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# Long-Term Acceptance of Fully Mhc-Mismatched Limb Allografts after a Short Course of Anti-αβ-T Cell Receptor Monoclonal Antibody and FK506

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**— Abstract —**

Whether a seven days course of anti-αβ-T cell receptor-antibody (αβ-TCRmAb) combined with FK506 therapy promotes survival of limb allografts in fully MHC-mismatched combination (Brown Norway → Lewis) was examined. Eight animals received 250 μg/kg/day of αβ-TCRmAb for 7 days and 2 mg/kg/day of FK506 postoperatively (Combination therapy group). Eight animals had FK506 only (Mono-therapy group) and five animals did not have treatment (Control group). Clinical signs of early rejection with edema or erythema in the skin occurred at an average of 8.6±1.5 days postoperatively in Control group and 59.0±8.3 days in Mono-therapy group, both of which proceeded to irreversible rejection with necrosis of the epidermis and finally mummification. In Combination therapy group, all animals showed evidence of early rejection at an average of 56.8 ±12.6 days postoperatively, however, in 4 of 8 limbs, early rejection resolved without any treatment and limbs survived >1 year. At 9 months postoperatively, donor skin grafts were accepted and third-party skin grafts were rejected by all four survivors, demonstrating donor-specific tolerance. Little or no detectable chimerism was observed in any of the 4 surviving animals at one-year postoperatively. Combination therapy of αβ-TCRmAb and FK506 resulted in long-term survival in fully MHC-mismatched limb transplants.

**Key Words:** Experimental limb transplantation, FK506 and rat

## Introduction

Over the past decade, the focus of experimental limb transplantation has been to obtain functional recovery and indefinite survival as reported in previous review articles.[1-7] These factors are of paramount importance in non-life-saving procedures such as hand transplantation, where quality of life is the main indication for reconstruction. There is an additional ethical issue in

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* 편집장 주: 본 영어 논문은 일본미세수술학회 회원이 제출한 것으로 복합조직이식 수술에 관한 실험을 다루고 있어 대한미세수술학회 이사회에서 본 학회의 학술적 발전에 도움이 될 것으로 판단되어 학회지에 게재되었습니다.

clinical composite tissue transplantation where continuous and extensive use of the powerful immunosuppressive drugs required to obtain long-term limb allograft survival might have serious and possibly fatal consequences. It is necessary to balance the opposing requirements for prolonged graft survival with the need to minimise immunosuppressive drug treatment. One approach to this problem is to establish an animal model of limb transplant rejection to develop potential treatment strategies that can reduce or eliminate the requirement for lifelong immunosuppression. However, there are only a few reports of tolerance induction across the strong histocompatibility barrier of Brown Norway (BN) donor to Lewis recipient without the need for chronic immunosuppression.[8,9] We have reported previously the indefinite survival of 26 % of limb allografts from BN donors to Lewis recipients resulting from triple combination therapy with the immunosuppressive drugs, FK506, Mycophenolate mofetil (MMF) and prednisone, which parallels the treatment for human hand transplantation.[8] This therapy had not only "marginal effects" but also suffers from substantial side effects in human hand transplantation, although it was used successfully. This indicated the necessity to combine with some other form of immunosuppression.

The anti-$\alpha\beta$-T cell receptor ($\alpha\beta$-TCR) on T cells plays an important role in recognition of foreign antigen and has been used as a target for immunosuppression. Experimental data published previously revealed that mono-therapy with monoclonal antibodies (mAb) directed at $\alpha\beta$-TCR led to long-term acceptance of allogeneic grafts.[10-13] Furthermore, combination of $\alpha\beta$-TCR mAb and conventional immunosuppresive drugs was demonstrated to induce indefinite survival in limb transpantation.[9] In this study, the effectiveness of combination therapy with $\alpha\beta$-TCR mAb and FK506 in a short 7 day course was examined to obtain long-term limb allograft survival in the strong histocompatibility barrier of BN donor to Lewis recipient.

## Materials and Methods

### Animals, experimental model and treatment

This study was performed in accordance with the guidelines of the Animal Care and Use committee of the Kobe University of Medical School and the Guide for the Care and Use of Laboratory Animals. Rat hind limb transplantation across the strong barrier of BN donor to fully allogeneic Lewis recipient was performed using previously published techniques.[8,14-16] Eight animals received subcutaneous dosage of 2 mg/kg/day of FK506 (Astellas, Osaka, Japan) and 250 ㎍/kg/day of TCRmAb (clone R73; Pharmingen, San Diego, CA) intraperitoneally from day 1 to day 7 postoperatively (Combination therapy group). Eight animals had monotherapy of FK506 for seven days postoperatively (Mono-therapy group) and five animals did not have any treatment (Control group).

