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Mechanism of suppression of cloned human suppressor T cells

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Abstract We report the mechanism of suppression of suppressor T cell clone III-1-C5 using helper T cell clone III-1-B6, mitogen responses and rIL-2. Clone III-1-C5 suppressed the mixed lymphocyte reaction (MLR) by secreting alloantigen non-specific, MHC non-restricted suppressor factor(s). Clone III-1-C5 did not suppress mitogen (PHA, Con A, PWM) response nor proliferation by exogeneous rIL-2. Clone III-1-C5 suppressed proliferation by clone III-1-B6, which augments proliferation by direct cell to cell contact with responder cells and not by soluble factors. These results indicated that suppressor T cells exhibit suppressive effects not only by inhibiting IL-2 synthesis but by inhibiting the direct effects of helper T-cells.

Key words Suppressor cell
Helper cell · Clone · IL-2
Adhesion molecule

Introduction

In long-surviving renal transplant recipients, unresponsiveness to donor cells is noted in some cases. Donor-specific suppressor cells of mixed lymphocyte reaction (MLR) and/or cell-mediated lympholysis (CML) have been observed in some of these patients. The appearance of suppressor cells may be the cause of donor-specific unresponsiveness in this group [1]. Therefore, the suppressor cell system is thought to be one of the most important factors that induces transplanted organ acceptance. Using a cloned human suppressor T cell [2], we reported a human suppressor T cell, in which the suppressor clone was a CD8-positive T lymphocyte, whose action was mediated via short-lived soluble suppressor factors. In the present study, we report the mechanism of action of suppressor cells using a helper T cell clone.

Materials and methods

Separation of mononuclear cells (PBMC)

Peripheral mononuclear cells were separated from whole blood obtained from healthy nontransfused donors using the Ficoll-Conray gradient. After washing three times, the PBMC were suspended in culture medium.

Priming mixed lymphocyte culture (MLC), cloning

Cloned human suppressor cell III-1-C5 and helper cell III-1-B6 were established as previously described [2]. Briefly, for the priming MLC, T lymphocytes of responder KH (HLA-A24,-; B7, W60; DR1, 4; DQW1, W3) and 2000 rad gamma-irradiated PBMC of stimulator HT (HLA-A26, 31; B7, w61; DR4, w8; DQw3, -) were mixed together in a culture flask (Falcon 3013), and cultured at 37 °C in a humidified incubator with 5% CO_2 in air for 6 days. From these primed lymphocytes, suppressor cell clone III-1-C5 and helper cell clone III-1-B6 were obtained by the limiting dilution technique. These clones were maintained with recombinant interleukin-2 (rIL-2, 120 JRU/ml, Takeda Co, Osaka, Japan) and feeder cells.

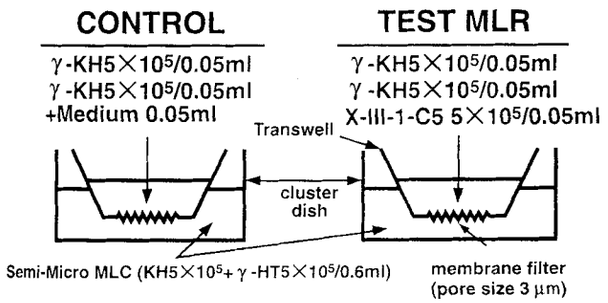

Fig. 1 Semi-micro MLR. The transwell, which contained 750-rad x-irradiated clone III-1-C5 and 2000-rad gamma-irradiated KH and HT, was placed in cluster dish containing semi-micro MLC cells or responder cells KH with mitogens. As the control, the Transwell without clone III-1-C5 was placed in the cluster dish

Suppressor cell assay

The function of the cloned suppressor cells was tested by adding $0.5 \times 10^5/0.05$ ml of 750-rad x-irradiated cloned suppressor cells to a fresh micro-MLC at its initiation. Micro MLC was established by adding $1 \times 10^5/0.1$ ml of responding cells KH and $1 \times 10^5/0.1$ ml of 2000-rad gamma-irradiated stimulating cells HT to flat-bottom wells of a microculture plate (Falcon 3072 culture plate), which was then cultured for 6 days. For the control, $0.5 \times 10^5/0.05$ ml of 750-rad x-irradiated responding cells KH were added to MLC in lieu of the suppressor clone. Using a liquid scintilation counter, MLR was determined by counting the incorportated 3 H-thymidine, which had been added in amounts of 0.5 μCi/well 18 h prior to harvesting.

