ENHANCEMENT BY CONCANAVALIN A OF THE SUPPRESSIVE EFFECT OF PRODIGIOSIN 25-C ON PROLIFERATION OF MURINE SPLENOCYTES

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Proliferation of concanavalin A (Con A)-activated nylon-wool purified murine splenic T cells was increasingly suppressed by prodigiosin 25-C as higher concentrations of Con A were used for the activation. Enhancement of suppressive effect of prodigiosin 25-C was not observed when T cells were stimulated with phytohemagglutinin (PHA), anti-CD3 antibody, or allogeneic splenic adherent cells. The suppressive effect of prodigiosin 25-C was enhanced by the addition of Con A in various T cell subpopulations as well as in LPS-activated splenic B cells. Lectins that recognize mannose residue of biantennary-complex-type sugar chains significantly enhanced the suppressive effect of prodigiosin 25-C, whereas a lectin that binds to *N*-acetylglucosamine did not. These results suggest that binding of lectins to the mannose residue of biantennary-complex-type sugar chains on cell surface of both T and B lymphocytes plays a central role on the enhancement of the suppressive effect of prodigiosin 25-C.

Prodigiosin 25-C is a T cell specific immunosuppressant that was found in a screening program for immunomodifying antibiotics using mitogen response of splenocytes[1,2]. When prodigiosin 25-C was administered to mice immunized with both sheep red blood cells (SRBC) and an allogeneic cell line, induction of specific cytotoxic T lymphocytes (CTL) was completely suppressed, whereas antibody production against SRBC was not affected[3,4]. Prodigiosin 25-C did not affect antibody production against a thymus dependent (TD) or a thymus independent antigen (TI)[4]. Thus, prodigiosin 25-C selectively inhibits the generation of killer T cells without affecting functions of helper T cells and B cells *in vivo*.

In the case of the *in vitro* Con A-induced proliferative response of T cells, prodigiosin 25-C suppressed neither IL-2 production nor expression of IL-2 receptor (IL-2R) and transferrin receptor at concentration that completely inhibited IL-2 dependent T cell proliferation[3,4]. In contrast, FK506[5] strongly inhibited IL-2 dependent T cell production as well as IL-2R expression[6~8]. This drug acts by inhibiting the translocation of a transcription factor subunit from the cytosol to the nucleus by blocking the phosphatase activity of calcineurin[9,10]. These results indicate that prodigiosin 25-C, unlike FK506, inhibits a later stage of T cell activation following the step of IL-2 binding to the IL-2R.

Mitogen response of murine splenocytes has been shown to be inhibited more strongly by prodigiosin 25-C when the cells were stimulated with high concentration of Con A[4]. This is a unique property of prodigiosin 25-C, distinct from other immunomodulators including cyclosporin A and FK506. In this report, the mechanism of enhancement of the suppressive effect of prodigiosin 25-C was studied using purified T and B cells.

Materials and Methods

Mice

Specific pathogen free BALB/c, C57BL/6 and C3H/He mice (female, 6 weeks old) were obtained from Charles River Japan Co. Ltd., Tokyo.

Chemicals

Prodigiosin 25-C was prepared from the extract of mycerial cakes of *Streptomyces hiroshimensis* as described previously[2]. Concanavalin A (Con A), *Lens culinalis* agglutinin (LCA), *Pisum sativum* agglutinin (PSA), *Vicia fava* agglutinin (VFA), Pokeweed agglutinin (PWM), chicken egg ovalbumin (OVA), A23187 and phorbol-12,13-dibutyrate (PDBu) were purchased from Sigma Chemical Co., St. Louis, USA. Phytohemagglutinin (PHA) and lipopolysaccharide (LPS) were the commercial products of Wellcome Diagnostics, Dartford, England and Difco Laboratories, Detroit, USA, respectively. Keyhole Limpet hemocyanin (KLH) was purchased from Calbiochem Corporation, La Jolla, USA.

