Transpl Int (2003) 16: 108–114
DOI 10.1007/s00147-002-0481-2

ORIGINAL ARTICLE

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The effects of immunosuppressants on FAS-mediated activation-induced cell death in human T lymphocytes

Received: 9 October 2001
Revised: 5 July 2002
Accepted: 8 July 2002
Published online: 17 December 2002

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Abstract The effects of cyclosporin A (CsA) and methylprednisolone (MP) on Fas-mediated activation-induced cell death (FMAICD) of T lymphocytes were examined. T lymphocytes were activated with the immobilized anti-CD 3 and CD 28 monoclonal antibodies (MoAbs) (activation phase) and incubated further with the agonistic MoAb against Fas (death phase). Cell proliferation and DNA fragmentation were measured by XTT and diphenylamine assay. CsA in the activation phase inhibited DNA fragmentation mediated by anti-Fas MoAb but not MP. The combination of CsA and MP at the lower concentrations had little effect on FMAICD, although they had similar degrees of suppression on T lymphocyte proliferation as the maximum obtained by CsA or MP alone. In the death phase, MP induced apoptosis without 7C11 and CsA had no effects. These results indicate that the combination of CsA and MP at low concentrations could maintain FMAICD with the suppression on T lymphocyte proliferation.

Keywords Activation-induced cell death · Fas · Cyclosporin A · Methylprednisolone · Human T lymphocyte

Introduction

Programmed cell death, or apoptosis, is an important regulatory mechanism in the peripheral immune system, as well as the deletion of self-reactive clones in the thymus. One of the mechanisms mediating peripheral immune homeostasis is an autocrine feedback death of clonally expanded T cells by the process of apoptosis [10]. Although freshly isolated T cells are largely resistant to apoptosis, T cells stimulated via the T-cell receptors (TCRs) not only proliferate but also undergo subsequent apoptosis by an active process termed "activation-induced cell death" (AICD) [14, 20]. Much evidence indicates the implication of AICD in the immune responses against alloantigens. Recently, studies have been accumulating, reporting that AICD can play a role in the clonal deletion or in the reduction of the allo-specific clonal size in the induction of transplantation tolerance [3, 11, 15, 19, 22]. Surprisingly, some reports demonstrated that cyclosporin A (CsA)

or steroids, used widely in clinical transplantation, broke down the transplantation tolerance by interfering with AICD [15, 19].

The Fas (CD95)/Fas ligand (Fas-L) pathway has been shown to be a key regulator of apoptosis and also a major AICD mediator of T cells [1, 2, 6]. Fas is constitutively expressed on multiple hemopoietic and non-hemopoietic cells and induced on T cells following activation [17]. Various kinds of cells expressing equivalent cell surface amounts of Fas antigens (Ag) show varying sensitivity to Fas/Fas L-mediated apoptosis [4]. It means that the Fas/Fas-L pathway is regulated by a number of implicating factors. The results of experiments examining the effects of the immunosuppressants on T-cell apoptosis are still inconclusive. The various results have been elucidated according to the cell types employed and the experimental systems [5, 8, 12, 16, 23, 24, 25, 26].

Given the above considerations, we aimed to examine the effects of the representative immunosuppressive drugs, CsA and methylprednisolone (MP), on Fas-mediated AICD (FMAICD) of human T cells. T cells isolated from peripheral blood were activated with anti-CD3 and anti-CD28 monoclonal antibodies (MoAbs), because this system provided the stimulation for T cells via the TCR/CD3 complex and co-stimulatory signals. Following the activation phase, the cells were further cultured in the presence of the agonistic MoAb against human Fas Ag (death phase), and DNA fragmentation of the cells were measured. The addition of CsA in the activation phase inhibited FMAICD. MP, on the other hand, had little effect or slightly augmented DNA fragmentation of T cells. MP induced apoptosis on activated T cells but not CsA in the death phase. The simultaneous addition of CsA and MP at lower concentrations had little effect on FMAICD in the activation phase, but caused a suppression of T-cell proliferation equivalent to that at the high concentration of CsA or MP alone. The significance of these results on clinical transplantation will be discussed below.

Materials and methods

Cells

Heparinized peripheral blood was obtained from healthy volunteers, and the mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway). The mononuclear cells were washed with PBS and re-suspended in RPMI 1640 medium (Sigma Chemical Co., St Louis, USA) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (complete medium). These cells were depleted of monocytes during 1-h incubation in plastic Petri dishes. T cell-rich populations were obtained by nylon-wool column elusion of the non-adherent cells. Flow cytometric analysis revealed that more than 95% of the cells obtained were CD3 positive.

