Studies on Thermophile Products. X. Further Biological Properties of Isofatty Acid-Containing Phosphatidylglycerol That Enhances the Induction of Suppressor T Cells

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Received March 28, 1994; accepted July 9, 1994

Isofatty acid-containing phosphatidylglycerol (Fr. 7-C), isolated from *Bacillus stearothersophilus* UBT8038, enhances the induction of concanavalin A (Con A)-activated suppressor T (Ts) cells in a dose dependent manner (0.01—1 $\mu\text{g/ml}$). Its further biological properties on mouse mixed lymphocyte reaction (MLR) has been demonstrated. Fr. 7-C (0.01—1 $\mu\text{g/ml}$) suppressed the MLR at 4 d in a dose-dependent manner when added at the start of splenocyte cultivation. Moreover, Fr. 7-C was effective in preventing the generation of cytotoxic T lymphocytes after the MLR. On the other hand, this fraction significantly enhanced the induction of Ts cells in the MLR carried out in any of the antigen-specific, antigen-nonspecific and major histocompatibility complex antigen-nonspecific fashions. Fr. 7-C increased prostaglandin E_2 (PGE_2) release approximately 2-fold in the culture supernatant of Con A-activated splenocytes, and PGE_2 release decreased dose-dependently when cultured with indomethacin. The inhibitory effect by Fr. 7-C on the MLR was abrogated by the addition of indomethacin. The enhancement by Fr. 7-C on Ts cell induction was blocked by indomethacin in a dose dependent manner. These results strongly suggest that Fr. 7-C suppresses the MLR via the enhancement of antigen-nonspecific Ts cell induction mediated at least partly by PGE_2 .

Keywords suppressor T cell; *Bacillus stearothersophilus*; phosphatidylglycerol; isofatty acid; mixed lymphocyte reaction; prostaglandin E_2

Suppressor T (Ts) cell activity plays an important role in the regulation of both humoral and cell-mediated immunity.¹⁾ In the course of our screening for immunosuppressive substances that include the effects on lymphocytes²⁾ and AP cells,³⁾ we previously reported the isolation of a *Bacillus stearothersophilus* UBT8038 lipid component, Fr. 7-C, that increased the induction of concanavalin A (Con A)-induced CD8 positive Ts cells, but did not affect helper T (Th) cells.⁴⁾ Fr. 7-C chemically consisted of phosphatidylglycerol with $\text{C}_{14:0}$ — $\text{C}_{18:0}$ isofatty acids. In this paper, we described further biological properties of this fraction on mouse mixed lymphocyte reaction (MLR).

MATERIALS AND METHODS

Mice Male BALB/c, C57BL/6, and CBA/N mice (6 to 8 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were maintained under specific-pathogen-free conditions.

Materials RPMI1640 was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Flow Laboratories (McLean, VA, U.S.A.). Tritiated thymidine ($[^3\text{H}]\text{TdR}$) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.), Con A from Sigma Chemical Co. (St. Louis, MO, U.S.A.), CytoTox 96TM non-radioactive cytotoxicity assay from Promega Co. (Madison, WI, U.S.A.), and prostaglandin E_2 (PGE_2) EIA kit from Amersham Life Science (Tokyo, Japan). Indomethacin and mitomycin C (MMC) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fr. 7-C was the preparation previously isolated from the debris of *Bacillus stearothersophilus* UBT8038. Cyclosporin A (CYA) was

a generous gift from Sandoz, Ltd., Biological and Medical Research (Basel, Switzerland). Lobenzalit (CCA) was a generous gift from Chugai Pharmaceutical Co., Ltd. (Gotenba, Shizuoka, Japan). Test samples were dissolved in ethanol and further diluted in a medium to the proper concentration in cultures. Other cultures received ethanol in the medium. The final concentration of ethanol in every culture was 0.1%.

MLR As previously described,^{2c)} the mouse MLR against alloantigen was performed in flat-bottomed microtiter plates with each well containing 5×10^5 C57BL/6 spleen cells (responder, H-2^b) and 5×10^5 25 $\mu\text{g/ml}$ MMC-treated BALB/c spleen cells (stimulator, H-2^d) in 0.2 ml RPMI1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol (2ME), 12 mM HEPES, 100 U/ml benzylpenicillin and 100 $\mu\text{g/ml}$ streptomycin (RPMI 1640 complete medium). The cells were incubated for 112 h at 37 °C in a humidified atmosphere of 5% CO_2 : 95% air. The cultures were pulsed with 0.5 μCi of $[^3\text{H}]\text{TdR}$ for 16 h before the end of culture, and were harvested onto glass fiber filter papers using an automated harvester LM 101 (Medi-Con Corp., Osaka, Japan). The papers were dried and processed for liquid scintillation counting.