Antibodies

Anti-CD4 antibody was purchased from Cedarlane Laboratories Ltd., Ontario, Canada. Monoclonal antibodies against CD8, 83-12-5[11], Thy1, 21-12, and CD3 ε-chain, 145-2C11[12], were also used. 21-12 is a monoclonal mouse IgM antibody against Thy1.2 established in the Department of Immunology, School of Medicine, Chiba University.

Preparation of Lymphocytes

RPMI1640 medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Gland Island, USA), 50 μM 2-mercaptoethanol, 50 μg/ml kanamycin, 8 μg/ml tylosin tartrate (RPMI1640 complete medium) was used for cell cultures. Nylon-wool purified T cells and spleen adherent cells (SAC) were prepared by the method described previously[4]. To prepare $CD4^+$ or $CD8^+$ T cell subsets, nylon-wool purified T cells (2×10^7 cells/ml) were incubated with anti-CD8 antibody (1/20 dilution of hybridoma 83-12-5 ascites) or 1/20 dilution of anti-CD4 antibody in RPMI1640 complete medium containing 10 mg/ml glucose (high glucose medium) for 15 minutes at 37°C[13], followed by the incubation with a 1/15 dilution of 21-day old rabbit fresh serum for 45 minutes for 37°C to remove antibody-sensitized cells. T cell populations enriched with $CD4^+$ cells contained 4% $CD4^-CD8^+$ cells and those enriched with $CD8^+$ cells contained 3% $CD4^+CD8^-$ cells, whereas the original T cell population contained 48% $CD4^+$ and 31% $CD8^+$ cells. To prepare a B cell enriched fraction, splenocytes (2×10^7 cells/ml) were depleted of T cells by incubating with anti-Thy1.2 antibody (1/400 dilution of hybridoma 21-12 ascites) and later with rabbit complement.

Cloned T Cell Lines

BK1[14] ($CD4^+$, KLH specific, I-A^d restricted killer T cell line), BO1[14] ($CD4^+$, OVA specific, I-A^d restricted killer T cell line), QM11[15] ($CD8^+$, I-A^k specific killer T cell line) and OE4[16] ($CD8^+$, L^d specific killer T cell line) were provided by N. SHINOHARA. 16, 38-1, 42-2, 47-3 (allogeneic I-A^b specific Th_1 cell lines), 35-9D, 35-8H[16], 34-7F[16] (OVA specific I-A^b restricted Th_1 cell lines), D10.G4.1[17], C3C, C3F (allogeneic I-A^b specific Th_2 cell lines) and 42-6A[18] (OVA specific I-A^d restricted Th_1 cell lines) were from our stock. T cell lines were maintained by repeated stimulations with specific antigens along with SAC followed by the incubation with 10 ng/ml IL-2 or 10% culture supernatant of rat spleen cells stimulated with Con A (rat conditioned medium). T cell lines were used for experiments at least two weeks after the antigen stimulation.

Proliferation Assay of T Cell Lines

T cell lines (1×10^4 cells/well) were stimulated with 20 μg/ml KLH or 200 μg/ml OVA together with SAC (2×10^4 cells/well), or with allogeneic SAC (2×10^4 cells/well) and cultured for 2 days in the presence of 10 ng/ml human recombinant IL-2 (for killer and Th_1 lines) or 10% rat conditioned medium (for Th_2 lines) in a 96 well culture plate. After being pulse-labeled with [^{3}H]thymidine (0.5 μCi/well) for 4 hours, cells were harvested and counted in a liquid scintillation counter.

