Action of Cytogenin on Lymphoid Cells and Their Cytokine Production

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Action of cytogenin on macrophages and T cells was investigated. Phagocytosis of yeast and production of PMA-elicited superoxide anion by macrophages taken from mice given cytogenin po were augmented. Cytogenin enhanced productions of IL-1α by macrophages and IFNγ and GM-CSF by spleen cells although it did not enhanced production of TNFα by macrophages and IL-6 by macrophages and spleen cells. Macrophages stimulated with cytogenin caused to stimulate proliferation of purified T cells in Intercell cultures in which each cell population was cultured without contact. Results suggest that cytogenin primarily activates macrophages to produce monokines such as IL-1α and it causes to stimulate proliferation and differentiation of T cells resulting in production of lymphokines such as IFNγ and GM-CSF.

Cytogenin is an antitumor antibiotic produced by *Streptoverticillium eurocidicum* MI43-37F11[1]. As reported previously[2], it exhibited antitumor effect against a syngeneic tumor, IMC carcinoma by the oral administration through activation of antitumor effector cells such as macrophages and cytotoxic T cells (CTL). It was suggested that cytogenin may primarily acts on macrophages since cytogenin did not show any effect against T cells directly. Therefore, the action of cytogenin on activation of macrophages and their cytokine production was investigated.

Materials and Methods

Cytogenin and Other Reagents

Cytogenin was prepared by Mercian Co., Ltd. (Tokyo, Japan) according to the methods as reported previously[1]. Nylon wool for preparation of T cells was purchased from Wako chemical Co. Ltd. (Osaka, Japan). RPMI1640 medium was purchased from Nissui Seiyaku Co. Ltd. (Tokyo, Japan). Concanavalin A (Con A) was purchased from Pharmacia (Sweden). Ferricytochrome C and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). [6-^{3}H]thymidine([^{3}H]TdR, specific activity 555KBq/mmol) was purchased from New England Nuclear (Boston, U.S.A). Intercell for separation culture was purchased from Kurabou Co. Ltd.(Osaka, Japan).

Mice

CDF_1 mice (6 weeks old, female) were purchased from Charls River Japan Inc. (Kanagawa, Japan) and were maintained under specific pathogen-free conditions at 23 ± 1°C and 55 ± 5% humidity. They were used for experiments at 8 to 10 weeks old.

Cell Preparation and Cultures

To obtain macrophages, peritoneal exudate cells (PEC) were collected from mice and suspended at 2×10^6 cells/ml in RPMI1640 medium supplemented with 10% FCS. PEC were plated on a microplate and cultured for 1 hour. Non-adherent cells were washed out with warmed RPMI1640 medium, and adherent cells were used as macrophages. T cells were prepared by passing spleen cells through a nylon wool column as reported previously[3]. Cells taken from mice were cultured with RPMI1640 medium supplemented with 10% FCS, 50 units/ml of penicillin and 50 μg/ml of streptomycin at 37°C in 5% CO_2 air.

Phagocytosis

Influence of cytogenin on phagocytosis of macrophages was examined as reported previously[4]. Briefly, cytogenin was administered po on days 3 and 1 before assay. PEC were collected from mice and macrophages were prepared as mentioned above. To macrophages, 50 μl of heat inactivated yeast suspension (7.5×10^6 cells/ml) was added and incubated for 45 minutes at 37°C in CO_2 air. After washing with medium thoroughly, adherent cells on the bottom of plate were stained with May-Grunwald and Giemsa solution and the number of phagocytic cells were counted.

Production of Superoxide Anion

Mice were given cytogenin po on days 3 and 1 before assay. Release of superoxide anion from macrophages was measured by the method reported by KITAGAWA and JOHNSTON[5]. Briefly, macrophages were incubated with 1.5 ml of the reaction mixture (80 mM ferricytochrome C, 100 ng/ml of PMA in Hank's balanced salt solution). After incubation for 60 minutes at 37°C, concentration of superoxide anion was determined as reduction of ferricytochrome C. Further, macrophages were solubi-

lized with 1 ml of 0.1 N NaOH and the solution were assayed for protein concentration by the method of LOWRY *et al.*[6] with bovine serum albumin as standard.

