Effect of Metacycloprodigiosin, an Inhibitor of Killer T Cells, on Murine Skin and Heart Transplants

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Metacycloprodigiosin is an antibiotic that has been shown to suppress T-cell proliferation induced by concanavalin A *in vitro*. We examined the effect of metacycloprodigiosin on murine allogeneic skin and heart transplantation models, and compared graft rejection with donor-specific cytotoxic T-cells and antibody activity. The antibiotic slightly prolonged the survival of C57Bl/6 heart and skin grafts in BALB/c mice, although the effect was less than that of cyclosporin A. The effect was more evident in Bm1 (H-2D mutant) skin grafts on C57Bl/6 hosts or in a minor histocompatibility antigen-mismatched model. In contrast, metacycloprodigiosin suppressed anti-graft cytotoxic T-cell activity of BALB/c spleen grafted with C57Bl/6 skin as comparable to cyclosporin A, but had only partial effect on antibody production. Thus, metacycloprodigiosin is more effective in reducing splenic cytotoxic T-cell activity than in prolonging murine skin or cardiac allografts.

Rejection of foreign grafts by allogeneic hosts is a problem that has fundamental and clinical relevance. T-lymphocytes have been shown to be important for initiating allograft rejection[1,2]. Subsequent to initial T-cell recognition, a number of effector mechanisms may be involved in rejection, including nonspecific inflammatory reactions and antigen-specific responses mediated by T-cells and B-cells. Among these, cytotoxic T lymphocytes (CTL) are considered to be a leading candidate as an effector for rejection[3,4]. However the conclusion that CTL is the major effector in allograft rejection has been questioned in a number of studies, and the effector mechanism mediating the actual tissue destruction has been a source of controversy[5~9].

Prodigiosin 25-C (PrG 25-C) and metacycloprodigiosin (MC-PrG) are red pigments produced by microbes including *Streptomyces* and *Serratia*[10,11]. These antibiotics specifically inhibited T-cell mediated mitogenic responses in the screening program using mitogenic responses of splenocytes[12]. Further studies demonstrated that PrG 25-C inhibited CTL induction *in vivo* without affecting antibody production, including antibodies against T-cell-dependent and T-cell-independent antigens[13,14]. This observation suggests that the antibiotic preferentially suppresses CTL without affecting functions of helper T-cells and B-cells. It also suppressed T-cell mediated immune responses, such as delayed type hypersensitivity reaction and major hisotcompatibility antigen complex (MHC)-mismatched skin graft rejection[15].

Reagents which specifically inhibit CTL activity *in vivo* are of use not only for elucidation of *in vivo* role of CTL, but also for clinical immunosuppressants which function in the effector phase of rejection. PrG 25-C suppresses growth of mammalian cell lines including T-cell lines and this inhibitory effect was synergistically enhanced by concanavalin A, partly because PrG 25-C increases binding of concanavalin A on cells[16,17]. Reagents that inhibit the acidification of intracellular organelles have a similar property[17] and, we found that PrG 25-C was an uncoupler of the vacuolar-type H^+-ATPase[18]. This enzyme is responsible for the acidification of intracellular organelles such as lysosomes, endosomes and Golgi apparatus[19]. Furthermore, we found that inhibitors of vacuolar-type H^+-ATPase blocked the killing activity of CTL[20]. These observations suggest that prodigiosins inactivate CTL by the inhibition of the acidification of cytotoxic granules of CTL.

Although *in vivo* acitivity of PrG 25-C has been extensively studied, little is known about the effect of MC-PrG *in vivo*, a derivative of PrG 25-C which has a cyclic hydrocarbon instead of hydrocarbon chain of

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PrG 25-C[10]. In this paper, we examined the effect of MC-PrG on CTL activity and graft rejection in allogeneic heart and skin transplantation models and found that MC-PrG only slightly prolonged survival time of grafts in spite of strong inhibition of splenic CTL activity.

Materials and Methods

Mice

All the mice used in these experiments were females, 6~10 week of age, obtained from the Animal Production Facility, NCI, Frederick, MD. Pregnant mice were received in our animal facility at two weeks of gestation. Hearts from the newborn mice were harvested within two days of delivery for the transplantation.

Chemicals

MC-PrG was purified from culture broth of *Streptomyces hiroshimensis* as described previously[12]. MC-PrG was dissolved in DMSO at 10 mg/ml, and the solution was diluted to 2 mg/ml with phosphate buffered saline (PBS, Gibco Lab., Gland Island, NY) containing 0.5% Tween 20 immediately before ip injection of 0.2 ml of the solution. Cyclosporin A (CsA) was kindly provided by Dr. DAVID WINTER (Sandoz, Hanover, NJ). The drug was diluted in olive oil (Sigma, St. Louis, MO) to 15 mg/ml and 0.05 ml of the material was injected ip.

