilibrated with 0.05 M Tris, pH 8.1, containing 0.5 M NaCl. One-milliliter samples were applied to the column, 0.9-ml fractions were collected, and

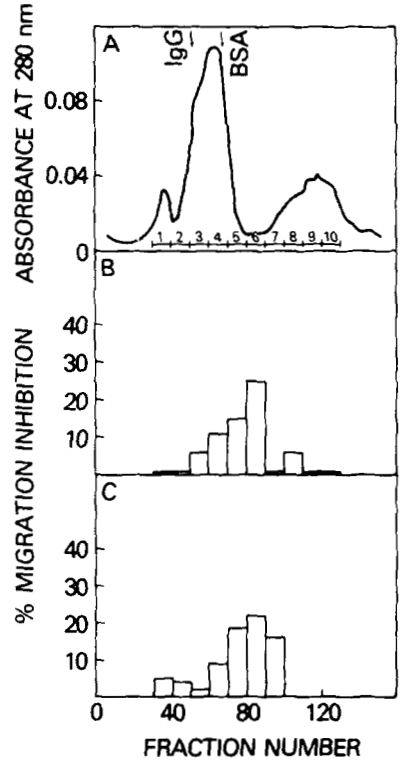

Figure 1. G-200 chromatography of high-titered LIF supernatants. *A*, protein profile characteristic of all supernatants tested and primarily a result of the exogenous fetal calf serum added during the generation phase. Elution positions of IgG and BSA are shown. Fractions were pooled as indicated. *B*, chromatography of a supernatant obtained by using PPD as the stimulating antigen. In this experiment, a conditioned medium supernatant was chromatographed on the same column and fractions were pooled and treated in a manner identical to that of the LIF supernatants. The pools of both fractionation procedures were assayed for their effects on PMN migration; migration inhibition due to the LIF supernatant fractions was calculated relative to the corresponding control fractions. *C*, chromatography of a supernatant obtained by using gp52 as the stimulating antigen. Details similar to those in panel B, except migration inhibition, were calculated relative to fresh (unfractionated) medium.

the column was calibrated with ^{125}I-IgG (provided by Dr. K. Kortright, Meloy Laboratories, Springfield, Virginia and BSA (Schwarz-Mann, Orangeburg, N.Y.). Fractions of 1.5 ml were collected, and the column was standardized with BSA and cytochrome c (both from Schwarz-Mann). The standard proteins were detected by their absorbance at 280 nm (and at 410 nm for cytochrome c) and by radioactivity measurement in a BioGamma counter. After chromatography, fractions were pooled as indicated and then dialyzed 16 hr at 4°C against 4 liters of 0.01 M sodium phosphate, pH 7.4. The LIF activity was tested after solid NaCl was added to a final concentration of 0.15 M. The samples could be frozen at least 2 weeks without appreciable loss of activity.

RESULTS

We were interested in determining whether, as reported by Philp *et al.* (12), high titers of LIF could also be produced in response to antigens. Supernatants were produced by stimulation of peripheral mononuclear cells from tuberculin skin reactive and negative donors by 25 μg/ml PPD, followed by incubation in 50-ml conical tubes. Tables I and II summarize data from two of the four experiments performed. In some experiments (e.g., Table I), most of the areas of migration in conditioned medium were close to those in fresh medium, whereas in other experiments (e.g., Table II), the PMN migrated substantially more in the higher concentrations of conditioned medium than in fresh medium. In each experiment, the areas of migration of PMN in supernatants of PPD-stimulated tuberculin-positive cells were substantially lower than the migration in conditioned medium from the same donor. When MI were calculated relative to the conditioned medium control, all skin-reactive donors gave positive results, with titers ranging from 10^4 to 10^7, whereas all of the tuberculin-negative donors had undetectable activity. The areas of migration in the presence of the stimulated supernatants from the tuberculin-positive donors gradually increased with higher dilutions, whereas those from the tuberculin-negative donors were fairly constant. In experiments in which conditioned medium did not induce increased migration relative to the fresh medium control, the titers based on MI relative to each control were similar. For example, in the experiment shown in Table I, the titer of the tuberculin positive donor was 10^7, according to either MI or

