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Analysis of suppressor T cells induced by donor-specific transfusion (DST): establishment of a human T cell hybridoma producing an antigen-nonspecific suppressor factor

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Abstract. Formation of suppressor T cells (Ts) induced by donor-specific transfusion (DST) is one of the most commonly suggested mechanisms for the beneficial effect of DST. In this study, we established a human T cell hybridoma derived from the peripheral blood lymphocytes (PBL) of a DST-treated patient, which produced an antigen-nonspecific suppressor factor. Post-DST PBL were fused with an azaguanine-resistant mutant of a human T cell leukemia cell line, CCRF-CEM^{AG}. After selection and cloning, we established one clone producing the mixed lymphocyte reaction (MLR) inhibitory factor (C524: 18%–43% suppression). Suppressive activity of the supernatant obtained from C524 after activation by PHA was highly augmented (64%–88% MLR suppression). This factor inhibited MLR dose-dependently in an antigen-nonspecific and HLA non-restricted manner. These results indicated that Ts clones could be generated in patients receiving DST and that the immunoregulatory factors produced by activated clones may play a role in the prolongation of renal allograft survival.

Key words: Donor-specific transfusion – Renal transplantation – Suppressor T cells – Donor-specific transfusion – T cell hybridoma – Suppressor T cells

The beneficial effect of donor specific transfusion (DST) on the survival of one-haplotype-identical living-related transplants is well-established [12], but the mechanisms responsible for the DST effect remain obscure. In our laboratory we have been investigating the mechanisms of the DST effect in human renal transplantation by examining the induction of antiidiotypic antibodies and suppressor T cells (Ts) to inhibit donor-specific primary mixed lymphocyte reaction (MLR) after DST. Previously, our studies have shown that DST can induce antiidiotypic antibodies and/or Ts and the induction of these cells and antibodies correlates well with the reduction of rejection episodes and with better graft survival [4, 9, 13]. Recently, we have reported the establishment of a human-mouse hybridoma [7] and human T cell hybridoma [5, 6] derived from patients preconditioned with DST, both of which secreted donor-specific MLR inhibiting factors. The human-mouse hybridoma produced IgG antibody reacting with the T cell antigen-specific receptors. The supernatant of the T cell hybridoma cocultured with donor cells inhibited donor-specific MLR in a dose-dependent manner when added during the early phase of MLR.

In this study, to explore immunoregulatory factors produced by DST-induced Ts, we established a novel T cell hybridoma (termed C524) propagated from peripheral blood lymphocytes (PBL) of a patient receiving DST. The culture supernatant of C524 (C524, CS) inhibited MLR in an antigen-nonspecific and HLA non-restricted manner. Interestingly, the culture supernatant of C524, after activation by PHA-P for 3 days (PHA-activated C524, CS), showed a marked inhibitory effect on MLR in the same manner as C524, CS. PHA-activated C524, CS did not affect the kinetics of MLR and showed its suppressive effect only when added early in the culture.

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Materials and methods

DST protocol and human subjects. A patient was transfused on three occasions with 200 ml of fresh whole blood from the same donor at 2-week intervals. To prevent sensitization, azathioprine was given orally at a dose of 150 mg/day on the day before and on each day of DST [4]. The recipient's PBL, collected pre-DST and post-DST, were cryopreserved in liquid nitrogen until use. PBL of three healthy volunteers from our laboratory staff were used as a control. Serological HLA-phenotyping of the recipient, donor and all volunteers was performed using a standard antibody-dependent microcytotoxicity test. The recipient (EK) was HLA-A –; B35, w48; C w3; DR 4,7 and the donor (TK) was HLA-A 2, 31; B w48, w42; C –; DR 2,4. The healthy volunteers' HLA-phenotypes were as follows: TF (HLA-A 24, –; B44, w62; C w4; DR 5,7; DQ w6); MU (HLA-A24, w3; B w57 –; C w1, w3; DR w14, w52; DQ w2); SK (HLA-A2,3; B44, w48; C –; DR w15, w12; DQ w6).