To determine whether or not clone III-1-C5 secretes suppressor factors, a semi-micro MLR suppression test was performed using a culture plate with a membrane filter cup. As shown in Fig. 1, x-irradiated clone III-1-C5 was mixed with gamma-irradiated KH and HT in the membrane filter cup (Transwell). The Transwell was then placed in a cluster dish containing semi-micro MLC cells at its initiation. As the control, the Transwell without a suppressor clone was placed in the cluster dish. With this system, humoral factors from the clone can move through the membrane without cell-to-cell contact between clone III-1-C5 and MLC cells.

MLR suppression values were obtained from the following formula:

MLR suppression (%) = (1-cpm of test MLC/cpm of control MLC) × 100 (%)

Helper cell assay

The function of cloned helper cells was examined by adding $0.5 \times 10^5/0.05$ ml of 750-rad x-irradiated cloned helper T-cell clone III-1-B6 to a fresh micro-MLC to which was added 5 μg of cyclosporin A (CsA) at its initiation. Instead of semi-micro MLC, mitogen response using Transwell was performed if needed.

Cell-mediated lympholysis (CML) assay

To check the cytotoxic function of clone III-1-C5, a CML assay was performed. The method used was the ^{51}Cr releasing assay. Target cells were cultured with PHA for 3 days and labeled with $Na_2{}^{51}CrO_4$. Labeled target cells ($1 \times 10^4/0.1$ ml) were mixed with effector cells ($5 \times 10^5/0.1$ ml) in round-bottom wells in a microculture plate and incubated at 37 °C for 4 h. After incubation, 0.1 ml of the supernatant was collected and counted using a gamma counter. The percentage cytotoxicity was calculated using the following equation:

CML (%) = (experimental release − spontaneous release/maximum release − spontaneous release) × 100 (%)

In all of these cultures, medium RPMI 1640 (GIBCO) which contained 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 20% pooled heat-inactivated human male AB serum, was used.

HLA typing

HLA was detected with the antisera presented at the 8th Japanese HLA workshop meeting.

Monoclonal antibody and complement treatment

We incubated $5 \times 10^5/1$ ml of clone III-1-C5 or III-1-B6 with 50 μl of monoclonal antibody at 24 °C. After 60 min, 2.5 ml of rabbit complement was added to the cells and further incubated for 60 min at 24 °C. The viability of treated cells was determined by Trypan blue exclusion test.

Results

Seventy-two clones were established by the limiting dilution technique. There were four clones that suppressed MLR of the original priming MLC pair and three clones that augmented the proliferation of the original priming MLC pair. Only one clone III-1-C5 suppressed the MLR of responder KH to stimulator HT without cytotoxicity against stimulator HT or responder KH. Clone III-1-B6 and clone IV-2-A5 augmented proliferation without cytotoxicity to KH or HT. Clone III-1-C5 was, thus, used as a suppressor clone and clone III-1-B6 as a helper clone.

Table 1 shows the specificity of MLR suppression of clone III-1-C5. Clone III-1-C5 suppressed MLR of responder KH to stimulator HT, but not to eight other stimulating cells. These results indicated that clone III-1-C5 is an alloantigen-specific suppressor cell clone. Next, we studied the mechanism of MLR suppression by clone III-1-C5 using the semi-micro MLR system.