TABLE I

Indirect migration inhibition of human PMN by PPD-stimulated supernatants[a]

Dilution of Supernatants	Tuberculin Skin-Negative Donor				Tuberculin Skin-Positive Donor			
	Area of migration of PMN in conditioned medium	Area of migration of PMN in PPD-stimulated supernatant	MI	(MI)[b]	Area of migration of PMN in conditioned medium	Area of migration of PMN in PPD-stimulated supernatant	MI	(MI)[b]
Undiluted	42.3[c]	64.5	1.52	1.27	43.7	29.7	0.68	0.59
10^{-1}	73.0	73.0	1.00	1.44	57.3	30.7	0.53	0.61
10^{-2}	59.0	68.3	1.16	1.34	57.3	31.3	0.55	0.62
10^{-3}	63.0	64.5	1.02	1.27	51.3	32.0	0.62	0.63
10^{-4}	51.7	59.3	1.15	1.17	48.3	33.3	0.69	0.66
10^{-5}	46.0	60.3	1.30	1.19	49.7	33.3	0.67	0.66
10^{-6}	52.3	57.3	1.10	1.13	49.3	37.3	0.76	0.74
10^{-7}	51.3	59.0	1.15	1.16	52.3	41.3	0.79	0.82
10^{-8}	50.0	60.5	1.21	1.19	54.3	53.0	0.98	1.05
10^{-9}	54.7	63.0	1.16	1.24	49.3	51.0	1.03	1.01
10^{-10}	53.0	60.0	1.13	1.18	52.3	51.3	0.98	1.02

[a] Supernatants generated for 48 hr with 25 μg/ml PPD as described in *Materials and Methods* with 50-ml plastic conical tubes. Supernatants were tested after 14 days of storage at −70°C.

[b] Average units of migration of PMN cells in presence of fresh media used to calculate this MI were 50.6.

[c] Planimetry units (average of four replicates) derived by projecting circles on paper, tracing their image, and measuring by planimetry as described in the *Materials and Methods* section.

TABLE II

Indirect migration inhibition of human PMN by PPD-stimulated supernatants generated with mononuclear cells from tuberculin skin test-positive and skin test-negative donors[a]

Dilution of Supernatants	Tuberculin Skin Test-Negative Donor				Tuberculin Skin Test-Positive Donor			
	Area of migration of PMN in conditioned medium	Area of migration of PMN in PPD-stimulated supernatant	MI	(MI)[b]	Area of migration of PMN in conditioned medium	Area of migration of PMN in PPD-stimulated supernatant	MI	(MI)
10^{-1}	78.0	79.3	1.02	1.36	105.0	62.4	0.59	1.07
10^{-2}	72.0	72.3	1.00	1.24	104.5	51.6	0.49	0.89
10^{-3}	74.3	82.0	1.10	1.41	107.5	54.0	0.50	0.93
10^{-4}	73.0	77.3	1.06	1.33	106.0	53.3	0.50	0.92
10^{-5}	66.5	75.3	1.13	1.29	92.0	61.7	0.67	1.06
10^{-6}	69.3	71.7	1.03	1.23	69.3	56.0	0.81	0.96
10^{-7}	71.5	71.3	1.00	1.22	66.7	59.5	0.89	1.02
10^{-8}	61.7	70.7	1.15	1.21	63.2	59.6	0.94	1.02
10^{-9}	69.3	67.3	0.97	1.15	60.2	55.7	0.93	0.96
10^{-10}	65.7	64.7	0.98	1.11	60.8	60.1	0.99	1.03

[a] Supernatants generated for 48 hr with 25 μg/ml PPD in 50-ml conical tubes.