Table 1. Inhibition of MLR by hybridoma supernatant

Responder[b]	Stimulator[b]	^{3}H-Thymidine incorporation (cpm)		
		Control (medium only)	Supernatant[a]	
			C524 (% suppression)	CEM (% suppression)
EK	TK	15331.7 ± 859.7	11257.0 ± 497.9* (26.5)	17145.7 ± 1648.8 (−12.1)
EK	TF	9533.5 ± 532.5	7055.7 ± 370.8* (26.0)	8330.3 ± 999.3 (12.6)
EK	SK	6641.7 ± 832.7	3748.3 ± 704.0** (43.6)	7859.6 ± 703.7 (−18.3)
TF	TK	31677.6 ± 2126.7	18137.5 ± 138.6* (42.6)	31381.8 ± 2992.5 (0.9)
MU	TK	27793.3 ± 1432.3	22796.5 ± 895.5** (18.0)	30803.7 ± 1654.6 (−10.8)
SK	TK	11322.2 ± 1041.8	9156.8 ± 620.8*** (19.1)	15320.7 ± 1538.9** (−35.3)
TF	MU	39745.1 ± 3258.8	22606.6 ± 883.8* (43.1)	35906.7 ± 1598.3 (9.7)
TF	SK	38161.7 ± 2415.6	26285.6 ± 1857.7** (31.1)	36440.3 ± 1937.3 (4.5)

[a] Fifty microliters of culture supernatant was added to the mixture (150 µl) of responder cells and stimulator cells
[b] HLA-typing of responder, stimulator cells were described iin Materials and methods section. EK is the recipient, FK is the donor and the others (TF, MU and SK) are controls
* $P < 0.001$ ** $P < 0.01$ *** $P < 0.05$

Table 2. Inhibition of MLR by PHA-activated C524 supernatant

Responder[b]	Stimulator[b]	3H-Thymidine incorporation (cpm)		
		Control (medium only)	Supernatant[a]	
			PHA-activated C524 (% suppression)	PHA-activated CEM (% suppression)
EK	TK	16978.2 ± 1240.3	6166.7 ± 258.2* (63.7)	17564.0 ± 1462.3 (−3.5)
EK	TF	8134.7 ± 1358.5	2928.7 ± 199.9* (64.0)	6236.1 ± 518.0 (23.3)
EK	MU	17251.6 ± 1694.9	3535.2 ± 160.6* (79.5)	15063.4 ± 2466.3 (12.7)
EK	SK	10446.0 ± 975.6	3494.2 ± 161.6* (66.5)	9994.2 ± 1041.0 (4.3)
TF	TK	27303.2 ± 1454.3	3748.8 ± 393.4* (86.3)	26692.6 ± 2256.3 (2.2)
MU	TK	13591.2 ± 1545.6	2507.3 ± 445.2* (81.6)	15276.8 ± 1286.3 (−12.4)
SK	TK	22323.4 ± 2200.4	4199.0 ± 123.2* (81.2)	20730.6 ± 828.5 (7.1)
TF	MU	20011.5 ± 2028.9	5095.1 ± 560.1* (74.5)	18252.4 ± 2241.5 (8.8)
TF	SK	29924.7 ± 3494.5	3600.6 ± 47.6* (88.0)	25822.6 ± 679.7 (13.7)

[a] Fifty microliters of PHA-activated C524 or CEM supernatant was added to the mixture (150 µl) of responder cells and stimulator cells
[b] EK is the recipient, FK is the donor, and the others are controls. Their HLA-typings were described in Materials and methods section
* $P < 0.001$

Cell fusion. PBL were isolated from heparinized venous blood by Ficoll-Conray density gradient centrifugation. Freshly prepared post-DST PBL were stimulated with mitomycin C (MMC)-treated donor PBL for 3 days. These allostimulated post-DST PBL were then hybridized with an azaguanine-resistant mutant of a human leukemic T-cell line, CCRF-CEM^{AG}, provided by DR Minowada (Hayashibara Biochemical Laboratories Inc., Okayama, Japan), in the presence of polyethylene glycol (m.w. 1450 BRL) according to a slightly modified technique of Okada et al. [11]. Fused cells were cultured overnight in a 24-well tissue plate (Falcon 3047; Becton Dickinson Labware, Oxnard, Calif.) in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with

10% heat-activated fetal calf serum (FCS). The culture medium was then replaced with hypoxanthine, aminopterin, and thymidine (HAT) medium. After 2 weeks, the HAT medium was replaced by RPMI 1640 medium containing 10% FCS, hypoxanthine, and thymidine for 1 week, and then the medium was switched to maintenance culture medium (RPMI 1640 medium plus 10% FCS without selection components). Supernatant fluids from cultures were filtrated through a 0.2 μm membrane and assayed for their suppressive activity in donor-specific primary MLR. Selected hybridomas were cloned using a limiting dilution method, and supernatant fluids were again tested for suppressive activity.

