Mini Review

Open Access Journal of
Biomedical Science
ISSN: 2690-487X

An Old Researcher Challenges the Empirical Timing of Cy-Treatment in the Clinical Haplo-Identical Bone Marrow Transplantation Followed by High-Dose Cyclophosphamide

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KEYWORDS: Cyclophosphamide-induced tolerance; Drug-induced tolerance; HaploBMT/PTCy; HaploBMT; PTCy; PTCy-haplo HSCT; Clonal destruction; Clonal deletion

ABBREVIATIONS: Cy: Cyclophosphamide; HaploBMT/PTCy: Haplo-Identical Bone Marrow Transplantation Followed by High-Dose Cyclophosphamide; BMT: Bone Marrow Transplantation; PTCy: Post-Transplantation Cyclophosphamide; TBI: Total Body Irradiation; Haplo BMT: HLA-Haploidentical Bone Marrow Transplantation; B6: C57BL/6J (H-2b); AKR: AKR/J (H-2^k); C3H: C3H/HeSnJ (H-2^k); SC: Spleen Cells; MMC: Mitomycin C; 5-FU: 5-Fluorouracil; CMC: Cell Mediated Cytotoxicity; mAb: Monoclonal Antibody; BMC: Bone Marrow Cells; MST±SD: Mean Survival Time±Standard Deviation; CBA: CBA/J (H-2^k)

MINI-REVIEW

In this article, I would like to propose the correction of the empirical and inappropriate timing for cyclophosphamide (Cy) administration among the widely spread clinical haplo-identical bone marrow transplantation followed by high-dose Cy (haploBMT/PTCy).

Haploidentical bone marrow transplantation (BMT) with high-dose post-transplantation cyclophosphamide (PTCy) is now becoming a safe, effective and inexpensive treatment for patients with hematologic malignancies or hemoglobinopathies and for the tolerance induction to transplants of solid organs [1] from the same donor (2). For the method to cross the haploidentical barrier in human BMT, the Johns Hopkins protocol comprising nonmyeloablative pretreatment with fludarabine on days -6 through - 2, Cy on days -6 and -5, total body irradiation (TBI) on day -1 (=preconditioning), and BMT on day 0 followed by Cy 50mg/kg on days 3 and 4 (=Cy-induced tolerance), and tacrolimus and mycophenolate mofetil from day 5 (=Post-immunosuppressive treatment) has been used in world-wide [2].

As was described in my recent overview paper [3], the central mechanism of this Johns Hopkins platform is the Cy-induced tolerance comprising BMT on day 0 followed by Cy 50mg/kg on days 3 and 4. This twice dosing of 50mg/kg Cy administration was initially on day 3 alone in the first report from the Johns Hopkins group [4]. This timing appeared to be decided from the basic murine (B10.BR (H-2^k) →B10 (H-2^b)) study performed by L. Luznik et al. [5] by obtaining a hint from our timing study for skin allograft tolerance induction in H-2-identical murine combinations [6]. Another Cy injection of 50mg/kg on day 4 was soon added to reduce both engraftment failure and severe acute GVHD [5] in the nonmyeloablative BMT from partially HLA-mismatched related donors using PTCy. To my knowledge, however, this additional Cy administration timing on day 4 is empirical to the last.

From our previously performed four studies (Table 1), the best intervals between viable cell injection and Cy-treatment were reviewed [6-9]. In the fully allogeneic murine donor→ recipient combination of C57BL/6 (B6; H-2^b)→AKR/J (AKR; H-2^k) [7], tolerance to the EL-4 tumor (originated from B6) was induced only when 40 x 10^6 live B6 spleen cells (SC) were injected i.v. into recipient AKR mice on day 0 followed by a single dose of 150mg/kg Cy i.p. on day 1, 2, or 3. Neither Cy on day -2, 0, 5 or 7 could induce tolerance (Experiment 1: Table 1).

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Received: November 18, 2021 **Published:** December 01, 2021

How to cite this article: Mayumi H. An Old Researcher Challenges the Empirical Timing of Cy-Treatment in the Clinical Haplo-Identical Bone Marrow Transplantation Followed by High-Dose Cyclophosphamide. 2021- 3(6) OAJBS.ID.000354. **DOI:** 10.38125/OAJBS.000354

Table 1: Review of the best intervals between viable cells and Cy among the various Cy-induced tolerance in mice.

