

# Immunosuppression in Dogs by Pretransfusions with Ultraviolet (UV)-Irradiated Whole Blood

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**ABSTRACT.** Ultraviolet (UV)-irradiated whole dog blood prevented the initiation of proliferative responses in allogeneic mixed lymphocyte reactions (MLR). When dogs were given 4 weekly intravenous transfusions of UV-irradiated allogeneic donor whole blood, peripheral blood lymphocytes (PBL) of the recipients responded less significantly to donor PBL in MLR for over one week after the final transfusion. Red blood cell (RBC)-crossmatching of these dogs was negative. Dogs treated with UV-irradiated blood did not produce anti-donor PBL antibody, or IgG, IgM and C3 determined by the indirect Coombs test. These dogs also had negligible delayed type hypersensitivity (DTH) responses to donor PBL at the end of the treatment period, recognized as slightly suppression of skin graft rejection. In contrast, six dogs receiving injections of untreated allogeneic whole blood evidenced high proliferation in MLR, agglutination in RBC-crossmatching, acute rejection of skin grafts and three of them produced C3 in the Coombs test.—**KEY WORDS:** crossmatching, delayed type hypersensitivity, dog, skin grafting, ultraviolet irradiation.

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Reversal of diabetes by transplanting islets of Langerhans isolated from adult pancreas or fetal pancreatic tissues has been successful in rodents, but is more complex in larger animals [1]. Mullen *et al.* [10] have reported the reversal of hyperglycemia in diabetic pigs by fetal pancreatic islet transplants, although long-term survival of the grafts required the continuous use of immunosuppressive drugs. Our long-term goal is to develop transplantation protocols that will allow transplantation without large doses of non-specific immunosuppressive agents. One approach may be the immunomodulation of recipient immune responses by treatment with donor specific antigens.

In rodents, graft survival has been prolonged by exposure to ultraviolet (UV) light, either directly by irradiation of islets before transplantation, or indirectly by transfusion of UV-irradiated whole blood or peripheral blood lymphocytes (PBL) [4].

In contrast, among large animals, the dog has been most commonly used for various experimental transplantation studies. However, though the modulation of immune function in dogs by UV light has been studied as a model system [3], allograft rejection cannot yet be eliminated without immunosuppression. UV-irradiated whole blood is doubtless a simpler procedure than that of PBL, since no separation is needed. If graft survival in the dog can be prolonged solely by immunomodulation with UV light, the need for immunosuppressive drugs and

their undesirable side effects will be reduced or eliminated.

According to the above rationale, the effect of transfusing UV-irradiated allogeneic donor whole blood was investigated in comparison with that of the same untreated allogeneic donor whole blood. Furthermore, the suppressive effect of preimmunization was examined by various immune parameters. These included the mixed lymphocyte reaction (MLR), red blood cell (RBC)-crossmatching, circulating levels of anti-donor PBL antibody, the indirect Coombs test, delayed type hypersensitivity (DTH), and skin grafting in order to determine immunity versus immunosuppression development in the dogs following specific alloantigen treatment.

## MATERIALS AND METHODS

**Animals:** Three mongrel dogs, weighing between 19 and 22 kg and ranging in age from 1 to 3 years were used as donors. Ten male and 8 female Beagles weighing between 9 and 14 kg and ranging in age from 1 to 6 years were the recipients. These were divided into three groups as follows: Group 1 (n=6), no treatment; Group 2 (n=6), injected with untreated fresh whole blood; Group 3 (n=6), injected with UV-irradiated whole blood.

