

Effects of Anti-Interleukin-2 Receptor Antibody and Cyclosporin A on Human T Cell Proliferation in Primary Mixed Lymphocyte Reaction

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ABSTRACT

Effects of anti-IL2 receptor antibody (anti-IL2 RAb) and cyclosporin A (CsA) on human T cell proliferation in primary mixed lymphocyte reaction (MLR) were investigated. Both agents inhibited the proliferative response induced by alloantigen in a dose dependent manner when they were added at the initiation of culture. We also analyzed the expression of activation antigen on responder cells and the kinetics of T lymphocyte subset proliferation during MLR using two-color flow cytometry. Cells expressing activation antigens, such as IL-2 receptor and HLA-DR, were found less frequently in anti-IL2 RAb-treated MLR culture (4.8%) than in CsA-treated MLR culture (16.5%) on day 6. Furthermore, proliferation of CD8⁺CD11⁻ cells, considered a cytotoxic T cell subset, were inhibited significantly more in anti-IL2 RAb-treated MLR culture than in CsA-treated MLR culture. We further demonstrate that CsA inhibits preferentially IL-2 production and anti-IL2 RAb inhibits T cell proliferation by blocking an absorption of IL-2 by activated lymphocytes. These data suggest that anti-IL2 RAb selectively inhibits alloantigen-activated T cells and may prove to be of significant value as an immunosuppressive agent in clinical organ transplantation.

Key words: *Anti-IL2 receptor antibody, Cyclosporin A, Mixed lymphocyte reaction, Two-color flow cytometry*

It is generally accepted that the T lymphocyte proliferative response to alloantigen requires a cascade of interactions between the antigen and monocytes and T cells, as well as lymphokine production, i.e., interleukin-1 (IL-1) and interleukin-2 (IL-2). IL-2, through binding to surface receptors of responding cells, induces cell cycle progression and T cell proliferation and IL-2-dependent effector activity, preventing graft rejection in clinical transplantation. It has been reported that the anti-IL-2 receptor antibody (anti-IL2 RAb) functionally blocked human IL-2 receptors and inhibited IL-2-dependent T cell proliferation^{3,6,15}. On the other hand, cyclosporin A (CsA) appears to inhibit the production of IL-2^{1,9,10}, including IL-2 messenger ribonucleic acid (mRNA) transcription¹². Although the effect of CsA on the expression on the IL-2 receptor is controversial, it has been demonstrated that CsA does not interfere directly with IL-2 receptor function¹⁰. In the present study, we analyzed the expression of activation antigens such as IL-2 receptor or HLA-DR and the proliferation of T lymphocyte subsets using two color flow cytometry. Furthermore, we investigat-

ed the effects of anti-IL2 RAb and CsA on the proliferative responses of allostimulated T lymphocytes in primary mixed lymphocyte reaction (MLR) and on IL-2 production.

MATERIALS AND METHODS

Cell preparation.

Peripheral blood mononuclear lymphocytes (PBL) were obtained from heparinized venous blood samples drawn from normal healthy donors. The PBL were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were resuspended at a density of 5×10^5 cells per ml in RPMI 1640 supplemented with L-glutamine (2.0 mM), 100 IU/ml of penicillin G, 100 μ g/ml streptomycin, and 10% heat-inactivated fetal calf serum.

Antibody and cyclosporin.

Anti-IL2 RAb was obtained from Becton Dickinson Immunocytometry Systems (Mountain View, CA). This antibody is derived from hybridization of mouse myeloma cell line NS-1 with spleen cells of BALB/c mice immunized with PHA activated human T cells. Immunoglobulin chain compositions are mouse IgG1 heavy chain and kappa light chains.

This antibody specifically reacts with human receptor for Interleukin-2 as well as the same molecule as anti-Tac-1. This antibody is supplied as 10 μg purified immunoglobulin in 2.0 ml (5 $\mu\text{g}/\text{ml}$) of phosphatebuffered saline containing 0.2% gelatin and 0.1% sodium azide. CsA was obtained from Sandoz Pharmaceuticals and was dissolved in absolute ethanol at 0.05 g/ml as a stock solution. Both agents were diluted to the appropriate concentration with RPMI 1640 medium and were added to the culture.

Culture and proliferation assays.