Experiment No	1	2	3	4
Experiment Title	Tumor allografting in a fully allogeneic combination	Skin allografting in an H-2 identical combination	*In vitro* tolerance in a fully allogeneic combination	Skin allografting in a fully allogeneic (major H-2 plus non-H-2) combination
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Combination of Mice	B6 (H-2^b) →AKR (H-2^k)	AKR (H-2^k) →C3H (H-2^k)	B6 (H-2^b) →C3H (H-2^k)	C3H (H-2^k) →B6 (H-2^b)
Tolerance Induction Procedure	B6SC+Cy	AKRSC+Cy	MMC-treated B6SC+5-FU	Ani-Thy-1.2 mAb+C3H(SC+BMC) +Cy
Allografts	Allo-tumor EL-4 (B6-origin; H-2^b)	AKR skin (H-2^k)	Coculture with MMC-treated B6SC (H-2^b)	C3H skin (H-2^k)
Successful Signs in Grafting	Growth to kill the recipients	Growth of donor-skin hair	CMC activity against B6 Con A blasts	Growth of donor-skin hair
Examined Intervals	-2, 0, 1, 2, 3, 5, or 7 days	-1, 0, 1, 2, 3, 4 or 5 days	1, 2, or 3 days	0, 1, 2, 3, 4 or 5 days
Succeeded Best Intervals	1, 2, or 3 days	⟨1⟩, 2 or 3 days	⟨1⟩, 2, or ⟨3⟩days	⟨1⟩, 2, or ⟨3⟩days
		The number in ⟨parenthesis⟩ means partial success.	The number in ⟨parenthesis⟩ means partial success.	The number in ⟨parenthesis⟩ means partial success.

In the H-2 identical allogeneic murine donor→recipient combination of AKR/J (AKR; H-2^k)→C3H/HeSnJ (C3H; H-2^k) [6], tolerance to the AKR skin was induced only when 50 x 10^6 live AKR spleen cells (SC) were injected i.v. into recipient C3H mice on day 0 followed by a single dose of 150mg/kg Cy *i.p.* on day ⟨1⟩, 2, or 3. Neither Cy on day -1, 0, 4 or 5 could induce tolerance (Experiment 2: Table 1). The AKR SC injection on day 0 followed by Cy on day 1 could induce tolerance only in one out of the 5 recipient C3H mice. Original data derived from [6].

In the *in vitro* system [8], C3H/HeSnJ (C3H; H-2^k) SC were cultured with mitomycin C (MMC)-treated C57BL/6J (B6; H-2^b) SC for 1-3 days, then 5-fluorouracil (5-FU) was added at a final concentration of 5μg/ml, and the preparations were cultured for another 9 hours. After washing with Hanks' balanced salt solution, the responder cells were restimulated with B6 SC for 5 days and then assayed for cytotoxicity. Although the activity of cell mediated cytotoxicity (CMC) against the allogeneic cells was suppressed in general, comparing with the control group, whenever the responders were pre-sensitized for 1-3 days, the CMC activity was completely suppressed when the responders were pre-sensitized for two days (Experiment 3; Table 1).

Our triumph of research was establishing a method of Cy-induced skin allograft tolerance in mice that can regularly overcome fully allogeneic (major H-2 plus non-H-2) antigen barriers in mice [9]. The final components of the method are *i.p.* administration of 50μg of anti-Thy-1.2 monoclonal antibody (mAb) one day before the cell injection, i.v. injection of 90 x 10^6 allogeneic SC mixed with 30 x 10^6 allogeneic bone marrow cells (BMC) from the same donor on day 0, and *i.p.* injection of 200mg/kg Cy on day 2. In this system also, the optimal timing of the Cy treatment in tolerance induction with C3H/HeSnJ (C3H; H-2^k) SC plus BMC in recipient C57BL/6J (B6; H-2^b) mice was examined [9] (Experiment 4; Table 1). When 50μg of anti-Thy-1.2 mAb on day -1, and C3H SC plus BMC were given on day 0 to B6 mice followed by Cy treatment on day 0, 1, 2, 3, 4 or 5, profound tolerance was induced only when Cy was given on day 1 (mean survival time±standard deviation (MST±SD)=64.4±10.3 days, *n*=5), day 2 (MST±SD >86.8±25.0 days, n=5), or day 3 (MST±SD >69.0±31.9 days, *n*=5). Again, the optimal timing interval of the Cy treatment is 1-3 days, whereas the two-day interval appeared to be the best [9].

