DELAMINOMYCINS, NOVEL NONPEPTIDE EXTRACELLULAR MATRIX RECEPTOR ANTAGONIST AND A NEW CLASS OF POTENT IMMUNOMODULATOR

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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In order to develop a new class of immunomodulator we have searched for a low molecular weight inhibitors of cell adhesion to components of extracellular matrix (ECM), fibronectin (FN), laminin (LM) and collagen type IV (CL), and succeeded to find a group of novel antibiotics, named delaminomycins. Delaminomycins were isolated from the mycelium and cultured broth of *Streptomyces albulus* MJ202-72F3. It was purified by use of centrifugal partition chromatography (CPC), preparative reverse phase HPLC and Sephadex LH-20 and was obtained as a white powder. Delaminomycins with inhibitory activity for cell adhesion to ECM components suppressed immune responses *in vitro* and *in vivo* and exhibited antimicrobial activity on Gram-positive bacteria.

It is known that most host-mediated responses involve immune responses, and metastases or invasion of tumors results from cell-cell interactions among various kinds of cells which interact with ECM receptors. In order to develop a new class of immunomodulator or an inhibitor of tumor metastases, we have sought a low molecular weight inhibitor of cell adhesion in microbial products to components of the ECM and found a novel antibiotic, named delaminomycin, in cultured mycelium of *Streptomyces albulus* MJ202-72F3. Delaminomycins are non-peptide[1~3] and inhibit the adhesion of tumor cells to components of the ECM. In this paper, we report the taxonomy, fermentation, isolation and biological activity of delaminomycins A, B and C.

Cell Adhesion Assay

We employed Con A-activated EL4 cells adhesion assay for screening of ECM antagonists in cultured broth of microorganisms and for assessment of the activity of each fraction during purification of delaminomycins. The inhibitory activity of EL4 cells adhesion assay was determined as follows.

Con A-activated EL4 cell adhesion assay: Cell adhesion assays were performed by the partially modified methods reported by GRAF *et al.*[4,5], SHIMIZU *et al.*[6] and HERSHKOVIZ *et al.*[7]. EL4 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). After cells were grown in culture bottles up to 80% confluence, they were washed with RPMI medium and resuspended in RPMI medium containing 1% FCS, 10 μg/ml of Con A and 10 mM HEPES. FN (porcine plasma, Iwaki Glass) or LM (mouse EHS sarcoma, Iwaki Glass) were added to the wells: 0.5 μg of FN or 1.25 μg of LM in 50 μl of PBS per well for 17 hours at 10°C. Protein-coated microwells were rinsed twice with PBS and blocked for

30 minutes with 1% bovine serum albumin (BSA) in PBS. 10 μl of cultured broth or test samples and 10^5 cells in 100 μl of the medium were added onto the protein-coated microwells and incubated for 1 hour at 37°C in 5% CO_2, 95% air. After incubation, plates were gently washed three times with warmed PBS (37°C) to remove non-adherent cells. Adherent cells were fixed with 20 μl of glutaraldehyde (GA) per well for 30 minutes at room temperature, then washed with tap water and dried in oven for 10 minutes at 60°C to remove GA. The fixed cells were stained with 100 μl of 0.4% crystal violet in 20% aqueous MeOH per well for 10 minutes at room temperature, then washed with tap water three times and dried. To extract the dye, 100 μl of 1 mM HCl in 30% aqueous EtOH was added into each well. The A_{540} of individual wells was measured using a microtiter plate reader (Titertek Multiskan). Each assay was performed in duplicates.

Taxonomy

The producing microorganism, strain MJ202-72F3, was isolated from a soil sample collected in Ohtsuki-shi, Yamanashi Prefecture, Japan. The cultural characteristics of strain MJ202-72F3 are shown in Table 1. Strain MJ202-72F3 has a branched vegetative hyphae and the end of aerial hyphae in the form of spiral. Mature spore chains were moderately long, with 10 to 50 or more spores per chain. Spore surface was spiny or warty. No sporangia, motile spores or synnemata were observed. Vegetative mass color was colorless to pale yellow on the various media and aerial mass color was in the gray color series on sucrose-nitrate agar and yeast extract-malt extract agar as described in Table 1. No pigment was found in the medium in sucrose-nitrate agar, yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, or glycerol-asparagine agar.