MLR was set up with 5×10^4 responding cells and 5×10^4 mitomycin C-treated stimulator cells in triplicate in 96-well round-bottom microtiter plates (Corning Glass Works, Corning, NY, U.S.A.) in the presence or absence of anti-IL2 RAb (0.000625 to 0.3 $\mu\text{g}/\text{ml}$) or CsA (0.05 to 2.5 $\mu\text{g}/\text{ml}$). Both agents were added at the initiation of culture in all cases. All cultures were incubated for 6 days at 37°C in 5% CO_2 in an air incubator, pulsed with 1 $\mu\text{Ci}/\text{well}$ of [^3H] thymidine (New England Nuclear, Boston, MA) during the terminal 18 to 24 hr of culture, cells were collected with a MASH multi-well harvester and incorporation of [^3H]TdR was assessed with a multi-channel liquid scintillation counter (Aloka, Tokyo, Japan). The results of triplicate cultures were obtained as counts per minute (cpm) \pm the standard deviation of mean. The percentage of suppression was calculated by the following formula.

$$\% \text{ suppression} = \left(1 - \frac{\text{cpm in experimental cultures with anti-IL2 RAb or CsA}}{\text{cpm in cultures with control medium alone}} \right) \times 100$$

Kinetic study.

Primary MLR was initiated at a 1:1 responder to stimulator ratio in 50 ml tissue culture flasks (Coster, Cambridge, MA) in the presence or absence of anti-IL2 RAb or CsA at 37°C and in 5% CO_2 . On days 1, 3, and 6 following the initiation of MLR, cultures were removed from the incubator. Cells were depleted by centrifugation on 30% percoll and the viable cells were prepared for cytofluorographic analysis.

Direct immunofluorescent staining of cells and analysis by fluorescence-activated cell sorter (FACS).

Cultures were harvested, and the cells were pooled and washed three times in RPMI 1640 medium. Direct immunofluorescence was performed by incubating 1×10^6 cells with a fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody at the appropriate dilution for 30 min at 4°C. Cells were washed twice, resuspended in 1 ml of staining buffer lysing reagent (NH_4Cl 8.3 g, KHCO_3 1.0 g, EDTA-4Na 37 mg, distilled water 1000 ml) and were examined using a Becton-

Dickinson FACS Analyser. OKT4 antibody has been shown to detect the helper-inducer phenotype and OKT8 antibody has been reported to react to the suppressor-cytotoxic phenotype (Ortho Diagnostics Co., NJ).

Two-color FACS systems analysis was utilized in these studies. Cells were double-labeled with the following strains of monoclonal antibody (MoAb) (CD4, CD8, DR; Becton Dickinson Monoclonal Center, Inc. CA) (CD11, T2H4; Coulter immunology, Hialeah, FL): CD4/DR (activated helper-inducer phenotype), CD8/DR (activated cytotoxic-suppressor phenotype), CD4/T2H4 (helper-inducer phenotype, $\text{CD4}^+\text{T2H4}^+$; suppressor inducer phenotype, $\text{CD4}^+\text{T2H4}^-$; helper phenotype), CD8/CD11 (cytotoxic-suppressor phenotype, $\text{CD8}^+\text{CD11}^+$; suppressor phenotype, $\text{CD8}^+\text{CD11}^-$; cytotoxic phenotype). The cells were resuspended in 50 ml of culture medium and then allowed to interact with FITC-conjugated MoAb and phycoerythrin-conjugated BoAb antibody. These were mixed gently and incubated for 1 h in an ice bath. Cells were washed three times in phosphate-buffered saline and resuspended in 1 ml of 1% formaldehyde solution for flow cytometry.

Assay of IL-2 activity.

A sample of supernatant fluids from these cultures was collected and IL-2 activity was determined in a standard bioassay by using the "IL-2-dependent" murine cytotoxic T cell line (CTLL)⁸.

RESULTS

Effect of anti-IL2 RAb and CsA on primary MLR.

Inhibition by anti-IL2 RAb and CsA in primary MLR was investigated. The data presented in Table 1 demonstrates that the addition of anti-IL2 RAb at the initiation of culture inhibited T cell proliferation in a dose-dependent manner. anti-IL2 RAb produced more than 50% inhibition of primary MLR at a concentration of 0.1 $\mu\text{g}/\text{ml}$. Table 2 presents representative data from several experiments in which the inhibitory effects of CsA on primary MLR were shown. CsA also showed dose-dependent inhibition of the proliferative response. Furthermore, we studied whether the addition of anti-IL2 RAb 0.1 $\mu\text{g}/\text{ml}$, which produced about 50% inhibition (Table 1), influenced inhibition by CsA. The addition of anti-IL2 RAb had no effect on T cell proliferation when MLR was set up in the presence of CsA at the highest doses (0.5 $\mu\text{g}/\text{ml}$), which inhibited proliferation by 90%. However, at the lower doses of CsA, it enhanced inhibition by CsA.