Assay for Cytokine Activities

Mice were administered po with cytogenin at 0.39 to 100 mg/kg on days 3 and 1 before assay. Macrophages and spleen cells were collected from mice and prepared at 1×10^6 cells/ml and 5×10^6 cells/ml, respectively. Macrophages were cultured for 1 day, and spleen cells were cultured for 3 days. Activities of cytokines in the cultured supernatant were assessed by enzyme linked immunoabsorbent assay (ELISA). ELISA kits were obtained from genzym (Cambridge, U.S.A.) for interleukin 1α (IL-1α), tumor necrosis factor α (TNFα) and interferon γ (IFNγ) and from ENDOGEN (Boston, U.S.A.) for granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 6 (IL-6).

Proliferation of T Cells Stimulated by Macrophages Taken from Mice Given Cytogenin

Mice were given cytogenin po at 6.25 mg/kg on days 3 and 1 before assay. Macrophages collected from mice were prepared at 1×10^6 cells/ml and 1 ml of macrophages was plated onto a microplate. Splenic T cells collected from normal CDF_1 mice were prepared at 5×10^6 cells/ml. T cells (0.6 ml) were plated onto Intercell and were put on each well of the microplate. These cells were cultured without contact for 5 days. Proliferation of T cells was assessed by measuring the incorporation of [^{3}H]TdR into cells.

Statistical Analysis

Statistical significance was analyzed by Student's *t*-test.

Results

Influence of Cytogenin on Macrophage Functions in Mice

To determine the influence of cytogenin on functions of macrophages, phagocytosis and production of superoxide anion by macrophages stimulated with cytogenin in mice were examined.

Mice were given cytogenin at 1.56 to 100 mg/kg, po, on days 3 and 1 before assay, and effect of cytogenin on phagocytosis against yeast by macrophages was examined. As shown in Table 1, cytogenin at 1.56 to 25 mg/kg increased the ratio of phagocytic cells significantly. In next, the effect of cytogenin on production of superoxide anion by macrophages was examined. Mice were given cytogenin at 1.56 to 100 mg/kg, po, on days 3 and 1 before assay. As shown in Table 1, the administration of cytogenin augmented production of PMA-induced superoxide anion at 6.25 mg/kg significantly. Results indicate that the administration of cytogenin po can modulate macrophage functions.

Table 1. Phagocytosis and production of superoxide anion by macrophages taken from mice given cytogenin.

Cytogenin (mg/kg)	Phagocytosing cells (%)±SD in macrophages		PMA-induced O_2^- (nmol/mg protein ±SD)	
0	34.8±4.8	(100)	24.0± 5.0	(100)
1.56	47.3±2.3*	(136)	29.8± 6.4	(124)
6.25	48.5±5.0*	(139)	53.0±13.3*	(221)
25	48.3±6.3*	(139)	30.2±13.5	(126)
100	40.0±2.0	(115)	25.8± 4.0	(107)

* $P<0.05$ against control group.

Table 2. IL-1α and TNFα production by macrophages taken from mice given cytogenin.

Cytogenin (mg/kg)	IL-1α (pg/ml)±SD		TNFα (pg/ml)±SD	
0	9.26±1.15	(100)	68.3± 2.8	(100)
0.39	15.80±1.40*	(170)	N.D.	
1.56	19.53±0.61**	(213)	82.5± 6.8	(121)
6.25	16.66±2.24	(180)	76.8±10.9	(111)
25	11.27±0.23	(120)	N.D.	
100	6.51±0.14	(70)	N.D.	

* $P<0.05$ and ** $P<0.01$ against control group.
N.D.: Not determined.

Table 3. Cytokine production by spleen cells taken from mice given cytogenin.

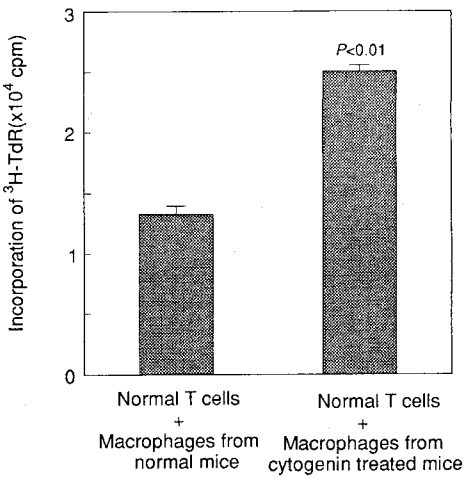
Cytogenin (mg/kg)	IFNγ (pg/ml)±SD		GM-CSF (pg/ml)±SD	
	T/C (%)		T/C (%)	
0	10,150±1,222	(100)	668±49	(100)
1.56	15,960± 436	(157)	883±31	(132)
6.25	16,842± 244*	(166)	992±37*	(149)

* $P<0.05$ against control group.

