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Succinoyl trehalose lipid induced differentiation of human monocytoid leukemic cell line U937 into monocyte-macrophages

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Abstract

A novel type of succinoyl trehalose lipid (STL-1) prepared from n-hexadecane-culture of *Rhodococcus erythropolis* SD-74 markedly inhibited the growth of a human monocytoid leukemic cell line, U937, and induced its morphological alteration along a monocyte-macrophage lineage. STL-1 markedly increased differention-associated characteristics in macrophage, such as nitroblue tetrazolium reducing ability, appearance of Fc receptor, phagocytic activities in U937. Furthermore, U937 cells, which were activated with STL-1 exhibited cytotoxic activity against human lung carcinoma cell line A549. However, STL-1 did not affect growth of a normal human fetal lung cell line TIG-1. The individual components of STL-1, neither sugar moiety nor fatty acids in the free form, were effective at inducing the differentiation of U937 cell. From these results, we concluded that STL-1 has low cytotoxicity against normal human cells and the ester molecule itself is responsible for the activity of inducing differentiation of human monocytoid leukemic cell line U937 into monocyte-macrophage which results in the stimulation of the production of some cytotoxic substances.

Introduction

Several species of bacteria are known to produce compounds which have surface active characteristics thus generally termed biosurfactants. One of them is succinoyl trehalose lipid (STL), an exolipid produced by *Rhodococcus erythropolis* and *Mycobacterium paraffinicum.* A strain of *Rhodococcus erythropolis*, SD-74, which was isolated from a soil sample reportedly produced two types of STLs namely 2,3,4,2′-di-*O*-succinoyl-di-*O*-alkanoyl-α-α-trehalose and 2,3,4-mono-*O*-succinoyl-di-*O*-alkanoyl-α-α- trehalose designated as STL-1 and STL-2, respectively (Figure 1) (Uchida *et al.*, 1989).

Under optimized culture conditions, *R. erythropolis*, SD-74, produced STLs, in concentrations of about 40 g per liter of culture broth. The yield of the exolipids were calculated to be 50% on the basis of n-hexadecane consumed. The amounts of STL-1 and STL-2 in the total exolipids were 67% and 18%, respectively (Uchida *et al.*, 1989). Studies on surface-active properties of these STLs showed their tension lowering, dispersing, dispersion-stabilizing, and emulsifying effects (Ishigami *et al.*, 1987).

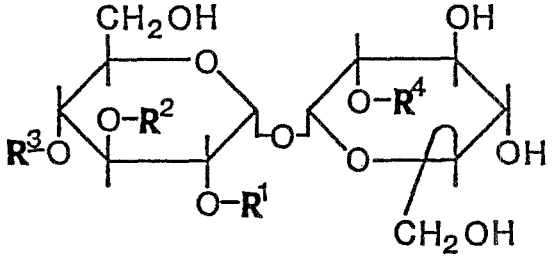

Figure 1. Structure of succinoyl trehalose lipids, STL-1 and STL-2, produced by *Rhodococcus erythropolis* SD-74 from n-hexadecane. STL-1: $R^1 \sim R^4 = 2 \times$ succinoyl + $2 \times$ alkanoyl; STL-2: $R^1 \sim R^3 = 1 \times$ succinoyl + $2 \times$ alkanoyl, R^4=H.

Threhalose conjugated glycolipids are one of the most characteristic components in the cell surface

lipid of *Mycobacterium, Nocardia*, and *Rhodococcus*, all belonging to *Actinomycetales*. Trehalose 6,6′-dimycolate (TDM, Cord factor) had already been recognized to be a toxic glycolipid related to the tuberculous infection and its physicochemical properties such as hydrophobicity and acid-fastness have been characterized (Goren *et al.*, 1972). Recently, attention has been extended to its various immunomodifying activities. It is reported that TDM increased the resistance against syngeneic tumors (Bekierkunst *et al.*, 1971; Leclerc *et al.*, 1976), and that mice inoculated intravenously with TDM acquired a high resistance to intranasal infection of influenza virus (Azuma *et al.*, 1988). An analogous compound, trehalose 2,3,6′-trimycolate (TTM) which is liposome-encapsulated had also been reported to have granuloma-forming activity in lungs and spleen of mice (Yokoi *et al.*, 1989) and induce peritoneal macrophages to express tumourlytic activity (Furukawa *et al.*, 1990). Furthermore, synthesized trehalose esters of fatty acids were reported to have anti-tumor activities against transplanted Ehrlich ascites tumor (Nishikawa *et al.*, 1977) and enhance phagocytic response of the reticuloendothelial system in mice (Nishikawa *et al.*, 1982). On STL, however, the detailed mode of biological actions has not yet been examined.

In this study, we focused our attention on STL-1 which is the major exolipid produced by *R. erythropolis* SD-74. We assessed its biological activities using human cell line, U937. It is a human monocytoid leukemic cell line that has monocytelike characteristics (Sundstrom *et al.*, 1976), and is known to differentiate into a macrophagelike cell line *in vitro* when incubated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Nilsson *et al.*, 1980), retinoic acid (Olsson *et al.*, 1982), 1α,25-dihydroxyvitamin D3 (Dodd *et al.*, 1983) or lymphokines (Koren *et al.*, 1979). In this report, we describe the STL-1 induced differentiation characterisstics of U937 cells, and discuss the immunological role of STL-1.