Inhibition of MLR by hybridoma supernatant. MLR was prepared in quaduplicate in 96-well, round-bottomed microculture plates in a final volume of 200 μl in RPMI 1640 HEPES medium (GIBCO) supplemented with 10% FCS. Were added 50 μl culture supernatants or control fluids to the mixture (150 μl) of 5×10^4 responder cells and 5×10^4 MMC-treated stimulator cells. Cultures were incubated for 5 days, pulsed with ^{3}H-thymidine (1 μCi/well) for 24 h, and harvested on glass fiber paper. Incorporation of ^{3}H-thymidine was measured with a β-scintillation counter, and expressed as mean counts per minute (cpm) ± SD. The percentage of suppression was calculated by the formula: [1 – (cpm in the presence of test supernatant/cpm in the presence of control medium)] × 100%.

Flow cytometric analysis of hybridoma cells. Hybridoma cells were immunostained with the following antibodies: Leu 2, Leu 3, Leu 4, Leu 8 and Leu 11 (Becton Dickinson, Mountainview, Calif.). Monoclonal antibodies were directly conjugated with fluorescein isothiocyate (FITC) or phycoerythin (PE). After staining, cell samples were analyzed using a FACscan (Becton Dickinson).

Preparation of PHA-activated hybridoma supernatant. Hybridoma cells were cultured at a density of 2×10^5/ml in the presence of PHA-P (10 μg/ml) for 3 days. Cells were then harvested, washed 3 times and resuspended with RPMI 1640 HEPES medium plus bovine albumin (10 mg/ml) (Sigma, Chemical Company, St. Louis, Mo.) at a density of 5×10^5/ml. After 48 h incubation at 37°C in 5% CO_2, cell free supernatants were filter-sterilized and stored at −37°C until use. The suppressive activity of PHA-activated hybridoma supernatant was also tested as described above.

The effect of PHA-activated hybridoma supernatants on spontaneous cytolysis. PHA-induced lymphoblasts were labeled with ^{51}Cr, suspended in culture medium, and distributed in 96-well, round-bottomed microculture plates (1×10^4/well). We added 100 μl PHA-activated hybridoma supernatant or control medium to the plates. Triplicate 100 μl aliquots of supernatant were harvested at the indicated times and ^{51}Cr release was determined using an auto-well gamma system. Maximum release was determined from ^{51}Cr labeled lymphoblasts exposed to 1*N* NaOH. Percentage cytolysis was calculated using the following formula: (experimental release/maximum release) × 100%.

Statistical analysis. Statistical analysis was performed by the paired *t*-test and $P < 0.05$ was considered significant.

