IMMUNOMODULATING PROPERTIES OF PRODIGIOSIN 25-C, AN ANTIBIOTIC WHICH PREFERENTIALLY SUPPRESSES INDUCTION OF CYTOTOXIC T CELLS

RYOHEI F. TSUJI[†], JUNJI MAGAE[††], MASAHIRO YAMASHITA[††], 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, 4259 Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa 227, Japan

(Received for publication February 12, 1992)

An antibiotic, prodigiosin 25-C, preferentially suppresses cytotoxic T lymphocytes (CTL) without affecting antibody production. Here, we investigated the effect of prodigiosin 25-C on delayed-type hypersensitivity (DTH), graft versus host reaction (GvHR) and allogeneic skin graft rejection. DTH reactions were markedly inhibited by ip treatment of the mice with prodigiosin 25-C. Cell transfer experiments indicated that prodigiosin 25-C exerted its suppressive effect on the late efferent phase rather than on the induction phase of DTH. Prodigiosin 25-C suppressed induction of anti-host CTL when GvHR was induced by iv inoculating splenocytes of parental C57BL/6 mice to adult unirradiated BDF_1 mice. It had little effect on GvHR-induced splenomegaly observed 2 weeks after the inoculation, but significantly delayed the subsidence of splenomegaly as revealed 8 weeks later, suggesting that suppression of CTL converts immunosuppressive GvHR to immunostimulative one as reported by G. M. SHEARER. However, reduction of interleukin-2 (IL-2) production and mitogen responses induced by GvHR were not rescued by prodigiosin 25-C treatment. Prodigiosin 25-C moderately prolonged survival of major histocompatibility (MHC)-mismatched skin grafts. Since the mode of action of prodigiosin 25-C is distinct from those of cyclosporin A and FK506, these results demonstrate potential usefulness of the antibiotic for a supplementary immunosuppressant.

Prodigiosin 25-C, a red pigment produced by *Streptomyces hiroshimensis*, was found to be a potent immunosuppressant with its primary effect on functions of T cells[1]. It was found to exert a significant inhibitory effect on induction of allogeneic cytotoxic T lymphocytes (CTL)[2]. Helper T cells did not appear to be the target of this immunosuppressive agent since the suppressive effect of prodigiosin 25-C on proliferative responses of T cells was not reversed by exogenous IL-2, and in addition, it did not suppress IL-2 production nor expression of IL-2 receptor by T cells[3]. Furthermore, prodigiosin 25-C had no effect on either T-dependent or T-independent antibody responses[3]. These observations suggest that prodigiosin 25-C acts primarily on CTL (or precursor CTL) rather than on helper T cells. Thus, the immunosuppressive property of prodigiosin 25-C appears quite distinct from that of cyclosporin A and FK506, which are believed to inhibit early events of T cell activation required for transcription of the IL-2 gene[4,5].

Because of the potential clinical usefulness of immunosuppressants of new types, we investigated the effect of prodigiosin 25-C on *in vivo* experimental models including allograft rejection, graft versus host reaction (GvHR) and delayed-type hypersensitivity (DTH).

[†] Present address: Noda Institute for Scientific Research; 399 Noda, Noda-shi, Chiba 278, Japan.

Materials and Methods

Animals

Specific pathogen-free female C57BL/6, BALB/c, BDF_1 and CDF_1 mice were obtained from Japan Charles River Co., Ltd., Tokyo.

Chemicals

Prodigiosin 25-C was prepared from the extract of the mycelial cake of *Streptomyces hiroshimensis* as described previously[1]. It was stored as dimethyl sulfoxide solution in the dark at $-20°C$ and was suspended in PBS (0.29% Na_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl, 0.02% KCl, pH 7.4) immediately before injection. It was stable during storage of more than 3 years.

DTH Assay

In the case of SRBC-induced DTH, sheep red blood cells (SRBC) (1×10^5 cells) suspended in 200 μl PBS were injected iv into the tail vein of female C57BL/6 mice. Five days later, 1×10^8 SRBC suspended in 20 μl PBS were injected into subcutaneous tissue of the left hind footpad. In the case of hapten-induced DTH, 200 μl of 10 mM 2,4,6-trinitrobenzene sulfonate (TNBS) in PBS was injected subcutaneously on the abdomen of BALB/c mice. Seven days later, 40 μl of the TNBS solution was injected into subcutaneous tissue of the left hind footpad. The increase in footpad thickness was measured 24 hours after elicitation with a dial-caliper (Ozaki, MFG Co., Ltd. Japan).

