AUGMENTATION OF NATURAL KILLER CELL ACTIVITY INDUCED BY CYTOMEGALOVIRUS INFECTION IN MICE TREATED WITH FK506

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Summary. – Comparable rates of patient and graft survival after FK506 and cyclosporine treatments have been reported in the prevention of liver allograft rejection. On this basis, we examined the effect of FK506 on pathogenesis of cytomegalovirus (CMV) infection in mice. FK506 induced apparent immunosuppression in mice which could be monitored by the level of antibody production. The effective dose of trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) for 50% reduction in antibody production was 0.9 mg/kg. Even in such an immunosuppressed status at this or higher dose of FK 506, CMV infection was relatively alleviated, which was observed by the frequency of virus isolation and the mean virus titer of the lungs of mice treated with 0.1 – 1 mg/kg FK506 in comparison to untreated mice. The dose of FK506 attaining 50% frequency of lung infection was 1.5 mg/kg. The activity of natural killer (NK) cells was enhanced in infected mice. This enhancement was stronger in infected mice treated with FK506 at 0.32 mg/kg and 10 mg/kg than in untreated infected mice on day 3 post infection (p.i.). Thus, an immunosuppressant FK506 augmented inducible NK cell activity and alleviated MCMV infection even under immunosuppression.

Key words: natural killer cell activity; FK506; cytomegalovirus infection

Introduction

FK506 (tacrolimus) has been used as one of the major immunosuppressants in liver allograft transplantation (European FK506 Multicentre Liver Study Group, 1994; Kino *et al.*, 1987a,b; Sakr *et al.*, 1992; Singh *et al.*, 1994; Starzl *et al.*, 1989; Umemoto *et al.*, 1993; Wasik *et al.*, 1991). A number of studies report a comparable rate of patient and graft survival after FK506 and cyclosporine treatments in the prevention of liver allograft rejection. In immunocompromized patients, such as transplant recipients and acquired immunodeficiency syndrome (AIDS) patients, CMV infection has been one of the troublesome infections (Betts and Hanshaw, 1977; Catignani *et al.*, 1989; Ho, 1977; Jacobson and Mills, 1988; Marker *et al.*, 1981; Zaia, 1993). In those cases, interestingly, there has been a lower incidence or severity of CMV infection after treatment with FK506 than with cyclosporine (Alessaiani *et al.*, 1990; Sakr *et al.*, 1992; Singh *et al.*, 1994). Recently, the European FK506 Multicentre Liver Study Group (1994) observed a significantly lower frequency of CMV infection with FK506 (29/185) than with cyclosporine (46/184) treatment. This indicates that FK506 may modify the course of CMV infection in transplant recipients. Therefore, we utilized the mouse lung infection model with attenuated mouse CMV (MCMV) grown in mouse embryo fibroblast (MEF) cultures (Osborn, 1982; Shanley and Presanti, 1985). Here, we report that low doses of FK506 reduced the severity of MCMV infection by the augmentation of NK cell activity induced by infection.

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Abbreviations: CMV = cytomegalovirus; MCMV = mouse cytomegalovirus; ELISA = enzyme-linked immunosorbent assay; FBS = foetal bovine serum; HCMV = human cytomegalovirus; IFN = interferon; MEF = mouse embryo fibroblast; NK = natural killer; TNP-KLH = trinitrophenyl-keyhole limpet hemocyanin

Materials and Methods

Cells, virus, and drugs. MEF cells were prepared from ICR mouse embryos. MEF and mouse L-929 cells were grown and maintained in Eagle's Minimum Essential Medium supplemented with 10% and 2% foetal bovine serum (FBS), respectively. The YAC-1 cell line, a Moloney leukaemia virus-induced T cell lymphoma line was propagated in RPMI 1640 medium supplemented with 10% FBS and used for the NK cell assay. The Smith strain of MCMV, provided by Dr. Y. Minamishima, Miyazaki Medical School, Japan, was propagated in MEF cell cultures (Hayashi *et al.*, 1985). To prepare MCMV stock, infected cultures were subjected to 3 cycles of freezing and thawing, centrifuged at 1,500 x g for 10 mins, and the resulting supernatants were stored at -85°C until use (Shiraki *et al.*, 1990, 1991a,b). FK506 was from Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan.