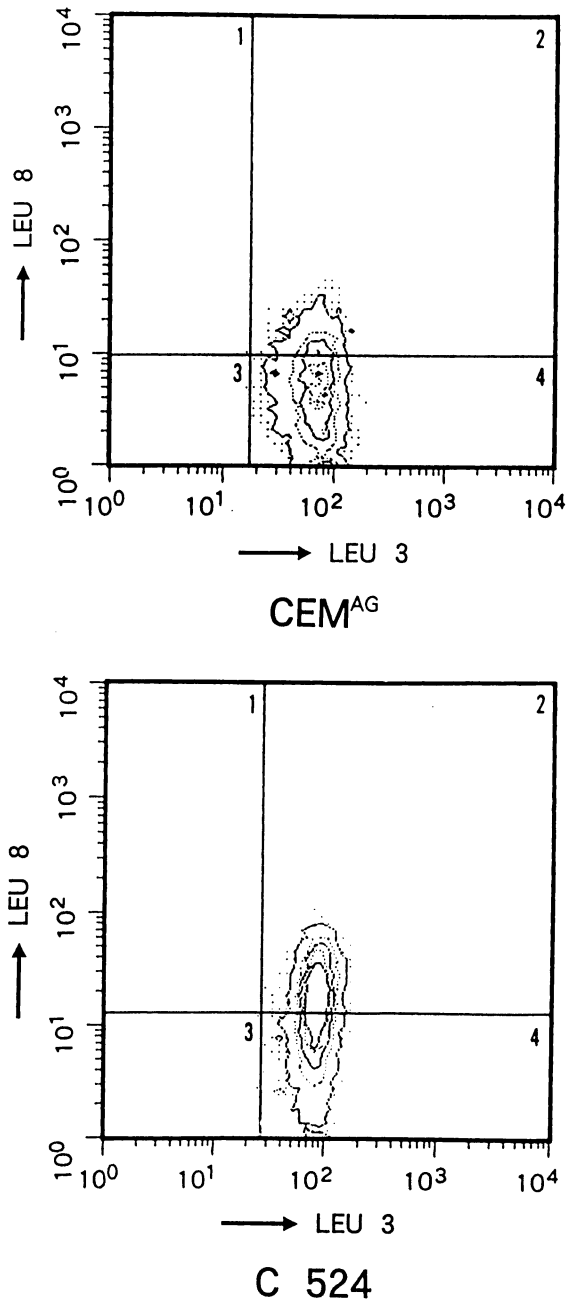

Fig. 1. Flow cytometric analysis of C524 clones: C524 and CCRF-CEM^{AG} cells were stained with FITC-conjugated Leu 3 antibody and PE-conjugated Leu 8 antibody and analyzed using a FACScan. Dot plots with markers set for control stained cells are shown

Results

Establishment of a human T-cell hybridoma

Following 3 weeks of culture, macroscopic evidence of hybridoma growth was observed in approximately 30% of the wells. After cloning, one clone producing MLR inhibiting factors was established (designated C524). This hybridoma has been stable in culture and has continuously produced MLR inhibiting factors for more than 6 months. As shown in Table 1, the addition of C524 culture supernatant (C524, CS) to donor-specific primary MLR resulted in a significantly reduced response (26.5% suppression, $P < 0.001$) relative to control cultures. Furthermore, C524, CS was able to inhibit significantly not only antigen-nonspecific MLR but also MLR between controls. Supernatant of CCRF-CEM^{AG} showed no inhibitory effect on any MLR.

Phenotypic analysis of C524 clone

C524 cells expressed Leu 4 and Leu 3 antigen on their cell surface, but were not stained with Leu 2 or Leu 11 antibodies (data not shown). The same findings were ob-

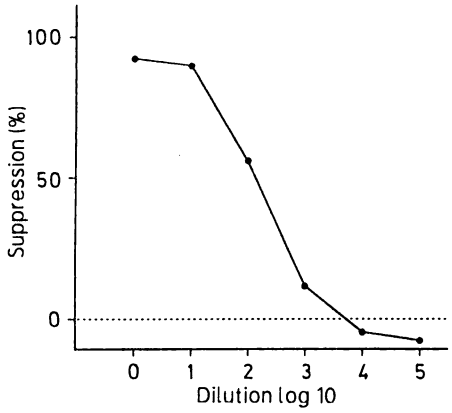

Fig. 2. Dose response of PHA-activated C524, CS to the percentage MLR suppression: Undiluted or 1:10 dilutions of PHA-activated C524, CS were added at 25% by volume to MLR culture (5×10^4 of TF-PBL and MMC-treated MU-PBL)

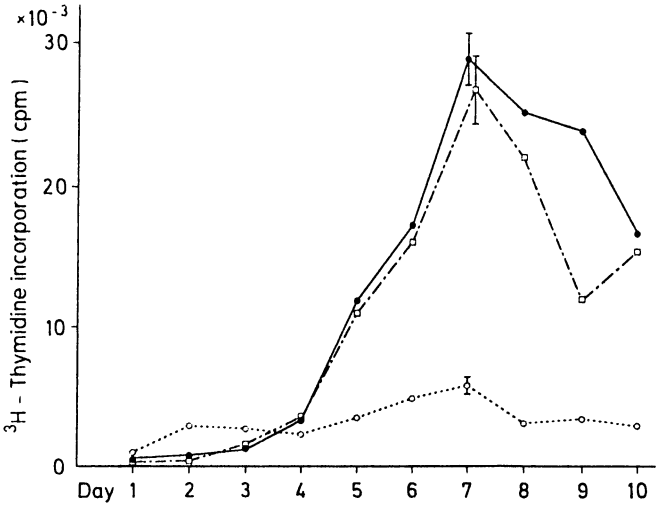

Fig. 3. Effect of PHA-activated C524, CS on the kinetics of MLR: 5×10^4 TF-PBL were cocultured with equal numbers of MU-PBL. Cells were harvested on the day indicated in medium only (●—●), with PHA-activated C524, CS (○---○) or with PHA-activated CCRF-CEM^{AG} (□---□). For graphical representation each point represents the mean of a quadruplicate sample. The standard deviation was calculated for each point. However, only the values on the day of maximal proliferation (day 7) are indicated for the sake of clarity

served on CCFR-CEM^{AG} cells. However, two-color analysis using FITC-conjugated Leu 3 and PE-conjugated Leu 8 showed that a higher expression of Leu 8 on C534 cells occurred compared with expression on CCRF-CEM^{AG} cells (Fig. 1).

