Early *In Vitro* Detection of Interleukin-2-Like Activities by Pretreatment with Allogeneic Blood Lymphocytes in Miniature Swine

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ABSTRACT. In order to determine the means of monitoring the immunological status of allograft recipients in miniature swine, an assay was developed to measure interleukin (IL)-2 production *in vitro* by pretreatment of donor peripheral blood lymphocytes (PBL). Miniature swine were given 0 to 4 weekly intravenous transfusions of $5–10\times10^7$ donor PBL incompatible at major histocompatibility complex (MHC) and assayed *in vitro* for donor specific immune IL-2-like activities. The results are summarized as follows: (1) IL-2-like activity in 24 hr and 48 hr supernatants from mixed lymphocyte cultures (MLC) with MHC-incompatible PBL was detected without pretreatment. The 48 hr MLC supernatant exhibited a high IL-2-like activity compared with the 24 hr; (2) IL-2-like activity after only one transfusion with MHC-incompatibile PBL was higher than that without pretreatment; (3) IL-2-like activity in 4 weekly transfusions was detectable slightly earlier than that without pretreatment or three transfusions with MHC-incompatibile PBL.—KEY WORDS: IL-2-like activity, *in vitro* assay, miniature swine, monitor.

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Interleukin (IL)-2 is a T-cell lymphokine secreted by activated T-cells. IL-2 is important for clonal expansion of T-cells and also as a helper factor for B cells in T-B collaborative immune responses [5]. Recently, it was reported that measurement of IL-2 could serve as a means of monitoring the immunological status of allograft recipients [1, 2, 8]. Aside from the fact that IL-2 levels in serum are elevated one to three days prior to those changes conventionally used to diagnose rejection, IL-2 levels were also elevated during infection and during dysfunction of human recipient [1, 2, 8]. The miniature swine is an excellent large animal model for transplantation because of the similarity to humans in their physiology, metabolism, and dietary habits [10]. Furthermore, the availability of genetically defined animals with known major histocompatibility complex (MHC) haplotypes [10] permits transplantation to be performed under defined conditions. We have recently reported the suppression of delayed type hypersensitivity (DTH) and mixed lymphocyte reaction (MLR) by treatment with ultraviolet (UV) light-irradiated peripheral blood lymphocytes (PBL) [11]. UV-irradiated stimulator PBL did not elicit either proliferation or IL-2 production with allogeneic responder PBL in MLRs [11]. Not being aware of any previous reports describing IL-2 during pretreatment with allogeneic peripheral blood lymphocytes in human and large animal models, we have reported the IL-2 production in 24 hr incubation of the 48 cultured mixed lymphocyte culture (MLC) supernatants and IL-2-dependent murine cell line [11]. We attempted to develop an assay to measure IL-2 *in vitro* production in order to monitor the early phase of anti-graft immune responses well before the clinical signs of rejection. In this study, miniature swine were given 0 to weekly intravenous transfusions of $5–10\times10^7$ donor PBL incompatible at MHC and tested for IL-2-like response with an *in vitro* assay and the MLR.

MATERIALS AND METHODS

Animals: Ten castrated males and four females of MHC-inbred miniature swine, descended from NIH stock (David H. Sachs. National Cancer Institute, Bethesda, MD) [9–11], weighing between 15 to 20 kg, and aged from 10 to 20 months were selected after tissue typing. The pigs were divided into the following groups: Group I, no pretreatment (n=6); Group II, pretreated once with donor PBL (n=3); Group III, 4 weekly pretreated with donor PBL (n=5) incompatible at MHC. Blood was collected from donor pigs with 2 histocompatibility (MHC), termed swine lymphocyte antigen (SLA) types, designated as *ag* and *dd*, by cranial vena cavae puncture and then processed by Ficoll (Pharmacia

LKB. NJ, specific gravity: 1.077) separation [9–11]. PBL (not purified) were then centrifuged and resuspended in 10 m*l* of PBS after washing twice. Each pig received 5–10×10^7 PBL through the ear vein on 0, 1 and 3 weeks after the first transfusion.