Induction of GvHR and Assessment of T Cell Functions

GvHR was induced by iv injection of 5×10^7 splenocytes from parental C57BL/6 mice into normal, unirradiated BDF_1 host mice. F_1 host mice injected with syngeneic splenocytes instead of parental splenocytes were served as controls. Two or eight weeks after the injection of the parental splenocytes, host spleens were removed and then weights measured. They were teased into single cell suspensions. T cell functions of GvHR mice were assessed as described previously[3] using RPMI1640 medium supplemented with 10% fetal calf serum (Gibco), 50 μM 2-mercaptoethanol, 50 μg/ml kanamycin, 8 μg tylosin tartrate. CTL activity of the splenocytes was determined with [^{3}H]thymidine-labeled target cells (1×10^4 cells/well) for 4 hours at 37°C in a 200 μl volume. At the end of the incubation, 100 μl of the supernatant was removed and the released radioactivity was counted by a liquid scintillation counter. Mitogen responses were determined by culturing splenocytes (1×10^5 cells/well) with 1 μg/ml concanavalin A (con A), 2 μg/ml phytohemagglutinin (PHA) or 5 μg/ml lypopolysaccharide (LPS) in a 100 μl volume for 48 hours. [^{3}H]Thymidine (0.5 μCi/well) was added for 4 hours at the termination of the culture. IL-2 was induced by stimulating the splenocytes (5×10^6 cells/ml) with 5 μg/ml con A for 24 hours. IL-2 contained in the supernatants was measured using a T cell clone, CTLL-2.

Skin Grafting

Transplantation of MHC-mismatched skin was carried out according to the method of INAMURA *et al.*[6]. A full thickness ear skin graft from C57BL/6 mouse was transplanted to the lateral thorax of a CDF_1 mouse and covered with sterile bactericidal gauze. The entire chest was wrapped with an elastic bandage which was removed on day 5. The grafts were inspected daily until rejection, which was defined as more than 50% necrosis of the graft epithelium.

Statistics

Statistical analysis was performed by using the one tailed STUDENT's *t*-test and $P < 0.05$ was taken as the level of significance.

Results

Effect of Prodigiosin 25-C on DTH Response

SRBC and TNBS were injected iv and sc, respectively. Experimental groups of animals re-

Fig. 1. Structure of prodigiosin 25-C.

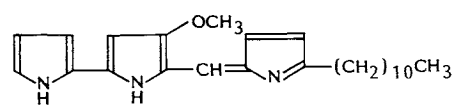


Table 1. Effect of prodigiosin 25-C on SRBC-induced DTH.

Immunization (SRBC, iv)	Prodigiosin 25-C (mg/kg)	Increase in footpad thickness[a] ($cm \times 10^{-2}$)
−	0	5.8 ± 1.2
+	0	11.9 ± 1.5
+	0.5	9.5 ± 3.8 (20.2)[c]
+	1.0	6.9 ± 2.1[b] (42.0)
+	2.0	1.2 ± 0.8[b] (89.9)

C57BL/6 mice (n=5) were iv immunized with SRBC on day 0 and prodigiosin 25-C was ip injected on days 0, 3, 5 (day 0 corresponded to 4 hours after immunization with SRBC). Animals were challenged with the antigen and footpad swelling was measured 24 hours later.

[a] Data represent as mean ± SD.

[b] Statistically significant ($P<0.05$) as compared to immunized mice which did not receive prodigiosin 25-C.

[c] The number in parentheses represents the inhibition (%) of footpad swelling.

Table 2. Effect of prodigiosin 25-C on TNBS-induced DTH.

Immunization (TNBS, sc)	Prodigiosin 25-C (mg/kg)	Increase in footpad thickness[a] ($cm \times 10^{-2}$)
−	0	0.1 ± 0.3
+	0	10.1 ± 1.3
+	0.5	$3.9 + 1.7$[b] (61.4)[c]
+	1.0	1.0 ± 1.1[b] (90.1)

BALB/c mice (n=4) were sc immunized with TNBS on day 0 and prodigiosin 25-C was ip injected on days 0, 2, 4, 6 (day 0 corresponded to 4 hours after immunization with TNBS). TNBS was challenged on day 5 and footpad swelling was measured 24 hours later.