As shown above and in Table 1, the best intervals between viable cells and Cy (antigen stimulation and 5-FU treatment *in vitro*) among the various Cy-induced tolerance in mice were 1-3 days, and the extremely optimal single dose interval appears to be two days. In the Johns Hopkins platform, however, Cy 50mg/kg is given twice on days 3 and 4 (Total Cy dose=100mg/kg) after BMT to suppress the unacceptable bone marrow graft rejection and GVHD in crossing the haploidentical barriers [5]. This twice-dosing of 50mg/kg Cy (50×2=100mg/kg) resembles to our previous study performed by my colleague Zhang QW [10] but its conclusion did not match with the Johns Hopkins regimen.

I have shown the key point summary of the Zhang's study in Table 2. Although injection of allo-SCs on day 0 followed by a single dose of Cy at 200mg/kg on day 1, 2, or 3 could induce tolerance of tumor and/or skin allografts in mice, it was investigated if fractionation of Cy can establish long-lasting skin graft survival, stable mixed chimerism, and intrathymic clonal deletion in the host as well, while minimizing the damage caused by Cy [10]. In an H-2-identical combination of AKR (H-2^k, Mls-1^a)→C3H (H-2^k, Mls-1^b), AKR skin graft survival was prolonged remarkably (80-90 days) in the SC+Cy 200×1 (Group 4), 100×2 (Group 5), and 66×3 (Group 6) groups (Table 2), but was prolonged moderately (20-60 days) in the SC+Cy 50×4 (Group 7) and 40×5 (Group 8) groups. In both of the SC+Cy 200×1 and 66×3 groups in the AKR→C3H combination, mixed chimerism was maintained for as long as 100 days after tolerance induction in the thymus, associated with intrathymic clonal deletion of Vβ6+ T cells (Groups 4 and 6; Table 2) [10,11]. The decreases in leukocyte count, hemoglobin level, spleen weight, SC count, and body weight were significantly smaller in the SC+Cy 66×3 group than in the SC+Cy 200×1 group. As shown in Experiment 2, the AKR-skin tolerance induced in C3H mice in both the AKR-SC+Cy 200×1 (Group 4) and AKR-SC+Cy 66×3 (Group 6) groups was tolerogen-specific because the third party skin from the H-2-identical CBA (H-2^k) was rejected almost normally in these tolerant groups (Groups 10 and 11; Experiment 2; Table 2).

The fractionated dosing of Cy was allotted to the days 1 and 2 in the Cy 100×2 group (Group 5) and to the days 1-3 in the Cy 66×3 group (Group 6) among the optimal three days of days 1-3. Although we do not have any data comparing the fractionated dosing allotted to days 1 plus 2 and to days 2 plus 3, allotting to days 2 plus 3 may be somewhat better considering from the results shown in Table 1. According to the data in both Table 1 and 2, the fractionated dosing

of Cy 50mg/kg allotted to day 4 by the Johns Hopkins regimen appears to be too late for the appropriate tolerance induction.

Table 2: Review of the optimally fractionated Cy and the optimal intervals between viable SC and Cy-treatment among the various Cy-induced tolerance in mice.