Transplantation

Skin grafting was performed as described previously[15] with slight modification. The recipients of the transplantation were anesthetized by the ip injection of 0.15 ml of PBS containing 20 mg/ml Ketamine (Aveco, Ft. Dodge, IO) and 0.4 mg/ml Xylazine (Haver, Shawnee, KA). Tail skin was removed and processed in about 50 mm^2 square and engrafted to the left flank of the recipients. Bandages were removed on day 5 and the grafts were monitored daily until rejection (defined as loss of >50% of the grafted tissue).

Heart grafting was performed according to the method reported by FULMER *et al.*[21]. The hearts of newborn mice sacrificed by decapitation were bisected along the longitudinal axis with a scalpel and each half was transplanted into a pocket prepared in the left ear of the adult recipients. The pulsatile of the grafts were observed under a stereomicroscope every other day using methoxylflurane (Pitman-Moore, Mundelein, IL) for anesthesia and graft rejection was determined by the cessation of beating.

CTL Assay

Spleens of recipients were removed and teased to make single cell suspension and incubated with ^{51}Cr-labeled target cells (5×10^3 cells/well) for 4 hours at 37°C in 7% CO_2 atmosphere in RPMI1640 medium supplemented with 5% fetal calf serum as described previously[13]. The isotope released in the culture supernatant was measured using a γ-counter. Maximum and spontaneous isotope release was determined as the cpm in the absence of effectors and in the presence of 1% SDS, respectively. Percent lysis was calculated by the formula.

$$\% \text{ lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

Anti-graft Antibody Titer

^{51}Cr-labeled splenocytes (1.25×10^5 cells) were incubated with serum from the allograft recipients which was serially diluted with medium in 96-well flat-bottom microtiter plates, as described previously[16]. After incubating for 15 minutes at 37°C, the plates were centrifuged at 1,000 rpm for 5 minutes and the pellet was further incubated with 10-fold diluted non-toxic rabbit complement for 45 minutes at 37°C. The radioactivity of the supernatant was measured and % lysis was calculated using above formula.

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

To confirm the effect of MC-PrG we induced CTL and antibody against allogeneic cells by ip injection of P815 ($H\text{-}2^d$) cells into C57Bl/6 mice ($H\text{-}2^b$) as reported previously[13]. As shown in Table 1, MC-PrG significantly suppressed anti-P815 CTL activity, whereas it had no effect on antibody titer against allogeneic spleen cells. In contrast, CsA suppressed both CTL activity and antibody titer. Thus MC-PrG, like PrG 25-C, preferentially suppressed CTL. Treatment with higher dose of MC-PrG resulted in the death of some mice.

Tail skin of C57Bl/6 mice was engrafted on the flanks of BALB/c mice. MC-PrG was administered every other day (Table 2, exp. 1). First, we used 1 mg/kg of MC-PrG, as we used for P815-immunized mice and observed no significant prolonged survival of the grafts. An increase in the dose to 2 mg/kg of MC-PrG resulted in some toxicity as mice exhibited a decrease in body weight but no death during the experiment. However, the antibiotic did not show significant suppressive activity in the skin graft model, although a slight prolongation of survival was consistently observed. In contrast, CsA showed a protective effect. Allogeneic skin grafts of the CsA-treated mice were not rejected during the experiments. A similar result was obtained in the heart transplant model (exp. 2). MC-PrG again prolonged graft survival only slightly, whereas CsA prevented rejection. Thus, the lack of extensive graft prolongation by MC-PrG was

Table 1. Inhibition of CTL induction of P815-immunized C57Bl/6 mice by MC-PrG.

P815	Treatment	CTL activity[a]		Antibody activity[b]	
		50:1	12.5:1	4%	1%
−	−	2.9±0.6	5.3±1.4	4.5±2.6	5.6±2.5
+	−	55.7±2.0	38.0±4.6	42.5±5.1	28.2±5.4
+	CsA	10.9±1.8	18.9±1.7	18.2±2.4	7.9±5.9
+	MC-PrG	15.6±1.2	12.0±2.9	45.7±8.4	38.4±2.9

C57Bl/6 (H-2^b) mice (3 mice/group) were immunized ip with P815 (H-2^d, 2×10^7 cells) and treated ip with MC-PrG (1 mg/kg) every other day or with CsA (30 mg/kg) every day from 24 hours after the immunization. Mice were killed on day 10 and CTL activity against P815 of pooled splenocytes or antibody activity of pooled serum were determined. Values are mean±SD of triplicate culture.