**RBC typing:** We examined the following seven systems of RBC groups (Table 1): DEA 1, DEA 3, D1, D2, J1, J2, J3, and J4. One donor in group 3

Table 1. Red blood cell typing of experimental dogs

Group	Dog No.	DEA 1	DEA 3	D1	D2	J1	J2	J3	J4
1	A	1-1	-	+	+	-	-	-	+
	10	1(-)	-	-	+	+	+	-	+
	27	1(-)	-	-	+	+	-	-	-
	14	1-2	-	-	+	-	+	-	+
	23	1-1	-	-	+	-	+	-	+
	24	1-1	-	-	+	-	+	-	+
	26	1-1	-	-	+	-	+	-	-
2	B	1-1	-	+	+	-	-	-	+
	2	1-1	-	-	+	+	+	-	+
	5	1-2	-	-	+	-	+	-	-
	11	1-1	-	-	+	+	-	-	+
	15	1-1	-	-	+	-	+	-	+
	17	1-1	-	-	+	-	+	-	-
	20	1-1	-	-	+	-	-	-	+
3	C	1-1	-	-	+	+	-	-	+
	1	1-2	-	-	+	+	+	-	+
	6	1-2	-	-	+	-	+	-	+
	7	1-2	-	-	+	-	+	-	+
	9	1-1	-	-	+	-	+	-	+
	12	1(-)	-	-	+	-	-	-	-
	25	1-1	-	-	+	+	-	-	-

(dog C) differed from the other donors in groups 1 and 2, in D1 and J1.

**UV light irradiation:** About 3 ml aliquots from 10 ml of heparinized whole blood in plastic culture dishes (60 mm, #3002, Falcon, U.S.A.) were placed 10 cm from a UV light source (Model UVM-57, 302 nm, UVP Inc., San Gabriel, CA., U.S.A.) then irradiated for 15 min. Each dog in groups 2 and 3 received 10 ml of normal or UV-irradiated whole blood at weekly intervals, through the cervical vein. For 4 weeks (starting with week 0, week 1, week 2, week 3) after the first transfusion, group 1 had DTH responses.

**Mixed lymphocyte reaction (MLR):** Five MLR were performed on weeks 0, 1, 2, 3 and 4 after the first transfusion using standard procedures [9, 15]. The final MLR was performed one week after the last injection. PBL were harvested by Ficoll separation (SG: 1.077, 17-0840-03 Pharmacia, U.S.A.) washed twice in phosphate buffered saline (PBS), and the procedure was repeated with RPMI 1640 (05911 Nissui pharmaceutical Co., Ltd., Japan) containing fetal calf serum (FCS, Bioproducts, U.S.A.). After the final washing, the cells were resuspended in culture medium (see below). Stimulator PBL were treated with mitomycin C (MMC, 0.04 mg/ml, Kyowa Hakko Kogyo Co., Ltd., Japan). The PBL were cultured in 96-well U-bottom microtiter plates (3910, Falcon) at  $5 \times 10^4$  stimulator cells and  $5 \times 10^4$  responder cells per well. The culture medium (0.2 ml/well) consisted of RPMI 1640

supplemented with 8% heat inactivated FCS, pyruvic acid (1.2 mM), L-Glutamine (3.4 mM), 2-mercaptoethanol (0.05 mM), penicillin (0.1 mg/ml), streptomycin (0.1 mg/ml), gentamicin (0.05 mg/ml), HEPES buffer (25 mM) and sodium bicarbonate (24 mM). Cultures were maintained at 37°C under 5% CO<sub>2</sub> in air for 5 days. Wells were washed 3 times with PBS, then 0.1 ml of PBS and 0.05 ml of sodium dodecyl sulfate (0.5 mg/ml, Wako Pure Chemical Industries Ltd., Japan) were added to each well. Plates were incubated at room temperature (24°C) for 30 min, then 0.05 ml of ethidium bromide (0.02 mg/ml, SIGMA, U.S.A.) was added to each well before a final incubation at 24°C for 15 min. Fluorescence intensity was measured with a microplate reader (MTP-32, Corona Electric, Japan). MLR was expressed as the stimulation index (SI), equal to the ratio of the experimental to the control intensity. The percentage of SI (%SI) was calculated as the ratio of SI at week 4 / SI of MLR at week 0. The control for MLR was the activity incorporated into an analogous culture consisting of untreated responder cells and stimulator cells from the same animals treated with MMC.