Reagents and MoAbs

CsA was purchased from Novartis Pharma K.K. (Basle, Switzerland) and MP, Solu-Medrol from Pharmacia K.K. (Tokyo, Japan). Orthoclone OKT3 (anti-CD3; IgG2a) was purchased from Janssen-Kyowa (Tokyo, Japan). CD28.2 (anti-CD28; IgG1) and 7C11 (anti-CD95; IgM) were purchased from Immunotech (Marseilles, France). All other materials were of the highest grade commercially available.

T-cell proliferation

T cells (1×10^5/well) were cultured with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) MoAbs in flat-bottomed, 96-well microplates for a final volume of 200 μl complete medium. Preliminary experiments determined the optimal concentrations of anti-CD3 and CD28 MoAbs for maximal T-cell proliferation. All experiments were performed in triplicate cultures. The viable cells were measured with the Cell Proliferation Kit (XTT) (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after incubation for the indicated days, 100 μl media were removed, and 50 μl XTT labeling mixture was added to each well. After overnight incubation at 37 °C, the ODs were measured at 450 nm. The proliferation index (PI) was calculated with the following formula:

$$\text{PI} = \text{mean OD with the immobilized MoAbs} / \text{mean OD without the MoAbs}$$

When CsA and/or MP was added to the wells at the beginning of culture, percent suppression was defined according to the following formula:

$$\%\ \text{suppression} = 100 \times [1 - (\text{PI in the presence of the immunosuppressant} / \text{PI in the absence of the immunosuppressant})].$$

Experimental scheme of FMAICD

T cells (1×10^6/well) were activated with immobilized anti-CD3 and CD28 in 24-well plates for the days indicated (activation phase). The T-cell cultures in the activation phase were performed in the presence or absence of CsA and/or MP added at the beginning of culture period. Following the activation phase, the cells were harvested, washed with PBS, and re-suspended in complete medium. They were further incubated at a density of 2×10^6/well in 12-well plates in the presence of the agonistic MoAb against human Fas, 7C11at 37 °C, for 24 h (death phase). In some experiments, CsA or MP was added to the cultures in the death phase, in the presence or absence of 7C11. Thereafter, DNA fragmentation of the cells was measured by the diphenylamine assay described below.

DNA fragmentation assay

The extent of DNA fragmentation was determined by spectrophotometric assay using diphenylamine as described previously [21]. Briefly, the cells (1.5×10^6) were harvested and washed with PBS. The pellet was lysed in 150 μl lysis buffer (10 mM Tri-HCl, pH 7.4, 10 mM EDTA and 0.5% Triton X-100) on ice for 20 min. The lysate was centrifuged at 15,000 *g* for 20 min to separate intact and fragmented chromatin. Both pellet and supernatant were precipitated at 4 °C for more than 30 min with 6% perchloric acid. The precipitates were sedimented at 15,000 *g* and 4 °C for 20 min.

The DNA precipitates were heated at 70 °C for 20 min in 50 μl of 6% perchloric acid and mixed with 100 μl of diphenylamine solution (1.5% (w/v) diphenylamine, 1.5% sulfuric acid and 0.01% acetaldehyde in glacial acetic acid). After overnight incubation, both ODs were determined at 600 nm. The percentage of DNA fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA.

Statistical analyses

We performed statistical analyses using the paired *t*-test, and *P* values of less than 0.05 were considered to be significant.

Results

Inhibitory effects of CsA and/or MP on T-cell proliferation

The kinetics study of T-cell proliferation revealed that the maximum PI was observed on day 4 of culture (data not shown). The addition of CsA or MP at the beginning of culture inhibited the PI of T cells in a dose-dependent manner; 100 ng/ml CsA or 100 μg/ml MP inhibited T-cell proliferation induced with the anti-CD3 and CD28 MoAbs with more than 70% suppression. Figure 1 shows the representative result of the experiments. As shown, the synergistic effects between CsA and MP were observed in the suppression of T-cell proliferation. The inhibition of the PI by the combination of 1 ng/ml CsA and 10 μg/ml MP or 10 ng/ml CsA and 1 μg/ml MP was approximately equal to that produced by the high concentration of CsA (100 ng/ml) or MP (100 μg/ml) alone.

