SELECTIVE IMMUNOSUPPRESSION OF PRODIGIOSIN 25-C AND FK506 IN THE MURINE IMMUNE SYSTEM

RYOHEI F. TSUJI, MASAKUNI YAMAMOTO, AKITO NAKAMURA, TAKAO KATAOKA, JUNJI MAGAE†, KAZUO NAGAI† and MAKARI YAMASAKI

Laboratory of Microbiology, Department of Agricultural Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan
†Department of Bioengineering, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152, Japan

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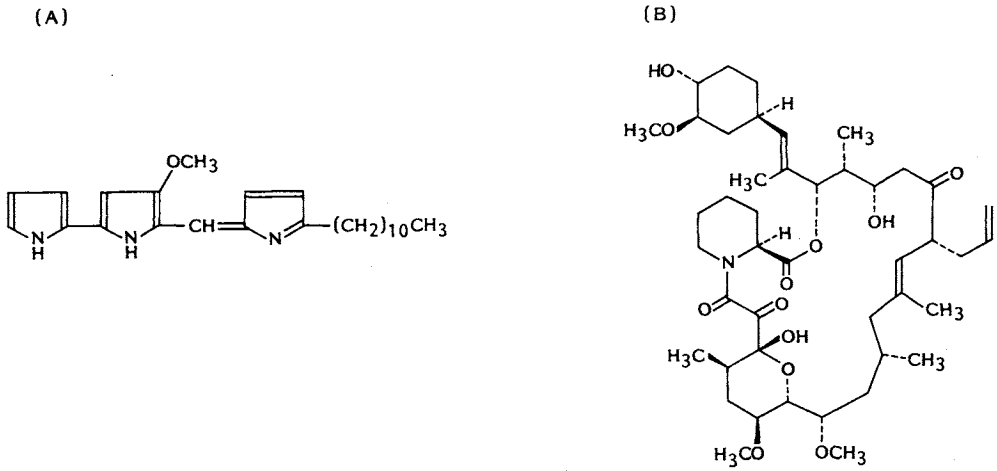
The immunosuppressive effects of prodigiosin 25-C were studied in comparison with FK506. Both prodigiosin 25-C and FK506 suppressed T cell proliferation in response to concanavalin A (con A) or phytohemagglutinin (PHA) more significantly than that to lipopolysaccharide. However, prodigiosin 25-C inhibited con A-mediated mitogenic response more strongly than PHA-mediated one. FK506 showed no selectivity among those responses. In addition, when higher concentration of con A was used an inhibitory effect of prodigiosin 25-C became more evident whereas that of FK506 became less evident. Furthermore, prodigiosin 25-C affected neither interleukin-2 (IL-2) production nor IL-2 receptor (IL-2R) and transferrin receptor (TF-R) expression *in vitro*, though FK506 extensively inhibited IL-2 production and significantly suppressed IL-2R and TF-R expression.

When comparing the effects of prodigiosin 25-C and FK506 *in vivo* by injecting antigens of different nature to a mouse, prodigiosin 25-C selectively inhibited cytotoxic T lymphocyte (CTL) activity induced by an allogenic mastocytoma, P815, without affecting production of antibody against a thymus dependent (TD) antigen, sheep red blood cell (SRBC). On the contrary, FK506 significantly inhibited both CTL induction and the antibody production. When *Brucella abortus*, a thymus independent (TI) antigen, and SRBC were simultaneously challenged to a mouse, neither prodigiosin 25-C nor FK506 affected antibody production against the TI antigen while the effect on the TD antigen were the same as described above. The present results revealed the unique immunosuppressive property of prodigiosin 25-C which was different from that of FK506.