[a] Data represent as mean ± SD.

[b] Statistically significant ($P<0.05$) as compared to immunized mice which did not receive prodigiosin 25-C.

[c] The number in parentheses represents the inhibition (%) of footpad swelling.

ceived graded amounts of prodigiosin 25-C by ip injection. Five or seven days later, the animals were challenged with SRBC and TNBS injected into left hind-footpad, respectively. As shown in Table 1, prodigiosin 25-C suppressed the SRBC-specific DTH response. Marked inhibition was observed at the doses over 1 mg/kg. When mice were treated with 2 mg/kg of prodigiosin 25-C, footpad swelling was even smaller than that of non-specific swelling observed in the non-immunized mice, suggesting that prodigiosin 25-C has a suppressive effect on non-specific components of inflammation. In the case of TNBS-specific DTH response, prodigiosin 25-C suppressed the footpad swelling at the doses over 0.5 mg/kg (Table 2). The LD_{50} of prodigiosin 25-C by a single ip dose was 7.1 mg/kg (data not shown). No immediately detectable effect on the animal health was observed even at the highest dose of prodigiosin 25-C used in this experiment.

Table 3. Prodigiosin 25-C suppressed the efferent phase of DTH induced by SRBC.

Immunization (SRBC, iv)	Prodigiosin 25-C	Increase in footpad thickness[a] ($cm \times 10^{-2}$)	
		Exp. 1	Exp. 2
−	—	1.8 ± 0.8	1.3 ± 0.8
+	—	4.0 ± 0.9	4.5 ± 0.5
+	Host	0.8 ± 1.9[b]	1.8 ± 1.0[b]
+	Donor	4.3 ± 0.6	3.5 ± 0.7

Donor C57BL/6 mice were immunized with 1×10^5 SRBC. Five days later, mice were killed and single cell suspension was prepared from the pooled spleens. The donor spleen cells (5×10^7 cells) were transferred intravenously into host mice (n=3) and DTH reaction was induced by challenging the host mice by injecting SRBC (1×10^8 cells) into left hind footpad. Footpad swelling was measured 24 hours after the challenging. Treatment of prodigiosin 25-C (1 mg/kg) was performed intraperitoneally, for successive 4 days from the immunization in the case of donor mice, or 6, 3, 1 day before and on the day of the transfer in the case of host mice.

[a] Data represent as mean ± SD.

[b] Statistically significant ($P<0.05$) as compared to immunized mice which did not receive prodigiosin 25-C.

Since our previous findings indicated that prodigiosin 25-C primarily affects CTL rather than helper T cells, which are believed to play the primary role in induction of DTH, a cell transfer experiment was carried out to determine whether prodigiosin 25-C inhibited the induction or the efferent phase of the DTH reaction. SRBC were injected iv and five days later, the spleen cells transferred intravenously to secondary non-immunized host animals. Twenty-four hours after the transfer, the host animals were chal-

lenged by injection of SRBC into footpads. As shown in Table 3, the treatment of the immunized donor mice with prodigiosin 25-C did not result in suppression of the DTH reaction detected in the secondary hosts. On the other hand, significant inhibition was observed when the secondary host mice were treated with prodigiosin 25-C. These results indicated that prodigiosin 25-C exerted its suppressive effect on the efferent phase of DTH rather than on the induction phase of the reaction, in which helper T cells play the major role. The result, nevertheless, did not reveal which are the target cells among the variety of cells involved in the efferent phase of the DTH reaction, including T cells and non-specific inflammatory cells.

Effect of Prodigiosin 25-C on GvHR

GvHR was induced in adult unirradiated (C57BL/6 × DBA/2)F_1 (BDF_1) mice by injecting intravenously parental splenocytes from C57BL/6 mice. The effect of prodigiosin 25-C on splenomegaly was examined (Table 4). Prodigiosin 25-C did not have a significant effect on the splenomegaly of F_1 hosts on day 14. Interestingly, the drug appeared to delay subsidence of the GvHR-induced splenomegaly detected as reduction in splenic size at 8 weeks of the experiment. When prodigiosin 25-C was injected ip into naive mice, no notable change in spleen size was observed, indicating that prodigiosin 25-C itself was not immunogenic and did not induce splenomegaly. We further investigated whether or not prodigiosin 25-C suppressed the induction of anti-host CTL in the GvHR-elicited mice. Since GvHR was induced in a combination, H-2^b anti-H-$2^{b/d}$, P815 cells (H-2^d, mastocytoma) were used as allogeneic targets for anti-host CTL. Increased anti-host killing was observed in spleen cells from the mice injected with parent spleen cells but not in those

Table 4. Effect of prodigiosin 25-C on splenomegaly induced by GvHR.