FMAICD of T cells

The activated T cells, harvested during days 6–8 of culture with the immobilized anti-CD3 and CD28

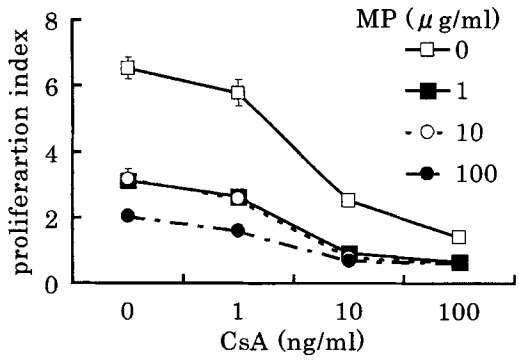

Fig. 1 Inhibitory effects of CsA and/or MP on T-cell proliferation. T cells (1×10^5) were cultured with the immobilized anti-CD3 and CD28 in the presence of CsA and/or MP. The PI was calculated according to the formula in the Materials and methods section

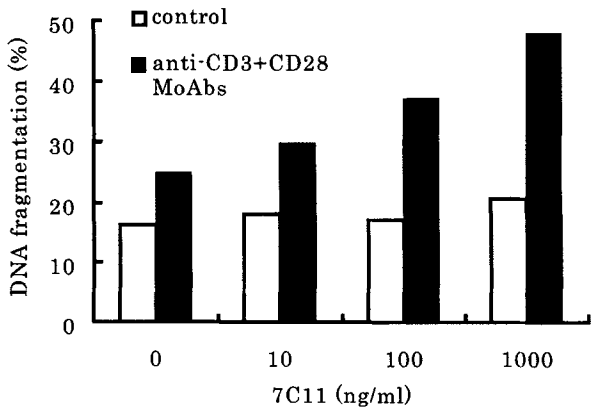

Fig. 2 FAICD of T cells. The activated T cells with (*closed bar*) or without (*open bar*) the immobilized anti-CD3 and CD28 were harvested on day 6 of culture. The cells were further incubated for 24 h in the presence of 7C11. Thereafter, % DNA fragmentation of the cells was determined by diphenylamine assay

MoAbs, were sensitive to apoptosis induced by the agonistic anti-Fas MoAb, 7C11 (data not shown). Figure 2 shows the representative results on the harvested cells after the 6-day culture. 7C11 induced DNA fragmentation on the activated T cells with the MoAbs dose-dependently, but not on the T cells cultured without the MoAbs. In the absence of 7C11 in the death phase, the percentage of DNA fragmentation of the activated T cells with the anti-CD 3 and CD28 was slightly greater than that of the not-activated cells. Although there were some variances within the experiments, the addition of 1 μg/ml 7C11 in the death phase induced DNA fragmentation of the activated T cells significantly, compared with that in the absence of 7C11 (% DNA fragmentation in the presence of 1 μg/ml 7C11 vs that in the absence of 7C11; 43.7 ± 9.5 vs 24.9 ± 8.6, $P<0.0001$ ($n=17$)). The difference between DNA fragmentation of the harvested cells following the 6-day culture in the presence and absence of 7C11 was more than that of the cells after the 8-day culture (data not shown). Therefore, we employed the activated T cells harvested on day 6 of culture in the following experiment. The DNA fragmentation of freshly isolated T cells was less than 10% in both the presence and the absence of 7C11 (data not shown).

Effects of CsA and of MP on FMAICD in the activation phase

In order to examine the effects of CsA and/or MP, which have immunosuppressive activity on T cells, on FMAICD, we used concentrations similar to those employed in the inhibition experiment of T-cell proliferation (Fig. 1). At first, the effects of the addition in the activation phase were investigated. The T cells cultured

with the MoAbs in the presence of CsA or MP were harvested on day 6 of culture, and were further incubated in the presence of 1 μg/ml 7C11. The effects of CsA or MP on DNA fragmentation of the T cells are shown in Fig. 3. The addition of CsA in the activation phase inhibited DNA fragmentation induced by 7C11, depending on the dose applied. On the other hand, MP had little effect on 7C11-mediated DNA fragmentation, and 100 μg/ml MP even augmented it slightly. It was noticeable that CsA or MP inhibited T-cell proliferation by the immobilized anti-CD3 and CD28 MoAbs approximately equally, but exhibited the diverse effects on DNA fragmentation induced by 7C11.

