PHYSICOCHEMICAL PROPERTIES OF A MITOGENETIC FACTOR FROM HUMAN LYMPHOCYTES INDUCED BY PHYTOHEMAGGLUTININ

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The physicochemical properties of a mitogenetic factor (MF) secreted by human lymphocytes *in vitro* under the influence of phytohemagglutinin were studied. Treatment of the culture medium with proteases was shown to reduce activity of the MF sharply. On gel chromatography on Sephadex and Biogel columns all the mitogenetic activity was concentrated in fractions corresponding to a molecular weight of 20,000-30,000 (peak at 25,000). On disk electrophoresis in 5% polyacrylamide gel MF migrated in the region of α_1 and α_2 globulins and transferrin and was separated into several fractions. On isoelectric focusing the MF was arranged as three fractions within the pH range 4.5-8.3. The functional heterogeneity of the MF is postulated.

KEY WORDS: *Lymphokines; phytohemagglutinin; mitogenetic factor of human lymphocytes.*

A study of the physicochemical properties of lymphokines, mediators of cellular immunity [6], and their isolation in the pure form have made it possible to compare the properties of various factors of this group and to study the biological action of the purified lymphokines.

In this investigation the physicochemical properties of a mitogenetic factor (MF) of human lymphocytes, liberated *in vitro* under the influence of phytohemagglutinin (PHA) [2], were studied.

EXPERIMENTAL METHOD

The model of induction of MF by PHA with inactivation of the latter by antisera against PHA (APS) was described by the writers previously [2, 3]. Lymphocytes isolated from donors' blood and purified from granulocytes on columns with polyacrylonitrile fiber, were incubated for 40 min with PHA-P (Difco), washed twice, and cultivated for 3 days in serum-free TC-199 medium (E-base, IBL), enriched with L-glutamine and antibiotics. The culture medium was used as the source of MF after removal of the cells. The remains of PHA present in the culture medium [2] were removed by incubation of the medium with immunosorbent against PHA based on γ globulins from APS, diazo coupled to an agar derivative [5] or neutralized by the addition of APS [2].

Treatment of MF with Proteases. 1) The culture medium containing MF was incubated at 37°C for 20 min with trypsin (Spofa, 12.5 µg/ml), after which antitrypsin (Reanal) was added in a dose of 15 µg/ml. In the control, trypsin and inhibitor were added simultaneously to the culture medium and incubated under the same conditions. 2) The culture medium was incubated at 37°C for 30 min with 5 or 2.5 mg/ml immobilized pronase. The pronase (Calbiochem) was diazo coupled to agar ester [5] and washed until proteolytic activity in the washings disappeared. The proteolytic activity of the enzymes was determined by a spectrophotometric method as casein hydrolysis products [1].

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TABLE 1. Effect of Treatment with Proteases on Activity of MF (thymidine-^{3}H, in counts/min/10^6 cells of test cultures; M±m; n=4 everywhere)

Enzyme	Culture medium containing MF	TC-199
	28 349±3 220	4 336±206
Trypsin (20 min) then inhibitor	7 153±2 068	3 517±287
Trypsin and inhibitor simultaneously	20 403±2 340	—
Immobilized pronase:		
5 mg/ml	8 423±991	2 800±1 150
2,5 mg/ml	11 043±2 401	—

Concentration of the Culture Medium. The culture medium was concentrated from 50-100 ml to 1-2 ml by ultrafiltration on PSAC filters (Millipore Co.) under nitrogen under pressure.

Gel-penetrating Chromatography (GPC). The concentrate of culture medium was fractionated at 5°C on calibrated columns (2 × 60 cm) with Sephadex G-75, G-100, and G-200 gels and with Biogels P-60 and P-150 (Bio-Rad). GPC was carried out by the method developed by the writers previously, by means of which chromatographic fractions can be obtained in nutrient medium, and consisting essentially of the use of bicarbonate buffer solution, corresponding in concentration to the $NaHCO_3$ content in complete nutrient medium TC-199 based on Earle's solution (0.275% allowing for a correction for subsequent dilution), as the eluent and restoring the isotonicity of the fractions with the appropriate amount of a fivefold concentrate of medium TC-199 without bicarbonate.