Physiological characteristics of strain MJ202-72F3 are shown in Table 2.

The whole cell hydrolysate contained LL-type of diaminopimelic acid. Melanoid pigments were not formed in peptone-yeast extract-iron agar, tyrosine agar, or tryptone-yeast extract broth.

Accordingly, strain MJ202-72F3 is considered to be in the genus *Streptomyces*. Among *Streptomyces*, *Streptomyces albulus*[8,9] and *Streptomyces natalensis*[10] are similar to strain MJ202-72F3. Strain MJ202-72F3 is a little different from both *S. albulus* and *S. natalensis* in liquefaction of gelatin, coagulation of milk, peptonization of milk and hydrolysis of starch. However, these characteristics are thought to easily change under the environmental conditions. Strain MJ202-72F3 is closely related to *S. albulus* in aerial

Table 1. Cultural characteristics of strain MJ202-72F3.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Colorless~pale yellow	Light gray (2fe, covert gray)	None
Yeast extract-malt extract agar (ISP No. 2)	Pale yellow (1½ic, lt antique gold)	Light gray (2fe, covert gray)	None
Oatmeal agar (ISP No. 3)	Pale yellow	Scant, white	None
Inorganic salts-starch agar (ISP No. 4)	Colorless~pale yellow	Scant, light gray (3fe, silver gray)	None
Glycerol-asparagine agar (ISP No. 5)	Pale yellow (2gc, bamboo)	Thin, white~light gray (3fe, silver gray)	None
Tyrosine agar (ISP No. 7)	Pale yellow (2gc, bamboo)	Thin, white~light gray (3fe, silver gray)	None
Glucose-asparagine agar	Pale yellow	None	None
Nutrient agar	Pale yellow	None	None

The color names used in this table were based on the Color Standard (Nihon Sikisai Co., Ltd.) and Color Harmony Manual (Container Corporation of America).

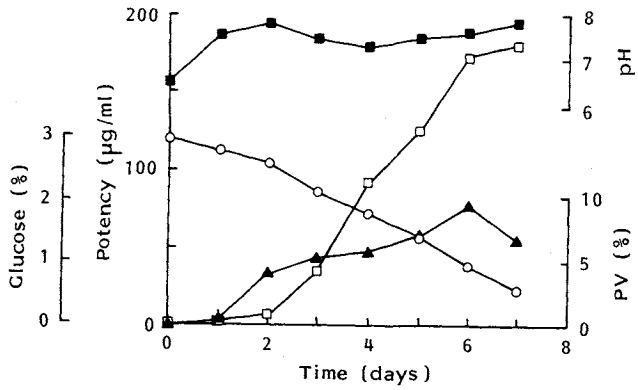
Table 2. Physiological characteristics of strain MJ202-72F3.

Temperature range for growth (°C)	20~37	Utilization of*	
Optimum temperature (°C)	27~30	D-Glucose	+
Formation of melanoid pigment		L-Arabinose	−
ISP No. 1	Negative	D-Xylose	d
ISP No. 6	Negative	D-Fructose	d
ISP No. 7	Negative	Sucrose	−
Liquefaction of gelatin	Negative	Inositol	+
Coagulation of milk	Negative	Rhamnose	−
Peptonization of milk	Variable	Raffinose	−
Hydrolysis of starch	Negative	D-Mannitol	−
Nitrate reduction	Negative		

* +; Growth, d; doubtful, −; no growth.

Fig. 1. Time course of delaminomycin A production.

■ pH, ▲ PV, ○ glucose, □ potency.



mass color and utilization of carbon sources. Therefore, it was designated as *S. albulus* MJ202-72F3. The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-12674.