Mixed lymphocyte reaction (MLR): MLRs were performed on 0, 1 and 4 weeks after the first transfusion using standard methods [9–11]. Pig PBL harvested by Ficoll separation were cultured in 96-well U-bottomed microtiter plates (Linbro Laboratories. McLean, VA) at concentrations of 5×10^5 responder cells and 5×10^5 stimulator cells per 200 μl RPMI1640 medium (Difco Laboratories, Grand Island, NY) supplemented with 8% (v/v) heat inactivated fetal pig serum, 2-mercaptoethanol (0.05 mM, Sigma), sodium pyruvate (1 mM, Sigma), nonessential amino acids (0.01 mM, Gibco), antimycotic-antibiotic solution (Gibco), gentamicin (0.05 mg/m*l*), HEPES (25 mM, Sigma) and $NaHCO_3$ (24 mM, Sigma). Stimulator cells were irradiated with 30 Gry γ-irradiation (Gammacell #220). The plates were cultured at 37°C in 5% CO_2 in air for 5 days at which time ^{3}H-thymidine (Tdr, ICN Radiochemicals, Irvine, CA) 18.5 kBq in 5 μl medium was added to each culture well. Six hours later, cultures were harvested onto glass fiber filter strips with a Mash II automated harvester and ^{3}H-Tdr incorporation was assessed by liquid scintillation counting (Beckman, LS-3100).

Assay for IL-2-like activity: PBL were collected from recipient pigs of all three groups at 0, 1 and 4 weeks after the first transfusion for MLR and IL-2-like activity. The MLR culture supernatants were taken at 0, 4, 6, 18, 24 and 48 hr to assay for IL-2-like activity. Mouse helper T lymphocytes (HT cells) were maintained in IL-2-dependent long-term proliferative cultures and used as the target cell populations in the IL-2 assay. Assays for IL-2-like activity were performed by the method of Gillis *et al.* [3]; HT-2 cells were kindly supplied by Dr. William Clark (Department of Biology. University of California, Los Angeles, CA) and used to measure IL-2 levels. The Culture supernatants from mitogen or antigen MLC-stimulated pig PBL were harvested and tested for IL-2-like activity. Pig IL-2-like activity was tested after 48 hr stimulation of normal pig PBL with concanavalin A (Con A, 100 μg/m*l*, Sigma) in a humidified atmosphere of 5% CO_2 in air at 37°C. HT-2 cells were washed twice and resuspended in RPMI 1640 supplemented medium (described above). Three thousand HT-2 cells suspended in 0.1 m*l* were added to 0.1 m*l* MLC supernatants in flat-bottomed 96-well Falcon plates (3072 Falcon R, Oxnard, CA). The plates were cultured at 37°C in 5% CO_2 in air for 24 hr at which time ^{3}H-Tdr of 18.5 kBq in 5 μl medium was added to each culture well. Six hours later, cultures were harvested as above. MLR and IL-2-like activity were expressed as a stimulation index (SI), equal to the ratio of experimental cpm to control cpm.

Statistical significant of the differences between the means of the sample groups was tested by Student's t test. The significance difference of $P<0.05$ was used as a criterion of statistical significance.

RESULTS

IL-2-like activity: Results of a typical IL-2 assay are shown in Table 1. HT-2 cells cultured in the presence of increasing concentrations of Con A-induced IL-2 incorporated ^{3}H-Tdr in a dose dependent manner. The control IL-2 was routinely harvested after a 48 hr stimulation of normal pig lymphocytes with Con A. The final Con A concentration was maximum stimulation for normal lymphocytes. HT-2 cells cultured for 26 hr in the absence of IL-2 incorporated only background levels of ^{3}H-Tdr. The viability of HT-2 cells within 26 hr was consistently greater than 90% by the trypan blue (0.2% in normal saline) exclusion test.

IL-2-like activity of MLC supernatant without pretreatment: Inducing effects of allogeneic donor PBL for *in vitro* IL-2-like activity were investigated and compared those of MLR. Table 2 shows that allogeneic (*ag* or *dd*) PBL stimulated responder (*cc,*

Table 1. ^{3}H-Tdr incorporation of HT-2 cells in 26-hr cultures in the presence of decreasing concentrations of control IL-2 (pig)

Dilution	^{3}H-Tdr incorporation (Mean cpm ± SE)
1	9155±157[a)]
2	8786±115
4	8326± 92
8	7383± 89
16	4607± 43
32	2284± 25
64	658± 15
128	29± 4
256	25± 4
512	23± 2
medium	8± 1

a) n=3.

