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INHIBITION OF HUMAN LEUKOCYTE MIGRATION INHIBITORY FACTOR (LIF) BY α -L-FUCOSE

By

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Interaction between sensitized lymphocytes and specific antigen *in vitro*, results in the generation of soluble factors exhibiting a variety of biological activities. These mediators, commonly known as lymphokines (7), are believed to participate in the *in vivo* expression of the cell-mediated immune response.

Isolation and characterization of lymphokines has been extremely difficult, mainly due to production of mediators in only minute quantities, and contamination by a variety of other products of cellular metabolism. Thus, human migration inhibitory factor (MIF) (14) and human leukocyte migration inhibitory factor (LIF) (12) have been characterized only in terms of general physicochemical properties (13, 14), response to enzymes, enzyme inhibitors (9, 13, 14) and treatment with various drugs and chemicals (3). Therefore, the necessity for the development of supplementary separation methods is obvious.

The monosaccharide, α -L-fucose, has been shown to neutralize the action of guinea-pig MIF, and the mediator activity was prevented by treating the migratory cells with α -L-fucosidase. Thus, α -L-fucose on the macrophage surface seems to be essential for the MIF response of this cell (11). The concept of α -L-fucose being a part of a receptor for MIF opens up

new possibilities for developing an affinity chromatography technique for the recovery of virtually purified guinea-pig MIF.

In the present article data are presented showing that human LIF, which in contrast to guinea-pig MIF is not thought to be a glycoprotein, is inhibited by α -L-fucose as well. The inhibition is specific for this sugar, and reversible. In a following article the development of an affinity column chromatography technique for purifying LIF will be described in detail.

MATERIAL AND METHODS

Lymphokine Production

Human mononuclear cells were obtained from heparinized peripheral blood from healthy adults according to methods previously described (4). The separated lymphocytes (2.5×10^6 cells per ml) containing only a few per cent monocytes and erythrocytes were then incubated in serum-free medium TC-199 with penicillin 67 i.u. per ml and streptomycin 67 μ g per ml (Difco Laboratories, Michigan, U.S.A.) (TC-199). After 22 h at 37° C in 2 per cent CO₂ in water-saturated atmospheric air in the presence or absence (control) of concanavalin A 80 μ g per ml (Pharmacia, Uppsala, Sweden) (Con-A), the supernatants were harvested, and the mitogen-free control supernatant was reconstituted with 80 μ g per ml of Con-A.

Both supernatants were subsequently applied on small columns of Sephadex G-100 (Pharmacia) equilibrated with volatile buffer (0.02 M ammonium bicarbonate; acetic acid, pH 7.0). Con-A was hereby removed by conjugation to the Sephadex matrix (1, 4). Using bed volumes, at least four times that of the supernatant volumes, the columns eluted with volatile buffer at the same time served to desalt the supernatants. The mitogen-free supernatants were then passed through Millipore filters (0.45 μ m pore size), lyophilized and stored at 4° C until use.

Assay for Leukocyte Migration Inhibition

The indirect leukocyte migration agarose technique described by Clausen (5) was employed using peripheral blood leukocytes from healthy, unrelated adults as migratory cells. 22×10^6 cells were suspended in 90 μ l culture supernatant and tested for migration under agarose as previously described (4). The LIF activity was determined as a migration index:

$$MI = \frac{\text{Mean area of migration in Con-A stimulated supernatants}}{\text{Mean area of migration in parallel control supernatants}}$$