Fermentation

The strain MJ202-72F3 on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of a seed medium and cultured at 30°C for 3 days on a rotary shaker (180 rpm). The seed medium consisted of glucose 1.5%, yeast extract (Nippon-Seiyaku) 0.25%, casamino acids (Difco) 0.25%, $CaCO_3$ 0.4% and pH of the medium was not adjusted. Two ml of this seed culture was inoculated into 110 ml of a production medium in 500-ml Erlenmeyer flask and cultured at 28°C for 6 days on a rotary shaker (180 rpm) for production. The production medium was consisted of glucose 3.0%, yeast extract 0.5%, casamino acids 0.5%, $NaNO_3$ 0.2%, KCl 0.2%, $CaCO_3$ 0.4% and pH of the medium was not adjusted. A typical fermentation profile for production of delaminomycin A in cultured mycelium is shown in Fig. 1. The activity of delaminomycins was assessed by the inhibitory activity of 10 μl of broth and MeOH extract of mycelium against Con A-activated EL4 cells adhesion assay. The amounts of delaminomycins were determined by HPLC using a CAPCELL PAK C_{18} column (Shiseido) with a mobile phase of MeOH - CH_3CN - 2-PrOH - 25 mM NH_4OAc (30 : 30 : 5 : 35) at 35°C. Although delaminomycins were found in both cultured broth and mycelium, the active principle was in the mycelium (data not shown).

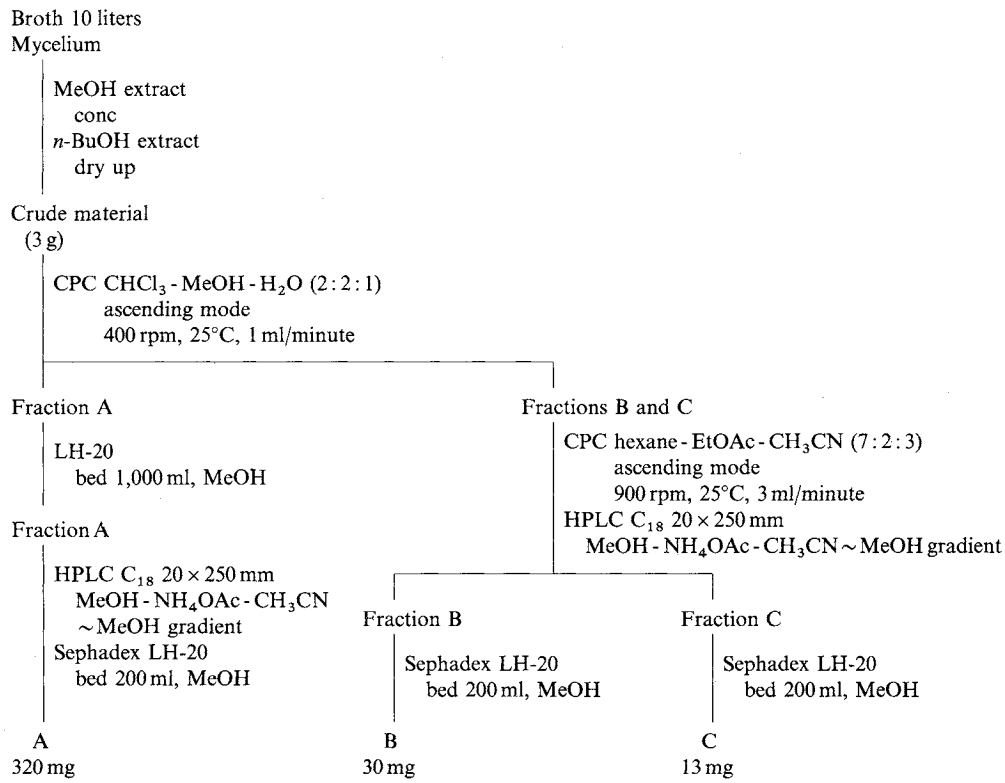
Delaminomycin A was obtained as the main component in delaminomycins, and the production ratio of delaminomycins A, B and C was approximately 100 : 10 : 5. After 6 days cultivation, delaminomycin A accumulated in mycelium up to 180 μg/ml.

