Tumor Res. **23**, 83—102 (1988)

Suppressor T cell Inducing Factor from a Human Macrophage Like Cell Line-U 937

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SUMMARY

U 987, a human histiocytic lymphoma cell line, spontaneously produces a factor(M-SF) which inhibits blastogenic responses of lymphocytes and Interleukin 2(IL-2) activated killer(IAK) induction from human peripheral blood lymphocytes (PBL).

We investigated the mechanism of the suppressor action and the physicochemical character of the M-SF. Suppressive activity of U 937 culture supernatant was absorbed with human peripheral blood T lymphocytes. When the M-SF pretreated T cells were added, the mitogenic response of fresh allogeneic or autologus PBL to phytohaemagglutinin(PHA), Concanavalin A(Con A) and pokeweed mitogen(PWM) were suppressed. In addition, IL-2 activated killer activity was suppressed when the M-SF pretreated T cells were present in the induction phase of IAK. These suppressions were mediated by soluble factors produced by M-SF treated T lymphocytes. These results suggest that the pretreatment of T lymphocytes with M-SF resulted in the induction of suppressor T lymphocytes.

M-SF also inhibited the protein kinase activity associated with T cell membrane. The intensity of phosphorylated T cell membrane proteins with the molecular weights of 110, 94, 42, 38 and 34 killodaltons on SDS PAGE were decreased. Dephosphorylation of these proteins may be related to the functional alteration of T lymphocytes.

CPG-10 Gel permeation and hydrophobic interaction column chromatographic analysis revealed that the M-SF was an extremely hydrophobic sialoprotein of which approximate molecular weight was 10,000 daltons.

Key words: Suppressor T cell, U-937, Immunosuppressive factor, IL-2, LAK

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INTRODUCTION

Macrophages are thought to play an important role for the regulation of cellular immunity(1). This regulation is mediated through humoral factors produced by macrophages(29). One of the major stimulating factors is interleukin 1(IL 1) (25). On the other hand, there are several reports concerning immunosuppressive factors derived from macrophage such as Prostaglandin E_2(5, 28), α-interferon(16), oxygen derived radicals(21), arginase(19) or other unidentified factors(3, 14, 22). However, the mechanism for the immunosuppression caused by factors other than prostaglandin E_2(5, 28) or α-interferon(16) have not yet been well investigated, mainly due to the insufficiency of materials obtained from peripheral blood macrophages.

In this regard, the established human macrophage cell lines(4, 18) are considered to be a good source for such an immunosuppressive factor to obtain sufficient amounts to investigate the character and the mode of action. Especially U 937(27), a human histiocytic lymphoma cell line, has been known to secrete spontaneously an immunosuppressive substance which was firstly reported by Willkins *et al*(31) in 1983. We also independently found a spontaneous secretion of immunosuppressive factor(M-SF) from U 937 in 1983(17). Recently Fujiwara(9) reported the immunosuppressive substance derived from U 937. However, the molecular weight and the physicochemical characters of the immunosuppresant derived from U 937 hitherto reported were different from one another.

The present study was performed to further charachterize the physicochemical properties and the mode of actions. We demonstrated here the M-SF is an extremely hydrophobic sialo-protein of which approximate molecular weight is 10,000 datltons and the suppression is mediated through treated T cells. The inhibitory effect of M-SF on the protein kinase activity of treated T cell membranes was also investigated.

MATERIALS AND METHODS

Medium

Complete culture medium(CM) consisted of RPMI 1640(Difco) supplemented with 25 mM HEPES(SIGMA), 60 μg/m*l* of Penicillin, 60 μg/m*l* of Gentamycine, 2 mM of L-Glutamine and 10%(v/v) heat inactivated fetal calf serum(Flow, USA).

Lymphocyte purification

Human peripheral blood mononuclear cells(PBMC) were obtained from heparinized venous blood of healthy adult volunteers by a density gradient centrifugation on Ficoll-Isopaque(Ficoll, Phamacia, Sweden). PBMC were washed three times

in RPMI 1640 medium and resuspended in CM at a concentration of 1×10^6/m*l*. PBMC were then depleted of adherent monocytes by incubation with CM in FCS coated plastic plate(MSP-plate, Japan Immuno Research Lab. Co. Takasaki, Japan) for 60 min at 37℃ in a humidified atmosphere of 5% CO_2 in air. Nonadherent mononuclear cells(NAMNC) were then collected, washed extensively and resuspended in CM at a concentration of 1×10^6/m*l*.

NAMNC were then incubated on Nylon-wool column(1 cm×5 cm) (Fenwall Laboratories, USA) filled with CM for 30 min at 37℃. T lymphocyte enriched population was recovered from the column by a drop wise elution with RPMI 1640 medium and resuspended in CM at a concentration of 1×10^6/m*l*. The T lymphocyte enriched fraction contained 98% of T lymphocytes estimated by their reactivity with anti-T monoclonal antibody(OKT 3). The monocyte contamination of these population was less than 2% as estimated by esterase staining.

Preparation of U 937 culture supernatant

Human histiocytic lymphoma cell line-U 937 was kindly provided from Prof. Masaki Saito, Jichi Medical School, JAPAN. U 937 cells were cultured in CM at 1×10^6 cells/m*l* in tissue culture flasks(Falcon, USA) or in a suspension culture flask (Shibata HCUO CULSTER-1602-400, 4 *l* volume, Tokyo, JAPAN.) at 37℃ in a humidified atmosphere of 5% CO_2 in air for 3 days.

U 937 culture supernatant was recovered by centrifugation at 1,500 xg for 10 min and were stored at-20℃ for the following experiments.

Mitogen stimulated lymphocyte DNA synthesis

PBMC(1×10^6/m*l*) were dispersed in 0.1 m*l* aliquots into flatbottomed multiwell tissue culture plates(Falcon, USA) and were cultured with 10 μg/m*l* of PHA-P(SIGMA, USA), 5 μg/m*l* of Concanavalin A(SIGMA, USA) or 10 μg/m*l* of Pokeweed mitogen(SIGMA, USA) in the presence or absence of U 937-CM(10% v/v) for 48 hrs. Cultures were pulsed with 1.0 μCi/well of [^{3}H] thymidine (specific activity 2 Ci/mmo*l*, NEN Research Products, USA) for 6 hrs before the cells were collected onto glass filter paper(Labomash filter, Labo science, Tokyo, JAPAN) to determine [^{3}H] thymidine incorporation by a liquid scintilation counter(LKB, USA).