During the course of our screening for new immunomodulatory substances, which utilizes mitogenic responses of mouse splenocytes to polyclonal mitogens[1], prodigiosin 25-C was found as a potent immunosuppressant[2]. Prodigiosin 25-C, MW 393, is a red pigment produced by *Streptomyces hiroshimensis*, which has three conjugated pyrrole rings and a hydrophobic hydrocarbon chain (Fig. 1). Prodigiosin 25-C inhibited the responses of murine splenocytes to T cell specific mitogens, concanavalin A (con A) and phytohemagglutinin (PHA), more extensively than to a B cell specific mitogen lipopolysaccharide (LPS)[2]. Prodigiosin 25-C inhibited both con A and interleukin-2 (IL-2)-dependent proliferative response of con A-primed splenocytes[3], which indicated that prodigiosin 25-C had a distinct property from a fungal metabolite, ciclosporin (cyclosporin A, CsA), which selectively inhibited con A-dependent proliferative response. It was also shown that prodigiosin 25-C inhibited the induction of H-2 specific cytotoxic T lymphocyte (CTL) both *in vitro* and *in vivo*, but not that of anti-sheep red blood cell (SRBC) antibody production at the same dose as suppression of CTL induction was observed *in vivo*[2,3]. Prodigiosin 25-C has little myelotoxicity because the numbers of peripheral blood leukocytes and splenocytes of prodigiosin 25-C treated mice were not affected (our unpublished results).

Fig. 1. Structure of prodigiosin 25-C (A) and FK506 (B).



Recently, we found that an unidentified streptomycetes produced another T cell specific immunosuppressant; we identified it as FK506, an antibiotic of the macrolide family, which was isolated from *Streptomyces tsukubaensis*[4]. It was shown that FK506 primarily inhibited early events in the T cell activation process that was required for transcription of the IL-2 gene[5] like the fungal metabolite CsA[6]. Recent studies have shown that their cellular binding proteins were different but had the same biological activities, *i.e.*, peptidyl-prolyl *cis-trans* isomerase activity[7~11]. In the present study, we comapred the immunosuppressive property of prodigiosin 25-C with that of FK506 *in vitro* and *in vivo*. The results demonstrated that prodigiosin 25-C but not FK506 selectively inhibited CTL induction without affecting functions of helper T cells and B cells.

Materials and Methods

Animals

Special pathogen-free female C57BL/6, CDF_1 and male Balb/c mice were obtained from Charles River Japan Co., Ltd., Tokyo. Female SD rat was also a product of Charles River Japan.

Preparation of Cells

RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Gibco), 50 μM 2-mercaptoethanol, 50 μg/ml kanamycin, 8 μg/ml tylosin tartrate was used for cell cultures throughout the experiments. Single cell suspension of spleen cells was prepared and erythrocytes in the suspension was removed as described previously[12]. T cells were purified by the method reported by JULIUS *et al.*[13] with slight modifications. Spleen cells (1×10^8 cells) were incubated at 37°C in the nylon wool (6 g, Wako Pure Chemical) which was packed in 10 ml syringe and preequilibrated with the medium. After 45 minutes incubation, cells were gently eluted with the medium. Those nylon-wool nonadherent cells contained more than 90% Thy 1^+ cells as analysed by a flow cytometer. Plastic adherent cells were separated as follows. Spleen cells (1×10^8) were incubated in a 100 mm-diameter culture dish for 2 hours at 37°C. At the termination of culture, the dish was gently washed three times with the medium. Adherent cells were detached by vigorous pippeting with 0.6 mM EDTA in phosphate buffered saline (PBS; 0.8% NaCl, pH 7.4). Resultant adherent cells were used as the spleen adherent cells (SAC).

Measurement of IL-2 Activity

IL-2 containing supernatant was diluted serially and incubated with 5×10^4 cells/ml CTLL-2 for 24

hours. [^{3}H]Thymidine (0.5 μCi/well) was pulsed during the final 4 hours-incubation and the incorporated radioactivity was counted by a liquid scintillation counter. Unit of IL-2 was determined as reported by GILLIS *et al.*[14]. One μg/ml corresponded to the concentration giving a half maximum incorporation in the CTLL-2 assay.