[b] Average units of migration of PMN in the presence of fresh medium were 58.3.

(MI). However, when conditioned medium caused increased migration of the PMN, only calculation of MI relative to comparable dilutions of the conditioned medium control gave satisfactory results. For example, in the experiment shown in Table II, the titer of the tuberculin-positive donor was 10^6 based on MI, but all (MI) were negative. With the tuberculin-negative donor, MI at all dilutions of the supernatant were close to 1.0, whereas (MI) were substantially above 1.0. Therefore, the conditioned medium control seemed to be more relevant and satisfactory, and in all subsequent experiments, data of stimulated supernatants were expressed relative to this control.

We were next interested in determining whether the high LIF activity of supernatants produced in conical tubes, relative to that seen in previous experiments with round-bottom tubes, was due to differences in numbers of cells in the cultures or to the shape of the tubes. Supernatants were produced by culturing 5×10^6 or 2×10^7 mononuclear cells from a tuberculin skin-reactive donor in 12 × 75 mm round-bottom and 50 ml conical tubes. The results are shown in Table III. As before, high activity (titer of 10^5) was obtained with the supernatant generated with 2×10^7 mononuclear cells/4 ml in the 50 ml conical centrifuge tube. In contrast, the supernatant generated with the same number of cells in the round-bottom tube or 5×10^6 cells/ml in the 50 ml conical tube had a titer of only 4. A repeat of this experiment (data not shown) yielded similar results, except that the supernatant generated in the round-bottom tube with 2×10^7 mononuclear cells/4 ml had activity at a 10^{-2} dilution (MI = 0.80). Therefore, both the number of cells and the shape of the tube appeared to be important factors in production of high titers of LIF.

A further variable in indirect LMI assays that might have an influence on the apparent reactivity of LIF-containing supernatants is the source of the PMN for the migration assay. Results presented in Table IV demonstrate the variability of PMN from different donors in their response to the same supernatants. The PMN of two donors responded well, showing similar high titers, and two failed to respond to any concentrations of the supernatant. Approximately 10% of PMN donors that we have tested failed to respond to positive supernatants.

For practical clinical applications of the indirect LMI assay, it would be helpful to perform the tests with small volumes of blood. Miniaturization of the assay would also allow more extensive evaluation of the specificity of reactions by testing a given cell population against a wide spectrum of antigens. We therefore studied whether high-titered supernatants could be produced in conical microtubes. Initially, we tested several cell concentrations ranging from 5×10^6 to 2.5×10^5 mononuclear cells/tube, and detected high titers of LIF at all cell concentrations in response to PPD. We then routinely used 5×10^5 mononuclear cells/tube in our subsequent studies, since this permits tests against eight to 10 antigens to be done with mononuclear cells that can be harvested from 5 ml whole blood. Table V illustrates the production of high-titered activity by 5×10^5 mononuclear cells in Microfuge tubes in response to PPD. Comparable reactivity was seen in the supernatants generated in the Microfuge tubes and in the 50 ml conical tubes containing 40 times the number of cells. Mononuclear cells from three tuberculin skin test-negative donors were unreactive when tested in Microfuge tubes (data not shown).

It was also of interest to determine whether cells cultured in microtubes would produce high levels of activity after exposure to lower concentrations of antigen than are required in the larger vessels. Results in Table VI show that optimal activity was obtained with a 50 or 25 μg/ml concentration of PPD, as was earlier seen in our studies with 50-ml conical tubes. Concentrations of 2.5 or 0.25 μg/ml PPD produced activity but with lower titers. Interestingly, prozone-like effects were observed with the supernatants generated with 50 and 25 μg/ml PPD, with no activity seen at low dilutions (1:3 to 1:10). We have

TABLE IV

Variation in the ability of PMN from different donors to be inhibited by the same supernatant[a]

Dilution of supernatant	MI with PMN from Donor No.			
	1	2	3	4
10^{-1}	0.61	1.04	0.67	1.02
10^{-2}	0.61	1.30	0.69	0.98
10^{-3}	0.80	1.19	0.74	1.03
10^{-4}	0.74	1.33	0.71	1.05
10^{-5}	0.80	1.20	0.67	1.01
10^{-6}	0.70	1.22	0.78	1.07
10^{-7}	0.95	1.12	0.84	0.99
10^{-8}	0.99	1.04	1.06	0.97
10^{-9}	1.00	1.03	1.13	1.03

[a] Supernatant generated for 48 hr with tuberculin skin-reactive donor mononuclear cells and 25 μg/ml PPD.