Donor splenocytes	Prodigiosin 25-C (ip, mg/kg)	Spleen weight (mg)[a]	
		2 weeks	8 weeks
BDF_1	—	69± 7[b]	83± 4
C57BL/6	—	194± 6	82±14
	0.5	201±15	59±12
	1.0	226±36	192±39[b]

Donor splenocytes were transferred into host BDF_1 mice on day 0. Prodigiosin 25-C was ip injected into host mice on days 0, 3, 5, 8 (day 0 corresponded to 4 hours after iv inoculation of parental splenocytes) then followed by injection every three days. Mice were sacrificed and their spleens were weighed 2 or 8 weeks after the transfer.

[a] Mean±SD (n=5).

[b] Statistically significant as compared to untreated GvHR mice.

Table 5. Prodigiosin 25-C suppressed anti-host CTL induction by GvHR.

Donor splenocytes	Prodigiosin 25-C (ip, mg/kg)	% lysis against P815[a]		
		Effector/Target ratio		
		80	40	20
BDF_1	—	4.8±4.9[b]	7.2±4.4[b]	3.7±3.2[b]
C57BL/6	—	30.6±4.2	20.3±1.5	13.4±1.9
	0.5	18.7±4.0[b]	11.9±2.5[b]	9.2±4.1
	1.0	16.1±5.3[b]	8.4±8.1[b]	10.2±2.1

GvHR was induced and prodigiosin 25-C was injected as described in the legend of Table 4. Two weeks after the injection of donor splenocytes, mice (5 mice/group) were killed and a single cell suspension was prepared from the pooled spleens. Percent lysis of the splenocytes against [^{3}H] labeled target cells was determined in independent triplicate cultures as described in Materials and Methods. CTL activity of splenocytes from untreated GvHR mice against syngeneic target cells (EL-4, H-2^b) was 7.5±5.1% (E/T ratio=40).

[a] % lysis (mean±SD, n=3).

[b] Statistically significant as compared to untreated GvHR mice.

Table 6. Effect of prodigiosin 25-C on suppression of mitogen responses by GvHR.

Donor splenocytes	Prodigiosin 25-C (ip, mg/kg)	[^{3}H]Thymidine incorporated (cpm)[a]			
		None	Con A (1 μg/ml)	PHA (2 μg/ml)	LPS (5 μg/ml)
BDF_1	—	1,876±326	189,032±14,099	53,812± 295	41,923±6,274
C57BL/6	—	3,227±665	85,405±13,359	37,689±5,537	13,850±4,133
	0.5	1,754±268	111,514± 9,301	24,043±1,569	23,085±2,089
	1.0	1,293±287	92,544±19,418	17,991±2,197	12,816± 462

GvHR was induced and prodigiosin 25-C was injected as described in the legend of Table 4. Two weeks after the injection of donor splenocytes, mice were killed and a single cell suspension was prepared from the pooled spleens. Proliferative responses of the splenocytes in the absence or presence of mitogens were determined in independent triplicate cultures as described in Materials and Methods. A representative of several independent experiments was shown.

[a] Mean±SD (n=3).

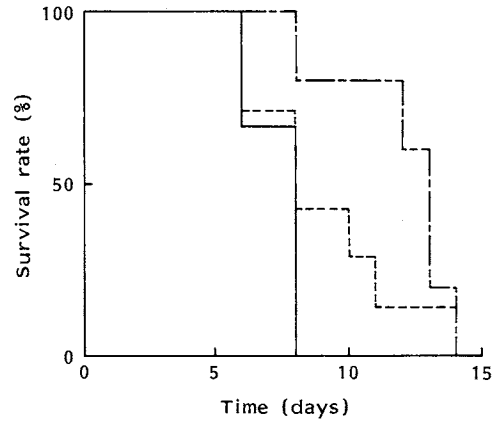
Table 7. Effect of prodigiosin 25-C on suppression of IL-2 production by GvHR.