Cytotoxic T Lymphocyte (CTL) Generation The cytotoxic activity of splenocytes was demonstrated as described by Nakamura *et al.*⁵⁾ with minor modifications.

Effector Cells: Single cell suspensions of effector cells were obtained from the bulk MLR (96 h). The collected cells were washed 3 times and finally resuspended in RPMI 1640 complete medium.

Target Cells: A suspension culture of DBA/2 mastocytoma P815 (H-2^d, Japan Cancer Resources Bank) cells maintained in RPMI 1640 complete medium was washed

3 times and resuspended in RPMI 1640 complete medium.

Effector cells (2×10^6 cells) and target cells (2×10^5 cells) were cultured in round-bottomed microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, U.S.A.) containing 0.1 ml RPMI 1640 complete medium for 4 h. At the end of the culture, 50 μ l of the supernatant was removed and the cytotoxic activities were determined using a CytoTox 96TM non-radioactive cytotoxicity assay. According to the recommended method of Promega, the percentage of cytotoxicity was calculated from the lactate dehydrogenase release in experimental, effector and target groups as follows: cytotoxicity (%) = $\{[(\text{experimental} - \text{effector spontaneous}) - \text{target spontaneous}] / (\text{target maximum} - \text{target spontaneous})\} \times 100$.

MLR-Activated Ts Cell Assay The cultures were comprised of a mixture of three spleen cell pools (effector cells, responder cells, and stimulator cells). Effector cells were prepared by bulk MLR cultures using C57BL/6 spleen cells (responder) and BALB/c spleen cells (stimulator) with or without test samples (primary MLR). They were incubated with 25 μ g/ml MMC at 37°C for 30 min, washed 3 times with FCS-free RPMI 1640 complete medium and resuspended in RPMI 1640 complete medium. Effector cells (5×10^4 cells) were added to fresh MLR (responder 5×10^5 cells, stimulator 5×10^5 cells) in flat bottom microtiter plates containing 0.2 ml RPMI 1640 complete medium. The cultures were incubated at 37°C in a CO₂-incubator for 4 d and pulsed with [³H]TdR 16 h before harvesting. The incorporated [³H]TdR was determined as described for the MLR. To determine the antigen specificity of the response, three groups of the fresh MLR were used. In the first group, the fresh MLR was autologous to the primary MLR. In the second group, the fresh MLR was autologous to the responder of the primary MLR but with a different stimulator (CBA/N, H-2^k). In the third group, the fresh MLR was heterogenous (CBA/N) to the responder of the primary MLR, but with an identical stimulator.

Con A-Activated Ts Cell Assay A Con A-induced Ts

cell assay was carried out as previously described.⁴⁾ Briefly, effectors were prepared by culturing the splenocytes (10^7 cells) of BALB/c mice in 24-well plates containing 1 ml RPMI 1640 complete medium with 2 μ g/ml Con A for 2 d and treated with 50 μ g/ml MMC. Fresh splenocytes (5×10^5 cells) were cultured in flat bottom microtiter plates containing 0.2 ml RPMI 1640 complete medium with effector cells (1.5×10^5 cells) and 2 μ g/ml Con A for 3 d, and pulsed with [³H]TdR which was determined as described for the MLR. Test samples were added during Ts induction cultures with or without 10^{-9} – 10^{-5} M indomethacin.

Con A Response As previously described,⁴⁾ splenocytes (10^7 cells) of BALB/c mice were cultured in 24-well plates containing 1 ml RPMI 1640 complete medium with 2 μ g/ml Con A for 2 d. In the indicated times of the Con A-responses, duplicate culture supernatant samples were assayed for PGE₂ level according to the manufacturer's instructions.

Statistical Analysis A student's *t*-test was used to determine the significance of differences between experimental groups.