Assay for Splenic T Cell Proliferation

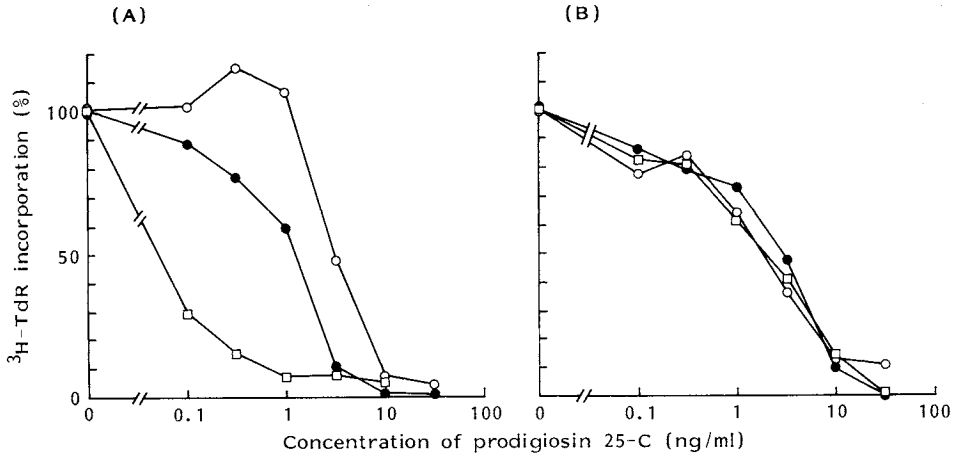
To assess the proliferative response of purified splenic T cells to Con A, PHA or other lectins, anti-CD3 antibody, or A23187 plus PDBu, T cells (1×10^5 cells/well) and SAC (2×10^4 cells/well) were cultured for 3 days with stimulants in a 96 well culture plate. They were pulsed with [^{3}H]thymidine (0.5 μCi/well) for the last 4 hours. When T cells were stimulated with allogeneic antigen, C57BL/6 mice splenic T cells (4×10^5 cells/well) were stimulated with various concentrations of SAC from C3H/He mice for 3 days.

Results

The degree of prodigiosin 25-C suppression of proliferation of nylon-wool purified T cells increased with increasing concentrations of Con A (Fig. 1A). The mitogenic response induced by 5 μg/ml Con A was inhibited by the addition of 1 ng/ml prodigiosin 25-C and that induced by 0.2 μg/ml Con A was inhibited by 10 ng/ml prodigiosin 25-C. Increased sensitivity to prodigiosin 25-C at high concentration of the stimulant was not observed when PHA (Fig. 1B), anti-CD3 antibody (Fig. 2B), or allogeneic SAC (Fig. 2C) was used as a stimulator. ID_{50} (50% inhibition dose) values of prodigiosin 25-C in these experiments were equivalent to those in the experiment where T cells were stimulated with 0.2 μg/ml Con A or with calcium ionophore plus phorbol ester, which stimulates T cells through the T cell receptor (TCR) independent pathway[19,20] (Fig. 2A). These results suggest that the increase in sensitivity to prodigiosin 25-C of T cell proliferation response to high concentration of Con A is not due to overstimulation to TCR.

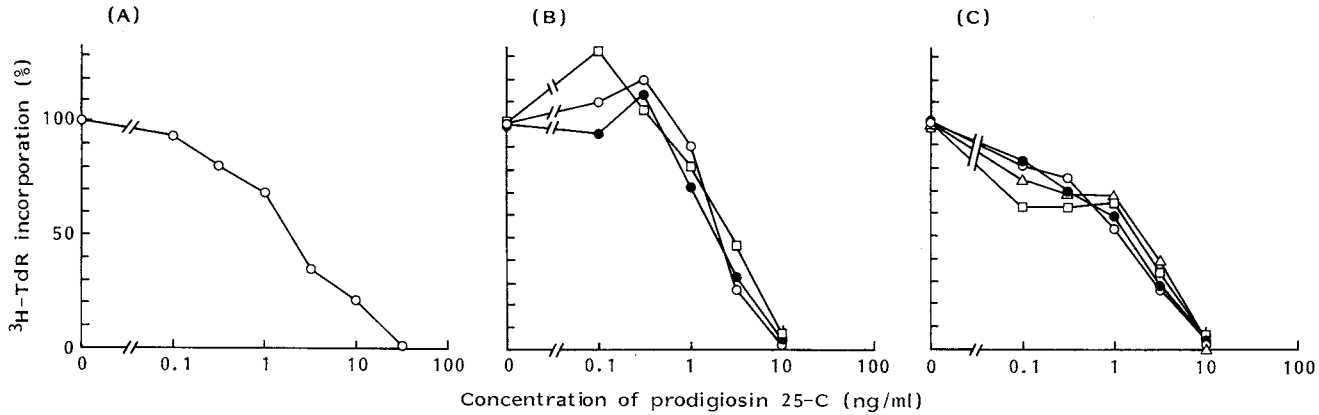
Both T cell preparations enriched with $CD4^-CD8^+$ (Fig. 3B) and $CD4^+CD8^-$ T cells (Fig. 3C) were shown to be more sensitive to prodigiosin 25-C, when they were stimulated with higher concentration of Con A. These two populations showed the same sensitivity to prodigiosin 25-C. Although most of the T