Table 2 shows the suppressive activity of the supernatant of clone III-1-C5. In experiment 1, 750-rad x-irradiated clone III-1-C5, with gamma-irradiated KH and HT in Transwell suppressed semi-micro MLR of responder KH to stimulator HT in a cluster dish. In experiment 2, clone III-1-C5 in Transwell suppressed semi-micro MLR of responder KH to third-party cells TY. In experiment 3, clone III-1-C5 in Transwell suppressed the semi-micro MLR of allogeneic responder EY to

Table 1 Specificity of MLR suppression of clone III-1-C5. We added 0.5×10^5/0.05 ml of 750-rad x-irradiated clone III-1-C5 to a micro MLC at its initiation. Micro MLC was established by adding 1×10^5/0.1 ml of responding cells KH and 1×10^5/0.1 ml of 2000-rad gamma-irradiated various stimulating cells (HT to EF)

	Stimulating cells				MLR suppression		
	HLA						
	A	B	DR	DQ	Test	Control	Suppression (%)
HT	26, 31	7, w61	4, w8	w3, –	13672	24666	44.6
EY	11, 24	w48, w61	2, w8	w1, –	12694	13554	6.3
HY	2, 24	44, w52	w8, –	w1, –	24279	21470	−13.1
BF	w33, –	44, 51	w8, –	w1, –	24399	24464	0.3
HF	2, 24	w54, 8 w59	4, NJ2	w3, –	17548	14446	−21.5
TY	2, 11	w67, –	2, –	w1, –	30253	23061	−31.2
AT	24, 31	51, 51	2, 4	w1, w3	20506	17909	−14.5
EO	9, 26	w52, w61	2, w9	w1, w3	22981	16541	−38.9
EF	24, –	w61, w62	w9, –	w3, –	36442	33765	− 7.9

Table 2 Specificity of MLR suppressor factors of clone III-1-C5. We mixed 750-rad x-irradiated clone III-1-C5 with 2000-rad gamma-irradiated KH and HT in Transwell. The Transwell was then placed in a cluster dish in which various kinds of semi-micro MLCs were cultured

Cluster			Transwell		MLR (cpm)	MLR Suppression (%)
Responder		Stimulator	Clone	Feeder		
Experiment 1	KH	γ-HT	(–)	γ-KH, γ-HT	181673	
	KH	γ-HT	x-III-1-C5	γ-KH, γ-HT	110601	39.9
Experiment 2	KH	γ-TY	(–)	γ-KH, γ-HT	189668	
	KH	γ-TY	x-III-1-C5	γ-KH, γ-HT	121983	35.7
Experiment 3	EY	γ-HT	(–)	γ-KH, γ-HT	110239	
	EY	γ-HT	x-III-1-C5	γ-KH, γ-HT	66694	39.5
Experiment 4	EY	γ-TY	(–)	γ-KH, γ-HT	131011	
	EY	γ-TY	x-III-1-C5	γ-KH, γ-HT	85079	35.1

Table 3 Effects of soluble factors of clone III-1-C5 on mitogen response. We mixed 750-rad x-irradiated clone III-1-C5 with 2000-rad gamma-irradiated KH and HT in Transwell. The Transwell was then placed in a cluster dish in which 5×10^5/0.6 ml o responder KH was cultured with mitogens or stimulator HT

Mitogen	Cluster	Transwell		3H-TdR incorporation (cpm)	Suppression (%)
		Clone	Feeder		
	KH	(–)	γ-KH, γ-HT	375281 ± 722	
PHA	KH	x-III-1-C5	γ-KH, γ-HT	370007 ± 9846	1.4
	KH	(–)	γ-KH, γ-HT	525815 ± 3214	
Con.A	KH	x-III-1-C5	γ-KH, γ-HT	483472 ± 3879	8.1
	KH	(–)	γ-KH, γ-HT	190316 ± 1564	
PWM	KH	x-III-1-C5	γ-KH, γ-HT	167627 ± 3115	12.0
	KH	(–)	γ-KH, γ-HT	302381 ± 27088	
MLR	KH	x-III-1-C5	γ-KH, γ-HT	204689 ± 38052	32.3

stimulator HT. In experiment 4, clone III-1-C5 suppressed semi-micro MLR of allogeneic responder EY to third-party cells TY. These results indicated that clone III-1-C5 suppressed MLR via humoral suppressor factors without cell-to-cell contact between clone III-1-C5 and MLC cells and that effects of suppressor factors of clone III-1-C5 were not alloantigen-specific or restricted by MHC.