### Assessment of rejection

The transplanted limbs were inspected daily for clinical signs of rejection using a visual scoring system of skin rejection based on that reported by Siemionow,[9] including edema, erythema, desquamation, hair loss, epidermolysis, exudation, and skin necrosis. Biopsies from transplanted limb skin were performed to confirm the visual assessment of rejection and also after reversal of the rejection episode. These were graded by a histological scoring system formulated by Saurat[17] as Grade 0, No rejection; Grade I, Mild rejection; Grade II, Moderate rejection; Grade III, Severe rejection.

### Skin grafting

Full thickness skin grafts were used to confirm systemic tolerance[18] in the rats with long-

surviving allografts at 9 months postoperatively. Four types of skin grafts: Lewis (autograft); BN (donor skin allograft); and Fischer344 and Sprague-Dawley (third-party skin allograft) were placed on the survivors using the standard technique.[19] Full thickness skin grafts 16 mm in diameter separated by a 10 mm skin bridge were placed onto the dorsal area.[9] As a control group, 4 age-matched Lewis non-transplanted rats received the same procedure. Rejection was monitored daily and defined as a necrosis involving more than 80% of the transplanted skin.[9]

### Immunohistochemical staining

Long-term surviving animals with good graft function were euthanased at the one-year end point. Frozen sections of spleen, mesenteric lymph nodes (MLN) and thymus were cut onto gelatin-coated slides and stained by an indirect immunoperoxidase method.[20] MHC Class I of donor (BN) origin was identified with MRC OX27 antibody (Serotec, Oxford, UK), which reacts with BN but not Lewis MHC. MOPC 21 (Sigma' St Louis, MO) was used as a negative control. Sources of antibody and the conditions used for staining have been previously described.[20] Extent of infiltrates was scored semi-quantitatively from-(no cells);+(〈1% of section area);++(1-5% of section area);+++(〉 5% of section area).

### Flow cytometric analysis of donor cells in recipient tissues.

At the one-year end point, spleen and thymus of long-term recipients were dissociated and mashed through a fine sieve and filtered. Leukocytes in bone marrow (BM) of transplanted femoral bone were purified by centrifugation for 5 min at 600×g and red blood cell lysis by re-suspension for 5 min in an aqueous solution consisting of 0.155M NH4Cl; 0.01M NaHCO3; 0.001M EDTA pH 7.35 followed by washing in PBS as has been previously described[20]. The cell suspension was centrifuged as above and the pellet was resuspended in 0.9M PBS plus 1% human serum. The phenotype of donor cells was analysed by single-colour staining and flow cytometry using a FacsCalibur flow cytometer and Cell Quest software (Becton Dickinson, Mountain view, CA) as described.[20] MRC OX 27 and anti-mouse FITC (Sigma) were used to identify donor cells. Dead cells were excluded by a combination of forward scatter and staining with propidium iodide. A total of 30,000 cells were analysed.

### Statistical analyses.

Comparison of survival data in control group, mono-therapy group and combination therapy group was by Kaplan and Meier analysis and by analysis of variance (ANOVA) using Fisher test.

## Results

There was no sign of graft-verus-host disease (GVHD) or side effects of the treatment, such as significant weight loss, hair loss or diarrhoea through the protocol in either treatment group.

Clinical signs of early rejection with edema or erythema in the skin occurred at an average of

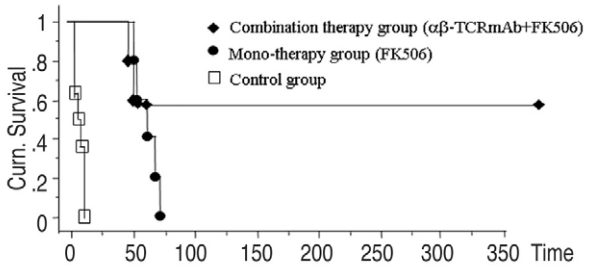


**Fig. 1.** Survival of BN to Lewis hind limb allografts. Control group (n=5) did not receive any immunosuppressive treatment while the mono-therapy group received FK506 for 7 days. The combination therapy group, received FK506 plus antibody to $\alpha/\beta$ TCR. All animals in this group showed evidence of early rejection at an average of 56.8±9.9 days, however, in 4 of 8 limbs, this resolved without any treatment.

8.6±1.5 days postoperatively in the control group and at an average of 59.4±7.9 days in the mono-therapy group. Both groups subsequently proceeded to irreversible rejection with necrosis of the epidermis and finally mummification (Fig. 1) and the animals were euthanased. In the combination therapy group, all animals showed evidence of early rejection at an average of day 56.9 ±10.4 postoperatively, however, in 4 of 8 limbs, early rejection resolved without any treatment (Fig. 2A, B) at an average of 144.5± 5.4 days and the 4 limbs survived until the end point of 1 year (Fig. 1) although one of the four long-term survivors had partial tissue damage due to the early rejection. Both treatment groups showed significant survival of transplanted limbs compared to control group ($p<0.0001$), however, there was no significant difference between the two treatment groups ($p=0.392$). At 9 months postoperatively, donor skin grafts were accepted and third-party skin grafts were rejected by all four survivors, demonstrating donor-specific tolerance (Fig. 2-C).