**RBC-crossmatching:** Heparinized blood was washed and diluted to 10% with physiological saline. Two drops of 10% donor RBC plus 2 drops of recipient serum were mixed. Agglutination was observed macroscopically after incubation at 24°C for 30 min and centrifuging at 1,000 rpm, using standard procedures [13]. The degree of agglutination was recorded from - to +++++.

**Complement mediated cytotoxicity test:** Sera were collected from recipients in all three groups before the first transfusion and at weeks 1 through 4 after the first transfusion. The sera were tested for cytotoxicity against donor PBL using the complement mediated cytotoxicity assay as described by Sachs *et al.* [9, 15]. Donor or control PBL were prepared as mentioned above and adjusted to  $5 \times 10^6$  cells/ml in RPMI 1640 with 1% FCS. Heat-inactivated test sera were diluted two fold using the same medium, and 0.025 ml aliquots placed into microtiter plate wells. Twenty five microliters of cell suspensions were then added. The mixtures were allowed to stand at room temperature (24°C) for 45 min, then centrifuged at  $400 \times g$  for 10 min. The supernatants were removed and 0.025 ml of ten-fold diluted rabbit complement (Cappel Products, U.S.A.) was added. The cells were incubated for 1 hr at 24°C, then placed on ice. Cytotoxicity was

scored microscopically as the percentage of cells stained by 0.2% trypan blue (in physiological saline). The antibody titer was expressed as the reciprocal of the serum dilution resulting in 50% cell death with rabbit complement.

**Indirect Coombs test:** Sera were collected from recipients in all three groups at weeks 0 and 4 after the first transfusion. RBC from the same species were washed with ethylenediamine tetraacetic acid-gelatin veronal buffer (EDTA-GVB), at 37°C for 30 min then incubated with the inactivated sera. RBC were washed and diluted to 2% with EDTA-GVB. IgG, IgM and C3 (the third component of complement) antisera were used as the Coombs reagents. IgG, IgM and C3 antisera were diluted two fold with GVB and 0.025 ml was added to each well. After 0.025 ml of 2% RBC were added, plates were incubated at 37°C for 15 min, 2 hr at 24°C and for 10 hr at 4°C. The cells were microscopically observed for agglutination and the degree was recorded from - to ++++.

**Delayed type hypersensitivity (DTH):** Ten days after the final transfusion, dogs were intradermally (ID) inoculated in the ear with  $5 \times 10^6$  cells PBL in 0.1 ml PBS, from the same donor dog as that used for the immunizations. Twenty four and 48 hr after the inoculation, DTH responses, such as induration of the skin, were measured by thickness gage (Mitutoyo, Japan) at the inoculation site. The ID injections of 0.1 ml physiological saline, phytohemagglutinin (Difco, U.S.A.) and Parvo vaccine (DUPHAR B. V., Holland) were given as negative and positive controls, respectively.

**Skin grafting:** Donor skin of full thickness was grafted at three months after the final transfusion. Grafts of 3.5 cm in diameter were cleaned by

removal of subcutaneous tissues, and sutured into a comparable defect made in the recipient flank. Survival of the graft was scored from - to ++++ by visual examination at three and seven days postgrafting.

Probability was determined by Student's *t*-test.

## RESULTS

**MLR responses directed to donor PBL in dogs by treatment with UV-irradiated blood:** The time course of MLR during and following transfusion is shown in Table 2. In group 3 (UV-irradiated), %SI at week 4 (mean %SI  $\pm$  SE =  $64.3 \pm 4.2$ ) was significantly ( $P < 0.01$ ) reduced compared with week 0 (%SI = 100). In contrast, the %SI at week 4 in group 2 (untreated blood) and group 1 (control),  $93.5 \pm 6.1$  and  $98.4 \pm 5.1$  with respectively, were not significant compared with week 0. And %SI at week 4 in group 3 was significantly ( $P < 0.01$ ) reduced compared with group 1 and 2.