Materials and methods

Materials

Succinic acid, trehalose and sodium dodecyl sulfate (SDS) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Hexadecanoate and tetradecanoate were purchased from Funakoshi (Tokyo, Japan).

Preparation of STL-1

STL-1 was prepared as described by Uchida *et al.* (1989). Seed cultures were prepared by inoculating loopfuls of cells of strain SD-74 grown on the slants into 300-ml Erlenmeyer flasks containing a seed culture medium (30 ml), followed by incubation at 30 °C for 2 days on the rotary shaker. The seed cultures (4 ml) were transferred to 500-ml Erlenmeyer flasks containing 50 ml of media with 10% (v/V) n-hexadecane, and shaken at 30 °C for 7 days on the rotary shaker (330 rpm). The culture broths were centrifuged at 10,000×*g* for 30 min. The aqueous layer was carefully siphoned, collected and acidified with 6 N HCl to pH 3.0. The acidic exolipids were precipitated as voluminous gel mass. The gel mass was washed twice with water and mixed with hot methanol (300 ml). The mixture was washed three times with n-hexane to remove minor lipids and residual n-hexadecane and then concentrated. The residue was dried over P_2O_5 under reduced pressure and used as the mixture of the main exolipids (STL-1 and STL-2) in the following experiments. This residue was dissolved in ethyl acetate (200 ml) and stood in cold room, STL-1 was mainly obtained as precipitates. After repeated recrystallization with ethyl ecetate, STL-1 was obtained as a colorless powder. STL-1 showed m.p. 169 to 171 °C and [α]+92.3 ° (C=0.9, $CHCl_3$/MeOH=2:1). The components of the long-chain fatty acids were n-hexadecanoate (87%) and n-tetradecanoate (13%). Found: C, 61.21; H,8.97%. Calculated for $C_{52}H_{90}O_{19}$ (hexadecanoate) :C, 61.27; H, 8.90%.

Cell and cell culture

The human monocytoid leukemic cell line U937 and the human lung carcinoma cell line A549 were obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). The human normal diploid cell line TIG-1 (Ohashi *et al.*, 1980) was kindly supplied from the Cell Biology Laboratory in National Institute of Bioscience and Human-technology (Tsukuba, Ibaraki, Japan). The cells were cultured in ERDF medium (Kyokuto Pharmaceutical Kogyo Co., Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum (CC Laboratories Cleveland, Ohio U.S.A). The cells were cultured under humidified 5% CO_2/95% air at 37 °C. Test samples, such as STL-1, their components and SDS, dissolved in Phosphate buffered saline (PBS) were sonicated, sterilized by passage through a MILLEX-HV 0.22 μm filter unit (Millipore Products

Diversion, Bedford, USA). They were added to the culture medium to the desired sample concentration. The final PBS concentration was kept at 0.1%. Control cultures were given the same volume of PBS (without samples treatment). After incubation with STL-1 for 2 days, cells that adhered to the culture vessels were removed with a Nunc 179693 plastic cell scraper (Nunc Inter Med., Roskilde, Denmark) after the addition of FCS containing medium, and each differentiation-associated characteristic was tested on total cells. In the assay of differentiation-associated characteristics, U937 cells cultured with 16nM of TPA (12-*O*-tetradecanoylphorbol- 13-acetate; Wako Pure Chemical Industries, Osaka, Japan) for 24hr examined as positive control.

Assay of the effects of STL-1 on cell growth

The effect of STL-1 on cell growth was measured according to WST-1 (Water Soluble Tetrazolium) method (Ishiyama *et al.*, 1993). U937 (1×10^5)cells were suspended in 100 μl of 5% FCS-ERDF medium and cultured with STL-1 in 96 well plates at 37 °C for 24 hr. Then, 10 μl of WST-1 working solution containing 0.1 mM WST-1 (2- (4-Iodophenyl)-3-(4-nitrophenyl)-5- (2,4-disulfophenyl)-2H- tetrazolium, monosodium salt; Dojin Laboratories, Kumamoto, Japan) solution and 0.02 mM 1-methoxy PMS (1-methoxy-5-methylphenazium methylsulfate; Dojin Laboratories, Kumamoto, Japan) was added. After 4 hr incubation, the amount of WST-1 formazan produced by the cells was determined photometrically (405 nm). The cells were also counted using with a hemocytometer, and, the viability was estimated by trypan blue dye exclusion.

Assay for nitroblue tetrazolium (NBT)-reducing activity

To assess the differentiation of U937 into macrophage-like cells, their ability to reduce the water-soluble nitroblue tetrazoium dye to insoluble intracellular blue-black formazan was investigated. The NBT-reducing ability was determined by the modified method of Takeda *et al.* (1988). Viable U937 cells (2×10^5) were washed once with ERDF medium and were suspended in 2 ml of ERDF medium containing 5% FBS, 0.1% NBT dye and 30 ng TPA in a 12-well plate and incubated for 60 minutes at 37 °C in a 5% CO_2 incubator. For each experimental point, the percentage of cells containing blue-black formazan deposits was determined by counting at least 200 cells under the microscope.