Next, we studied whether or not clone III-1-C5 suppressed the proliferation of lymphocytes by mitogen stimulation. Table 3 shows the supppressive activity of clone

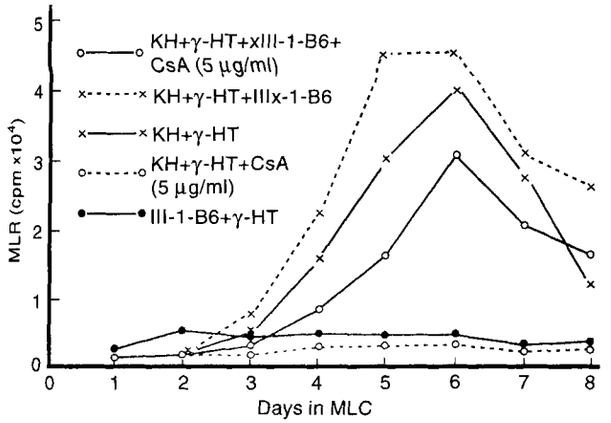

Fig. 2 Effect of clone III-1-B6 on MLR that was suppressed by CsA. Irradiated or nonirradiated clone III-1-B6 was added to a micro-MLC at its initiation. Incorporation of 3 H-TdR of micro-MLC was determined daily up to the 8th day

III-1-C5. Gamma-irradiated KH and HT mixed with 750-rad x-irradiated clone III-1-C5 in Transwell did not suppress the proliferation of PBMC of KH by PHA, Con A or PWM. This indicated that clone III-1-C5 suppressed the proliferation of PBMC stimulated by alloantigen but not the proliferation of PBMC stimulated by mitogens. This means that clone III-1-C5 suppressed the antigen-specific response but not the nonspecific response.

Next, we studied the action of clone III-1-C5 on helper T cell clone III-1-B6. Figure 2 shows the augmentative effect of clone III-1-B6 on MLR. CsA (5 μg/ml) inhibited the proliferation of responder KH to stimulator HT, and 750-rad x-irradiated clone III-1-B6 augmented the suppressed proliferation of responder KH by CsA. Clone III-1-B6, itself, showed no proliferation to stimulator HT. This indicated that clone III-1-B6 has the ability to help lymphocyte proliferation.

Table 4 shows the augmentative effect of clone III-1-B6 on cytotoxicity. CsA suppressed the cytotoxicity of responder KH to stimulator HT and K562. Clone III-1-B6 augmented the proliferation, CML to stimulator HT and the cytotoxicity to K562 of responder KH without CsA. Clone III-1-B6 augmented proliferation and cytotoxicity to K562 of responder KH, but did not enhance suppressed CML to stimulator HT of responder KH with CsA. These results indicated that clone III-1-B6 did not help suppressed CML by CsA, which requires alloantigen recognition. This augmentative effect resembled that of rIL-2.

Table 5 shows the function of clone III-1-B6 treated with monoclonal antibodies and complement. In experiment 1, clone III-1-B6 treated with monoclonal antibody OKT3, OKT4, and complement did not augment the 3 H-TdR incorporation of responder KH that was suppressed by CsA. Clone III-1-B6 treated with monoclonal antibody OKT8 and complement showed helper activity. In experiment 2, clone III-1-B6 treated with monoclonal antibody OKDR, anti-Leu-M3, Hansen 20.2, and complement did not augment the 3 H-TdR incorporation of responder KH to mediate its helper function, x-irradiated treatment with anti-Leu-8 and complement. These results indicated that clone III-1-B6 is a CD4, DR, Leu-M3 positive helper T lymphocyte.

