EFFECTS OF PRODIGIOSIN 25-C ON CULTURED CELL LINES: ITS SIMILARITY TO MONOVALENT POLYETHER IONOPHORES AND VACUOLAR TYPE H^+-ATPase INHIBITORS

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Prodigiosin 25-C inhibited the proliferation of various cultured cell lines more strongly when concanavalin A (Con A) was added to the cultures. The increase in sensitivity was most evident in T lymphoma YAC-1 cells. The combination of prodigiosin 25-C and Con A induced characteristic morphological changes in these cells. In the presence of Con A, monovalent polyether ionophores and vacuolar type H^+-ATPase inhibitors induced effects similar to those of prodigiosin 25-C on YAC-1 cells. Prodigiosin 25-C had neither K^+ionophore activity nor inhibitory effect on vacuolar type H^+-ATPase. A Golgi mannosidase II inhibitor, swainsonine, inhibited the proliferation of YAC-1 cells only when Con A was added. Prodigiosin 25-C and swainsonine increased Con A binding receptors on the surface of YAC-1 cells. These results suggest that prodigiosin 25-C affects the intracellular transport and/or processing of glycoproteins.

Prodigiosin 25-C is an immunosuppressant that inhibits the proliferation of T cells induced by the plant lectins, Con A and phytohemagglutinin, more strongly than the proliferation of B cells induced by lipopolysaccharide[1]. In a mixed lymphocyte reaction, prodigiosin 25-C suppressed cytotoxic T lymphocytes induction[1,2]. When prodigiosin 25-C was administered to mice, the compound completely suppressed cytotoxic T lymphocytes induction, but antibody production was unaffected[2,3]. These results indicate that prodigiosin 25-C inhibits cytotoxic T lymphocytes induction without affecting the *in vivo* functions of helper T cells and B cells.

The mitogen response of murine splenocytes was inhibited more strongly by prodigiosin 25-C when the cells were stimulated with higher concentration of Con A[3]. In both T cells and B cells purified from murine splenocytes, the inhibitory effect of prodigiosin 25-C was enhanced by the addition of Con A[4]. The increased sensitivity was also observed in the lectins which bind to mannose residues of biantennary-complex-type sugar chains[4]. These results suggested that the inhibitory effect of prodigiosin 25-C was closely related to the expression and the structure of cell surface glycoproteins. In this paper, we further examined the effects of prodigiosin 25-C on cultured cell lines.

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Materials and Methods

Cells

T lymphoma YAC-1 cell, T lymphoma EL-4, thymoma L5178-Y, B lymphoma A20.2J, mastcytoma P815 and macrophage cell line J774A.1 were maintained in RPMI1640 medium supplemented with 10% (v/v) fetal calf serum (GIBCO Laboratories, Gland Island, U.S.A.), 50 μM 2-mercaptoethanol, 50 μg/ml kanamycin and 8 μg/ml tylosin tartrate. T cell line CTLL-2, helper T cell line HT-2 and natural killer cell line SPB2.4[5] were maintained in the presence of human recombinant IL-2. Myeloid cell line FDC.P2 was maintained in the presence of WEHI-3 supernatant.

Proliferation Assay

Cells (1,000 or 5,000 cells/well, 0.1 ml) were incubated with inhibitors at 37°C for 48 or 72 hours in a microtiter plate. After being pulse-labeled with [^{3}H]thymidine (0.5 μCi/well) for 4 hours, cells were harvested and the radioactivity of them was measured in a liquid scintillation counter.

Measurement of Ionophore Activity[6]

A half ml of aqueous buffer (25 mM Tris-HCl, pH 8.5) containing radioactive potassium ion (^{42}K) and 0.5 ml of 30% *n*-buthanol - 70% toluene mixture (v/v) containing an antibiotic were mixed vigorously and the radioactivities of both phases were measured in a gamma counter.

Measurement of H^+-ATPase Activity

Plant vacuole from malt was prepared by the method of MATSUURA-ENDO *et al.*[7]. Vacuolar membrane was incubated in 30 mM MES-Tris (pH 7.5), 50 mM KCl, 3 mM $MgSO_4$, 3 mM ATP, 50 mM ammonium molybdate and 1 μM gramicidin D at 38°C for 30 minutes at a final volume of 0.5 ml. The reaction was terminated by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid and the amount of free phosphate was measured.