Fig. 1. Suppression of T cell proliferation induced with Con A or PHA by prodigiosin 25-C.



Nylon-wool purified T cells (1×10^5 cells/well) and SAC (2×10^4 cells/well) from C57BL/6 mice were incubated with Con A (A) or PHA (B) at 37°C in the presence of prodigiosin 25-C for 3 days. The concentrations of Con A and PHA used were 0.2 μg/ml (○), 1 μg/ml (●) and 5 μg/ml (□). Mean ± SD cpm of [^{3}H]thymidine incorporation of T cells stimulated in the absence of prodigiosin 25-C with Con A were 15,643 ± 1,234 (0.2 μg/ml), 73,291 ± 6,698 (1 μg/ml), and 9,701 ± 1,603 (5 μg/ml). When they were stimulated with PHA, the incorporations were 1,517 ± 157 (0.2 μg/ml), 49,812 ± 5,623 (1 μg/ml), and 10,873 ± 2,850 (5 μg/ml).

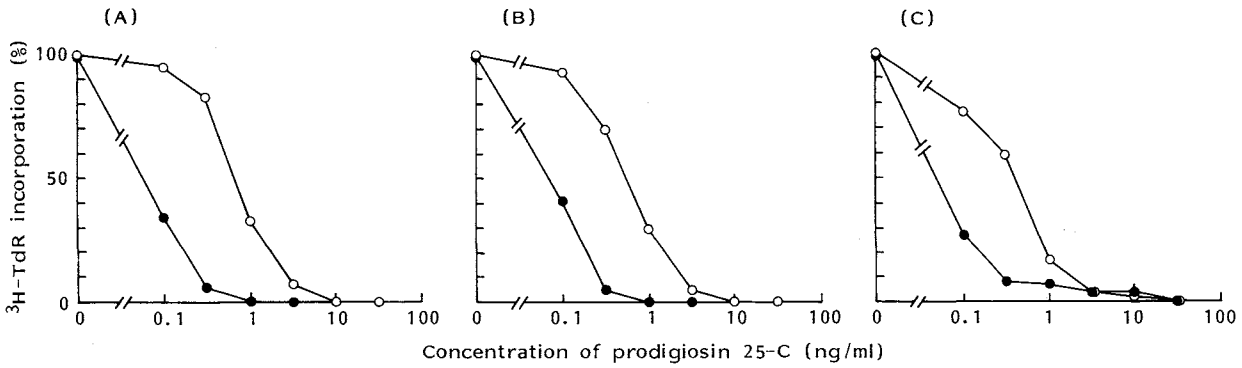
Fig. 2. Suppression of prodigiosin 25-C of T cell proliferation induced by TCR independent or TCR dependent stimulators.