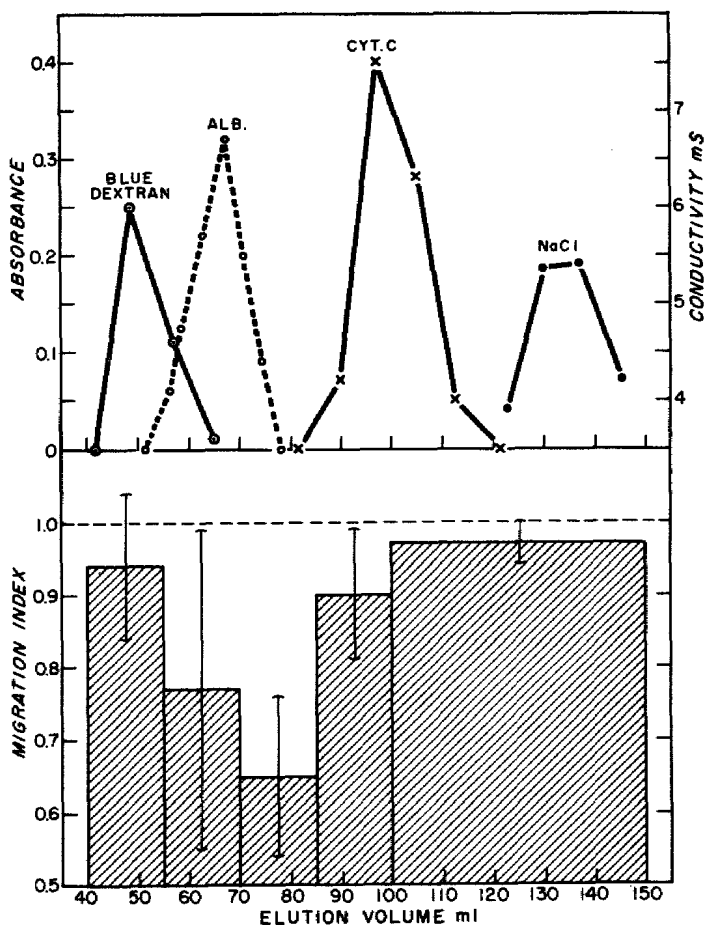


Fig. 1.

Top: Calibration of Sephadex G-100 columns (1.6×90 cm) with blue dextran (mol. wt. 2×10^6), human albumin (mol. wt. 69×10^3), cytochrome C (mol. wt. 12×10^3) and NaCl. The albumin values are given in arbitrary units as measured by fused rocket immunoelectrophoresis (15) with rabbit anti-human albumin antibody incorporated in the gel. —○— Absorbance at 610 mμ (blue dextran).

—x— Absorbance at 412 mμ (cytochrome C). —●— Conductivity (saline).

Bottom: The elution pattern of human leukocyte migration inhibitory factor.

Mean values of five different experiments. Vertical lines indicate $\pm$ SD.

Fractionation of Supernatants on Sephadex G-100

Lyophilized Con-A preincubated and Con-A reconstituted supernatant material was dissolved in isotonic 0.1 M phosphate-buffered saline (PBS) to achieve a 70-fold concentration of the initial supernatant fluid. Two ml of the concentrated supernatant, cleared of any insoluble material by centrifugation, were then applied on Sephadex G-100 glass columns (1.6×90 cm) (Pharmacia) equilibrated and eluted with PBS at 4° C with a flow rate of 8 ml per h. Fractions of 4 ml were collected and the total effluent was divided into 5 fractions as shown in Fig. 1. Unconcentrated fraction material was then tested for LIF activity.

Calibration of the Columns

A mixture of 500 μ l human serum, 100 μ l blue dextran (2 mg per ml) (Pharmacia), 50 μ l cytochrome C (Sigma, St. Louis, Mo., U.S.A.) and 1350 μ l 1.0 M NaCl was applied on the columns and eluted under similar conditions as described above. The serum was included in order to permit the determination of the elution profile of albumin (mol. wt. 69,000 daltons) by means of the fused rocket immunoelectrophoresis technique described by Svendsen (15). Blue dextran (mol. wt. 2×10^6 daltons) which appeared in the void volume and cytochrome C (mol. wt. 12×10^3 daltons) were measured by light absorbance at 610 m μ and 412 m μ respectively. The total bed volume, corresponding to the appearance in the eluate of a higher salt concentration, was determined by conductivity measurements.

TABLE 1

Effect of Monosaccharides on Leukocyte Migration Inhibitory Factor (LIF).