Donor splenocytes	Prodigiosin 25-C (ip, mg/kg)	IL-2 (unit/ml)	
		Exp 1	Exp 2
BDF_1	—	17.3	44.8
C57BL/6	—	2.3	4.3
	0.5	3.0	4.1
	1.0	2.3	4.2

GvHR was induced and prodigiosin 25-C was injected as described in the legend of Table 4. Two weeks after the injection of donor splenocytes, mice were killed and single cell suspension was prepared from the pooled spleens. IL-2 was induced and measured as described in Materials and Methods.

Fig. 2. Effect of prodigiosin 25-C on survival of MHC-mismatched skin graft.

Median survival times (mean±SD); control group (n=6, —, 7.3±1.0); 0.2 mg/kg prodigiosin 25-C treated (n=7, – – –, 8.9±2.6); 0.5 mg/kg prodigiosin 25-C treated (n=5, —·—, 12.2±2.6).



Ear skin grafts from C57BL/6 (H-2^b) mice were transplanted on CDF_1 (H-2^d) mice as described in Materials and Methods. Prodigiosin 25-C was ip injected into host mice every other day from the operating day.

Prolongated survival time by the treatment of prodigiosin 25-C (0.5 mg/kg) were statistically significant. Body weights of mice during the experiment were not significantly different among the three groups.

from the mice injected with F_1 spleen cells. This killing activity was H-2^dspecific, because no increase was observed when EL-4 (H-2^b, thymoma) cells were used as the target cells. The anti-host CTL induction was significantly prevented by ip treatment of prodigiosin 25-C at doses over 0.5 mg/kg (Table 5).

Consistent with the previous reports[7~9], mitogenic responses of splenocytes from GvHR mice stimulated with con A, PHA and LPS were suppressed. The suppression was not restored by ip treatment with prodigiosin 25-C (Table 6). Spleen cells from GvHR mice produced less IL-2 (Table 7) in response to con A which is also consistent with the previous studies[9,10]. Prodigiosin 25-C did not rescue the deficiency of IL-2 production by GvHR. Prodigiosin 25-C did not have any influence on con A-induced proliferation and IL-2 production of splenocytes when the agent was injected into naive mice. The percentage of $Thy1^+$ cells and surface Ig^+ cells were not extensively changed by treatment of prodigiosin 25-C (data not shown).

Prodigiosin 25-C Prolonged Allograft Survival

MHC-mismatched skin grafts from C57BL/6 mice were transplanted to CDF_1 mice. The mice treated with PBS every other day rejected their grafts within 8 days with a median survival time of 7.3 days (Fig. 2). Prodigiosin 25-C, when injected intraperitoneally to the host mice prolonged skin graft survival in a dose-dependent manner. With 0.5 mg/kg of prodigiosin 25-C, the median survival time was prolonged to 12.2 days and with 0.2 mg/kg, 8.9 days. Increasing the doses of prodigiosin 25-C above 1 mg/kg did not cause further prolongation of graft survival time (data not shown). All the mice treated with 2 mg/kg of prodigiosin 25-C appeared to be healthy.

Discussion

Prodigiosin 25-C has been shown to be an immunosuppressant with preferential effect on CTL. Its mode of action is different from those of cyclosporin A or FK506 which selectively inhibit the function of helper T cells, causing deficiency of both CTL induction and antibody production. In the present study, effects of prodigiosin 25-C on murine experimental models of DTH, GvHR and graft rejection were studied to test the *in vivo* efficacy of its immunosuppressive property. DTH response has been thought to be mediated by Type 1 helper T cells which secrete IL-2[11~13]. Therefore, we considered that prodigiosin 25-C would not suppress DTH reaction since it does not inhibit secretion of IL-2. However, the drug significantly suppressed DTH responses to both SRBC and TNBS. The results of cell transfer experiments indicated that prodigiosin 25-C suppressed the efferent phase rather than the induction phase of DTH (Table 3). Thus it appears that prodigiosin 25-C suppressed DTH by affecting functions of non-specific inflammatory cells, such as polymorphonuclear leukocytes and macrophage. An alternative possibility is that prodigiosin 25-C caused perturbation of late components of T cell function necessary for initiation of inflammation.