**RBC crossmatching responses to donor dogs in dogs by treatment with UV-irradiated blood:** RBC-crossmatching responses of group 3 dogs were all negative throughout the experimental duration (Table 3). In contrast, sera of three dogs (No. 5, 15 and 17) in group 2 induced agglutination of donor RBC starting from week 2. The degree of agglutination in group 2 increased with time (++++ = 6/6 at week 4). We detected anti-D1 antibody in the sera of all dogs at week 4. We also detected a minor antibody from the sera of four dogs (No. 5, 11, 15 and 20) in group 2 at week 4 (data not shown).

**Complement mediated cytotoxicity test in dogs by treatment with UV-irradiated blood:** Cytotoxicity in all groups was generally absent when the sera were

Table 2. The time course of mixed lymphocyte reaction (MLR) as the percent stimulation index

Group (treat- ment)	% change in MLR stimulation index <sup>a)</sup> Weeks after first transfusion					
	0	1	2	3	4	
1 (None)	100.0	109.0 $\pm$ 4.9 <sup>b)</sup>	119.8 $\pm$ 9.3	105.6 $\pm$ 7.0	98.4 $\pm$ 6.1	
2 (Fresh)	100.0	111.7 $\pm$ 7.4	96.8 $\pm$ 4.5	124.2 $\pm$ 22.2	93.5 $\pm$ 5.1	
3 (UV)	100.0	150.1 $\pm$ 26.1	100.3 $\pm$ 9.0	99.0 $\pm$ 7.8	64.3 $\pm$ 4.2 <sup>**c)</sup>	

a) SI=intensity of allogeneic MLR/intensity of autologous MLR.

b) Mean  $\pm$  SE (N=6).

c) Significant difference,  $P < 0.01$  compared with week 0.

d) Significant difference. \*\*:  $P < 0.01$ .

Table 3. The time course of red blood cell crossmatching

Group	Dog No.		Degree of agglutination <sup>u)</sup> at week				
	Serum	RBC	0	1	2	3	4
1	10	A	—	—	—	—	—
	27	A	—	—	—	—	—
	14	A	—	—	—	—	—
	23	A	—	—	—	—	—
	24	A	—	—	—	—	—
	26	A	—	—	—	—	—
2	2	B	—	—	±	++	+++
	5	B	—	—	++	++	+++
	11	B	—	—	—	+	+++
	15	B	—	—	+++	+++	+++
	17	B	—	—	++	++	+++
	20	B	—	—	—	—	+++
3	1	C	—	—	—	—	—
	6	C	—	—	—	—	—
	7	C	—	—	—	—	—
	9	C	—	—	—	—	—
	12	C	—	—	—	—	—
	25	C	—	—	—	—	—

a) Degree of agglutination is recorded from — to +++.

Table 4. Cytotoxicity patterns of dog sera against donor peripheral blood lymphocytes

Group	Dog No.		Antibody titer <sup>a)</sup> at week				
	Serum	Target Cell	0	1	2	3	4
1	10	A	0	0	0	0	0
	27	A	0	0	0	0	0
	14	A	0	0	0	0	0
	23	A	0	0	0	0	0
	24	A	0	0	0	0	0
	26	A	0	0	0	0	0
2	2	B	0	0	0	0	0
	5	B	0	0	0	0	0
	11	B	0	0	0	0	0
	15	B	0	0	0	0	0
	17	B	0	0	0	0	0
	20	B	0	0	0	0	0
3	1	C	0	0	0	0	0
	6	C	0	0	0	0	0
	7	C	0	0	0	0	0
	9	C	0	0	0	0	0
	12	C	0	0	0	0	0
	25	C	0	0	0	0	0

a) Titers are the reciprocal of the serum dilution giving 50% lysis with rabbit complement.

tested against cells of the donor (Table 4).

*Indirect Coombs test in dogs by treatment with UV-irradiated blood:* IgG, IgM and C3 were not detected in groups 1 and 3, whereas C3 was detected in the sera of three dogs (No. 2, 15 and 17) in group 2 (Table 5).