		LIF - control (without sugar)	LIF + 0.1 M monosaccharide	P
α -L-fucose	(12)*	0.57 ± 0.17 §	0.74 ± 0.21	< 0.05
D-(+)-fucose	(5)	0.56 ± 0.19	0.65 ± 0.18	ns†
L-rhamnose	(4)	0.62 ± 0.21	0.64 ± 0.17	ns
D-(+)-mannose	(4)	0.77 ± 0.06	0.77 ± 0.08	ns
N-acetyl-D-mannosamine	(4)	0.58 ± 0.27	0.63 ± 0.24	ns
N-acetyl-D-glucosamine	(4)	0.66 ± 0.19	0.65 ± 0.19	ns
N-acetyl-D-galactosamine	(4)	0.71 ± 0.14	0.71 ± 0.13	ns
α -methyl-D-mannoside	(4)	0.65 ± 0.21	0.63 ± 0.20	ns

* Number of experiments.

§ Mean migration index $\pm$ SD.

† ns = not significant.

Effect of Monosaccharides on LIF Activity

All sugars tested (see Table 1) were obtained in analytical grade form from Sigma. Three different batches of α -L-fucose were tested. The carbohydrates were dissolved in TC-199 at concentrations outlined in "Results" and added to the lyophilized material from both LIF and control supernatants before assay. Identical LIF and control supernatants were dissolved in the same amount of TC-199 and assayed in parallel for LIF activity. As a further control, cells were migrated in TC-199 both with and without sugar.

Statistical Methods

The statistical significance of difference was determined by the Wilcoxon rank sum test. The significance of correlation was assessed by means of Spearman's coefficient of rank correlation (6).

RESULTS

Sephadex Gel Filtration

Lyophilized material from 140 ml lymphokine-rich or control supernatants was filtered on Sephadex G-100. LIF activity eluted both with and slightly after the albumin marker (2nd and 3rd fraction respectively). If LIF is assumed to be a globular protein the estimated molecular weight was 40,000 to 50,000 daltons. The results of five such experiments are summarized in Fig. 1.

The mean percentage of inhibition found in the second fraction was 23 per cent ($P < 0.05$) and in the third fraction 35 per cent ($P < 0.01$). No significant inhibitory activity was found in any other fractions. In particular, no activity could be detected in fractions containing molecules of the same size as human MIF (mol.wt. 23,000 daltons).

Monosaccharides and LIF Activity

The effect of monosaccharides on human LIF is presented in Table 1. In all experiments LIF-rich and control supernatants, with and without the sugars, were set up in parallel. The sugars by themselves did not inhibit the migration of leukocytes. Only α -L-fucose showed a significant reduction in LIF activity but the leukocytes in an average of twelve differ-

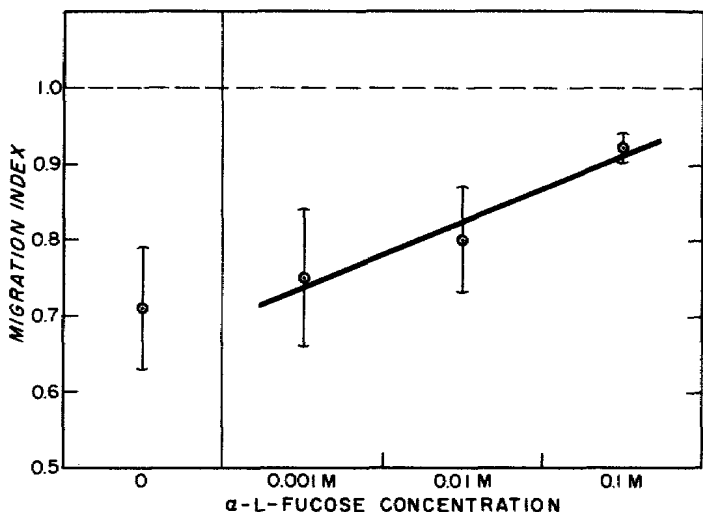


Fig. 2.