CPG 10 permeation column chromatography

Sixty m*l* of U 937 culture supernatant was concentrated to 30-fold by vaccume dialysis, and 2 m*l* of the concentrate was applied to a CPG-10 (pore size 120 Å, Electro-Nucleonics, USA) column(1×100 cm) in PBS containing 0.1% glucose to avoid nonspesific binding. Each fraction(2 m*l*) was tested at a 1/10 dilution for

the effects on the Con-A induced blastogenesis of PBMC.

Partial purification of M-SF

We started purification of M-SF from a faily large quantity(60 *l*) of the culture supernatant derived from 6×10^{10} cells in a suspension culture flask, because, in a preliminary experiment, the suppressive activity tends to diminish easily during purification process. The culture supernatant was centrifuged at 1500 xg for 10 min and then filtered through a 0.45 μm filter(Millipor Products division, USA).

The filtered culture supernatant was applied to the hollow fibre column(>MW 30,000 cut off, Amicon Corp.) to concentrate 15 x fold.

Ammonium sulfate was added to this fraction at a final concentration of 2M, and chromatographed on a phenyl sepharose(Pharmacia Fine Chemicals, USA) column(2×25 cm) equilbriated with 10 mM phosphate buffer containing 2M ammonium sulfate, pH 7.4. Then the column was eluted by 50 m*l* of 10 mM phosphate buffer without ammonium sulfate, followed by 50 m*l* of 100% ethyleneglycol. The eluate was adjusted to pH 5.0 with 1N HCI and kept at 4℃ for 20 hrs to precipitate out the M-SF. After centrifugation at 3,000 xg for 60 min at 4℃, the Precipitate was dissolved in PBS, pH 10.0 to give a concentration of 24.9 mg/m*l*.

Inhibitory activity of M-SF on blastogenic response of PBMC

Human peripheral blood mononuclear cells(PBMC) were suspended in CM at a concentration of 1×10^6/m*l*.

PBMC(1×10^5/well) were cultured for 48 hrs with or without 10%(v/v) of partially purified M-SF in the presence of 10 μg/m*l* of PHA-P. Blastogenic response of PBMC was determined by the method described above.

Inhibitory activity was expressed as a unit which represents a dilution of sample giving 50% inhibition of the [^{3}H] thymidine incorporation of PBMC stimulated with PHA.

Absorption of immunosuppressive factor by PBMC or T lymphocyte

One m*l* of U 937-CM was cultured with 9 m*l* of PBMC, T lymphocyte enriched fraction or B lymphocyte enriched fraction(1×10^6/m*l*) in CM in tissue culture flasks(Falcon, USA) for 90 min at 37℃ in a humidified atmosphere of 5% CO_2 in air. Culture supernatants were recovered by centrifugation and their inhibitory effect on PHA stimulated blastogenesis of PBMC were examined. The 100% inhibition was defined as the effect of 10 times diluted U 937 culture supernatant with RPMI 1640 medium on PHA stimulated blastogenesis of PBMC.

Preparation of membrane proteins of T lymphocytes

One m*l* of 0.05 M borate buffer containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ were added to 2×10^5 of T lymphocytes. T lymphocytes were homogenized in 20 m*l* of 0.02 M borate, 0.2 mM EDTA, pH 10.2(extraction solution) with Dounce homogenizer(WHEATON, USA). Then the suspension was mixed with 1.5 m*l* of 0.5 M borate solution, pH 10.2 and filtered through two layers of nylon gauze(average mesh size, 900 μm). After centrifugation at 12,000 xg for 30 min at 2℃, membrane fraction was separated by 35%(w/w) sucrose density gradient centrifugation at 24,000 xg for 1 hr at 2℃.

Membrane fraction was resuspended in PBS and ultracentrifuged at 100,000 xg for 10 min at 2℃. The supernatant was kept at −70℃ for the following experiments.

Analysis of phosphorylated membrane proteins from T lymphocytes on SDS PAGE

Membrane proteins of T lymphocytes were solubilized with 10% TritonX100 on ice for 15 min. Then 17 μl of partially purified M-SF obtained by phenyl Sepharose column chromatography(Suppressive activity, 8 units/m*l*) or equal volume of RPMI 1640 medium, 0.4 $\mu g/4\lambda$ of PHA-P, 50μCi/5 μl of [γ-^{32}P]ATP (specific activity 50 Ci/mmo*l*, NEN Research Products, USA) and 4 μl of 10 times concentrated buffer (20 mM Hepes, 0.06 M NaCl, 0.1% BSA, and 3 mM $MnCl_2$, pH 7.4) were added to 10 μl of solubilized T cell membrane proteins and kept on ice for 10 min. The phosphorylated membrane proteins were separated by 10% SDS/polyacrylamide gel electrophoresis(PAGE) and subjected to autoradiography.

Treatment of T lymphocytes with M-SF

T lymphocytes were cultured in CM for 90 min at 37℃ with or without 10% (v/v) of partially purified M-SF from phenyl sepharose column. T lymphocytes were then washed for 3 times with 50 m*l* of PRMI medium for each washing. Washed T lymphocytes were suspended in CM at a concentration of 1×10^6/m*l* and recultured at 37℃ in 5% CO_2 in the atmosphere for 0, 12, 24, 48, 72 and 96 hrs. The T lymphocytes were then collected and their inhibitory effect on PHA stimulated blastogenesis, IL 2 production of PBMC and LAK cell induction from PBMC were examined.

Culture supernatant of these T lymphocytes were collected at the same time and their effects on LAK cell induction from PBMC were also examined.

PHA induced IL 2 production of PBMC

PBMC were cultured at the concentration of 1×10^6 cells/m*l*(1 m*l*/well) in 24

well tissue culture plate(Falcon, USA) with M-SF treated or nontreated T lymphocytes ranging from 0.4×10^4 to 3.2×10^4/m*l* in the presence or absence of PHA-P(10μg/m*l*) for 72 hrs. Culture supernatant were then recovered by centrifugation and were stored at −20℃ for the assay of IL 2 activity.