Analysis of Surface Antigens

The expression of surface antigen was analysed by a flow cytometer (EPICS, Coulter Electronics, Hialeah, FL). All experiments were carried out in the phenol red-free Hanks balanced salt solution containing 2% FCS, 0.2% NaN_3. Con A-primed T cells (1×10^6) were washed and incubated with 100-fold diluted mouse monoclonal antibody to the rat IL-2 receptor (IL-2R, MRC OX-39) or that to the rat transferrin receptor (TF-R, MRC OX-26) in a 100-μl volume for 45 minutes on ice. Cells were washed and again incubated with 20-fold diluted FITC-conjugated anti-mouse IgG antibody in a 50-μl volume for 45 minutes on ice. Cells were washed several times with the medium and the intensity of their fluorescence was analysed by the flow cytometer. Small particles including erythrocytes were eliminated by gating on the front light scattering. Percent positive cells were calculated by subtracting the background positive cells which were determined by staining only with the second antibody.

Agglutination Assay of Antibody Titer

Serum was incubated with or without 0.1 M 2-mercaptoethanol for 60 minutes at 37°C. They were serially diluted with PBS and incubated with 1.5×10^7 SRBC in a round bottomed microtiter well or 2.5×10^8 *Brucella abortus* in a V-shape bottomed microtiter well for 20 hours at 37°C. Antibody titer defined as half maximum agglutinating ability was expressed as $\log_2$. IgG antibody titer was measured after inactivating IgM by the treatment of the serum with 2-mercaptoethanol.

Determination of CTL Activity

Cytotoxic actvity of splenocytes was determined as described previously[2,3] with slight modifications. Tritium-labeled P815 cells were prepared by incubating the cells for 18 hours in the presence of [^{3}H]thymidine (1 μCi/ml). They were washed three times before use. 4×10^5 splenocytes from primed mice were added to microtiter wells with 1×10^4 target cells and incubated at 37°C for 4 hours. At the end of the incubation period, 100 μl of the supernatant was removed and the released radioactivity was counted by a liquid scintillation counter. Percent lysis was calculated[2] from the experimented release in comparison with the maximum (100%) and spontaneous (0%) releases which were determined by incubating the target cells with 0.5% sodium dodecylsulfate or target cells alone, respectively.

Chemicals

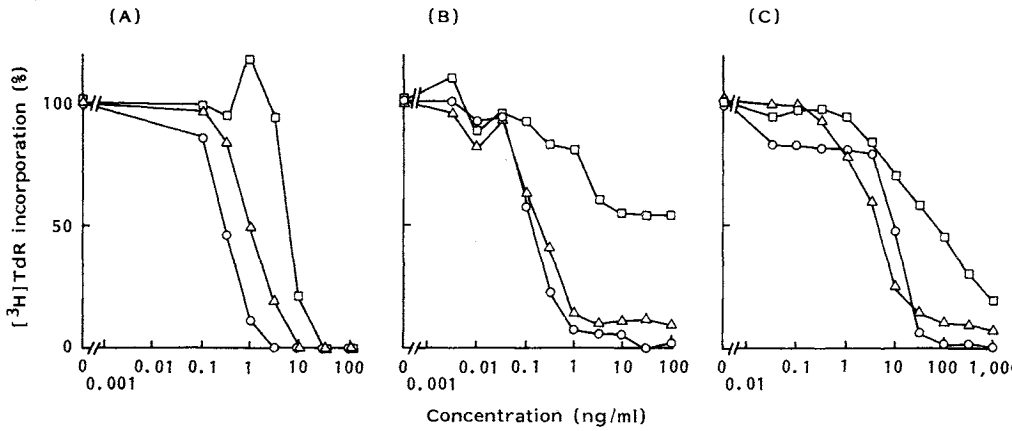
Prodigiosin 25-C was prepared from the extract of the mycelial cake of *S. hiroshimensis* as described previously[2]. It was dissolved in DMSO and was suspended in PBS for injection. FK506 was purified from the mycelial cake of an unidentified streptomycetes through extraction with ethyl acetate, silica gel and Sephadex LH-20 column chromatography. Active materials were finally purified through reverse phase HPLC using a ODS-H451 column (Senshu Kagaku, Tokyo, Japan) and eluting with 80% methanol. Resultant white powder was used for *in vitro* experiments. FK506 which was used for *in vivo* administration was a kind gift from Fujisawa Pharmaceutial Co., Ltd., Osaka, Japan. Monoclonal antibody to rat IL-2R (MRC OX-39) and rat TF-R (MRC OX-26) were purchased from Serotec Corp. (Cambridge, UK) and FITC-conjugated anti-Fc portion of mouse IgG (Fab fragment) was obtained from TAGO, Inc. CA. SRBC was a product of Nippon Bio-Supp. Center (Tokyo, Japan). *B. abortus* was a kind gift from Dr. K. HIROTA (National Institute of Animal Health, Tsukuba-shi, Japan.)