In the 4 surviving animals at the one-year end-point in the combination therapy group, little or no detectable donor cell chimerism was observed in the BM on the proximal side of the transplanted femoral bone (Fig. 3). No detectable chimerism was found in the spleen, thymus, peripheral blood or recipient (contralateral femur) bone marrow by flow cytometry. This was also the case in the spleen, mesenteric lymph nodes (MLN) and thymus, where no detectable donor cells were identified by immunochemical staining.

## Discussion

Ideally, limb transplantation should be followed by an effective anti-rejection therapy that

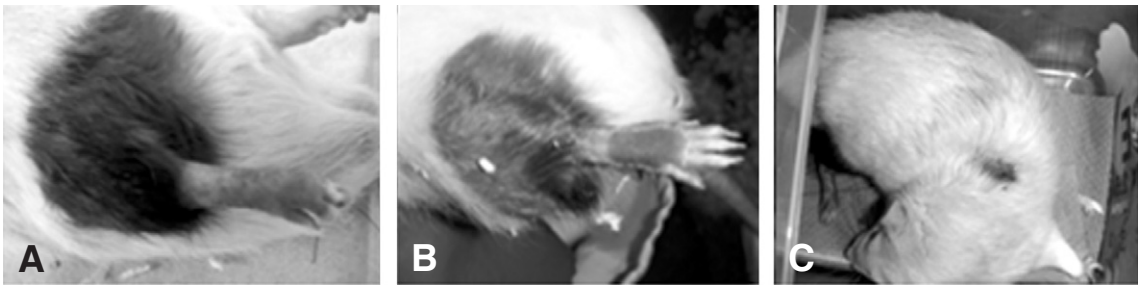


**Fig. 2.** Spontaneous resolution of rejection after combination therapy. **(A)** Early rejection at day 62 in a limb allograft in the combination therapy group. **(B)** Spontaneous reversal of rejection without any treatment. **(C)** Acceptance of donor skin graft 9 months after hind limb transplantation.

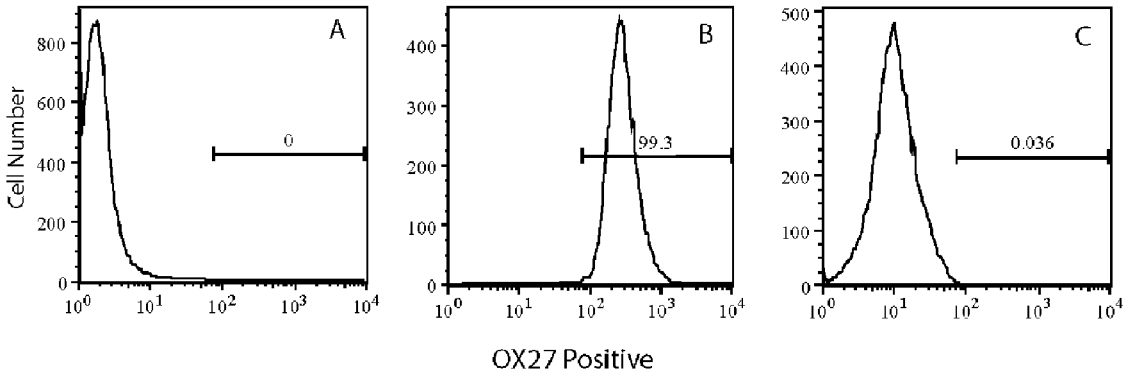


**Fig. 3.** Flow cytometry analysis of donor chimerism in bone marrow of the limb transplant. **(A)** Normal Lewis bone marrow is not stained by anti-BN antibody OX27. **(B)** All normal BN bone marrow cells react with OX27. **(C)** No cells of BN origin are observed in the transplanted BN limb after one year.

can be quickly tapered to a maintenance dose and then stopped.[21] This would then justify performance of a non-life-saving procedure by an attempt to minimize possible side effects or life-threatening complications. The complete withdrawal of immunosuppression would be feasible only in cases demonstrating tolerance, where the functional recovery of the transplanted limb would not be jeopardized by its withdrawal. In our search for achieving this goal, we set out experimentally to identify the optimal available treatment in a stringent, high-responder rat model in order to obtain an effective immunosuppressant action without side effects and withdrawal of the treatment thereafter.