Next, we studied the suppressive effect of clone III-1-C5 on the augmentative effect by helper T cell clone III-1-B6, which augments the proliferation of responder KH. Because clone III-1-B6 requires cell-to-cell contact with responder KH to mediate its helper function, x-irradiated clone III-1-B6 was added to KH in the cluster. Table 6 shows that x-irradiated clone III-1-C5 suppressed the proliferation of KH by x-irradiated clone III-1-B6 but not the proliferation of KH by rIL-2. These results indicated that suppressor clone III-1-C5 suppressed the T cell mediated response by suppressing helper T cells that augmented the cellular response and did not mediate its suppressive function following the secretion of IL-2.

Table 4 Effect of clone III-1-B6 on cytotoxicity. We added 750-rad x-irradiated clone III-1-B6 and/or rIL-2 to semi-micro MLCs with or without CsA at its initiation. Semi-micro MLC was established by adding 1×10^6 of responder KH and 1×10^6 of 2000-rad gamma-irradiated stimulator HT. The number of harvested cells and cytotoxicity were measured after culture for 5 days

Responder	Stimulator	CsA (5 ug/ml)	Added materials	Number of harvested cells	Cytotoxicity to (%)		
					KH	HT	K562
KH	HT	(−)	(−)	2.0×10^6	4.8	56.4	81.0
KH	HT	(+)	(−)	0.7×10^6	N.D	2.0	37.3
KH	HT	(+)	x-750 III-1-B6	1.5×10^6	N.D	10.0	74.5
KH	HT	(+)	rIL-2 (80JRU/ml)	2.6×10^6	2.0	14.3	83.7
KH	HT	(+)	x-750 III-1-B6 rIL-2 (80JRU/ml)	3.4×10^6	0.2	11.6	79.7
KH	HT	(−)	rIL-2 (80JRU/ml)	4.0×10^6	3.2	51.6	80.3

Table 5 Function of clone III-1-B6 treated with monoclonal antibody and complement. Clone III-1-B6 treated with various monoclonal antibodies and complement was 750-rad x-irradiated and added to the micro MLC

Responder		Stimulator	CsA (5 ug/ml)	Added materials	MLR (cpm)
Experiment 1	KH	HT	(−)	(−)	43897
	KH	HT	(+)	(−)	4702
	KH	HT	(+)	x-III-1-B6 (C)	34914
	KH	HT	(+)	x-III-1-B6 (OKT3+C)	3960
	KH	HT	(+)	x-III-1-B6 (OKT4+C)	3213
	KH	HT	(+)	x-III-1-B6 (OKT8+C)	48061
Experiment 2	KH	HT	(−)	(−)	36556
	KH	HT	(+)	(−)	2916
	KH	HT	(+)	x-III-1-B6 (C)	14614
	KH	HT	(+)	x-III-1-B6 (OKDR+C)	6241
	KH	HT	(+)	x-III-1-B6 (anti-Leu-M3+C)	5585
	KH	HT	(+)	x-III-1-B6 (anti-leu-M3)	19460
	KH	HT	(+)	x-III-1-B6 (Hansen 20.2+C)	3797
	KH	HT	(+)	x-III-1-B6 (anti-Leu-8+C)	24251

Table 6 Suppressive effect of clone III-1-C5 on augmentative function of clone III-1-B6 and rIL-2. We mixed 750-rad x-irradiated clone III-1-C5 with 2000-rad gamma-irradiated KH and HT in a Transwell. The Transwell was then placed in a cluster dish in which responder KH was cultured with 750-rad x-irradiated clone III-1-B6 or 80 JRU/ml of rIL-2

Added cells	Cluster	Transwell		3H-TdR incorporation	Suppression (%)
		Clone	Feeder		
	KH	(–)	r-KH, r-HT	22341 ± 3760	
	KH	x-III-1-C5	r-KH, r-HT	26166 ± 3062	
x-III-1-B6	KH	(–)	r-KH, r-HT	642632 ± 50216	
x-III-1-B6	KH	x-III-1-C5	r-KH, r-HT	385260 ± 4223	40.0
r-IL-2	KH	(–)	r-KH, r-HT	483622 ± 52323	
r-IL-2	KH	x-III-1-C5	r-KH, r-HT	506218 ± 40015	− 4.7