Assay for IL 2 activity

IL 2 activity was determined by their ability to support the growth of the murine IL 2 dependent cell line(CTLL-2) as previously described(11). Briefly, CTLL-2 cells were cultured at 10^5 cells/well with different concentrations of the test supernatant in total volume of 0.2 m*l* and incubated at 37℃ in 5% CO_2 for 24 hrs. During the final 6 hrs of culture, cells were pulsed with [^{3}H]-thymidine(1 μ Ci/well). Then cells were collected on glass filter paper and their radioactivities were determined by a liquid scintilation counter.

Induction of Lymphokine activated killer cell(LAK)

PBMC were suspended in CM at a concentration of 1×10^6 cells/m*l* in 10×10 mm round bottomed culture flask(Falcon, USA) with rIL 2(TGP 3, specific activity 52.45×10^6 Jurkat Units(J. U.)/mg protein, TAKEDA, JAPAN) at a concentration of 2 units/m*l* in the presence of 1×10^5 cells/m*l* of M-SF treated or nontreated T lymphocytes, or 10%(v/v) of culture supernatant of M-SF treated T lymphocytes or nontreated T lymphocytes for 5 days at 37℃ in a humidified atmosphere of 5% CO_2 in air. During the culture period, CM and rIL 2 were added into the flask every other day. The cultured cells were resuspended at a concentration of 1×10^6/m*l* in CM before analysis of their cytolytic activity.

Assay of LAK activity

Daudi cells, NK resistant Burkitt's lymphoma cell line, were used for target cells. LAK activities were determined by the standard 4 hr ^{51}Cr-release assay as previously described(15). The cytotoxicity assay was performed in triplicate in 96 well round bottomed microtiter plates by adding effector cells producing effector : target cell(E : T) ratio of 40 : 1 or 20 : 1 in a final volume of 0.2 m*l* of CM. Mean percent lysis in triplicate culture was calculated as follows

$$\%\ \text{lysis}=\frac{\text{Experimental }^{51}\text{Cr release}-\text{Spontaneous }^{51}\text{Cr release}}{\text{Maximum }^{51}\text{Cr release}-\text{Spontaneous }^{51}\text{Cr release}}\times100$$

Maximum release was determined by lysis of ^{51}Cr labelled target cells in 1% TritonX100.

Spontaneous release from target cells was evaluated by incubating these cells in culture medium.

Analysis of the effect of soluble factor derived from M-SF treated T lymphocytes on LAK cell induction from PBMC.

Each well of 24 well culture plate(Falcon, USA) was divided into 2 compartments with 0.45 μm filter using a Millicell H A(Millipore Products division, USA). PBMC(1×10^6/ml) were cultured with 2 units/ml of human rIL 2 in an inner compartment, and in an outer compartment 1×10^6 cells of M-SF treated T cells (1×10^6/ml) or control nontreated T cells(1×10^6/ml) were cultured in a humidified atmosphere of 8% CO_2 in air at 37℃. After 5 days, cells in an inner compartment were collected and LAK activity was examined as described above.

RESULTS

Effect of U 937 conditioned medium on mitogenic response of human PBMC

U 937 culture supernatant significantly inhibited [^{3}H]-thymidine incorporation of PBMC stimulated with PHA, Con A or PWM at a concentration of 10%(v/v) (Table 1). To analyse the target cells for this immunosuppressive factor(M-SF), 10%(v/v) of U 937-CM was incubated for 90 min with 1×10^6 of PBMC, T lymphocytes or B lymphocytes, then the cells onwhich the putative M-SF was absorbed were removed by centrifugation. The remaining M-SF activity of the supernatant was determined by assessing the ability to suppress [^{3}H] thymidine incorporation of PBMC stimulated with PHA. As shown in Fig. 1, the remaining M-SF activity after absorption with PBMC, T cells and B cells was 31.9%, 21.5% and 90.8%, respectively. It is noteworthy that almost 78.5% of this suppressive activity was absorbed with T lymphocytes, while only 9.2% was absorbed with B lymphocytes enriched fraction.

Table 1 *Effect of U 937 conditioned medium on PBMC responses to polyclonal T cell activators.*

	U 937 conditioned medium	
	(−)	10% (v/v)
PHA	45,050.3±7,135.5	1,608.7±257.8
Con A	13,471.0±2,359.7	1,138.7± 83.3
PWM	17,312.0±4,511.0	1,606.7±422.7
Mitogen (−)	621.0± 36.0	592.0± 42.0

Results were expressed as cpm (mean of three cultures±S. D.)

Effect of U 937 conditioned medium(M-SF) on PBMC responses to polyclonal T cell activators. 1×10^5 cells of PBMC were cultured with the indicated mitogen alone, or with 10%(v/v) M-SF for 48 hr and labeled for the final 6 hr of culture with 1 μCi of [^{3}H] thymidine.

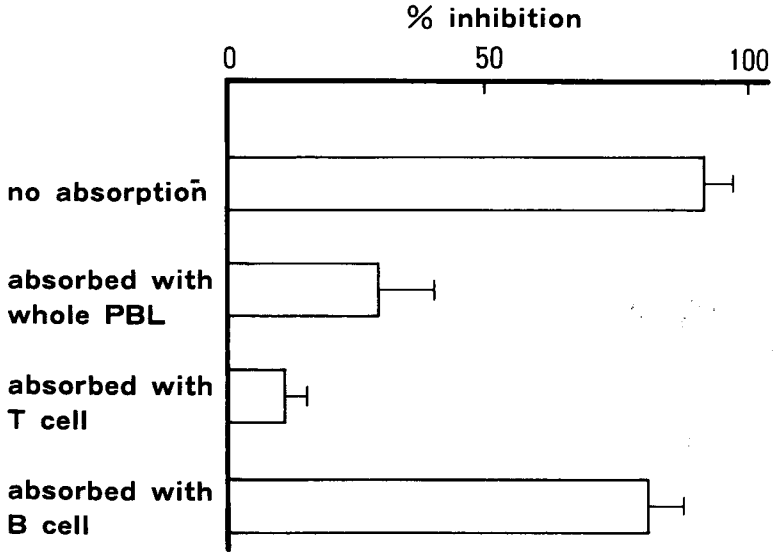

Fig. 1 Absorption of immunosuppressive activity with PBMC or T lymphocytes. Inhibitory effects of U 937-CM(1 m*l*) after incubation with 9×10^6 cells of PBMC, T lymphocytes or B lymphocytes on PHA induced blastogenesis of PBMC(1×10^5 cells) were examined. Horizontal column represents % inhibition calculated as follows.