Kinetics of inhibition by anti-IL2 RAb on MLR-induced proliferation.

We also investigated the effect of delayed addition of anti-IL2 RAb on T cell proliferation occurring in cultures. As shown in Fig. 1, more than

Table 1. Anti-IL2 receptor antibody inhibitory activity in primary MLR^a

Anti-IL2 RAb concentration ($\mu\text{g/ml}$) ^b	[³ H] TdR uptake (cpm)		(% suppression)	
	Exp.1		Exp.2	
0.3	5622.4 \pm 279.7 ^c	(82.7)	10544.9 \pm 214.3	(75.3)
0.2	8007.3 \pm 950.6	(73.9)	14942.9 \pm 3397.8	(65.0)
0.1	10052.9 \pm 905.1	(67.2)	23683.4 \pm 2942.4	(44.5)
0.05	17259.9 \pm 1947.9	(43.7)	34956.6 \pm 6083.7	(18.1)
0.025	22537.3 \pm 878.1	(26.5)	45266.8 \pm 4345.6	
0.0125	24485.8 \pm 1856.1	(20.2)	46188.9 \pm 2935.9	
0.00625	27589.9 \pm 908.1	(10.0)	48250.8 \pm 5679.1	
0.2% gelatin containing control culture ^d	30667.4 \pm 1013.5		45679.2 \pm 3205.2	

^a: Fresh MLR were established, to which various concentration of anti-IL2 RAB were added initiation of culture.

^b: Anti-IL2 RAB was diluted with RPMI 1640 medium.

^c: Proliferation was assayed after 6 days incubation. [³H]TdR uptake was measured 18-24 hr pulse before the end of the incubation period.

Results represent mean cpm of triplicate cultures \pm SD.

^d: Control MLR was established, to which containing 0.2% gelatin was added.

Table 2. CsA inhibitory activity in primary MLR^a and effect of anti-IL2 receptor antibody addition

CsA concentration ($\mu\text{g/ml}$) ^b	[³ H] TdR uptake (cpm)		(% suppression)	
	CsA only		CsA + anti-IL2 RAB	
2.5	624.7 \pm 354.8 ^c	(96.9)	657.6 \pm 61.6	(96.9)
1.0	1579.7 \pm 551.8	(89.5)	767.2 \pm 280.8	(94.9)
0.5	1632.9 \pm 708.3	(89.2)	1680.1 \pm 207.1	(88.9)
0.1	5374.6 \pm 1277.6	(64.4)	1801.9 \pm 344.5	(88.1)
0.05	6580.4 \pm 764	(63.7)	1930.9 \pm 163	(87.8)
None	15100.9 \pm 1359.5		5528.2 \pm 248.4	(63.4)

^a: Fresh MLR were established, in the presence of either various concentration of CsA or CsA and anti-IL2 RAB (0.1 $\mu\text{g/ml}$) added at the initiation of culture.

Results of one typical experiment are presented.

^b: CsA was diluted with RPMI 1640 medium.

^c: Results represent mean cpm \pm SD of triplicate cultures. [³H] TdR uptake was measured at 6 days.

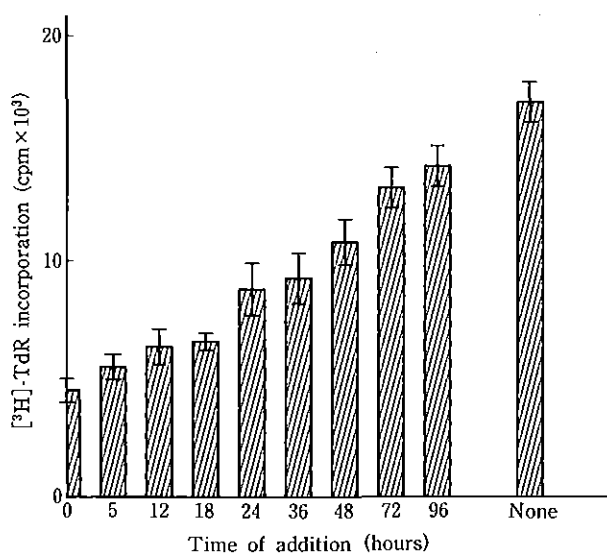


Fig. 1. Effect of delayed addition of anti-IL2 receptor antibody to MLR. Anti-IL2 RAB and added at different times after initiation of MLR as indicated on the abscissa. [³H] TdR incorporation was measured on day 6. MLR after 6 days in media only: 17112.5 \pm 2656.6 cpm.

60% inhibition occurred only when anti-IL2 RAB was added within the first 18 hr of the 6-day culture period. If the addition of the antibody to the MLR was delayed beyond this period, inhibition was less.