The dose-response of different concentrations of α -L-fucose on moderate LIF activity. The reduction of LIF activity is significant in the presence of 0.1 M α -L-fucose ($P < 0.05$). The correlation between decrease in LIF activity and increase in α -L-fucose concentration is significant ($P < 0.01$). Mean values of six different experiments. Vertical lines indicate $\pm$ SD.

ent experiments were still significantly inhibited, even in the presence of 0.1 M α -L-fucose.

The experiments could be divided into two groups:

a) When unconcentrated supernatant material was tested in the absence or presence of α -L-fucose, the LIF activity (MI: 0.71) was repeatedly and completely inhibited (MI: 0.92) as shown in Fig. 2. Furthermore, the blocking of LIF was dose-dependant and a significant linear correlation between increase in α -L-fucose concentration and decrease in LIF activity was found ($P < 0.01$) (Fig. 2).

b) When concentrated LIF-rich and control materials were tested only a slight decrease in LIF activity could be seen. The results of six different experiments are presented in Fig. 3.

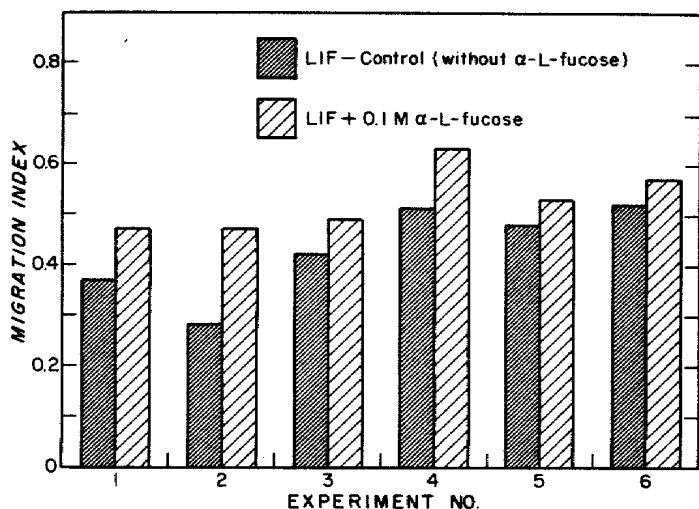


Fig. 3.

The effect of 0.1 M α -L-fucose on strong LIF activity.

TABLE 2

Reversibility of the α -L-fucose Effect on Leukocyte Migration Inhibitory Factor (LIF).

		Migration Index
LIF	Control (without α -L-fucose)	0.61*
	+ α -L-fucose 0.1 M	0.90
	Preincubated 1 h 37° C without sugar, then passed on Sephadex G-50§	0.60
	Preincubated 1 h 37° C with 0.1 M α -L-fucose, then passed on Sephadex G-50	0.65

* Mean of two experiments.

§ The sugar was removed by Sephadex G-50 column chromatography.

Even high concentrations of α -L-fucose (0.33 M) did not significantly block strong LIF activity.

Experiments were then undertaken to investigate whether the effect of α -L-fucose was reversible. LIF and control super-

natants were incubated with 0.1 M α -L-fucose for 1 h at 37° C. The sugar was then removed by passage through Sephadex G-50, equilibrated and eluted with TC-199. As shown in Table 2, LIF activity was blocked when assayed in the presence of sugar, but when LIF was preincubated with α -L-fucose and the sugar removed, the LIF activity was still present.

Likewise, human buffy coat cells were incubated for 1 h at 37° C with 0.1 M α -L-fucose. The indicator cells were then washed free of sugar and assayed with lymphokine and control supernatants. The cells were still inhibited by LIF as shown in Table 3.

TABLE 3
Effect of Pulse Treatment of Peripheral Blood Leukocytes with α -L-fucose.

		Migration Index
Buffy coat cells	Preincubated 1 h 37° C without sugar, then washed three times and assayed with LIF	0.64*
	Preincubated 1 h 37° C with 0.1 M α -L-fucose, then washed three times and assayed with LIF	0.63

* Mean of two experiments.

