CYTOBLASTIN, A LOW MOLECULAR WEIGHT IMMUNOMODULATOR PRODUCED BY *Streptoverticillium eurocidicum*

Sir:

In the course of screening for immunomodulators modulating T cell functions, we found a novel, low MW immunomodulator named cytoblastin in a culture filtrate of the strain, MI43-37F11 which was isolated from the soil sample collected in Yokohama City, Kanagawa Prefecture, Japan. The strain was identified as *Streptoverticillium eurocidicum* on the basis of its cultural properties[1]. Cytoblastin showed low cytotoxicity and no antimicrobial activity and promoted the proliferation of T cells. In this communication, the production, isolation, physico-chemical properties, structure and biological properties are reported.

A slant culture of the strain, MI43-37F11 was inoculated into 110 ml of the medium consisting of galactose 2.0%, dextrin 2.0%, soy peptone 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, silicone oil 0.003% (adjusted to pH 7.4 before sterilization) and incubated at 30°C for 2 days on rotary shaker (180 rpm). For production of cytoblastin, 2.5 ml of the culture was transferred to 125 ml of the production medium consisting of glycerol 2.0%, soy bean meal (Ajinomoto Co., Inc.) 1.5%, KH_2PO_4 0.1%, $CoCl_2 \cdot 6H_2O$ 0.0005%, silicone oil 0.003%, (pH 6.2 adjusted with 1 N K_2HPO_4 before sterilization) in Sakaguchi flask (500 ml) and cultured at 27°C for 4 days on a reciprocating shaker at 120 times per minute.

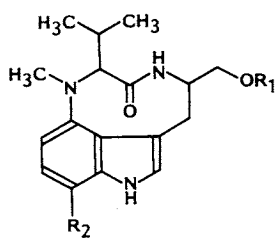
The culture filtrate (9 liters) was extracted with EtOAc. The EtOAc extract was concentrated under a reduced pressure to give an oily residue (2.0 g) and it was applied to silica gel column. After washing the column with chroloform - methanol (10 : 1), the

Table 1. Physico-chemical properties of cytoblastin.

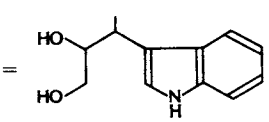
Appearance	White powder
FAB-MS (m/z)[a]	
Positive	491
Negative	489
HREI-MS (m/z)	
Calcd for $C_{28}H_{34}N_4O_4$:	490.2580
Found:	490.2605
MP	177~182°C (dec)
$[\alpha]_D^{21}$ (c 0.493, MeOH)	−110°C
UV λ_{max}^{MeOH} nm (log ε)	202 (5.06), 225 (4.96), 283 (4.33), 290 (sh, 4.21), 300 (sh, 4.21)
IR (KBr) cm^{-1}	3480, 1680, 1630, 1570, 1510, 1230, 1190, 1170, 1110, 1090, 980, 860, 840, 710, 690
Solubility:	
Easily soluble	MeOH, DMSO
Soluble	Acetone, $CHCl_3$, EtOAc
Insoluble	n-Hexane, H_2O
Color reaction	Ehrlich, $FeCl_3$ - $HClO_4$
Rf value[b]	0.50

[a] Glycerol matrix.
[b] Silica gel TLC (Merck Art. No. 5715), $CHCl_3$ - MeOH (8 : 2).

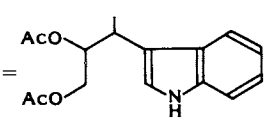
Fig. 1. Structure of cytoblastin (**I**), triacetylcytoblastin (**II**) and indolactam V (**III**).



Cytoblastin (**I**) $R_1 = H$ $R_2 =$



Triacetylcytoblastin (**II**) $R_1 = Ac$ $R_2 =$



Indolactam V (**III**) $R_1 = R_2 = H$

active substance was eluted with the solvent ratio at 8:2. The active eluate was concentrated under a reduced pressure to give a crude powder (0.5 g) and subjected to a reverse phase HPLC (Senshu pak ODS330IN, 20×250 mm). It was eluted with linear gradient solution of 50 to 100% methanol in H_2O. After concentration of the active fraction, the resultant yellow powder (10 mg) was applied on Sephadex LH-20 column (MeOH). Active fraction was evaporated to dryness *in vacuo*. Cytoblastin was obtained as white powder (3 mg).