$$\%\ \text{inhibition}=\frac{\%\ \text{suppression}^a\ \text{of}\ [^3\text{H}]\text{thymidine incorporation of PHA stimulated PBMC by tested samples}}{\%\ \text{suppression of}\ [^3\text{H}]\text{thymidine incorporation of PHA stimulated PBMC by 1/10 diluted U 937-CM}}\times100$$

[a]% suppression was calculated as follows

$$\%\ \text{suppression}=\frac{[^3\text{H}]\text{thymidine incorporation of PHA stimulated PBMC in the presense of 10\%(v/v) tested samples}}{[^3\text{H}]\text{thymidine incorporation of PHA stimulated PBMC}}\times100$$

Physicochemical characterization

Table 2 shows the susceptibility of M-SF to low pH, trypsin, neuraminidase, and freezing and thawing by measuring the suppression of [^{3}H] thymidine incorporation of PBMC stimulated with PHA. Exposure of M-SF at pH 2.0 for 1 hr resulted in inactivation of the suppressive activity. Heating at 56℃ for 30 min, treatment with trypsin or neuraminidase completely eliminated the activity of M-SF. On the other hand, suppressive activity of M-SF was almost fully maintained (83.9%) after freezing and thawing for 5 times. Fig. 2 shows the gel permeation profile at 280 nm by CPG 10 column chromatography and the inhibitory activity of each fraction on Con A induced blastogenesis of PBMC. The main inhibitory activity for blastogenesis was eluted at the inverted peaks with a Mr. of 67,000 and 10,000.

Table 2 *Physicochemical characters of immunosuppressive factor in U-937 conditioned medium.*

		cpm	% suppression
(1)	heat treatment.		
	control	31,690.0±2,692.7	
	no treated	1,421.6± 127.4	
	heat treated (56℃, 30 min)	31,746.7±5,721.5	(0.0%)
(2)	pH titration (for 60 minutes)		
	control	24,681.7±3,494.0	
	no treated	1,852.7± 187.8	(92.5%)
	pH 6	2,093.3± 456.1	(91.5%)
	pH 5	2,032.3± 233.1	(91.8%)
	pH 4	4,864.3±1,282.7	(80.3%)
	pH 3	6,659.0± 386.0	(73.0%)
	pH 2	8,381.3±1,048.4	(66.0%)
	pH 1	10,606.7±2,225.7	(57.0%)
(3)	freeze and thawing		
	control	67,112.3±1,383.3	
	no treated	960.0± 110.9	(98.6%)
	1 x	954.7± 375.1	(98.6%)
	2 x	984.7± 101.8	(98.5%)
	3 x	1,279.7± 11.2	(98.1%)
	4 x	3,464.7±1,471.3	(94.8%)
	5 x	10,833.0±2,856.9	(83.9%)
(4)	trypsin or neuraminidase treatment		
	control	112,682.0±4,440.1	
	non-treated	7,114.3±1,309.9	(93.7%)
	trypsin treated	101,633.3±6,100.3	(9.8%)
	neuraminidase treated	93,119.7±1,223.8	(17.4%)

Data were expressed as [^{3}H] thymidine incorporation (cpm±S. D.)

Effect of U 937-CM treated with heat, acid, freeze and thawing, trypsin or neuraminidase on PHA induced blastogenesis in PBMC. U 937CM were incubated at 56℃ for 30 min(1); U 937-CM were titrated with 10 N HCl to pH 6 to 1 for 60 min. Then the pH were titrated with 10 N NaOH to pH 7.4(2); U 937-CM were frozen at −90℃ in liquid nitrogen for 1 hr and then thawed in water. The same procedure was undertaken up to five times(3); 20 U of insoluble trypsin (Sigma) or 50 U of insoluble neuraminidase (Sigma) was incubated with 1 m*l* of U 937-CM at 37℃ for 4 hr(4). PHA induced blastogenic response of PBMC was assessed as described in materials and methods. The results are the mean±S. D. of triplicate wells of a representative experiment.

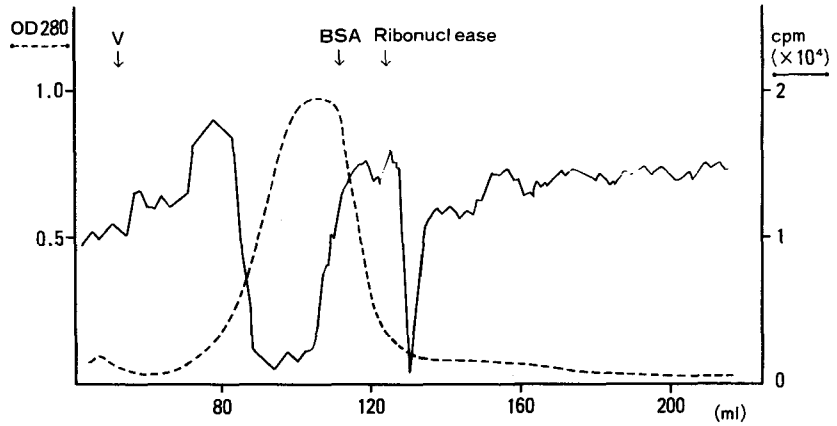

Fig. 2 Molecular sieve chromatography of M-SF
Two milliliters of 30-fold concentrated U 937-CM were subjected to molecular sieve chromatography. M-SF was fractionated on a CPG 10 column prepared in PBS containing 0.1% glucose(pH 7.4). Each fraction(2 m*l*) was tested at a 1/10 dilution for M-SF activity. Solid bar(———) indicates [^{3}H]thymidine incorporation of 1×10^5 cells of PBMC stimulated with 5 μg/m*l* of Concanavalin-A(Con A) in the presense of each fraction. Broken bar(- - - - - -) indicates a protein concentration as indicated by absorbance at 280 nm. The column was calibrated with Blue dextran, bovine serum albumin (BSA) and ribonuclease. V indicates the void volume.