DISCUSSION

Sephadex G-100 Chromatography and LIF Activity

Human MIF and LIF so far seem to be two different products of activated lymphocytes (12). LIF selectively inhibits the migration of polymorphonuclear leukocytes but does not inhibit the migration of human monocytes or guinea-pig macrophages. The main physiochemical difference between the two mediators was shown to be the elution pattern in Sephadex G-100 gels. Thus, LIF eluted with albumin (68,000 daltons) differing from the estimated molecular weight of human MIF (23,000 daltons). Antigen-induced and Con-A-induced LIF behaved alike with regard to Sephadex chromatography, acrylamide gel electrophoresis, isopycnic centrifugation and susceptibility to chymotrypsin and neuraminidase (13).

Peak LIF activity appeared from Sephadex G-100 gels with molecules of an approximate molecular weight of 40,000 to 50,000 daltons. No activity was present at around 23,000 daltons while some activity was demonstrable around 68,000 daltons. The apparent difference in molecular weight between this mediator and LIF described by Rocklin (13), and the elution in a rather broad area, could be due to the presence of aromatic substances in the lymphokine molecule. These substances show greater affinity to the Sephadex gel phase than to the aqueous phase and are retarded to varying extents, depending on even slight changes in experimental conditions. However, this is only speculative, and the fact that the mediators were tested using different assay methods must also be considered. Thus, microcinematographic studies have revealed two distinctly different ways of cell migration in capillary and agarose migration assays (2). In the capillary assay, cells are tumbling passively out from the tubes showing only little autonomous movement. The inhibition phenomenon was seen to be initiated by the spreading and clumping of indicator cells which became stuck to glass surfaces and to each other. In the agarose assay the polymorphonuclear cells migrate by an active movement, the motion being slowed down more and more under the influence of the inhibitory factor. Therefore, the possibility of the existence of several molecular species of LIF, or of LIF being made up of subunits undergoing association-dissociation reactions cannot be excluded.

The Blocking of LIF Activity by α -L-fucose

The studies indicate that α -L-fucose, a well-known component of mammalian cell membranes and bloodgroup substances, blocks LIF activity reversibly whereas other monosaccharides do not. Consequently, α -L-fucose, either on LIF or on the polymorphonuclear cells and the macrophages, seems to be necessary for the mediator-cell interaction. The findings of Remold (11) that α -L-fucose blocked the effect of guinea-pig MIF on macrophages, and that macrophages incubated

with α -L-fucosidase no longer responded to MIF, seems to favour the hypothesis that α -L-fucose is in fact an important part of the receptor for guinea-pig MIF. However, the blocking of LIF activity by α -L-fucose in only high concentrations and the fact that strong LIF activities could not be blocked even by 0.33 M solutions of the sugar suggests that α -L-fucose is merely a part of the binding receptor.

Recently, two independent laboratories have stressed the significance of α -L-fucose in mediator-membrane biology by the documented blocking effect of this sugar on MIF-like activity derived from fetal calf serum (8) and from virus transformed mouse cells (10).

If LIF can be shown to bind reversibly to α -L-fucose without being destroyed, affinity chromatography might be used for achieving separation of lymphokines which is difficult or even impossible when using less specific techniques.

SUMMARY

Peak leukocyte migration inhibitory activity was produced *in vitro* by human lymphocytes stimulated with concanavalin A and eluted from Sephadex G-100 gel columns in fractions containing molecules of mol. wt. 40,000–50,000 daltons. No activity was found in fractions containing molecules of mol. wt. 23,000 daltons which is the estimated size of the human macrophage migration inhibitory factor (MIF). In the present experiments migration inhibition was tested on human peripheral blood leukocytes migrating under agarose.

Unconcentrated supernatant activity was reduced in a dose-related manner by α -L-fucose. At a concentration of 0.1 M α -L-fucose, the mediator activity was completely neutralized. The effect was reversible and specific for α -L-fucose. Concentrated supernatant activity, however, was only slightly reduced even in the presence of a higher concentration of sugar. The possible role of α -L-fucose as a part of a mediator receptor on the indicator cells is discussed.

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