Table 2. ^{3}H-Tdr incorporation of HT-2 cells in the presence of MLC supernatant and MLR in non-pretreated pigs (Group-I)

Responder Stimulator		HT-2 ^{3}H-Tdr (Mean cpm ± SE)				MLR ^{3}H-Tdr (Mean cpm ± SE)	
(SLA)[a]	(SLA)	24 hr	SI[b]	48 hr	SI	120 hr	SI
# 1(*cc*)	#10(*ag*)	60± 1	1.9	1956±310	38.4	29360±1965[c]	115.1
# 2(*cc*)	# 9(*dd*)	52±38	1.6	488± 7	15.3	103412±9147	236.1
# 7(*ac*)	# 9(*dd*)	31± 2	1.2	563± 25	21.8	20861±1315	98.4
# 8(*cc*)	# 9(*dd*)	45± 4	2.1	497± 41	23.2	13476±4800	112.3
#11(*cc*)	#13(*dd*)	36± 3	1.4	321± 27	12.5	16416±3200	68.4
#12(*cd*)	#14(*ag*)	38± 4	1.3	476± 15	16.3	22082±6430	70.1
Mean SI ± SE		1.6±0.1		21.3±3.8		116.7±25.2	

a) SLA: miniature swine MHC. b) SI= cpm of allogeneic MLR/cpm of autologus MLR. c) n=3.

Table 3. ^{3}H-Tdr incorporation of HT-2 cells in the presence of MLC supernatants at 48 hr of culture and MLR in pretreated pigs with allogeneic donor PBL (Group-II)

Recipient Donor		SI[a] of HT-2		SI of MLR	
(SLA)	(SLA)	pretrans-fusion	week 1	pretrans-fusion	week 1
#3(*cc*)	#9(*dd*)	12.0	17.1	10.1	11.8
#4(*cc*)	#9(*dd*)	12.8	26.1	18.1	19.3
#5(*cc*)	#9(*dd*)	1.9	38.4	11.5	12.1
Mean SI ± SE		8.9±3.5	27.2±6.2	13.2±2.5	14.4±2.5

a) SI = cpm of allogeneic MLR/cpm of autologus MLR.

ac and *cd*) PBL to produce IL-2-like activity into 48 hr cultured MLC supernatant and in 120 hr MLR without pretreatment. However, there was little or no IL-2-like activity at 24 hr.

Stimulation of IL-2-like activity by one transfusion with MHC-incompatible PBL: Table 3 shows that allogeneic (*dd*) PBL stimulated responder (*cc*) PBL to produce IL-2-like activity into 48 hr cultured MLC supernatant and in MLR on one week. The MLC supernatant exhibited a higher ($P<0.05$) activity of IL-2 after one transfusion with allogeneic PBL than that of the pretransfusion. There were no differences between MLR of the pretransfusion and that of the one week.

Detection of IL-2-like activity on 4 weekly transfusions with MHC-incompatible PBL: Four weekly pretreatments with allogeneic donor PBL stimulated IL-2-like activity detected in 24 and 48 hr MLC supernatants and 120 hr MLR at 4 week (Table 4). The 48 hr MLC supernatant exhibited a high IL-2-like activity compared to that of 24 hr.

The time course of IL-2-like activity and MLRs during and following the series of transfusions is shown in Table 5. SIs of HT-2 cells increased, starting at week 0. Pigs 8, 11, and 12, which had received a fourth injection of $5–10\times10^{7}$ fresh PBL, showed great production. Although SIs of HT-2 cells of all pigs in this group increased after the 4th transfusion, the response of pig 7 was still low with high MLR responses. The detection of IL-2-like activity at the 4th week, was slightly trending to

Table 4. ^{3}H-Tdr incorporation of HT-2 cells in the presence of MLC supernatants at 24 and 48 hr of culture and MLR in pretreated pigs with 4 weekly administrations of allogeneic donor PBL on week 4 (Group-III)

Recipient Donor		SI[a] of HT-2		SI of MLR
(SLA)	(SLA)	24 hr	48 hr	120 hr
# 6(*cg*)	#10(*ag*)	7.3	96.4	111.7
# 7(*cc*)	# 9(*dd*)	29.5	105.7	109.9
# 8(*cc*)	# 9(*dd*)	65.2	84.4	103.2
#11(*cc*)	#13(*dd*)	14.8	30.4	84.2
#12(*cd*)	#14(*ag*)	16.4	24.9	88.6
Mean SI ± SE		26.6±10.3	68.4±17.0	99.5±5.6

a) SI = cpm of allogeneic MLR/cpm of autologus MLR.