Disk Electrophoresis in Polyacrylamide Gel. The apparatus designed by B. A. Ustinnikov and G. A. Ermolin, by means of which preparative and analytical electrophoresis can be carried out in the same plate of gel, was used. A 5% separating gel in Tris–HCl buffer, pH 8.9, a concentrating gel of 2.5% acrylamide in Tris–HCl buffer, pH 6.7, and an electrode buffer of Tris–glycine, pH 8.3, were used. The separating gel was polymerized using, as catalyst, ammonium persulfate which was removed electrophoretically (before photopolymerization of the concentrating gel). At the end of electrophoresis, carried out at 5°C, the gel was cut into 15 or 16 fractions which were homogenized, the protein extracted, and the extract dialyzed against TC-199.

Isoelectric Focusing (IEF). IEF was carried out in a 1% solution of ampholines (LKB) with Biogel P-60 as the anticonvection medium [11]. The electrode buffers were: anode 0.2 M H_2SO_4, cathode 0.4 M ethylene diamine. IEF was continued for 7-14 h at 5°C, after which the gel was divided into 16-18 fractions, their pH was measured, and proteins were extracted from them. The extract was dialyzed for 48 h against TC-199 (five changes of medium).

The test culture media and fractions were sterilized by filtration through 0.3 μ millipore filters (Millipore Co.).

Mitogenetic activity in the culture media and fractions were investigated in test cultures of human lymphocytes (four cultures in one determination), isolated by the method described above [2, 4], or in a "Ficoll-Triosil" density gradient [4, 7]. The cells were cultivated directly in the nutrient medium fractions obtained after the addition of 10% heated human serum, glutamine, and antibiotics. On the seventh day, DNA synthesis was investigated with the aid of thymidine-^{3}H (2 μCi/ml, 17 Ci/mmole, 4-h label), the labeled cells were treated by a modified method of Robbins [12], and radioactivity was measured on a β spectrometer (Nuclear Chigago).

EXPERIMENTAL RESULTS

As Table 1 shows, treatment of the culture medium from PHA lymphocytes with trypsin and with immobilized pronase sharply reduced its mitogenetic activity, which indicates that the MF is proteinaceous in nature.

During fractionation of the culture medium containing MF by the GPC method it was found that the elution profile of MF virtually coincided with that of chymotrypsinogen A, with a

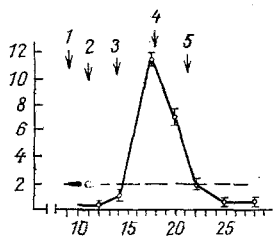

Fig. 1

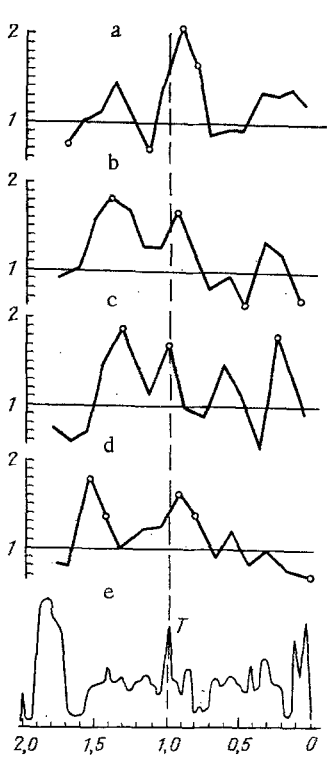

Fig. 2

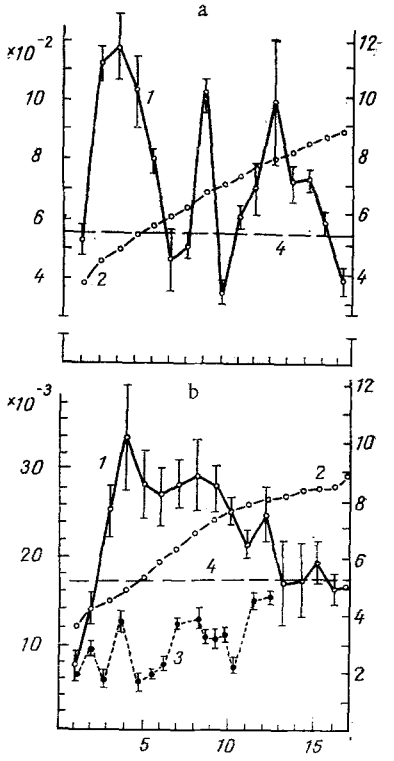

Fig. 3

Fig. 1. Elution profile of MF during GPC on Biogel P-150. Arrows indicate regions of elution of molecular weight standards: 1) blue dextran (Ferak), mol. wt. $2 \cdot 10^6$; human serum albumin (Reanal), mol. wt. $69 \cdot 10^3$; 3) ovalbumin, mol. wt. $45 \cdot 10^3$; 4) chymotrypsinogen A (Serva), mol. wt. $25 \cdot 10^3$; 5) cytochrome c (Serva), mol. wt. $12 \cdot 10^3$. Abscissa, No. of fractions; ordinate, mitogenetic activity (in counts/min$\cdot 10^{-4}$).