Effects of CsA or MP on FMAICD in the death phase

The activated T cells following the 6-day culture with the MoAbs were harvested and incubated with 7C11 (1 μg/ml) in the presence or absence of CsA or MP for 24 h. As shown in Fig. 4a, the addition of CsA in the death phase had no effect on DNA fragmentation. MP induced DNA fragmentation on the activated T cells in the absence of 7C11, but did not interfere with the 7C11-mediated DNA fragmentation, as shown in Fig. 4b.

Effects of the combination of CsA and MP on FMAICD

Since the inhibitory effects of the combination of CsA and MP at the low concentrations on T-cell proliferation were almost equal to those of high concentration of CsA or MP alone (Fig. 1), we investigated the effects of their simultaneous addition in the activation phase on FMAICD. Following the 6-day culture with the MoAbs in the presence of CsA and/or MP, the harvested T cells were incubated in the presence of 7C11 for 24 h, and DNA fragmentation of the cells was analyzed. The simultaneous addition of 1 μg/ml MP only slightly, but not significantly, augmented the DNA fragmentation of the harvested cells at the indicated concentrations (0.1 to 10 ng/ml) of CsA addition (Fig. 5a). Conversely, 1 ng/ml CsA inhibited very slightly the DNA fragmentation of the cells harvested from the activation culture in the presence of the indicated concentrations (0.1 to 10 μg/ml) MP (Fig. 5b).

The inhibitory effects on T-cell proliferation and the effects on FMAICD of CsA, MP or their combination are summarized in Table 1. The percentages of suppression on T-cell proliferation of four groups (100 ng/ml CsA, 100 μg/ml MP, 1 ng/ml CsA + 10 μg/ml MP and 10 ng/ml CsA + 1 μg/ml MP) were approximately

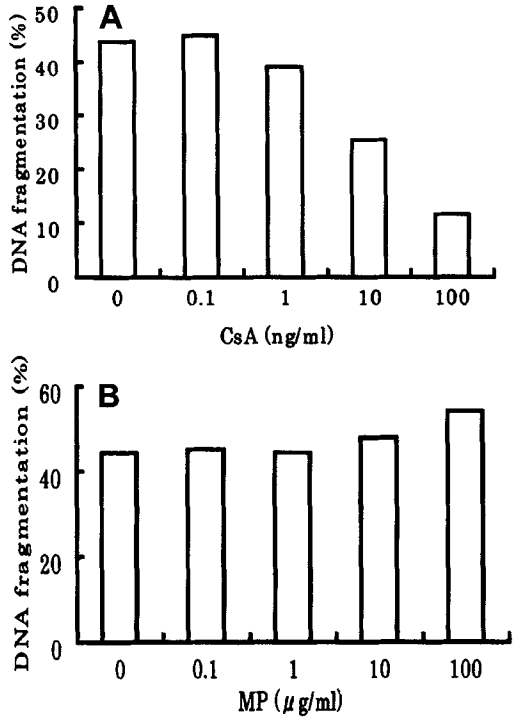

Fig. 3 Effects of CsA or MP on FMAICD in the activation phase. The T cells after 6-day culture with the MoAbs in the presence of CsA or MP were harvested and further incubated in the presence of 7C11. **A** CsA addition in the activation phase, **B** MP addition in the activation phase

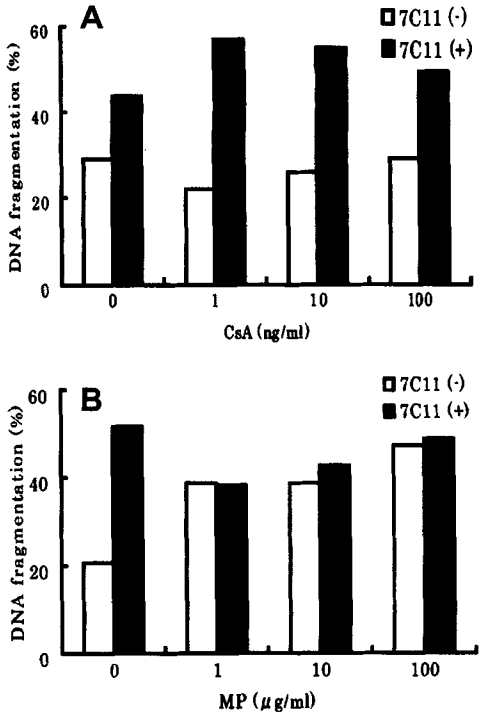

Fig. 4 Effects of CsA or MP on FMAICD in the death phase. The activated T cells after 6-day culture with the MoAbs were harvested and then incubated in the presence of CsA (**A**) or MP (**B**) with (*closed bar*) or without (*open bar*) 7C11