Results

In the course of our screening for immunomodulators[1], we found that an unidentified streptomycetes produced a T cell-specific immunosuppressant which differed from prodigiosin 25-C. After isolation of the active component we identified it as FK506 from its physico-chemical properties. Then we examined

Fig. 2. Suppression of mitogenic responses of splenocytes by prodigiosin 25-C, FK506 and CsA.

(A) Prodigiosin 25-C, (B) FK506, (C) CsA. ○ con A, △ PHA, □ LPS.



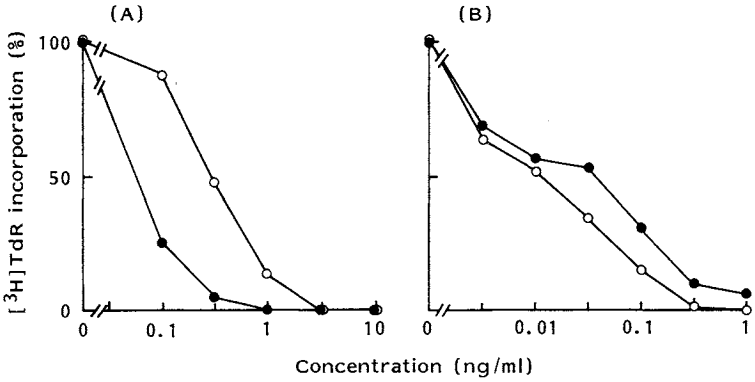
Proliferative response of splenocytes was carried out as described previously[1]. Splenocytes from 6 week old male Balb/c mice (1×10^6 cells/ml) were cultured in a 100-μl volume of the medium containing 1 μg/ml con A, 1 μg/ml PHA or 2 μg/ml LPS, in the presence of prodigiosin 25-C, FK506 or CsA for 48 hours. Four hours prior to harvesting, [^{3}H]thymidine ([^{3}H]TdR) (0.5 μCi/well) was pulsed.

the suppressive effects of prodigiosin 25-C and FK506 on mitogenic response. As shown in a previous report[2], prodigiosin 25-C inhibited con A- and PHA-induced proliferative responses of splenocytes more significantly than LPS-induced one. Furthermore, prodigiosin 25-C inhibited the con A-induced mitogenic response more extensively than PHA-induced one (Fig. 2A). Although FK506 and CsA showed no selectivity between con A- and PHA-induced mitogenic responses, they, like prodigiosin 25-C, inhibited the mitogenic responses of T cells more significantly than that of B cells, which was induced by LPS (Fig. 2B and 2C). It should be noted that the specificity of FK506 in inhibition of T cells was far more evident than CsA, in spite of their resemblance in the mechanism of the T cell specific immunosuppression. ID_{50} of FK506 against con A-, PHA- and LPS-induced proliferative responses were 0.1, 0.1 and >100 ng/ml, respectively, while those of CsA were 3, 6 and 30 ng/ml, respectively.