MCMV infection and FK506 treatment of mice. Ten female ICR mice aged 4 weeks were injected subcutaneously (s.c.) with various doses of FK506 once a day for 17 days, starting on the day before the intraperitoneal (i.p.) infection, with 1,000 PFU of MCMV. On day 16 p.i., the lungs were isolated under ether anesthesia and subjected to the virus isolation. Lung homogenates (10%) were serially diluted and inoculated into MEF cultures in 60 mm-Petri dishes, and then overlaid with a nutrient methylcellulose medium. On day 3-4 p.i., the cultures were fixed with 5% neutral formalin, stained with Methylene Blue (Shiraki *et al.*, 1990, 1991a,b) and plaques were counted. The frequency of infection was assessed simultaneously on infected mouse lungs by the same method.

Antibody production in mice treated with FK506. Antibody production to TNP-KLH (Good *et al.*, 1980; Henry and Kimura, 1980) was examined in 5 mice treated with FK506. ICR mice were injected s.c. with FK506 daily for 17 days, starting on the day before immunization (day -1), and immunized i.p. with 20 μg of TNP-KLH mixed with 4 mg of alum adjuvans on day 0. Antibody titer to TNP-KLH was assessed by an enzyme-linked immunosorbent assay (ELISA) on day 16 after immunization. A goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma Chemical Co., St Louis, MO) was used to assess the amount of the antibody bound to TNP-KLH in a sample specimen.

NK cell activity in mice treated with FK506. Female ICR and BALB/c mice at the age of 4 weeks were injected s.c. with 0, 0.32, and 10 mg/kg FK506 daily, starting on the day before the infection, and infected i.p. with 10^5 PFU of MCMV. NK cell activity of spleen cells was determined by the chromium-release cytotoxicity assay on day 4 in ICR mice and on days 1 and 3 in BALB/c mice p.i., respectively. Microcytotoxicity assays were performed as described earlier (Nagasaka *et al.*, 1995; Yamamoto *et al.*, 1990). Briefly, target cells were labelled with sodium [^{51}Cr]chromate (ICN, Costa Mesa, CA) for 2 hrs at 37°C at a concentration of 3.7 MBq/10^6 cells/ml. Splenic effector cells were prepared as described (Bancroft *et al.*, 1981; Nagasaka *et al.*, 1995). Briefly, the spleens were harvested, teased apart in the complete medium, and filtered through a nylon mesh. The obtained cells were centrifuged at 400 x g for 5 mins, resuspended in 0.83% NH_4Cl to lyse erythrocytes, sedimented, and washed in the complete medium. Effector to target cell ratios, ranging from 100:1 to 8:1, were tested in microtiter plates with 10^4 target cells per well. Only the medium instead of the effector cell suspension was added to target cells for the determination of spontaneous lysis, and 1% Nonidet P-40 was added shortly before the harvest for the determination of total (100%) cell lysis. The mixture of target and effector cells was incubated for 4 hrs at 37°C in a CO_2 incubator and centrifuged at 400 x g for 5 mins. An aliquot (0.1 ml) of the culture supernatant was taken for the counting of radioactivity with an Aloka Autowell gamma counter. The data are expressed as follows:

$$\text{specific } ^{51}\text{Cr release (\%)} = \frac{\text{(tested sample cpm)} - \text{(medium control cpm)}}{\text{(Nonidet P-40 control cpm)} - \text{(medium control cpm)}} \times 100$$

Results

MCMV infection in the lungs of mice treated with FK506

In the time course study on MCMV infection in the lungs, the frequency of infection and the virus yield were maximal on day 16 p.i. (data not shown). Fig. 1 shows the frequency of virus isolation in the lungs of mice treated with various doses of FK506. Interestingly, the frequency of MCMV infection decreased with increasing dose of FK506 from zero to 0.1 mg/kg, and reached a minimum between 0.1 mg/kg and 1 mg/kg. However, this tendency reversed at higher doses of FK506 (1 mg/kg to 10 mg/kg, which led to higher frequencies of virus isolation. The FK506 dose attaining the 50% frequency of lung infection was 1.8 mg/kg and 2.1 mg/kg in Exp. 1 and 2, respectively. The frequencies of infection in mice treated with 10 mg/kg FK506 (the maximal dose examined in this study) in two experiments were 82 and 83%.