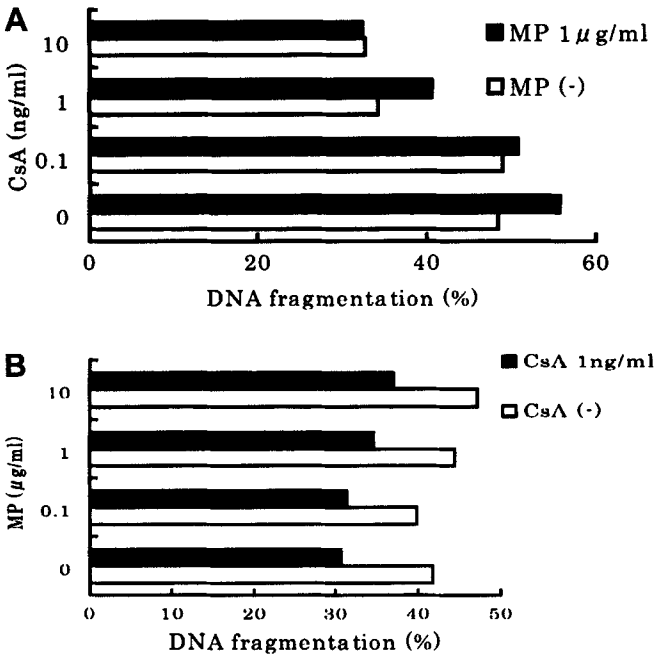

Fig. 5 Effects of the combination of CsA and MP on FMAICD in the activation phase. The T cells after 6-day culture with the MoAbs in the presence of the combination of CsA and MP were harvested and further incubated in the presence of 7C11. **A** The addition of the indicated concentrations of CsA with (*closed bar*) or without (*open bar*) 1 μg/ml MP in the activation phase. **B** The addition of the indicated concentrations of MP with (*closed bar*) or without (*open bar*) 1 ng/ml CsA in the activation phase

comparable. The percentages of DNA fragmentation of the combination of CsA and MP were slightly low, compared with the control, but higher than that of 100 ng/ml CsA.

Discussion

The data reported in this study demonstrated the following three striking findings:

1. CsA inhibited effectively both T-cell proliferation induced by the immobilized anti-CD3 plus CD28 and the following DNA fragmentation mediated by the agonistic MoAb against human Fas, 7C11.
2. MP had little effect on FMAICD in the activation phase but induced DNA fragmentation on the activated T cells as well as 7C11 in the death phase.
3. The combination of CsA and MP at low concentrations inhibited only slightly FMAICD in the activation phase, while keeping the suppressive activity on T-cell proliferation.

These results, combined, suggest that CsA might inhibit FMAICD, while MP could induce apoptosis in human T cells. It is speculated that the suppression by CsA of FMAICD may be, at least in part, due to lack or shortage of interleukin-2 (IL-2). IL-2 is reported to predispose T cells to apoptosis [9, 10]. Our preliminary experiments indicated that the CsA suppression of FMAICD could be restored fully by the simultaneous addition of recombinant IL-2 in the activation phase (data not shown). MP is known to have suppressive effects on T-cell function via various mechanisms, including the inhibition of cytokine production. In addition, MP itself has been reported to have the ability to induce T-cell apoptosis [5, 8, 12]. The addition of MP in the death phase induced DNA fragmentation of the activated T cells. The neither synergistic nor additive effect between MP and 7C11 was observed in the death phase, because 100 μg/ml MP or 1 μg/ml 7C11 might induce the maximal level of DNA fragmentation of the activated T cells with the anti-CD3 and CD28 MoAbs. The relationship between MP- and 7C11-mediated apoptosis pathways could not be elucidated in this study.