Assay for Fc receptor appearance

The appearance of Fc receptors on the cell surface of U937 was assayed by erythrocyte antibody (EA) rosette formation (Takeda *et al.*, 1982). U937 cell (2×10^5) were washed with ERDF medium and suspended in 2 ml of 0.9% NaCl and then were incubated with suspension of bovine erytrocytes (5×10^6/ml) sensitized with rabbit IgG antibodies (Funakoshi, Tokyo, Japan) at 22 °C for 60 min. Cells with more than 3 attached erythrocytes were counted as positive for Fc receptor appearance. At least 200 cells were examined for each experimental point.

Assay of phagocytic activity

The phagocytic activity of U937 cells was measured by counting the percentage of the cells that phagocytosed Zynaxis fluorescent tracking dye (PK-2 dye; Dainihon Pharmaceutical Co. Ltd., Osaka, Japan) or yeast particles. Viable U937 cells (2×10^5) were washed once with ERDF medium and were suspended in 2 ml of ERDF medium containing 5% FBS, 100 μl of 5×10^{-4} nM of PK-2 dye solution in a 12-well plate, and incubated for 60 min at 37 °C in a 5% CO_2 incubator. For each experiment point, at least 200 viable cells were observed under fluorescence microscope and cells containing PK-2 dye were counted as being phagocytic. On the other hand, the yeast-phagocytic activity was investigated using opsonized yeast. Heat-killed yeast particles (*Saccharomyces cerevisiae*; Wako Pure Chemical Industries Ltd., Osaka, Japan) were incubated with 3 volumes of mouse serum (Funakoshi, Tokyo, Japan) for 30 min at 37 °C and washed with Hank's balanced salt solution (HBSS). Viable U937 cells (2.0×10^5) pellet was mixed with 5.0×10^6 of opsonized yeast particles suspended in 2 ml of HBSS and incubated for 60 min at 37 °C. Cells were washed again and 2 ml of 0.01% fuchsin solution was added to the cell pellet. At least 200 cells were counted for each experimental point and the cells containing yeast particles were scored as phagocytosis positive. (Takeda *et al.*, 1988).

Determination of cytotoxic activity of U937 cells against A549

Monocyte-macrophage cells secrete some cytotoxic substances such as tumor necrosis factor α (TNF α) and active oxygens which are expected to kill tumor

cells. To assess the cytotoxic activity of the U937 cells activated by STL-1 against tumor line the following experiment was performed.

Human lung carcinoma cell line A549 maintained in ERDF medium supplemented with 5% FBS was inoculated 3 ml in the 6-well plates at the cell density of 5×10^4 cells/ml and cultured for 24 hr. After changing the spent medium into a new one, the plates were incubated in a CO_2 incubator at 37 °C for 2 hr. On each plate, a 0.45 μm filter cup with a diameter of 30 mm (Millicell-HA, Millipore Products Sivision, Bedford, USA), which contained the U937 cells (2×10^4 cells/ml) in 3 ml of ERDF-5% FBS medium and STL-1 solution at a concentration of 2.0, and 5.0 μM were placed. The plates were then incubated in a CO_2 incubator at 37 °C for 48 hr. The viability of A549 cells was estimated by trypan blue dye exclusion.

Results

Effect of STL-1 on U937 cell growth

Figure 2 shows the effects of STL-1 on the growth of U937. Growth inhibition of cells was found to be dependent on the concentration of STL-1. STL-1 at a concentration of 2.0 μM inhibited almost 50% of cell growth, and was toxic to the U937 cells at higher concentrations (>25 μM) (Figure 2 *Left*). Based on these results, U937 cells were cultivated in the presence of STL-1 at the concentrations of 2.0, and 5.0 μM, and viable cells were counted. During the 5 days of culture, STL-1 at a concentration of 5.0 μM inhibited the growth of U937 almost completely, while the addition of 2.0 μM resulted that the growth was inhibited at early times, but after 2 days, the cells appeared to have overcome the growth inhibition. (Figure 2 *Right*).

On the second day of cultivation, morphological changes of U937 were observed in all STL-1 treated cultures Figure 3. shows the effect of STL-1 addition on the morphology of U937 cells. Significant cell aggregation and adhesion were evident in the cells added with 5 μ M STL-1 (Figure 3B).

Appearance of differentiation-associated characteristics of U937

The observed morphological changes of U937 cells are assumed to be manifestation of the differentiation of these cells to macrophage like cells. To confirm this assumption, the following differentiation-associated characteristics of U937 cells were investigated: NBT-reducing activity, appearance of Fc receptor and phagocytic activity.

NBT-reducing activity is often used as a marker for neutrophile, monocyte, macrophage and lymphocyte which are known to exhibit toxicity on the numerous pathogenic bacteria or cancer cells. This toxic response is demonstrated by their ability to reduce the water-soluble nitroblue tetrazolium dye to insoluble intracellular blue-black formazan due to O_2^- and H_2O_2 released upon activation. NBT-reducing activity was examined to assess the differentiation of U937 into macrophage-like cells. As shown in Figure 4, NBT reducing ability of STL-1 treated U937 cells was higher than that of TPA (16 nM) treated cells. The percentage of NBT reducing cells treated with 5 μM of STL-1 reached approximately 33%, which was twice as much as that of TPA treatment.