Table 1 shows the virus yields in the lungs of infected mice treated with various doses of FK506. Similarly, the virus titer in the lungs decreased as FK506 increased from zero to 1 mg/kg and increased as FK506 increased from 1 mg/kg to 10 mg/kg. Thus, low doses of FK506 reduced both the frequency of infection of the lungs and the virus titer in the lungs, which was corroborated by repeated experiments.

Production of antibody to TNP-KLH in mice treated with FK506

Antibody production to TNP-KLH was determined in mice treated with various doses of FK506 on day 16 after immunization (Table 2). FK506 suppressed the production of the antibody to TNP-KLH dose-dependently. The effective FK506 dose for 50% reduction (ED_{50}) in antibody production was 0.9 mg/kg. These results indicate that the mice could be severely immunosuppressed by the FK506 treatment in this system.

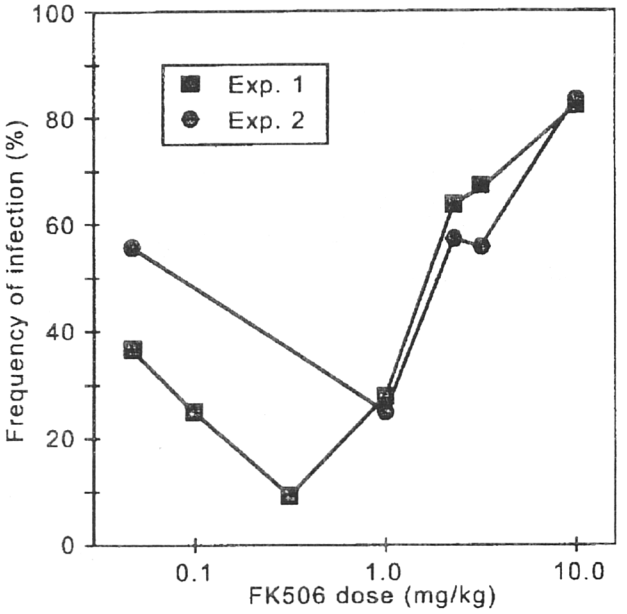

Fig. 1
The frequency of lung infection in infected mice treated with FK506
Ten mice were injected s.c. with various doses of FK506 daily for 16 days, starting on the day before infection, with 1,000 PFU of MCMV. Lung infection was evaluated on day 16 p.i.. The frequency of infection was determined from the number of mice with lung infection in the same treatment group.

NK cell activity in mice treated with FK506

Fig. 2 shows the NK cell response of spleen cells in BALB/c mice examined. The NK cell activity was low in all uninfected mice on day 1, and relatively enhanced in mice treated with 0.32 mg/kg FK506 as compared with untreated mice or mice treated with 10 mg/kg FK506 on day 3 ($p < 0.01$). In contrast, the NK cell activity was markedly elevated in infected mice on day 3 p.i.. This activity was significantly elevated in infected mice treated with 0.32 mg/kg or 10 mg/kg FK506 on day 3 as compared with infected mice without FK506 treatment. However, there was no significant difference between FK506 treatments with 0.32 mg/kg and 10 mg/kg. This induction of NK cell activity apparently reached a plateau at the range of concentrations of FK506 of 0.32 mg/kg to 10 mg/kg.

In ICR mice, similar results were obtained. In infected mice, the NK cell activity was elevated dose-dependently by FK506 (Table 3). After the administration of FK506 at 0.32 mg/kg and 10 mg/kg, the NK cell activity of infected mice was significantly elevated ($p < 0.05$ for infected mice treated with 0.32 mg/kg FK506 versus untreated mice, and $p < 0.01$ for mice treated with 10 mg/kg FK506 versus untreated mice), while that of uninfected mice remained very low even after the administration of FK506. Thus, the induction of NK cell activity by MCMV infection was augmented by FK506 also in ICR mice.