According to the classification of GvHR by VIA and SHEARER[14], a combination C57BL/6 anti-BDF_1 induces immunosuppressive GvHR which is characterized by reduction of spleen size, appearance of anti-host CTL and chimerism of spleen cells. However, KINO *et al.*[15] and ABE *et al.*[16] reported that GvHR by this combination resulted in splenomegaly. We also observed marked splenomegaly in this combination. Administration of prodigiosin 25-C had little effect on splenomegaly two weeks after cell transfer but caused delay of its subsidence. The administration of prodigiosin 25-C also significantly suppressed induction of CTL specific to $H\text{-}2^d$. Immunosuppressive GvHR was reported to be converted to immunostimulatory GvHR which is characterized by increase in spleen size and appearance of autoantibody when development of anti-host CTL in the host mice was blocked by depletion of $Lyt2^+$ cells in donor splenocytes[17]. Therefore, our observations that prodigiosin 25-C converted the immunosuppressive GvHR to immunostimulatory GvHR resulting in sustained splenomegaly suggests the preferential effect of prodigiosin 25-C on the development of CTL in the GvHR model. However, reduction of mitogen response and IL-2 production of T cells, observed in this GvHR were not reversed by prodigiosin 25-C, suggesting that the reduction was not mediated by activation of anti-host CTL. Based on these results, we feel that the mode of immunosuppression by prodigiosin 25-C on GvHR shares common features with that of L-leucyl-L-leucine methyl ester (Leu-Leu-OME). Leu-Leu-OME is reported to be selectively toxic to CTL and natural killer cells without affecting helper T cells[18]. Pretreatment of donor splenocytes with Leu-Leu-OME enhanced splenomegaly, inhibited induction of anti-host CTL and prevented lethality induced in the same GvHR model we employed[19,20]. However, the effect of prodigiosin 25-C was not the same as that of Leu-Leu-OME, in that it strongly inhibited response of spleen cells to con A *in vitro*, whereas Leu-Leu-OME affected that only slightly[20].

Recently, it was shown that allograft rejection is mediated by T cell populations that contained both helper T cells and cytotoxic T cells[21]. Since DTH and anti-host CTL in GvHR was substantially suppressed by prodigiosin 25-C, we tested the efficacy of prodigiosin 25-C on MHC-mismatched skin graft survival and found that it significantly prolonged survival time of the graft at the dose where prodigiosin 25-C did not affect body weight. Furthermore, even when 2.0 mg/kg of prodigiosin 25-C was used we did not observe

any toxicity in the mice. Usually rejection against allogeneic skin is stronger than that against other organs, such as heart, kidney and liver. Even FK506 and cyclosporin A can not lead to complete tolerance in the case of skin graft. In this respect, the effect of prodigiosin 25-C is hopeful. The effect on graft rejection was not as striking as with FK506 or cyclosporin A[6,22] indicating that suppression of CTL activity is not sufficient to prevent graft rejection. Nevertheless, since the mode of action of prodigiosin 25-C is distinct from that of cyclosporin A and FK506, and it may have a use as a supplementary immunosuppressant.

Acknowledgment

We thank N. SHINOHARA for the critical discussion. We also thank M. SCHAECHTER for the help of preparation of the manuscript. This work was partly supported by the grant for "Biodesign Research Program" from RIKEN to K. NAGAI.