The process of phagocytosis by macrophage seems to involve two steps. The first step is bacterial recognition by macrophage through bacterial surface immunoglobulin molecule (Fc). This step requires Fc receptor expression on the cell surface. The second is the uptake of the bacteria into the macrophage.

Receptors for the Fc region of IgG on surface of the STL-1 treated cells had also increased. As shown in Figure 5, 47% of cells treated with 5.0 μM of STL-1 were found to have Fc receptors on their surfaces. This value is comparable to that of TPA treated cells. Even at a lower concentration (2.0 μM), 25% of cells were observed to possess Fc receptors.

A similar relationship was observed in the capacity for phagocytosis as reflected in the uptake of PK-2 dye or opsonized yeast particles. As shown in Figure 6, phagocytic activity was increased with the addition of STL-1, and phagocyted more yeast particles than PK-2 dye.

Cytotoxic effect of U937 cells activated by STL-1 on human lung carcinoma cell line A549

The monocytes and macrophages have an important role to produce cytotoxic such as TNFα. To confirm whether or not U937 cells activated by STL-1 produce cytotoxic molecules, cultured U937 cells with STL-1 and human lung carcinoma A549 cells were cultured together separated by a filter membrane and were examined for any cytotoxic effect.

The results are shown in Figure 7. When A549 cells were treated with the 5.0 μM of STL only, the viability decreased by about 18%. Whereas the A549

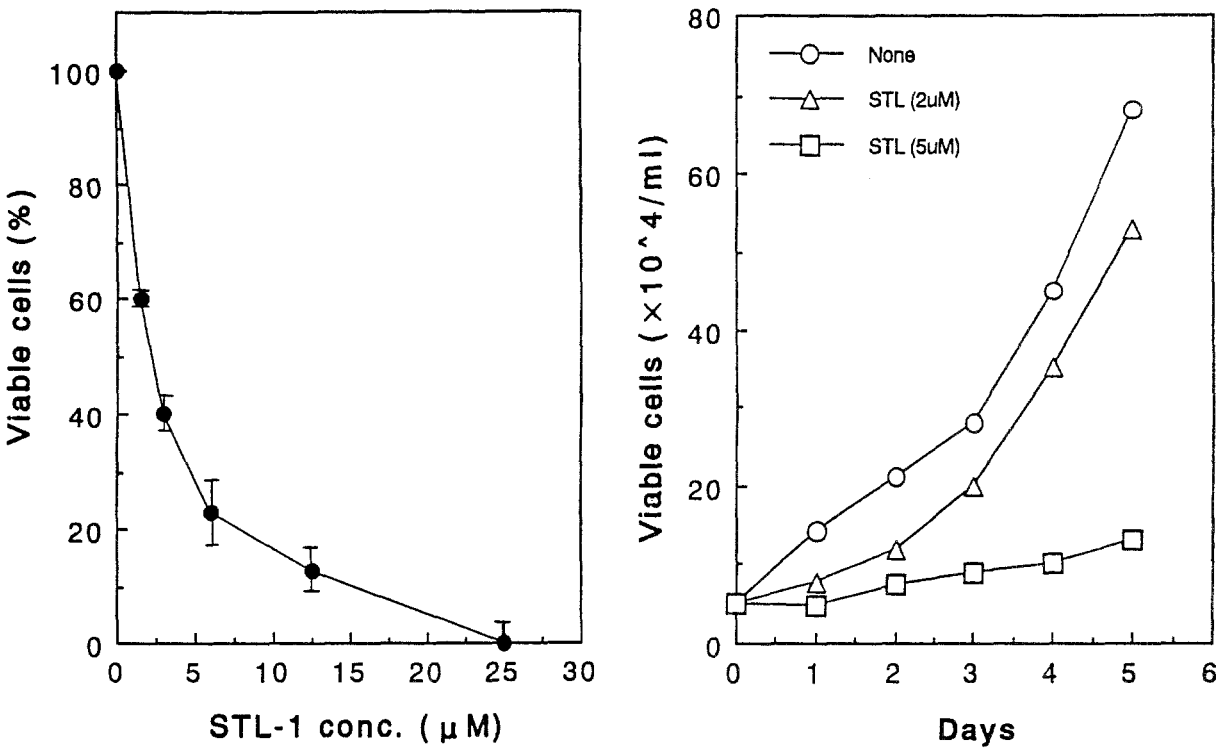

Figure 2. Dose-dependent effect of STL-1 on cell growth of U937 cells. (Left) Viable cells were measured by WST-1 method described in Materials and Methods. Each point represent the mean of three determinations, and vertical bars show standard deviations. (Right) U937 cells were seeded at a initial concentration of 5.0×10^4 cells per ml and grown in the eRDF-5% FCS medium with or without STL-1. Cells were counted in a hemocytometer, and viability was estimated by trypan blye dye exclusion. Each data point represents the mean of three measurements. Standard deviations were <10%. The viability was >90% in each group throughout the culture periods.

cells were cultured with U937 cells without STL treatment, the viability was almost the same as that of control. However, the viability of A549 cells cultured with STL treated U937 cells decreased markedly. From these observations, it was found that STL activated the U937 cells to release a cytotoxic molecule.