Table 3 *Partial purification of the immunosuppressive factor from U 937 conditioned medium.*

	volume (m*l*)	protein (mg)	units/m*l*	total units	units/mg protein	fold	yield (%)
Concentrated CM(x 15)	400	7,480	10	4,000	0.53		
eluate from phenyl Sepharose column	55	340	8	440	1.29	2.43	11.0
precipitate at pH 5.0	6.7	166.8	64	428.8	2.49	4.70	10.7

Suppressive activity was expressed as a unit.
1 unit=volume of U 937 conditioned medium which suppress PHA induced blastogenesis of PBMC by 50%.

Partial purification of M-SF

The results of a partial purification of M-SF are summarized in Table 3. Sixty *l* of culture supernatant was collected from 6×10^{10} of U 937 cells. The medium was concentrated 15 times by hollow fibre column and subjected to Phenyl Sepharose column chromatography. Eleven % of initial activity was recovered from the column by 100% ethyleneglycol and the activity concentrated 36.45%. Then, the M-SF was precipitated at pH 5.0 and 10.7% of the M-SF activity was recovered.

The M-SF activity was concentrated to have an activity of 70.5 times of the starting material. The total yield of the activity was 1.14%.

Effect of M-SF treated T lymphocytes on blastogenesis and IL 2 production by PBMC stimulated with PHA

In order to study the immunosuppressive activity of T lymphocytes treated with M-SF, a different number of T lymphocytes treated with 10%(v/v) of M-SF for 90 min were incubated with 1×10^5 cells of PBMC and were stimulated with PHA.

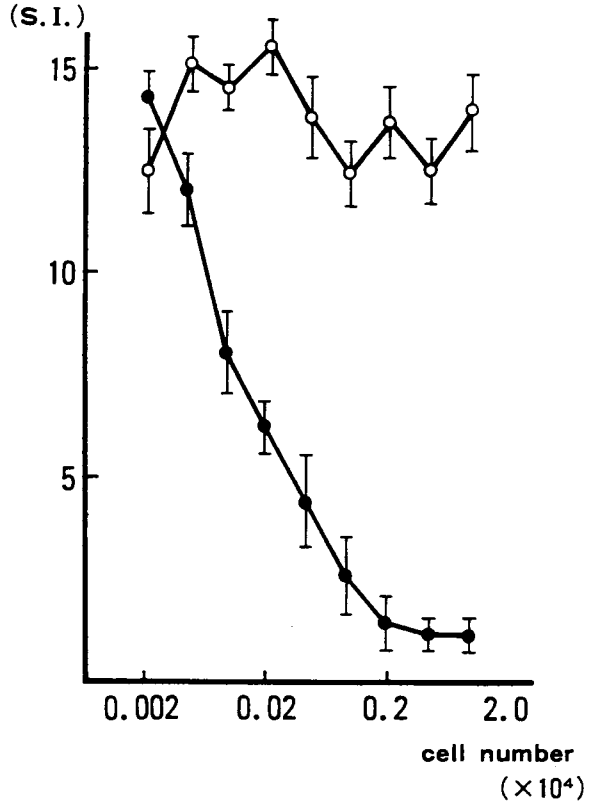

Fig. 3 Effect of the M-SF treated T lymphocytes on PHA induced blastogenesis of PBMC. T lymphocytes were treated with 10%(v/v) of U 937-CM(M-SF) for 90 min. These M-SF treated T lymphocytes(●——●) or nontreated T lymphocytes(○——○) at a number from 2.0×10 to 2.0×10^4 were incubated with 1×10^5 cells of PBMC for 48 hr with a [^{3}H]thymidine pulse added during the last 6 hr. Vertical column represents stimulation index(S. I.) calculated as follows

$$\text{S. I.} = \frac{\text{[}^3\text{H]thymidine incorporation of PBMC cultured with M-SF treated T lymphocytes or nontreated T lymphocytes stimulated with PHA}}{\text{[}^3\text{H]thymidine incorporation of PBMC cultured with M-SF treated T lymphocytes or nontreated T lymphocytes}}$$

The inhibition of [^{3}H] tymidine incorporation was dose dependent manner inhibition by added T cells treated with M-SF was observed from 2.0×10 to 2.0×10^4 cells shown in Fig. 3. The maximum suppression was obtained at the concentration of 1.4×10^3 cells/m*l*. Fifty % suppression of mitogenic response was observed at such low concentration as 1.4×10^2 cells/m*l*. In contrast, non-treated T lymphocyte did not inhibit [^{3}H] thymidine incorporation of PBMC at any concentration from 0.002 to 2.0×10^4 cells.

Effects of M-SF treated T cells on IL 2 production by PHA stimulated PBMC was also investigated. As shown in Fig. 4, M-SF treated T lymphocytes inhibited

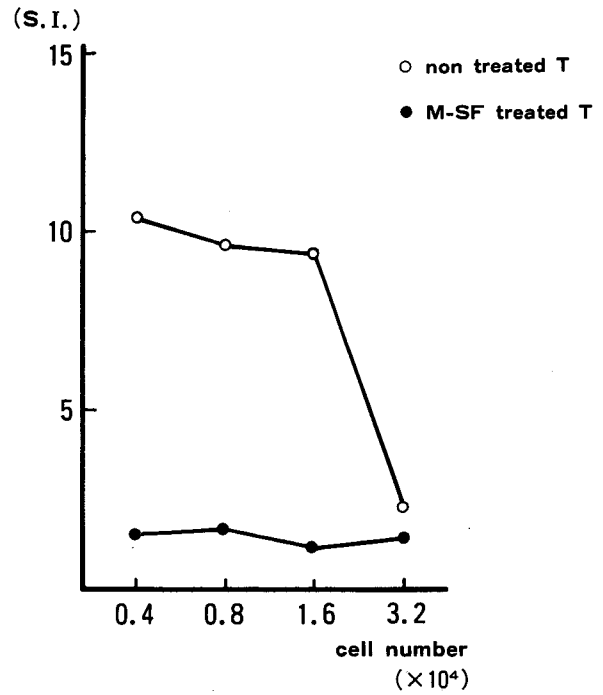

Fig. 4 Effect of M-SF treated T lymphocytes on IL 2 production of PBMC stimulated with PHA. T lymphocytes(1×10^6/m*l*) were incubated with 10%(v/v)of U 937-CM(M-SF) for 90 min. 1×10^6 cells of PBMC were cultured with M-SF treated or nontreated T lymphocytes at a number from 0.4×10^4 to 3.2×10^4 in the presense of PHA for 72 hr. 1×10^5 cells of CTLL-2 were cultured with 50%(v/v) of culture supernatant obtained from PBMC incubated with M-SF treated T lymphocytes(●——●) or nontreated T lymphocytes(○——○) for 24 hr with a [^{3}H]thymidine pulse added for final 6 hr. Vertical column represents stimulation index(S. I.) calculated as follows