Fig. 2. Investigation of MF by disk electrophoresis: a, b) chromatographic purification of MF; c, d) whole culture medium containing MF; e) densitogram of normal serum. Points signify statistically significant differences from control. Abscissa, electrophoretic mobility relative to human serum transferrin (T), calculated in each experiment; ordinate, ratio between values of DNA synthesis under the influence of PAG fraction and DNA synthesis in control cultures.

Fig. 3. Distribution of mitogenetic activity in pH gradient during isoelectric focusing. Abscissa, No. of fraction; ordinate: left, mitogenetic activity (in counts/min/10^6 cells); right, pH. 1) DNA synthesis under the influence of MF fractions; 2) pH gradient; 3) DNA synthesis under the influence of ampholine fractions from control pH gradient; 4) DNA synthesis in control culture: a) whole medium with MF, b) chromatographically purified MF.

molecular weight of 25,000 daltons (Fig. 1). Consistent results were obtained in eight experiments during GPC on Sephadex and Biogel columns (not shown in Fig. 1), so that error in the determination of the volume yield of MF through its interaction with the matrix of the gel could be ruled out, for biogels are chemically inert "molecular sieves." In the GPC experiments in all cases it was found that fractions corresponding to a molecular weight of 50,000-100,000 daltons had a marked inhibitory effect on "background" DNA synthesis in test cultures of lymphocytes ($P<0.02$).

Investigation of the properties of MF by disk electrophoresis in 5% polyacrylamide gel showed that the factor could be separated into several discrete fractions (Fig. 2), migrating toward the anode with the speed of α_1 and α_2 globulins and transferrin. Chromatographically purified MF (peak of mitogenetic activity, see Fig. 1) formed the same fractions on electrophoresis as regards the size of their molecules, but they differed in the size of their elec-

tric charges. Despite some variability in the results of disk electrophoresis, the mobility of the individual peaks was quite constant (Fig. 2), evidently reflecting the true heterogeneity of the MF.

During isoelectric focusing 3 peaks of mitogenetic activity were found in different pH zones (Fig. 3) both for chromatographically purified and for "unpurified" MF. Similar results were obtained in four experiments. The less clearly defined distribution of chromatographically purified MF in the pH gradient was evidently caused by the too short "focusing" time (7 h compared with 14 h for the "unpurified"). Investigation of the IEF fractions in the cultures was made more difficult by the fact that the pH gradient consisted of polyaminopolycarboxylic amino acids with different structures. It was noted (Fig. 3b) that the ampholine fractions from the control pH gradient, even after dialysis for 48 h, inhibited DNA synthesis by the lymphocytes irregularly (without any significant decrease in viability of the cells; $P > 0.5$).

It can be concluded from the results of these experiments that MF of human lymphocytes, induced by PHA, is a protein (at least its activity is connected with its protein structure) with a molecular weight of 25,000. Horvat et al. [8] showed previously by the GPC method that MF of human lymphocytes induced by concanavalin A has a molecular weight of about 100,000 daltons [8]. It is difficult to interpret these findings because concanavalin A, which was used in the work cited for primary stimulation of the lymphocytes, has a molecular weight of about 80,000 daltons and there are reports that it can be washed out of Sephadexes [13]. The PHA used for primary stimulation of lymphocytes in the present experiments has a molecular weight of 100,000 daltons [11], and no mitogenetic effect was observed in this region in any of the experiments. Considering the data of Peter et al. [9] on the molecular weight of human lymphotoxin induced by PHA (80,000-100,000 daltons, results of GPC), it can be concluded that lymphotoxin and MF from human lymphocytes are different substances.

The results of investigations of the properties of MF by disk electrophoresis in polyacrylamide gel and by the IEF method correlate with each other and indicate that the MF of human lymphocytes is heterogeneous. Our preliminary observations show that individual MF fractions differ in their ability to exert their mitogenetic action in different subpopulations of lymphocytes. The final solution of this problem could be of great importance to the understanding of the mechanisms of intercellular interaction during the immune response.

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