TABLE III

Influence of cell numbers and tube configuration on LIF activity of supernatants from cultures of mononuclear cells from tuberculin skin-reactive donor and PPD

Dilution of Supernatants	Supernatants Generated with 25 μg/ml PPD in							
	12 x 75 mm Round-bottom tubes				50-ml Conical tubes			
	5 x 10^6/ml		2 x 10^7/4 ml		5 x 10^6/ml		2 x 10^7/4 ml	
	MI	Conditioned media areas	MI	Conditioned media areas	MI	Conditioned media areas	MI	Conditioned media areas
Undiluted	0.64	(2991)[a]	0.67	(3157)	0.63	(2989)	0.44	(3026)
1:2	0.86	(2670)	0.77	(2841)	0.73	(2731)	0.52	(2889)
1:4	0.95	(2646)	0.83	(2952)	0.83	(2919)	0.76	(2745)
10^{-1}	1.02	(2578)	0.92	(2789)	0.99	(2496)	0.80	(2660)
10^{-2}	1.20	(2023)	1.07	(2187)	1.20	(2062)	0.82	(2105)
10^{-3}	1.05	(2163)	0.96	(2260)	1.16	(2004)	0.84	(2196)
10^{-4}	1.03	(2186)	1.17	(2043)	1.07	(2072)	0.78	(2228)
10^{-5}	1.01	(2154)	0.93	(2300)	0.94	(2361)	0.73	(2295)
10^{-6}	1.06	(2162)	1.02	(2206)	1.10	(2151)	0.91	(2176)
10^{-7}	1.01	(2088)	1.07	(2037)	1.00	(2173)	0.93	(2149)

[a] Units derived by the second method of calculation (i.e., two-diameter measurements) as described in the *Materials and Methods* section.

TABLE V

Comparison of supernatants of cultures generated in 50-ml conical tubes and Microfuge tubes with PPD-stimulated mononuclear cells from a tuberculin skin-reactive donor

Dilution of Supernatant	Experiment 1				Experiment 2			
	Microfuge tubes (5 x 10^5 cells/0.2 ml)		50-ml conical tubes (2 x 10^7 cells/4 ml)		Microfuge tubes (5 x 10^5 cells/0.2 ml)		50-ml conical tubes (2 x 10^7 cells/4 ml)	
	MI	Control migration area	MI	Control migration area	MI	Control migration area	MI	Control migration area
10^{-1}	0.73	(2214)[a]	0.73	(2248)	0.64	(1914)	0.53	(2161)
10^{-2}	0.81	(2202)	0.84	(2356)	0.71	(2162)	0.66	(2041)
10^{-3}	0.81	(2189)	0.84	(2348)	0.68	(2058)	0.71	(2032)
10^{-4}	0.82	(2097)	1.00	(1904)	0.77	(2002)	0.70	(2069)
10^{-5}	0.95	(1969)	0.98	(1787)	0.79	(2144)	0.73	(2141)
10^{-6}	0.90	(2060)	0.95	(1925)	0.84	(1984)	0.79	(2019)
10^{-7}	0.91	(2207)	1.04	(1753)	0.96	(1944)	0.86	(1993)

[a] Units derived by the second method of calculation (i.e., two-diameter measurements) as described in the *Materials and Methods* section. Supernatants were generated for 24 hr after stimulation with 25 μg/ml PPD.