Con A Binding Assay

YAC-1 cells (5×10^6 cells/ml, 0.2 ml) were incubated with [^{3}H]Con A (8.5 μCi/mmol, Amersham International plc, Buckinghamshire, England) in HANKS' balanced salt solution containing 2% (v/v) fetal calf serum and 0.02% (w/v) sodium azide at 37°C for 40 minutes. Cell suspension was overlaid onto 80% di-*n*-butyl phthalate - 20% olive oil (v/v) and centrifuged (2,000 $\times$ *g*, 2 minutes). The cell pellet was dissolved in 0.3 ml of 10% (w/v) Triton X-100 and the radioactivity was measured in ten volumes of Aquasol-2 (New England Nuclear, Boston, U.S.A.). To distinguish nonspecific binding from specific binding, YAC-1 cells were incubated with [^{3}H]Con A in the presence of 100 mM α-methyl-D-mannoside.

Chemicals

Prodigiosin 25-C, bafilomycin B_1 and ML-236B were prepared in our laboratories. Brefeldin A and tunicamycin were the gifts from A. TAKATSUKI. Concanamycin A was provided by Taisho Pharmaceutical Co., Ltd., Tokyo. Other reagents including Con A, antibiotics and inhibitors were purchased from Sigma Chemicals Co., St. Louis, U.S.A.

Results

Ten cultured cell lines were incubated with prodigiosin 25-C in the presence or absence of Con A for 72 hours (Table 1). ID_{50} values of prodigiosin 25-C on these cell lines ranged from 3.2 ng/ml to 35 ng/ml, but there was no correlation between the origin of cell lines and the sensitivity to prodigiosin 25-C. By the addition of Con A, the sensitivity to prodigiosin 25-C was increased in all of these cell lines tested in Table 1, but the degree of the enhancement varied from 1.3-fold in J774A.1 cells to 60-fold in YAC-1 cells. The sensitivities to prodigiosin 25-C in CTLL-2, J774A.1, P815, FDC.P2 and SPB2.4 increased by only about 2-fold, whereas HT-2, L5178-Y, EL-4 and A20.2J were sensitive to more than 5-fold as low as prodigiosin

Table 1. Effect of prodigiosin 25-C on the proliferation of various cultured cell lines in the presence of Con A.

Cell lines	[^{3}H]Thymidine incorporation (cpm)		ID_{50} (ng/ml)	
	None	Con A	None	Con A
CTLL-2	23,252±2,149	21,670±1,238	3.2	1.5
HT-2	89,919±7,970	77,634±3,896	16.0	2.7
YAC-1	54,137±4,650	35,001±2,581	12.0	0.2
L5178-Y	127,008±1,643	114,293±4,080	15.5	1.5
EL4	89,029±6,758	65,518±2,490	11.5	2.2
A20.2J	42,365±2,022	27,538±2,328	35.0	4.2
J774A.1	84,413±4,201	86,387±3,102	21.0	16.0
P815	105,200±3,713	81,610±3,112	6.0	2.7
FDC.P2	96,373±2,613	87,459±2,888	18.0	10.5
SPB2.4	26,285± 332	23,523±3,447	27.0	11.5

Cells (1,000 cells/well, 0.1 ml) were incubated with prodigiosin 25-C in the presence or absence of Con A 5 μg/ml for 72 hours. Mean ±SD cpm of [^{3}H]thymidine incorporation in triplicate cultures and ID_{50} values of prodigiosin 25-C on them were presented.

Table 2. Effect of various inhibitors on the proliferation of YAC-1 cells in the presence of Con A.