Inhibition of MLR by PHA-activated C524 supernatant

To determine whether the suppressive activity of C524 was augmented after activation of mitogen, PHA-activated C524 culture supernatant (PHA-activated C524, CS) was prepared as described in Materials and methods section, and tested in MLR. Interestingly, a maked suppression of MLR was observed when PHA-activated C524, CS was added to MLR (64–88% suppression, $P < 0.001$). Suppression by PHA-activated C524, CS was nonspecific for stimulator alloantigens and was not HLA-restricted (Table 2). Therefore, MLR between controls (TF-PBL as responder and MU-PBL as stimulator cells) was performed in the following assay. The supernatant of PHA-activated CCRF-CEM^{AG} had little effect on MLR.

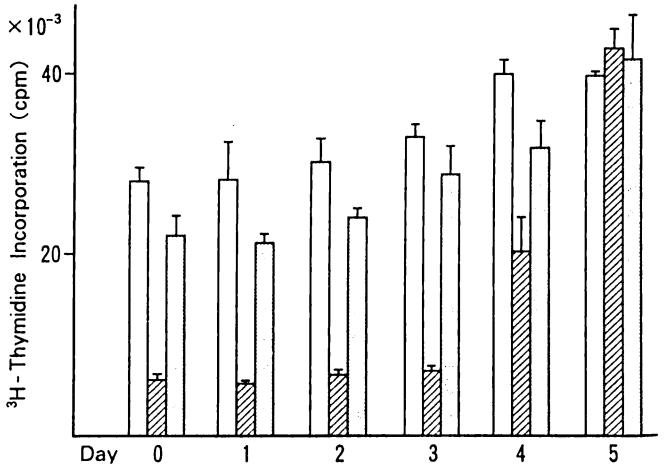

Fig. 4. Kinetics of the inhibitory effect of PHA-activated C524, CS on MLR: 5×10^4 TF-PBL was cocultured with MMC-treated MU-PBL for 6 days. On the days indicated, 50 µl medium (□), PHA-activated C524, CS (▨), or PHA-activated CCRF-CEM^{AG}, CS (□) were added to MLR

Serial 1:10 dilutions of PHA-activated C524, CS were added at 25% by volume to MLR. Figure 2 shows that suppressive activity of PHA-activated C524, CS was dose-dependent, with no suppression seen at dilutions greater than 1:100.

To determine whether PHA-activated C524, CS affected the kinetics of MLR, supernatants of PHA-activated C524, CCRF-CEM^{AG}, and control medium were added at the beginning to replicate MLR cultures which were harvested serially at 1–10 days. As shown in Fig. 3, PHA-activated C524, CS significantly inhibited MLR but failed to alter its kinetics.

Furthermore, MLR was suppressed when PHA-activated C524, CS was added between days 0–3, but its addition after day 4 resulted in little or no suppression (Fig. 4).

The effect of PHA-activated C524, CS on spontaneous cytolysis

To examine the cytotoxicity of PHA-activated C524, CS, it was added at 50% by volume to the incubation of PHA-induced lymphoblasts labeled with ^{51}Cr. Figure 5 shows that PHA-activated C524, CS did not affect spontaneous cytolysis of lymphoblasts during the observation period. Therefore, the suppression by PHA-activated C524, CS was not considered to be due to cytotoxic effects.