RESULTS

MLR Fr. 7-C enhanced the induction of Con A-activated Ts cells in a dose dependent manner (0.01–1 μ g/ml). We examined the effect of Fr. 7-C (0.01–1 μ g/ml) on the proliferative response of mouse splenocytes to alloantigen stimulation. The result is illustrated in Fig. 1. Fr. 7-C (0.1 and 1 μ g/ml) significantly inhibited the MLR in a dose dependent manner. CYA (0.012 and 0.12 μ g/ml) and CCA (10 μ g/ml) also inhibited the MLR.

CTL Generation The MLR resulted in the generation of a cytotoxic response against target cells restricted by the same MHC haplotype as that of the effector. As shown in Fig. 2, a significant reduction in the degree of cytotoxicity was observed in effector cells previously incubated with 1 μ g/ml Fr. 7-C, which was effective in

Fig. 1. Effect of Fr. 7-C on MLR

The data show the mean $\pm$ S.E. of 3 determinations. The [³H]TdR uptake for unstimulated lymphocytes was 1619 ± 757 cpm. a) μ g/ml. Significantly different from the control, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$.

Fig. 2. Effect of Fr. 7-C on CTL Generation

The data show the mean $\pm$ S.E. of 4 determinations. a) μ g/ml. Significantly different from the control, b) $p < 0.05$.

TABLE I. Effect of Fr. 7-C on Induction of MLR-Activated Suppressor Cells in Antigen Specific Fashion

Sample	Concentration (μ g/ml)	Primary MLR		Fresh MLR		$[^3\text{H}]\text{TdR}$ uptake (cpm)
		Res	Sti	Res	Sti	
Control I	—	—	—	A	B	14607 $\pm$ 1752
Control II	—	A	B	A	B	3777 $\pm$ 148
Fr. 7-C	1	A	B	A	B	2898 $\pm$ 117 ^{a)}
CCA	10	A	B	A	B	3364 $\pm$ 304

Induction of MLR-activated suppressor cells was assessed by adding effector cells from sample-treated primary MLR to fresh MLR. Data show the mean $\pm$ S.E. ($n=6$). Significantly different from control II; a) $p < 0.05$. The abbreviations used were: A, C57BL/6 (H-2^b); B, BALB/c (H-2^d) treated with MMC; Res, responder cells; Sti, stimulator cells.

TABLE II. Effect of Fr. 7-C on Induction of MLR-Activated Suppressor Cells in Antigen Nonspecific Fashion

Sample	Concentration (μ g/ml)	Primary MLR		Fresh MLR		$[^3\text{H}]\text{TdR}$ uptake (cpm)
		Res	Sti	Res	Sti	
Control I	—	—	—	A	C	11322 $\pm$ 496
Control II	—	A	B	A	C	8977 $\pm$ 607
Fr. 7-C	1	A	B	A	C	6939 $\pm$ 653 ^{a)}
CCA	10	A	B	A	C	8405 $\pm$ 876

Induction of MLR-activated suppressor cells was assessed by adding effector cells from sample-treated primary MLR to fresh MLR. Data show the mean $\pm$ S.E. ($n=6$). Significantly different from control II; a) $p < 0.05$. The abbreviations used were: A, C57BL/6 (H-2^b); B, BALB/c (H-2^d) treated with MMC; C, CBA/N (H-2^k) treated with MMC; Res, responder cells; Sti, stimulator cells.

inhibiting MLR. CYA (0.12 μ g/ml) also inhibited the generation of CTL, whereas CCA (10 μ g/ml) did not.

MLR-Activated Ts Cells The effect of Fr. 7-C on the induction of Ts cells by the MLR was investigated. A dose of 1 μ g/ml Fr. 7-C, which was effective in enhancing the induction of Con A-activated Ts cells and in inhibiting MLR, was used for the MLR-activated Ts cell induction assay. The result is illustrated in Table I. When MLR-activated effector cells were added to a fresh MLR (control II), an inhibition of MLR proliferation was seen compared with control I, to which effector cells had not

TABLE III. Effect of Fr. 7-C on Induction of MLR-Activated Suppressor Cells in MHC Nonrestricted Fashion

Sample	Concentration (μ g/ml)	Primary MLR		Fresh MLR		$[^3\text{H}]\text{TdR}$ uptake (cpm)
		Res	Sti	Res	Sti	
Control I	—	—	—	C	B	9901 $\pm$ 620
Control II	—	A	B	C	B	11921 $\pm$ 494
Fr. 7-C	1	A	B	C	B	9584 $\pm$ 498 ^{a)}
CCA	10	A	B	C	B	10905 $\pm$ 829

Induction of MLR-activated suppressor cells was assessed by adding effector cells from sample-treated primary MLR to fresh MLR. Data show the mean $\pm$ S.E. ($n=6$). Significantly different from control II; a) $p < 0.05$. The abbreviations used were: A, C57BL/6 (H-2^b); B, BALB/c (H-2^d) treated with MMC; C, CBA/N (H-2^k); Res, responder cells; Sti, stimulator cells.