Effect of Cytogenin on Cytokine Production by Macrophages and Spleen Cells

Mice were given cytogenin po on days 3 and 1 before assay and production of cytokines by macrophages and spleen cells were determined. As shown in Table 2, production of IL-1α by macrophages was significantly augmented by cytogenin but not TNFα. On the other hand, production of IFNγ and GM-CSF, which are known as differentiation factors for hematopoietic cells and lymphocytes, by spleen cells were augmented significantly (Table 3). The production of IL-6 by both cells was not augmented by cytogenin. These results suggest that monokine production by macrophages stimulated with cytogenin activates T cells to produce some lymphokines such as IFNγ and GM-CSF and it may cause proliferation of T cells. Thus, the effect of

Fig. 1. Enhancement of T cell proliferation by macrophages taken from mice given cytogenin in intercell culture.



macrophages stimulated with cytogenin on proliferation of T cells was examined.

Effect of Cytogenin-stimulated Macrophages on Proliferation of T Cells

Macrophages taken from mice given cytogenin at 6.25 mg/kg were cultured with T cells from normal mice without contact with each cell population in Intercell and the effect of macrophages on proliferation of T cells was examined. As shown in Fig. 1, the proliferation of T cells cultured with macrophages stimulated with cytogenin was significantly enhanced in comparison to T cells cultured with non-stimulated macrophages. These results indicate that cytogenin activates macrophages to produce some monokines like IL-1α which stimulates proliferation of T cells.

Discussion

Cytogenin was found in products of *Streptoverticillium eurocidicum* MI43-37F11 primarily as an antitumor antibiotic exhibiting antitumor activity against Ehrlich carcinoma by oral administration[1]. As the antitumor activity of cytogenin had been studied[2], it was shown that the antitumor activity of cytogenin is due to its immunomodulatory activity revealing generation antitumor effector cells such as macrophages and cytotoxic T cells in tumor bearing mice and results on action of cytogenin in tumor bearing mice suggest that cytogenin might activate macrophages to stimulate proliferation and/or differentiation of T cells. Thus, the activity of cytogenin on functions of macrophages and splenic lymphocytes was investigated. Phagocytosis and production of superoxide anion, which are known as indicators of activation of macrophages, were stimulated by macrophages taken from mice given cytogenin as well as forphenicinol[4,7], a low molecular weight immunomodulator. Results suggest that cytogenin activated macrophages and the activation may cause to show antitumor activity by cytogenin[8,9]. This conclusion is supported by the result that cytogenin activated antitumor effector activity of macrophages in tumor bearing mice as reported previously[2].

As mentioned above, cytogenin activate macrophages and it is well known that activated macrophages produce monokines such as IL-1 and TNF. IL-1 is a major mediator of inflammation[10] and an important signal for activation and differentiation of lymphoid cells[11]. TNF is a major candidate for the toxic mediators produced by macrophages[12]. Therefore, production of IL-1α and TNFα by macrophages and production of some cytokines by spleen cells was investigated. Results as shown in Tables 2 and 3 indicate that the administration of cytogenin stimulated to produce IL-1α but not TNFα by macrophages and IFNγ and GM-CSF by spleen cells. It is of note that production of IL-6, which is known as inflammatory cytokine[13] as well as IL-1, by macrophages and spleen cells was not enhanced by cytogenin. Results suggest that cytogenin enhances IL-1α production by macrophages without inflammatory effect and the monokine stimulates proliferation and production of IFNγ and GM-CSF by T cells.

Then, macrophages treated with cytogenin and non-treated T cells were cultured without contact and proliferation of T cells was examined in Intercell. It was shown that macrophages taken from mice given cytogenin caused to stimulate proliferation of T cells taken from normal mice significantly. Results suggest that the proliferation of T cells might be stimulated by some monokines such as IL-1α produced by macrophages.

It can be concluded from results shown in this paper that cytogenin primarily activates macrophages to stimulate production of monokine and it causes to stimulate proliferation and/or differentiation of T cells like CTL. Activation of macrophages and T cells by cytogenin may cause to show antitumor activity of cytogenin.

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