Many studies have tried to develop a high responder animal model of limb transplantation in which the limb survives long-term after treatment with a short course of immunosuppressive drugs. We have previously reported that 26% of limb allografts survive across the strong histocompatibility barrier of BN donor to LEW recipient when treated using triple therapy comprising FK506, MMF and prednisone, which parallels the treatment administered to human hand transplant recipients.[8] This result showed the marginal effects of conventional immunosuppression therapy and suggested a need to improve the treatment protocol by combining with other forms of therapy such as antibody to TCR. Heidecke[10,11] reported the effectiveness of mono-therapy of αβ-TCR mAb treatment which resulted in long-term cardiac graft survival when initiated prior to transplantation. The therapeutic effect was associated with creation of an environment in adult animals where encounter with alloantigen results in unresponsiveness or incomplete T cell activation in the pre-transplant period. Furthermore, they reported that post-transplant administration of αβ-TCR mAb developed a transient state of hyporesponsiveness with blockade of TCR-mediated activation during the period of αβ-TCR mAb administration, where there is a possibility of tolerance induction by being combined with immunosuppression drugs. In this study, we examined this possibility by combining αβ-TCR mAb with FK506 treatment. It would be interesting to investigate numbers and activity of αβ-T-cells in blood and tissue.

To our knowledge, Sieminow et al.[9] reported the first successful survival across the strong histocompatibility barrier of BN to Lewis using combination therapy with αβ-TCRmAb and Cyclosporine A (CsA). They demonstrated indefinite, 100% survival (〉350 days) of all 8 rats after withdrawal of immunosuppression 7 days postoperatively in combination with significant numbers of donor cells in the thymus at day 120. Interestingly, in contrast to their report, our treatment using αβ-TCRmAb and FK506 showed a different outcome, with a lower survival rate of 4 out of 8 rats (50%). This may be due to the different adjunctive immunosuppression used, although CsA and FK506 have a similar mechanism of action through inhibition of calcineurin.

Another possibility is the different therapeutic dosages of CsA and FK506. FK506 has an effective dose rate 20 to 50 times lower than CsA clinically,[22] where 16 mg/kg of CsA corresponds to between 0.32-0.8 mg/kg of FK506. In this study, we administered 2 mg/kg of FK506 based on our previous reports of optimal dosage of FK506 to maintain rejection free conditions in limb allogratfts.[8, 14,15] Sieminow[9] administered 16 mg/kg of CsA in their protocol and reported that limbs were rejected between 13 to 14 days by mono-therapy of 16 mg/kg of CsA. This compared to an average of 59 days using mono-therapy of 2 mg/kg of FK506 in our study. It is possible that this difference in the potency of adjunctive immunosuppression might account for the difference in outcome. It would be interesting to examine whether a lower dose of FK506 less than 2 mg/kg/day might combine optimally with αβ-TCRmAb therapy and lead to increased survival. In support, in some situations,

increased dose rates of CsA have been shown to reverse the salutary effects of low-dose CsA treatment.[23]

Another reason for the lower survival in our results is the difference between CsA and FK506 in distribution into skin. Skin is the major barrier to tolerance induction in composite tissue allografts (CTA) because of its strong antigenicity. FK506 distributes mainly in lung, pancreas and liver tissue,[24] while CsA goes to liver, kidney and skin.[25]

Transplantation of limbs leads to transfer of donor BM cells in association with transplantation of the long bones of the leg and could promote mixed hematopoietic chimerism. The observation that mixed hematopoietic chimerism strongly promotes transplant tolerance[26] has led to investigation of the extent of donor-specific mixed allogenic chimerism as a marker for limb transplant tolerance.[27] Initially, there are high levels of donor cell chimerism identified in the recipient lymphoid tissues, which peak on days 2 to 4 after limb transplantation then decline[28] and there is often a correlation between detectable chimerism and graft acceptance.[9,13,29] We were unable to find significant levels of mixed chimerism at one-year postoperatively in any of the tissues we investigated despite the presence of stable transplant tolerance. This was similar to the findings of Kubitskiy et al.[30], which demonstrated low levels of long-term chimerism associated with limb graft acceptance despite manipulations to increase its incidence.

In this study, we observed the spontaneous reversal of episodes of early rejection in the αβ-TCR antibody and FK506 treated group. To our knowledge, this is the first report of spontaneous reversal of episodes of early rejection in experimental CTA. A similar phenomenon has already been reported previously in liver transplantation where it occurs in the first two weeks of transplantation[31,32] which is much earlier than the delayed reversal observed here.

In conclusion, this study demonstrated that a short course of combination therapy of αβ-TCRmAb and FK506 was effective for long-term survival in fully MHC-mismatched limb transplants without recipient conditioning and side effects. We believe our animal model of drug administration compares favorably with previous studies and allows for speculation that this approach can be tested in humans.

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