Since prodigiosin 25-C preferentially inhibited con A-induced T cell proliferation, the inhibitory effect of prodigiosin 25-C was compared at different concentration of con A which was used for inducing the mitogenic response (Fig. 3). Prodigiosin 25-C inhibited the mitogen response more strongly when higher concentration of con A was used. Only 1 ng/ml was enough for the complete inhibition when the mitogenic response was induced by 5 μg/ml con A. In contrast, inhibitory effects of FK506 and CsA turned to be weaker when con A concentration was increased. Dexamethasone, a clinical immunosuppressant, and cycloheximide, a potent inhibitor of protein synthesis had the same property as FK506 and CsA in influencing the dependency of con A concentration (data not shown). These results suggested that the immunosuppressive manner of prodigiosin 25-C was unique and different from that of FK506, CsA and dexamethasone. We previously showed that prodigiosin 25-C inhibited IL-2-dependent proliferation of splenocytes which had been primed by con A and the effect was comparable to the case of con A-induced mitogenic response[3]. In contrast, CsA did not significantly inhibit the IL-2 dependent proliferation. The results indicate that CsA affected the stage of IL-2 production induced by con A while prodigiosin 25-C suppressed the later stage of T cell proliferation. To further investigate the inhibitory stage of prodigiosin

Fig. 3. Suppression of mitogenic responses of splenocytes to different concentrations of con A by prodigiosin 25-C and FK506.

Proliferative responses to con A (5 μg/ml (●) and 1 μg/ml (○)) were determined in the presence of prodigiosin 25-C (A) or FK506 (B).



The experiment was carried out as described in the legend of Fig. 2.

Table 1. Effects of prodigiosin 25-C and FK506 on IL-2 production and expression of IL-2R and TF-R.

Treatment	IL-2 production (U/ml)	Expression of surface antigen (%)	
		IL-2R	TF-R
None	<1.5	2.2	3.6
Con A (5 μg/ml)	18.6	30.7	30.8
+prodigiosin 25-C (ng/ml)			
1.0	30.2	25.0	27.8
10.0	15.5	17.0	27.4
+FK506 (ng/ml)			
1.0	<1.5	7.0	17.3
10.0	<1.5	9.5	13.8

Nylon-wool purified T cells (1×10^6 cells/ml) and spleen adherent cells (1×10^5 cells/ml) from a 6-week old female SD rat were incubated with 5 μg/ml con A at 37°C in the presence of antibiotics for 24 hours. IL-2 activity in the culture supernatant and expressions of IL-2R and TF-R were determined as described in Materials and Methods.

25-C on T cell activation, the effects of prodigiosin 25-C and FK506 on the production of IL-2 and the expression of IL-2R in response to con A were compared. The expression of TF-R was also examined to determine whether prodigiosin 25-C inhibits a signal transduction pathway from IL-2, because TF-R expression following the interaction of IL-2 and IL-2R was shown to be necessary for T cell activation[15]. FK506 strongly inhibited IL-2 production and IL-2R expression as reported by other investigators[5,16], although the expression of TF-R was only partially inhibited. In contrast, prodigiosin 25-C inhibited neither IL-2 production nor IL-2R expression at 1 ng/ml, where the agent completely inhibited the con A-induced proliferative response (Table 1). IL-2 production was rather stimulated at this concentration. At a higher concentration of prodigiosin 25-C (10 ng/ml), the expression of IL-2R was partially inhibited although that of TF-R was not significantly affected. This indicated that prodigiosin 25-C partially inhibited the up-regulation of IL-2R by IL-2 at higher concentrations. These results demonstrated that the suppressive effect of prodigiosin 25-C was not resulted from the inhibition of IL-2 production and ex-

Table 2. Effects of prodigiosin 25-C and FK506 on CTL induction and anti-SRBC antibody production in an identical mouse.