Effect of anti-IL2 RAB and CsA on the kinetics of lymphocyte proliferation and the expression of various surface antigens in MLR.

Using the concentration of anti-IL2 RAB or CsA that produced about 50% inhibition of MLR, we investigated the effects of both agents on the kinetics of lymphocyte proliferation and the expression of various surface antigens in MLR. The proliferative response increased with the progression of culture and reached a maximum on day 6 of culture. Both anti-IL2 RAB and CsA suppressed the proliferative response during the course of MLR (Table 3). Figures 2A, B and C illustrate representative data from several experiments in which cells expressing either the IL-2 receptor or HLA-DR were measured. Anti-IL2 RAB diminished levels of IL-2 receptor- and HLA-DR-positive cells by 80-85% compared to control MLR at 6 days, while suppression by CsA was 40-50%. The presence of

Table 3. Effect of anti-IL2 receptor antibody and CsA on the kinetics of T cell proliferation in MLR^a

	Anti-IL2R Ab	CsA	[³ H] TdR uptake (cpm) ^b		(% suppression)
			Day 1	Day 3	Day 6
Exp. 1	—	—	1169.6 ± 151.5	10796.1 ± 914.1	40560.5 ± 3434.2
	+	—	1201.6 ± 315.9	4902.7 ± 284.0	4520.6 ± 452.6 (88.9)
	—	+	881.3 ± 230.1	2724.4 ± 607.6	5744.5 ± 1176.0 (83.4)
Exp. 2	—	—	720.7 ± 83.9	1582.7 ± 389.9	28643.3 ± 1986.5
	+	—	454.2 ± 69.1	492.7 ± 108.4	730.1 ± 173.4 (97.5)
	—	+	453.4 ± 33.9	1125.0 ± 679.0	1837.4 ± 298.4 (93.6)

^a: 5×10^4 PBL were mixed with 5×10^4 mitomycin C-treated allogeneic PBL and cultured with 0.1 μ g/ml anti-IL2 RAb or 0.1 μ g/ml CsA for 24h to 6 days. 18-24h before termination of culture, each well was pulsed with 1.0 μ Ci of [³H] thymidine.

^b: Data represents mean cpm ± standard deviation of quadruplicate samples.

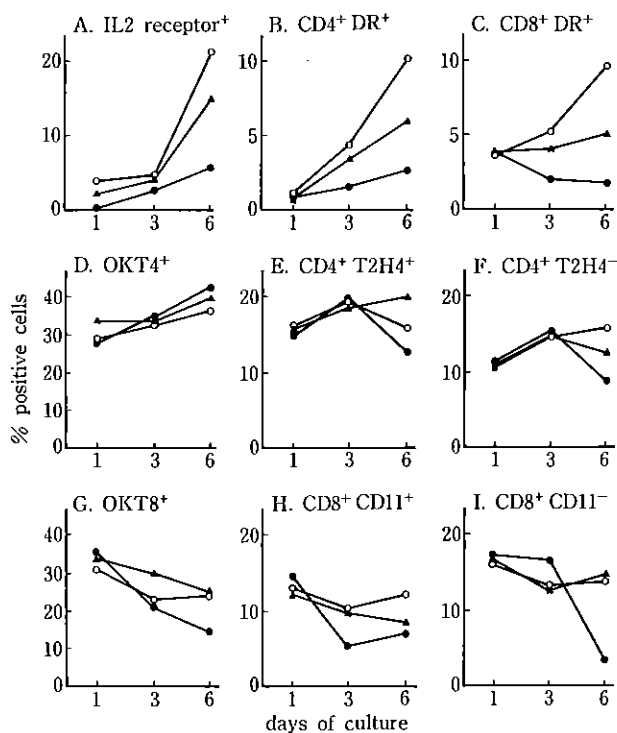


Fig. 2. Differential effect of anti-IL2 receptor antibody and CsA on kinetics of activation antigen expression and T lymphocytes subpopulations proliferation in MLR. MLR flask cultures were prepared in media only (○), anti-IL2 RAb 0.1 μ g/ml (●) and CsA 0.1 μ g/ml (▲) and incubated for 24-144 h. Cells were washed, dead cells sedimented and analyzed by flow cytometry. Data shown here are representative of three experiments and express percentage.

anti-IL2 RAb resulted in stronger inhibition than with CsA on the activation of T lymphocytes as indicated by expression of activation antigens.

Differential effect of anti-IL2 RAb and CsA on T lymphocyte subsets.