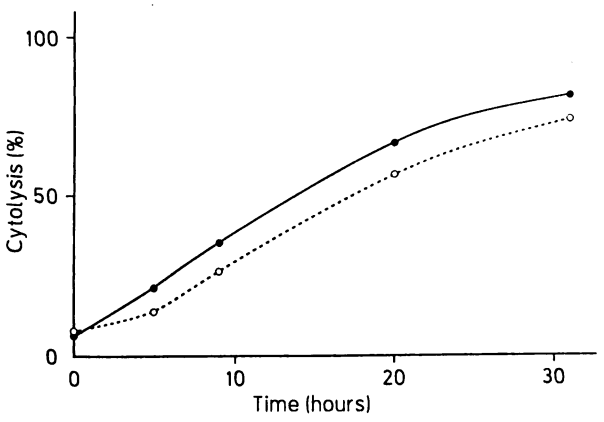

Fig. 5. The effect of PHA-activated C524, CS on spontaneous cytolysis: ^{51}Cr release of ^{51}Cr labeled lymphoblasts was measured at the indicated times in the absence (●—●) or the presence (o---o) of PHA-activated C524, CS (50% by volume). Percentage cytolysis was calculated using the formula described in Material and methods section

Discussion

DST has been used extensively prior to transplantation to improve the results of one-haplotype-identical living-related donor transplants. Recently Grailer et al. have reported the development of donor-specific cytotoxic T lymphocyte hyporesponsiveness obtained up to 2 years post-transplant in patients preconditioned with DST plus azathioprine and withdrawn from steroids 14 days following transplantation [3]. They also state that the DST effect might be most significant in the context of a reduction of immunosuppressive drug therapy.

Although several hypotheses have been proposed to explain the beneficial effect of DST, the exact mechanisms behind this effect is as yet unknown. In this study, we established a human T cell hybridoma, termed C524, producing MLR inhibiting factors by the fusion of post-DST PBL with an azaguanine-resistant mutant of a human leukemic T-cell line. The suppressive activity of the supernatant obtained from C524 was highly augmented after activation by PHA (PHA-activated C524, CS). This factor inhibited MLR dose-dependently in an antigen-nonspecific and HLA non-restricted manner. Furthermore, PHA-activated C524, CS did not affect the kinetics of MLR and showed its suppressive effect only when added early in the culture. Suppression by PHA-activated C524, CS was not considered to be due to cytotoxic effects since it did not influence spontaneous cytolysis of ^{51}Cr-labeled lymphoblasts. These results indicated that Ts clones could be induced by DST and that the immunoregulatory factors produced by activated clones may play a role in the DST effect.

Flow-cytometric analysis showed that C524 cells expressed Leu 3 and Leu 8 antigen on their cell surface. The subpopulation of T cells bearing Leu 3^+, 8^+ phenotype is reported by Mohagheghpour et al. to have suppressor inducer function [10]. They have demonstrated that the activation of Leu 2^+ suppressor effector cells generated in vitro is dependent on Leu 3^+, 8^+ cells. It is possible that suppressor effector cells are generated and inhibit proliterative responses to alloantigen when PHA-activated C524, CS is added early during the culture period.

Various antigen-nonspecific suppressor factors have been reported to inhibit the proliterative response of T cells [1, 2, 8, 14]. For example, Maki et al. have demonstrated that a murine T cell hybridoma produced "contra-IL 2", a suppressor lymphokine that inhibited interleukin 2 (IL-2) activity [8]. However, we did not observe that PHA-activated C524, CS directly inhibited IL-2 activity in the bioassay using IL-2 dependent cytotoxic T lymphocyte cell line (CTLL-2) (data not shown). In addition, PHA-activated C524, CS was thought to have the chemical properties of a protein since its suppressive activity was abolished after heat treatment or filtration, with a limiting core size of 10000 m.w. (data not shown), and thus was not likely to represent a low molecular-weight immunoregulatory substance such as prostaglandin [2, 14]. Emara and Sanfilippo have recently reported the soluble factor produced by suppressor cell line derived from a renal transplant recipient (TsEEF) [1]. TsEEF inhibited the generation of MLR and cytotoxic T cell responses, as well as mitogen-induced proliferative responses to PHA and PWM. The way in which TsEEF inhibited MLR seems to be the same as that of PHA-activated C524, CS. The other suppressive effects on the immune system of PHA-activated C524, CS and the mechanisms by which it exerts suppression are now being investigated.

Finally, although biochemical characterization and purification of the factor was not performed in this study, we consider that these approaches might be useful in explaining the mechanism of DST effect, and may also be helpful for in vivo preconditioning or immunosuppression for organ transplantation. More details and precise analysis of immunoregulatory factors produced by human suppressor clones will provide a means of applying these factors to clinical transplantation.

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