*DTH responses in dogs by treatment with UV-*

Table 5. Changes of titers in indirect Coombs test

Group	Dog No.	IgG		IgM		C3	
		Pre	Post <sup>a)</sup>	Pre	Post	Pre	Post
1	10	—	—	—	—	—	—
	27	—	—	—	—	—	—
	14	—	—	—	—	—	—
	23	—	—	—	—	—	—
	24	—	—	—	—	—	—
	26	—	—	—	—	—	—
2	2	—	—	—	—	—	×32
	5	—	—	—	—	—	—
	11	—	—	—	—	—	—
	15	—	—	—	—	—	×128
	17	—	—	—	—	—	×32
	20	—	—	—	—	—	—
3	1	—	—	—	—	—	—
	6	—	—	—	—	—	—
	7	—	—	—	—	—	—
	9	—	—	—	—	—	—
	12	—	—	—	—	—	—
	25	—	—	—	—	—	—

a) One week after final transfusion.

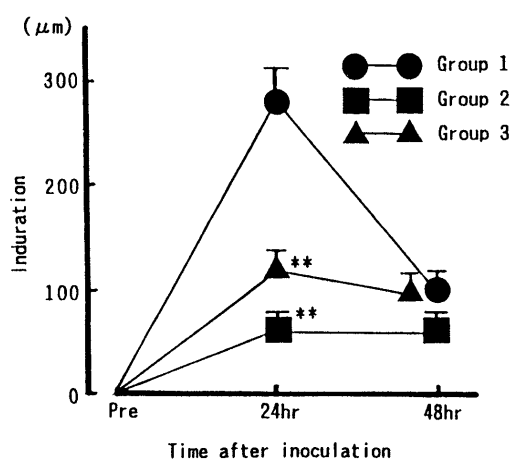


Fig. 1. Delayed type hypersensitivity (DTH) responses with donor peripheral blood lymphocytes (PBL) at one week after the final transfusion. DTH of 24 hr in group 1 all strongly responded on challenge with allogeneic donor PBL. That in group 2 and 3 reacted less significantly group 1. \*\*:  $P < 0.01$  Significant difference compared with group 1.

*irradiated blood:* No responses was visible at the sites of physiological saline, whereas phytohemagglutinin and Parvo vaccine produced strong reactions (data not shown). DTH of 24 hr in group 1 (control) all strongly responded on challenge with allogeneic donor PBL (Fig. 1). In contrast, that in groups 2 (untreated blood) and 3 (UV-irradiated)

Table 6. Delayed type hypersensitivity (DTH) responses at ten days after the final transfusion

Group	Transfused PBL <sup>a)</sup>		DTH responses at 24 hour		DTH responses at 48 hour
	No.	Treatment	Induration ( $\mu$ m)		Induration ( $\mu$ m)
1		None	280 $\pm$ 30 <sup>b)</sup>	<div style="display: inline-block; vertical-align: middle;"> <div style="border-left: 1px solid black; border-right: 1px solid black; height: 40px; margin: 0 auto;"></div> <div style="text-align: center; margin-top: -10px;"> ** *  </div> </div> <div style="display: inline-block; vertical-align: middle; margin-left: 5px;"> **c) </div>	100 $\pm$ 20
2	B	Fresh $\times$ 4	70 $\pm$ 10		60 $\pm$ 20
3	C	UV $\times$ 4	120 $\pm$ 20		100 $\pm$ 20

a) Peripheral blood lymphocytes.

b) Mean $\pm$ SE(N=6).

c) Significant difference. \*: P&lt;0.05. \*\*: P&lt;0.01.

Table 7. The time course of skin allograft rejection at three months after the final transfusion

Group	Dog No.	Days after skin grafting			
		0	3	7	7
1	10	- <sup>a)</sup>	-	+++	5/6 <sup>b)</sup>
	27	-	-	+++	
	14	-	-	+++	
	23	-	-	+++	
	24	-	-	+++	
	26	-	-	+	
2	2	-	-	+++	5/6
	5	-	-	+	
	11	-	++	++	
	15	-	++	+++	
	17	-	-	++	
3	20	-	++	+++	3/6
	1	-	-	++	
	6	-	-	+	
	7	-	-	+++	
	9	-	-	++	
	12	-	-	++	
	25	-	++	++	

a) Grade of rejection. -: 0~10% +: 11~30% ++: 31~70% +++: 71~90% ++++: 91~100%

b) Rejection (&gt;+++)/normal.

reacted less significantly group 1 (Table 6).