OKT4-positive cells (helper phenotype) expanded during the time elapsed in all cultures (Fig. 2D). Further, two-color flow cytometry permitted clear analysis of functional T lymphocytes subsets. Hence, CsA tended to increase the percentage of CD4⁺T2H4⁺ cells but inhibited the increase in

Table 4. Effect of anti-IL2 receptor antibody and CsA on IL-2 production in MLR^a

Supernatants ^b	IL2 (U/ml)		
	Day 1	Day 3	Day 6
Control MLR culture	0	11.5	4.5
+ anti-IL2 RAb	0.2	6.1	8.3
+ CsA	0	0.1	0

^a: Bulk MLR were established, to which 0.1 μ g/ml concentration of anti-IL2 RAb or CsA were added at the initiation of culture.

^b: The supernatants obtained on day 1, 3, 6 of culture were harvested and tested for IL-2 activity. This experiment was performed three times with similar results.

CD4⁺T2H4⁻ cells. In contrast, anti-IL2 RAb tended to inhibit both cell types (Fig. 2E and F). It is also noteworthy that anti-IL2 RAb inhibited the CD8 lineage, especially the induction of CD8⁺CD11⁻ cells, considered to be cytotoxic T cells, to a greater extent than CsA did (Fig. 2I).

Effect of anti-IL2 RAb and CsA on IL-2 production.

IL-2 activity was assessed in these cultures and the results are shown in Table 4. Anti-IL2 RAb didn't inhibit IL-2 activity in the presence of anti-IL2 RAb, and it became greater as progression of MLR culture. In contrast, CsA inhibited IL-2 activity almost completely.

DISCUSSION

Anti-IL2 receptor antibody and CsA are known to exert immunosuppressive effects on T cell proliferation induced by lectin and alloantigen^{1,3,6,9,12,15}. We observed inhibition by these agents of T cell proliferation in a dose-dependent manner in primary MLR. This effect of anti-IL2 RAb was demonstrated only when it was added to the culture within 18 h after initiation of the culture. CsA was reported to have an inhibitory effect only during the early inductive phase of the cell cycle^{1,9}. Our results indicate that IL-2-IL-2 receptor interaction occurs at an early phase in the immune response. In MLR, activated lymphocytes

express activation antigen on their surface. IL-2 receptor is expressed on the surface of activated T cells before the onset of DNA synthesis, and HLA-DR antigen is expressed after DNA synthesis⁹. In the present study, we observed that anti-IL2 RAb and CsA have different effects on distinct steps during the T cell activation process: anti-IL2 RAb reduced the levels of IL-2 receptor and HLA-DR expression on both CD4⁺ and CD8⁺ subsets by 80-85%, while CsA reduced the levels of IL-2 receptor and HLA-DR expression by only 40-50%. As to the expression of the activation antigen, anti-IL2 RAb exhibited preferential inhibition to CsA in both T cell subsets. Hence, this low dose of ethanol in which CsA was dissolved had no effect on MLR response.

In addition, we examined the effects of both agents on the kinetics of functional T lymphocyte subsets. Anti-IL2 RAb markedly inhibited CD4⁺T2H4⁻ cells, considered to recognize helper function, and CD8⁺CD11⁻ cells, considered to recognize cytotoxic function. Consistent with the effect of CsA on the T4 subset suggested by previous investigators^{7,11}, we also observed inhibition by CsA of surface marker expression on helper T cells. We showed in this report that anti-IL2 RAb preferentially inhibits proliferation of helper and cytotoxic T cells, which are IL-2-dependent, compared with CsA. Proliferation of suppressor T cells (CD8⁺CD11⁺ cells) also was inhibited by both agents less than proliferation of the helper-cytotoxic lineage. This finding is consistent with that of Schneider et al¹³, who suggested that the suppressor cells are inhibited less than cytotoxic cells by anti-IL2 RAb. Our results are rather favorable in terms of preventing transplant rejection, because development of antigen-specific suppressor T cell plays an important role in the prolongation of allograft survival and in the reversal of the acute rejection process². Furthermore anti-IL2 RAb did not inhibit IL-2 production and IL-2 was gradually accumulated during 6 days of culture, because of blocking of absorption of IL-2 by IL-2 receptor bearing cells. On the other hand, CsA inhibited IL-2 production almost completely.

In summary, our studies indicate that anti-IL2 RAb and CsA can function by interfering with distinct steps in the T cell activation process. Anti-IL2 dependent immune responses act selectively at the receptor stage and should be more effective in reducing T lymphocyte activation and helper-cytotoxic pathway than CsA. From this study we suggest that not only CsA, but also anti-IL2 RAb may possess valuable immunosuppressive potential in clinical transplantation.

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