Table 1. MCMV yields in the lungs of infected mice treated with FK506

FK506 (mg/kg)	Number of mice	Mean PFU titer per 0.1 g of lungs	SD
0	10	153*	293
0.1	12	31*	86
0.32	11	7	23
1	11	14	26
2.5	11	289	455
3.2	12	844	1729
10	11	1545	1545

Ten mice were injected s.c. with various doses of FK506 once a day for 16 days, starting on the day before infection, with 1,000 PFU of MCMV. Lung infection was evaluated on day 16 p.i.. Representative data are shown in this table. *The difference between the indicated values was statistically significant ($p = 0.05$).

Table 2. Suppression of antibody production to TNP-KLH in mice treated with FK506

FK506 (mg/kg)	Suppression of antibody production (%)	SD
0.1	9.2	18.8
0.32	8.6	18.8
1	72.8	20.5
3.2	85.8	17.4
10	95.2	11.4

Five mice were injected s.c. with various doses of FK506 once a day for 16 days, starting on the day before immunization, and immunized with TNP-KLH. Antibody titer to TNP-KLH was assessed by ELISA on day 16 after immunization.

Discussion

FK506 suppressed the T cell-mediated immune response dose-dependently as assessed by antibody production. However, the MCMV infection was milder with low doses of FK506 which corresponded to the range of T cell-mediated immune suppression in the mouse model used in this study. This discrepancy may be explained by the NK cell activity augmented by FK506. In this study we used an attenuated MCMV strain grown in MEF cultures to assess the load of MCMV in immunosuppressed mice. The frequency of virus infection and the mean virus yield in the lungs in this mouse model may represent the incidence and severity of human CMV (HCMV) infection of transplant recipients, respectively (Selgrade *et al.*, 1984; Shanley and Presanti, 1985).

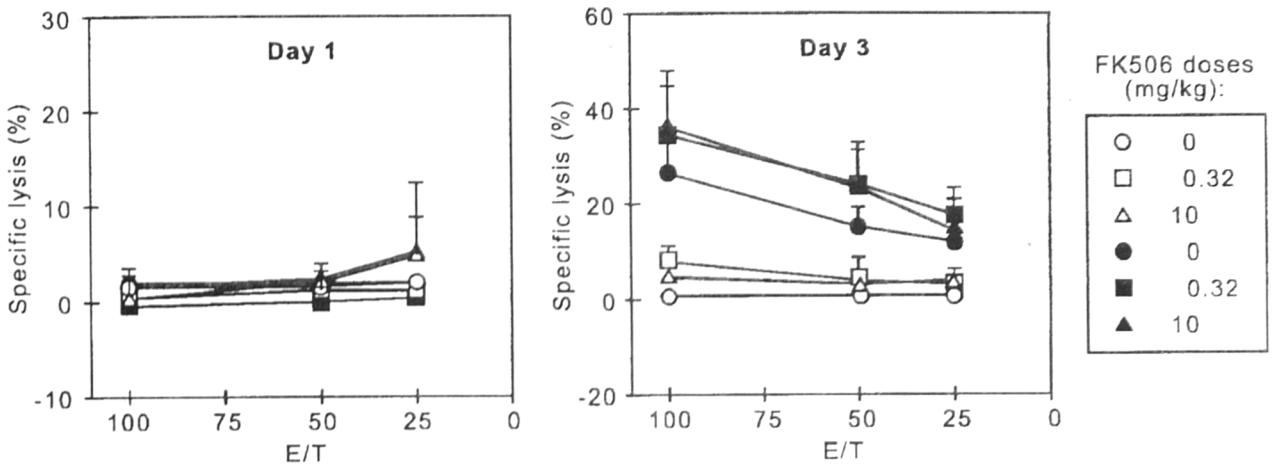

Fig. 2
NK cell activity of spleen cells in BALB/c mice treated with FK506 on day 1 and day 3 p.i.
NK cell activity is expressed as specific lysis (%) of YAC-1 cells. Mean values from 3 experiments are shown. MCMV-infected (full symbols) and uninfected (empty symbols) mice. E/T = the ratio of the effector to the target cells.