Compounds (concentration)	ID_{50}		Ratio (None/ Con A)	Compounds (concentration)	ID_{50}		Ratio (None/ Con A)
	None	Con A			None	Con A	
Mitomycin C (ng/ml)	3.0	2.4	1.3	Prodigiosin 25-C (ng/ml)	18	0.62	29
Actinomycin D (ng/ml)	0.44	0.52	0.85	Nigericin (ng/ml)	54	15	3.6
Cycloheximide (ng/ml)	9.4	7.2	1.3	Nonactin (ng/ml)	19	24	0.79
Cerulenin (μg/ml)	1.8	1.3	1.4	Valinomycin (ng/ml)	1.6	3.1	0.52
ML-236B (μM)	4.5	30	0.15	A23187 (μM)	0.32	0.27	1.2
Amphotericin B (μg/ml)	1.5	1.6	0.90	Bafilomycin B_1 (ng/ml)	1.9	0.44	4.3
Antimycin A (ng/ml)	2.8	2.4	1.2	Concanamycin A (ng/ml)	0.43	0.12	3.6
Gramicidin S (ng/ml)	11	11	1.0	Oligomycin (ng/ml)	4.3	4.3	1.0
Cytochalasin D (μg/ml)	1.3	1.0	1.3	Ouabain (μg/ml)	58	52	1.1
Vincristine (ng/ml)	4.4	4.9	0.90	Chloroquine (μg/ml)	5.0	3.9	1.3
Brefeldin A (μg/ml)	0.26	0.23	1.1	Methylamine (%)	0.032	0.016	2.0
Tunicamycin (ng/ml)	4.5	3.6	1.3	Swainsonine (μg/ml)	>10	0.078	>128
Monensin (ng/ml)	52	9.4	5.5				

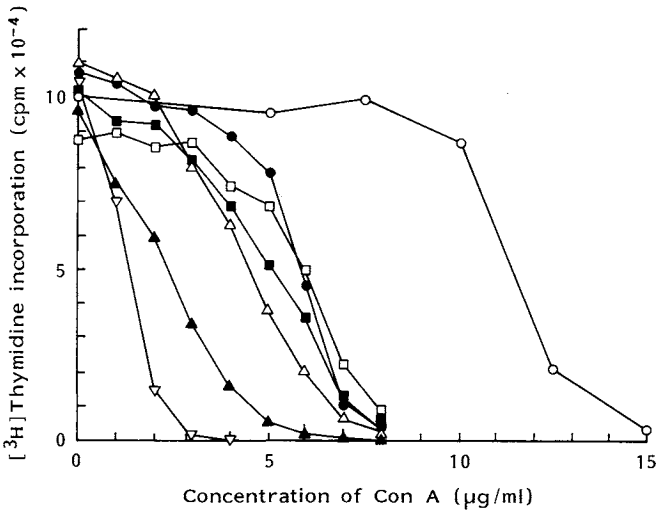
YAC-1 cells (5,000 cells/well, 0.1 ml) were incubated with prodigiosin 25-C in the presence or absence of Con A 5 μg/ml for 48 hours in triplicate cultures. ID_{50} values of various inhibitors on them and ID_{50} ratio were presented.

25-C in the presence of Con A. The increase of sensitivity of T lymphoma YAC-1 cells was most evident, and the cells were used for the following experiments.

The primary target of prodigiosin 25-C is still unknown and the increased sensitivity in the presence of Con A was not observed with other immunomodulators such as FK506 and cyclosporin A[3]. Inhibitors which have different mechanisms of action were examined to determine whether they become more effective when combined with Con A (Table 2). Among the inhibitors tested in Table 2, monovalent polyether ionophores, monensin and nigericin, a vacuolar type H^+-ATPase inhibitor bafilomycin B_1[8], its structurally related compound concanamycin A[9] and a Golgi mannosidase II inhibitor swainsonine[10] significantly inhibited the proliferation of YAC-1 cells in the presence of Con A. It should be noted that swainsonine had no inhibitory effect on YAC-1 cells by itself at 10 μg/ml. None of the other types of ionophores including A23187, nonactin and valinomycin, Na^+/K^+-ATPase inhibitor ouabain, and F_1F_0-ATPase

Fig. 1. Inhibitory effect of Con A on the proliferation of YAC-1 cells in the presence of various inhibitors.

○ None, □ monensin 20 ng/ml, ■ nigericin 10 ng/ml, △ bafilomycin B_1 1 ng/ml, ▲ concanamycin A 0.3 ng/ml, ● swainsonine 1 μg/ml, ▽ prodigiosin 25-C 10 ng/ml.