Effect of STL-1 on normal human fetal lung cells

To confirm whether or not STL-1 has cytotoxicity to normal human cells, the effect of STL-1 on the growth of human normal diploid cell line TIG-1 was examined. STL-1 did not inhibit the growth of TIG-1 and the viability was almost 88% even under concentration of 5.0 μM (data not shown).

Effect of STL-1 components and surface activity

In order to assess the mechanisms of U937 cell differentiation-induction by STL-1, we examined the effect of STL-1 componnents and surface activity. The various components of STL-1 molecule, namely, succinic acid, trehalose, fatty acids, and sodium dodecyl sulfate (SDS) which is an analogue of STL-1 as an anionic surfactant were added to U937 cell cultivation separately. The growths of the cells treated with various components of STL-1 and SDS were not inhibited and no morphological changed of them were detected (Figure 8).

Discussion

In the present study, we evaluated the biological activities of STL-1 using human monocytoid leukemic cell line U937. Our results showed that STL-1 strongly inhibited the growth of human monocytoid leukemia cell line U937 *in vitro*. The increase of differentiation-associated characteristics of U937 cells, such as NBT-reducing ability, appearance of Fc receptor, and phagocytic activity indicated that STL-1 induced differentiation of U937 cells to monocyte-macrophage. NBT-

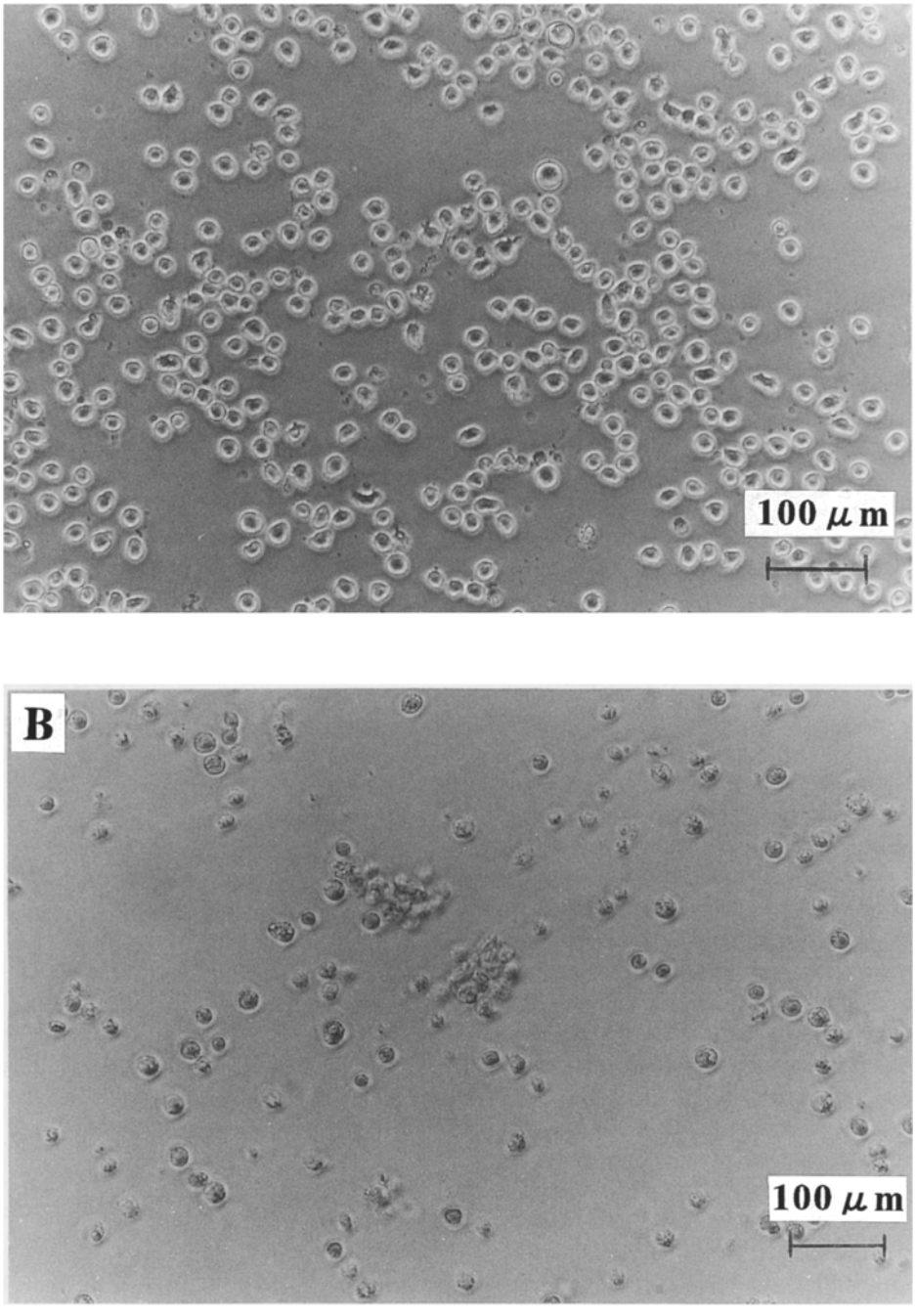

Figure 3. Morphological change of U937 cells treated with STL-1. (A) Untreated U937 cells cultured in the ERDF-5% FCS medium for 2 days. (B) U937 cells cultured in the ERDF-5% FCS medium with 5 μM of STL-1 for 2 days.