[a] % Lysis at effector to target ratio at 50:1 and 12.5:1.

[b] % Lysis of BALB/c splenocytes treated with 4% or 1% serum.

Table 2. Prolongation of graft survival by MC-PrG.

Exp.	Host (K,I,D)[e]	Graft	Treatment[a]	Survival (day)[b]	Ratio (%)[c]
1	BALB/c	C57Bl/6 skin	None	8.3±0.5	
	(d, d, d)	(b, b, b)	CsA	>30[d]	>261
			MC-PrG	9.3±0.9	13
2	BALB/c	C57Bl/6 heart	None	11.4±0.9	
	(d, d, d)	(b, b, b)	CsA	>30[d]	>163
			MC-PrG	13.0±1.6	14
3	C57Bl/6	Bm1 skin	None	18.6±2.2	
	(b, b, b)	(Bm1, b, b)	MC-PrG	27.5±4.9[d]	48
4	C57Bl/6	Bm12 skin	None	7.8±1.2	
	(b, b, b)	(b, bm12, b)	MC-PrG	8.8±2.1	13
5	B10.A(2R)	B10.A skin	None	11.0±1.2	
	(k, k, b)	(k, k, d)	MC-PrG	11.3±2.9	3
6	C3D2F1	B10BR skin	None	13.0±1.3	
	(k/d, k/d, k/d)	(k, k, k)	MC-PrG	16.8±2.5[d]	29

[a] Host (5 mice per group) were treated ip with MC-PrG (2 mg/kg) every other day or with CsA (30 mg/kg) everyday from 24 hours after the transplantation.

[b] Mean±SD.

[c] Percent of prolongation compared to untreated group.

[d] Statistically significant ($P<0.05$, Student t-test) compared to untreated group.

[e] Haplotype of MHC.

Table 3. Suppression of CTL activity of allogeneic skin-transplanted mice by MC-PrG.

B6 Skin	Treatment	CTL activity[a]		Antibody activity[b]	
		100:1	50:1	10%	3%
−	−	0.1±2.0	0.9±1.9	<0[c]	5.2±1.4
+	−	30.6±1.7	21.1±1.5	32.7±2.5	22.8±2.4
+	CsA	1.0±1.8	1.9±2.1	<0	5.2±2.4
+	MC-PrG	4.9±1.9	1.7±1.8	10.1±1.4	16.2±1.5

BALB/c (H-2^d, 3 mice per group) were engrafted with C57Bl/6 skin (H-2^b) and treated ip with MC-PrG (2 mg/kg) every other day or CsA (30 mg/kg) every day from 24 hours after the transplantation. Mice were killed on day 10 and CTL activity against EL-4 (H-2^b) of pooled splenocytes and antibody activity of pooled serum were determined. Values are mean±SD of triplicate culture.

[a] % Lysis at effector to target ratio 100:1 and 50:1.

[b] % Lysis of C57Bl/6 splenocytes treated with 10% or 3% of serum.

[c] Less than spontaneous cpm.

independent of the tissue transplanted.

Because majority of CTL is $CD8^+$ cells restricted by class I MHC, the effect of MC-PrG was compared in class I- or class II-mismatched transplants, using mice expressing MHC mutation. A significant prolongation of graft survival by MC-PrG was observed with class I-mismatched model (Bm1 into C57Bl/6, exp.3) but not in the class II-mismatched model (Bm12 into C57Bl/6, exp. 4). However, such prolongation was not observed in the class I-mismatched model using congeneic mice that involved an H-2D and L difference (exp. 5). Finally we used the antibiotic in a minor histocompatibility antigen-mismatched model (B10.BR into C3D2F1) and observed a significant prolongation of the allografts. Since the rejection of minor histocompatibility antigen-different graft is dependent on $CD4^+$ T-cells[22~25], the result suggests that the effectiveness of MC-PrG is dependent on the strength of the rejection and not on differences in MHC type.

We determined the CTL activity of spleen from BALB/c mice that were grafted with C57Bl/6 skin. The CTL activity in the spleen was observed maximally 10 days after transplantation, and the activity declined gradually thereafter (data not shown). MC-PrG again inhibited the CTL activity on day 10, which was comparable to that of CsA (Table 3). Antibody titer against allogeneic spleen cells was also reduced by MC-PrG treatment, but was not as dramatic as the effect of CsA on antibody production.