A, B: Proliferative response of T cells was induced by a combination of A23187 (0.2 μM) and PDBu (10 ng/ml) (A) or anti-CD3 antibody (B). In panel B, T cells were incubated with 20,000- (○), 160,000- (●) and 320,000-fold dilution (□) of anti-CD3 ascites. Mean $\pm$ SD cpm of [^{3}H]thymidine incorporation of T cell stimulated in the absence of prodigiosin 25-C were 39,055 $\pm$ 6,066 (A23187 plus PDBu), 44,510 $\pm$ 1,853 (1/20,000 dilution anti-CD3), 33,532 $\pm$ 2,081 (1/160,000 dilution anti-CD3), and 16,533 $\pm$ 2,676 (1/320,000 dilution anti-CD3).

C: Nylon-wool purified T cells (4×10^5 cells/well) from C57BL/6 mice and allogeneic SAC from C3H/He mice were incubated for 3 days. Responder to stimulator ratios were 3:1 (○), 5:1 (●), 10:1 (□) and 25:1 (△). Mean $\pm$ SD cpm of [^{3}H]thymidine incorporation of cultures incubated without addition of prodigiosin 25-C were 100,998 $\pm$ 8,842 (3:1), 50,208 $\pm$ 1,804 (5:1), 26,600 $\pm$ 5,497 (10:1) and 6,662 $\pm$ 1,896 (25:1). Other experimental conditions, including panels A, B and C, were the same to those described in Fig. 1.

Fig. 3. Suppression of Con A-induced proliferation of CD4-depleted or CD8-depleted T cells by prodigiosin 25-C.



Nylon-wool purified T cells were treated with complement alone (A), complement plus anti-CD4 antibody (B) and complement plus anti-CD8 antibody (C). After washing, proliferative response of these populations were induced by 1 μg/ml Con A (○) or 5 μg/ml Con A (●) in the presence of 40 ng/ml human recombinant IL-2. Mean $\pm$ SD cpm of [^{3}H]thymidine incorporations of T cells stimulated with 1 μg/ml Con A in the absence of prodigiosin 25-C were 28,162 $\pm$ 2,381 (A), 76,387 $\pm$ 8,989 (B) and 18,480 $\pm$ 2,315 (C) and with 5 μg/ml Con A: 7,710 $\pm$ 393 (A), 20,517 $\pm$ 496 (B) and 2,553 $\pm$ 227 (C).

Table 1. Effect of Con A on growth inhibition of various cloned T cell lines by prodigiosin 25-C.

Cell lines	[^{3}H]Thymidine incorporation (cpm)		ID_{50} of prodigiosin 25-C (ng/ml)	
	Con A (−)	Con A (+)	Con A (−)	Con A (+)
BK1 (Killer)	15,950± 695	2,178± 1,000	11.0	1.3
BO1 (Killer)	18,058± 480	1,435± 106	2.0	0.39
QM11 (Killer)	32,765± 2,652	26,355± 1,810	4.1	3.7
OE4 (Killer)	29,770± 721	8,305± 871	13.0	0.16
16 (Th_1)	53,970±12,841	39,585±12,389	2.8	0.41
35-9D (Th_1)	50,815± 431	7,737± 2,585	8.6	<0.1*
35-8H (Th_1)	33,120± 2,573	2,102± 186	0.64	0.58
34-7F (Th_1)	47,050± 4,388	4,175± 659	1.0	0.36
42-6A (Th_1)	103,295± 4,253	7,689± 255	5.5	0.16
38-1 (Th_1)	52,438± 8,277	13,955± 2,173	14.0	0.26
42-2 (Th_1)	9,485± 877	2,552± 150	3.9	0.49
47-3 (Th_1)	10,750± 1,215	2,918± 39	6.0	0.84
D10.G4.1 (Th_2)	17,312± 1,829	15,755± 2,527	4.5	2.1
C3C (Th_2)	17,500± 340	2,373± 18	0.31	<0.1*
C3F (Th_2)	20,200± 1,322	2,050± 106	0.32	0.14

* ID_{50} values are less than 0.1 ng/ml.