reducing ability of STL-1 treated cells were particularly higher than that of TPA treated cells. It seems that STL-1 activated U937 cells resulting in the subsequent release of H_2O_2 or O_2^-. The percentage of U937 cells that possessed Fc receptor and of those that exhibited phagocytic activity were also significantly increased by STL-1 treatment. Human lung carcinoma A549 cells were cocultured with STL-1 treated U937 cells, and

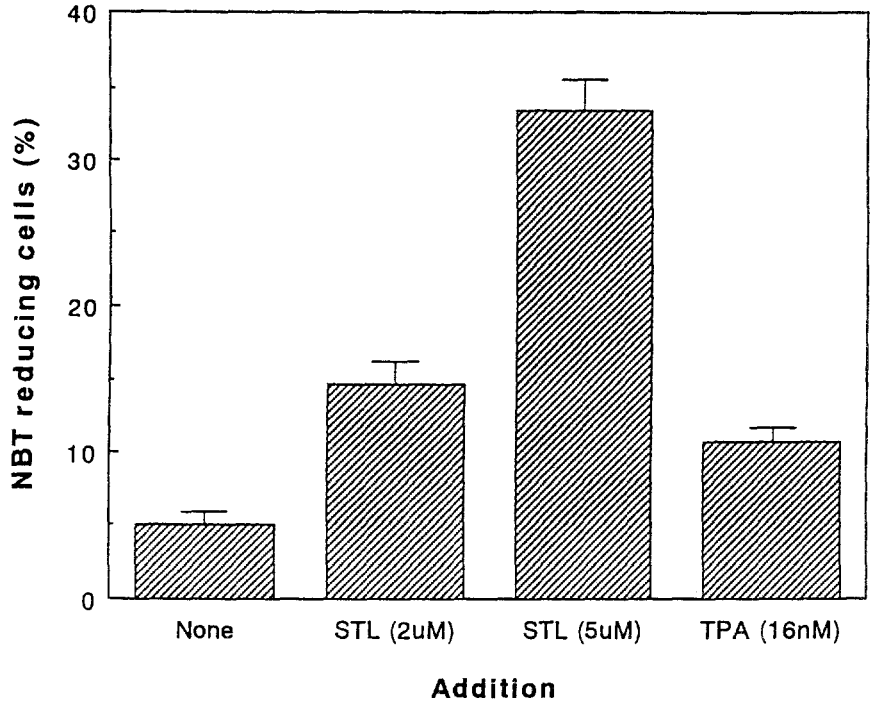

Figure 4. Effect of STL-1 on NBT-reducing activity of U937 cells. NBT-reducing activity was determined by colorimetric assays described in Materials and Methods. Values are mean of three determinations. Vertical bars on columns show standard deviations.

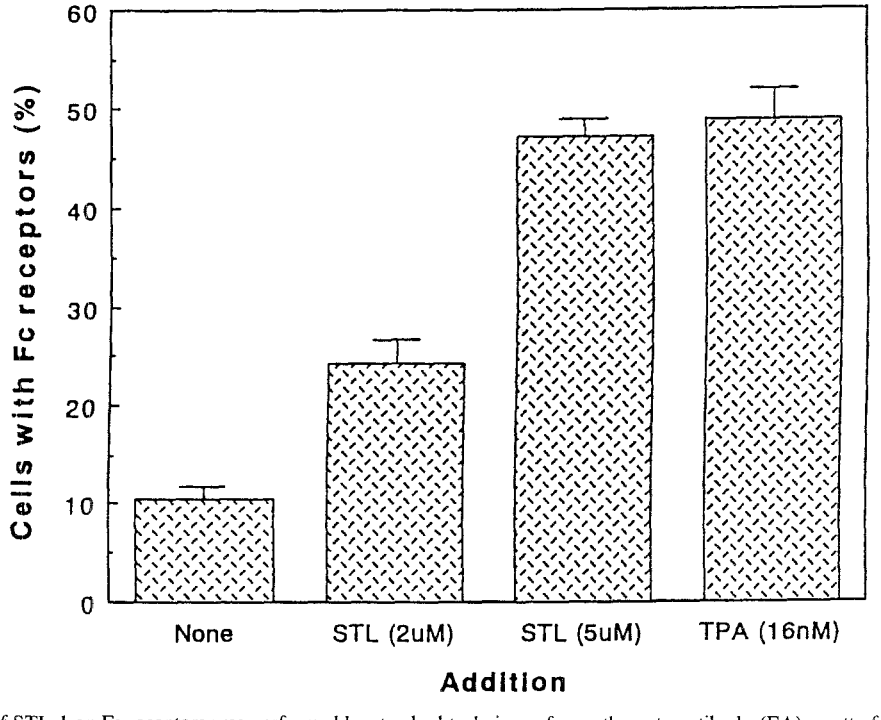

Figure 5. Effect of STL-1 on Fe receptors was performed by standard techniques for erythrocyte-antibody (EA) rosette formation described in Materials and Methods. Values are mean of three determinations. Vertical bars on columns show standard deviations.