References

1) NAKAMURA, A.; K. NAGAI, K. ANDO & G. TAMURA: Selective suppression by prodigiosin of the mitogenic response of murine splenocytes. J. Antibiotics 39: 1155~1159, 1986
2) NAKAMURA, A.; J. MAGAE, R. F. TSUJI, M. YAMASAKI & K. NAGAI: Suppression of cytotoxic T cell induction *in vivo* by prodigiosin 25-C. Transplantation 47: 1013~1016, 1989
3) TSUJI, R. F.; M. YAMAMOTO, A. NAKAMURA, T. KATAOKA, J. MAGAE, K. NAGAI & M. YAMASAKI: Selective immunosuppression of prodigiosin 25-C and FK506 in the murine immune system. J. Antibiotics 43: 1293~1301, 1990
4) KRONKE, M.; W. LEONARD, J. DEPPER, S. ARYA, F. WONG-STAAL, R. GALLO, T. WALDMANN & W. GREENE: Cyclosporine A inhibits T-cell growth factor gene expression at the level of mRNA transcription. Proc. Natl. Acad. Sci. U.S.A. 81: 5214~5218, 1984
5) TOCCI, M. J.; D. A. MATKOVICH, K. A. COLLIER, P. KWOK, F. DUMONT, S. LIN, S. DEGUDICIBUS, J. J. SIEKIERKA, J. CHIN & N. I. HUTCHINSON: The immunosuppressant FK506, selectively inhibits expression of early T cell activation genes. J. Immunol. 143: 718~726, 1989
6) INAMURA, N.; K. NAKAHARA, T. KINO, T. GOTO, H. AOKI, I. YAMAGUCHI, M. KOHSAKA & T. OCHIAI: Prolongation of skin allograft survival in rats by a novel immunosuppressive agent, FK506. Transplantation 45: 206~209, 1988
7) SHEARER, G. M. & R. B. LEVY: Graft-vs-host-associated immune suppression is activated by recognition of allogeneic murine I-A antigens. J. Exp. Med. 157: 936~946, 1983
8) WALL, D. A.; S. D. HAMBERG, D. S. REYNOLDS, S. J. BURAKOFF, A. K. ABBAS & J. L. M. FERRARA: Immunodeficiency in graft-versus-host disease I. Mechanism of immune suppression. J. Immunol. 140: 2970~2976, 1988
9) GIBBONS, Jr. J. J. & J. LUCAS: Immunomodulation by low-dose methotrexate I. Methotrexate selectively inhibits Lyt-2$^+$ cells in murine acute graft-versus-host reactions. J. Immunol. 142: 1867~1873, 1989
10) JOSEPH, L. J.; T. IWASAKI, T. R. MALEK & G. M. SHEARER: Interleukin-2 receptor dysfunction in mice undergoing a graft-vs-host reaction. J. Immunol. 135: 1846~1850, 1985
11) WRIGHT, T. & I. A. RAMSHAW: A requirement for helper T cells in the induction of delayed-type hypersensitivity. J. Immunol. 130: 1596~1599, 1983
12) MILLER, S. D. & M. K. JENKINS: *In vivo* effects of GK1.5 (anti-L3T44a) monoclonal antibody on induction and expression of delayed-type hypersensitivity. Cell. Immunol. 92: 414~426, 1985
13) CHER, D. J. & T. R. MOSSMANN: Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by Th1 clones. J. Immunol. 138: 3688~3694, 1987
14) VIA, C. S. & G. M. SHEARER: T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. Immunol. Today 9: 207~213, 1988
15) KINO, T.; H. HATANAKA, M. HASHIMOTO, M. NISHIYAMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & H. IMANAKA: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physico-chemical and biological characteristics. J. Antibiotics 40: 1249~1255, 1987
16) ABE, F.; M. HAYASHI, H. HORINISHI, A. MATSUDA, M. ISHIZUKA & H. UMEZAWA: Enhancement of graft-versus-host reaction and delayed cutaneous hypersensitivity in mice by ubenimex. J. Antibiotics 39: 1172~1177, 1986
17) VIA, C. S.; S. O. SHARROW & G. M. SHEARER: Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-vs-host disease. J. Immunol. 139: 1840~1849, 1987
18) THIELE, D. L. & P. E. LIPSKY: The immunosuppressive activity of L-leucyl-L-leucine methyl ester: Selective ablation of cytotoxic lymphocytes and monocytes. J. Immunol. 136: 1038~1048, 1986
19) CHARLEY, M.; D. L. THIELE, M. BENNETT & P. E. LIPSKY: Prevention of lethal murine graft versus host disease by treatment of donor cells with L-leucyl-L-leucine methyl ester. J. Clin. Invest. 78: 1415~1420, 1986

20) THIELE, D. L.; M. R. CHARLEY, J. A. CALOMENI & P. E. LIPSKY: Lethal graft-vs-host disease across major histocompatibility barriers: Requirement for leucyl-leucine methyl ester sensitive cytotoxic T cells. J. Immunol. 138: 51～57, 1987

21) ROSENBERG, A. S.; T. MIZUOCHI, S. O. SHARROW & A. SINGER: Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. J. Exp. Med. 165: 1296～1315, 1987

22) WHITE, D. J. G.: Cyclosporin A: Clinical pharmacology and therapeutic potential. Drugs 24: 322～334, 1982