Isolation and Purification

The procedure for isolation purification of delaminomycins is shown in Fig. 2. The activity of delaminomycins was assessed Con A-activated EL4 cells adhesion assay. Each fraction in the process of isolation was diluted with MeOH and assessed.

The cultured broth (10 liters) was centrifuged and the mycelium was harvested. The mycelium was extracted twice with five volumes of MeOH per wet weight of mycelium. The extract was concentrated under reduced pressure to give an aqueous solution. The aqueous solution was extracted twice with *n*-BuOH and the organic layer was evaporated under reduced pressure. The crude material (3 g) was subjected to centrifugal partition chromatography (CPC, Sanki Engineering) previously equilibrated with the lower layer of $CHCl_3$ - MeOH - water (2 : 2 : 1) at 25°C, 400 rpm. Delaminomycin A was eluted with the mobile phase of the upper layer of $CHCl_3$ - MeOH - water (2 : 2 : 1) in the ascending mode and evaporated under reduced pressure. Delaminomycins B and C remained in the immobile phase in the ascending mode, and were eluted with the lower layer of $CHCl_3$ - MeOH - water (2 : 2 : 1) in descending mode and evaporated under reduced pressure. The residue containing delaminomycin A was applied to a reverse phase HPLC column (CAPCELL PAK C_{18} 20 × 250 mm, flow rate 5 ml/minute) and eluted with a gradient of MeOH - 25 mM NH_4OAc - CH_3CN (10 : 60 : 30 ~ 60 : 10 : 30 linear gradient in 90 minutes). The active fractions

Fig. 2. Purification of delaminomycins A, B and C.



containing delaminomycin A were concentrated under reduced pressure and then loaded onto a Sephadex LH-20 column. The activity was eluted with MeOH and concentrated under reduced pressure to give pure delaminomycin A as a colorless powder (320 mg). The fraction containing delaminomycins B and C was injected to CPC previously equilibrated with the lower layer of *n*-hexane - EtOAc - CH_3CN (7 : 2 : 3) at 25°C, 900 rpm. Delaminomycin B and C fractions remaining in the immobile phase in ascending mode, were eluted with the lower layer of *n*-hexane - EtOAc - CH_3CN (7 : 2 : 3) in the descending mode, and were concentrated under reduced pressure. The resulting residue was applied to a reverse phase HPLC C_{18}-column and eluted by the same gradient system as described above. Active fractions B and C were collected and concentrated under reduced pressure, and then loaded onto a Sephadex LH-20 column and eluted with MeOH. Then each active fraction was concentrated to dryness and 30 mg of delaminomycin B and 13 mg of delaminomycin C were obtained as a colorless powder.

Biological Activity

Con A-activated EL4 Cell Adhesion Assay

Various amounts of delaminomycins and 10^5 cells in 100 μl of assay medium were added onto the protein-coated microwells and incubated for 1 hour at 37°C in 5% CO_2, 95% air. Each assay was performed in triplicate. The viability of EL4 cells treated with test samples for 60 minutes was checked by the trypan blue dye exclusion test. As shown in Table 3, all delaminomycins inhibited Con A-activated adhesion of EL4 cells to each ECM component at 4.8 to 13.7 μg/ml.

B16 Cell Adhesion Assay

B16 melanoma cell adhesion assays were performed using the modified method reported by GRAF *et al.*[4,5]. Briefly, B16 cells were maintained in DULBECCO's modified EAGLE's medium (DMEM) supplemented with 10% FCS. The cells grown up to 80% confluence in culture bottles were detached with a solution of 2 mM EDTA, after washing, resuspended in serum-free DMEM containing 0.1% BSA, 25 μg/ml of cycloheximide and 20 mM HEPES. Delaminomycins at different concentrations and B16 cells (10^5 in 100 μl) were added to the ECM protein-coated wells including CL. The plate was incubated for 1 hour at 37°C in 95% air and 5% CO_2 and adhesion assays were performed in triplicate as described above. As shown in Table 4, delaminomycins A and B inhibited adhesion of B16 melanoma cells to FN, LM and CL, although C showed no inhibition on FN at 100 μg/ml. Delaminomycin A showed stronger inhibitory activity than B and C.