YAC-1 cells (5,000 cells/ml, 0.1 ml) were incubated with Con A in the presence of various inhibitors for 48 hours in triplicate cultures.

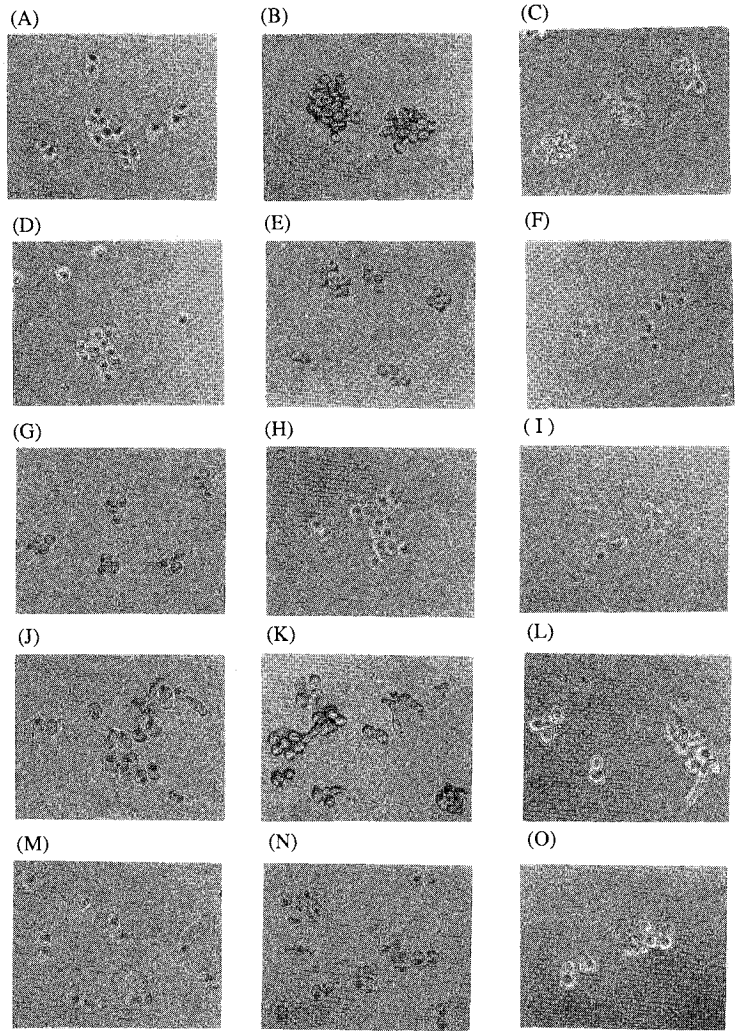
inhibitor oligomycin showed such effect. On the contrary, YAC-1 cells were rendered more resistant to ML-236B[11] by the addition of Con A. Preliminary experiments showed that Con A inhibited the incorporation of exogenous cholesterol (data not shown). These findings suggest that HMG - CoA reductase, a target enzyme of ML-236B, increases in the presence of Con A and YAC-1 cells consequently become resistant to the drug.

The ID_{50} value of Con A on YAC-1 cells decreased from 11.5 to 6 μg/ml in the presence of swainsonine, whereas it decreased to 1.5 μg/ml following the addition of prodigiosin 25-C (Fig. 1). Polyether ionophores, monensin and nigericin and vacuolar type H^+-ATPase inhibitor bafilomycin B_1 also increased the sensitivity of the cells to Con A by about 2-fold, but a 4.6-fold increase was observed in the case of concanamycin A (Fig. 1).

Con A induced the agglutination of YAC-1 cells without affecting the cell shapes (Fig. 2B and 2C) and 10 ng/ml of prodigiosin 25-C did not change their shape (Fig. 2D). But, with the combination of prodigiosin 25-C and Con A, elongated cells and enlarged cells appeared (Fig. 2J). Similar morphological changes were induced by monensin (Fig. 2K), nigericin (Fig. 2L), bafilomycin B_1 (Fig. 1M) and concanamycin A (Fig. 2N) only in the presence of Con A. In contrast, swainsonine and Con A induced merely enlarged cells (Fig. 2O). Thus, the effect of prodigiosin 25-C was similar to that of polyether ionophores and vacuolar type H^+-ATPase inhibitors rather than that of swainsonine.