Various cloned T cell lines were stimulated with their specific antigens together with SAC in the presence or absence of 5 μg/ml Con A for 2 days. Results are presented as mean±SD cpm of [^{3}H]thymidine incorporation of triplicated assay in the absence of prodigiosin 25-C and ID_{50} values of prodigiosin 25-C.

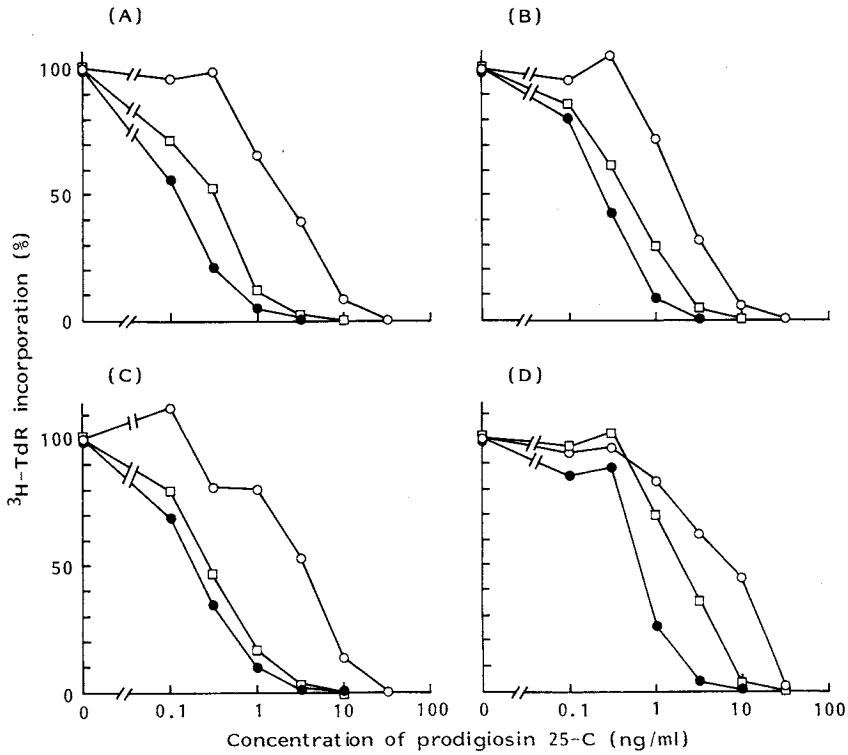
cell lines listed in Table 1 were inhibited by Con A itself, these cell lines were also more sensitive to prodigiosin 25-C in the presence of high concentration of Con A. Thus, there was no difference in T cell subsets regarding the effects of Con A on their sensitivity to prodigiosin 25-C. When cells were stimulated in the presence of higher concentrations of Con A, all T cell populations examined were more sensitive to prodigiosin 25-C regarding their proliferative response, irrespective of their surface marker, $CD4^+CD8^-$ or $CD4^-CD8^+$ status, cytokine secretion phenotype, Th_1 or Th_2, and their function as helper or killer cells. T cell blasts obtained by stimulating splenocytes with anti-CD3 antibody, low concentration of Con A or PHA, were more sensitive to prodigiosin 25-C in their proliferation in the presence of Con A (Fig. 4). It should be noted that the proliferation of B cell blasts was also more strongly inhibited by prodigiosin 25-C in the presence of a high concentration of Con A (Fig. 4D). These results suggest that the increased sensitivity to prodigiosin 25-C in high concentration of Con A was neither due to too much stimulation of TCR nor to different sensitivity to the drug among the T cell subsets.

Effects of other lectins on the sensitivity of T cells to prodigiosin 25-C were also examined. T cells were shown to be sensitive to prodigiosin 25-C in the presence of high concentration of PSA, VFA, and LCA, which bind to mannose residues of biantennary-complex-type sugar chains as Con A (Fig. 5), although the effects of these lectins were less pronounced than that of Con A. On the other hand, PWM, which is known to bind to *N*-acetylglucosamine, exerted no effect on the suppressive effect of prodigiosin 25-C.