Mixed Lymphocytes Culture Reaction (MLCR)

MLCR assays were performed by the method described previously[11]. Spleen cells (nylon wool-passed)

Table 3. Inhibition of adhesion of Con A-activated EL4 cells to ECM components by delaminomycins.

Compounds	IC_{50} (μg/ml)	
	Fibronectin	Laminin
A	6.7	13.7
B	4.8	13.4
C	11.3	6.8

Table 4. Inhibition of adhesion of B16 melanoma cells to ECM components by delaminomycins.

Compounds	IC_{50} (μg/ml)		
	Fibronectin	Laminin	Collagen type IV
A	6.0	6.5	3.3
B	17.0	17.0	14.0
C	>100	25.0	14.5

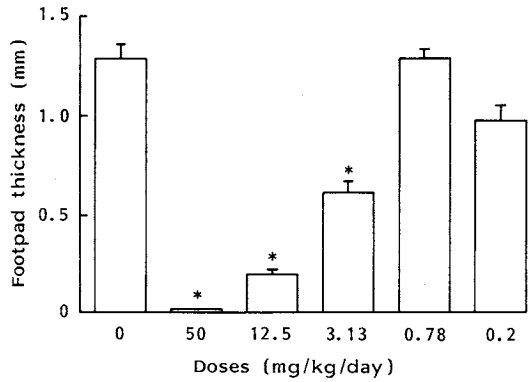
Table 5. Inhibitory effect of delaminomycins on MLCR.

Compound	Dose (μg/ml)	Mean $\pm$ S.D. ($\times 10^3$ cpm)	Inhibition (%)	IC_{50} (μg/ml)
A	100	0.13 $\pm$ 0.04	96.4[a]	
	25	0.18 $\pm$ 0.04	95.0[a]	
	6.25	2.37 $\pm$ 0.50	35.3[b]	8.6
	1.56	3.01 $\pm$ 0.89	17.5	
	None	3.65 $\pm$ 0.29	0	
B	25	0.26 $\pm$ 0.15	95.0[a]	
	6.25	0.25 $\pm$ 0.02	95.1[a]	
	1.56	2.15 $\pm$ 0.43	58.4[a]	1.1
	0.39	3.73 $\pm$ 0.53	27.9[c]	
	None	5.18 $\pm$ 0.18	0	
C	6.25	0.14 $\pm$ 0.07	97.3[a]	
	1.56	0.57 $\pm$ 0.23	89.0[a]	
	0.39	3.02 $\pm$ 0.72	41.6[c]	0.5
	0.10	4.17 $\pm$ 0.45	19.5[c]	
	None	5.18 $\pm$ 0.18	0	

[a] $P<0.001$ as compared with control.
[b] $P<0.01$.
[c] $P<0.05$.

taken from Fisher F344 rats as the responder were mixed with spleen cells taken from WKY rats as the stimulator which had been previously incubated with 50 μg/ml of mitomycin C at 37°C for 20 minutes. The mixed cells were cultured with or without drugs in medium containing 10% FCS at 37°C for 5 days in 5% CO_2 in air and [^{3}H]thymidine was added 16 hours before assay. MLCR was determined by measuring the incorporation of [^{3}H]thymidine into the cultured cells. Delaminomycins dissolved in MeOH were diluted with RPMI-1640 and added to cultures. Triplicate determinations were made. As shown in Table 5, delaminomycins A, B and C inhibited MLCR in a dose dependent manner and the IC_{50} values were 8.6 μg/ml, 1.1 μg/ml and 0.5 μg/ml, respectively.