TABLE VI

Activity of supernatants generated in Microfuge tubes by using tuberculin skin-reactive mononuclear cells and various concentrations of PPD

Dilution of Supernatant	Concentrations of Tuberculin (μg/ml) Used (5 x 10^5 cells/0.2 ml)				
	50	25	2.5	0.25	0.025
1:3	0.92[a]	0.88	0.79	0.85	0.95
10^{-1}	0.90	0.73	0.77	0.83	1.03
10^{-2}	0.64	0.81	0.79	0.94	0.98
10^{-3}	0.67	0.81	0.84	1.03	0.94
10^{-4}	0.83	0.82	0.83	1.00	1.02
10^{-5}	0.84	0.83	0.91	1.11	1.05
10^{-6}	0.89	0.90	0.96	1.03	1.01

[a] MI. Supernatants were generated for 24 hr after stimulation with 25 μg/ml PPD.

observed such prozones on a number of occasions in response to PPD and also to tumor antigens.

Gel filtration of high-titered LIF supernatants. High-titered LIF supernatants and appropriate conditioned medium controls were subjected to gel filtration in order to estimate the molecular size of the factor(s) responsible for the inhibition of PMN migration. Conventionally generated LIF has been reported to elute from molecular sieve columns with (4) or slightly after (10) molecules the size of serum albumin (M_r = 68,000). Thus, it was of interest to see whether the high activity LIF studied here was similar in size to the LIF described in previous reports.

Gel filtration of high-titered LIF is shown in Figure 1. Panel A indicates the protein profile representative of the crude supernatants and the fractions that were pooled for analysis of LIF activity. Chromatography of high-titered LIF generated with PPD by using mononuclear cells from a tuberculin skin test-positive donor is shown in panel B. Panel C demonstrates the pattern by using high-titered LIF generated with gp52 and mononuclear cells from a breast cancer patient (14). In both cases, the maximum inhibition of migration was found in fractions 80 through 90 (pool 6), corresponding to molecules slightly smaller than serum albumin. In panel B each pool was diluted 2000-fold before assaying for activity. Since 1 ml of supernatant was applied to the column, and since each pool contained 10 ml, pools with an overall dilution of 1:20,000 still contained demonstrable LIF activity. Pool 6 from Figure 1, panel B, was then titered in 10-fold dilutions in comparison with the unfractionated sample and was found to have activity at one dilution lower than the crude sample (10^{-6} *vs* 10^{-7}, data not formally presented). Since pool 6 represents only a portion of the peak G-200 region containing LIF activity, it follows that much of the LIF activity was recovered after gel filtration.

Monosaccharide inhibition of high-titered LIF activity. In a further attempt to compare high-titered LIF with conventional LIF, we examined the sugar specificity of the interaction between LIF and the target cell. It has previously been reported that L-fucose could prevent the inhibition of migration by human LIF (10), and that this sugar, when covalently linked to gel beads, could remove LIF activity from crude supernatants (6).

Table VII shows the results of an experiment in which D-glucose, L-fucose, and *N*-acetyl-D-glucosamine were tested at three concentrations in the presence of three dilutions of LIF-containing or control supernatants. In the absence of exogenous sugar the supernatants showed migration inhibition at dilutions of 10^{-2} and 10^{-4} M. In all experiments and at concentrations as low as 10^{-6} M, L-fucose inhibited the effect of the lymphokine. In the presence of fucose, the migration areas of the PMN in the PPD-stimulated (LIF-containing) supernatants were about the same as the migration areas in conditioned medium, and the latter were virtually unaffected by the sugar. Similar results were obtained with *N*-acetyl-D-glucosamine, but at a concentration of 10^{-6} M this sugar was incapable of blocking LIF activity. Glucose at any concentration had no effect on migration of PMN in either the conditioned medium or PPD-stimulated supernatant.

