ISOLATION OF *BACILLUS STEAROTHERMOPHILUS* CONSTITUENT THAT SUPPRESSES MOUSE MIXED LEUKOCYTE REACTION *IN VITRO*

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A novel immunosuppressant, Fr.5-B, has been isolated from the debris of *Bacillus stearothermophilus* UK563 autolysate. Fr.5-B suppressed mouse mixed leukocyte reaction at doses ranging from 10^{-4} to 1 $\mu\text{g/ml}$, but not proliferation stimulated by concanavalin A and lipopolysaccharide.

KEYWORDS thermophile; *Bacillus stearothermophilus*; immunosuppressant; mixed leukocyte reaction; cyclosporin A; T cell proliferation

Cyclosporin A (CYA), a fungal metabolite, is an effective immunosuppressant with low myelotoxicity¹⁾ and has been used successfully as the primary drug to suppress the rejection of transplants.²⁾ The immunosuppressive agents which would attack specific target sites of cells are expected to provide a useful prototype of drugs for immunotherapy. Accordingly, we focused on thermophile that contains a variety of components such as lipids and polyamines which are chemically different from those of mesophile.³⁾ We have tested products and constituents of *B. stearothermophilus* for specific inhibitory effects on mixed leukocyte reaction (MLR). As a result, the strain UK563 was found to contain the potent immunosuppressive compound designated as Fr. 5-B. In this paper, we describe the isolation of Fr. 5-B. Biological properties and structural elucidation of Fr. 5-B will be published elsewhere.

EXPERIMENTAL

Isolation *B. stearothermophilus* UK563 (Fermentation Research Institute, Deposit No. 7275) was cultured in a continuous fermenter at 60°C at a flow rate of 400 l/h in a medium (adjusted to pH 7.0) of the following composition: glucose (0.35%), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1%), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005%), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.0001%) and Na_2MoO_4 (0.0001%).⁴⁾ After 4 days culture, the culture broth was separated into cells and fluid by centrifugation. The collected cells were autolyzed by incubation in 25 mM phosphate buffer (pH 8.0)-2 mM ethylenediaminetetraacetic acid at 40°C for 2 h. Supernatant was removed by centrifugation. The cell debris (equivalent to approximately 500 g wet weight of cells) was extracted with hot EtOH. After removal of the solvent, the extract was suspended in deionized water, and the suspension was mixed and extracted with CH_2Cl_2 , three times. The prepared CH_2Cl_2 extract was dissolved in CHCl_3 and fractionated by silica gel column chromatography, which was successively developed with CHCl_3 , CHCl_3 -MeOH (100:1), CHCl_3 -MeOH (50:1), CHCl_3 -MeOH (10:1) and MeOH. Fraction 5 eluted with CHCl_3 -MeOH (100:1) was collected and concentrated under reduced pressure to give a yellow oil (1.1 g) containing the substance that shows appreciable suppression of MLR. The active fraction was dissolved in n-hexane and refractionated by silica gel column chromatography, which was successively developed with n-hexane-AcOEt (2:1) and n-hexane-AcOEt (1:1). Fraction 5-B (Fr.5-B) eluted with n-hexane-AcOEt (2:1) was collected and concentrated under reduced pressure to give Fr.5-B in the form of white powder (91 mg) showing MLR suppression activity.

Drugs Cyclosporin A (CYA) was a generous gift from Sandoz Ltd., Biological and Medical Research (Basel Switzerland). Fr.5-B and CYA were dissolved in ethanol and further diluted in medium to the proper concentrations in culture.

Suppression of MLR The MLR test was performed in flat-bottomed microtiter plates, with each well containing 5×10^5 C57BL/6 responder cells (H-2^b) and 5×10^5 mitomycin C-treated (incubated with 25 $\mu\text{g/ml}$ mitomycin C at 37°C for 30 min and washed 3 times with Hank's balance salt solution) BALB/C stimulator cells (H-2^d) in 0.15 ml PRMI 1640 medium (Nissui) supplemented with 10% fetal calf serum, 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 at 37°C in a humidified atmosphere of 5% CO₂ : 95% air for 112 h pulsed with [³H] thymidine ([³H] TdR, American Radiolabeled Chemicals, Inc., 0.5 $\mu\text{Ci/well}$) 16 h before the end of culture, and harvested onto glass fiber filter papers. The papers were dried and processed for liquid scintillation counting.