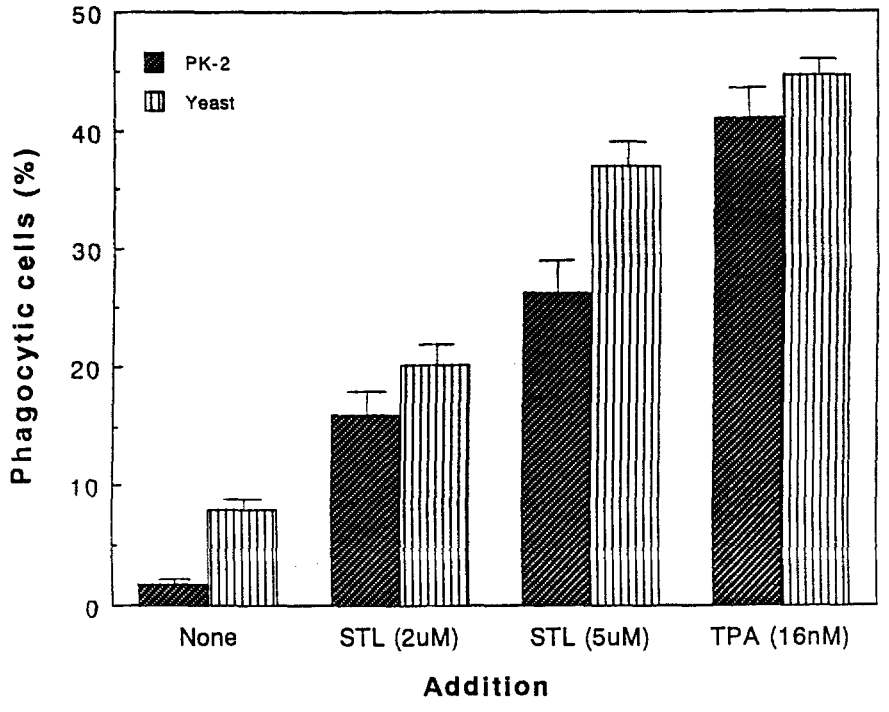

Figure 6. Effect of STL-1 on phagocytic activity of U937 cells. Phagocytic activity is expressed as the percentage of cells ingesting PK-2 dye or yeast particles. Values are mean of three determinations. Vertical bars on columns show standard deviations.

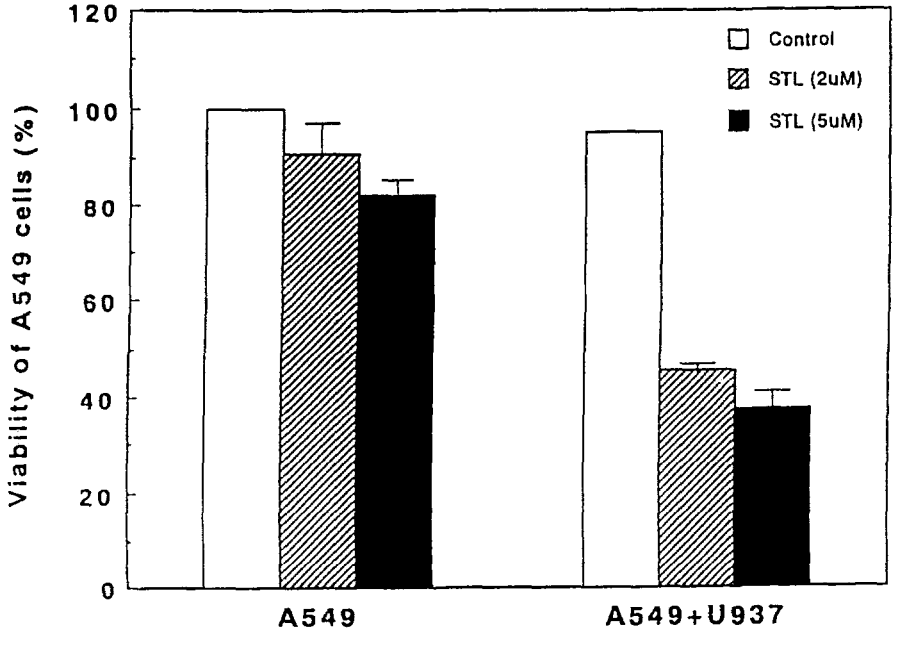

Figure 7. Cytotoxic effect of U937 cells activated by STL-1 on A549 cells. The cytotoxic activity of U937 cells activated by STL-1 against A549 cells was assayed as described in Materials and Methods. Values are mean of three determinations. Vertical bars on columns show standard deviations.