Physico-chemical properties of cytoblastin (**I**) are summarized in Table 1. The FAB-MS spectra of cytoblastin revealed that the molecular ion peaks at *m/z* 491 ($(M+H)^+$) and *m/z* 489 ($(M-H)^-$). The molecular formula of cytoblastin was confirmed as $C_{28}H_{34}N_4O_4$ by HREI-MS.

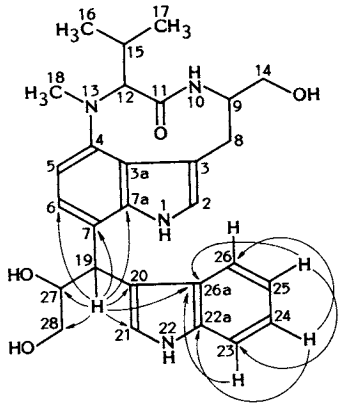
The presence of an indole moiety in cytoblastin was indicated by its UV spectrum at 225, 283 and 290 nm and positive color reaction (purple) with Ehrlich reagent on silica gel TLC. The ^1H NMR spectra of cytoblastin were measured in acetone-d_6, methanol-d_4, pyridine-d_5 and acetic acid-d_4. All spectra showed the existence of two conformers changing the ratio of the mixture in these tested solvents. The ^1H NMR and UV spectra of cytoblastin resembled substances of the teleocidin group. In the ^1H NMR spectra of teleocidin de-

Table 2. NMR data of cytoblastin and indolactam V.

	Cytoblastin (Acetone-d_6)			Indolactam V ($CDCl_3$)	
Ratio[a]		3:1		4:1	
Position	^{13}C	^1H	J (Hz)	^1H	J (Hz)
1	—	(~10.11) br	—	8.05 br	—
		(~10.01) br	—		
2	122.81	6.95 br	—	6.88 s	—
3	114.81	—	—	—	—
3a	118.26	—	—	—	—
4	146.92	—	—	—	—
5	106.57	6.42 d	8.0	6.50 d	8.0
6	123.94	7.11 d	8.0	7.06 t	8.0
7	119.28	—	—	6.90 d	8.0
7a	140.00	—	—	—	—
8	35.05	3.00 d, d	3.2, 17.0	3.06 dd	17.0, 3.0
		3.08 br d	17.0	3.17 br d	17.0
9	56.66	4.16 m	—	4.31 m	—
10	—	6.38 br	—	7.42 br	—
11	173.06	—	—	—	—
12	71.83	4.36 d	10.0	4.40 d	9.0
13	—	—	—	—	—
14	65.39	3.42 m	—	3.57 dd	8.0, 7.0
		3.63 m	—	3.74 dd	8.0, 7.0
15	29.19	2.54 m	—	2.59 m	—
16	19.96	0.56 d	6.5	0.63 d	6.5
17	21.86	0.86 d	6.5	0.93 d	6.5
18	33.08	2.84 s	—	2.91 s	—
19	42.12	4.77 d	6.0		
20	117.32	—	—		
21	123.85	7.44 s	—		
22	—	~10 br	—		
22a	137.51	—	—		
23	111.98	7.32 d	8.0		
24	121.92	7.01 t	8.0		
25	119.16	6.86 t	8.0		
26	120.17	7.46 d	8.0		
26a	128.49	—	—		
27	75.55	4.58 m	—		
28	65.82	3.52 m	—		

[a] Ratio = major conformer: minor conformer in the solution. The assignment of the major component are shown.

Fig. 2. A part of HMBC correlation observed in cytoblastin (acetone-d_6).



rivatives, ENDO *et al.*[2] reported the existence of the two stable conformational states in the solution.

The ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and heteronuclear multiple-bond correlation (HMBC) spectra of **I** in deuteroacetone were analyzed to elucidate the structure of cytoblastin. The presence of indolactam V moiety in cytoblastin molecule is obvious from the NMR data (Table 2). Connectivity of the indolactam V skeleton to a side chain containing another indole moiety was established by HMBC spectroscopy: cross peaks were observed between 19-H and the following signals of C-6, C-7, C-7a, C-27, C-28, C-20, C-21, and C-26a, as shown in Fig. 2. Upon treatment with acetic anhydride and pyridine at room temperature, **I** gave a triacetylcytoblastin (**II**) (EI-MS, *m/z* 617 $(M+1)^+$). The ^{1}H NMR spectrum of **II** in $CDCl_3$ showed three acetyl groups at 2.08, 2.02 and 1.94 ppm, and two set of signals corresponding to a major and a minor conformer (1.7 : 1). We propose the structure of cytoblastin as 7-[2,3-dihydroxy-1-(3-indolyl)propyl]indolactam V. Determination of the absolute structure of cytoblastin remains to be elucidated.

According to the method described previously[3], the activity of cytoblastin was determined on incorporation of [^{3}H]thymidine into nylon wool-passed rat spleen cells (T cell-rich preparation) treated with concanavalin A (Con A). Con A-treated cells were cultured with cytoblastin and pulsed with [^{3}H]thymidine 18 hours before termination of culture. The incorporation of [^{3}H]thymidine into cultured cells was measured by a liquid scintillation counter. The effect of cytoblastin was shown in Table 3. Cytoblastin at 0.39 to 6.25 μg/ml increased the incorporation but not at 25 μg/ml. The stimulatory

Table 3. Effect of cytoblastin on the proliferation of T cells pretreated with Con A.

Dose (μg/ml)	cpm (mean $\pm$ SD)	T/C (%)
0	789 $\pm$ 84	100
0.1	900 $\pm$ 30	114
0.39	1,749 $\pm$ 433	222
1.56	4,760 $\pm$ 762	603
6.25	2,807 $\pm$ 268	356
25.0	258 $\pm$ 135	33

index (cpm of treated/cpm of non-treated) was 2 to 6.

As described above, the structure of cytoblastin is resemble to teleocidins and indolactam V, and those are known to be a tumor-promoter as well as an inflammatory agent[4]. To estimate the tumor-promoter activity of cytoblastin, we tested the effect of cytoblastin on the inhibition of [^{3}H]phorbol-12,13-dibutyrate (PDBu) binding to A431 cells[5] and on the translocation of protein kinase C from cytosol to membrane in A431 cells[6]. At the doses (0.4 $\sim$ 1 μg/ml) promoting the proliferation of T cells, cytoblastin did neither inhibit PDBu binding and nor induce the translocation. Next, the inflammatory activity of cytoblastin on mouse ear skin was tested. Each dose of cytoblastin and of teleocidin B and indolactam V as control was dissolved in 10 μl of MeOH and put on mouse ear skin (ICR, female, 6 weeks old). After 24 hours, inflammatory response of mouse ear skin (ICR, female, 6 weeks old) with a redden swelling was examined. Although teleocidin B and indolactam V were inflammatory at 0.2 μg/ear and 15 μg/ear, respectively, cytoblastin did not show any response up to 640 μg/ear. The acute toxicity (LD_{50}) of each substance by single ip injection to ICR mice (female, 6 weeks old) was as follows; $>$ 100 mg/kg in cytoblastin, 0.25 mg/kg in teleocidin B, 1.25 mg/kg in indolactam V. These results indicate that cytoblastin has different biological properties from teleocidin B and indolactam V.

Cytoblastin at 100 μg/ml showed no cytotoxicity against L1210, P388, EL-4, IMC carcinoma cells and human stomach cancer cells, SC-6 and had no antimicrobial activity against bacteria and fungi.

The immunomodulating activity of cytoblastin is now under study.

Acknowledgment

We would like to thank Dr. MASAYA IMOTO for his

advice and encouragement. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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(Received April 22, 1991)

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