Fig. 3. Effect of Fr. 7-C on PGE₂ Release in Culture Supernatant of Con A-Activated Splenocytes

The data are the mean of duplicate determinations. B is the magnification of the part indicated by a dotted square in A. $\circ$, control; $\bullet$, Fr. 7-C (1 μ g/ml).

been added, thus identifying the presence of effectors with suppressor activity. The effector cells treated with Fr. 7-C (1 μ g/ml) significantly enhanced suppressor activity when added to the fresh MLR in an antigen-specific fashion. The effector cells treated with CCA (10 μ g/ml) did not.

The enhancement of suppressor activity by Fr. 7-C was also observed when the effector cells treated with Fr. 7-C were added to the fresh MLR in an antigen-nonspecific fashion (Table II).

The above effector cells did not display suppressor activity in the fresh MLR with an allogenic responder in a major histocompatibility complex (MHC)-nonrestricted fashion. However, the primary MLR in the presence of Fr. 7-C induced effector cells displaying significant suppressor activity (Table III).

PGE₂ Release in the Culture Supernatant of Con A-Activated Splenocytes We measured the level of PGE₂ in the supernatant of the Con A response of cultured splenocytes in a high density. As shown in Fig. 3, the level of PGE₂ continued to increase 1 to 48 h after the onset of cultivation. Fr. 7-C elevated PGE₂ release approximately 2-fold as compared with the control group, all measured during periods after the addition.

Fig. 4. Effect of Indomethacin on Elevation of PGE₂ Release by Fr. 7-C in Culture Supernatant of Con A-Activated Splenocytes

The data are the mean of duplicate determinations. ○, control (without Fr. 7-C); ●, Fr. 7-C (1 μg/ml); ×, non-activated (without Con A).

TABLE IV. Effect of Indomethacin on Inhibition by Fr. 7-C of MLR

Sample	Concentration (μg/ml)	Indomethacin (μM)	[³ H]TdR uptake (cpm)
Control	—	—	18937 ± 1214
Fr. 7-C	0.01	—	15942 ± 1529 (19) ^c
	0.1	—	14949 ± 674 ^a (25)
	1	—	8620 ± 837 ^b (65)
	0.12	—	6118 ± 557 ^b (81)
CYA	0.12	—	24724 ± 752
Control	—	10	23084 ± 1094 (8)
Fr. 7-C	0.01	10	21261 ± 2086 (16)
	0.1	10	23485 ± 1461 (6)
	1	10	7910 ± 587 ^b (77)
CYA	0.12	10	

Data show the mean ± S.E. (n=4). Significantly different from control; a) $p < 0.05$, b) $p < 0.001$. c) % inhibition.

TABLE V. Effect of Indomethacin on Enhancement by Fr. 7-C on Induction of Con A-Activated Suppressor Cells

	Indomethacin (μM)	[³ H]TdR uptake (cpm)
Non-activated		70422 ± 1014
Con A-activated		
Control	—	35895 ± 1045 (49) ^b
	0.001	41302 ± 458 (41)
	0.01	37940 ± 964 (46)
	10	44750 ± 1225 (37)
Fr. 7-C	—	20322 ± 763 ^a (71)
(1 μg/ml)	0.001	48541 ± 1167 ^a (31)
	0.01	50060 ± 1508 ^a (29)
	10	44420 ± 1399 (37)

Data show mean ± S.E. (n=8). Significantly different from control; a) $p < 0.001$. b) % suppression.

PGE₂ release decreased dose-dependently when cultured with 10^{-9} – 10^{-5} indomethacin (Fig. 4).

Neutralization of Fr. 7-C Action by Indomethacin As shown in Table IV, the inhibition of the MLR by Fr. 7-C (0.1 and 1 μg/ml) was almost abolished by the addition of 10^{-5} M indomethacin.

The enhancement by Fr. 7-C (1 μg/ml) on Con A-activated Ts induction was also blocked by incubation with 10^{-9} – 10^{-5} M indomethacin in a dose dependent manner (Table V). The effect of CYA was not influenced by indomethacin.