Table 5. Changes of HT-2 cell ^{3}H-Tdr incorporation of MLC supernatant at 4, 6, 18 and 24 hr culture and MLR in pretreated pigs with 4 weekly administrations of allogeneic donor PBL (Group-III)

Recipient (SLA)	Donor (SLA)	Donor PBL Pre-treatment[c]	SI[a] of HT-2 Weeks	4	6	18	24	48 hr	SI of MLR 120 hr
# 7(*ac*)	# 9(*dd*)	None	0	1.0	1.3	1.5	1.2	21.8	98.4
		Fresh×3	3	1.0	1.1	2.9	4.1	NT[b]	NT
		Fresh×4	4	NT	NT	2.9	29.5	105.7	109.9
# 8(*cc*)	# 9(*dd*)	None	0	1.3	1.1	1.1	2.1	23.2	112.3
		Fresh×3	3	1.2	1.4	9.0	4.5	NT	NT
		Fresh×4	4	1.2	2.1	34.0	65.2	84.4	103.2
#11(*cc*)	#13(*dd*)	None	0	1.2	1.1	1.6	1.4	12.5	68.4
		Fresh×3	3	1.0	1.6	1.3	2.9	NT	NT
		Fresh×4	4	1.6	1.8	10.2	16.2	30.4	84.2
#12(*cd*)	#14(*ag*)	None	0	1.1	1.0	1.2	1.3	16.3	70.1
		Fresh×3	3	1.6	2.1	4.2	3.4	NT	NT
		Fresh×4	4	1.3	3.1	11.1	18.3	24.9	88.6

a) SI = cpm of allogeneic MLR/cpm of autologus MLR. b) NT: Not Tested. c) PBLs were i.v.-injected fresh weekly for 3 or 4 weeks.

earlier than that of pretransfusion and week 3. As compared with MLRs performed prior to the first injection, 3 pigs at week 4 (one week after the 4th transfusion) showed increases of MLR responses.

DISCUSSION

Late detection of allograft rejection has been implicated as an important factor causing allograft failure. Organ dysfunction other than rejection, such as inflammation or toxicity of immunosuppression, can affect parameters such as serum or urinary emzymes. IL-2 or IL-2 receptor assays in serum as a means of monitoring allograft rejection has been studied extensively in human and a lesser extent in pigs [1, 2, 4, 8, 11]. Cornaby *et al.* have suggested that serial measurement of lymphokines, especially IL-2, can provide clinically relevant information regarding the state of the transplanted organ [1]. It would be useful to continue to study serum IL-2 levels in the setting of clinical transplantation [2]. Georgi *et al.* also suggested that serum IL-2 levels were elevated during infection and during episodes of non-specific inflammation.

In miniature swine, stimulator PBL of SLA type *ag* elicited IL-2 production in allogeneic responder PBL of SLA type *cc*, whereas γ-irradiated (30Gy) PBL stimulated high responses in the 48 hr MLC supernatant (Table 2), an observation originally described in the pig by Taura *et al.* [11]. IL-2 production after only one transfusion with allogeneic PBL was higher than that without-pretreatment (Table 3). The detection of IL-2 production by the *in vitro* assay was earlier than that of PBL non-pretreated pigs from 48 hr to 18 hr after the PBL were 4 weekly injected into pigs of a different SLA type (Tables 4, 5). MLRs can also provide clinically relevant information, but the incubation period, 120 hr, is too long to use as clinical monitoring. If we can detect the early phase of anti-graft immune responses, immune therapy for rejection is possible before the clinical signs of rejection.

IL-2 was produced by T cells in response to two triggers provided by antigen activated T cells ($CD4^{+}$): antigen in context with T cell MHC and IL-1 from antigen presenting cells [5]. CD4 expresses IL-2 receptors after contact with specific antigens [5]. It is necessary to determine the concentration of IL-2, the number of IL-2 receptors per cell and the continued expression of receptors in order to monitor of donor specific IL-2 response, because IL-2 acts on any IL-2 receptor positive cell, in *in vitro* assays.

Whereas our future studies will include the transplantation of whole pancreata and islets into diabetic pigs, it is still difficult to monitor the early phase of an anti-graft immune responses. Evidence of mild rejection and spontaneous resolution occasionally has been observed with adequate immuno-

suppression [9]. In this preliminary study with a limited number of animals, these IL-2 *in vitro* assays may help to determine when to initiate immunosuppressive therapy, and help to monitor the effectiveness of various pretreatment procedures. This study suggests that PBL treatment has the potential to shift the recipient IL-2 response toward early production *in vitro* under the appropriate conditions.

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