Ionophore activity against potassium ions was measured using a two phase distribution system (Table 3). Nigericin efficiently transported ^{42}K from an aqueous phase to an organic phase. In the case of valinomycin, small but significant radioactivity was detected in an organic phase. Valinomycin had only a little ionophore activity as compared with nigericin, probably because the dissociation constant for the interaction of potassium ions with valinomycin is much larger than that with nigericin[12]. Prodigiosin 25-C showed no ionophore activity against potassium ion under these experimental conditions.

Fig. 2. Morphological changes of YAC-1 cells.



YAC-1 cells were incubated with various inhibitors in the presence or absence of Con A for 24 hours. A: control, B: Con A 5 μg/ml, C: Con A 7.5 μg/ml, D: prodigiosin 25-C 10 ng/ml, E: monensin 20 ng/ml, F: nigericin 20 ng/ml, G: bafilomycin B_1 1 ng/ml, H: concanamycin A 1 ng/ml, I: swainsonine 1 μg/ml, J: prodigiosin 25-C 10 ng/ml plus Con A 5 μg/ml, K: monensin 20 ng/ml plus Con A 5 μg/ml, L: nigericin 20 ng/ml plus Con A 5 μg/ml, M: bafilomycin B_1 1 ng/ml plus Con A 5 μg/ml, N: concanamycin A 1 ng/ml plus Con A 5 μg/ml, O: swainsonine 1 μg/ml plus Con A 7.5 μg/ml.

The activity of plant vacuolar type H^+-ATPase was calculated by assaying the amount of free phosphate produced through hydrolysis of ATP (Table 4). HNO_3 and bafilomycin B_1, known inhibitors of the enzyme, significantly inhibited the activity. Concanamycin A also inhibited ATPase activity almost completely, suggesting that concanamycin A is an inhibitor of vacuolar type H^+-ATPase similar to bafilomycin B_1. However, prodigiosin 25-C had only a small effect at 20 μg/ml.

YAC-1 cells were incubated with prodigiosin 25-C or swainsonine for 72 hours and the amount of Con A binding receptors on cell surface were estimated (Fig. 3). Prodigiosin 25-C and swainsonine increased Con A binding receptors to about twice the control level.

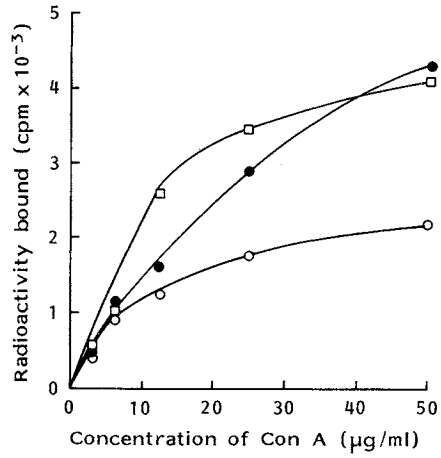
Table 3. Ionophore activity against potassium ion.

Compounds	Radioactivity of ^{42}K (cpm)	
	Toluene/buthanol phase	Aqueous phase
None	11	1,208
Nigericin	609	567
Valinomycin	58	1,102
Prodigiosin 25-C	9	1,141

Table 4. Plant vacuolar type H^+-ATPase activity.

Treatment	Specific activity (μmol/min/mg)	T/C (%)
Control	0.464	100
Vanadate (50 μM)	0.396	85
HNO_3 (50 mM)	0.058	12
Bafilomycin B_1 (2 μg/ml)	0.027	6
Concanamycin A (2 μg/ml)	0.015	3
Monensin (2 μg/ml)	0.387	83
Prodigiosin 25-C (20 μg/ml)	0.404	87

Fig. 3. Effect of prodigiosin 25-C on the expression of Con A binding receptors on YAC-1 cells.



YAC-1 cells were incubated with prodigiosin 25-C 10 ng/ml (closed circle), swainsonine 1 μg/mol (open square) or no addition (open circle) for 72 hours and the amount of Con A binding receptors was measured.