The sugar specificity of this reaction was further investigated by using a series of monosaccharides at a concentration of 10^{-4} M. As shown in Table VIII, L-fucose inhibited the effect of the lymphokine, whereas all other sugars were ineffective. In another experiment, it was found that the concentration of glucose had to be increased to 10^{-1} M before inhibition of LIF activity was seen. *N*-acetyl-D-glucsamine was inconsistently effective at 10^{-4} M, as shown in Tables VII and VIII. In Table VII, *N*-acetyl-glucosamine was effective at 10^{-2} and 10^{-4} M but not at 10^{-6} M, whereas in Table VIII this sugar was incapable of preventing LIF activity at 10^{-4} M. This variability may be due to interassay variation, with the 10^{-4} M concentration of sugar at the borderline of effective inhibition. In all experiments, however, L-fucose was consistently capable of blocking LIF action at concentrations as low as 10^{-6} M.

The potency of L-fucose was also compared with the D- form (data not shown). L-Fucose blocked LIF activity at sugar concentrations as low as 10^{-8} M, whereas D-fucose exhibited only weak blocking activity from 10^{-3} to 10^{-5} M. We have observed

TABLE VII

Inhibition of activity of high-titer LIF supernatants with soluble monosaccharides

Dilution of Supernatant	Sugar Tested																							
	None			D-glucose						L-fucose						*N*-acetyl-D-glucosamine								
				10^{-2} M			10^{-1} M			10^{-6} M			10^{-2} M			10^{-4} M			10^{-6} M			10^{-2} M			10^{-1} M			10^{-6} M		
	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI	CM	PPD	MI
10^{-2}	1823[a]	1486	0.82[b]	1799	1401	0.79	1824	1522	0.83	1881	1596	0.85	1761	1663	0.94	1844	1691	0.92	1901	1827	0.96	1718	1622	0.94	1911	1800	0.94	1887	1456	0.77
10^{-4}	1719	1262	0.73	1882	1362	0.72	1761	1291	0.73	1753	1301	0.74	1627	1648	1.01	1707	1663	0.97	1733	1817	1.04	1648	1714	1.04	1787	1648	0.93	1729	1322	0.76
10^{-6}	1700	1562	0.92	1751	1626	0.93	1629	1599	0.98	1715	1686	0.98	1663	1714	1.04	1641	1688	1.03	1747	1789	0.97	1583	1667	1.05	1744	1722	0.99	1671	1573	0.94

[a] Values given are areas of PMN cell migration in the presence of conditioned medium (CM) or antigen-stimulated supernatants (PPD).

[b] MI is the M.I. obtained as the ratio of these two areas $\left(\frac{PPD}{CM}\right)$.

TABLE VIII

Effect of various monosaccharides on LIF activity

Sugar[a]	M.I.
None[b]	0.70
L-Fucose	1.01
L-Rhamnose	0.72
Galactose	0.73
Glucose	0.80
Mannose	0.70
α-Methyl mannoside	0.65
N-acetylgalactosamine	0.72
N-acetylglucosamine	0.72

[a] All sugars were tested at 10^{-4} M.

[b] Conditioned medium and LIF supernatants (generated in response to PPD) were tested at a final dilution of 10^{-2}.

blocking of LIF activity by D-fucose in other experiments but only at concentrations greater than 10^{-5} M. Therefore, we conclude that L-fucose is a potent inhibitor of high-titered LIF activity and that D-fucose and *N*-acetylglucosamine, while also apparently effective, are at least a thousand times less potent.

DISCUSSION

Supernatants containing high titers of LIF activity were successfully generated by culturing mononuclear cells in conical tubes, at a higher concentration than previously used (2, 4, 6, 15). Previously, LIF supernatants were produced in round-bottom tubes (2, 4, 6) or flasks (15) by using 2.5 to 5 $\times$ 10^6 mononuclear cells/ml. Under those conditions, LIF activity was only detectable in undiluted supernatants (4, 6, 15) or at low dilutions (2).