Treatment		CTL activity (% lysis)	Anti-SRBC antibody (mean $\log_2$ titer)	
			No treatment	2ME treatment
Saline		50.4±18.0	4.5	2.3
Prodigiosin 25-C	0.5 mg/kg	11.1± 5.2[a]	4.4	2.0
	1.0 mg/kg	5.5± 4.2[a]	4.6	1.8
Saline		79.0± 2.0	5.0	4.0
FK506	50 mg/kg	64.0± 3.6[a]	5.0	0.2[a]
	200 mg/kg	12.2± 6.9[a]	0.5[a]	<0.2[a]

C57BL/6 mice were injected ip with 2×10^7 P815 cell (H-2^d), mastocytoma, to induce H-2 specific CTL and iv with 1×10^8 SRBC to produce anti-SRBC antibody together. Ten days later, mice were sacrificed. CTL activity of splenocytes and anti-SRBC antibody titer were determined as described in Materials and Methods. The values shown are the mean±SD of three C57BL/6 mice. Prodigiosin 25-C and FK506 were administered ip and po respectively on days 0, 3, 5, 8.

[a] Statistically significant compared with saline-treated group ($P<0.01$, Student's test).

Table 3. Effects of prodigiosin 25-C and FK506 on anti-TD and TI antibody production in an identical mouse.

Treatment		Anti-TD antibody (mean $\log_2$ titer)		Anti-TI antibody (mean $\log_2$ titer)
		No treatment	2ME treatment	
Saline		5.8	4.3	4.8
Prodigiosin 25-C	1.0 mg/kg	4.7	3.3	4.8
	2.0 mg/kg	5.3	3.8	5.0
FK506	30 mg/kg	5.2	1.3[a]	4.7
	100 mg/kg	4.8	0.7[b]	4.8

CDF_1 mice were injected iv with 1×10^8 SRBC as a TD antigen and 1×10^9 *Brucella abortus* as a TI antigen together. Six days later, 1×10^9 *B. abortus* were secondarily immunized. Twelve days later, the serum of these mice were obtained and antibody titer was assayed as described in Materials and Methods. Prodigiosin 25-C and FK506 were administered ip and po, respectively on days 1, 5, 8. Values shown are the mean of three CDF_1 mice.

[a,b] Statistically significant compared with saline-treated group ([a] $P<0.05$, [b] $P<0.01$, Student's test).

pressions of IL-2R or TF-R, which are thought to be major principles of immunosuppression of FK506 and CsA[5,6,17~19].

To demonstrate the selective effect of prodigiosin 25-C on the immune system *in vivo*, we compared the effects of prodigiosin 25-C and FK506 on the induction of CTL and the production of antibody in the system in which two antigens, P815 allogenic cells for the CTL induction and SRBC for the antibody production, were simultaneously immunized to a mouse. As shown in Table 2, prodigiosin 25-C inhibited the CTL induction at the dose above 0.5 mg/kg whereas prodigiosin 25-C influenced neither anti-SRBC titer of whole antibody nor that of IgG antibody. The induced CTL was H-2 specific, because it was reactive to H-2^d but not to H-2^b and H-2^k (data not shown) as previously shown[3].

In contrast, FK506 inhibited the allospecific CTL induction and the anti-SRBC antibody production at the same level and depending on the applied dose. FK506 inhibited the lymphokine production by helper T cells[4,17~19], which was thought to be a main reason for its inhibitory effects on the CTL induction

and the anti-SRBC antibody production.