Discussion

Donor-specific MLR, CML unresponsiveness is commonly seen in renal transplant recipients [1]. MLR and CML unresponsiveness is easily abolished by the addition of exogenous rIL-2 at its initiation [1]. This phenomenon shows the need for IL-2 synthesis unresponsiveness; thus, in long-surviving recipients, although the recipient's PBMC can recognized the donor's alloantigen, they cannot synthesize IL-2 against the donor alloantigen.

A number of authors have discussed the mechanism of action of human suppressor T cells. Loertscher et al. [3] have reported that suppressor cells directly suppress the effects of IL-2, while Haisa et al. [4] have observed that their suppressor clone mediated suppression by inhibiting IL-2R expression. We have documented donor-specific MLR-, CML-suppressor T cells that suppressed IL-2 synthesis in a renal transplant recipient [5]. These results indicate that MLR-, CML-suppressor cells mediate suppression by inhibiting the IL-2 system. The fact that the suppressive effect of clone III-1-C5 was abolished by the addition of exogenous rIL-2 indicated that the principal mechanism of action of suppressor clone III-1-C5 is suppression of IL-2 synthesis.

Clone III-1-B6 has a unique character. It possesses CD3, CD4, Leu-M3, Hansen 20.2 surface antigens. As Leu-M3 antigen is thought to be a marker of monocytes, clone III-1-B6 has both T cell and monocyte antigens. The supernatant of clone III-1-B6 did not cause cell proliferation and clone III-1-B6 required cell-to-cell contact with responder cells to exhibit helper activity. This indicated that clone III-1-B6 does not indirectly augment cell proliferation by secreting cytokines, such as IL-2 but transmits signals for proliferation by direct contact with responder cells. These results are similar to the observation by Poo et al. [6] that CD4-bearing cells cause proliferation of B cells by direct interaction with adhesion molecule. Clone III-1-C5 failed to suppress mitogen responses that did not require antigen-presenting cells. Clone III-1-C5 suppressed the helper activity of clone III-1-B6 that requires cell-to-cell contact with responder cells. These results indicated the possibility that clone III-1-C5 suppresses proliferation by blocking adhesion molecules as well as inhibition of IL-2 synthesis. These results suggested that suppressor T cells mediate suppression by various mechanisms.

References

1. Lantz O, Alard M, Ben Aribia H, Chavanel G, Fries D, Senik A, Charpentier B (1990) Persistence of donor-specific IL-2-secreting cells and cytotoxic T lymphocyte precursors in human kidney transplant recipients evidenced by limiting dilution analysis. J Immunol 144:3748–3755
2. Yahata H, Fukuda I, Ishikawa T, Ono E, Asahara T, Dohi K (1989) Characterization of cloned human suppressor T cells generated from one-way MLC. Transplant Proc 21:224–227
3. Loertscher R, Williams JM, Guttmann RD, Strom TB (1987) Modification of the mixed lymphocyte reaction by alloactivated suppressor T lymphocytes and exogenous interleukin-2. Transplant Proc 14:3385–3387
4. Haisa M, Sakagami K, Kawamura K, Niguma T, Fujiwara T, Kusaka S, Matsuoka J, Shiozaki S, Fujiwara T, Onoda T, Orita K (1991) Induction of suppressor T cells by donor-specific blood transfusions: establishment of a human T-cell hybridoma producing an MLR suppressant factor. Transplant Proc 23:196–199
5. Okimoto T, Yahata H, Fukuda Y, Hayamizu K, Shimatani K, Dohi K (1993) Donor-specific suppressor T cells in a renal transplant recipient with a well-functioning kidney. Transplant Proc Jpn (in press)
6. Poo W-J, Conrad L, Janeway CA (1988) Receptor-directed focusing of lymphokine release by helper T cells. Nature 332:378–438