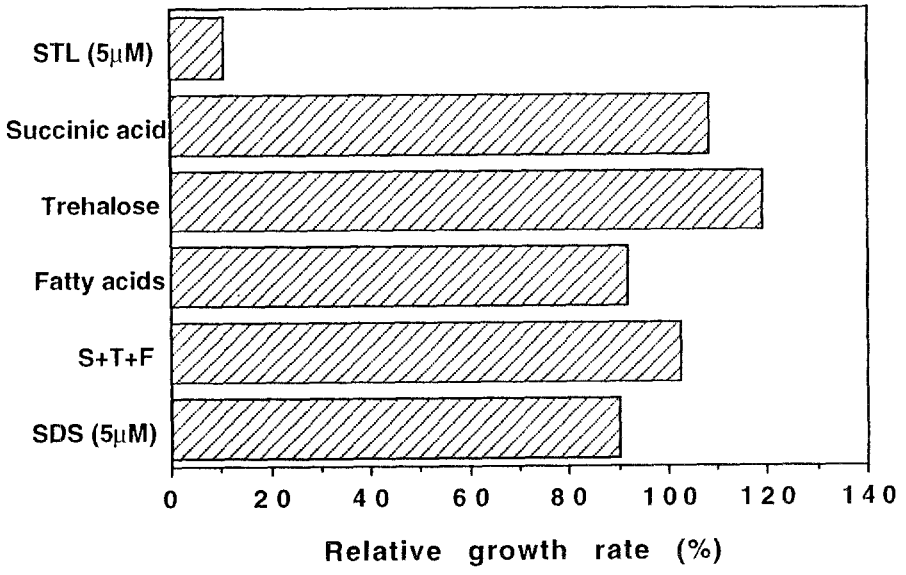

Figure 8. Effect of STL-1 components and surface activity. U937 cells were seeded at an initial concentration of 5.0×10^4 cells per ml and grown in the ERDF-5%FCS medium with or without various components of STL-1 molecule at the concentration of which contained in 5μM of STL-1, and 5μM of SDS. S+T+F means that succinic acid, trehalose and fatty acids were added to U937 cell cultivation separately. Cells were counted in a hemacytometer, and viability was estimated by trypan blue dye exclusion. The relative growth rate percentage indicates the comparison of value at cell numbers in the control (PBS) with that in each additives. Each point represents the mean of three determinations, and vertical bars show standard deviations.

STL-1 was also found to activate the U937 cells to cause a cytotoxic effect on A549 cells. This observation suggests that STL-1 promoted the secretion of a water-soluble cytotoxic cytokine such as TNFα. Furthermore, STL-1 showed lower toxicity against human normal fetal lung cells than human lung carcinoma cells even at concentration (5 μM).

Recently, it was reported that trehalose conjugated glycolipids have immunological activities. Fujita *et al.* (1990) reported that the increase in activities of interferons and TNF was approximately paralleled with granuloma formation in spleen of mice primed with trehalose 2,3,6′-trimycolate (TTM) derived from *R. aurantiacus*, and TTM was also shown to prime strong activation of macrophages, spenic T cells and to inhibit *in vivo* growth of syngeneic tumour cells in the peritoneal cavity either directly or by released factors (Ohtsubo *et al.*, 1991). Furthermore, stimulation of cord blood with trehalose 6,6′-dimycolate (TDM) increased T-cell receptor (TCR) γ/δ expression on T cells (Tsuyuguchi *et al.*, 1991). Sazaki *et al.* (1992) also suggested that the recovered anti-infuluenza virus resistance on Cyclosporin A (CsA)-treated mice by treatment with TDM emulsion was caused by elicitation of macrophages with TDM, followed by activation of γ δ TCR+YT lymphocytes.

These previous works on similar glycolipids suggest the need to conduct *in vivo* studies on STL-1 to determine its possibility for application in the treatment of cancer and infections diseases.

Nishikawa *et al.* (1982) examined the effects of synthesized disaccharide esters of fatty acids on the phagocytic response of the reticuloendothelial systems, and found that disaccharide containing di- and polystearate or -palmitate were particularly active. It is suggested that the ester molecule itself was responsible for the apparent activity, since neither disaccharide nor fatty acid in the free form proved to be effective. As shown in Figure 8, the components of STL-1 molecule separately added to U937 cell cultivation were not active, and therefore this case also indicated the ester molecule was responsible for the activity. Ishigami *et al.* (1987) reported that STL-1 can act as anionic surfactant with detergent property and the critical micelle concentration (CMC) of STL-1 is 960 μM. To confirm whether or not the ability of inducing differentiation of U937 cells could be attributed to surface activity of STL-1, an analogue of STL-1, sodium dodecyl sulfate (SDS) which is a typical anionic surfactant was used. The result showed that no surface activity to the induction of differentiation on the basis of the complete ineffectiveness of the SDS tested (Figure 8). As

the effective concentration of STL-1 (5 μM) is far less than the CMC, STL-1 is assumed not to form micelle, but it could act on the plasma membrane specifically.

A lot of biological in the pathogenesis on Trehalose, 6,6′-dimycolate (TDM, Cord factor), including antitumour activity, immunomodulation, and granulomagenic activity have been uncovered and indirect evidence has been provided that TDM might be responsible for inhibiting fusion between phospholipid vesicles (Spargo *et al.*, 1991). They suggested that TDM could affect fusion by increasing the hydration force that is known to be an important primary barrier, or by acting as a steric block to fusion. Because STL-1 also has trehalose moiety and fatty acid moiety, a similar mode of action on marcrophage action as TDM is expected.

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