To study the direct effect of antibiotics on B cells *in vivo*, we challenged a thymus independent (TI) antigen[20], *B. abortus*, as well as a thymus dependent (TD) antigens, SRBC, to an identical mouse. In the case of *B. abortus*, only IgM production was observed probably because IgG antibody production requires the help of T cells. Prodigiosin 25-C did not inhibit the production of antibody against these two antigens while FK506 inhibited the antibody production against SRBC, but not that to *B. abortus*. Furthermore, FK506 inhibited IgG production more intensively than IgM production (Tables 2 and 3). Thus, it was strongly suggested that prodigiosin 25-C affects neither helper T cells nor B cells whereas FK506 inhibits helper T cell function without affecting the function of B cells.

Discussion

The immunological network is in a dynamic equilibrium regulated by various kinds of cells. To control the balance of the network, low MW agents that have specificity to the function of each group of cells are of use. We have been screening for such compounds from the culture broths of microorganisms in a screening system using spleen cells and specific mitogens and have found prodigiosin 25-C and FK506 as T cell-specific immunosuppressants. However, they are different in some respects. Prodigiosin 25-C but not FK506 inhibited the T cell response to con A more siginificantly than that to PHA (Fig. 2). Furthermore, prodigiosin 25-C inhibited proliferative response more significantly when con A concentration used for the stimulation was increased, while the inhibitory effect of FK506 turned to be weaker at a higher concentration of con A (Fig. 3). A further experiment showed that the immunosupression induced by prodigiosin 25-C as different from FK506, was not attained by inhibiting the IL-2 production, IL-2R expression and TF-R expression (Table 1). It can be considered that prodigiosin 25-C inhibits the later step of the T cell activation while FK506 inhibits its earlier stage.

Such differences were also demonstrated *in vivo*. Prodigiosin 25-C specifically inhibited the H-2-restricted CTL induction without inhibiting the antibody production against SRBC and *B. abortus* while FK506 inhibited both the CTL induction and the anti-SRBC antibody production *in vivo* (Tables 2 and 3). Since SRBC is a TD antigen and the participation of helper T cells is required to produce specific antibody, FK506 may inhibit this response by eliminating the function of helper T cells. This was further confirmed by the experiment using *B. abortus* which is a TI antigen and does not need the help of T cells. Neither prodigiosin 25-C nor FK506 affected the antibody production against *B. abortus* at all. These results clearly demonstrated that prodigiosin 25-C strongly inhibits the CTL induction without affecting the functions of helper T cells and B cells while FK506 inhibits the CTL induction and the antibody production against the TD antigen by inhibiting the function of helper T cells without affecting the function of B cells.

Recent studies on immunosuppressive antibiotics revealed that some antibiotics have a T cell specific immunosuppressive activity. Among them, deoxyspergualin is similar to prodigiosin 25-C because it does not inhibit IL-2 production but suppresses mixed lymphocyte response and CTL induction[21]. Furthermore, IL-2 induced proliferation of con A blasts and CTLL-2 are inhibited at low concentration of the agent[22]. So, deoxyspergualin, like prodigiosin 25-C, is thought to affect the proliferative stage after IL-2 and IL-2R binding assembly. However, deoxyspergualin inhibits antibody production against both TD and TI antigens *in vivo* and also inhibits B cell proliferation and antibody production to LPS *in vitro*[23] while prodigiosin 25-C did not affect the functions of B cells.

Recent studies showed that there are helper T cells bearing CD8 surface molecule[24] and also CTL bearing CD4 surface molecule[25~27]. Though CD4 helper T cells and CD8 CTL are dominant subsets, it is now clear that functions of T cells cannot be distinguished with surface markers such as CD4 or CD8. Since the effect of prodigiosin 25-C seems to be specific on CTL the agent will be useful to study the role of CTL in the immune network. It is of interest to investigate how much CTL-mediate cytotoxicity contributes to the allograft rejection, the graft *versus* host reaction and delayed type-hypersensitivity by using prodigiosin 25-C. Furthermore, defining the nature of the molecular target of prodigiosin 25-C

would serve as a useful probe for analysing the CTL activation pathway.

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