Fig. 3. Suppressive effect of delaminomycin A on DTH response to SRBC in mice.



Delaminomycin A was dissolved in DMSO-Tween 80-saline (9:1:90) and given ip dialy from days 0 to 4 after immunization. Vehicle (0.25 ml) was given ip as same schedule for drug. $P<0.001$ (*) as compared with vehicle group.

Effect of Delaminomycin A on Immune Responses to Sheep Red Blood Cells (SRBC) in Mice

The effect of delaminomycin A on immune responses to SRBC in mice was investigated as follows.

Antibody formation: Female CDF_1 mice (10 weeks old) were immunized on day 0 with 1×10^8 SRBC iv. Antibody formation was determined on day 4 by enumerating plaque forming cells (PFC) according to the method described previously[12]. Drug was administrated ip daily from days 1 to 3 after immunization. Delaminomycin A in doses of 0.2 to 50 mg/kg did not suppress antibody formation.

Delayed-type hypersensitivity response (DTH): CDF_1 mice were immunized iv with 1×10^5 SRBC. Four days later, 1×10^8 SRBC was injected subcutaneously into the left hind footpad. Twenty four hours

after the elicitation, footpad thickness was measured with a caliper[13]. Drug was injected ip daily for 5 days starting from the day of immunization. As shown in Fig. 3, delaminomycin A at doses of 3.13 to 50 mg/kg strongly suppressed the DTH response.

Cytotoxicity

The cytotoxicity of delaminomycins on tumor cells is shown in Table 6. Tumor cells were cultured in RPMI-1640 containing 10% FCS with test samples for 72 hours and cytotoxicity was determined by MTT assay. Among delaminomycins, C showed the strongest cytotoxic activity at concentration of lower than 10 μg/ml on the tumor cells tested except for L1210. L1210 cells were more resistant to delaminomycins than other tumor cells.

Table 6. Cytotoxicity of delaminomycins on tumor cells in cultures.

Tumor cells	IC_{50} (μg/ml)		
	A	B	C
L1210	>100	30.0	42.0
P388D1	14.0	13.3	3.1
EL4	17.9	11.1	1.9
B16	21.1	>100	7.4

Antimicrobial Activity

Antimicrobial activity of delaminomycins was examined by the serial agar dilution method using Mueller Hinton agar (Difco) for antibacterial tests with incubation at 37°C for 18 hours and a nutrient agar containing 1% glucose for antifungal tests with incubation at 27°C for 42 hours. The minimum

Table 7. Antimicrobial activity of delaminomycins.