DISCUSSION

In recent years, the immunosuppressive activity of several novel immunomodulating agents (e.g. FK-506, rapamycin, 15-deoxyspergualin, and morpholinoethyl ester of mycophenolic acid, RS-61443) have been studied extensively.⁶⁾ Although relatively free of toxicity at low doses, they may cause side effects with long-term use.^{6,7)} Immunomodulating substances which attack specific target sites of cells are expected to be potent tools for specific disease responses, and also to provide a useful prototype of drugs for immunotherapy.^{6,7)} Few immunosuppressive agents which specifically affect the induction and/or function of Ts cells in subsets of lymphocytes have been reported.⁶⁾

Fr. 7-C, phosphatidylglycerol with C_{14:0}–C_{18:0} isofatty acids, enhances the induction of Con A-activated CD8 positive Ts cells.⁴⁾ The present study revealed that Fr. 7-C inhibited the MLR and the generation of CTL, generally regarded as *in vitro* correlates of allograft rejection.⁸⁾ Thus, it was investigated whether the immunosuppression by Fr. 7-C was related to the induction of Ts cells. The MLR cultures with Fr. 7-C generated effector cells with significant suppressor activity, as compared to control MLR cultures, when added to a fresh autologous MLR. Thus, it is probable that Fr. 7-C exhibits immunosuppression at least partly *via* the enhancement of Ts cell induction. Effector cells treated with Fr. 7-C also enhanced suppressor activity on a fresh MLR with a different stimulator. Moreover, the effector cells showed suppressor activity when added to a fresh MLR with an allogenic responder. Therefore, it seems that antigen-nonspecific Ts cells, which act despite the restriction of major histocompatibility molecules, are induced by Fr. 7-C.

Ts cells are classified into two groups, i.e., antigen specific Ts cells and antigen-nonspecific Ts cells.⁹⁾ A common feature of these Ts cells is that their suppressor functions are at least partly mediated by soluble factors.¹⁰⁾ However, the mechanism of immunosuppression by Ts cells and the biological significance of Ts cells have not been fully elucidated. It is well known that the presence of monocytes is essential to the induction of Ts cells,¹¹⁾ and PGE can induce Ts cells in mice.¹²⁾ PGE has direct immunosuppressive activity,¹³⁾ and reducing PGE levels can alleviate PGE-mediated immunosuppression.¹⁴⁾ Further, PGE can bind directly to lymphoid cells and thereby inhibit their proliferation.¹⁵⁾ It was investigated whether PGE₂ participated in the immunosuppression by Fr. 7-C. Fr. 7-C apparently elevated PGE₂ release in the supernatant of the Con A response of cultured splenocytes which activated Ts cells. PGE₂ release decreased dose-dependently when cultured with indomethacin. The suppression of the MLR and the enhancement of Ts cell induction by Fr. 7-C was abrogated by the addition of indomethacin. Further, the enhancement of Ts cell

induction by Fr. 7-C was blocked in a dose dependent manner by indomethacin. Also, the enhancement of Con A-activated Ts cell induction by Fr. 7-C disappeared by depleting cell populations of Thy-1.2 or CD8 positive lymphocytes.⁴⁾ There is no doubt that Fr. 7-C enhances the induction of antigen-nonspecific Ts cells mediated at least partly by PGE₂. This fraction may act on macrophages that can mainly synthesize PGE among splenocytes.

The immunosuppressive drugs, CYA¹⁶⁾ and CCA,¹⁷⁾ which act on helper T cells and Ts cells, respectively, were used as contrasts to Fr. 7-C. CCA strongly inhibited MLR and CTL, and the inhibition on MLR was not by indomethacin, as expected. CCA showed mild inhibition on MLR and CTL and no effect on MLR-activated Ts cell induction. CCA would stimulate a T cell population that is more weakly activated, thus resulting in the modulation of the immunoresponse to a normal level.¹⁷⁾ The mode of action of Fr.7-C seems to be different from that of CYA or CCA. Further investigations are in progress to clarify the immunosuppressive mechanism of Fr. 7-C.

Acknowledgement We are grateful to the Biochem. Dept., Research and Development Center, Unitika, Ltd., for the supply of materials; to the Biological and Medical Research Dept. of Sandoz, Ltd., for the supply of CYA; and to Chugai Pharmaceutical Co., Ltd., for the supply of CCA.

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