Table 3. NK cell activity of spleen cells in ICR mice

FK506 (mg/kg)	Specific lysis in % (SD)		
	1:8*	1:30*	1:100*
0	4(3)	7(4)	15(8)
0.32	7(4)	13(3)	24(5)
10	9(5)	15(3)	24(3)

ICR female mice aged four weeks were injected s.c. once a day with various doses of FK506, starting on the day before infection, (day -1) for 5 days. MCMV was injected i.p. at 2 x 10^5 PFU per mouse on day 0. Spleen lymphocytes were collected on day 4 p.i. and used as the effector cells. Labelled YAC-1 cells were used as the target cells. *The ratio of the effector to the target cells.

Quinnan *et al.* (1981, 1982) have reported that the pre-existing NK cell activity and antibody dependent cell-mediated cytotoxicity play an important role as one of the major defense mechanisms in the acute phase of HCMV infection. The earliest host responses to MCMV infection, the interferon (IFN) production and subsequent augmented NK cell activity, play crucial roles in the recovery from acute MCMV infection (Bancroft *et al.*, 1981; Catignani *et al.*, 1989; Selgrade *et al.*, 1982; Shanley and Presanti, 1985; Shellam *et al.*, 1981, 1982; Yung *et al.*, 1985). FK506 has little effect on the HCMV growth *in vitro* (Shiraki *et al.*, 1991a) and may not work as an anti-MCMV agent, which made us to speculate that this immunosuppressive drug might work as an immunological modulator. In this study, we observed a milder lung infection in mice treated with low doses of FK506 than in untreated mice, and evaluated both the serum IFN level (data not shown) and the NK cell activity as the possible factors affecting the severity of MCMV infection. We used a higher dose of MCMV inoculum for the NK cell assay than Markus *et al.* (1991) have used for the lung infection to facilitate the effect of those factors. Thus, the NK cell activity can be induced strongly enough to characterize the action of FK506 on the MCMV-induced NK cell activity.

Welsh *et al.* (1991) have reported that, in SCID mice, the IFN levels continued to rise over a 10-day-period (Welsh *et al.*, 1991), whereas the NK cell response peaked on days 3-5 and gradually tapered, which indicated that there would be a discrepancy between the IFN titer and the level of NK cell activity. In our study, there were too small differences in the serum IFN levels in FK506-treated groups (data not shown) to postulate the modulation of lung infection by the induction of IFN. In this case, the NK cell activity was significantly augmented in infected mice treated with FK506 as compared with untreated mice. The augmentation of the MCMV-induced NK cell activity by FK506 must play an important role in a mild MCMV infection in the lungs in this system. The high dose (10 mg/kg) FK506 treatment similarly augmented the MCMV-induced NK cell activity, although the lung infection was severe possibly because of strong immunosuppression. In contrast, the low dose (0.32 mg/kg) FK506 treatment significantly augmented the MCMV-induced NK cell activity without severe impairment of the host immune system. This augmentation may have resulted in a milder lung infection in treated mice as compared with untreated mice.

There have been reported several differences between two immunosuppressants, FK506 and cycrosporine, in the induc-

tion of the NK cell activity. Cyclosporine has suppressed the induction of NK cell activity in MCMV-infected rats (Selgrade *et al.*, 1992). While the NK cell activity was sensitive to a preincubation of rat spleen cells or human peripheral mononuclear cells with cyclosporine, it was resistant to the preincubation of those cells with FK506 (Markus *et al.*, 1991; Wasik *et al.*, 1991). Thus, these opposite effects of FK506 and cyclosporine on the NK cell activity may modify the course of CMV infection as observed clinically (Alessaiani *et al.*, 1990; European FK506 Multicentre Liver Study Group, 1994; Sakr *et al.*, 1992; Singh *et al.*, 1994). Our study showed the augmentation of NK cell activity in the case of MCMV infection, which may be a clue to clarification of the mechanism of suppression of clinical CMV proliferation by FK506.

We showed one of the beneficial activities of an immunosuppressant, FK506, which augmented the CMV-induced NK cell activity to prevent the CMV infection. This type of augmentation of the NK cell activity by FK506 may be associated with a significant reduction in the frequency of bacterial and fungal infection in liver transplantation as observed clinically (Alessaiani *et al.*, 1990; European FK506 Multicentre Liver Study Group, 1994; Sakr *et al.*, 1992; Singh *et al.*, 1994). The precise mechanism of augmentation of NK cell activity by FK506 remains to be determined.

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