Microorganisms	MIC (μg/ml)			Microorganisms	MIC (μg/ml)		
	A	B	C		A	B	C
Staphylococcus aureus FDA209P	12.5	6.25	3.12	*Providencia rettgeri* GN466	>100	>100	>100
				Serratia marcescens	>100	>100	>100
S. aureus Smith	25	6.25	3.12	*Pseudomonas aeruginosa* A3	>50	>50	>50
S. aureus MS9610	25	6.25	3.12	*P. aeruginosa* GN315	>100	>100	>100
S. aureus No. 5 (MRSA)	25	6.25	3.12	*Klebsiella pneumoniae* PC1602	>100	>100	>100
S. aureus No. 17 (MRSA)	25	6.25	3.12				
Micrococcus luteus FDA16	6.25	3.12	1.56	*Mycobacterium smegmatis* ATCC 607	>100	>100	>100
M. luteus IFO3333	6.25	3.12	1.56				
M. luteus PCI1001	100	6.25	1.56	*Candida tropicalis* F-1	>100	>100	>100
Bacillus anthracis	3.12	1.56	<0.78	*C. pseudotropicalis* F-2	>100	>100	>100
B. subtilis NRRL B-558	6.25	6.25	3.12	*C. albicans* 3147	>100	>100	>100
B. subtilis PCI219	6.25	6.25	3.12	*C.* Yu-1200	>100	>100	>100
B. cereus ATCC 10702	6.25	3.12	1.56	*C. krusei* F-5	>100	>100	>100
Corynebacterium bovis 1810	3.12	6.25	3.12	*Saccharomyces cerevisiae* F-7	>100	>100	>100
Escherichia coli NIHJ	>100	>100	>100				
E. coli K-12	>100	>100	>100	*Cryptococcus neoformans* F-10	>100	>100	>100
E. coli K-12 ML1629	>100	>100	>100				
E. coli BEM11	>100	>100	>100	*Cochliobolus miyabeanus*	>100	>100	>100
E. coli BE1121	>100	>100	>100	*Pyricularia oryzae*	>100	>100	>100
E. coli BE1186	>100	>100	>100	*Pellicularia sasakii*	>50	>50	>50
Shigella dysenteriae JS11910	>100	>100	>100	*Xanthomonas citri*	>100	100	100
S. flexneri 4b JS11811	>100	>100	>100	*X. oryzae*	>100	100	100
S. sonnei JS11746	>100	>100	>100	*Trichophyton asteroides* 429	>100	100	>100
Salmonella typhi T-63	>100	>100	>100	*T. mentagrophytes* F-15 (833)	>100	>100	>100
S. enteritidis 1891	>100	>100	>100				
Proteus vulgaris OX19	>100	>100	>100	*Aspergillus nigar* F-16	>100	>100	>100
P. mirabilis IFM OM-9	>100	>100	>100	*A. fumigatus* F-181	>100	>100	>100
Providencia rettgeri GN311	>100	>100	>100	*Pseudomonas fluorescens*	>100	>100	>100

inhibitory concentration (MIC) value is expressed as the minimum concentration which inhibits growth of the microorganisms. As shown in Table 7, delaminomycins A, B and C showed antibacterial activity only against Gram-positive bacteria but not against fungi.

Toxicity

The LD_{50} value of delaminomycin A was found to be more than 500 mg/kg ip to ICR mice.

Discussion

In order to develop a new class of immunomodulators and an inhibitor of tumor metastases from microbial products, we have sought ECM receptor antagonists from cultured broths of 3,600 strains and found a low molecular weight inhibitor, named delaminomycins.

When EL4 cells were activated by Con A, they showed the same ability to adhere to LM and FN as that of T cells. Adhesion of T cells to ECM components requires stimulation of T cells by antigen or mitogen[6,7]. Since adhesion of non-activated EL4 cells to ECM protein coated wells was not observed (data not shown), we employed Con A-activated EL4 cells instead of T cells for assay. As shown in Table 3, delaminomycins inhibited adhesion of Con A-activated EL4 cells to ECM components. In this assay, however, it was not excluded whether the effect might be due to inhibition of the binding of Con A to EL4 cells and/or of signal transduction induced by Con A. To elucidate this possibility, we tested cell adhesion assay using B16 melanoma cells instead of EL4 cells because B16 cells have an active receptor for FN, LM and CL[4,5,14,15]. Results shown in Table 4 indicate that delaminomycins inhibited adhesion of B16 melanoma cells to the ECM components to the same degree as EL4 cells. Thus, it can be concluded that delaminomycins are neither inhibitors of Con A binding to EL4 cells nor inhibitors of signal transduction induced by Con A, but rather are inhibitors of cell adhesion to ECM components.

To determine the effect of delaminomycins against immune systems *in vivo* and *in vitro*, we investigated effects of delaminomycins on MLCR *in vitro* and delaminomycin A on antibody formation and DTH *in vivo*. Results suggest that delaminomycins suppressed T cell-mediated immune responses although delaminomycin A did not inhibit antibody formation to a T-dependent antigen, SRBC in mice. Thus, it can be considered that delaminomycin A may inhibit generation of effector T cells or may block the effector-target interaction. Therefore, we concluded that delaminomycins, novel antagonists for ECM receptors, are a new class of immunomodulator.

The physico-chemical properties and structural elucidation of the delaminomycin A will be reported[16].

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