$$\text{S. I.} = \frac{\text{[}^3\text{H]thymidine incorporation of CTLL-2 cultured with tested samples}}{\text{[}^3\text{H]thymidine incorporation of CTLL-2 cultured with RPMI 1640 medium}}$$

almost completely the IL 2 production by PHA-stimulated PBMC(1×10^6 cells). In contrast, non-treated T lymphocytes with concentration of 0.4×10^4, 0.8×10^4, 1.6×10^4 cells did not exert inhibitory activity at all, although those with higher concentration(3.2×10^4) exhibited some full suppression possibly due to the over

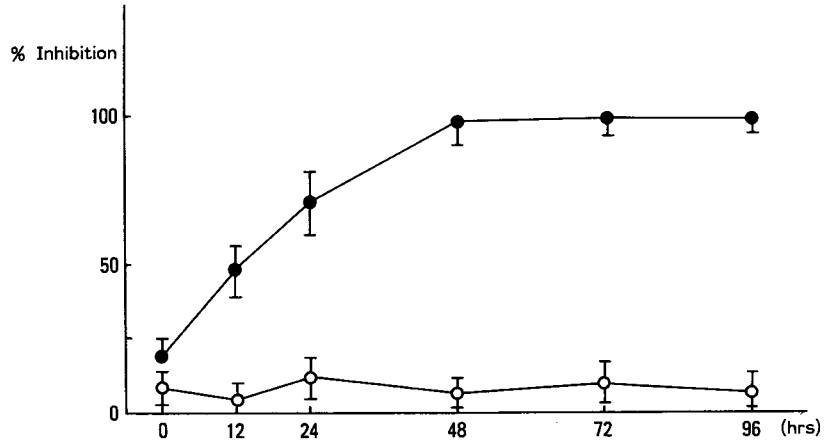

Fig. 5 Effect of M-SF treated T lymphocytes on LAK cell induction from PBMC.
T lymphocytes were incubated with or without 10%(v/v) of U 937-CM for 90 min. The cells were washed, recultured in CM and then collected 0, 12, 24, 48, 72, 96 hr after reculturing. 2×10^6 cells of PBMC were cultured with 2×10^5 of M-SF treated(●——●) or nontreated(○——○) T lymphocytes for 5 days in the presense of 2 units/m*l* of rIL 2. The whole cells were recovered and their cytolytic activity against Daudi cells were examined as described in materials and methods. Vertical column represents % inhibition calculated as follows.

$$\%\ \text{inhibition} = \frac{\%\ \text{lysis}^a\ \text{of LAK induced with M-SF treated or nontreated T lymphocytes}}{\%\ \text{lysis of LAK}} \times 100$$

[a]% lysis was calculated as described in materials and methods.

Table 4 *Effect of soluble factor derived from M-SF treated T lymphocytes on LAK cell induction from PBMC.*

inner chamber	outer chamber	LAK activity (% cytotoxicity)
PBL	T cell	42.9
PBL	M-SF treated T cell	0.3

A well of culture plate was devided into 2 chambers with 0.45 μm filter membrane. In an inner chamber, 1×10^6 cells of PBMC were cultured with 2 units of rIL2. In an outer chamber, 1×10^6 cells of M-SF treated or non-treated T lymphocytes were cultured. After 5 days, the cytolytic activity of the cells in an inner chamber against Daudi cells were examined. Data represents % cytotoxicity as described in materials and methods.

concentration effect.

Effect of M-SF treated T lymphocytes on LAK induction

The effect of T lymphocytes treated with 10%(v/v) of M-SF on LAK cell induction was investigated. As shown in Fig. 5, LAK activity was suppressed by 1×10^4 of M-SF treated T lymphocytes. The suppressive activity reached its maximum after 48 hrs of cultivation. In contrast, nontreated T lymphocytes did not inhibit LAK induction.

Effect of suppressive factor(T-SF) derived from M-SF treated T cells on LAK induction

To investigate the possibility that the inhibition of M-SF treated T lymphocytes was mediated by the soluble factor, a culture well divided into two compartments with filter membrane was devised. As shown in Table 4, LAK activity in the inner conpartment was suppressed from 42.9% to 0.3% when co-cultured with 1×10^6 of M-SF treated T lymphocytes in the outer compartment, suggesting that this suppression is mediated by soluble factors derived from M-SF treated T lymphocytes (T derived suppressor factor, T-SF).

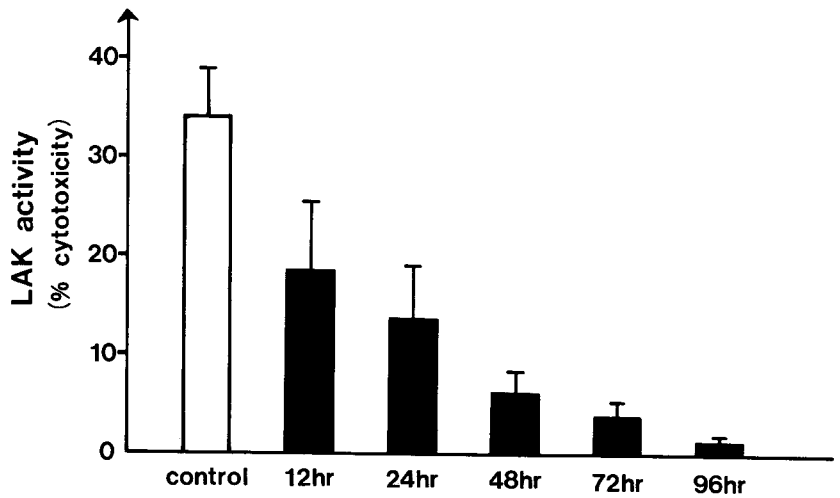

Fig. 6 Effect of culture supernatant of M-SF treated T lymphocytes on LAK cell induction from PBMC.
2×10^5 cells of LAK[a] were incubated with 1×10^4 cells of ^{51}Cr-labeled Daudi cells in the presense(■) or absense(□) of 10%(v/v) culture supernatant of M-SF treated T lymphocytes obtained at a different time after reculturing. LAK activity was expressed as % cytotoxicity[b] in the vertical column.
[a]LAK cells were induced as described in materials and methods.
[b]% cytotoxicity was calculated as described in materials and methods.