There exist contradictions regarding the effects of CsA or steroids on AICD of T cells. Although many papers demonstrated that CsA inhibited AICD [16, 23, 24] and steroids could induce apoptosis of T cells, contrary results were also reported [5, 18, 26]. Interestingly, there are also studies reporting the diverse effects of exogenous IL-2 on T-cell apoptosis. IL-2 is generally considered to make activating T cells become susceptible to apoptosis [9, 10], however, Mor and Cohen demonstrated that IL-2 could rescue antigen-specific T cells

Table 1 The effect of CsA, MP or their combination on proliferation and Fas-mediated DNA fragmentation

	Control (no addition)	CSA 100 ng/ml	MP 100 μg/ml	CsA 1 ng/ml + MP 10 μg/ml	CsA 10 ng/ml + MP 1 μg/ml
Suppression (%)[a]	–	73.8 ± 9.7 ($n=7$)	71.3 ± 7.3 ($n=7$)	70.0 ± 5.5 ($n=4$)	76.2 ± 2.1 ($n=4$)
DNA fragmentation (%)[b]	43.7 ± 9.5 ($n=17$)	18.5 ± 5.1* ($n=4$)	49.3 ± 6.4 ($n=5$)	34.4 ± 7.8 ($n=4$)	72.0 ± 8.5 ($n=4$)

The number of experiments is given in parentheses
*$P < 0.001$ difference from the corresponding value of the control
[a]T cells were cultured with the immobilized anti-CD3 and CD28 MoAbs in the presence or absence of CsA and/or MP. Percentage suppression of T-cell proliferation was calculated according the formula in the Materials and methods section
[b]The T cells after 6-day culture with the MoAbs in the presence or absence of CsA and/or MP were harvested and incubated further in the presence of 7C11. DNA fragmentation of the cells was determined by the diphenylamine assay

from radiation or dexamethasone-induced apoptosis by inducing *bcl*-2 [12]. The situation at present seems to be very complicated. In the present study we used human T cells isolated from peripheral blood and stimulated them with the immobilized anti-CD3 and CD28 for the activation via both TCR/CD3 complex-mediated and co-stimulatory signals. The agonistic MoAb against Fas, 711, was added to the culture of the activated cells to determine the effects of CsA and/or MP on FMAICD. Repeatedly, in this system, CsA inhibited FMAICD but MP had little influence on the activation phase addition. MP could induce DNA fragmentation on the activated T cells but not CsA in the death phase.

Recently, more attention has been focused on the implications of FMAICD in organ transplantation, especially in tolerance induction [3, 11, 15, 19, 22]. The clonal deletion or reduction of the clonal size of allo-reactive T cells is thought to be mainly dependent on FMAICD. Li et al. reported that, in mice, cardiac transplantation tolerance induced by the blocking of both CD28-B7 and CD40-CD40 ligand interactions using the specific MoAbs was broken down by treatment with CsA [11]. Sharland et al. demonstrated that spontaneous tolerance of PVG to DA rat liver transplantation was prevented by peri-transplant administration of MP [15]. Both studies proposed that the tolerance prevention was due to the blocking of FMAICD of allo-reactive T cells by CsA or MP. These statements are very surprising, considering that both CsA and MP are widely utilized in clinical organ transplantation to prevent allograft rejection. In our system, MP did not interfere with FMAICD, but CsA inhibited it. One hundred micrograms per milliliter of MP slightly augmented the DNA fragmentation mediated by 7C11 with the maximum suppression of T-cell proliferation. However, the administration of the high dose of MP would be required to obtain the concentration of 100 μg/ml in serum or grafted organ. On this point, our data suggested that the combination of CsA and MP at the low dosages were more beneficial for clinical usage, because the administration of large doses of the immunosuppressant has been known to increase the risk of morbidity and mortality of recipients, especially viral or fungal infection.

Cocktails of immunosuppressants, including anti-metabolic agents in addition to calcineurin inhibitors (CsA or tacrolimus) and steroids, are used almost universally in clinical organ transplantation. The agents interfering with T-cell functions have complex effects on the FMAICD of T cells, as mentioned above. Furthermore, the pathways besides the Fas/Fas L system in the AICD of T cells have been reported [7, 13]. Of importance also is investigation of the actions of the immunosuppressants on the systems that mediate tolerance of allografts, e.g., suppressor-cell systems. Based on the results of the studies determining the above issues, a re-evaluation of the immunosuppressants used in clinical transplantation protocols will be required, to achieve, as a final goal, clinical allograft tolerance.

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