Discussion

Prodigiosin 25-C was found as an immunosuppressant which inhibited the proliferation of T cells more strongly than that of B cells[1]. When Con A was added to the culture, the inhibitory effect of prodigiosin 25-C was markedly enhanced in splenic B cells as well as in splenic T cells[3,4]. The increased sensitivity was also observed in several cultured cell lines including lymphoid and myeloid cells, and was most evident in T lymphoma YAC-1 cells.

We found that monovalent polyether ionophores, vacuolar type H^+-ATPase inhibitors and Golgi mannosidase II inhibitor inhibited the proliferation of YAC-1 cells more strongly in the presence of Con A as did prodigiosin 25-C. Swainsonine inhibited the proliferation of YAC-1 cells only when Con A was added to their culture. Prodigiosin 25-C and Con A induced characteristic morphological changes on YAC-1 cells such as the appearance of enlarged cells and elongated cells but these changes were not observed in other cell lines listed in Table 1. Monovalent polyether ionophores and vacuolar type H^+-ATPase inhibitors also induced similar morphological changes, whereas swainsonine induced only the appearance of enlarged cells. These results suggest that the effect of prodigiosin 25-C resembles that of monovalent polyether ionophores and vacuolar type H^+-ATPase inhibitors rather than that of swainsonine. However, prodigiosin 25-C had no ionophore activity against potassium ion in a two phase distribution system. Since the structure of prodigiosin 25-C differs from that of all known ionophores, which contain several oxygen atoms to complex the ions, it is unlikely that prodigiosin 25-C has ionophore activity against cations.

In this paper, we showed that concanamycin A, which is structurally related to bafilomycin B_1, inhibited plant vacuolar type H^+-ATPase. However, prodigiosin 25-C had no inhibitory effect on plant vacuolar type H^+-ATPase. Vacuolar type H^+-ATPase generates an internal acidic environment in the intracellular organella such as endosome, lysosome and Golgi apparatus[13]. We have recently found that bafilomycin B_1[14] and concanamycins[15,16] are potent inhibitors of ATP-dependent acidification of endosomes and lysosomes in mammalian cells. Monovalent polyether ionophores can transport not only cations but also protons across the lipid bilayer and monensin is known to perturb the structure and function of the Golgi apparatus by raising the vacuolar pH, resulting in the inhibition of glycoprotein transport[17,18]. Again, the increased sensitivity and the morphological changes with Con A in YAC-1 cells

were observed with only monovalent polyether ionophores, but not with A23187, nonactin and valinomycin, which are inert to transport proton across membrane. Thus, these results suggest that prodigiosin 25-C affects vacuolar acidification especially in the Golgi apparatus.

Asparagine-linked sugar chains of glycoproteins are divided into three groups termed high-mannose-type, hybrid-type and complex-type. Con A binds to biantennary-complex-type, high-mannose-type and hybrid-type sugar chains[19,20]. Swainsonine converts complex-type into hybrid-type sugar chains and increases cell surface expression of Con A binding receptors[21]. Prodigiosin 25-C also increased Con A binding receptors in YAC-1 cells by about 2-fold as did swainsonine. Since swainsonine itself was not toxic to cultured cell lines and increased the sensitivity to Con A of YAC-1 cells by 2-fold, YAC-1 cells may be preferentially killed by the toxic effect of Con A in the presence of swainsonine. Prodigiosin 25-C induced on YAC-1 cells a 7.7-fold increase of sensitivity to Con A, which was much greater than that expected from the 2-fold increase of Con A binding receptors and elongated morphology in the presence of Con A unlike swainsonine. These results suggest that both swainsonine and prodigiosin 25-C cause increase of the number of Con A binding receptors on YAC-1 cells, but prodigiosin 25-C had some additional effect involved in the synergistic effect with Con A.

Taken together the results presented in this paper suggest that prodigiosin 25-C affects the intracellular transport and processing of glycoproteins and change their expression and structure through raising vacuolar pH. We are now carrying out some biochemical experiments including the isolation of the prodigiosin 25-C binding protein, which will make an important clue to address the mode of action of prodigiosin 25-C on the molecular level. The mechanism of vacuolar acidification will be elucidated by studying the mode of action of prodigiosin 25-C.

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