Discussion

As a substitute for antigen stimulation, Con A can induce quiescent T cells to proliferate by crosslinking of TCR. In the present paper, we studied the mechanism by which the addition of a high concentration of Con A to cultures enhances the suppressive effect of prodigiosin 25-C on T cell proliferation. Such

Fig. 4. Growth suppression of T cell blasts and B cell blasts by prodigiosin 25-C in the presence of Con A.

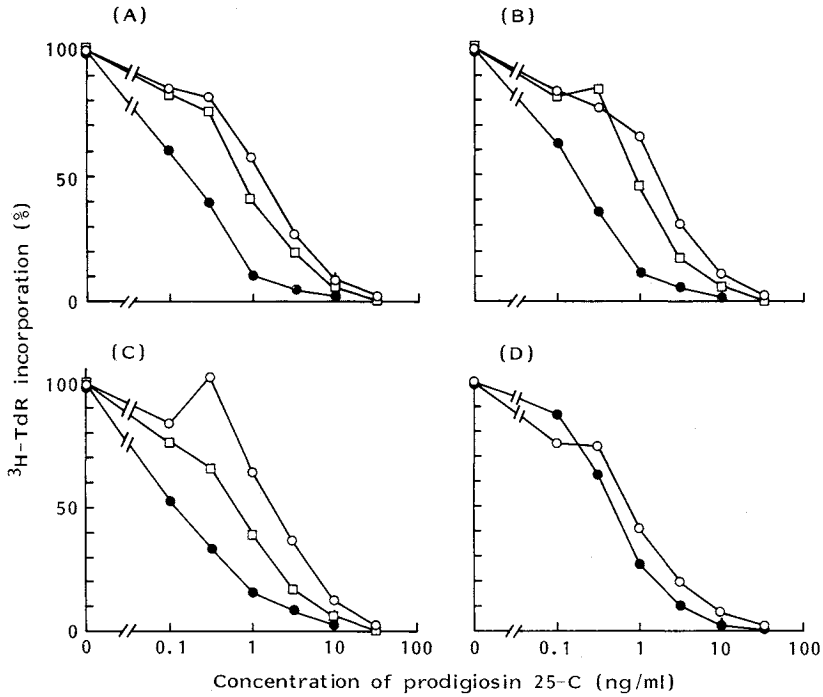


Nylon-wool purified T cells were stimulated in the presence of SAC with 1 μg/ml Con A (A), 1 μg/ml PHA (B) or 1/2,000 dilution of anti-CD3 ascites (C) for 2 days (T cell blasts). After washing, cells were further incubated with prodigiosin 25-C and Con A in the presence of 40 ng/ml IL-2 for 2 days. The B cell enriched fraction was stimulated with 5 μg/ml LPS for 2 days (B cell blasts) and cells were further incubated with prodigiosin 25-C and Con A in the presence of 5 μg/ml LPS for 2 days (D). Con A was added to T and B cell blasts at final concentration of 0 μg/ml (○), 2 μg/ml (□) or 5 μg/ml (●). Mean ± SD cpm of [^{3}H]thymidine incorporation of cells cultured in the absence of prodigiosin 25-C in each panel were as follows: A; 70,434±695 (○) 44,738±4,596 (□) 31,610±1,514 (●), B; 45,338±2,167 (○) 35,110±3,026 (□) 27,883±2,328 (●), C; 54,365±1,848 (○) 34,068±2,820 (□) 18,520±2,115 (●) and D; 23,703±1,462 (○) 18,948±3,900 (□) 10,667±566 (●).

enhancement was not observed with T cells stimulated with anti-CD3 antibody or allogeneic SAC. ID_{50} values of prodigiosin 25-C against the splenic T cell proliferation induced by those stimulants were almost equivalent to those against mitogenic response induced by A23187 and PDBu which stimulate T cells in a TCR independent manner[19,20]. In addition, Con A enhanced the inhibitory effect of prodigiosin 25-C on IL-2 dependent proliferation of T cell blasts as well as LPS-induced mitogenic response of B cells. These results suggest that TCR is not involved in the mechanism of the increased sensitivity of lymphocytes to prodigiosin 25-C in the presence of Con A. It has been reported that Con A induces membrane freezing, microtubular aggregation, receptor capping, adenylate cyclase activation and cellular cyclic AMP increase[21]. These cellular physiological changes may be closely related to the suppressive effect of prodigiosin 25-C on cell proliferation.