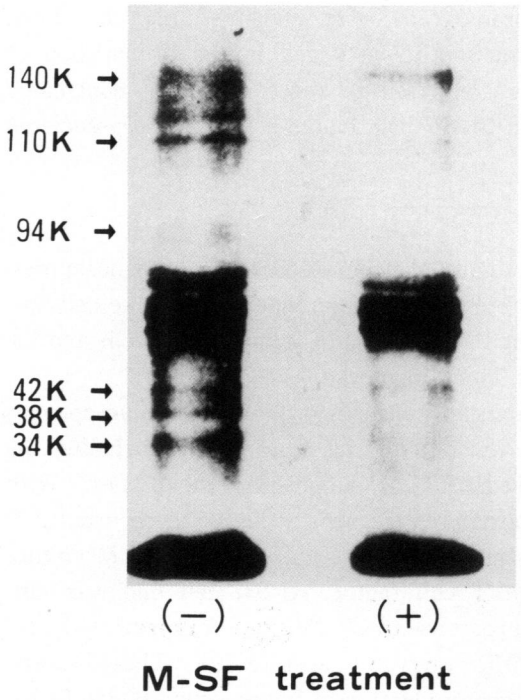

Fig. 7-A The autoradiogram of phosphorylated T cell membrane proteins. 40 μl of solublized T cell membrane proteins were incubated with 5 μl(50 μCi) of [γ-^{32}p] ATP and 2 μl of partially purified M-SF(8 units[a]/ml) (+) or PRMI 1640 medium(−) on ice for 10 min. The phosphorylated proteins were separated by 10% SDS/PAGE and subjected to autoradiography.

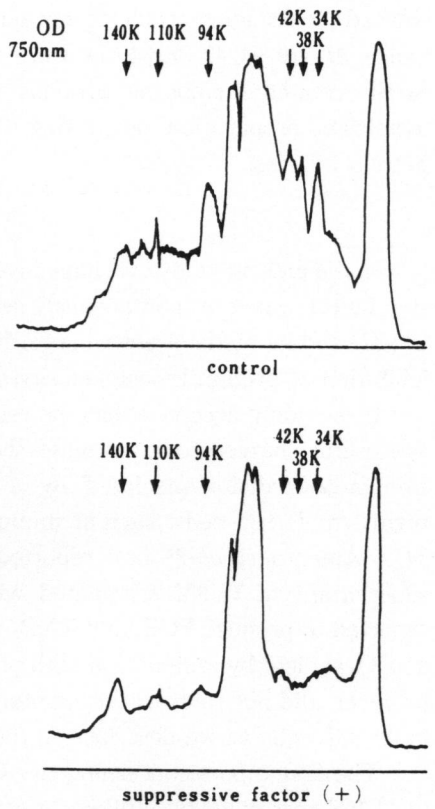

Fig. 7-B Scanning profile of the same autoradiogram by gel scanner (Bio-Rad) at 750 nm. Scanning speed is 2.5 cm/min. Vertical column represents the optical density expressed by an absorbance at 750 nm.

Inhibitory activity of conditioned medium from M-SF treated T cells on LAK induction was then explored(Fig. 6). The culture medium of T cells treated with M-SF for 12, 24, 48, 72, 96 hrs suppressed LAK activity to 41.3%, 55.4%, 73.1%, 82.9%, 94.1% indicating the T-SF activity in culture medium accumulated as the culture time was prolonged.

Effect of M-SF on phosphorylation of membranic protein of T lymphocytes

In view of interest to clarify the mechanisms of induction of suppressive T cells by M-SF, the effects of M-SF on the autophosphorylation of T cell membrane proteins were studied. Fig. 7-A showed the autoradiogram of membrane proteins of T cells which were treated with M-SF in the presence of γ-^{32}P ATP. Fig. 7-B

showed the scanning profile of this autoradiogram. The intensity of phosphorylated bands at 110, 94, 42, 38, 34 kD. were apparently weakened in the preparation of M-SF treated membrane proteins as compared to those in M-SF nontreated membrane preparation, suggesting that M-SF inactivated a membrane-associated protein kinases.

DISCUSSION

In the present study, we have investigated the characters of the immunosuppressive factor which is spontaneously secreted from human macrophage-like cell line, U 937, and its mode of action regarding the induction of suppressor T cells and the inhibition of protein kinase activity of T cell membrane.

It is widely accepted that the established cell lines originating in macrophages are useful sources to investigate the function of the macrophage, including the immunosuppression mediated by a secretion of suppressive monokines. With regard to U 937 cells, several immunosuppressive substances were reported(2, 3, 24). Amento *et al*(2) first reported the presence of anti IL 1 factor in culture supernatant of U 937 stimulated with T cell factor. U 937 cell line was also reported to produce PGE_2 and TXB_2 in response to C3 cleavage fragment, C_3B(24) and O_2 radical by stimulation with phorbor myrystate acetate(20). These reports, however, did not refer to the spontaneous production of immunosuppressive factor by U 937 cells as we described in this report.

The first report concerning the spontaneous production of immunosuppressant by U 937 was made by Willkins *et al*(31) in 1983. They described the spontaneous production of immunosuppressant by U 937 along with the other factor secreted by mitogen stimulation, although they have not extended their investigation to explore its physichochemical nature and mode of action. More recently, we(17) and Fujiwara et al.(9, 10) independently found the immunosuppressive factor spontaneously produced by U 937.

The physicochemical characterization by our experiments revealed that this factor is labile to acidification(pH 2.0 for 1 hr), heating(56℃ for 30 min), trypsin and neuraminidase treatment, and resistant to freezing and thawing, which are similar to Fujiwara's observation(10).