The generation of supernatants with high titers of LIF activity in conical tubes appeared antigen-dependent, since culturing unstimulated cells failed to produce LIF. Also, PPD produced high LIF activity in the presence of mononuclear cells from tuberculin skin-reactive donors but failed to induce activity with mononuclear cells from tuberculin skin-negative donors. In the cultures in large tubes, induction of augmented LIF was not solely dependent on either increased cell numbers or the conical shape of the tubes. Supernatants from round-bottom tubes containing the higher number (2×10^7/4 ml) of mononuclear cells failed to produce titers of LIF higher than 10^2, and a low cell number (5×10^6) in 50-ml conical tubes also failed to induce titers above 4. However, in cultures performed in microtubes, the shape seemed to be the main variable, since similar high titers of LIF were seen over a range of cell concentrations. Although the mechanisms underlying the ability to generate such remarkably high titers of activity by simple changes in culture conditions are not clear, these observations point toward the critical importance of optimal cell-cell contact, as suggested by Philp *et al.* (12). For induction of MIF (see 22) and LIF (McCoy, J. L. *et al.*, unpublished observations) by soluble antigens or mitogens, presentation by macrophages or monocytes appears to be required, and the degree of contact between the lymphocytes and auxiliary cells may have a major influence on the level of response.

Whether the increased activity in the supernatants represents the production of quantitatively more LIF or the synthesis of biologically more active LIF molecules, as has been described for insulin (16), remains to be determined.

In several experiments, PMN had increased migration in supernatants with the higher concentrations of conditioned media than they had in the presence of fresh media. This

enhancing activity of some supernatants may have accounted for the prozone effects in some of our experiments (e.g., Table VI), with activity in supernatants of PPD-stimulated cells not detectable at low dilutions but clearly seen at higher dilutions. We considered the possibility that the high titers of LIF activity that appeared to be present in the supernatants of stimulated cells might actually be due to production of less "enhancing factor" in antigen-containing cultures rather than to LIF itself. This possibility can largely be dismissed by the observations in some experiments (see Tables I and II) that even calculation of MI values relative to fresh medium indicated high titers of migration-inhibitory activity.

The elaboration of migration-enhancing factors in addition to LIF may also account for the narrow range of positive MI values that were frequently seen over a wide range of dilutions of supernatants from stimulated cells. This observation indicates that the degree of migration inhibition is not a reliable indicator of the amount of LIF produced. Assessment of the titer of LIF in a supernatant would appear to be a better method for quantitating the degree of reactivity of mononuclear cells.

Another interesting observation in these studies was that the PMN of some donors consistently did not respond. This may lead to some false negative results in indirect tests. In addition, in direct LMI tests it would not be possible to determine whether negative results were due to failure of the mononuclear cells of the donors to make LIF in response to stimulation or to lack of response of the PMN to LIF. This is clearly an advantage of an indirect assay, in which the two phases can be separately examined. The reasons for unresponsiveness of PMN of some donors to LIF are not known. Possible explanations are a) the lack of sufficient LIF receptors on the PMN, b) nonspecific release of proteases from PMN (17) that may degrade LIF, and c) suppressive factors that may interfere with the action of LIF on the PMN. The lack of responsiveness of PMN of some donors to LIF may be analogous to the lack of responsiveness to MIF of macrophages from some mouse strains (18), where suppressor cells appear to play a role.