Since high concentration of Con A is toxic to murine splenocytes and various T cell lines, it is possible that prodigiosin 25-C enhances the suppressive effect of Con A. Recently, we found that prodigiosin 25-C increased the number of Con A binding sites on cell surface of a cultured cell line, and that the enhancement of suppressive effect of prodigiosin 25-C was much greater than that predicted from the increase of

Fig. 5. Suppression by prodigiosin 25-C of T cell proliferation induced with lectins.



Nylon-wool purified T cells and SAC were incubated with PSA (A), VFA (B), LSA (C) and PWM (D) in the presence of prodigiosin 25-C for 3 days at 37°C. Mean ± SD cpm of [^{3}H]thymidine incorporation were A: PSA 0.5 μg/ml (○) 61,389 ± 9,500 1 μg/ml (□) 68,060 ± 3,479 10 μg/ml (●) 35,888 ± 6,098, B: VFA 0.2 μg/ml (○) 20,237 ± 1,390 1 μg/ml (□) 48,627 ± 2,279 5 μg/ml (●) 32,638 ± 1,260, C: LCA 0.5 μg/ml (○) 34,427 ± 2,511 1 μg/ml (□) 38,923 ± 2,606 4 μg/ml (●) 14,740 ± 1,620 and D: PWM 1 μg/ml (○) 13,293 ± 685 10 μg/ml (●) 16,953 ± 1,082.

Con A receptors (KATAOKA, T., MAGAE, J., KASAMO, K., YAMANISHI, H., ENDO, A., YAMASAKI, M. & NAGAI, K., unpublished data). These findings suggest that prodigiosin 25-C and Con A enhance their suppressive effects each other and synergistically suppress the cell proliferation.

Asparagine-linked sugar chains of glycoproteins are divided into three groups, high-mannose-type, complex-type and hybrid-type. Cells express a wide variety of these glycoproteins on their surface. Con A preferentially binds to mannose residues of asparagine-linked glycoproteins and has affinity to high-mannose-type, biantennary-complex-type and hybrid-type sugar chains[22,23]. Proliferation of splenic T cells was more sensitive to prodigiosin 25-C in the presence of higher concentration of LCA, PSA and VFA which bind to mannose residue of biantennary-complex-type sugar chains[22,23]. On the other hand, sensitivity of T cells to prodigiosin 25-C was not changed when the mitogenic response was induced with PHA and PWM, which bind to *N*-acetylgalactosamine and *N*-acetylglucosamine, respectively. Thus, binding of Con A to the mannose residue of biantennary-complex-type sugar chains seems to be required to enhance the inhibitory effect of prodigiosin 25-C.

The enhancement of the growth inhibitory effect of prodigiosin 25-C by Con A was observed in most of the T cells examined, including both $CD4^+CD8^-$ and $CD4^-CD8^+$ subsets and various T cell lines, although their sensitivities differed depending on the population. LPS-induced B cell blasts had greater sensitivity to prodigiosin 25-C in the presence of Con A. Thus, sensitivity to prodigiosin 25-C can be assumed to be affected by the activation stage of cells.

The present results indicate that the effect of prodigiosin 25-C is not selective for particular lymphocyte populations. However, *in vivo* the compound has been shown to suppress preferentially the generation of killer T cell[3,4]. Lymphocytes *in vivo* interact with extracellular matrix as well as various other cells[24].

Thus, the microenvironmental conditions in the stimulation or differentiation may be important for prodigiosin 25-C to exert its effect.

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