On the other hand, there are several differences regarding the approximate molecular weight and the activity of immunosuppression in these two reports. A peak with major inhibitory activity reported by Fujiwara was eluted at fractions between 67,000 and 13,000 by AcA 54 gel chromatography. On the way to partial purification of this suppressive factor, we found that this factor adheres to the gel matrix such as Sephadex gel and to dialyzing membrane made of Celophane, especially one starting from a small volume(unpublished obserevation). In order to

avoid the nonspecific adhesion to gel matrix, we used glass bead chromatography (CPG 10) and eluted with buffers with high ionic strength containing high concentrations of glucose. The molecular weight of immunosuppressive fraction which we obtained by this procedure was eluted at two main fractions located at Mr. of 10,000 and 64,000. It can be estimated that a Mr. of the M-SF is around 10,000 and the activity around 64,000 is its aggregate, although the data did not exclude the possibility that more than one factor contributes to the immunosuppressive activity of U 937 supernatant. Another different point is the strength of suppressive activity in the supernatant. The M-SF activity was observed even at 1/100 dilution (Table 1), while that obtained by Fujiwara was not seen at 1/16 dilution. This difference may be explained by the different quantity of secreted suppressive factors or by the heterogeneity of U 937 cells as described by Gitter *et al*(12).

It is also well documented that macrophages produce prostaglandins(PG) (5, 28) which suppress the immunoreactivity of PBMC. The concentrations of PG in these conditioned medium were in the range suppressed only by 50% the proliferation of splenic lymphocytes stimulated with Con A.

Furthermore, even after stimulation by phorbor myrystate acetate, U 937 produces only 6.35×10^{-9}M of PGE_2(24) which is still inadequate to suppress the mitogenic response of PBMC. The supernatant of our U 937 did not produce a detectable amount of PGE_2 by the radioimmunoassay procedure and the addition of indomethacin did not affect the production of M-SF by U 937 (unpublished observation). These results cleary suggests that M-SF is different from the prostaglandins.

Moreover the extremely strong activity, *i. e.* the addition of 10%(v/v) of culture supernatant obtained from 1×10^6 cell/m*l* suppress almost 95% of the mitogenic response of PBMC(Fig. 1), was incomparable with any other suppressive factors hitherto reported(2, 3, 5, 6, 14, 16, 19, 24, 26, 28).

The M-SF was quite adhesive to gel matrix and dialyzing membrane, so that the activity would be completely lost during the purification process unless the quantity of the starting materials were sufficiently large. For that reason, we started the purification from 60 *l* of culture supernatant of U 937 cells. The M-SF could be eluted out from phenyl Sepharose column not by lowering the ionic strength but by lowering the polarity of the eluent with ethyleneglycol, suggesting that the M-SF is strongly hydrophobic. The isoelectric point of the M-SF was estimated about 5.0 because the isoelectric point precipitation of this particular protein occured at pH 5.0(Table 3).

Several characters of the M-SF described above also indicated that it is a novel immunosuppressive substance derived from U 937 and is distinct from other numerous factors such as thymidine(26), prostaglandins(5, 28), α-interferon(16), arginase(19), oxygen derived radicals(21) and immunoregulatory α-globulin(19).

Until now, there are no reports concerning the mode of immunosuppression by the factor produced by U 937. The present result in which the M-SF activity was absorbed with peripheral blood T lymphocytes but not with B lymphocytes(Fig. 1) is suggestive of T lymphocytes as the target cells for M-SF. Indeed, the treatment with M-SF rendered T lymphocytes to suppress the mitogenic response of PBMC or IL 2 production of T lymphocytes induced with PHA in a dose dependent manner (Fig. 3, 4). In this regard, the M-SF we reported here seems to have the same mechanism for the immunosuppression with that of prostaglandine(8, 30). However, immunosuppressive activity of PGE_2 was definitely not of the magnitude observed with the M-SF as described above.

M-SF treated T lymphocytes also suppress the Lymphokine activated killer (LAK) induction from PBMC(Fig. 5). This indicated that the suppression does not simply reflect the decrease of IL 2 production by T lymphocytes in PBMC since the induction was inhibited in the presence of sufficient exogenous IL 2 in the medium. Furthermore the fact that M-SF treated T lymphocytes did not interfere with the LAK activity itself when they were added in an effector phase(data not shown) disclosed that the suppressive mechanism involved in the induction phase of LAK from its precurser cells.

This immunosuppression was mediated by the soluble factor from the M-SF treated T lymphocytes because the M-SF treated T lymphocytes separated from PBMC with 0.45 μm filter of Millicell could suppress LAK induction from PBMC (Table 4) and immunosuppressive factor(T-SF) was indeed identified in the medium of T lymphocytes treated with M-SF.

It is well documented that tyrosine phosphokinase or Ca^{2+} and phospholipid dependent protein kinase C activity has an important role for the activation of lymphocytes in response to several cytokines such as IL 2 or BSF-1.

In this study, we also found that the M-SF affected the membranic proteins of T lymphocytes. The M-SF dephosphorylated the several membranic proteines of T lymphocytes whose Mr. were 110 k. D., 94 k. D., 42 k. D., 38 k. D., 34 k. D.. Recently, protein kinase activity is thought to be responsible for activation or growth of lymphocytes in response to several soluble mediators including IL 2. Farrar *et al*(7) reported that phytohemagglutinin(PHA) and phorbol esters stimulated de novo acquisition of Tac antigen, which was associated with the subcellular redistribution of protein kinase C(PK-C) from cytosol to the particulate membrane of human T lymphocytes. They speculated that PK-C activation is necessary for the regulation of Tac antigen expression. On the other hand, Fujiwara observed decreased Tac antigen expression of T lymphocytes treated with U 937 conditioned medium(10). Taking these reports into consideration, our preliminary observation that the M-SF decreased protein kinase activity of T lymphocytes may explain the decrease of Tac

expression of T lymphocytes. However, at the present time we have no direct evidences to indicate the linkage between dephosphorylations of membrane proteins and the change of T cell function, aquisition of an immunosuppressive activity.

Further studies are presently being conducted in our laboratory to purify the M-SF from large amount of culture supernatat of U 937. These studies to examine the M-SF and the suppressor T cells induced by the M-SF may provide further understanding of the mechanism of immunosuppression by suppressor T cells induced by macrophage derived factor.

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