Discussion

We compared the effect of MC-PrG in both heart and skin transplantation models and found that the antibiotic significantly suppressed the CTL induced by allogeneic graft as well as allogeneic tumor immunization. Although the antibiotic prolonged the graft survival, the effect was low compared with CsA. It prolonged the graft survival only in strain combinations that exhibit weak rejection patterns, such as Bm1/C57Bl/6 or minor histocompatibility antigen-mismatched differences. It is unlikely that residual negligible CTL activity is sufficient to effect rejection, because CsA which suppressed CTL activity as strongly as MC-PrG completely prevented graft rejection. Our results suggest that suppression of splenic CTL is not sufficient for prolongation of graft rejection involving strong immunologic differences.

These results are consistent with previous reports that anti-CD4 antibody is more effective than anti-CD8 antibody for suppression of graft rejection[23~26], and that MHC-deficient mice still reject allografts despite the lack of specific CTL activity[27]. This latter report suggests that some effectors other than T-cells also play significant roles in graft rejection. Since antibody against tumor necrosis factor, an major cytotoxic effector molecule of macrophages, effectively suppresses graft rejection[28,29], immunosuppressants that suppress macrophage function might help the suppressive activity of MC-PrG.

PrG 25-C inhibits intracellular acidification through inhibition of proton pumping of vacuolar-type H^+-ATPase[18]. We found that an inhibitor of vacuolar-type H^+-ATPase also inhibits the acidification of cytotoxic granules and induces destruction of their structure and degradation of perforin[20], one of the effector molecules in the granules[30]. However, it affected neither granule exocytosis in response to stimulation of T-cell receptor nor granzyme activity in the granules[20]. Recent reports suggest that Fas-dependent pathway contributes to killing activity of CTL in addition to perforin dependent pathway[31,32]. Since PrG 25-C does not affect Fas-dependent pathway (T. KATAOKA, unpublished result), it is possible that molecules other than perforin which are active even in the presence of MC-PrG induce the rejection.

The suppressive effect of MC-PrG is dependent on the kind of allo-stimulation. CTL induced by P815-immunization was almost completely inhibited by 0.5 mg/kg whereas this dose only partially suppressed CTL induced by allogeneic skin or heart (data not shown). Since bioavailability of the antibiotic might differ from tissue to tissue, it is possible that CTL in the graft is sufficiently active to provoke rejection even if that in spleen is suppressed. Although MC-PrG exhibits high toxicity which prevented our treatment of animals with higher doses, chemical modification of the antibiotic might improve the bioavailability and toxicity, and thus the efficacy for suppression of graft rejection.

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References

1) ROSENBERG, A. S. & A. SINGER: Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. Annu. Rev. Immunol. 10: 333~358, 1992
2) LAFFERTY, K. J.; S. J. PROWSE, C. J. SIMEONOVIC & H. S. WARREN: Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. Annu. Rev. Immunol. 1: 143~173, 1983
3) MINTZ, B. & W. K. SILVERS: "Intrinsic" immunological tolerance in allophenic mice. Science 158: 1484~1487, 1967
4) MINTZ, B. & W. K. SILVERS: Histocompatibility antigens on melanoblasts and hair follicle cells. Cell-localized homograft rejection in allophenic skin grafts. Transplantation 9: 497~505, 1970
5) HALL, B. M. & S. E. DORSCH: Cells mediating allograft rejection. Immunol. Rev. 77: 31~59, 1984