Determination of Lymphocyte Proliferation *in Vitro* The splenocytes obtained from a BALB/C mouse were cultured in RPMI 1640 complete medium, at a concentration of 2.5×10^6 cells/ml (5×10^5 cells/well) with or without 1.25 $\mu\text{g/ml}$ concanavalin A (Con A) or 50 $\mu\text{g/ml}$ lipopolysaccharide (LPS) and a test sample in flat-bottom microtiter plates. Cells were cultured for 64 h at 37°C in a humidified atmosphere of 5% CO₂ : 95% air and pulsed with [³H] TdR (0.5 $\mu\text{Ci/well}$) 16 h before the end of the culture. The incorporated [³H] TdR was determined as described for suppression of MLR.

Fig.1. HPLC of Fr.5-B

Column, TSK gel silica - 150 (0.78 X 30 cm); eluant, n-hexane : AcOEt (4 : 1); flow rate, 1.0 ml/min; detection, refractive index. Fr. 5-B (25 μl) was dissolved in n-hexane : AcOEt (4 : 1). Peak 1 is due to the injected solution.

Fig.2. Effect of Fr. 5-B and CYA on Mouse MLR

The data are presented as the percentage of inhibition based on response in the control diluent. Mean cpm of [³H]TdR uptake for mouse MLR was $28,832 \pm 1646$. Unstimulated lymphocytes average 2,726 cpm of [³H]TdR.

RESULTS AND DISCUSSION

In the course of our screening for immunomodulators, we found that *B. stearotherophilus* UK563 contained a T cell-specific immunosuppressant. An active component, Fr.5-B, in the debris of the strain UK563 autolysate for inhibition of the MLR was purified by successive silica gel column chromatographies. The homogeneity was confirmed by HPLC as shown in Fig.1. Fr. 5-B and CYA were tested for ability to suppress the *in vitro* proliferative response of mouse lymphocytes to alloantigen stimulation. The results of representative experiments in which various quantities of Fr. 5-B and CYA were added to mouse MLR at the initiation of culture are illustrated in Fig. 2. The IC₅₀ values of Fr. 5-B and CYA were 1 ng/ml and 11 nM (13 ng/ml), respectively. Then we examined the suppressive effects of Fr. 5-B and CYA on mitogenic response. While Fr. 5-B did not inhibit Con A- and LPS-induced proliferative responses of splenocytes, CYA inhibited both mitogenic responses (Table I). These results suggest that the

TABLE I. Effect of Fr.5-B and CYA on Mitogenic Responses of Mouse Splenocytes

Sample	Concentration (ng/ml)	[³ H]TdR uptake (cpm, mean ± s.e., n = 4)			
		Con A		LPS	
Control	—	57963 ± 2632		32001 ± 1012	
Fr.5-B	0.001	60494 ± 3674	(0) ^{a)}	32008 ± 893	(0)
	0.1	60494 ± 1941	(0)	31401 ± 1127	(2)
	10	60999 ± 973	(0)	33127 ± 1498	(0)
	1000	61361 ± 2447	(0)	27496 ± 991*	(14)
CYA	10 ^{b)}	40929 ± 2573**	(30)	24105 ± 1052**	(25)
	100	13660 ± 696***	(77)	15380 ± 1418***	(53)

a) % Inhibition. b) nM. * p < 0.05, ** p < 0.01, *** p < 0.001; versus control.

immunosuppressive mechanism of Fr. 5-B was unique and different from those of CYA and FK506. CYA and FK506 affected mainly the stage of interleukin-2 production induced by alloantigen and Con A stimulation.⁵⁾ As far as the cytotoxicity tests are concerned, Fr. 5-B was non-toxic at less than 1 µg/ml against splenocytes used in the MLR (data not shown). Fr. 5-B specifically inhibited the proliferative response of lymphocytes to alloantigen stimulation at non-toxic concentrations. The action mode and immunosuppressant activity *in vivo* of Fr.5-B should be further studied.

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