Upon gel filtration of antigen-stimulated supernatants containing high-titered inhibitory activity, one inhibitory peak of activity was found in fractions eluting with globular proteins slightly smaller than serum albumin. These results are quite compatible with previous studies with LIF. Bendtzen (10), using LIF supernatants from Con A-stimulated lymphocytes, found inhibitory activity to elute between the albumin and cytochrome c markers on a Sephadex G-100 column. Rocklin found that LIF produced by antigen (4) or Con A stimulation (19) eluted in the same region as albumin. However, since broad regions of the profile were pooled for analysis, the precise elution volume was not accurately defined. In our studies as well, chromatographic resolution was limited by the pooling of fractions, although we did make as many as 10 pools across the working volume of the profile. Although technically feasible with high-titered LIF supernatants, we have not attempted to assay each individual fraction for LIF activity. This type of study would be necessary for a better definition of gel chromatographic behavior of this lymphokine and could provide a value for the Stokes radius of the LIF molecule. One obstacle to this approach is the limited effectiveness of the semi-quantitative cell migration assay in producing a LIF activity profile. Thus, the present state of the art allows for only an approximation of the elution position of human LIF by gel filtration chromatography.

Another criterion for the high-titered migration inhibitory activity being due to LIF was the ability of L-fucose to specifically block its effects. Bendtzen (10) demonstrated that 10^{-1} M L-fucose, but not D-fucose, could inhibit LIF activity. Our results again were compatible with this feature of LIF, but with effective inhibition by concentrations of L-fucose as low as 10^{-8} M. As predicted by the results of Bendtzen (10), the biologically inactive D- form of the sugar was ineffective at comparable concentrations but showed some ability to inhibit LIF activity at much higher concentrations.

As a further demonstration of specificity, another 5-methylpentose, α-L-rhamnose, was found to be ineffective, as were a number of hexoses and hexose derivatives. *N*-acetyl-D-glucosamine consistently blocked LIF activity at 10^{-3} M. At 10^{-4} M its effect was inconsistent and at 10^{-6} M it became totally ineffective. This result is consistent with those of Rocklin (11) who found that LIF activity could be blocked by 10^{-1} M *N*-acetyl-D-glucosamine.

The previous results on sugar inhibition of LIF activity were somewhat contradictory. Bendtzen (10) failed to observe inhibition by *N*-acetylglucosamine, and Rocklin (11) failed to observe an effect by L-fucose. The explanation for this discrepancy is presently unknown but might be due to the different assay methods used by these investigators, which may have reflected somewhat different phenomena. The agarose microdroplet assay used here is different from that used by either Bendtzen or Rocklin. As measured here, LIF activity was considerably more susceptible to inhibition by the active sugars, and this greater degree of sensitivity probably accounts for our observation of inhibition by *N*-acetylglucosamine as well as by much lower concentrations of L-fucose.

These studies implicate L-fucose as part of a receptor on the surface of PMN that is involved in the regulation of cell migration. In further support of this, a fucose-binding lectin from *Lotus tetragonolobus* has been shown to affect the movement of human PMN (20). It was demonstrated that this lectin could bind fucosyl residues on PMN regardless of the donor's blood type. Thus, fucose is present on the PMN surface and plays a role in the transmission of a signal that alters cellular migration. This role may involve the very earliest step in events of this kind, namely that of recognition and binding, as in the formation of a hormone-receptor complex (21).

There are a number of issues that remain unresolved in the present study or that await further investigation. For example, even though we detect incredibly high titers of LIF in our active supernatants, we found no significant increase in the protein content of these supernatants. The Sephadex G-200 protein profile (Fig. 1) was essentially the same for both active and control supernatants. It is conceivable that orders of magnitude more of one or more of a few proteins are being produced, but if originally present in extremely low concentrations, they may still represent a small fraction of the total, and this increase would not be detected. Further, presumably other lymphokines (e.g., interferon) may be present in our active supernatants in high titers, and demonstration of their possible enhanced activities would strengthen our observations with LIF. We are quite cognizant of the importance of these types of issues that would help further substantiate the issue that our data reflect a true production of high titers of LIF and would further rule out the possibility that we are dealing with some unidentified technical artifact.

In conclusion, our results indicate the presence of a factor in high-titered LIF supernatants with properties similar to conventional LIF. This factor is relatively stable and will withstand gel chromatography, dialysis, and general handling procedures.

Furthermore, the ability to detect its activity at high dilution should facilitate attempts at purification and characterization of this lymphokine.

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