6) MASON, D. W.; M. J. DALLMAN, R. P. ARTHUR & P. J. MORRIS: Mechanisms of allograft rejection: the roles of cytotoxic T-cells and delayed type hypersensitivity. Immunol. Rev. 77: 167～184, 1984
7) STEINMULLER, D.: Which T cells mediate allograft rejection? Transplantation 40: 229～233, 1985
8) MASON, D. W. & P. J. MORRIS: Effector mechanisms in allograft rejection. Annu. Rev. Immunol. 4: 119～145, 1986
9) HALL, B. M.: Cells mediating allograft rejection. Transplantation 51: 1141～1151, 1991
10) HARASHIMA, K.; N. TSUCHIDA, T. TANAKA & J. NAGATSU: Prodigiosin 25-C: Isolation and the chemical structure. Agric. Biol. Chem. 31: 481～489, 1967
11) WASSERMAN, H. H.; G. C. RODGERS & D. D. KEITH: Undecylprodigiosin. Tetrahedron 32: 1851～1854, 1976
12) NAKAMURA, A.; K. NAGAI, S. SUZUKI, K. ANDO & G. TAMURA: Selective suppression by prodigiosin of the mitogenic response of murine splenocytes. J. Antibiotics 39: 1155～1159, 1986
13) 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
14) 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
15) TSUJI, R. F.; J. MAGAE, M. YAMASHITA, K. NAGAI & M. YAMASAKI: Immunomodulating properties of prodigiosin 25-C, an antibiotic which preferentially suppresses induction of cytotoxic T cells. J. Antibiotics 45: 1295～1302, 1992
16) KATAOKA, T.; J. MAGAE, H. NARIUCHI, M. YAMASAKI & K. NAGAI: Enhancement by concanavalin A of the suppressive effect of prodigiosin 25-C on proliferation of murine splenocytes. J. Antibiotics 45: 1303～1312, 1992
17) KATAOKA, T.; J. MAGAE, K. KASAMO, H. YAMANISHI, A. ENDO, M. YAMASAKI & K. NAGAI: Effects of prodigiosin 25-C on cultured cell lines: its similarity to monovalent polyehter ionophores and vacuolar type H^+-ATPase inhibitors. J. Antibiotics 45: 1618～1625, 1992
18) KATAOKA, T.; M. MUROI, S. OHKUMA, T. WARITANI, J. MAGAE, A. TAKATSUKI, S. KONDO, M. YAMASAKI & K. NAGAI: Prodigiosin 25-C uncouples vacuolar type H^+-ATPase, inhibits vacuolar acidification and affects glycoprotein processing. FEBS lett. 359: 53～59, 1995
19) MELLMAN, I.; R. FUCKS & A. HELENIUS: Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. 55: 663～700, 1986
20) KATAOKA, T.; K. TAKAKU, J. MAGAE, N. SHINOHARA, H. TAKAYAMA, S. KONDO & K. NAGAI: Acidification is essential for maintaining the structure and function of lytic granules of cytotoxic T lymphocytes. J. Immunol. 153: 3938～3947, 1994
21) FULMER, R. I.; A. T. CRAMER, R. A. LIEBELT & A. G. LIEBELT: Transplantation of cardiac tissue into the mouse ear. Am. J. Anat. 113: 273～281, 1963
22) 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
23) WHEELAHAN, J. & I. F. C. MACKENZIE: The role of $T4^+$ and Lyt-2^+ cells in skin graft rejection in the mouse. Transplantation 44: 273～280, 1987
24) COBBOLD, S. P. & H. WALDMANN: Skin allograft rejection by L3/$T4^+$ and Lyt-2^+ T cell subsets. Transplantation 41: 634～639, 1986
25) MADSEN, J. C.; W. M. PEUGH, K. J. WOOD & P. J. MORRIS: The effect of anti-L3T4 monoclonal antibody treatment on first-set rejection of murine cardiac allografts. Transplantation 44: 849～852, 1989
26) COBBOLD, S. P.; A. JAYASURIYA, A. NASH, T. D. PROSPERO & H. WALDMANN: Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*. Nature 312: 548～551, 1985
27) GRUSBY, M. J.; H. AUCHINCLOSS Jr., R. LEE, R. S. JOHNSON, J. P. SPENCER, M. ZIJLSTRA, R. JAENISCH, V. E. PAPAIOANNOU & L. H. GLIMCHER: Mice lacking major histocompatibility complex class I and class II molecules. Proc. Natl. Acad. Sci. U.S.A. 90: 3913～3917, 1993
28) IMAGAWA, D. K.; J. M. MILLIS, K. M. OLTHOFF, P. SEU, R. A. DEMPSEY, J. HART, P. I. TERASAKI, E. M. WASEF & R. W. BUSULTIL: The role of tumor necrosis factor in allograft rejection: II. Evidence that antibody therapy against tumor necrosis factor-alpha and lymphotoxin enhances cardiac allograft survival in rats. Transplantation 50: 189～193, 1990
29) SCHRINGE, M.; R. W. F. DE BRUIN, H. JEEKEL & R. L. MARQUET: Anti-tumor necrosis factor alpha serum prolongs heart allograft survival in rat. Transplant. Proc. 23: 547～548, 1991
30) TSCHOPP, J. & M. NABHOLZ: Perforin-mediated target cell lysis by cytolytic T lymphocytes. Annu. Rev. Immunol. 8: 279～302, 1990
31) LOWIN, B.; M. HAHNE, C, MATTMANN & J. TSCHOPP: Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature 370: 651～652, 1994
32) KÄGI, D.; F. VIGNAUX, B. LEDERMANN, K. BÜRKI, V. DEPRAETERE, S. NAGATA, H. HENGARTNER & P. GOLSTEIN: Fas and perforin pathways as major mechanism of T cell-mediated cytotoxicity. Science 265: 528～530, 1994