*Skin grafting in dogs by treatment with UV-irradiated blood:* Three dogs in group 2 (No. 11, 15 and 20) and one in group 3 (No. 25) had begun to reject three days after skin grafting. The ratio of rejection(>+++)/normal, in groups 1 and 2 however, was 83.3% (5/6) at seven days after skin grafting. In contrast, that of group 3 was 50% (3/6) (Table 7).

## DISCUSSION

Immune function modulation by UV light has

been studied extensively in rodents and to a lesser extent in dog and non-human primates [2, 5]. The suppression of allogeneic responses by UV-light that has been demonstrated directly *in vitro*, and indirectly *in vivo* by transfusion of UV-treated blood elements (PBL, plasma, or platelets, etc.) has been in transplantation [3, 4, 6, 11, 12, 17]. Directly UV-irradiated graft tissue or transfusion of UV-irradiated donor specific blood have been used in pancreatic islet allografts in some rodents [6, 7]. Allograft survival without immunosuppression remains difficult. The requirement for immunosuppressive drugs has been reduced by pretransplant transfusions [8, 11]. In our investigation using dogs, slightly suppression of skin graft rejection was presumed in group 3 (UV-irradiated) (Table 7). Bone marrow or skin transplantation is more complex due to strong immunogenicity and graft versus host disease (GVHD). Bone marrow allograft prolongation and GVHD prevention could be improved by pretransplant treatment with UV-irradiated blood elements [3, 14], or by the total body irradiation of the recipient [17].

UV-irradiation of whole blood did not negatively affect blood elements in our investigation. More importantly, when UV-irradiated whole blood was injected into dogs, proliferative responses of recipient PBL in MLR (Table 2), DTH responses (Table 6) and skin graft rejection (Table 7) were reduced. %SI of group 3 at week 4 in MLR was significantly (P<0.01) reduced compared with group 1 and 2. Although group 2 dogs significantly exhibited minimum DTH responses, suppression of skin graft rejection was not observed. The main reason may be that immunoalteration occurred within the three months between DTH and skin grafting. We speculate that immunosuppression may

be more remarkable if more UV-treated whole blood could be transfused.

Suppression of RBC-crossmatching is important in blood transfusion and for treating anemia and other blood diseases (Table 3). The group 3 donor differed from the other donors in two RBC group systems (D1, J1). Therefore, anti-D1 antibody was detected in the sera of all group 2 dogs at week 4. RBC-crossmatching responses of the dogs in group 3 were all negative, although RBC group systems differed between donor and recipient, and four dogs in group 2 (No. 5, 11, 15 and 20) produced the minor antibody (data not shown). The study of anti-D1 antibody suppression has just begun in our laboratory.

Anti-donor PBL antibody was raised in miniature swine which had been pretransfused with UV-treated lymphocytes [16]. Anti-donor PBL antibodies were not detected in this study. We speculated that the C3 detected was involved with agglutination in RBC-crossmatching without cytotoxic antibody (Table 4).

The usefulness of UV-treated whole blood transfusion in transplantation has been shown repeatedly [6], but little has been studied in larger animals such as dogs [3]. Our results demonstrated that dogs treated with UV-irradiated allogeneic whole blood have a beneficial effect on alloimmune responses. UV-treated whole blood, however, may require immunosuppressive drugs to prolong graft survival in high responder animals with more complex immune systems. Therefore, using these treatments with conventional therapy (i.e. immunosuppressive drugs) in rat [12] may allow successful transplantation without requiring high levels of these frequently toxic substances, in larger animal in future.

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