Experiment	Recipient	Group No.	Group	Treatment						Skin graft survival				Intrathymic chimerism and intratymic clonal deletion
				AKR-SC on day 0	Cy on day Xs					Donor of skin graft	No. of mice	Median (days)	Mean survival time ± SD (days)	
					Day 1	Day 2	Day 3	Day 4	Day 5					
1	C3H(H-2^k)	1	Control	-	-	-	-	-	-	AKR(H-2^k)	15	11	11.3±1.5	-
		2	AKR-SC control	100×10^6	-	-	-	-	-		6	10	9.2±1.5	-
		3	Cy control	-	Cy 50	Cy 50	Cy 50	Cy 50	-		6	12	12.5±1.2	ND
		4	AKR-SC+Cy 200×1	100×10^6	-	Cy 200	-	-	-		9	65	82.2±57.2	Yes
		5	AKR-SC+Cy 100×2	100×10^6	Cy 100	Cy 100	-	-	-		5	72	90.8±64.3	ND
		6	AKR-SC+Cy 66×3	100×10^6	Cy 66	Cy 66	Cy 66	-	-		11	65	92.9±49.8	Yes
		7	AKR-SC+Cy 50×4	100×10^6	Cy 50	Cy 50	Cy 50	Cy 50	-		7	49	63.9±48.7	ND
		8	AKR-SC+Cy 40×5	100×10^6	Cy 40	Cy 40	Cy 40	Cy 40	Cy 40		6	26	23.2±5.8	ND
2	C3H(H-2^k)	9	Control	-	-	-	-	-	-	CBA(H-2^k)	5	11	11.0±0	
		10	AKR-SC+Cy 200×1	100×10^6	-	Cy 200	-	-	-		5	18	16,2±2.7	
		11	AKR-SC+Cy 66×3	100×10^6	Cy 66	Cy 66	Cy 66	-	-		5	17	15.0±3.2	

The basic and central mechanism of the cells-followed-by-Cy system was named as "clonal destruction". This most important mechanism is considered the destruction, with Cy, of the antigen-stimulated, and thus-proliferating, cells. Namely, the mature T (or B) cells reactive against the allo-antigens clonally expand after the injection of allogeneic cells. The DNA contained in the proliferating blast cells is especially sensitive to Cy, an alkylating agent, and thus the clones are selectively destroyed with this agent given 1-3 days later while leaving the other resting clones intact.

A fraction of mature T cells in the recipient, however, is less proliferative against the antigen stimulation, mature quickly before the Cy-treatment given 1-3 days later, and thus remain in an anamnestic state after the Cy-treatment resulting in a split tolerance state especially when the donor-recipient combination is disparate in major H-2 and minor H antigens [3]. Interestingly, such anamnestic memory T cell activities remained after the Cy-treatment are not augmented by the subsequent immunization with responsible allogeneic SC, skin allografting, or tumor allografting [12].

In the cells-followed-by-Cy system providing successful skin tolerance, five mechanisms were identified using the correlation between super-antigens and T-cell receptor (TCR) Vβ segments mainly in the H-2-identical murine combinations [3, 11].

Those consist of:

1) clonal destruction of antigen-stimulated-thus-proliferating mature T cells with Cy [12]

2) peripheral clonal deletion associated with immediate peripheral chimerism [13]

3) intrathymic clonal deletion associated with intrathymic chimerism [11]

4) delayed generation of suppressor T cells [14]

5) delayed generation of clonal anergy [15]

These five mechanisms are insufficient to induce tolerance when the donor-recipient combinations are disparate in MHC antigens plus minor H antigens as is seen in haploBMT [2]. Clonal destruction is incomplete, resulting in a split tolerance, when the antigenic disparity is too strong to establish intrathymic mixed chimerism because of the existence of the less-proliferative T cell population [3]. Although this incomplete clonal destruction leaves the less-proliferative, antigen-stimulated T cells behind, these cells may confer graft-versus-leukemia (GVL) effects after haploBMT/PTCy. Too late administration of additional Cy, however, may be generating insufficient tolerant state in the Johns Hopkins protocols. In the clinical haploBMT/PTCy, therefore, the best timing

of the drug-treatment should be reconfirmed in clinical trials, or at least *in vitro* by using our 5-FU-induced *in vitro* tolerance system [8] in humans.

In summary, the timing of BMT on day 0 followed by Cy 50mg/kg on days 3 and 4 among the widely spread Johns Hopkins Platform for clinical haploBMT/PTCy appears empirical to the last. According to our previous murine studies, a single dose Cy 100 mg/kg should be given on day 2 alone, or a fractionated dose of Cy 50 mg/kg should be given twice on days 2 and 3 when the BMT is performed on day 0. Such appropriate BMT-Cy interval will decrease the number of residuals less-proliferative, antigen-stimulated T cells left behind the tolerance induction among the HLA-haploidentical donor-recipient combinations.

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